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The time-profile of the PBMC HSP70 response to in vitro heat shock appears temperature-dependent

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Summary. Heat shock proteins (HSPs) are synthesised by cells subsequent to a stress exposure and are known to confer protection to the cell in response to a second challenge. HSP induction and decay are correlated to thermotolerance and may therefore be used as a biomarker of thermal history. The current study tested the temperature-dependent nature of the heat shock response and characterised its time profile of induction. Whole blood from 6 healthy males (Age: 26 ± (SD) 2 yrs; Body mass 74.2 ± 3.8 kgs; VO_{2max}: 49.1 ± 4.0 ml · kg^{-1} · min^{-1}) were isolated and exposed to in vitro heat shock (HS) at 37, 38, 39, 40, and 41 °C for a period of 90 min. After HS the temperature was returned to 37 °C and intracellular HSP70 was quantified from the leukocytes at 0, 2, 4, and 6 h after heat treatment. The concentration of HSP70 was not different between temperatures ($P > 0.05$), but the time-profile of HSP70 synthesis appeared temperature-dependent. At control (37 °C) and lower temperatures (38–39 °C) the mean HSP70 concentration increased up to 4 h post HS ($P < 0.05$) and then returned towards baseline values by 6 h post HS. With in vitro hyperthermic conditions (40–41 °C), the time-profile was characterised by a sharp rise in HSP70 levels immediately after treatment ($P < 0.05$ for 40 °C at 0 h), followed by a progressive decline over time. The results suggest a temperature-dependent time-profile of HSP70 synthesis. In addition, the temperature at which HSP70 is inducted might be lower than 37 °C.

Keywords: Heat shock protein 70 – Temperature – In vitro, PBMCs

Introduction

Heat shock proteins (HSPs) are universally expressed in cells and organs in response to a physiologically stressful exposure. In addition to being highly inducible, there is also a constitutively expressed isoform of each family of HSPs, which are categorised by their molecular weight (Locke, 1998). The most highly conserved family of HSPs is the 70 kDa family (HSP70), and therefore this group are the most commonly studied (Kregel, 2002). During and after stress, HSP70 and its constitutive isoform (HSC70) migrate and protect the nucleus, nucleolus and cytoskeleton (Welch and Feramisco, 1984). This protection is conferred by chaperoning and folding nascent polypeptides (Beckman et al., 1990), which prevents improper folding and aggregation (Zietara and Skorkowski, 1995). The precise stimulus for the redistribution and synthesis of HSPs is unknown (Skidmore et al., 1995). The term heat shock was derived from the original stressor that was shown to produce a stress protein response (hyperthermia; Ritossa, 1962), but other physiological stressors such as pH alterations (Weitzel et al., 1985; Gapen and Moseley, 1995), calcium accumulation (Kiang et al., 1994), energy depletion (Febbraio and Koukoulas, 2000; Febbraio et al., 2004), abnormal protein generation (Chiang et al., 1989), hypoxia (Iwaki et al., 1993), and ROS generation (Wallen et al., 1997) have since been implicated.

The role of temperature *per se* on the heat shock response (HSR) has been previously investigated in different experimental models. Induction and decay of HSP70 has been shown to correlate with thermotolerance (Landry et al., 1982; Li and Mak, 1989), which occurs within several hours and may last 3–5 days (Kregel, 2002), allowing the cell to become more resistant to potentially lethal heat stress. With in vivo (Skidmore et al., 1995; Walters et al., 1998; Oishi et al., 2002) and in vitro (Salo et al., 1991) animal studies, passive exposure to hyperthermic temperatures has produced a HSR in various tissues, and this response was amplified at higher heating rates (Flanagan et al., 1995). Furthermore, acute exercise at elevated temperatures has produced a greater HSR in comparison to exercise in normothermic ambient

