Amino Acids (2008) 34: 203–211
DOI 10.1007/s00726-007-0507-2
Printed in The Netherlands

Amino Acids

Effects of active and passive hyperthermia on heat shock protein 70 (HSP70)

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Received July 27, 2006
Accepted February 13, 2007
Published online October 12, 2007;

Summary. The purpose of this study was to delineate the effects of hyperthermia and physical exercise on the heat shock protein 70 (HSP70) response in circulating peripheral blood mononuclear cells (PBMCs). Six healthy, young (age: 24 ± 3 yrs), moderately trained males (VO_{2max}: $48.9 \pm 2.7\,ml \cdot kg \cdot min^{-1}$) undertook two experimental trials in a randomised fashion in which the core temperature (T_c) was increased and then maintained at 39 °C during a 90 min bout by either active (AH) or passive (PH) means. AH involved subjects cycling at 90% of their lactate threshold in attire designed to impede heat loss mechanisms. In the PH trial, subjects were immersed up to the neck in a hot bath (40.2 ± 0.4 °C), once the critical T_c was achieved, intermittent cycling and water immersions were prescribed for the AH and PH conditions, respectively, to maintain the T_c at 39 °C. HSP70 was measured intracellularly pre, post and 4 h after trials, from circulating PBMCs using an ELISA technique. T_c reached 39 °C quicker in PH than during AH trials (PH: 21 ± 4 min vs. AH: 39 ± 6 min; $P < 0.01$), thereafter T_c was maintained around 39 °C (PH: 39.1 ± 0.2 °C; AH: 38.8 ± 0.3 °C; $P > 0.05$). AH induced a marked leukocytosis in all sub-sets ($P < 0.05$). PH generated significant monocytosis and granulocytosis ($P < 0.05$), without changes in lymphocyte counts ($P > 0.05$). There were no significant increases in intracellular HSP70 at 0 h (AH: $\Delta -21.1 \pm 44.8$; PH: $\Delta +12.5 \pm 32.4$ ng/mg TP/10^3/µl PBMCs; $P > 0.05$) and 4 h (AH: $\Delta -30.0 \pm 40.1$; PH: $\Delta +36.3 \pm 70.4$ ng/mg TP/10^3/µl PBMCs; $P > 0.05$) post active and passive heating. Peak HSP70 expressed as a fold-change from rest was also not increased by AH (1.1 ± 0.9; $P > 0.05$) or PH (3.2 ± 4.8; $P > 0.05$). There were no significant differences between the AH and PH trials at any time-point, and the HSP70 response appeared to be individual specific. These results did not allow us to delineate the effects of hyperthermia and other exercise associated stressors on the heat shock response and therefore further work is warranted.

Keywords: Exercise – Leukocytes – Temperature – Heat – Stress proteins

Introduction

Heat shock proteins are synthesised by cells upon exposure to stress. They are known as molecular chaperones since their functions are to re-assert cellular stasis through translocation, re-folding or dis-assembly of nascent polypeptides (Morimoto et al., 1994). A number of changes in the intracellular milieu have been shown to produce an accumulation of HSPs, a process known as the HSR. The term 'heat' shock proteins was applied as a heat shock of ~42 °C was shown to produce an accumulation of these proteins in *drosophilia* (Ritossa, 1962). Since then, alterations in pH (Gapen and Moseley, 1995), increased calcium accumulation (Kiang et al., 1994), glucose depletion (Febbraio et al., 2004), and oxidative stress (Benjamin et al., 1990), have been shown to produce a HSR. Each of these conditions are associated with the physiological response to exercise, which has been shown to induce a marked acute HSR in organ (Walters et al., 1998; Kim et al., 2004) and muscle tissues (Skidmore et al., 1995; Khassaf et al., 2001) and also circulating leukocytes (Ryan et al., 1991; Fehrenbach et al., 2000a, b).

The role of temperature in the HSR has been determined passively in in vivo and in vitro models. Rats exposed to high ($0.166\,°C \cdot min^{-1}$) and low ($0.045\,°C \cdot min^{-1}$) whole-body heating rates produced a greater accumulation of HSP70 in the small intestine, liver and kidney following the high body heating rate condition (Flanagan et al., 1995). In addition, whole blood exposed to high temperatures (42–43 °C) produced a HSR in leukocytes (Oehler et al., 2001; Schneider et al., 2002). However, it is not clear whether temperature is the major contributor to the HSR during physical exercise, or whether the cumulative effects of metabolic changes such as alterations in pH and calcium, oxidative stress or a reduced energy availability are the primary signals. There are few studies that have attempted to delineate the effects of hyperthermia and exercise on the HSR. Those that have tried have produced contradictory findings using various tissue extracts from

animal models (Skidmore et al., 1995; Walters et al., 1998; Harris and Starnes, 2001; Kim et al., 2004). To our knowledge, no study has yet investigated the role of temperature during exercise on the HSR in humans. If temperature is the primary stimulus for HSP70 up-regulation, these can be used as molecular markers of thermal history in populations that work/perform in high ambient temperatures. Therefore it was the aim of the current study to delineate the effects of hyperthermia and physical exercise on the HSP70 response in circulating leukocytes. Since circulating leukocytes visit all organs and tissues their HSR may give an overall marker of stress (Sonna et al., 2002).

