Effect of exercise with and without a thermal clamp on the plasma heat shock protein 72 response

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Whitham M, Laing SJ, Jackson A, Maassen N, Walsh NP. Effect of exercise with and without a thermal clamp on the plasma heat shock protein 72 response. J Appl Physiol 103: 1251–1256, 2007.—The contribution of heat and exercise related stress to the release of heat shock protein 72 (HSP72) is currently unknown. The purpose of the present study was to determine the combined and independent effects of heat and exercise on the extracellular (e)HSP72 response. Eleven moderately trained male volunteers [means ± SD: age 21 ± 4 yr; body mass 75.7 ± 7.7 kg; maximal oxygen uptake (Vo2max) 57.8 ± 3.3 ml·kg⁻¹·min⁻¹] completed four 2-h, heat-manipulated, water-immersion trials. Trials were exercise-induced heat (EIH; rectal temperature change +2.2°C), clamped exercise (CEx; 0°C), passive heating (PHT; +2.3°C), and control (Con; 0°C). Exercise trials (EIH and CEx) comprised deep-water running at 58.5 ± 2.4 and 59.1 ± 1.7% Vo2max. eHSP72 and catecholamine concentrations were determined by ELISA and HPLC, respectively, pre- and postimmersion. All trials induced an eHSP72 response (P < 0.05) with postimmersion values significantly greater on EIH compared with other trials (6.0 ± 3.4; CEx 3.8 ± 2.6; PHT 2.7 ± 2.1; Con 2.2 ± 1.9 ng/ml). Exercising with a thermal clamp blunted the eHSP72 response, but postimmersion values were also greater than Con. PHT induced a large catecholamine response, but postimmersion eHSP72 values did not reach significance vs. Con. Given that exercising with a thermal clamp evoked a significant increase in plasma eHSP72 concentration, exercise-related stressors other than heat appeared influential in stimulating HSP72 release. Moreover, the catecholamine data from PHT suggest neither epinephrine nor norepinephrine was solely responsible for eHSP72 release.

HEAT SHOCK PROTEIN 72 (HSP72) is the highly stress-inducible isoform of the heat shock protein 70 family. As such, HSP72 has been detected in the circulation of humans in response to exercise (12, 26, 46) and trauma (35). While commonly considered an intracellular chaperone with roles in protein homeostasis, the appearance of HSP72 in the extracellular environment has generated interest in the possible systemic roles of this protein. For example, extracellular (e)HSP72 has been hypothesized to play a number of roles in modulating the immune system, such as the stimulation of proinflammatory cytokines (1), activation of the complement cascade (36) and activation of CD40+ antigen-presenting cells (47). Furthermore, in vitro experiments suggest that exogenous HSP72 can bind to cells, penetrate the plasma membrane and protect cells from cytotoxicity (19, 33). Indeed, correlative data exist that suggest that extracellular HSP72 concentration is associated with improved survival in severe trauma patients (35). Moreover, resting eHSP72 appears to decrease with repeated heat stress, perhaps as a consequence of increased uptake by cells requiring improved stress tolerance (28). While extracellular HSP72 appears to play an important role in the response to stress, the mechanisms involved in the release of HSP72 into the circulation remain to be elucidated.

The term “heat shock protein” is perhaps misleading, because it is apparent that the intracellular concentration of HSP72 can be increased following nonthermal stressors such as glycogen depletion (11), high acidity (48), Ca2+-increasing agents (8), and a number of stress hormones such as corticosterone (43) and epinephrine or norepinephrine (29, 45). Furthermore, data from rats suggest that while the combined stressors of 60 min of treadmill running and whole body hyperthermia (41°C) induced the largest intracellular HSP72 response, exercising in a cool environment, with no measurable increase in core temperature, also induced a significant increase in skeletal muscle and cardiac HSP72 (40). Therefore, by measuring the HSP72 response to exercise during a thermal clamp, the authors where able to assert that in this animal model, factors other than heat stress contribute to the intracellular HSP72 response to exercise. Conversely, in repeatedly (3 × 30 min/day) exercising rats in thermally clamped conditions, Hamilton et al. (20) did not observe an increased myocardial HSP72 content compared with rats exercising at room temperature. The effect of heat and exercise may therefore be dependent on the tissue in which HSP72 is measured and the frequency or duration of the exercise (16, 30).