conditions (Skidmore et al., 1995; Walters et al., 1998). However, investigating the HSR to heat stress in biopsy samples from skeletal muscle or organ tissues is an invasive procedure, and therefore are not practical sampling sites for large-scale or serial monitoring. A limited number of studies have begun to measure HSP accumulation kinetics from circulating leukocytes (Fehrenbach et al., 2002a, b; Sonna et al., 2002; Oehler et al., 2001), which may give more valid indications as to the level of stress endured by the whole organism (Sonna et al., 2002). These reports have tended to corroborate the results from tissue samples, and showed that in vitro heat shock of isolated leukocytes at high temperatures (41–43 °C) produced an accumulation of inducible HSP70 (Oehler et al., 2001; Sonna et al., 2002). Whilst previous studies have shown that hepatic intravascular (Rowell et al., 1968) and deep muscle temperatures (Asmussen and Boje, 1945) to be 1.0–1.5 °C greater than the body core temperature, it is unlikely that the circulating leukocyte temperatures would maintain this intensity for prolonged periods, even during febrile illness (Eichna et al., 1951) or heavy exercise (Sproule and Archer, 1959; Robinson et al., 1965; Aulick et al., 1981), when core temperatures are elevated to 39–40 °C (Eichna et al., 1951; Robinson et al., 1965). Furthermore, the threshold temperature at which HSP70 is inducted is unknown, so work is required to determine the effects of temperature on the HSR of leukocytes at lower temperatures within the homeotherm range.

An important and often neglected methodological aspect of HSP70 measurements is the time at which they are sampled, subsequent to a sub-lethal cellular stressor. Whilst the time profile of HSP70 accumulation in skeletal muscle (Khassaf et al., 2001, 2003), cardiac tissue (Maloyan et al., 1999), skin (Wilson et al., 2000) and organ cells (Flanagan et al., 1995) have been studied, the kinetics vary between sampling sites, species, and the nature of the stress (Maloyan et al., 1999; Sonna et al., 2002). In circulating leukocytes, the peak HSP70 response has been observed at 4 h in peripheral blood mononuclear cells (PBMCs; Sonna et al., 2002), and at 24 h (Fehrenbach et al., 2000a) in granulocytes after a heat shock and physical exercise, respectively. The sampling frequencies adopted in previous work may have been insensitive to the peak HSP70 response (Fehrenbach et al., 2000a; Schneider et al., 2002), and the time-profile of the accumulation kinetics in circulating leukocytes is not well characterised. Therefore, this investigation aimed to determine the time-profile of HSP70 expression in circulating PBMCs in response to a sub-lethal heat shock at a range of temperatures within the homeotherm range (37–41 °C). If HSP70 expression is to be applied as a stress biomarker and cytoprotective therapeutic agent in the medical field, information of this nature would have economic implications for its measurement.

Materials and methods

With institutional ethics committee approval, six moderately-trained male subjects (Age: $26 \pm$ (SD) 2 yrs; Body mass 74.2 ± 3.8 kgs; VO_{2max}: $49.1 \pm 4.0\,ml \cdot kg^{-1} \cdot min^{-1}$), who had not undertaken warm weather training or had febrile illness in the previous 12 weeks were recruited. Participants reported to the laboratory at 0900 in a 2 h post absorptive state, having been requested not to perform any moderate or high intensity exercise, or unaccustomed activity in a 48 h period prior to measurement. Upon arrival at the laboratory, and after 30 min of supine rest, a 70 ml blood-sample was collected aseptically from the ante-cubital vein in the non-dominant arm of each participant using an EDTA vacutainer system. The sample was immediately analysed for resting blood lactate (BLa), glucose (Glu), pH and ionized calcium (iCa^+) levels as well as a full haematological profile. The BLa and Glu were measured using a YSI 2300 $Stat^+$ (YSI Inc, Yellow Springs, OH), with pH and iCa^+ levels assessed using the AVL OMNI 4 (Roche Diagnostics Ltd., Sussex, UK). The haematological profile was performed using the fluorescence flow cytometry method (Sysmex XT 2000, Sysmex UK Ltd., Milton Keynes, UK). The remaining blood was separated into five equal aliquots (1.3 ml) and incubated at 37, 38, 39, 40, or 41 °C for 90 min in a block heater with a stability and uniformity of ± 0.1 °C (SBH130DC, Stuart Scientific Co. Ltd., Surrey). The duration of time from the blood draw to the in vitro graded thermal exposure was always $\leq$10 min. A pre-heat shock baseline HSP sample was taken and after the heat exposure, samples were maintained at 37 °C and measured at 0, 2, 4, and 6 h thereafter. At each time-point, whole blood was diluted 1:1 with phosphate buffered saline (PBS) and layered on an equal volume of histopaque (10771, Sigma, UK). Samples were then centrifuged at $400 \times g$ for 30 min. PBMCs in the plasma were separated and the overlay was washed with phosphate buffered saline (PBS) at $400 \times g$ for 10 min. The supernatant was aspirated leaving the PBMC pellet, which was re-suspended with HSP70 extraction reagent (Stressgen Biotechnologies, Canada) supplemented with protease inhibitors. The cell suspension was homogenized and incubated on ice for 30 min, followed by spinning at $21{,}000 \times g$ for 30 min in a 4 °C refrigerated centrifuge. The cell lysate was then extracted in the supernatant and 250 µl was transferred to polypropylene tubes and then frozen rapidly to −80 °C for later sampling.