Materials and methods

Subjects

With ethical approval and written informed consent, six moderately trained young male subjects with the following mean (±SD) physical characteristics, age: 24 ± 3 yrs; body mass: 76.4 ± 8.6 kg; VO_{2max}: 48.9 ± 2.7 ml · kg · min^{-1}, were recruited to participate in this study. The selection criteria included having not contracted febrile illness and not undertaken warm weather training in the twelve weeks prior to the study.

Experimental design

The testing procedures consisted of four laboratory visits that were performed within a four week period, with 5–10 days between conditions. Maximal oxygen capacity (VO_{2max}) was determined using an incremental test to exhaustion on an electrically braked cycle ergometer (Schroberer Rad MeBtechnik (SRM), Weldorf, Germany). The incremental exercise test began at a power output of 100 W for two min, thereafter the workload was increased by 25 W · min^{-1} until volitional exhaustion. Breath by breath expired gas analysis was assessed for oxygen uptake, carbon dioxide output and ventilation (Cosmed Quark b^2, Cosmed, Rome, Italy). Flow rate was measured using a bi-directional digital turbine which has an accuracy of 2% and was calibrated with a 3-l syringe (Cosmed, Italy). The gas analysers were calibrated prior to each test using ambient air that was assumed to be 20.93% O_2 and 0.03% CO_2 and alpha standard gases (16.00% O_2 and 4.96% CO_2; BOC, Guilford, UK).

On the next visit the lactate threshold (LT) was established for each individual by performing a second incremental test to exhaustion in which the initial workload was set at 100 W for 4 min, and every four min thereafter, the workload was increased by 15 W until exhaustion. In the last 15 sec of each stage, finger-prick blood lactate (BLa) samples were taken and immediately analysed using the YSI 2300 STAT + (YSI Inc., Yellow Springs, OH). The lactate threshold was determined using the D-max method (Cheng et al., 1992).

The experimental trials were designed in an attempt to delineate the effects of temperature and physical exercise on the HSP response. In these trials the body core temperature (T_c) was increased to 39 °C as soon as possible, and then was maintained for the duration of the session, which was 90 min in total. In the active heating (AH) trial the subjects were dressed in a whole-body wet-suit and an all-in-one body boiler suit with a hood to inhibit sensible heat dissipation mechanisms. Subjects cycled on the SRM ergometer at a self-selected cadence at 90% of the power output that corresponded to the LT (140 ± 13 W). In the passive heating (PH) trial subjects were immersed in an upright seated position, up to the neck in a hot bath (40.2 ± 0.4 °C). The AH and PH trials were administered in a randomised order, and in both trials once the target temperature was attained (39 °C), exercise and hot water immersion were prescribed in an intermittent fashion in the two trials, respectively, so that T_c remained at 39 °C for the rest of the bout. During this stage the exercise intensity was reduced in the AH trials to maximise the time spent exercising whilst maintaining the T_c at 39 °C.

Protocol

Subjects arrived at the laboratory at 10:00 AM, in a two-hour post absorptive state to standardise resting physiological status. In the 48 h prior to arrival at the laboratory, it was requested that no exercise or unaccustomed activity be undertaken, and caffeine and alcohol were not ingested in the 24 h before experimental trials. The pre-exercise dietary intake was recorded over 24 h before the first experimental trial and the subjects were instructed to mimic this for the second trial. Upon arrival subjects confirmed that they adhered to the experimental controls, were weighed, and donated a urine sample prior to the trials to assess hydration status. Urine specific gravity (USG) was measured using a digital refractometer (Wolf Laboratories LTD, York, England). The T_c was measured using a small ingestible temperature sensor pill, which had been taken three hours prior to arrival. This pill transmitted temperature signals from the gut to an external receiver placed at the small of the back (CorTemp, HQ Inc., Florida, USA), and was measured at 1 min intervals during the PH and AH trials and averaged over 5 min periods. Heart rate (HR) was monitored continuously throughout the duration of the test (S810i, Polar Electro, Kempele, Finland). Upon cessation of the trials the participants were towel dried and re-weighed to determine sweat losses (kg). During the AH trials, breath-by-breath expired gas analysis (Cosmed Quark, Rome, Italy) was undertaken at five min epochs throughout the bout to estimate the exercise intensity. At five min intervals during AH conditions, ratings of perceived exertion (RPE; CR 6–20 scale; Borg, 1998) and temperature (PT; CR 1–10 scale; Gagge et al., 1967) were recorded, whereas only PT ratings were recorded during PH.

Venous blood samples were obtained at rest, immediately after (0 h Post), and four hours after cessation (4 h Post) of the passive and active heating trials. A 10 ml blood sample was collected via venepuncture from the ante-cubital vein in the non-dominant arm using a vacutainer system. The whole blood was treated with either EDTA or heparin and inverted. Heparinised blood was used to determine pH and ionized calcium (iCa^{2+}) using a blood gas analyser (AVL OMNI 4, Roche Diagnostics Ltd, Sussex). The EDTA-treated blood was used to measure BLa and glucose (YSI 2300 STAT+, YSI Inc., Yellow Springs, OH), leukocyte numbers and differential counts using the fluorescence flow cytometry method (Sysmex XT 2000, Sysmex UK Ltd, UK), and intracellular HSP70 and total protein levels (see below).