If the extracellular concentration of HSP72 were to simply reflect a spillover of intracellular concentrations, then one might expect to see similar trends in extracellular HSP72 that are seen in the intracellular protein concentrations in response to exercise. Indeed, one mechanism by which eHSP72 might increase in the circulation is via leakage from cellular necrosis (2). However, experiments demonstrating eHSP72 responses to exercise have used protocols not thought to cause significant cell damage (10, 32), and the induction of eHSP72 in response to psychological (rather than physical) stress in animals (15) suggests there is an alternative means of HSP72 trafficking. In vitro data from a number of laboratories suggest that the release of HSP72 from monocytes (23, 25) and epithelial cells (4) is via a nonclassical secretory pathway. Furthermore, through the use of α- and β-adrenergic blockade in rats, Johnson et al. (24) were able to provide convincing data to support the involvement of catecholamines in the eHSP72 response to tail shock. While the involvement of catecholamines in the stimulated release of eHSP72 has been...

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supported in a human exercise model (49), the role of hyperthermia in the specific release of eHSP72 during exercise is unclear.

The aim of the present research was first to determine the combined and independent effects of exercise and hyperthermia on the eHSP72 response in humans. We hypothesized that exercise inducing whole body hyperthermia would result in the largest eHSP72 response, whereas exercise alone and hyperthermia alone would also induce significant but smaller HSP72 responses. Because exercise with and without a thermal clamp induces different stress hormone responses (38), the second aim was to further examine the role of stress hormones in the eHSP72 response to stress.

MATERIALS AND METHODS

Subjects. Eleven healthy, moderately trained men volunteered to participate following local ethics approval and written informed consent. Subject characteristics were as follows (means ± SD): age 21 ± 4 yr; body mass 75.7 ± 7.7 kg; maximal oxygen uptake (V\textsubscript{O\textsubscript{2max}}) 57.8 ± 3.3 ml·kg\textsuperscript{-1}·min\textsuperscript{-1}.

Preliminary testing and experimental design. Before experimental trials, all subjects carried out a standard continuous incremental treadmill exercise test to volitional exhaustion to determine individual V\textsubscript{O\textsubscript{2max}}. Expired gases were analyzed continuously using an online gas analysis system (metalyser 3B, Cortex, Leipzig, Germany) with the V\textsubscript{O\textsubscript{2max}} values derived thereby used to determine the work rate of subsequent exercise trials. At this initial visit, subjects were also familiarized with deep-water running exercise, completing a 30-min exercise bout immersed in water to the shoulder level and supported by a harness and ropes to aid buoyancy. Subjects were then asked to return to the laboratory a further four times to carry out the experimental immersion trials in a randomized order. Each trial was separated by 7 days.