Quantification of inducible HSP70 from PBMC lysate was achieved by a sandwich enzyme-linked immunosorbent assay (ELISA). A HSP70 specific monoclonal antibody is pre-coated on the wells of the immunoassay plate (EKS-700, Stressgen Biotechnologies, Canada) which binds and detects inducible HSP70. The intra- and inter-assay coefficient of variation is <10% and it is specific for the inducible isoform HSP70. The ELISA has been shown to be more sensitive and more quantifiable than Western Blotting (Milne and Noble, 2002), the ELISA also does not require user definable parameters, which is a limitation of flow cytometry techniques.

The HSP70 concentrations were calculated by interpolating the absorbance readings from a standard curve developed with the 7-point standards. The absolute values of HSP70 were normalised per mg of the total protein content of the supernatant, which was determined via the Bradford method (Bradford, 1976). Briefly, 10 µl of samples were added with 30 µl of Coomassie Plus Assay Reagent (Pierce Biotechnology Inc., IL, USA), and the absorbance was measured at 595 nm (Biotek Synergy HT-R, Biotek Instruments, Vermont, USA). The absorbance of the samples was plotted against that of albumin standards ranging from 100 to $1500\,\mu g \cdot ml^{-1}$.

Statistical analysis was performed using the SPSS (version 12.0) package. A mixed factorial two-way ANOVA was performed to determine the

effects of time, temperature, and their interaction. One-way ANOVA and Fishers PLSD were used as *post hoc* tests for temperature and time, respectively. Data are presented as mean ± standard deviation, and the alpha level was set at $P = 0.05$ throughout.

Results

Pilot data indicated that the isolation procedure did not affect the viability of the PBMCs, for each temperature and time-point analysis. Dead cells accounted for ≤5% of the total PBMC count. There were no changes in iCa^{+} during the in vitro heat shock and in the hours thereafter ($P > 0.05$). As expected, blood pH decreased progressively with time ($P < 0.01$), although this response was not temperature dependent ($P > 0.05$). Blood lactate increased progressively with time ($P < 0.01$) and averaged 8.5 ± 1 mmol across temperatures 6 h post HS (see Table 1). Blood GLU, decreased over time to 0.5 ± 0.8 mmol at 6 h ($P < 0.01$). The changes in BLa and GLU were not temperature dependent ($P > 0.05$), but over time were indicative of glycolysis.

Table 2 shows the actual and relative changes of neutrophils, lymphocytes, and monocytes during the heat treatment. The HS did not result in leukocytosis in the subsequent 6 h ($P > 0.05$), and there were no changes in the number or proportion of the leukocyte sub-sets ($P > 0.05$).

Table 1. In vitro response of BLa and GLU to heat shock

Temp. (°C)	Time	BLa ($mmol \cdot l^{-1}$)	GLU ($mmol \cdot l^{-1}$)
	Rest	1.2 ± 0.3	3.88 ± 0.71
37	0 h	3.25 ± 0.71	2.97 ± 0.78
	2 h	5.42 ± 1.09	1.96 ± 0.85
	4 h	7.43 ± 1.32	0.98 ± 0.90
	6 h	8.57 ± 1.01	0.44 ± 0.62
38	0 h	3.53 ± 0.67	2.76 ± 0.90
	2 h	5.66 ± 1.01	1.95 ± 1.48
	4 h	7.43 ± 1.21	1.09 ± 1.46
	6 h	8.37 ± 1.17	0.65 ± 1.10
39	0 h	3.63 ± 0.77	2.87 ± 1.42
	2 h	5.95 ± 1.06	1.46 ± 1.04
	4 h	7.56 ± 1.07	0.78 ± 0.98
	6 h	8.45 ± 1.14	0.37 ± 0.58
40	0 h	3.78 ± 0.70	2.63 ± 0.96
	2 h	6.10 ± 1.00	1.56 ± 1.01
	4 h	7.77 ± 1.08	0.73 ± 0.95
	6 h	8.47 ± 1.02	0.37 ± 0.58
41	0 h	3.90 ± 0.83	2.46 ± 1.01
	2 h	6.28 ± 1.08	1.40 ± 1.08
	4 h	7.88 ± 1.07	0.90 ± 1.30
	6 h	8.50 ± 0.92	0.59 ± 0.99