At each time-point, whole blood was diluted 1:1 with PBS, layered on to histopaque (10777, Sigma, UK), and centrifuged at 400 × *g* for 30 min. The PBMCs were isolated and washed with PBS at 400 × *g* for 10 min. The supernatant was aspirated and the PBMC pellet was re-suspended with HSP70 extraction reagent (Stressgen Biotechnologies, Canada) and protease inhibitors (Cocktail inhibitor set III, Calbiochem, Germany). The cell suspension was mixed, left on ice for 30 min and spun in a 4 °C refrigerated centrifuge at 21,000 × *g* for 30 min. 250 µl of the cell supernatant was then removed and frozen at −80 °C.

A sandwich enzyme linked immunosorbent assay (ELISA) was used to quantify the inducible HSP70 from the PBMC lysate. An HSP70 specific monoclonal antibody is pre-coated on the immunoassay plate (EKS-700, Stressgen Biotechnologies, Canada), from which inducible HSP70 can be quantified, without detecting the constitutive isoform (HSC70) and the glucose regulated (GRP78) proteins from the same 70 kDa family. The intra- and inter-assay coefficient of variation is <10%. The ELISA has been shown to be more sensitive and quantifiable compared to the Western Blotting technique (Milne and Noble, 2002), and also does not require user definable parameters, which is a limitation of flow cytometry techniques.

The HSP70 ELISA was carried out according to manufacturer's instructions. Absorbance readings were measured at 450 nm (Biotek Synergy HT-

Table 1. Resting physiological measures between the two experimental trials

	Thermoregulatory		Hydration		Metabolic	
	HR (beats · min^{-1})	T_c (°C)	USG	Body mass (kg)	BLa (mM)	GLU (mM)
AH	62 ± 4	37.0 ± 0.3	1.017 ± 0.011	76.7 ± 10.9	1.45 ± 0.65	4.18 ± 0.35
PH	61 ± 5	37.2 ± 0.2	1.012 ± 0.001	76.6 ± 10.2	1.28 ± 0.84	4.03 ± 0.95

AH Active heating; *PH* passive heating; *HR* heart rate; T_c core temperature; *BLa* blood lactate; *GLU* blood glucose

R, Biotek Instruments, Vermont, USA) and HSP70 concentrations were calculated by interpolating these readings from a 7-point standard curve (0.78–50 ng/ml). The absolute values of HSP70 were normalised per mg of total protein content, per PBMC. Leukocyte numbers and differential counts were determined using the fluorescence flow cytometry method (Sysmex XT 2000, Sysmex UK Ltd, UK). Leukocyte counts were adjusted for changes in PV that were calculated from hematocrit and haemoglobin concentrations (Dill and Costill, 1974). The total protein content of the supernatant was determined by the Bradford Method (1976). A 300 µl of Coomassie Plus Assay Reagent (Pierce Biotechnology Inc., IL, USA) was added to 10 µl of the samples and the absorbance was measured at 595 nm. Protein concentrations were calculated by interpolating the readings from albumin standards ranging from 100 to 1500 µg/ml.

Statistics

The SPSS package (version 12.0) was used to perform all statistical techniques. The normality of all data was assessed with the Shapiro–Wilk test. Sphericity was assessed using Mauchly's test, and where this assumption was violated, the Greenhouse-Geisser estimates of sphericity were employed. A two-way mixed factorial (time × trial) ANOVA was used to detect any interactions or main effects for the independent variables. Paired-samples *t*-tests were used to analyse changes in the physiological parameters over time, whilst any between trial differences were observed using independent samples *t*-test post hoc. Independent samples *t*-tests were also used to detect any differences in resting hydration, temperature and metabolic status. Data are presented as mean ± standard deviation, and the alpha level was set at $P \leq 0.05$.

Results

A comparison of resting physiological measures is provided in Table 1. There were no differences in resting thermoregulatory, hydration or metabolic variables. Ambient laboratory temperature and relative humidity was 19.7 ± 0.8 °C and 38.5 ± 3.2% with AH, but was higher during PH due to a combination of evaporation of hot water from the bath, coupled with the restricted space in the bathroom (temperature: 23.0 ± 1.8 °C; relative humidity: 78.2 ± 9.3%). In AH trials the subjects spent a total time of 62.8 ± 7.8 min exercising, which was significantly greater than the total time immersed in PH trials (48.8 ± 8.0 min; $P < 0.05$).

