Amino Acids (2010) 38:1423–1428
DOI 10.1007/s00726-009-0354-4

ORIGINAL ARTICLE

In vitro heat shock of human monocytes results in a proportional increase of inducible Hsp70 expression according to the basal content

Rebecca V. Vince · Katherine Oliver · Adrian W. Midgley · Lars R. McNaughton · Leigh A. Madden

Received: 11 August 2009 / Accepted: 12 September 2009 / Published online: 25 September 2009

Abstract Heat shock proteins play an important role as molecular chaperones of the cell. Inducible heat shock protein 70 is rapidly synthesised in response to numerous stressors and monocytes are sensitive to changes in core temperature resulting in a circadian variation of Hsp70 expression. Monocytes were isolated via density centrifugation from nine healthy male volunteers at 5 am, 1 pm and 9 pm, representing the nadir (5 am), peak (9 pm) and intermediate (1 pm) of Hsp70 expression in the 24-h cycle. Analysis of freshly isolated monocytes for Hsp70 expression confirmed Hsp70 levels at the three selected time points. Monocytes were subjected to in vitro heat shock at 40°C (±0.1) for 90 min with a 90 min 37°C (±0.1) exposure acting as a control. A significant increase in Hsp70 was observed at 5 am ($p < 0.001$) and 1 pm ($p = 0.028$) at 40°C when compared to 37°C but not at 9 pm ($p = 0.19$). A significant increase was also observed from the basal levels of Hsp70, measured on freshly isolated monocytes and the levels detected after heat shock at 40°C at 5 am ($p < 0.001$) and 1 pm ($p = 0.001$), which was not observed at 9 pm ($p = 0.15$). Furthermore, a significant correlation was observed in the heat shock response at 40°C and that obtained at 37°C ($p < 0.001$). In conclusion, the heat shock response in monocytes is directly proportional to the amount of Hsp70 present in the cells and the stress response may be much higher at different times of the day.

Keywords Heat shock · Stress response · Hsp70 · Monocytes · Diurnal variation

R. V. Vince · A. W. Midgley · L. R. McNaughton
Department of Sport, Health and Exercise Science, The University of Hull, Hull HU6 7RX, UK

K. Oliver · L. A. Madden
Postgraduate Medical Institute, The University of Hull, Hull HU6 7RX, UK

L. A. Madden (✉)
Room 003, Hardy Building, University of Hull, Hull HU6 7RX, UK
e-mail: l.a.madden@hull.ac.uk

Introduction

Heat shock proteins (Hsp) are ubiquitous and perform an essential cellular function, the chaperoning of nascent proteins, preventing them from degradation (Bukau and Horwich 1998). Hsp are a family of stress proteins with molecular mass from 10 to 170 kDa (Snoeckx et al. 2001). The most highly inducible Hsp has a molecular mass of 72 kDa (Hsp70), belongs to the Hsp70 family of proteins and is well characterised (Madden et al. 2008a). Inducible Hsp70 can re-fold damaged proteins, inhibit protein aggregation and translocate proteins to different cellular compartments (Kregel 2002). Hsp70 expression is increased markedly in response to temporary heat shock and has shown to be cellularly protective against subsequent stresses such as heat (Locke and Noble 1995), hypoxia (Patel et al. 1995) and oxidative stress (Kukreja et al. 1994). Also, Hsp70 is capable of rescuing cells from programmed death by disrupting the apoptotic cascade (Garrido et al. 2001).

Various strategies aimed at increasing basal Hsp70 levels have been used to attempt to confer cellular protection in an exercise setting, such as acquisition of thermal tolerance (McClung et al. 2008) prior to prolonged bouts of competitive exercise in a hot environment (Sandstrom et al. 2008). Thus, using the basal levels of Hsp70 is thought to

be an acceptable outcome marker for preconditioning regimes (Madden et al. 2008a).

Many studies have demonstrated increased Hsp70 expression in vitro following heat shock; however, most groups exposed cells to extreme temperatures that could ultimately cause immediate death in humans (up to 45°C) (Oehler et al. 2001; Sonna et al. 2002; McClung et al. 2008). The amount of Hsp70 induction is temperature and time dependant (Lovell et al. 2007).