Intracellular HSP70 expression

Figure 1 shows the individual and mean proportional changes in expression of HSP70 at different temperatures

Table 2. Changes of leukocytes and their sub-populations during the heat treatment

Temp. (°C)	Time	Total WBC	Granulocyte		Lymphocyte		Monocyte	
			#	%	#	%	#	%
	Rest	5.2 ± 1.1	3.1 ± 0.9	60.0 ± 6.5	1.6 ± 0.3	31.5 ± 6.4	0.5 ± 0.2	8.6 ± 2.2
37	0 h	5.1 ± 1.1	3.1 ± 1.0	60.0 ± 7.0	1.6 ± 0.3	31.1 ± 6.1	0.4 ± 0.1	8.5 ± 1.7
	2 h	5.2 ± 1.2	3.2 ± 0.9	60.7 ± 6.6	1.6 ± 0.3	31.0 ± 6.5	0.4 ± 0.2	8.3 ± 2.0
	4 h	5.1 ± 1.1	3.1 ± 0.9	59.8 ± 6.8	1.5 ± 0.3	31.2 ± 6.7	0.5 ± 0.2	9.0 ± 2.0
	6 h	5.2 ± 1.1	3.1 ± 1.0	60.1 ± 6.6	1.6 ± 0.3	30.8 ± 6.4	0.5 ± 0.2	9.1 ± 1.7
38	0 h	5.1 ± 0.8	3.0 ± 0.8	59.4 ± 6.4	1.6 ± 0.3	31.5 ± 6.3	0.5 ± 0.2	9.1 ± 2.3
	2 h	5.0 ± 0.9	3.0 ± 0.8	59.7 ± 6.3	1.6 ± 0.2	31.6 ± 6.0	0.5 ± 0.1	8.8 ± 1.8
	4 h	4.9 ± 1.0	3.0 ± 0.8	60.0 ± 5.9	1.5 ± 0.2	31.6 ± 5.8	0.4 ± 0.2	8.5 ± 1.7
	6 h	4.9 ± 1.0	3.0 ± 0.9	59.9 ± 6.5	1.5 ± 0.2	31.1 ± 6.2	0.5 ± 0.2	9.0 ± 2.1
39	0 h	5.0 ± 1.0	3.0 ± 0.9	59.7 ± 6.3	1.5 ± 0.2	31.5 ± 6.4	0.5 ± 0.2	8.8 ± 2.2
	2 h	5.1 ± 1.0	3.1 ± 0.9	60.3 ± 6.5	1.6 ± 0.2	31.1 ± 6.3	0.5 ± 0.2	8.6 ± 1.8
	4 h	5.2 ± 1.0	3.1 ± 0.9	59.9 ± 6.6	1.6 ± 0.3	31.3 ± 6.4	0.5 ± 0.2	8.8 ± 2.1
	6 h	5.0 ± 0.9	3.0 ± 0.8	59.1 ± 7.5	1.6 ± 0.3	31.7 ± 6.5	0.5 ± 0.2	9.3 ± 2.6
40	0 h	4.9 ± 0.8	3.0 ± 0.7	60.0 ± 6.1	1.5 ± 0.3	31.5 ± 6.0	0.4 ± 0.2	8.5 ± 2.2
	2 h	5.2 ± 1.2	3.1 ± 1.0	59.5 ± 6.5	1.6 ± 0.3	31.8 ± 6.3	0.5 ± 0.2	8.7 ± 2.1
	4 h	5.1 ± 1.2	3.1 ± 1.0	59.6 ± 7.3	1.5 ± 0.4	31.0 ± 7.0	0.5 ± 0.2	9.4 ± 2.4
	6 h	5.0 ± 1.1	3.0 ± 0.9	59.4 ± 6.6	1.6 ± 0.3	31.6 ± 5.8	0.5 ± 0.2	9.0 ± 1.6
41	0 h	5.1 ± 1.2	3.1 ± 1.0	60.4 ± 6.9	1.5 ± 0.3	30.5 ± 6.6	0.5 ± 0.2	9.1 ± 2.3
	2 h	5.0 ± 1.1	3.0 ± 0.9	60.6 ± 6.5	1.5 ± 0.3	31.3 ± 6.0	0.4 ± 0.2	8.2 ± 2.3
	4 h	5.0 ± 1.2	3.1 ± 1.0	60.7 ± 6.9	1.5 ± 0.3	30.4 ± 6.7	0.5 ± 0.2	8.9 ± 2.2
	6 h	4.9 ± 1.1	3.0 ± 1.0	60.0 ± 6.1	1.5 ± 0.2	31.2 ± 6.0	0.4 ± 0.2	8.8 ± 2.5