Thermoregulation

The T_c response and the T_c changes from rest across the 90 min trials are shown in Fig. 1. The PH increased the T_c quicker than AH, and T_c reached 39 °C after 21 ± 4 min in PH compared with 39 ± 6 min during AH ($P < 0.01$). In the AH trial, three subjects failed to reach a T_c of 39 °C at

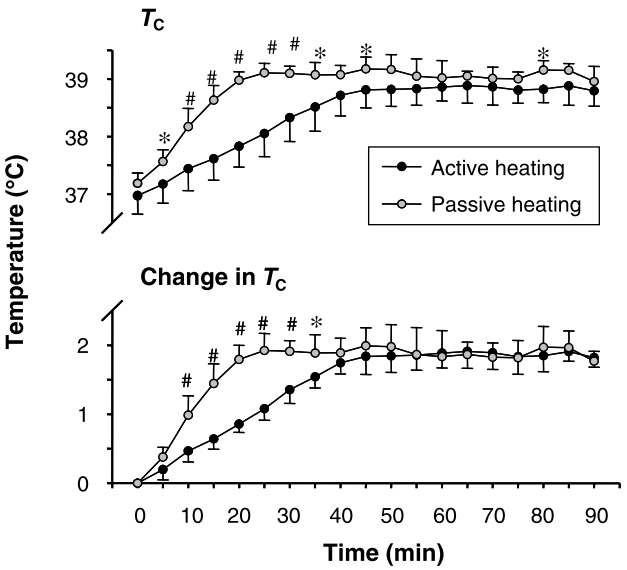

Fig. 1. Core temperature (top) and core temperature changes from rest during active and passive heating trials. ∗Denotes $P < 0.05$ between trials, # denotes $P < 0.01$ between trials. T_c core temperature

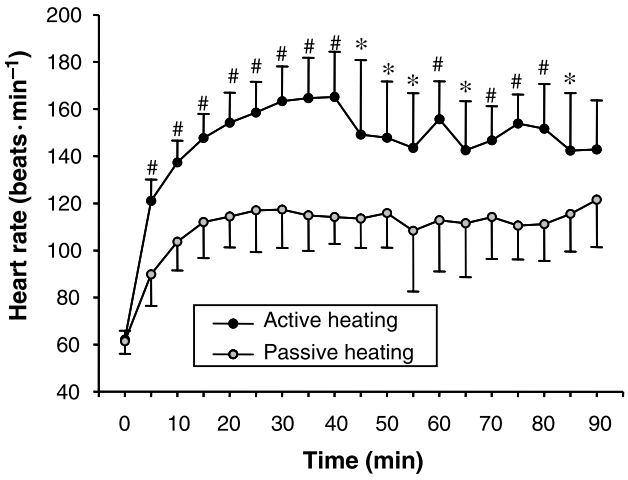

Fig. 2. The HR response to active and passive heating modes. ∗Denotes $P < 0.05$ between trials, # denotes $P < 0.01$ between trials. *HR* Heart rate

all during the 90 min, the average peak T_c of these individuals was 38.7 ± 0.1 °C. Once the critical T_c (CT_c) was achieved, the T_c rarely differed between the two heating modes; however, the higher rate of heating during PH lead to an increased exposure time at 39 °C (69 vs. 51 min).

Body weight changes did not differ between the two trials ($P > 0.05$). Subjects lost 1.5 ± 0.6 kg in AH, and 1.6 ± 0.2 kg during PH, which accounted for losses of 2.0 ± 0.9 and $2.1 \pm 0.5\%$ body mass, respectively. Plasma volume changes also did not differ between experimental conditions (AH: $-13.8 \pm 3.0\%$ vs. PH: $-11.4 \pm 3.8\%$; $P > 0.05$).

The HR was higher during AH ($P < 0.01$) as opposed to PH (149.3 ± 17.4 vs. 112.1 ± 16.4 beats $\cdot$ min^{-1}). In the AH trial a high level of circulatory strain was produced and HR peaked at 171.6 ± 16.5 beats $\cdot$ min^{-1}, thereafter when intermittent exercise was prescribed after the critical T_c was reached, average HR reduced to 147.6 ± 20.7 beats $\cdot$ min^{-1}. In contrast, after the critical T_c was attained during PH, intermittent immersion did not reduce the cardiovascular strain (see Fig. 2).

Metabolic

The VO_2 during AH averaged $65.3 \pm 4.6\%$ VO_{2max} until CT_c was obtained, thereafter during intermittent work, the workload decreased to $50.8 \pm 10.5\%$ VO_{2max}. The

