Exercise-heat acclimation in humans alters baseline levels and ex vivo heat inducibility of HSP72 and HSP90 in peripheral blood mononuclear cells

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McClung JP, Hasday JD, He J, Montain SJ, Cheuvront SN, Sawka MN, Singh IS. Exercise-heat acclimation in humans alters baseline levels and ex vivo heat inducibility of HSP72 and HSP90 in peripheral blood mononuclear cells. Am J Physiol Regul Integr Comp Physiol 294: R185–R191, 2008. First published October 31, 2007; doi:10.1152/ajpregu.00532.2007.—The induction of cellular acquired thermal tolerance (ATT) during heat acclimation (HA) in humans is not well described. This study determined whether exercise-HA modifies the human heat shock protein (HSP)72 and HSP90 responses and whether changes are correlated with physiological adaptations to HA. Using a 10-day HA protocol comprising daily exercise (treadmill walking) in a hot environment (T_e = 49°C, 20% RH), we analyzed baseline and ex vivo heat-induced expression of HSP72 and HSP90 in peripheral blood mononuclear cells (PBMCs) isolated prior to exercise from eight subjects on day 1 and 10 of the HA protocol. Classical physiological responses to HA were observed, including significantly reduced heart rate and core body temperature, and significantly increased sweating rate. Baseline levels of HSP72 and HSP90 were significantly increased following acclimation by 17.7 ± 6.1% and 21.1 ± 6.5%, respectively. Ex vivo induction of HSP72 in PBMCs exposed to heat shock (43°C) was blunted on day 10 compared with day 1. A correlation was identified (r² = 0.89) between changes in core temperature elevation and ex vivo HSP90 responses to heat shock between days 1 and 10, indicating that volunteers demonstrating the greatest physiological HA tended to exhibit the greatest degree of physiological strain. Few studies have examined the coincident induction of HA and ATT in humans and the relationship between HA and expression of cytoprotective heat shock proteins (HSPs) (22, 38). HSPs are highly conserved proteins that serve as molecular chaperones, sequester denatured peptides, block cell death, and accelerate cellular repair from heat stress, ischemia, and endotoxic shock in cells and in animal models (17, 18). The expression of mammalian HSP genes is regulated by the heat/stress-activated transcription factor, heat shock factor-1 (HSF-1), which, upon exposure to heat/stress, trimerizes, translocates to the nucleus, binds to its cognate binding sites in HSP gene promoters, and activates gene transcription (5). Increased expression of HSPs is evident within an hour of exposure to heat stress, and elevated HSP levels persist for several days. The rate of HSP synthesis correlates with the severity of heat stress and the cumulative heat stress exposure in rats (21). Expression of HSP genes is central to the cellular response against stress, including heat stress, and contributes to the development of ATT (11, 35, 36). In recent animal studies, Horowitz et al. (13) demonstrated in rats that HA involves changes in the expression pattern of cytoprotective genes, including antiapoptotic, antioxidant, and HSP genes. Moreover, the same study found HSP expression to temporally correlate with heat acclimation and acquisition of the ATT state (13). Although the human HSP response to acute exercise and environmental heat stress has been the focus of some studies (8, 22–24, 26, 27), changes in the human HSP response that occur during either passive HA or exercise-HA are poorly understood. To our knowledge, only two studies have examined HSP expression in humans during heat acclimation. Marshall et al. (22) examined baseline peripheral blood mononuclear cells (PBMC) HSP72 changes during the first 2 days of exercise-heat exposure and found no differences in protein expression. However, it should be noted that full heat acclimatization generally requires 5–10 days (28, 30). Yamada et al. (38) examined PBMC HSP72 expression during a 10-day HA program and found elevated baseline levels from day 6 through day 10. The present study expands upon those two recent studies by including HSP90 and determining the response of HSP72 and HSP90 to ex vivo exposure to heat shock during HA in humans. The ex vivo experiments enable determination of HA with the former reducing physiological strain and the latter providing cellular protection against serious heat injury for any degree of physiological strain.

