Downhill running and exercise in hot environments increase leukocyte Hsp72 (HSPA1A) and Hsp90 α (HSPC1) gene transcripts

James A. Tuttle,¹ Paul C. Castle,¹ Alan J. Metcalfe,^{1,4} Adrian W. Midgley,³ Lee Taylor,^{1*} and Mark P. Lewis^{2*}

¹Muscle Cellular and Molecular Physiology Research Group, Institute of Sport and Physical Activity Research, Department of Sport Science and Physical Activity, University of Bedfordshire, Bedford, United Kingdom; ²National Centre for Sport and Exercise Medicine, School of Sport, Exercise and Health Sciences, Loughborough University, Loughborough, United Kingdom; ³Department of Sport and Physical Activity, Edgehill University, Ormskirk, United Kingdom; and ⁴School of Exercise and Health Sciences, Edith Cowan University, Perth, Australia

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Tuttle JA, Castle PC, Metcalfe AJ, Midgley AW, Taylor L, Lewis MP. Downhill running and exercise in hot environments increase leukocyte Hsp72 (HSPA1A) and Hsp90 α (HSPC1) gene transcripts. *J Appl Physiol* 118: 996–1005, 2015. First published February 26, 2015; doi:10.1152/jappphysiol.00387.2014.—Stressors within humans and other species activate Hsp72 and Hsp90 α mRNA transcription, although it is unclear which environmental temperature or treadmill gradient induces the largest increase. To determine the optimal stressor for priming the Hsp system, physically active but not heat-acclimated participants (19.8 ± 1.9 and 20.9 ± 3.6 yr) exercised at lactate threshold in either temperate (20°C , 50% relative humidity; RH) or hot (30°C , 50% RH) environmental conditions. Within each condition, participants completed a flat running (temperate flat or hot flat) and a downhill running (temperate downhill or hot downhill) experimental trial in a randomized counterbalanced order separated by at least 7 days. Venous blood samples were taken immediately before (basal), immediately after exercise, and 3 and 24 h postexercise. RNA was extracted from leukocytes and RT-quantitative PCR conducted to determine Hsp72 and Hsp90 α mRNA relative expression. Leukocyte Hsp72 mRNA was increased immediately after exercise following downhill running (1.9 ± 0.9 -fold) compared with flat running (1.3 ± 0.4 -fold; $P = 0.001$) and in hot (1.9 ± 0.6 -fold) compared with temperate conditions (1.1 ± 0.5 -fold; $P = 0.003$). Leukocyte Hsp90 α mRNA increased immediately after exercise following downhill running (1.4 ± 0.8 -fold) compared with flat running (0.9 ± 0.6 -fold; $P = 0.002$) and in hot (1.6 ± 1.0 -fold) compared with temperate conditions (0.9 ± 0.6 -fold; $P = 0.003$). Downhill running and exercise in hot conditions induced the largest stimuli for leukocyte Hsp72 and Hsp90 α mRNA increases.

heat shock response; exercise heat stress; thermotolerance

THE HEAT SHOCK PROTEIN (HSP) system has a crucial role in acquired thermotolerance via the protein chaperone (22) and antiapoptotic functions (3) of HSP72 (commonly known as HSPA1A) and HSP90 α (HSPC1). Modulation of kinase signaling along with assembly of gene transcription and protein translation machinery by HSP90 α is also a crucial aspect of both the cellular stress response and cellular adaptation to exercise heat stress (54, 61). These functions help attenuate the pathophysiological events (tissue damage and the systemic

inflammatory response syndrome) associated with multiorgan failure syndrome, which is central within exercise heat stress and exertional heat illness-specific morbidity and mortality (13). Both HSP72 and HSP90 α protein concentrations are elevated proportionally to increased cellular stress (increased cellular temperature) following ex vivo heat shock (39). Therefore, elucidating the in vivo cellular stressor(s), which induces the largest Hsp72 and Hsp90 α mRNA, increases could indicate the stressor(s) most likely to elevate HSP72 and HSP90 α protein concentrations and, thus, potentially attenuate exertional heat illness risk by developing a thermotolerant phenotype. This thermotolerance, from a whole body perspective, may include delaying thermal injury (33) by elevating the core body temperature during exercise heat stress at which exercise becomes physiologically limited (1). For practical purposes, this could enhance athletic performance and extend occupational pursuits (fire-fighting, industrial work, and military training or operations) in challenging (hot and humid) environments.

