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ORIGINAL ARTICLE

Daily quadratic trend in basal monocyte expressed HSP72 in healthy human subjects

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Abstract The inducible human stress protein heat shock protein 72 (HSP72) performs vital roles within the body at rest and during periods of stress. Recently it was shown over a 24 h period that basal HSP72 followed a diurnal variation. However, these results and previous literature demonstrate noticeable inter-subject variation in basal HSP72 expression. The notion of intra/inter-day variation in basal HSP72 expression has not been explored in detail. Basal monocyte expressed HSP72 was determined every 3 h, over a 9 h period in 12 healthy male subjects (20.2 ± 1.9 years, 178.7 ± 5.6 cm, 75.1 ± 6.0 kg) within a temperature controlled laboratory. A significant quadratic trend was observed for time ($F = 26.0$, $P = 0.001$, partial $\eta^2 = 0.74$), where HSP72 decreased between 0800 and 1100 hours ($P < 0.001$) and then increased between 1100 and 1400 hours ($P = 0.015$). The main effect for day ($F = 2.6$, $P = 0.14$) and the day × time interaction effect ($F = 3.9$, $P = 0.08$) were not significant. There was no correlation between serum and monocyte expressed HSP72, with no significant effect for time ($F = 2.0$, $P = 0.21$) in serum HSP72 expression. The results support findings by others that basal monocyte expressed HSP72 follows a diurnal variation which incorporates a quadratic trend, which is not compromised by any significant daily variation and that serum HSP72 expression has no endogenous circadian rhythm. The significant quadratic trend in basal monocyte HSP72 expression shown here highlights the need to tightly control variables, such as timing of sample collection, as it is known basal values influence the magnitude of HSP72 expression post-stressor/intervention.

Keywords HSP70 · HSP72 · Daily variation · Methods

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Introduction

Heat shock protein 72 (HSP72) is a highly conserved and inducible member of the heat shock protein 70 (HSP70) family, which performs vital roles during episodes of stress (Kregel 2002; Katschinski 2004) and under homeostatic conditions (Kiang and Tsokos 1998). Notable roles include rescuing cells from programmed cell death (Garrido et al. 2001), protein folding (Hartl 1996; Fink 1999) and acting as a molecular chaperone (Bukau and Horwich 1998). Factors, such as hypoxia (Patel et al. 1995; Weinstein et al. 2004), exercise (Locke and Noble 1995), oxidative stress (Kukreja et al. 1994), ischaemia (Richard et al. 1996), hyperbaria (Yogaratnam et al. 2007), hypothermia (Yang et al. 1996) and hyperthermia (Ritossa 1962; Cairo et al. 1985) have been shown to induce HSP72 expression. Extracellular HSP72 (ECHSP72) is known to initiate a pro-inflammatory immune response (Pockley 2003; Hickman-Miller and Hildebrand 2004; Asea 2005), whilst intracellular HSP72 (ICHSP72) was demonstrated to activate an endogenous anti-inflammatory pathway (Ianaro et al. 2001).

The important role of HSP72 in various biological systems and cascades has ensured a significant increase in the scientific research in the area, particularly exercise

performance (Locke and Noble 1995; Madden et al. 2008a), role in heat acclimation strategies (Marshall et al. 2007; Yamada et al. 2007; McClung et al. 2008; Sandstrom et al. 2008) and as a biological tool for cancer treatments and vaccines (Brahimi-Horn et al. 2007; Sherman and Multhoff 2007; Bolhassani and Rafati 2008). Inter-subject variation in basal HSP72 values has been demonstrated within the muscle (Khassaf et al. 2001; Morton et al. 2006) and/or peripheral blood mononuclear cells (PBMC) in human studies (Sandstrom et al. 2008, 2009). Both tissues have shown inter-subject variation in expression at numerous time points with basal/resting values demonstrating the most notable inter-subject variation, within muscle (Khassaf et al. 2001; Morton et al. 2006) and PBMCs (Sandstrom et al. 2009). Under normal resting conditions HSP72 is present within the circulation (Pockley et al. 1998) and recently within our laboratories basal monocyte expressed HSP72 was shown to follow a diurnal variation in resting healthy subjects over a 24 h period (Sandstrom et al. 2009). It is plausible that despite this quadratic trend in diurnal variation, inter-day or intra-day expression could be present and potentially underline the quadratic trend illustrated previously (Sandstrom et al. 2009), disclosure of such fluctuations may strengthen/weaken those novel findings and contribute to future work within the area. It is important to note the extent of HSP72 response to exercise is correlated to its basal value (Gjovaag and Dahl 2006), therefore, when measuring HSP72 acutely or chronically daily fluctuations in basal values would affect the magnitude of the HSP72 response. Any such variation would be relevant to sporting performance, training schedules, heat acclimation strategies and the methodologies employed to measure HSP72 in vivo.