Previously we have observed a diurnal expression pattern of Hsp70 expression within monocytes and linked this to changes in core temperature (Sandstrom et al. 2009). Hsp70 was found to peak at both 9 am and 9 pm with a nadir at 5 am and low expression levels between 1 pm and 5 pm. Although recently no circadian variation was observed in serum Hsp70 (Fortes and Whitham 2009), the source of serum Hsp70 is unknown and monocytes have been shown to be the most sensitive cell type for Hsp70 expression analysis (Sandstrom et al. 2009; Bachelet et al. 1998).

A circadian rhythm has been demonstrated in core temperature and vascular tone (Refinetti 1998; Krauchi and Wirz-Justice 2001). Furthermore, we reported a circadian rhythm in endothelial microparticles within the circulation, which may be a marker of endothelial function (Madden et al. 2008b). These studies clearly demonstrate that performing exercise or indeed surgery at different times of the day may have implications in terms of cellular response.

The aim of this study was to characterise Hsp70 expression within monocytes in response to a non-lethal (40°C) in vitro heat shock and relate the magnitude of this response to the amount of Hsp70 present prior to the heat shock. To gain the broadest range of baseline Hsp70 levels in humans within a 24 h cycle, samples were taken when monocyte Hsp70 expression has been shown to be highest (9 pm), lowest (5 am) and low (1 pm).

Materials and methods

Subjects

Nine healthy non-smoking male volunteers (18–24 years) took part in this study. Departmental ethics was granted and written informed consent given. All subjects were treated in accordance with the Declaration of Helsinki.

Blood sampling

Blood was drawn by venipuncture into potassium EDTA tubes (Greiner, Stonehouse, UK). After mixing, 100 μL was taken for immediate Hsp70 analysis whilst the remaining blood was used for peripheral blood mononuclear cell (PBMC) isolation. All blood samples were taken on the same day.

Live inducible Hsp70 assay

Inducible Hsp70 was determined by flow cytometry immediately following the collection of blood as previously reported (Sandstrom et al. 2009) and detailed below. Furthermore, PBMC was isolated by density gradient centrifugation (Histopaque, Sigma, Poole, UK) according to the manufacturers' instructions and stored in liquid nitrogen until in vitro heat shock testing was carried out.

In vitro heat shock assay

Peripheral blood mononuclear cell was defrosted on ice, washed with PBS (20 mL) and resuspended in 1 mL RPMI1640 media (PAA, Yeovil, UK) supplemented with 10% (*v*/*v*) foetal calf serum. Viability was assessed by trypan blue exclusion. Each PBMC sample was then divided into two 500 μL aliquots and transferred in 2-mL round bottom polypropylene tubes (to allow for adequate gas exchange). One aliquot of each sample was placed in a heating block set at 37°C (±0.1°C) and the other in a heating block at 40°C (±0.1°C) for 90 min. Heat shock was chosen at 40°C as we have previously demonstrated maximal response at this temperature immediately following exposure (Lovell et al. 2007). Samples were then centrifuged (400×*g*, 3 min) and the PBMC pellet resuspended in fixation buffer (AbD Serotec, Oxford, UK) for 15 min at ambient temperature. PBS (3 mL) was added and PBMC was then pelleted by centrifugation (400×*g*, 3 min), resuspended in permeabilisation buffer (100 μL) then divided into two aliquots (50 μL), to which 5 μL of either isotype-matched negative control:FITC (AbD Serotec) or anti-Hsp70:FITC (Assay Designs, Ann Arbor, MI) was added to the same final concentration. Samples were incubated in the dark at ambient temperature for 30 min prior to washing with PBS (3 mL), centrifuged (400×*g*, 3 min) and finally the pellet was resuspended in 300 μL PBS prior to flow cytometry analysis.

Flow cytometry analysis

Samples were analysed on a BDFACSCalibur (Becton–Dickinson, Oxford, UK) running CELLQuest software and 20,000 events were counted. Monocytes were gated according to their scatter properties in relation to lymphocytes and Hsp70 expression determined by mean fluorescence of monocytes incubated with the anti-Hsp70 antibody relative to monocytes incubated with negative control antibody. The experimentation and subsequent flow

cytometry analysis were carried out by different authors in a blinded fashion.