Experimental trials. During all trials, chamber water was manipulated to the desired temperature and monitored continuously using temperature thermistors (Edale, Cambridge, UK) at various positions in the chamber. All trials were 2 h in duration. To determine the effects of hyperthermia and exercise on the outcome variables, subjects carried out the exercise-induced heat trial (EIH; water temperature 35.3 ± 0.2°C) during which rectal temperature was intended to significantly increase. To investigate the effect of hyperthermia alone, the passive heating trial (PHT; 38.5 ± 0.2°C) involved seated immersion in hot water during which rectal temperature was also intended to significantly increase. To determine the effects of exercise alone (i.e., a thermally clamped exercise bout), subjects carried out the clamped exercise trial (CEx; 23.5 ± 0.9°C). Finally, to control for time of day effects and the influence of immersion alone, subjects carried out the control trial (Con; 35.3 ± 0.2°C). Before all trials, subjects were asked to refrain from carrying out vigorous physical activity and consuming alcohol and caffeine for the preceding 24 h. To standardize nutritional status, subjects were asked to record and replicate dietary intake the day before each trial. At 0800 of the morning of each trial, subjects were provided with a standardized breakfast (816 kcal; carbohydrate 78%, fat 14%, and protein 8%) and consumed only water before beginning each trial at 1200. Before immersion, each subject was asked to empty their bladder and bowels before nude body mass was determined (model 705, Seca, Hamburg, Germany). To prevent dehydration, urine osmolality was determined using freezing-point depression osmometry (Advanced Instruments, Needham Heights, MA) using the threshold for hydration of 700 mosmol/kgH\textsubscript{2}O (39). Subjects were then seated for 15 min before a resting blood sample was taken by venipuncture of an antecubital vein. On commencement of each and every trial, subjects were immersed to the shoulder level suspended by a harness and ropes to aid buoyancy. The exercise trials (EIH and CEx) comprised deep water running (37) at ~60% V\textsubscript{O\textsubscript{2max}}. Oxygen consumption during these bouts was monitored via Douglas bag collections every 10 min (Harvard Apparatus, Edenbridge, UK) and measured using a dry-gas meter (Harvard Apparatus) and combined paramagnetic oxygen and infrared carbon dioxide analyzer (1420B, Servomex, Crowborough, UK). On immediate completion of all trials, subjects were removed from the immersion chamber, and another venous blood sample was taken in a seated position. Nude body mass was assessed as described above to determine body mass losses corrected for ad libitum water consumption throughout all trials. All subjects remained fasted for a further 60 min before the final venous blood sample was taken.

Measures. Throughout all trials, heart rate was measured by telemetry (Polar Electro Oy, Kempele, Finland) and subjects were asked to rate their perceived exertion (RPE) (3) and thermal comfort (22). Rectal temperature was monitored using a thermistor probe (model 4000A, Yellow Springs Instruments, Dayton, OH) inserted 10 cm beyond the anal sphincter. Expired gas, heart rate, RPE, thermal comfort, and rectal temperature were recorded every 10 min.

Analytical methods. Blood samples were collected into EDTA (1.6 mg EDTA/ml blood)- and heparin (1.5 IU heparin/ml blood)-coated tubes. Following centrifugation at 1,500 g for 10 min (4°C), resulting plasma samples were frozen and stored at −80°C for later analysis. Plasma epinephrine and norepinephrine were determined by high-pressure liquid chromatography (Clinreg complete kit for catecholamines, Recipe Chemicals, Munich, Germany) as previously described (5). Plasma cortisol was determined in duplicate in heparinized plasma by ELISA (DRG Diagnostics, Marlburg, Germany). EDTA plasma was analyzed for HSP72 in duplicate by a commercially available ELISA (Stressgen, Victoria, Canada). Intra-assay coefficient of variation was 3.1, 7.8, 11, and 3.2% for cortisol, epinephrine, norepinephrine, and HSP72, respectively. Hemoglobin and hematocrit were determined from whole blood treated with EDTA using an automated cell counter (Gen S, Beckman Coulter, Fullerton, CA). Using these data, plasma volume change was calculated as previously described (7), and all hormone and HSP72 data were corrected accordingly.

Statistics. Adequate sample size was calculated a priori using means and SDs of plasma HSP72 exercise responses reported previously (49). Using a freely available web-based power calculator (www.dssresearch.com/toolkit/sscale), a total of 11 subjects was deemed to provide adequate statistical power (α = 0.01, β = 0.9). Data were examined using trial (4) × time (3) fully repeated-measures ANOVA and eta squared effect size (η\textsuperscript{2}) for homogeneity and sphericity using the Greenhouse-Geisser method, where appropriate. Significant interactions were further examined by post hoc Tukey’s honestly significant difference test or Bonferroni pairwise comparisons depending on the value of epsilon (42). Statistical significance was accepted at P < 0.05, and data are presented as means ± SE unless otherwise stated.