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effects on HSP regulation (e.g., threshold temperature for induction and magnitude of HSP expression for a given temperature challenge).

The purpose of this study was to determine whether exercise-HA modifies the human HSP72 and HSP90 responses (baseline and in response to ex vivo heat shock) and whether those responses are correlated with physiological adaptations to HA. We hypothesized that exertional HA would enhance baseline HSP72 and HSP90 levels but blunt the ex vivo heat shock response and that those changes might correlate with parameters of physiological adaptation. We focused on two HSPs, namely HSP72 and HSP90. HSP72, which is virtually absent under normal conditions, is the predominant stress-induced molecular chaperone that prevents aggregation of stress-damaged proteins and assists in refolding of denatured polypeptides (25). Unlike HSP72, HSP90 is constitutively expressed and is distinguished from other chaperones in that most of its known substrates are signal transduction proteins like steroid hormone receptors and signaling kinases (39). HA was achieved by repeated daily exercise at high ambient temperature for 10 days, which causes integrated changes in thermoregulatory control, fluid balance, and cardiovascular responses typical to HA (28, 30). We analyzed the baseline and ex vivo heat shock-induced levels of expression of HSP72 and HSP90 in PBMCs isolated before and after completion of the HA protocol.

METHODS

Volunteers

Eight healthy soldier volunteers (mean [range]: age = 23 [18–29] yr, ht = 176 [169–182] cm, wt = 71 [60–85] kg, body surface area = 1.86 [1.64–2.06] m²) participated in this study. The volunteers (7 men, 1 woman) were physically active, taking no medications, non-smokers, otherwise healthy as determined by physical exam, and had comparable physical fitness levels. The study protocol was approved in advance by the Human Use Review Committee at the U.S. Army Research Institute of Environmental Medicine and the Human Subjects Research Review Board at the U.S. Army Medical Research and Materiel Command.

Each volunteer provided written informed consent before participating. The investigators adhered to the policies for protection of human subjects as prescribed in Army Regulation 70–25, and the research was conducted in adherence with the provisions of 32 CFR Part 219.

Heat Acclimation

Each volunteer completed a 10-day HA protocol (19) that consisted of walking (1.56 m/s, 4% grade) in a hot environment (49°C, 20% relative humidity) until reaching one of the three criteria: 1) 100 continuous minutes of exercise, 2) core temperature of 39.5°C, or 3) voluntary cessation. The total exercise time was defined as the daily endurance. Experiments were conducted in the early spring months before volunteers were exposed to warm weather; therefore, their natural heat acclimatization state should have been at the annual nadir. Each morning, volunteers were provided with ad libitum fluids and a small meal ~3h before testing. An additional 250 ml of water was given 1 h before testing as a way of standardizing hydration state. All experimental testing was conducted at the same time of day to control for circadian fluctuations in body temperature.

Sweat losses were determined by the change in nude body mass pre- to post-exercise, as previously described (3). Sweat volume and mass were considered equivalent (i.e., 1 ml = 1 g) and were expressed as a rate (volume per unit time, l/h). Heart rate (HR) (Polar â„¢, Polar Electro, Inc, Woodbury, NY) and core (intestinal) body temperature (Tc) (Jonah core body temperature capsule, Mini Mitter, Bend, OR) were measured continuously and recorded at 10-min intervals. Pills were ingested the evening before exercise to provide sufficient time to be located in intestinal tract and the most accurate “core” temperature. The core temperature area under the curve (AUC) was calculated when core temperature exceeded 38°C using a modification to the trapezium rule (14), where AUC ([°C × min] = Σ time interval (min) × 0.5 [°C above 38°C at start of interval + °C above 38°C at end of interval].

A core temperature of 38°C was selected as an approximate minimum for intolerance (29), as it was well enough below (~1.5°C) our laboratory safety threshold to allow an ample time × temperature interaction for AUC calculation, which is a more accurate index of total heat strain than peak body temperature (14).