Statistical analysis

All statistical analyses were performed using PASW statistics 17 (SPSS Inc., Chicago, IL). Quantile–quantile plots indicated that the normality assumption for Hsp70 expression was plausible. The effects of condition (Pre, 37°C, 40°C) and time (5 am, 1 pm and 9 pm) on Hsp70 expression were analysed using linear mixed models, as were the effects of condition and group (none, medium and high Hsp70 expression). Condition, group and time were modelled as fixed effects and subject as a random effect. Various plausible covariance structures were assumed and the one that minimised the Hurvich and Tsai's criterion (AICC) value was chosen as the main selection criterion for the final fitted model. Fisher's least significant difference test was used for post hoc pairwise comparisons where a significant F ratio was observed. Correlations between Hsp70 expression in vivo, in vitro at 37°C, and in vitro at 40°C were investigated using the Pearson correlation coefficient. Two-tailed statistical significance was accepted as $p < 0.05$.

Results

In vivo Hsp70 determined immediately after each blood draw is shown in Fig. 1. Data are shown as mean fluorescence obtained with the anti-Hsp70 relative to that gained with negative control. Therefore, a ratio of 1 indicates no detectable Hsp70 within monocytes, as mostly observed at 5 am. Compared to 5 am, no significant change in Hsp70 expression within monocytes was observed at 1 pm ($p = 0.087$); however, a significant increase was observed at 9 pm ($p < 0.001$), in accordance with our previous observations (Sandstrom et al. 2009).

The minimum observed PBMC viability prior to heat shock was 90%. The main effects of condition ($F = 35.3$, $p < 0.001$) and time ($F = 24.5$, $p < 0.001$), and the condition × time interaction ($F = 35.5$, $p < 0.001$) were all statistically significant.

After in vitro heat shock at 37°C, monocytes taken at 5 am ($p = 0.002$) and 1 pm ($p = 0.036$) exhibited up to a 1.5-fold increase in expression; however, monocytes taken at 9 pm showed a significant decrease in Hsp70 expression ($p < 0.001$; Fig. 1). In vitro heat shock at 40°C resulted in an over twofold increase in monocyte Hsp70 expression in samples taken at 5 am ($p < 0.001$), a 1.8-fold increase at 1 pm ($p = 0.001$) and no significant change at 9 pm ($p = 0.15$; Fig. 1).

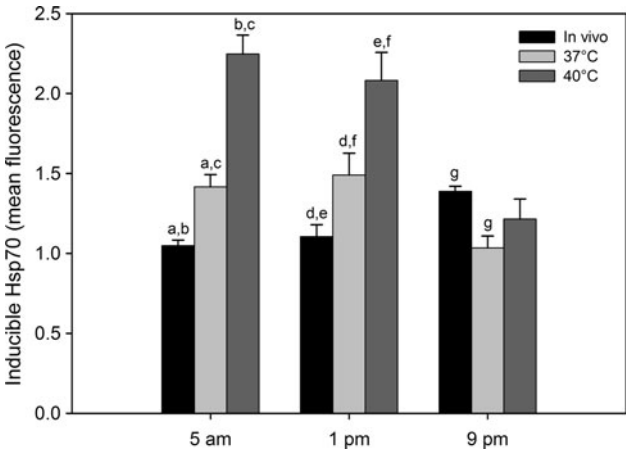

Fig. 1 *Bar chart* showing in vivo levels of Hsp70 expression (*black bars*) as well as relative increases in expression in response to incubation at 37°C (*light grey bars*) and heat shock at 40°C (*dark grey bars*) in monocytes isolated at 5 am, 1 pm and 9 pm. Data are presented as mean fluorescence intensity in relation to that obtained with a negative control antibody ± SEM ($n = 9$). Like *letters* above the *error bars* represent statistically significant differences between the means ($p < 0.05$)

Refinement of the samples into groups according to the expression of Hsp70 observed 'live' resulted in three groups. The first was samples with no detectable Hsp70 ($n = 11$) taken from the 5 am and 1 pm samples. The second group was designated low Hsp70 expression (with a mean increase over the negative control of 1.01–1.30, i.e. 1–30% increase), again made up from samples at 5 am and 1 pm ($n = 8$) and finally a third group of relatively high (ratio over 1.35) basal expression ($n = 8$). The main effects of condition ($F = 39.4$, $p < 0.001$) and group ($F = 16.4$, $p < 0.001$), and the condition × group interaction effect ($F = 17.8$, $p < 0.001$) were all statistically significant. The largest increase following in vitro heat shock was observed in monocytes with no detectable Hsp70 expression prior to shock (Fig. 2), where significant differences were observed between each condition ($p < 0.001$). The group with moderate Hsp70 expression prior to heat shock exhibited significant increases in expression in the 40°C condition ($p < 0.001$), but not at 37°C ($p = 0.38$). A small significant increase was observed in Hsp70 expression in subjects with a high basal monocyte Hsp70 content for the 37°C condition ($p = 0.01$), but not at 40°C ($p = 0.20$). Three samples from the study showed a reduction in Hsp70 expression following heat exposure at 40°C and all of these samples were from the 9 pm/high basal groups. All other samples ($n = 24$) showed an increase in Hsp70 after heat shock at 40°C.