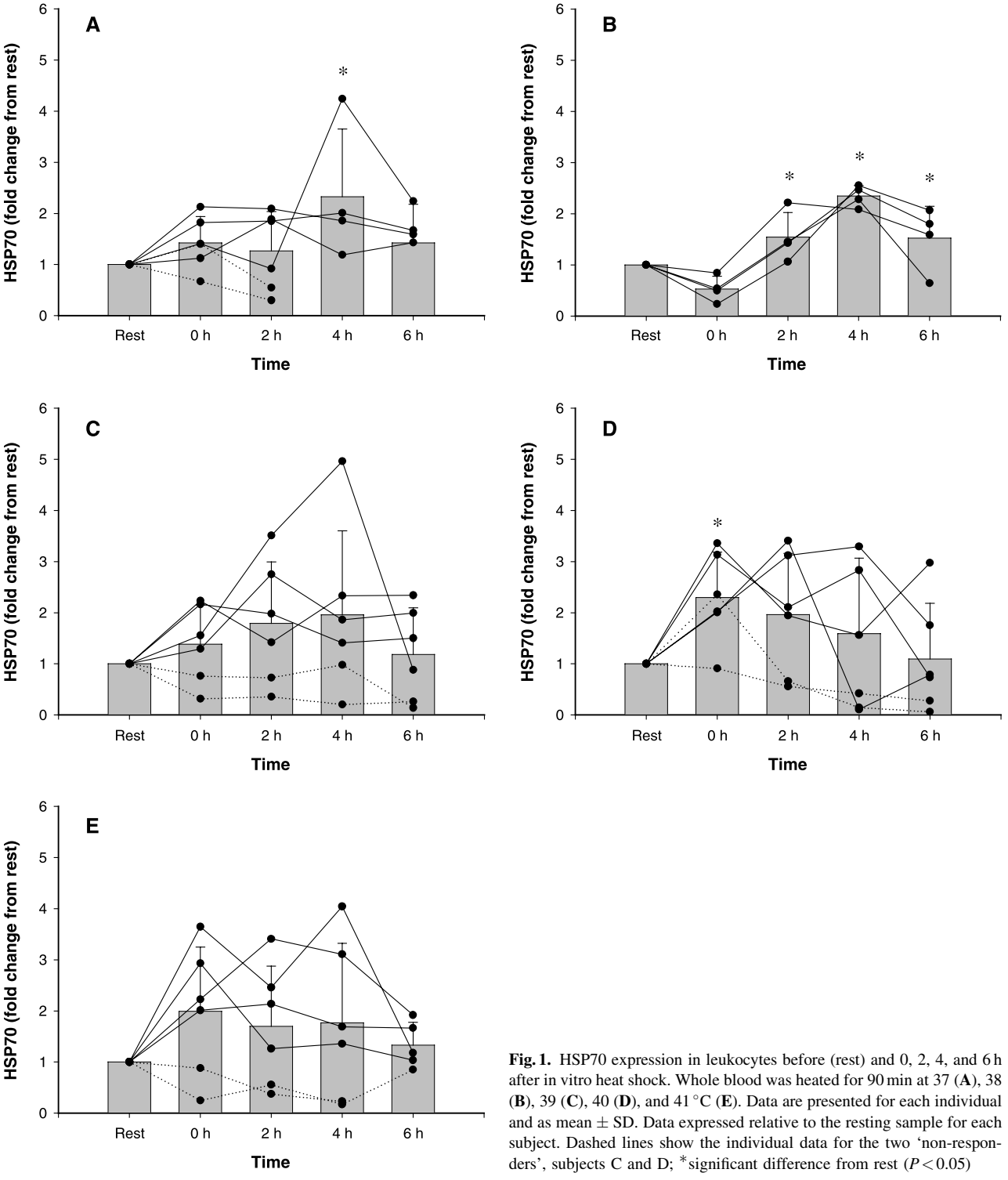

Fig. 1. HSP70 expression in leukocytes before (rest) and 0, 2, 4, and 6 h after in vitro heat shock. Whole blood was heated for 90 min at 37 (**A**), 38 (**B**), 39 (**C**), 40 (**D**), and 41 °C (**E**). Data are presented for each individual and as mean ± SD. Data expressed relative to the resting sample for each subject. Dashed lines show the individual data for the two 'non-responders', subjects C and D; *significant difference from rest ($P < 0.05$)

(37–41 °C), relative to baseline. HSP70 data was unavailable for 2 subjects at 4 and 6 h after 37 °C exposure, and throughout the 38 °C HS. The concentrations of HSP70 are presented relative to total protein (TP) per PBMC ($ng \cdot mg^{-1}$ TP per 10^9 PBMC; see Fig. 2). Mean resting HSP70 concentrations were 6.8 ± 3.3. At 37 °C HSP70 increased 2.3 ± 1.3 fold 4 h later to $10.5 \pm 5.1\ ng \cdot mg^{-1}$ TP per 10^9 PBMC ($P < 0.05$). After HS at 38 and 39 °C, HSP70 also peaked 4 h later, increasing 2.3 ($P < 0.01$) and 2.0 fold ($P > 0.05$, NS), respectively. At 37 and

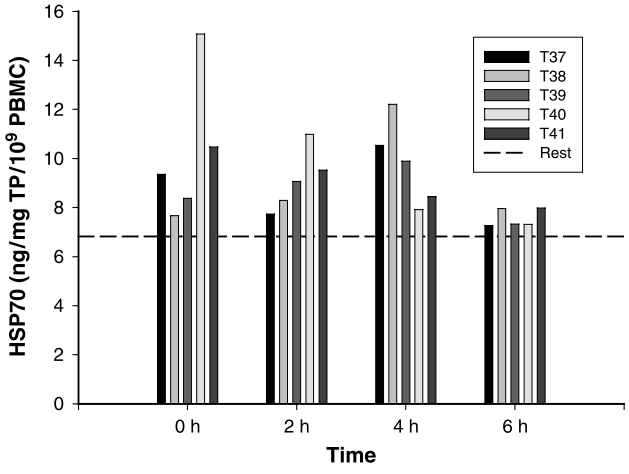

Fig. 2. HSP70 expression leukocytes before (rest) and 0, 2, 4, and 6 h after in vitro heat shock at different temperatures in the homeotherm range (37–41 °C). Dashed line represents resting values