Isolation and Incubation of PBMCs

On the mornings of HA days 1 and 10, volunteers reported to the laboratory for phlebotomy before eating or drinking (overnight fast). Therefore, all blood data represent pretest values on days 1 (zero heat exposure days) and 10 (nine heat exposure days).

Venous blood was collected into Ficoll-Hyapaque-containing cell separation tubes (BD Vacutainer CPT; Becton-Dickinson, Franklin Lakes, NJ) prior to exercise on days 1 and 10 of the HA protocol. PBMCs were isolated in the CPT tubes, according to the manufacturer’s protocol and resuspended in RPMI 1640 medium (Cambrex Bioscience, Walkersville, MD) with 10% FBS (Cambrex). For baseline comparisons, PBMCs were lysed immediately after isolation (0 h). To determine the effect of heat shock on these cells ex vivo and to determine whether HA altered the thermal threshold for HSP expression, we exposed the cells to heat shock (43°C) or to intermittent temperatures between 37°C and 43°C. Cells in culture tubes were incubated in fixed temperature water baths for 1 h at 43°C then 5 h at 37°C, or for 6 h at one of three temperatures (37°, 39.5°, or 41°C) and lysed for immunoblotting. The heat shock treatment (1 h at 43°C then 5 h at 37°C) was selected to provide sufficient stress to produce robust HSP responses without inducing cell death.

Immunoblotting

PBMCs were collected by centrifugation at 500 g for 5 min at 4°C, washed twice with 4°C PBS, and lysed in a modified RIPA cell lysis buffer containing 50 mM sodium β-glycerophosphate, 2 mM dithiothreitol, 10 mM MgCl2·6H2O, 5 mM EGTA, 10 mM KH2PO4, 1 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride (Calbiochem, San Diego, CA), 1 mM benzamidine, 0.1 μM microcystin-LR, and 10 μg/ml each of leupeptin and aprotinin. Except where noted, all reagents were obtained from Sigma Chemical (St. Louis, MO). The PBMCs in modified RIPA buffer were shaken for 10 min at 4°C, clarified by centrifugation at 12,000 g for 10 min, and the supernatants were assayed for protein content using the Bradford method (Bio-Rad, Hercules, CA) with BSA as the standard.

For immunoblotting, aliquots of cell extract containing 10 μg of protein were resolved on 10% SDS-PAGE gels and electrostatically transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). Membranes were blocked with 5% nonfat dry milk and exposed to anti-HSP72 and HSP90 (rabbit polyclonal) antibodies (Stressgen, Ann Arbor, MI) followed by anti-rabbit peroxidase conjugated secondary antibody (Bio-Rad), as previously described (15, 34). As a loading control, the membranes were also immunoblotted for β-tubulin (Chemicon International, Temecula, CA). The blots were developed using chemiluminescence detection (Renassiance; New England Nuclear, Boston, MA), quantified by direct imaging (Fuji gel documentation system and ImageGauge software) and subsequently exposed to X-ray film. Band intensities for HSPs were normalized to β-tubulin bands.
Statistical Analysis

Sample size analysis indicated that 6–10 subjects would provide sufficient power ($\beta = 0.20$) to detect a biologically significant (two-fold) change in HSP expression (35) for an anticipated effect size $\approx 1.0$ (Glass’ Delta). Similarly, this sample size is sufficient to detect a meaningful difference in $T_c$ between days 1 and 10, defined herein as a value twice (0.40°C) the typical standard deviation (0.20°C) (4), thus allowing for unique and additive response variability, resulting from experimental perturbations.

