Effect of caffeine supplementation on the extracellular heat shock protein 72 response to exercise

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Whitham, Martin, Gary J. Walker, and Nicolette C. Bishop. Effect of caffeine supplementation on the extracellular heat shock protein 72 response to exercise. J Appl Physiol 101: 1222–1227, 2006.—The stimulus for the release of 72-kDa heat shock protein (HSP72) during exercise in humans is currently unclear. Recent evidence in an animal model is suggestive of an involvement of catecholamines. The present study, therefore, investigated the effect of caffeine supplementation, a known stimulator of sympathetic activity, on the extracellular (e)HSP72 response to prolonged exercise. Ten healthy male endurance-trained cyclists were recruited (age: 21 ± 1 yr, maximum O2 uptake 61.1 ± 1.7 ml·kg⁻¹·min⁻¹, mean ± SE). Each subject was randomly assigned to ingest either 6 mg/kg body mass of caffeine (Caff) or placebo (Pla) 60 min before one of two 90-min bouts of cycling at 74 ± 1% maximum O2 uptake. Trials were performed at least 7 days apart in a counterbalanced design. Venous blood samples were collected by venepuncture at pre-treatment, preexercise, postexercise, and 1 h postexercise. Serum caffeine uptake was determined by ELISA. Serum caffeine and plasma catecholamines were determined using a spectrophotometric assay and high-performance liquid chromatography, respectively. Plasma HSP72 and cortisol were determined by ELISA. Serum caffeine concentrations were significantly increased throughout Caff, while no increases were detected in Pla. Caffeine supplementation and exercise was associated with a greater eHSP72 response than exercise alone (postexercise Caff 8.6 ± 1.3 ng/ml; Pla 5.9 ± 0.9 ng/ml). This greater eHSP72 response was associated with a greater epinephrine response to exercise in Caff. There was a significant increase in norepinephrine and cortisol, with no intertrial differences. The present data suggest that, in humans, catecholamines may be an important mediator of the exercise-induced increase in eHSP72 concentration.

catecholamines; stress; immune

cascade (27), and activation of CD40⁺ antigen presenting cells (32). Taken together, data such as these support the hypothesis that heat shock proteins act as “danger” signals to alert the immune system during times of stress (23).

While research has provided suggestions as to the role of eHSP72, little is known as to the stress-induced release signal and mechanism. Human exercise studies that have demonstrated an elevation in eHSP72 have yet to delineate the aspects of exercise that induce a release of HSP72 into the general circulation. Since studies have incorporated exercise without eccentric or potentially cell necrosing elements (5, 19), authors have proposed a specific exocytosis pathway by which cells are able to release HSP72 (2, 15, 18). In an animal model, Fleshner and colleagues (8, 16) have conducted a series of elegant experiments outlining possible stress hormone roles in the stimulation of HSP72 release. The authors were able to show an attenuated, but not eliminated, eHSP72 response in adrenalectomized rats following cat exposure stress (8). This suggested a role of adrenal hormones in the release of HSP72 into the circulation. Furthermore, pharmacological blockade of adrenergic-mediated pathways suggests a key involvement of catecholamines in the stimulation of eHSP72 during stress, particularly in humans.

Caffeine supplementation during exercise causes a robust stimulation of the sympathetic nervous system (13). Accordingly, during exercise with a caffeine supplement, plasma epinephrine concentrations are characteristically higher than during exercise with a placebo. While similar increases have been observed for norepinephrine, results are inconsistent (20). Since caffeine supplementation induces greater epinephrine and possibly norepinephrine responses to exercise, the use of a caffeine supplementation model provides an attractive in vivo method to assess the role of catecholamines in the stimulation of HSP72 release.

The present study, therefore, examined the effect of caffeine supplementation on the eHSP72 response to exercise. It was
hypothesized that there would be greater catecholamine and eHSP72 concentrations following exercise with caffeine supplementation than following a placebo exercise trial.