38 °C, HSP70 was still greater than baseline levels at 6 h. The 40 °C HS prompted a 2.3 fold increase to $15.1 \pm 9.9\,\text{ng} \cdot \text{mg}^{-1}$ TP/10^9 PBMC at 0 h ($P < 0.05$). Thereafter, HSP70 concentration decreased progressively to baseline levels $7.3 \pm 9.8\,\text{ng} \cdot \text{mg}^{-1}$ TP per 10^9 PBMC. The 41 °C HS induced a 2 fold increase in HSP70 at 0 h which also steadily declined after 6 h, although this initial increase in HSP70 was not statistically significant ($P > 0.05$). There were no statistically significant differences between HS temperatures, possibly due to the large inter-individual responses to HS, as demonstrated in Fig. 1. However, there was a trend towards a greater HSP70 induction at 0 h after 40 °C HS, compared with control temperatures (37 °C; $P = 0.064$).

Discussion

The aims of the current study were to determine the time profile of the HSP70 response in circulating PBMCs after an in vitro heat shock. Our results indicated that the time-profile of the HSP70 response might be temperature dependent. At lower temperatures (37–39 °C), HSP70 increased approximately two-fold from resting levels and peaked at 4 h post HS. At higher temperatures (40–41 °C), HSP70 induction peaked immediately post HS (0 h), and steadily declined thereafter. However, although the time-profile of HSP70 appeared to shift to the right after exposure to higher temperatures, there were no differences between the magnitude of HSP70 accumulation between the temperatures at any time-point. Another important finding in this study was the increased HSP70 response to control temperatures (37 °C), which increased by two-fold at 4 h post HS.