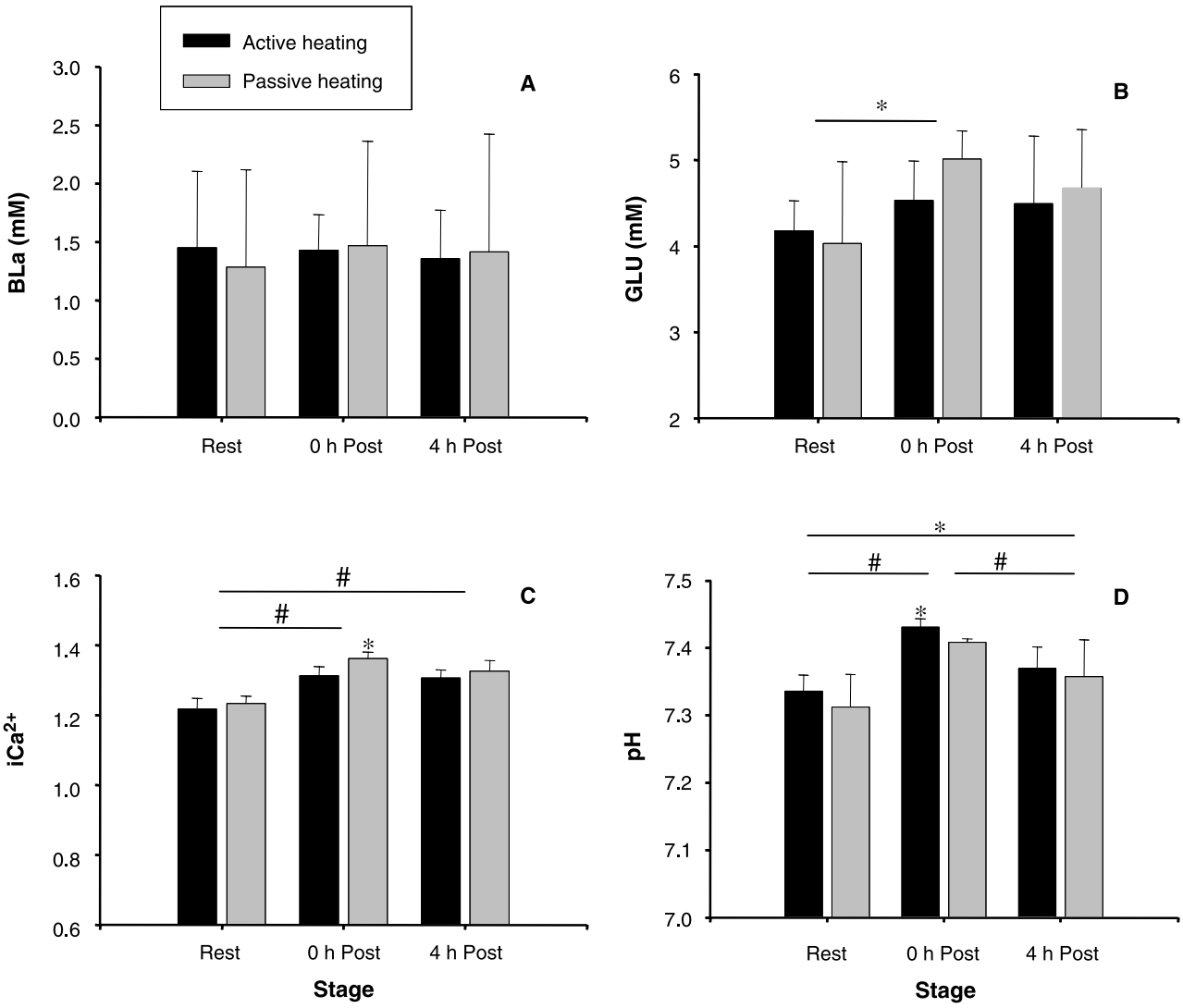

Fig. 3. Influence of active and passive heating modes on BLa (**A**), GLU (**B**), iCa^{2+} (**C**), and pH (**D**) at rest, 0 h and 4 h Post trials. $*$ Denotes $P < 0.05$, # denotes $P < 0.01$, horizontal lines represent differences between time-points. *BLa* blood lactate; *GLU* blood glucose; iCa^{2+} ionised calcium

Table 2. Influence of active and passive hyperthermia on WBC counts

Cell type		Time					
		Rest		0 h Post		4 h Post	
		AH	PH	AH	PH	AH	PH
Total WBC (10^9/L)		4.99 ± 1.03	5.40 ± 1.21	$10.48 \pm 1.9^{*,\ddagger}$	$7.76 \pm 1.69^{\ddagger}$	$9.30 \pm 1.01^{\ddagger}$	$8.05 \pm 1.67^{\ddagger}$
Granulocytes	#	2.89 ± 0.69	3.41 ± 1.02	$6.30 \pm 1.69^{\ddagger}$	$4.46 \pm 1.33^{\ddagger}$	$7.03 \pm 0.60^{*,\ddagger}$	$5.16 \pm 1.49^{\ddagger}$
	%	57.5 ± 3.4	61.9 ± 6.6	$67.2 \pm 7.7^{\ddagger}$	63.5 ± 7.4	$73.8 \pm 3.0^{*,\ddagger}$	65.1 ± 8.3
Lymphocyte	#	1.65 ± 0.32	1.54 ± 0.32	$2.35 \pm 0.47^{\ddagger}$	1.94 ± 0.41	$1.91 \pm 0.50^{\dagger}$	2.02 ± 0.51
	%	33.4 ± 3.4	29.0 ± 5.6	$26.3 \pm 6.9^{\ddagger}$	28.4 ± 5.8	$19.8 \pm 3.2^{\ddagger}$	$26.4 \pm 6.3^{*}$
Monocyte	#	0.45 ± 0.13	0.48 ± 0.08	$0.58 \pm 0.13^{\ddagger}$	$0.57 \pm 0.13^{\ddagger}$	$0.60 \pm 0.10^{\ddagger}$	$0.64 \pm 0.09^{\ddagger}$
	%	9.1 ± 2.0	9.2 ± 2.1	$6.5 \pm 1.6^{\ddagger}$	8.4 ± 2.7	$6.3 \pm 0.9^{\ddagger}$	8.5 ± 2.4