Induction of Hsp72 mRNA occurs following exercise (69), exercise-induced muscle damage (EIMD) (52), and exercise heat stress (44), while Hsp90 α mRNA is induced after exercise (12, 38) and exercise heat stress (44). These stressors were measured in isolation, precluding interstudy comparison because exercise intensity (34) and duration (58), environmental conditions (40), participant training state (46), sampling time courses, tissue of interest, and measurement techniques [Northern blot analysis, RT-quantitative PCR (RT-QPCR), and gene arrays] that were used to measure Hsp72 and Hsp90 α mRNA were not standardized. Consequently, the most appropriate stressor or combination of stressors (e.g., downhill running and exercise heat stress) to increase Hsp72 and Hsp90 α mRNA transcription (and, thus, potentially enhance thermotolerance via elevated basal HSP72 and HSP90 α protein concentrations) following an acute preconditioning stressor was unknown.

Therefore, the primary purpose of the current study was to determine the environmental temperatures and treadmill gradients (singularly and in combination; downhill running in a hot environment) that induced the largest leukocyte Hsp72 and Hsp90 α mRNA increases. The current study also aimed to determine the physiological [rectal temperature (T_{re}) and heart rate (HR)] and perceptual responses (delayed onset muscle soreness, DOMS) to each condition to determine the physiological stressors associated with Hsp72 and Hsp90 α mRNA induction. It was hypothesized that downhill running

* L. Taylor and M. P. Lewis are to be considered senior authors of this article.

Address for reprint requests and other correspondence: J. A. Tuttle, Muscle Cellular and Molecular Physiology Research Group, Institute of Sport and Physical Activity Research, Dept. of Sport Science and Physical Activity, Univ. of Bedfordshire, Bedford, United Kingdom (e-mail: james.tuttle1987@gmail.com).

Table 1. Participant demographic characteristics

	Temperate Group (TEMP)	Hot Group (HOT)	Group Significance ($P < 0.05$)
Age, yr	19.8 ± 1.9	20.9 ± 3.6	$P = 0.563$
Height, m	1.8 ± 0.1	1.74 ± 0.05	$P = 0.398$
Body weight, kg	71.7 ± 7.4	70.9 ± 10.4	$P = 0.880$
$\dot{V}O_{2\max}$, ml·kg ⁻¹ ·min ⁻¹	55.6 ± 5.7	55.5 ± 5.3	$P = 0.991$
% Lean mass	87.0 ± 8.1	87.2 ± 5.5	$P = 0.507$
% Body Fat	13.0 ± 8.1	12.8 ± 5.5	$P = 0.951$
Training, hr/wk	2.4 ± 0.8	2.6 ± 0.5	$P = 0.689$

Values are expressed as means ± SD. $\dot{V}O_{2\max}$, maximum oxygen uptake.

and exercise within hot environmental conditions would offer the greatest stimuli to increase Hsp72 and Hsp90α mRNA. Therefore, downhill running in a hot environment would be the best condition to induce Hsp72 and Hsp90α mRNA transcription.

METHODS

Ethical Approval

The protocol was approved by the University of Bedfordshire's Sport and Exercise Science Departmental Human Ethics Committee, and all participants signed informed consent in accordance with the ethical standards outlined in the 1964 Declaration of Helsinki.