Paired t-tests were used to compare baseline HSP expression on days 1 and 10. Systems physiological changes (e.g., $T_c$) on days 1, 5, and 10 were compared using a one-way repeated measures ANOVA, while ex vivo HSP expression was analyzed using two-way ANOVA (temperature $\times$ day) for repeated measurements. Violations of sphericity were handled by adjusting the F value using the Greenhouse-Geisser correction factor. When appropriate, Tukey’s HSD procedure was used to identify differences among means following significant main and/or interaction effects. Relationships between HSP responses and the core temperature AUC were examined using Pearson’s correlation analysis. All data are presented as means $\pm$ SD. Statistical significance was accepted at $P < 0.05$.

RESULTS

Physiological Adaptations to HA

Classical physiological adaptations to the 10-day HA protocol were observed (Table 1). Because of the inability of two subjects to exercise on day 10, physiological comparisons among days 1, 5, and 10 were made for six volunteers only. The 40-min exercise time period was selected because this was the minimum time to exhaustion on day 1 for any volunteer. Heart rate and core body temperature were significantly lower following 40 min of heat and exercise exposure on days 5 and 10 of the HA protocol compared with day 1. Sweat rate was greater on day 10 of the HA protocol compared with day 1 (1.09 ± 0.21 vs. 1.30 ± 0.2) as was exercise time to exhaustion (79.3 ± 21.1 vs. 95.5 ± 11).

Baseline Expression of HSPs Following HA

The baseline expression of HSPs 72 and 90 in freshly isolated PBMCs obtained from volunteers prior to heat and exercise exposure on days 1 and 10 appears in Fig. 1, A and B. The day 10 values reflect levels present $\sim 22$ h after nine consecutive days of exercise-HA. Baseline expression of both HSP72 and HSP90 was significantly increased following HA. HSP72 expression was 17.7% greater on day 10 compared with day 1. Likewise, expression of HSP90 was 21.1% greater on day 10 compared with day 1. Levels of $\beta$-tubulin, a housekeeping protein that was used to control for cell number and gel loading were comparable in pre- and post-HA cells. The post-HA increase in HSP72 and HSP90 expression tended to be positively correlated ($P < 0.15$; $r^2 = 0.313$; Fig. 1C).

HSP72 and 90 Expression in Response to Ex Vivo Heat Shock

To determine whether completion of the HA program affected the ex vivo heat shock expression of HSPs, freshly isolated PBMCs collected on days 1 and 10 were exposed to 37°C, 39.5°C, and 41°C water baths for 6 h or to heat shock (43°C for 1 h followed by 5 h recovery at 37°C), and HSP72 and HSP90 protein levels were analyzed by Western blot analysis. For HSP72, the ex vivo exposure to heat shock stimulated a 3.3-fold increase on day 1, compared with only a 2.2-fold increase on day 10 ($P < 0.05$; Fig. 2A). For HSP90, the ex vivo exposure to heat shock stimulated an 89% increase above baseline levels on day 1 ($P < 0.05$, Fig. 2B) but did increase above baseline levels on day 10.

Relationship between HA and Ex Vivo HSP72 and HSP90 Responses

Pearson’s correlations were used to examine possible relationships between HA physiological adaptations and ATT cellular adaptations. Because a blunted core temperature elevation during exercise-heat stress is the benchmark measure of HA, $AUC > T_c$ 38.0°C was used as an index for the calculation of AUC. Like the physiological data in Table 1, AUC data are for $n = 6$ on day 10. Correlation analysis of the change in ex vivo inducibility of HSP90 between days 1 and 10 and the change in AUC $> T_c$ 38.0°C between days 1 and 10 indicated a significant correlation ($r^2 = 0.89$, $P < 0.05$; Fig. 3A), while a similar analysis of HSP72 showed no significant correlation ($r^2 = 0.21$, $P = 0.36$) (Fig. 3B). Analysis of the relationship between an interval change in AUC $> T_c$ 38.0°C between days 1 and 10 and the changes in ex vivo HSP90 and HSP72 inducibility between days 1 and 10 revealed a similar trend ($r^2 = 0.45$, $P = 0.07$ for HSP90; $r^2 = 0.46$, $P = 0.065$ for HSP72; Fig. 3, C and D). These results suggest that volunteers demonstrating the greatest physiological HA (minimal elevation in core temperature during exercise) tend to exhibit the greatest blunting (least inducibility) of ex vivo HSP induction in response to heat shock.