AH Active heating; *PH* passive heating; *WBC* white blood cell. # Denotes leukocyte sub-set count ($10^3/\mu$l); * denotes $P<0.05$ between trials; ‡ denotes $P<0.05$ from rest; † denotes $P<0.05$ from 0 h Post

changes in BLa, GLU, iCa^{2+}, and pH are shown in Fig. 3. The BLa was not increased by either AH or PH. The post GLU increased from resting levels in both trials ($P<0.05$), but there was no difference between AH and PH ($P>0.05$). iCa^{2+} increased after both AH and PH immediately after exposures ($P<0.01$), and remained elevated above baseline levels 4 h later ($P<0.01$). The PH had a greater concentration of iCa^{2+} than AH procedures immediately after the treatments ($P<0.05$). Blood pH increased after both heating modes ($P<0.01$), but was greater after AH ($P<0.05$). Although pH declined at 4 h Post, it remained higher than resting levels ($P<0.05$), but no differences between trials was observed.

RPE and PT

The RPE increased steadily over time in AH trials ($P<0.01$) until the CT_c was reached, after which RPE reached a plateau. The PT increased over time in both trials ($P<0.01$), and AH had greater ratings of PT in comparison with PH (AH: 6.6 ± 0.7 vs. PH: 6.0 ± 0.8; $P>0.05$).

WBC count and leukocyte differentials

The absolute and relative white blood cell (WBC) and differential leukocyte counts were not different at rest between the two experimental trials (see Table 2). A marked leukocytosis was observed after both methods of heating ($P<0.01$), and remained elevated above resting conditions 4 h Post ($P<0.01$). The AH procedures induced a greater WBC count immediately after exposure. The increased WBC count observed was accompanied by granulocytosis, lymphocytosis and monocytosis, which also persisted 4 h Post trials ($P<0.01$). The AH induced a greater proliferation of WBCs at the end of the exposure ($P<0.05$), and had a greater granulocyte count at 4 h Post ($P<0.05$), in comparison with PH. No other differences existed in leukocytes or their subsets between trials ($P>0.05$). The relative amounts of granulocytes increased with hyperthermia ($P<0.05$), and therefore the percentages of lymphocytes and monocytes were generally reduced ($P<0.05$).

HSP70

Hyperthermia (39 °C) did not statistically increase the mean intracellular stock of HSP70 in either passive or active heating methods ($P>0.05$). Immediately after trials HSP70 was 1.0 ± 0.9 and 1.8 ± 2.2 fold of resting levels in AH and PH, respectively (see Figs. 4 and 5). Four hours

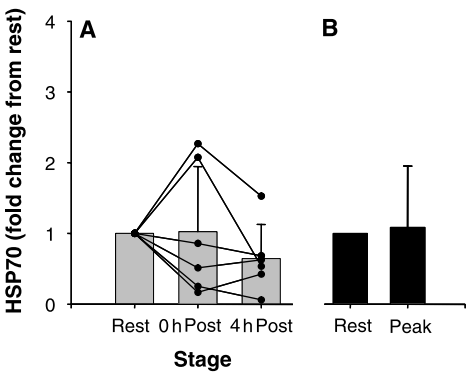

Fig. 4. HSP70 expression in PBMCs at rest, 0 h and 4 h Post active heating in vivo to 39 °C. Data are presented for each individual (line) and as group mean ± SD (bar). Data expressed relative to the resting sample for each subject

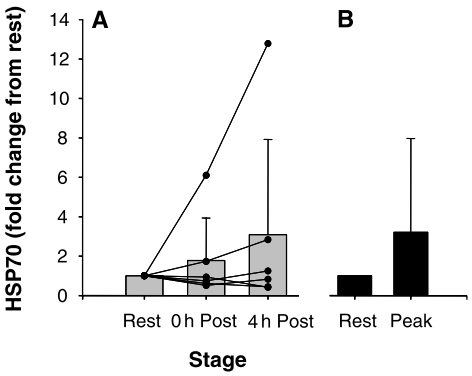

Fig. 5. HSP70 expression in PBMCs at rest, 0 h and 4 h Post passive heating in vivo to 39 °C. Data are presented for each individual (line) and as group mean ± SD (bar). Data expressed relative to the resting sample for each subject

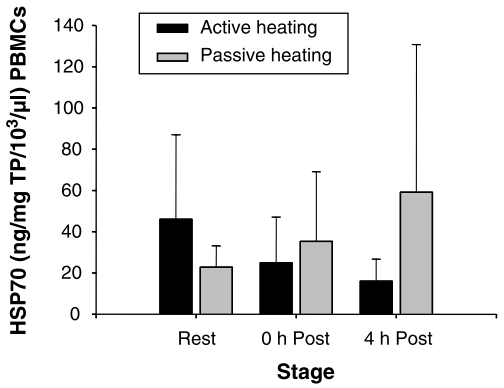

Fig. 6. HSP70 expression at rest, 0 h and 4 h Post passive and active heating protocols

after AH, HSP70 was 0.6 ± 0.5 fold of baseline levels, whereas in PH HSP70 was 3.1 ± 4.8 fold with respect to resting levels. However, no statistical difference was found either between trials or time-points, either when HSP70 was expressed as a fold change from baseline, or as absolute levels normalised per mg of total protein, per $10^3/\mu$l PBMC (see Fig. 6; $P > 0.05$).