Participants

Demographic variables were recorded in a population of 14 male Caucasian participants who were team game players and were non-smokers (see Table 1). All of the participants were not accustomed to downhill running or regular eccentric exercise and were not heat-acclimated [testing conducted between December and March within the United Kingdom; average temperature range was 1.5°C–7.9°C (41)]. Body mass (kg) and height (cm) were measured using mechanical scales (Weylux Marsden 424, London, UK) and a stadiometer (Harpender HAR-98.602, Crymych, UK), respectively. Body composition was measured using air displacement plethysmology (Bod Pod 2000A, Cranlea, UK). The lactate threshold (LT) and maximum oxygen uptake ($\dot{V}O_{2\max}$) were determined using a graded treadmill test (30). This test consisted of 6–8 incremental 3-min stages at a 1% gradient. Participants started running at 8–9 km/h, and running velocity was increased by 1 km/h per stage until exhaustion (30). Finger tip capillary blood samples (40 µl) were taken at rest and at the end of each 3-min stage to determine blood lactate concentrations (B[La]). Blood lactate concentrations were plotted against running velocity to determine LT, which was defined as the first sustained B[La] increase above baseline (30). Pulmonary gas exchange was measured breath by breath using an online gas analysis system (Cortex Metalyser 3B, Biophysik, Leipzig, Germany) to determine changes in oxygen uptake ($\dot{V}O_2$) with the highest $\dot{V}O_2$ attained over a 30-s period accepted as $\dot{V}O_{2\max}$ (30).

Participants ran at the running velocity, which elicited their individualized LT during the graded treadmill test throughout the flat running trials. For the downhill running trials, participants exercised at the running velocity that required the same oxygen uptake as running at the LT during flat running (1% gradient). This was determined during familiarization and was typically 3–3.5 km/h faster than LT on a 1% gradient. This velocity was maintained throughout each downhill running trial. This difference between flat and downhill running is similar to that found in previous literature (51).

Sample size calculations were determined a priori (G.Power 3.1, Universität Dusseldorf, Germany) (14) for exercising T_{re} , HR (31) and Hsp72 mRNA (49) using data from previous papers. Two-tailed tests with alpha set at 0.05 and power at 0.8 suggested that a sample

size of six was required within each group to detect significant ($P < 0.05$) differences in exercising T_{re} (0.8°C) and HR (7 beats/min) between environmental conditions and an Hsp72 mRNA increase of 489% between basal (immediately before exercise) and immediately after exercise.

Experimental Design

Participants were split into two groups that exercised in different environmental conditions (see Fig. 1).

The temperate environmental condition featured two experimental trials separated by 7 days: 1) the temperate flat (TEMP_{FLAT}) experimental trial involved 30 min of running at the LT on a 1% gradient in 20°C, 50% RH; and 2) the temperate downhill (TEMP_{DOWN}) experimental trial involved 30 min downhill running at the LT on a –10% gradient to induce muscle damage (14) in 20°C, 50% RH.

The hot environmental condition featured two experimental trials separated by 7 days: 1) The hot flat (HOT_{FLAT}) experimental trial involved 30 min of running at the LT on a 1% gradient in 30°C, 50% RH; and 2) The hot downhill (HOT_{DOWN}) experimental trial, which involved 30 min of downhill running at the LT on a –10% gradient to induce muscle damage (14) in 30°C, 50% RH.

A counterbalanced experimental design was used in which the experimental trials were completed in a randomized order, at the same time of day and at the running velocity, which elicited the LT to minimize differences in metabolic strain between individuals (6). However, environmental temperature-mediated differences still remained as relative exercise intensity is higher at the same velocity during exercise in hot environments (35). The confounding variables of caffeine and alcohol (72 h), nonsteroidal anti-inflammatory medications (48 h) (48, 65), dietary supplementation (vitamins, ergogenic aids; 30 days), exercise (7 days) (45), nonexercise-based thermal stressors (3 mo), and hypoxic and hyperbaric stressors (3 mo) were controlled for via abstinence prior to testing and throughout the testing period. A questionnaire was administered prior to each testing session and sampling time point to determine adherence to the aforementioned experimental control measures. Adherence was 100% in all participants. Participants were instructed to drink 500 ml of water 2 h before each experimental trial in line with the American College of Sports Medicine position stand (55) and recent work in the field (26, 27). Hydration status was assessed via urine osmolality (UOsm). All

Fig. 1. Experimental design. HOT_{DOWN} (hot downhill), HOT_{FLAT} (hot flat), LT (lactate threshold), TEMP_{DOWN} (temperate downhill), and TEMP_{FLAT} (temperate flat).

participants were euhydrated [UOsm was below 600 mosmol/kgH₂O (2)] prior to all experimental trials.