METHODS

Subjects. Ten endurance-trained men volunteered to participate in the study following local ethics approval and written, informed consent. Subject characteristics were as follows: (mean ± 5E) age 21 ± 1 yr; body mass 71.9 ± 2.3 kg; maximum O2 uptake (V\text{O}_2\text{max}) 61.1 ± 1.7 ml·kg\(^{-1}\)·min\(^{-1}\); peak power output 321 ± 8 W; habitual caffeine intake 183 ± 43 mg/day.

Preliminary Testing and Experimental Design

Approximately 1 wk before experimental testing, each subject carried out a continuous incremental exercise test on an electromagnetically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands) to determine V\text{O}_2\text{max}. Heart rate was monitored throughout using short-range radiotelemetry (Polar Beat, Polar Electro Oy, Kempele, Finland). Expired gas samples were collected in Douglas bags during the 3rd min of every 3 min, 35-W increment stages, and they were measured using a paramagnetic oxygen analyzer (Servomex 1420B; Crowborough, UK), infrared carbon dioxide analyzer (Servomex 1415B), and dry-gas meter (Harvard Apparatus, Edenbridge, UK) to determine minute ventilation, O2 uptake, and CO2 production. The work rate equivalent to 70% V\text{O}_2\text{max} was then calculated for subsequent exercise trials. Within 2 days of this preliminary visit, all subjects were familiarized with the exercise trial protocol, cycling for 90 min at 70% V\text{O}_2\text{max}. Expired gas samples were collected as described above after the first 10 min and at 20-min intervals to ensure subjects were exercising at the correct intensity. The remaining two visits to the laboratory consisted of the experimental trials during which subjects were assigned to either the caffeine (Caff) or placebo (Pla) in a crossover random block design. Each trial was separated by a period of 1 wk.

Experimental Trials

To control for previous habitual caffeine use, all subjects were instructed to abstain from caffeine containing products for 60 h preceding each trial. This period of time was for washout, since half-lives reported for moderate doses of caffeine are between 2.5 and 4.5 h (11). Subjects were also required to avoid vigorous physical activity and alcohol for the 24 h before commencing experimental trials. Diet sheets were completed before the first trial, and subjects were asked to replicate foods consumed before the second experimental trial. On both occasions, subjects reported to the laboratory (21.1 ± 0.8°C and 52 ± 5% relative humidity) at 0800 following an overnight fast. Subjects were asked to empty the bladder, after which body mass was recorded. After 10 min of sitting quietly, an initial resting blood sample was taken by venepuncture of the antecubital vein. Subjects were, at this point, given either a caffeine supplement of 6 mg/kg body mass, dissolved in 3 ml/kg body mass of artificially sweetened (aspartame) grapefruit juice, or placebo consisting of the same volume of grapefruit-flavored water. After 1 h of resting in the laboratory, another blood sample was taken. Immediately after this, subjects began the exercise trial, cycling on an electromagnetically braked cycle ergometer (Lode Excalibur Sport) at 70% V\text{O}_2\text{max} (222 ± 33 W) for 90 min. Expired gas samples were taken as previously described to monitor exercise intensity, and heart rate was recorded at 15-min intervals. To standardize fluid intake, subjects consumed 2 ml/kg body mass of plain water, also at 15-min intervals. On completion of the exercise trial, a further blood sample was taken before body mass was recorded. A final postexercise blood sample was taken 1 h after completion of each experimental trial. No food was permitted during this 1-h postexercise period, but subjects were provided with 3 ml/kg body wt of plain water.

Plasma Hormones, Serum Caffeine, and HSP72.