Discussion

The aim of the current study was to delineate the effects of hyperthermia and exercise on the accumulation of HSP70 in circulating PBMCs. Passive and active heating strategies that elevated T_c to 39 °C did not significantly increase the mean intracellular HSP70 stock. The HSR appeared to be individual specific, with a small number of subjects showing a strong HSP response to the treatments, whilst the majority demonstrated no induction of HSP70. The individual specific nature of the HSR in leukocytes has been observed previously (Ryan et al., 1991; Fehrenbach et al., 2000b; Morton et al., 2006), including in our laboratory, from PBMCs exposed to in vitro heat shock (Lovell et al., 2007).

The current observations that exercise and hyperthermia did not induce a HSR is in contrast to previous work (Fehrenbach et al., 2000a, 2001; Oehler et al., 2001). Subjects passively heated via warm water immersion to a tympanic temperature of 39 °C, increased HSP70 in all leukocyte subsets by 15–60% (Oehler et al., 2001). In addition, endurance exercise increased cytoplasmic HSP70 expression immediately after the bout, and remained upregulated for 24 h (Fehrenbach et al., 2000a, b).

The role of internal temperature on the HSP70 response to exercise has been investigated in animals (Skidmore et al., 1995; Ruell et al., 2004). The magnitude of HSP70 induction has been shown to be correlated with the thermal load (Walters et al., 1998; Ruell et al., 2004), and is dependent upon the intensity of the passive heating protocol (Flanagan et al., 1995). This certainly suggests a role of temperature on the HSR, but studies investigating the role of temperature per se have reported conflicting results. Skidmore et al. (1995) exposed male adult rats to passive and active heating protocols in different ambient temperatures (36–42 vs. ~14 °C). Although heating per se increased HSP70 expression in soleus, gastrocnemius and left ventricle tissues, HSP70 increases were greater in the active heating trials and exercise in the cold. These findings suggest that factors associated with exercise have a greater effect on the HSR than the independent influence of heat. In contrast, Sprague-Dawley rats exposed to acute (Kim et al., 2004), and chronic (Harris and Starnes, 2001) exercise, demonstrated a HSR in myocardial tissue when the activity was accompanied by increases in T_c. Yet, when these regimes were undertaken in cool rooms (4–11 °C) to attenuate the exercise-associated rise in T_c, HSP70 was not inducted. Walters (1998) found that although HSP70 increased similarly with both active and passive heat stress, HSP70 was correlated with brain temperature. Furthermore, AH in moderate ambient temperatures (~24 °C) did not evoke a central HSP70 response, and exercise per se did not increase HSP70 in the brain without hyperthermia (Walters et al., 1998).

In humans, subjects that exercised at 90% of the individual anaerobic threshold for 60 min, showed a greater and more prolonged HSP70 response in leukocytes when the activity was performed in warm (28 °C) as opposed to moderate (18 °C) ambient temperatures (Fehrenbach et al., 2003). Whereas, subjects exercising at a low intensity

(walking at 4–6 $km \cdot h^{-1}$) for 2 h in the heat only mildly increased HSP70 expression (Ryan et al., 1991). However, to our knowledge the current study is the first to attempt to delineate the effects of temperature and exercise on the HSR in human subjects. The data presented here do not support the hypothesis that temperature per se is responsible for the HSP70 induction. Moreover, the finding that prolonged moderate intensity exercise did not evoke a mean HSR in leukocytes, is contradictory to the majority (Fehrenbach et al., 2000a, b, 2001; Neiss et al., 2002), but not all (Shastry et al., 2002) of the previous work. The reason for this disparity could be due to a number of factors, including the muscle, organ or cell type examined, training status, exercise intensity, large inter-individual variability, and the method of quantifying HSP70. The majority of previous studies have utilised western blotting or flow cytometry techniques, which are not as sensitive or quantifiable as the ELISA technique which was used in the current study.

The current findings suggest that HSP70 was not synthesised in response to the stresses induced by the experimental trials. The reason for this is unclear, given that the intensity of the thermal stress prescribed in the PH trials was sufficient to induce a HSR in previous work (Oehler et al., 2001). However, whilst the degree of granulocytosis and monocytosis was similar to that observed by Oehler et al. (2001), our subjects did not demonstrate lymphopenia which suggests that the current trials induced a lower inflammatory response, which in turn may not require an increase in HSP70 synthesis. The same trends were also denoted when comparing the leukocyte response from the AH trials with other studies that administered higher exercise intensities (Fehrenbach et al., 2000a, b, 2001; Neiss et al., 2002). Therefore, perhaps the level of stress endured in this study was comparatively low, and if the exercise and its associated hyperthermia were within normal stress ranges for the moderately trained individuals, the stimulus for HSP70 production in PBMCs may have been absent. Given that the magnitude of the HSR is intensity dependent (Liu et al., 1999; Milne and Noble, 2002), this hypothesis seems rational. Alternatively, HSP70 may have been released into the extracellular environment via exocytosis (Hunter-Lavin et al., 2004), the rate of which has been suggested to increase with elevated temperatures (Hunter-Lavin et al., 2004), in the presence of adrenaline and noradrenaline (Fleshner et al., 2003), and/or via cell death (Basu et al., 2000). As such, a number of studies have measured HSP70 in the serum (Walsh et al., 2001; Banfi et al., 2004; Febbraio et al., 2004; Lancaster et al., 2004). However, during exercise it would be unclear which tissues contribute to the extracellular HSP given that some have high constitutive levels and they can accumulate from brain (Lancaster et al., 2004), and hepatosplanchnic release (Febbraio et al., 2004). Furthermore, HSP's can be released from the cell via exocytosis (Fleshner et al., 2003; Hunter-Lavin et al., 2004), and if the cell dies a necrotic or lytic death (Basu et al., 2000). Therefore increased extracellular HSP's may occur without de novo synthesis and measurements from the serum should be interpreted with caution. In contrast, the concentration of intracellular HSP70 represents the constitutive and inducible isoforms synthesised by the cell to serve protective functions, and can be used as a biomarker of thermal history (Ryan et al., 1991; Fehrenbach et al., 2001).