The aims of this study were therefore to identify any intra/inter-day variation in basal monocyte expressed HSP72, and to rationalise the impact of such variation for human in vivo studies. Additionally, the confirmation and repeatability of the previously disclosed quadratic trend in HSP72 expression was examined.

Methods

Subjects

Twelve healthy recreationally active non-smoking male subjects [mean ± SD 20.2 ± 1.9 years, 178.7 ± 5.6 cm, 75.1 ± 6.0 kg, physical activity (PA/h week^{-1}) 5.2 ± 1.9] volunteered to participate in the study. Caffeine (Lu et al. 2008) and glutamine (Rhoads et al. 1996; Lindemann et al. 2001; Singleton et al. 2004) consumption were barred from all meals for 7 days prior to each of the three trial days with abstinence from prolonged thermal exposures (baths, saunas, steam rooms, tanning devices, etc.), vigorous PA and alcohol also requested, compliance was monitored via a pre-study questionnaire. Subjects that had resided or visited a temperate climate (>30°C) prior to the study and smokers were barred from the study as it has been shown that smoking may illicit HSP72 expression as a consequence of oxidative stress (Anbarasi et al. 2006). Subject's were collected and delivered to the lab at identical times by a mini-bus on each morning of the three study days. The protocol was approved by the Departmental ethics committee and all subjects signed informed consent following the principles outlined in the Declaration of Helsinki prior to any experimental procedures taking place.

Blood sampling and experimental protocol

Subjects consumed identical meals for each of the three experimental days, which were provided at 0830 and 1200. Subjects remained within the temperature controlled laboratory (average WBGT 20.9 ± 0.3°C, humidity 48 ± 3%) throughout the course of the study. Study days were separated by 3 days. On each of the study day's subjects spent their time under resting conditions within the temperature controlled laboratory. All blood samples were preceded by 10 min in the supine position with samples drawn from the antecubital vein into potassium EDTA Vacuette tubes (Vacuette®, Greiner BIO-one, UK). Fortes and Whitham (2009) showed that repeated venepuncture demonstrated no stress induced changes in ECHSP72 in comparison to repeated cannulation blood draws. Samples were processed immediately for measurements of monocyte expressed HSP72. It is important to note that EDTA coated tubes were utilised as these have been shown to yield higher HSP72 values than other anti-coagulation tubes, which for low basal HSP72 values is an important factor (Whitham and Fortes 2006). Blood samples were taken at 0800, 1100 and 1400. Methods, procedures and restrictions replicated those used previously to gain serial data for HSP72 and other physiological markers of homeostasis (Madden et al. 2008b; Sandstrom et al. 2009; Vince et al. 2009) with sample frequency being increased to 3 h increments compared to the 4 h collections used previously. This increase in sampling frequency was designed to achieve greater insight into the expression kinetics of HSP72 over the targeted (9 h) period.