The Hsp70 expression at 37°C was significantly positively correlated with that at 40°C (Fig. 3), whereas significant negative correlations were observed between Hsp70 expression in vivo and in vitro at 37°C ($r = -0.43$,

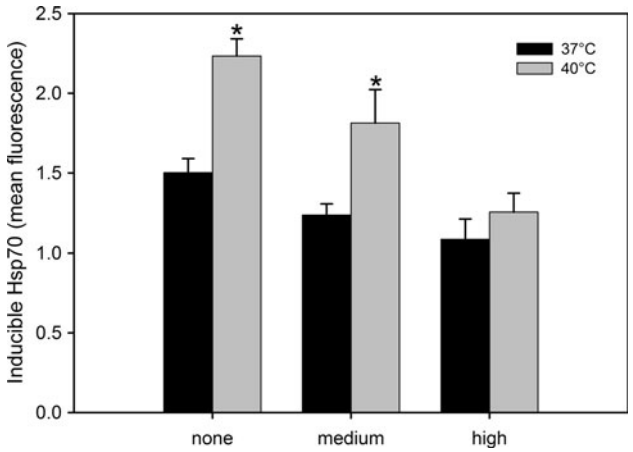

Fig. 2 *Bar chart* showing the relative increase in Hsp70 expression following incubation at 37°C (*black bars*) and heat shock at 40°C (*grey bars*) in monocytes. Data are presented according to basal levels, as determined at the time of isolation. *Significantly different from the 37°C value ($p < 0.05$)

$p = 0.026$), and between in vivo and in vitro expression at 40°C ($r = -0.67$, $p < 0.001$).

Discussion

Data presented in this study clearly show a demonstrable link between the quantity of Hsp70 induced via heat shock and the amount already present with monocytes. Also, a correlation exists between the amounts of Hsp70 synthesised at 40°C relative to 37°C, again based on the amount of Hsp70 already present. When monocytes have no detectable Hsp70, at the nadir of a diurnal variation (5 am), the induction in response to stress is significantly increased in comparison to that observed when more Hsp70 is present (1 and 9 pm). Furthermore, the amount of Hsp70 present

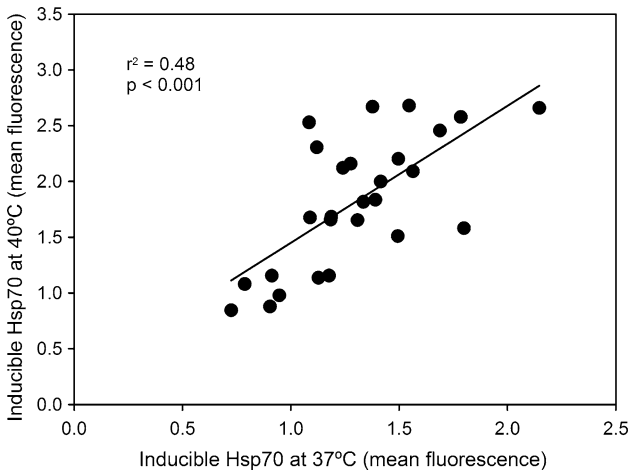

Fig. 3 Correlation between the heat shock response at 40°C in relation to Hsp70 expression following incubation at 37°C

within monocytes at 9 pm appears to confer substantial protection resulting in a minor increase in Hsp70 expression after a 40°C heat shock. Monocytes isolated at 5 am and to a lesser extent at 1 pm demonstrated increased Hsp70 expression even at 37°C. Based on our previous study (Sandstrom et al. 2009), this may be due to a raise in temperature compared to the environment of their isolation. Core temperature has been shown to be at its lowest value around 5 am (Waterhouse et al. 2005) and below 37°C (Sandstrom et al. 2009), therefore any induction of Hsp70 expression at this time could be due to a raise in core temperature in vivo (Sandstrom et al. 2009) and in this study in vitro compared to in vivo.