Blood samples were collected into four separate tubes (Sarstedt, Leicester, UK), one uncoated serum tube, one containing K\text{3},EDTA (1.6 mg EDTA/ml blood), and two containing lithium heparin (1.5 IU heparin/ml blood). A heparin tube was spun at 1500 g for 10 min in a refrigerated centrifuge (4°C) within 5 min of sampling. Two milliliters of the plasma derived were immediately added to chilled tubes containing 200 ml of preservative (pH 6.5) containing EGTA (100 mM) and glutathione (100 mM). Tubes were then mixed and frozen at −80°C for later determination of norepinephrine and epinephrine concentrations by HPLC, as described previously (10). Remaining heparinized plasma was stored at −80°C for later determination of plasma cortisol by ELISA (DRG Diagnostics, DRG Instruments). Whole blood taken into the EDTA monovette was used to assess hematoцит and hemoglobin by a Sysmex SE9000 cell counter (Sysmex, Milton Keynes, UK). These data allowed calculation of plasma volume, as described previously (3). Remaining EDTA blood was spun at 1500 g in a refrigerated centrifuge for 10 min, and the resulting plasma was frozen at −80°C for later determination of plasma HSP72. Plasma HSP72 concentrations were measured by ELISA (Stressgen, Victoria, Canada). Whole blood in the serum tube was left to clot on ice for 1 h, and then it was spun at 1,500 g for 10 min at 4°C. Serum was transferred to Eppendorf tubes and frozen at −80°C. Serum caffeine concentrations were determined using a commercially available assay (Emit Caffeine Assay, Dade-Behring, Milton Keynes, UK) on an automatic photometric analyzer (COBAS Miras Plus, Roche Diagnostic Systems, Rotkreuz, Switzerland). The intra-assay coefficient of variation was 1.9% and 6.6% for norepinephrine and epinephrine, respectively. In addition, the intra-assay coefficient of variation was 2.4% for cortisol and 6.3% for HSP72.

Statistics

Sample size calculation. Adequate sample size was determined using means and standard deviations of HSP72 exercise responses reported previously (7) and a freely available web-based power calculator (http://calculators.stat.ucla.edu/powercalc/). This gave a required n of 9.1 (α = 0.001, β = 0.9). A total of 10 subjects was, therefore, deemed appropriate to provide adequate statistical power.

Data analysis. Data were examined using a two-factor repeated-measures ANOVA with post hoc Bonferroni-adjusted t tests or Tukey’s honestly significant difference where appropriate. Assumptions of homogeneity and sphericity in the data were checked, and adjustments in the degrees of freedom for the ANOVA were made using the Greenhouse Geisser correction, where appropriate. Statistical significance was accepted at P < 0.05. Data are presented as means ± SE, unless otherwise stated.

RESULTS

Mean exercise intensity was 74% on both trials (74.1 ± 1.0 on Caff and 74.4 ± 0.7 on Pla). Similarly, heart rates during each trial were comparable (mean of all recordings: Caff 160 ± 3 beats/min, Pla 158 ± 3 beats/min). After exercise, changes in body mass (corrected for fluid intake) were similar in both trials (−1.62 ± 0.13 and −1.67 ± 0.16 kg on Caff and Pla, respectively). Furthermore, there was no significant time × trial interaction (P = 0.36) for changes in plasma volume relative to pretreatment. Serum caffeine concentrations were, as expected, significantly elevated in Caff preexercise, postexercise, and 1 h postexercise (Fig. 1). In contrast, there was no effect of Pla on serum caffeine concentrations.
Effect of Caffeine Supplementation on Exercise Catecholamine and Cortisol Responses

Supplementation with caffeine has been consistently shown to increase plasma epinephrine concentrations during exercise (20). Consistent with this, a significant group × time interaction was observed for epinephrine \( (P = 0.018, \text{ Fig. 2A}) \). Follow-up tests showed that, while an elevated postexercise epinephrine concentration was evident in both Pla \( (P = 0.007) \) and Caff \( (P = 0.005) \), there were significantly greater postexercise epinephrine concentrations in Caff \( (P < 0.05) \). Although no between-trial differences were observed, there was a trend toward a greater norepinephrine response in Caff \( (\text{group } \times \text{time interaction}, P = 0.09, \text{ Fig. 2B}) \). There was, however, a main effect of time \( (P < 0.001) \). In addition, exercise induced an increase in cortisol in both trials \( (\text{main effect of time}, P = 0.002, \text{ Fig. 3}) \), but no between-trial differences were observed \( (\text{group } \times \text{trial interaction}, P = 0.11) \). These data suggest that, while catecholamine and cortisol concentrations were elevated as a result of exercise \( (\text{regardless of trial}) \), caffeine supplementation induced a greater epinephrine response to exercise stress.