Monocyte HSP72 assay

The choice and justification of monocyte expressed HSP72 has been discussed in detail previously (Sandstrom et al. 2009). Whole blood (200 μL) from EDTA tubes was incubated with erythrolyse red blood cell lysing buffer (AbD Serotec, UK) for 20 min, washed with 2 mL

phosphate buffering solution (PBS) and spun at 400×*g* for 3 min. The resulting supernatant was discarded and cells then fixed (Leucoperm, AbD Serotec, UK) and incubated for a further 20 min. Post-incubation samples were washed, spun and the supernatant was discarded as previously stated. Cells were then permeablised (AbD Serotec, UK) and a negative control (FITC, AbD Serotec, UK) or anti-HSP72 antibody (SPA-810, Assay Designs, USA) was added and then incubated for 30 min in the dark. After incubation, samples were washed, spun and the supernatant was discarded, with cells re-suspended in 150 µL of PBS. Samples were then analysed on a BDFACSCalibur (BD Biosciences) by flow cytometry with monocytes gated by forward/side scatter properties and further discriminated by CD14 expression. Mean fluorescence intensity (MFI) was then calculated using CELLQuest software (BD Biosciences) with a total of 50,000 cells counted. Plasma HSP72 was analysed in duplicate using a commercially available high-sensitivity sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturers protocol (Assay Designs EKS-715, MI, USA). EDTA plasma was obtained via centrifugation of the EDTA blood tube (10 min, 1,500×*g*), stored at −80°C and analysed at a later date for plasma HSP72.

Statistical analysis

Statistical analyses were performed using PASW statistics 17 (SPSS Inc., Chicago, IL, USA). Polynomial contrasts were used to identify linear or quadratic trends in basal HSP72 expression at the same three times (0800, 1100, 1400 hours) on three different days. The HSP72 expression at 1100 and 1400 hours was expressed as a percentage change from 0800 hours for each day (Morton et al. 2007). Polynomial contrasts were also used to identify linear or quadratic trends in serum HSP72 at the same three times on a single day. In the event of a significant F statistic, Fisher's least significance test was used to locate significant paired differences. Two-sided statistical significance was accepted as $P < 0.05$.

Results

Figure 1 shows the mean ± SEM basal percentage change in monocyte HSP72 expression for each time and for each day. A significant quadratic trend was observed for time ($F = 26.0$, $P = 0.001$, partial $\eta^2 = 0.74$), where HSP72 decreased between 0800 and 1100 hours (mean difference = −17%, 95% CI = −24 and −10%, $P < 0.001$) and then increased between 1100 and 1400 hours (mean difference = 8%, 95% CI = 2 and 14%, $P = 0.015$). The main effect for day ($F = 2.6$, $P = 0.14$) and the day × time interaction effect ($F = 3.9$, $P = 0.08$) were not significant. Figure 2 shows the mean ± SEM serum HSP72 for each time point on day 2. No significant effect of time was observed ($F = 2.0$, $P = 0.21$). There was no correlation between serum and monocyte expressed HSP72.

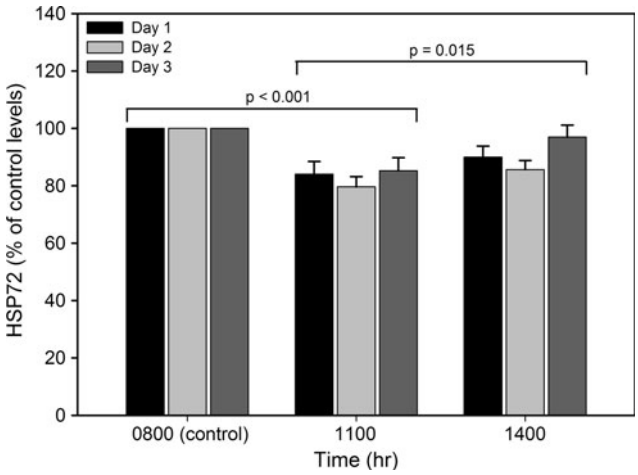

Fig. 1 Mean basal HSP72 expression at 0800, 1100 and 1400 hours on three separate days. The *error bars* represent the standard error of the mean. Data at 1100 and 1400 hours are represented as the percentage change from 0800 hours (control). The P values represent significant differences in HSP72 between times ($n = 12$)

Discussion

This study was conducted to ascertain whether an intra/inter-day variation existed in basal monocyte expressed HSP72 and found that there was no significant difference in expression between days. However, a significant quadratic trend in basal HSP72 values was seen within each experimental day. The significant quadratic trend for time (a decrease followed by an increase) is consistent for all days and nearly all subjects.