RESULTS

In the two exercise trials, mean exercise intensity was 58.5 ± 2.4 and 59.1 ± 1.7% V\textsubscript{O\textsubscript{2max}} in EIH and CEx, respectively. There was a significant time × trial interaction for rectal temperature (P < 0.001, η\textsuperscript{2} = 0.87). As expected, follow-up tests revealed that (after 20 min) EIH and PHT induced significantly greater rectal temperatures than CEx and Con (Fig. 1). Because there were no significant increases in rectal temperature in CEx, the protocol was successful in clamping core temperature. Furthermore, there were no significant differences in rectal temperature between EIH and PHT at any time point (P < 0.05). As anticipated, thermal sensation reflected these trends in core temperature (Fig. 2). There was a significant increase in heart rate in all trials except Con (Fig. 2). As expected, heart rates recorded during exercise trials were
significantly greater than seated immersion trials and in line with established literature: heat stress induced a greater heart rate response than non-heat-stress trials (EIH > CEx; PHT > Con). Heat stress also significantly increased ratings of perceived exertion in EIH and, unexpectedly in PHT (Fig. 2). The increase in RPE in PHT likely reflects an increase in perceived dyspnea during this seated trial. There were no significant differences in percentage of body mass loss between EIH, CEx, and PHT. Additionally, there were no differences in plasma volume change observed between trials (P = 0.071, \(\eta^2 = 0.41\)).

Stress hormone responses. It is well established that both exercise (38) and heat stress (31) induce a stress hormone response. Consistent with this, there was a significant time × trial interaction for epinephrine (P < 0.001, \(\eta^2 = 0.41\)) with no intertrial differences observed at preexercise or 1 h postexercise (Table 1). Post-trial values were significantly increased from preimmersion in EIH, PHT, and CEx. These epinephrine responses were significantly greater than Con in EIH and PHT, whereas the difference between CEx and Con approached significance. There was a significant time × trial interaction (P < 0.001, \(\eta^2 = 0.54\)) for norepinephrine, with post hoc tests revealing significant increases from baseline in all trials (Table 1). EIH and PHT caused a significantly greater increase in noradrenaline than Con in EIH and PHT, whereas the difference between CEx and Con approached significance. Whole body hyperthermia, therefore, induced the largest increases in circulating catecholamine concentrations whether induced by exercise (EIH) or passive heating (PHT). As expected, there were also significant effects of trial and time on plasma cortisol concentration (P < 0.001, \(\eta^2 = 0.68\) Fig. 3). Once again, hyperthermia was associated with the largest increases from baseline in EIH and PHT, which remained significantly elevated 1 h post immersion. There was no significant increase in cortisol associated with exercising in a cool environment (CEx; \(P = 0.14\)), and there was a significant drop in cortisol observed in Con (\(P = 0.004, \eta^2 = 0.43\)). The decrease in cortisol from baseline in this control condition likely reflects the commonly observed circadian rhythm of this hormone (50).

Extracellular HSP72 responses. The eHSP72 data exhibited a significant trial × time interaction (P < 0.001, \(\eta^2 = 0.55\); Fig. 4), with post hoc tests revealing a significant increase in eHSP72 in all trials (including Con) that remained elevated 1 h postimmersion in EIH and CEx. As expected, and in line with our hypothesis, the combined stress of exercise and hyperthermia resulted in the largest increase in eHSP72 in EIH. Indeed, eHSP72 concentrations in EIH were significantly higher than all other trials. The independent effects of passive heating (PHT) and exercise alone (CEx) also induced significantly increased eHSP72 in circulation, with CEx eHSP72 concentrations significantly higher than Con. Although mean postimmersion concentrations in PHT were higher than Con, these differences did not reach significance. A repeated-measures
**Table 1. Catecholamine responses to EIH, PHT CEX, and Con immersion trials**