To our knowledge, this is the first study that has quantitatively measured the intracellular HSP70 concentrations at various temperatures within the homeothermic range (37–41 °C). Previous work has typically exposed isolated blood samples to higher temperatures (42–43 °C). Schneider et al. (2002) observed an increased HSP70 in PBMCs after incubation at 42.5 °C. Sonna et al. (2002) also showed an increased HSP70 expression in PBMCs after an acute HS at 43 °C. In addition, Fehrenbach et al. (2000b, 2003) performed an in vitro HS at 42 °C, which produced an up-regulation of HSP70 mRNA. These studies exposed blood samples to very high in vitro temperatures, which would be rarely experienced in the intravascular environment. During heavy exercise or fever, although body core temperature and muscle temperatures may increase to 40 °C, the intravascular temperature has been shown to be approximately 0.5 °C lower than that of rectal temperature in the femoral vein (Robinson et al., 1965; Aulick et al., 1981) and the right heart (Eichna et al., 1951). Although the temperature of the veins draining the muscle and liver (Hepatic) can be 1.0–1.5 °C higher than the core temperature (Asmussen and Boje, 1945; Rowell et al., 1968), mean intravascular temperatures are likely to be lower. Therefore, measuring the HSP response in leukocytes or serum to a HS of greater than ~41 °C has limited clinical relevance.

Previous research has suggested that one of the primary stimuli for HSP response is an increase in tissue temperature (Flanagan et al., 1995; Walters et al., 1998; Oishi et al., 2002). Walters et al. (1998) compared the HSP70 response in the brains of rats exposed to either exercise at room (23 °C) or warm (34 °C) temperatures, or passive heating protocols. Passive and active heat stress resulted in similar HSP70 increases, and HSP70 expression was significantly correlated with brain temperature. Similarly, rats passively heated at high ($0.166\,^{\circ}\text{C} \cdot \text{min}^{-1}$) and low ($0.045\,^{\circ}\text{C} \cdot \text{min}^{-1}$) rates until colonic temperature reached 42 °C, showed a greater HSP72 expression in the liver, small intestine and kidney, in the high heating rate conditions (Flanagan et al., 1995). If temperature is responsible for the up-regulation in HSP70 and can be used as an indicator of thermal history (Ryan et al., 1991), we hypothesised that by measuring the HSP70 response to HS at a range of temperatures, a dose-dependent HSP response would be observed, and that an induction temperature could be established. However, our results did not demonstrate differences in the HSP70 response to in vitro HS at different temperatures. This finding may be due to the large inter-individual variation in the HSP70 accumlation kinetics (see Fig. 1). Contrary to previous work (Oehler

et al., 2001; Sonna et al., 2002), we found that the HSP70 increased in response to exposure at 37 °C. It has been shown that peripheral intravascular temperatures at rest range between 33–35 °C in the saphenous (Robinson et al., 1965) and axillary veins (Aulick et al., 1981) respectively, under resting euthermic conditions. It is possible that the 37 °C HS increased the temperature of the whole blood compared to its resting in vivo temperatures, and that this mild increase was sufficient to induce HSP70 synthesis. However, the isolation procedure used here may have caused other stresses such as alterations in pH and substrate availability, which have been shown to cause a HSR (Weitzel et al., 1985; Gapen and Moseley, 1995; Febbraio and Koukoulas, 2000; Febbraio et al., 2004).

The results of the present study do not conclusively support the theory that HSP70 induction is caused by temperature increases *per se*, however this is the first investigation, to our knowledge, to report a temperature-dependent time-profile of the HSR. Previous work has shown that an in vitro HS ranging between 39 and 42.5 °C resulted in an up-regulation of HSP70 that was observed at 1 h (Schneider et al., 2002) and 3 h (Oehler et al., 2001) later. However, the time-course of the HSP70 response has not been well documented. Sonna et al. (2002) showed that HS at 43 °C for 20 min increased the intracellular concentrations of HSP70 after 1 h, and peaked with a three-fold increase 4 h post HS. We observed a similar time-to-peak (4 h) HSP70 concentrations to those reported by Sonna et al. (2002) in PBMCs shocked at 37–39 °C, although the intensity of the response was lower (1.3–2.3 fold increase). However, at higher temperatures (40–41 °C), the time-profile graph shifted to the right, such that the peak HSP was denoted immediately after the HS=(0 h). Perhaps, at such hyperthermic temperatures there is a greater demand for the protective properties of HSP70 earlier in the recovery period. Exposure to severe temperatures fluidises the cell membrane, increasing the permeability, which allows an influx of calcium and proton concentrations (Weitzel et al., 1987; Koratich and Gaffin, 1999); collapses the cytoskeleton around the nucleus (Warters et al., 1986); and inhibits protein turnover (Parag et al., 1987).