It is well documented that disease (Collins and Hightower 1982) and the classic stressor hyperthermia, increase HSP72 expression (Staib et al. 2007; Lovell et al. 2008). These factors, along with HSP72 increases in response to exercise (Locke and Noble 1995; Madden et al. 2008a; Sandstrom et al. 2008; Yamada et al. 2008; Morton et al. 2009) have been explored at length and in detail (Kregel 2002; Katschinski 2004). Recently, we showed that over a 24 h period the basal levels of monocyte expressed HSP72 followed a diurnal variation when analysed via flow cytometry, which was composed of two, 12 h components, which effectively mirrored each other (Sandstrom et al. 2009). Utilising the high sensitivity ELISA is the most popular method of measuring ECHSP72, however, basal values often fall at or below the 90 pg/mL sensitivity with

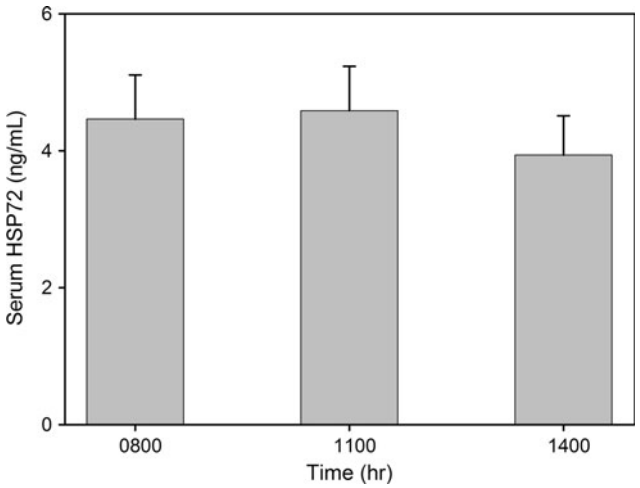

Fig. 2 Mean serum HSP72 at 0800, 1100 and 1400 hours on a single day. The *error bars* represent the standard error of the mean ($n = 12$)

inter and intra assay variation seen (Whitham and Fortes 2006), therefore protocols utilising ELISA potentially miss subtle basal variations in expression. This may account for the lack of a diurnal variation in basal HSP72 values shown by others (Fortes and Whitham 2009). Additionally, differences between commercially available and in-house ELISAs are often large. Analysis of HSP72 via ELISA is less time consuming compared to flow cytometry, however, its cost is greater (Yamada et al. 2008). Additionally, we observed (Fig. 2) ELISA measurements to be a relatively insensitive measure of basal HSP72 expression with large inter-subject variation, in comparison to flow cytometry. Western blotting has proved a popular analytical technique and has previously shown inter-subject variations in basal HSP72 values within muscle (Khassaf et al. 2001), potentially due to a lenient exclusion protocol (PA) and experimental restrictions pre, during and post-study. Recently, stronger research methods were utilised by Morton et al. (2006), with exclusion criteria, subject restrictions and specifically PA tightly controlled. Despite more rigorous control methods, this inter-subject variation in basal muscle HSP72 was still evident (Morton et al. 2006). However, due to western blotting being less sensitive than ELISA to changes in HSP72 (Yamada et al. 2008) its ability to detect subtle changes, i.e. daily variation in basal HSP72 expression is questionable. It is important to gain an accurate basal value as pre exercise values have been correlated to the magnitude of HSP72 response post-stressor (Maloyan et al. 1999; Gjovaag and Dahl 2006; McClung et al. 2008; Sandstrom et al. 2008).