Effect of Caffeine Supplementation on Exercise HSP72 Responses

The HSP72 data exhibited a significant time × trial interaction \( (P = 0.02, \text{ Fig. 4}) \). Post hoc analysis confirmed that both Caff \( (P < 0.001) \) and Pla \( (P < 0.001) \) trials induced an increase in plasma HSP72. However, plasma HSP72 concentrations were significantly higher in Caff \( (P < 0.05) \) immediately postexercise and 1 h after cessation of the exercise bout. These data suggest that caffeine supplementation augments the eHSP72 response to prolonged exercise stress.

DISCUSSION

The main finding of the present study was that the combined stimulus of caffeine and exercise induced greater postexercise eHSP72 concentrations than exercise alone. The significant increase shown in plasma HSP72 in both Caff and Pla exercise trials is in support of previous studies suggesting that, in nonclinical disease states, the release of HSP72 into the circulation following acute exercise stress may occur as part of the normal stress response \( (5, 7, 31) \). The present data are consistent with the hypothesis that catecholamines are involved in the stimulation of eHSP72 during prolonged exercise in humans.

There are two possible means by which HSP72 may appear in the extracellular environment. Cellular damage may cause a passive release of intracellular proteins into the circulation. However, release of HSP72 during moderate-intensity cycling through cell necrosis is unlikely \( (5, 26) \), and the induction of eHSP72 in response to psychological \( (\text{rather than physical}) \) stress \( (8) \) suggests there is an alternative means of HSP72 trafficking. Indeed, early in vitro experiments in rat embryo cells provided evidence that cells exposed to heat shock are able to specifically release proteins, including HSP72 \( (14) \). Through the experimental use of agents that inhibit endoplasmic reticulum/Golgi protein transport in monocyte and epithelial cell types, a handful of laboratories have suggested that this specific release is via a nonclassical secretory pathway \( (2, 15, 16) \).
One explored possibility is the release of proteins in exosomes, small membrane vesicles that have been shown to contain heat shock proteins (21). Indeed, Lancaster and Febbraio (18) were able to show increased HSP70 within isolated exosomes from monocytes exposed to heat shock. Interestingly, the rate of cellular exosome release is affected by intracellular calcium levels (14), and an alteration in calcium signaling is one pathway by which α1-adrenergic receptors mediate their actions (12). These findings provide a biological rationale for the hypothesis that catecholamines mediate HSP72 release during exercise.

In the present study, supplementation with caffeine was associated with a greater eHSP72 response to exercise. A direct effect of caffeine on HSP72 release is plausible, since caffeine is known to increase intracellular calcium concentration via an action on ryanodine receptors in the brain (25). However, this is thought to occur only at millimolar concentrations (11), in excess of those attained in the present study (Fig. 5). Since caffeine consumption stimulates sympathetic activation, it is possible that higher eHSP72 concentrations observed in the Caff trial were due to the increased concentration of catecholamines. Of note, greater epinephrine concentrations observed in Caff were indeed associated with greater eHSP72 responses to exercise. Furthermore, the interaction between time and trial for norepinephrine approached significance (P < 0.09). This finding is in support of previous data suggesting a prominent role of α1-adrenergic receptors in the active release of HSP72 following tail shock stress in rats (16). Interestingly, while the present data are suggestive of a significant role of epinephrine in eHSP72 release in humans, adrenalectomy in rats (which depletes the majority of secreted epinephrine) had no effect on the eHSP72 response to tail shock stress (16). As norepinephrine has a higher affinity for α1-adrenergic receptors, Fleshner and colleagues (9, 16) asserted that it is norepinephrine, and not epinephrine, that mediates eHSP72. The present study was not able to support this assertion in a human exercise model. There was, however, a trend for a greater norepinephrine response in Caff, which, with greater statistical power, may confirm a greater influence of norepinephrine in

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**Fig. 3.** Cortisol responses to 90-min cycling at 70% \(V_{O2\text{max}}\), with and without caffeine supplementation. Values are means ± SE. †Main effect of time (P < 0.05).