DISCUSSION

This study determined the effect of heat acclimation on both baseline and ex vivo heat shock responses in humans and examined whether these changes were related to physiological adaptations. An exercise-HA protocol was employed that was similar to that employed by occupational and military workers exposed to heat. Only two recent studies have previously examined human HSP responses to heat acclimation (22, 38), and the present study expanded upon those findings. The following new observations were made: 1) exercise-HA resulted in increased baseline expression of HSP72 and HSP90, 2) ex vivo inducibility to heat shock of HSP72 was blunted after HA, and 3) volunteers demonstrating the greatest physiological HA tended to exhibit the greatest blunting of ex vivo HSP induction in response to heat shock.

HA is a reversible systemic response stimulated by prolonged exposure to elevated body temperatures and character-
ized by adaptive physiological changes that increase the capacity of an organism to function in the face of elevated temperatures (26, 28). Animal models of HA demonstrate altered gene expression profiles in critical tissues (13, 31), including genes encoding elements of the heat shock response. Activation of such genes by HA also confers protection against ischemia-reperfusion injury in the rat heart (13). Compared with HA, the heat shock response is a more primitive, evolutionarily conserved cellular response. It is rapidly activated by exposure to thermal or other stress and is characterized by a stereotypical gene expression profile (9). Studies in ectothermic and endothermic animals have demonstrated an overlap between HA stimulated by continuous exposure to hyperthermia and expression of genes that comprise the heat shock response (2, 6, 10, 13, 20, 21, 37). We have extended these studies by showing a similar overlap between HA and the heat shock response in humans who are repeatedly exposed to exercise/environmental hyperthermia.

We studied eight volunteers who completed a heat acclimation protocol traditionally used for occupational and military workers. The experiment was performed in the winter and spring months using volunteers without previous HA exposure to ensure a common preacclimation baseline physiological response. In addition, hydration and time-of-day were carefully controlled. As expected, on the basis of previous studies of HA in humans (28, 30), these volunteers exhibited a reduction in core temperature and heart rate and increased sweating following repeated exposure to exercise-heat stress. The 10-day HA involved exercise-heat exposure of 100 min in duration with core temperature elevations up to 39.6°C. These procedures are consistent with scientific recommendations to induce HA (26). It is generally agreed that most of human physiological adaptation to HA occurs during the first 4 or 5 days (28, 30), which was corroborated herein (Table 1) and generally completed by 10 days. Exposure to these conditions has been shown to elicit changes in HSP expression in animal models. In passively warmed mice, a 3-h exposure to core temperatures similar to those reached in our human subjects activates expression of HSPs (15). In a rat model of HA, Maloyan et al. (21) showed that continuous 30-day exposure to passive mild hyperthermia increases baseline HSP72 levels in heart muscle but blunts additional HSP72 expression in response to a classic heat shock exposure (43°C for 2 h). Marine snails (37), intertidal mussels (2), and fish (20) each exhibit a similar pattern of increased HSP72 and HSP90 expression following chronic exposure to elevated temperatures.
In the present study, we analyzed HSP expression in PBMCs from human volunteers undergoing HA. Circulating PBMCs are easily accessible, exposed to core and tissue-organ temperatures in vivo, and exhibit a stress response when collected from human volunteers exposed to exercise hyperthermia (27) or individuals suffering from exertional heat injury (36). We found that PBMCs isolated from subjects that were exposed to intermittent exercise-heat-mediated hyperthermia for 10 days exhibited a pattern of HSP expression that was similar to the cardiac HSP expression pattern in rats subjected to passive HA (21). In both studies, HA was associated with increased baseline expression and reduced inducibility of HSP72 and HSP90.