Notably on three separate days, the quadratic trend demonstrated by Sandstrom et al. (2009), in this instance is shown to be consistent and repeatable, supporting the previously disclosed diurnal variation in basal monocyte expressed HSP72 (Sandstrom et al. 2009). The presence of such a quadratic trend impacts upon any study that measures HSP72 expression in human subjects, especially those which look to take serial measurement across different days, as differing basal values due to the quadratic trend need controlling for. It is particularly relevant for studies that explore HSP72 expression in response to a stressor (various interventions) for example exercise or hyperthermia, where the quadratic trend in basal HSP72 expression could ultimately affect the speed and magnitude of the SP response and consequently the duration and maximal expression of HSP72 seen. Elevated muscle HSP72 is present 7 days post-exercise (Morton et al. 2006; Paulsen et al. 2007; Tupling et al. 2007), therefore any PA 7 days prior to HSP72 sampling may illicit undue effects on basal values potentially producing erroneous values. Contrary to much of the literature some research (Shastry et al. 2002; Liu et al. 2004; Watkins et al. 2007) has demonstrated minimal or no increase in HSP72 in response to stress, whereas similar protocols have demonstrated a conflicting significant increase in stress protein expression post-stressor (Walsh et al. 2001; Thompson et al. 2002). These methodological flaws in establishing an accurate basal HSP72 value could account for previous research demonstrating no elevation in HSP72 expression in response to exercise (Salo et al. 1991; Puntschart et al. 1996). In this study, PA was tightly controlled for 7 days prior to study commencement and thus previous inter-subject variation in expression may be due to inadequate controls for physical exercise being utilised.

Every effort was made by the research team in an attempt to exclude all relevant factors which could theoretically induce a HSP72 response and hence impact basal HSP72 expression. Psychological stress has been shown to induce HSP72 expression (Hoekstra et al. 1996; Isosaki and Nakashima 1998), however, subjects reported no impending deadlines, personal issues or other psychological stressors in recruitment or throughout the study.

Basal expression is indicatively linked to the magnitude of the HSP72 response to a stressor (Maloyan et al. 1999; Gjovaag and Dahl 2006; McClung et al. 2008; Sandstrom et al. 2008), therefore the quadratic trend in basal monocyte HSP72 expression should be considered when designing studies. It requires careful postulation when protocols utilise separate days of testing where the stress response may differ to an identical stressor, on a separate day, due to the timing of the intervention with respect to the quadratic trend shown here. This quadratic trend in basal HSP72 expression could impact upon performance, whereby training or performance may be augmented or hindered dependant on the basal level of endogenous HSP72. This paradigm would be of particular concern during heat acclimation strategies or hyperthermic exercise performance/competition as high levels of endogenous HSP72

are implicitly linked to increased cellular survival (McMillan et al. 1998) and ultimately augmented hyperthermic athletic performance (Madden et al. 2008a; Sandstrom et al. 2008, 2009). As discussed previously changes in time zone could mean an athlete is competing when their HSP72 expression is at its lowest point [0500 (Sandstrom et al. 2009)] in their diurnal variation/quadratic trend of expression which has been shown to coincide with increased rates of perceived exertion and impaired thermoregulation versus the same exercise bout at other time points (Waterhouse et al. 2004, 2005). It could be hypothesised that hyperthermic exercise performance will be impaired when basal levels of HSP72 are low due to the quadratic trend in HSP72 expression repeatedly demonstrated here and previously on one occasion (Sandstrom et al. 2009). Whether the quadratic trend associated changes in HSP72 expression would manifest a significant impact upon exercise performance needs further elucidation. The notion of low basal levels of HSP72 as a marker of training status or acclimation status requires further investigation. It could potentially be used if an athlete was having regular basal HSP72 values taken longitudinally and an accurate non-trained/non-acclimated range of values could be ascertained.

In summary, the impact of the basal quadratic trend in monocyte expressed HSP72 requires careful consideration when designing human studies to explore HSP72 expression in vivo. The importance of gaining a true resting basal HSP72 value, with respect to HSP72 in response to a stressor, is paramount due the relationship between basal values and the induction of HSP72 post-stressor. Future work should look to examine the link between muscle and PBMC expressed HSP72 and whether the two demonstrate comparable or opposing quadratic trends in basal expression.

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