A number of investigations have managed to determine the cell type specific variation of HSP70 induction in leukocytes (Oehler et al., 2001; Fehrenbach et al., 2000b). The constitutive expression of HSP70 has been shown to be ∼2-fold greater in granulocytes as opposed to monocytes (Oehler et al., 2001; Fehrenbach et al., 2000b), with lymphocytes expressing a very low levels (Oehler et al., 2001). Heat shock treatment (42 °C for 2 h) induced a rapid expression of HSP70 in monocytes (∼10-fold) and lymphocytes (∼5-fold), with only a 30% increase above resting levels observed in granulocytes (Oehler et al., 2001). In the above study, the HSR on leukocyte subdivisions was also shown to be temperature sensitive, as a 39 °C HS did not increase HSP70 above resting concentrations in lymphocytes and granulocytes, whereas monocytes showed a 6-fold increase over 37 °C controls. These results suggested that lymphocytes and granulocytes may only respond to very strong heat stress, and monocytes have intermediate constitutive HSP70 with the strongest increases with HS. In the current study, the HS treatment at all temperatures did not induce leukocytosis, or changes in the relative number of the leukocyte subsets, therefore the cell-type specific variations in the HSR did not account for the results presented here.

The inter-individual HSP70 response to in vitro HS observed in our results demonstrated some interesting patterns (see Fig. 2). Two subjects (dashed lines) consistently showed a down-regulation of HSP70 that persisted throughout the entire time-course analysis. The resting levels of HSP70 were very high (24.3 and 36.2 $ng \cdot mg^{-1}$ TP per 10^9 PBMC, respectively), despite the strict control procedures that were set prior to arrival at the laboratory. If these subjects had undertaken some form of preconditioning, such as exercise or thermal stress, this may have impaired HSP70 synthesis to a secondary challenge. Leukocytes pre-treated with either HS (41 °C) or endurance exercise, manifested a decreased HSP70 synthesis after administration of a secondary heat challenge in comparison with non-treated cells (Ryan et al., 1991). The results of both these two trials supported the use of HSP kinetics as assays of thermal history, and further suggested that HSP's might be responsible for regulating their own synthesis. Perhaps the high constitutive expression observed in subjects C and D, was caused by a psychological stress met by an adrenal hormone secretion, which have been implicated in the HSP response (Matz et al., 1996a, b; Fleshner et al., 2004). The decreased intracellular content of HSP70 could have resulted due to a release into the extracellular milieu after either cell death (Basu et al., 2000) or via exocytosis after an induction of adrenal hormones (Febbraio et al., 2002; Fleshner et al., 2004). Since the cell viability after the in vitro HS procedures was $\geq$95% and the PBMCs were isolated, it is unlikely that HSP70 release was the cause of the down-regulation of HSP70 in subjects C and D.

In conclusion, it appears that the time-profile of the HSP70 response might be dependent upon the intensity

of the stressor. The results of the current study demonstrated a quicker time-to-peak HSP70 concentration after in vitro heat shock at higher temperatures (40–41 °C). At lower temperatures (37–39 °C) the HSR appears to peak at 4 h before returning towards baseline levels. The magnitude of the HSR was not different between temperatures, however the induction temperature for HSP70 synthesis in isolated PBMCs was shown to be as low as 37 °C, since resting in vivo blood temperature might range between 33 and 37 °C dependent upon the tissues it perfuses, the induction temperature may be lower than previously thought. Based on the current findings, further work needs to be undertaken to establish the time-profile of the HSR following exposure to in vitro heat shock and other physiological stressors such as exercise, this will allow the future use of HSP70 as a biomarker for the stress response. Furthermore, future work should concentrate on delineating the effects of temperature and other cellular insults, such as oxidative stress and alterations in pH and calcium accumulation, in vivo.

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