<table>
<thead>
<tr>
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<th>Preimmersion</th>
<th>Postimmersion</th>
<th>1 h Postimmersion</th>
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<tr>
<td><strong>Epinephrine, pmol/l</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>EIH</td>
<td>194±100</td>
<td>640±212±1†</td>
<td>242±109</td>
</tr>
<tr>
<td>PHT</td>
<td>256±112</td>
<td>696±485±1†</td>
<td>213±147</td>
</tr>
<tr>
<td>CEX</td>
<td>211±123</td>
<td>490±289*</td>
<td>241±104</td>
</tr>
<tr>
<td>Con</td>
<td>200±60</td>
<td>224±112</td>
<td>218±75</td>
</tr>
<tr>
<td><strong>Norepinephrine, pmol/l</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EIH</td>
<td>1,054±380</td>
<td>3,114±1,097*†</td>
<td>1,256±358</td>
</tr>
<tr>
<td>PHT</td>
<td>786±355</td>
<td>2,666±1,198*†</td>
<td>923±333</td>
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<tr>
<td>CEX</td>
<td>1,027±376</td>
<td>1,984±573*</td>
<td>1,417±496*†‡</td>
</tr>
<tr>
<td>Con</td>
<td>935±387</td>
<td>1,314±581*</td>
<td>926±451</td>
</tr>
</tbody>
</table>

Values are means ± SD for 9 subjects. EIH, exercise-induced heat; CEx, clamped exercise; PHT, passive heating; Con, control (thermoneutral). *Significant difference from preimmersion (P < 0.05). †Significant difference from Con, P < 0.05. ‡Significant difference from CEx, P < 0.05. §Significant difference from PHT, P < 0.05.

ANOVA on resting eHSP72 concentration revealed no main effect of time (P = 0.68), suggesting that there were no latent effects of previous trials on the eHSP72 response. In all, these data suggest that both heat and exercise factors are involved in the release of eHSP72 into the circulation.

**DISCUSSION**

The present research aimed to determine the combined and independent effects of hyperthermia and exercise on the eHSP72 response in humans. There were significant eHSP72 responses to exercise in a thermoneutral environment (EIH), passive heating (PHT), exercise with no increase in core temperature (CEx), and immersion alone (Con). As expected, and in line with data from animal skeletal muscle HSP72 (40), exercise and whole body hyperthermia combined to induce the largest eHSP72 response. The finding of a significant increase in eHSP72 following exercise with a thermal clamp supports the notion that exercise factors other than heat can stimulate the release of HSP72. Interestingly, the effect of passive heating did not induce a large eHSP72 response, despite rectal temperatures reaching similar values to those in the thermoneutral exercise condition (Fig. 1). Furthermore, the difference in postimmersion values between PHT and Con did not reach significance. Indeed, Marshall et al. (28) have also reported that 2 h of passive heating at 38°C and 60% relative humidity had no effect on eHSP72 concentrations. Heat per se, therefore, appeared to have less influence on the release of HSP72 than factors associated with exercise.

The exact stimuli of HSP72 release are currently unclear. Data reported in both animal and human models support the role of catecholamines in the stimulated release of HSP72 (24, 49). Through the use of adrenergic blockade, Johnson et al. (24) were able to assert that HSP72 release during tail shock in rats is mediated by a mechanism involving α1-adrenoceptors. Furthermore, using the adrenergic stimulating properties of caffeine, Whitham et al. (49) were able to show significantly greater eHSP72 responses to exercise with caffeine vs. exercise without. Although not implying cause and effect, the present data can also provide some support for the role of catecholamines in the stimulated release of eHSP72. In particular, there were significant norepinephrine responses in all trials that were associated with increases in eHSP72. The observed increase in eHSP72 in the control trial was unexpected and could reflect an effect of time of day or psychological stress associated with confinement in the immersion chamber. Indeed, data in an animal model support the view that eHSP72 can be released following nonphysical stressors (15). Current work is underway in our laboratory to determine whether there are time of day effects for eHSP72. That postimmersion norepinephrine concentrations were also elevated in the control condition provides support for the role of this hormone in the stimulated release of HSP72. However, as indicated, eHSP72 responses to passive heating were not substantial and indeed, did not reach significance when compared with Con. Because catecholamine responses to passive heating were generally large, these data suggest that neither epinephrine nor norepinephrine were solely responsible for the stimulation of eHSP72, and other...
factors were likely to have contributed. Similarly cortisol responses were not associated with alterations in eHSP72 concentrations, which supports previous data in predatory and tail shock-stressed animals (15, 24) and exercising humans (49) that cortisol is not primarily involved in the stimulation of HSP72 release.