The constitutive expression of HSP70 is considered to be approximately 2-fold greater in granulocytes as opposed to monocytes (Fehrenbach et al., 2000b; Oehler et al., 2001), with lymphocytes expressing a very low levels (Oehler et al., 2001). After exposure to a stressor, monocytes have the strongest inducible response, whilst lymphocytes and granulocytes only respond to intense stress (Oehler et al., 2001). Quantification of HSP70 concentrations from granulocytes is confusing since it is unclear how granulocytosis influences the HSR. Therefore, in the current study, HSP70 concentrations of PBMCs were measured since we did not expect changes in PBMC counts. Indeed, there were no differences between the trials in the number of monocytes and lymphocytes. Lymphocytes show low levels of HSP70 up-regulation, even during intense stress (Oehler et al., 2001), and any de novo lymphocyte HSP70 synthesis may be off-set by their ability to release HSP70 into the serum (Hunter-Lavin et al., 2004). Therefore any changes in PBMC HSP70 levels in this study was the product of the highly inducible HSR in monocytes, and the release of these proteins by lymphocytes.

During active and passive heat stress only two subjects in each trial appeared to show a HSR, one subject demonstrated a response in both trials, although it was greater with PH (12.8 vs. 1.5 fold increase at 4 h). Although not statistically significant, the greater induction of HSP70 after PH might have been caused by the greater exposure time experienced at 39 °C (68 ± 4 vs. 51 ± 5 min). Acidosis causes a cellular stress response (Gapen and Moseley, 1995) yet in the current study blood pH was increased by both trials, possibly cause by respiratory-induced alkalosis. The iCa^{2+} also increased with both exposures although it was higher after PH, and this has also been demonstrated to induce a HSR (Kiang et al., 1994). Oxi-

dative stress (Benjamin et al., 1990) and glucose depletion (Febbraio et al., 2004) are also known to induce HSR, however in the current study BLa did not increase above baseline levels and GLU depletion did not occur. Therefore it is unlikely that any changes in BLa, GLU, iCa^{2+} and pH were responsible for any induction of HSP70.

The reason for the large inter-individual response observed here is unclear and should be interpreted with caution given the small sample size used in the current study. Nonetheless, in both the AH and PH trials, the intermittent exercise/immersions were different between subjects whilst attempting to maintain T_c at $\geq 39\,^{\circ}C$. During the AH trial three subjects did not attain a T_c of $39\,^{\circ}C$, although the change in T_c was not different between trials (see Fig. 1). In PH, the thermoregulatory response was much more consistent, yet there was no consistent up-regulation of HSP70. Taken together, this suggests that mechanisms other than hyperthermia may have been responsible for any up-regulation of HSP70 in the 'responders'. Alternatively, the inter-individual response may be indicative of the individuals' level of TT or pre-conditioning. This variation has been a feature of HSP70 in human exercise studies (Ryan et al., 1991; Fehrenbach et al., 2000b; Khassaf et al., 2001; Morton et al., 2006). The animal HSP70 response to exercise has been much more consistent, perhaps because humans have relatively higher constitutive expression which may require a greater intensity or frequency of stressor to induct stress proteins. Prior exposure to a thermal stressor has been shown to impair the HSR to a secondary challenge (Ryan et al., 1991), which suggests that HSP kinetics could be used as assays of thermal history, and that HSP's might be responsible for regulating their own synthesis. The individual response patterns observed might be indicative of their capacity to tolerate high body temperatures, but further work is required in this area.

In summary, we aimed to delineate the effects of temperature and physical exercise on the HSR. Active and passively-induced hyperthermia ($39\,^{\circ}C$) did not evoke a consistent intracellular HSP70 induction from circulating PBMCs. The findings are contradictory to the majority of previous work, and could be due to many factors such as training status, exercise duration and intensity, species, muscle or cell type examined, and the method of HSP70 quantification. Therefore further work is warranted to classify the cell-type specific stress response to hyperthermia, and to determine if HSP70 can be used as a biomarker of thermal history and/or tolerance given that the HSP70 response is highly individual specific.

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