Further selection of samples, based on the Hsp70 levels as measured live resulted in samples allocated into three groups, those with no detectable Hsp70 and those with a low and a high amount. It is clear from the data obtained that the degree of response to heat stress is dependent upon the amount present prior to the stress, in agreement with previous studies (Maloyan et al. 1999; McClung et al. 2008). The findings reported here also clearly show that monocytes are highly sensitive to changes in temperature and the magnitude of the heat shock response is time-of-day dependent.

The implications of these findings would be primarily for those undertaking exercise but also for scheduling of surgery where Hsp70 has been shown to confer protection in, for example, cardiac surgery (Alex et al. 2005). Previously, Waterhouse et al. (2004) have shown that undertaking exercise at times of low core temperature and therefore low monocyte Hsp70 expression results in a significantly increased rate of perceived exhaustion in comparison to exercise at different times of the day and therefore of the circadian rhythm. This was accompanied by the most rapid rise in core temperature (Waterhouse et al. 2004) and presumably therefore increased expression of Hsp70.

Elite athletes crossing time zones should therefore allow enough time to adjust the natural circadian rhythm otherwise a high stress response may be seen. Although no evidence linking Hsp70 expression and exercise performance exists, due to difficulties in defining increases in performance and, in particular, interpreting any performance advantage in relation to Hsp70 expression, more likely a disruption to homeostasis occurs following exercise at times when the body is not prepared and heat shock proteins are a measure of the degree of this shift. Therefore, strategies aimed at increasing basal Hsp70 expression are most probably valid. The data also suggest that the stress response would be minimal when undertaking intense exercise, especially when a rise in core temperature is expected, when basal Hsp70 is at its highest, i.e. 9 am or 9 pm. Conversely, the stress response could also be

affected when undertaking mild exercise in harsh environments, e.g. extreme cold or in cold water (SCUBA or commercial diving), where core temperature may be expected to decrease.

McClung et al. (2008) reported a similar response based on a heat acclimation (HA) protocol and western blot analysis, where Hsp70 expression in PBMC after in vitro heat shock at 43°C was blunted in humans displaying the highest basal levels following HA. This extreme heat shock was outside the homoeothermic range and may not fully reflect PBMC response to an in vivo raise in core temperature. Classical heat stroke occurs with a core temperature of 41°C and above (Parsons 2003) and exercise regimes in our laboratory are ceased when a core temperature of 40°C is reached. Furthermore, Hsp70 has been suggested to play a role in prevention of heat stroke (Ruell et al. 2009). Small increases in Hsp70 expression have been previously reported in response to heat shock and high basal levels at the sampling times could explain these findings (Shastry et al. 2002; Liu et al. 2004; Watkins et al. 2007).

This study is the first to characterise the monocyte response to in vitro heat shock within a normal circadian rhythm in healthy humans, using a non-lethal heat stress. In conclusion, the data presented in this study corroborate previous findings and clearly defines a direct proportional response within the blood to heat shock and further adds to the view that monocytes are highly sensitive to heat changes and can be a model cell type for assessment of the stress response in vivo and in vitro.

Acknowledgments The authors wish to thank Mr Lee Taylor for logistical support, Dr's Ric Lovell, Jason Siegler and Marie Sandstrom for useful discussions and blood collection.

References

Alex J, Laden G, Cale ARJ, Bennett S, Flowers K, Madden L, Gardiner E, McCollum PT, Griffin SC (2005) Pretreatment with hyperbaric oxygen and its effect on neuropsychometric dysfunction and systemic inflammatory response after cardiopulmonary bypass: a prospective randomized double-blind trial. J Thorac Cardiovasc Surg 130:1623–1630

Bachelet M, Mariethoz E, Banzet N, Souil E, Pinot F, Polla CZ, Durand P, Bouchaert I, Polla BS (1998) Flow cytometry is a rapid and reliable method for evaluating heat shock protein 70 expression in human monocytes. Cell Stress Chaperones 3:168–176

Bukau B, Horwich AL (1998) The Hsp70 and Hsp60 chaperone machines. Cell 92:351–366

Fortes MB, Whitham M (2009) No endogenous circadian rhythm in resting plasma Hsp72 concentration in humans. Cell Stress Chaperones 14:273–280

Garrido C, Gurbuxani S, Ravagnan L, Kroemer G (2001) Heat shock proteins: endogenous modulators of apoptotic cell death. Biochem Biophys Res Commun 286:433–442

Krauchi K, Wirz-Justice A (2001) Circadian clues to sleep onset mechanisms. Neuropsychopharmacology 25:S92–S96

Kregel KC (2002) Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. J Appl Physiol 92:2177–2186