Our findings extend those of two recent publications examining baseline HSP72 levels in humans (22, 38). Marshall et al. (22) recently reported that humans exposed to an exercise-HA protocol failed to exhibit increased baseline HSP72 protein after 2 days of HA. That study only monitored HSP72 expression during the first 2 days of an exercise-HA protocol. However, it is generally agreed that adaptation to HA requires at least 5–10 days in normal subjects (28, 30). Yamada et al. (38) recently showed that baseline levels of HSP72 were increased following 10 days of HA. Our study measured baseline levels of both HSP72 and HSP90 and analyzed the induction of both genes in response to ex vivo heat shock exposure. We found that HA increased baseline levels of both HSP72 and HSP90. Furthermore, our ex vivo experiments demonstrated a blunted HSP72 induction in response to heat shock following HA and a possible change in HSP90 induction.

The coincidence of increased baseline expression and reduced ex vivo inducibility of HSP72 is consistent with previous studies demonstrating that HSP72 and HSP90 each repress HSF-1 activity (1, 7, 12, 16). The actions of HSP72 on HSF-1 are mediated through multiple pathways, including direct binding to HSF-1 (32) and dephosphorylation of HSF-1 through the actions of a serine-threonine phosphatase (7). The effects of HSP90 are mediated, in part, by sequestering HSF-1 in large multichaperone complexes (12, 40). Because denatured proteins generated during stress release inactive HSF-1 from multichaperone complexes by competing for HSP binding (32), higher baseline HSP levels would increase the threshold for HSF-1 activation. This mechanism is thought to underlie the increased thermal threshold for induction of the heat shock response that follows continuous exposure to ≥10°C elevations in body temperature in eurythermal ectothermic goby fish, snails, and mussels (2, 20, 37).

To the best of our knowledge, this was the first study to use correlation analysis to explore the relationships between HA physiological adaptations and cellular adaptations. In conducting this analysis, AUC > Tc 38.0°C was used as an index for physiological adaptation, and the percentage change in HSP72 and HSP90 expression in response to heat shock was used as an indicator of cellular adaptations. We found that correlations between the percentage change in AUC > Tc 38.0°C and ex vivo HSP90 inducibility between days 1 and 10 showed a significant correlation (P < 0.05) and a similar analysis of HSP90 and HSP72 inducibility and percentage change in AUC > Tc 38.0°C between days 1 and 5 showed a strong trend toward correlation. It is likely that some of our findings failed to reach statistical significance because of the limited sample size and also individual variation during HA. For example, in the correlations presented in Fig. 3B, one volunteer increased...
endurance time and AUC between days 1 and 10, whereas all of the others reduced AUC during the same time period. Taken together, our findings suggest that individuals with the greatest physiological adaptation to the exercise-HA protocol may, in fact, be least likely to generate a cellular heat shock response, although future studies with larger populations will be required to confirm this novel finding.

Perspectives and Significance

The present study is among the first to describe the complementary adaptations of human exercise-HA and ATT. Previous studies found that baseline HSP72 levels did not change after 2 days (22) and increased following 10 days (38) of exercise-HA. The present study extends upon those findings by examining baseline HSP90 (which unlike HSP72 is constitutively expressed) and examining HSP72 and HSP90 induction in response to ex vivo heat shock exposure. The present study found that 1) exercise-HA increased baseline levels of HSP72 and HSP90, 2) inducibility of HSP72 to ex vivo heat shock was bluntet after HA, and 3) volunteers demonstrating the greatest physiological HA tended to exhibit the greatest blunting of ex vivo HSP induction in response to heat shock. These data demonstrate that physiological adaptations in humans undergoing HA are accompanied by both increases in baseline levels and changes in regulation of cytoprotective HSPs. The observations gleaned from this human model are consistent with the conclusion that the cellular protective adaptations and physiological strain reductions are complementary in exercise-HA.

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