**Fig. 4.** Plasma heat shock protein 72 (HSP72) responses to 90-min cycling at 70% \(V_{O2\text{max}}\), with and without caffeine supplementation. Values are means ± SE. *Significant difference from pre (P < 0.05); #significant between-trial differences (P < 0.05).

**Fig. 5.** Individual epinephrine (A) and extracellular HSP72 (B) responses to prolonged cycling with caffeine supplementation. Each line represents a subject.
the release of HSP72 in humans. Although the present study does not establish a causal link between epinephrine and HSP72 release, the data provide evidence that epinephrine has a prominent role in the stimulation of eHSP72 in humans. This finding is in contrast to that found in animals (16), which could be due to species differences. Indeed, there are differences in \( \alpha_1 \)-adrenergic receptor subtype expression that may limit extrapolation of findings from nonhuman models (12). Furthermore, adrenalectomy in rats exposed to predator stress caused an attenuated but not eliminated eHSP72 response (8), suggesting the type of stressor might alter the contribution of epinephrine and norepinephrine to HSP72 release.

The use of caffeine and exercise is a known model to effectively increase epinephrine concentrations (13). Because caffeine is a known stimulator of epinephrine secretion, and we have put forward the hypothesis that catecholamines stimulate the release of HSP72 in humans, one might expect caffeine alone to stimulate HSP72 release. However, despite significant increases in serum caffeine due to supplementation (Fig. 1), increases in eHSP72 and catecholamines before exercise were not evident (Figs. 2 and 4). The effects of caffeine alone on catecholamine release are generally small compared with exercise (13), and both catecholamine secretion and eHSP72 responses show individual variability. Therefore, while differences in epinephrine and eHSP72 did not reach significance when subjected to a minor stimulus such as caffeine alone, the implementation of the greater stimulus of exercise and caffeine allowed significant differences to be observed. Concentrations of epinephrine and eHSP72 rose in parallel, and these trends provide support for the link between epinephrine and HSP72 release.

Cortisol is characteristically released in response to stress. Accordingly, it is possible HSP72 release during exercise may be stimulated by glucocorticoids. Indeed, exercise, regardless of trial, was associated with an increase in cortisol, and an increase in plasma HSP72 was observed in both Pla and Caff trials. However, the greater eHSP72 response in Caff could not be explained by differences in cortisol, since no intertrial differences were observed. This is in support of data in rats suggesting that adrenalectomy, which completely removes circulating cortisone, has no effect on eHSP72 responses to tail shock (16).

A limitation of the present study is that it was not able to determine the source of the eHSP72. This is significant, as the release mechanism of eHSP72 may be dependent on the cell type. For example, Broquet et al. (2) presented evidence for the involvement of lipid rafts in the release of HSP72 from epithelial cells, and the possible involvement of exosomes in hematopoietic cells has already been discussed. Heat, glucose deprivation, oxidative stress, and changes in pH join a multi-type of stressors capable of inducing intracellular HSP72 accumulation, and, as these stresses are likely relevant in the exercising muscle, it seems logical that muscle may contribute to the eHSP72 response to exercise. However, by determining the arterial-venous balance of an exercising leg, Febbraio et al. (5) were able to assert that the intact muscle is impermeable to HSP72. Subsequent research by the same authors has suggested possible contributions of the hepatosplanchnic tissues (5) and brain (19) to the extracellular concentration of HSP72 during exercise. In view of the present and previous (17) data suggesting a role for catecholamines in HSP72 release, and the apparently ubiquity of HSP72 (24), it is possible that HSP72 is released from cells in tissues innervated by sympathetic neurons, as suggested by Johnson and Fleshner (17).

In summary, the aim of the present study was to determine the effect of caffeine supplementation on the eHSP72 response to prolonged exercise. The combined stimulus of caffeine and exercise induced greater eHSP72 concentrations than exercise alone, and the presented data support the hypothesis proposed in an animal model that catecholamines are at least partly responsible for the stimulated release of HSP72 in humans. Further research is required to confirm the contribution of catecholamine stimulated adrenergic receptors in the eHSP72 response to exercise. In particular, pharmacological adrenergic blockade may provide further insights into the involvement of sympathetic activation in HSP72 release during exercise in humans.

REFERENCES