Kukreja RC, Kontos MC, Loesser KE, Batra SK, Qian YZ, Gbur CJ, Naseem SA, Jesse RL, Hess ML (1994) Oxidant stress increases heat-shock protein-70 messenger-rna in isolated-perfused rat-heart. Am J Physiol Heart Circ Physiol 267:H2213–H2219

Liu YF, Lormes W, Wang LL, Reissnecker S, Steinacker JM (2004) Different skeletal muscle HSP70 responses to high-intensity strength training and low-intensity endurance training. Eur J Appl Physiol 91:330–335

Locke M, Noble EG (1995) Stress proteins—the exercise response. Can J Appl Physiol 20:155–167

Lovell R, Madden L, Carroll S, McNaughton L (2007) The time-profile of the PBMC HSP70 response to in vitro heat shock appears temperature-dependent. Amino Acids 33:137–144

Madden LA, Sandstrom ME, Lovell RJ, McNaughton L (2008a) Inducible heat shock protein 70 and its role in preconditioning and exercise. Amino Acids 34:511–516

Madden LA, Vince RV, Sandstrom ME, Taylor L, McNaughton L, Laden G (2008b) Microparticle-associated vascular adhesion molecule-I and tissue factor follow a circadian rhythm in healthy human subjects. Thromb Haemostasis 99:909–915

Maloyan A, Palmon A, Horowitz M (1999) Heat acclimation increases the basal HSP72 level and alters its production dynamics during heat stress. Am J Physiol Regul Integr Comp Physiol 276:R1506–R1515

McClung JP, Hasday JD, He JR, Montain SJ, Cheuvront SN, Sawka MN, Singh IS (2008) 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

Oehler R, Pusch E, Zellner M, Dungel P, Hergovics N, Homoncik M, Eliasen MM, Brabec M, Roth E (2001) Cell type-specific variations in the induction of hsp70 in human leukocytes by feverlike whole body hyperthermia. Cell Stress Chaperones 6:306–315

Parsons K (2003) Human thermal environments. Taylor & Francis, London

Patel B, Khaliq A, JarvisEvans J, Boulton M, Arrol S, Mackness M, McLeod D (1995) Hypoxia induces HSP 70 gene expression in human hepatoma (HEP G2) cells. Biochem Mol Biol Int 36:907–912

Refinetti R (1998) Body temperature and behavior of tree shrews and flying squirrels in a thermal gradient. Physiol Behavior 63:517–520

Ruell PA, Thompson MW, Hoffman KM (2009) Heat shock proteins as an aid in the treatment and diagnosis of heat stroke. J Thermal Biol 34:1–7

Sandstrom ME, Siegler JC, Lovell RJ, Madden LA, McNaughton L (2008) The effect of 15 consecutive days of heat-exercise acclimation on heat shock protein 70. Cell Stress Chaperones 13:169–175

Sandstrom ME, Madden LA, Taylor L, Siegler JC, Lovell RJ, Midgley A, McNaughton L (2009) Variation in basal heat shock protein 70 is correlated to core temperature in human subjects. Amino Acids 37:279–284

Shastry S, Toft DO, Joyner MJ (2002) HSP70 and HSP90 expression in leucocytes after exercise in moderately trained humans. Acta Physiol Scand 175:139–146

Snoeckx L, Cornelussen RN, Van Nieuwenhoven FA, Reneman RS, Van Der Vusse GJ (2001) Heat shock proteins and cardiovascular pathophysiology. Physiol Rev 81:1461–1497

Sonna LA, Gaffin SL, Pratt RE, Cullivan ML, Angel KC, Lilly CM (2002) Effect of acute heat shock on gene expression by human peripheral blood mononuclear cells. J Appl Physiol 92:2208–2220

Waterhouse J, Edwards B, Bedford P, Hughes A, Robinson K, Nevill A, Weinert D, Reilly T (2004) Thermoregulation during mild exercise at different circadian times. Chronobiol Int 21:253–275

Waterhouse J, Drust B, Weinert D, Edwards B, Gregson W, Atkinson G, Kao SY, Aizawa S, Reilly T (2005) The circadian rhythm of core temperature: origin and some implications for exercise performance. Chronobiol Int 22:207–225

Watkins AM, Cheek DJ, Harvey AE, Goodwin JD, Blair KE, Mitchell JB (2007) Heat shock protein (HSP-72) levels in skeletal muscle following work in heat. Aviat Space Environ Med 78:901–905