Despite the apparent ubiquity of intracellular HSP72, the origins of eHSP72 during exercise are currently unclear. Unfortunately the present study is unable to further add to the literature in this regard. However, this is important, because the specific releasing mechanism appears to some degree to depend on the cell from which HSP72 is released. For example, while Broquet et al. (4) provided support for the involvement of lipid rafts in the specific release of HSP72 from epithelial cells, Lancaster and Febbraio (25) demonstrated increased exosomal HSP72 content following heat shock of peripheral blood mononuclear cells. Because the rate of exocytosis of these vesicles appears calcium dependent (21) and α-adrenergic receptors are thought to mediate their actions through alteration of intracellular calcium (17), adrenergic stimulation of exosomal release may well be a mechanism by which various cells release HSP72. To date, it has not been possible to pinpoint the exact tissues or cells that add to circulating HSP72 concentration during exercise. However, through elaborate cannulation across various tissue beds, experiments carried out by Febbraio et al. (10, 11, 26) suggested the brain, hepatosplanchnic tissues, but not intact skeletal muscle at least partly contributed to the circulating HSP72 concentration during prolonged cycling. Interestingly, by supplementing with exogenous glucose and reducing metabolic stress on the liver, the authors were able to blunt the hepatosplanchnic release of HSP72 during moderate exercise (9). Therefore, heightened metabolic stress and subsequent HSP72 release from the liver may partly explain the larger eHSP72 responses to exercise trials observed in the present study. Tissues and cells may have different reserves of HSP72 (14), perhaps dependent on the nature of the stress incurred. This may subsequently be reflected in circulating eHSP72 concentrations and in this regard, further research to determine the potential origin of eHSP72 is warranted.

While the present research has suggested a lessened effect of passive heating on the eHSP72 response, it should be acknowledged that the rectal temperatures recorded at the end of the trial (38.55°C) were lower than have been presented in previous literature. Furthermore, although Marshall et al. (28) concluded that 2 h of passive heating in a 38°C environment had no effect on eHSP72 concentration, no core temperature data were included. Indeed, when clamping core temperature in exercising rats, studies have shown little or no cardiac HSP72 expression (20, 44). In addition, passive heating to tympanic temperatures of 39°C increased HSP72 expression in the leukocytes of humans (34). While the intracellular HSP72 concentrations in these tissues and cells might not necessarily reflect circulating concentrations, it should be considered that the core temperatures reached in the present study were less effective in inducing a heat shock protein response. The finding of Ruell et al. (6) of higher concentrations of eHSP72 in athletes suffering exertional heat illness (41°C) vs. controls (39.8°C) adds weight to this assertion. As the name suggests, heat has a strong influence on heat shock protein transcription. Although the mechanisms involved are unconfirmed, data suggest the regulator of heat shock protein synthesis, heat shock transcription factor 1, is directly activated by temperature (18, 41, 51). So, although the present study intimates that the release of HSP72 when passively heated is relatively small, the effect might be more pronounced at core temperatures approaching 40°C. Nevertheless, the current data are still applicable to previous literature demonstrating an increase in eHSP72 in response to prolonged exercise during which core temperatures did not reach levels associated with heat illness (10, 12, 13, 46).

Despite this limitation, the present investigation’s finding of a significant eHSP72 response to exercise with a thermal clamp provides support for the notion that factors other than heat stimulate the release of HSP72. Cellular upregulation of HSP72 occurs following many stresses, including oxidative stress, pH disturbances, hypoxia, and glucose deprivation (27), all of which could occur during the disturbed homeostasis caused by exercise. In particular reference to eHSP72, Fischer et al. (13) were able to demonstrate reduced serum HSP72 in exercising participants supplementing with antioxidants. Therefore, reactive oxygen species may well be one of the factors stimulating HSP72 release during exercise.

To conclude, the present investigation suggests that because exercising with a thermal clamp did not abolish the eHSP72 response, factors other than heat are capable of stimulating the release of HSP72 during exercise. The present data also suggest that neither epinephrine nor norepinephrine was solely responsible for the increase in eHSP72; hence it is likely other stimulators are additionally involved.

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