Physiological Measures

Rectal temperature (T_{re}) was continuously measured using a rectal thermistor (Henleys 400H/4491H, Welwyn Garden City, UK) inserted 10 cm past the anal sphincter. The signal was amplified and measured using a temperature monitor (ET402; Libra Medical, Reading, UK). Participants were removed from the environmental chamber if exercising T_{re} reached the ethical safety limit of 39.7°C or T_{re} increased by 2°C from resting value, as recommended by the institutional ethical board. Heart rate was recorded continuously (Cortex Metalyser 2B, Biophysik, Leipzig, Germany) from a Polar telemetric HR monitor (attached to the chest), while ratings of perceived exertion (RPE) (9) and thermal sensation (TS) (70) were measured at rest and every 5 min during experimental trials. Pulmonary gas exchange was measured breath by breath using an online gas analysis system (Cortex Metalyser 2B; Biophysik, Leipzig, Germany) to measure changes in oxygen uptake ($\dot{V}O_2$) and was averaged over 5-s periods. The average of six 5-s periods (30 s total) from 15 s before to 15 s after each time interval was taken as the oxygen uptake at that time interval. Fingertip capillary whole blood samples were obtained in duplicate before (basal) and immediately after exercise to determine B[La] (Analox, Stokesley, UK; coefficient of variation, 2.65%).

Measurements of Delayed Onset Muscle Soreness

Noninvasive measures of DOMS were recorded immediately before (basal), immediately postexercise, 3 h postexercise, and 24 h postexercise to infer whether EIMD had occurred. Quadriceps tenderness (QT) using an analog force gauge was measured in accordance with a previously validated method (10). This method had an intratester intraclass correlation coefficient (ICC) of 0.76 (10). Sampling locations were always within the belly of each muscle as distal and proximal locations have a lower pain threshold at rest and a more variable tenderness response (5). Perceived muscle soreness was measured using the visual analog scale of pain (VAS) and was assessed along a 10-cm scale (0 = no soreness, 10 = extreme soreness). The VAS has previously been validated as a reliable measure (ICC of 0.97) of muscular pain (8).

Molecular Physiology Measures

Leukocyte isolation and RNA extraction. Venous blood was obtained from the antecubital region into a 6-ml EDTA tube immediately before (basal), immediately postexercise, 3 h postexercise, and 24 h postexercise. Using an adaptation of a previously validated method (62), we pipetted 500 μ l of venous blood into 10 ml of 1 in 10 red blood cell lysis solution (10 $\times$ red blood cell lysis solution; Miltenyi Biotec, Surrey, UK). Samples were incubated for 15 min at room temperature and then isolated via centrifugation at 400 g for 5 min and washed twice in 2 ml of PBS at 400 g for 5 min. The TRIzol method was then used to extract RNA from the leukocytes, in accordance with manufacturer's instructions (Invitrogen; Life Technologies, Carlsbad, CA). Quantity was determined at an optical density of 260 nm, while quality was determined via the 260/280 and

260/230 ratios using a Nanodrop spectrophotometer (Nanodrop 2000c; Thermo Scientific, Horsham, UK).

One-step RT-quantitative PCR. Primers (see Table 2) were designed using primer design software (Primer Quest and Oligoanalyzer-Integrated DNA technologies). During primer design, sequence homology searches were performed against the GenBank database to ensure the primers matched the gene of interest. Primers were designed to span exon-intron boundaries and avoided three or more guanine-cytosine bases within the last five bases at the 3' end of primer to avoid nonspecific binding. Further searches were performed to ensure primers did not contain secondary structures and intermolecular or intramolecular interactions (hairpins, self-dimer, and cross dimers), which can inhibit product amplification. Relative Hsp mRNA expression was then quantified using RT-QPCR. Reactions (20 μ l) containing 10 μ l of SYBR Green RT-PCR Mastermix (Quantifast SYBR Green kit; Qiagen, Manchester, UK), 0.15 μ l of forward primer, 0.15 μ l of reverse primer, 0.2 μ l of reverse transcription mix (Quantifast RT Mix, Qiagen), and 9.5 μ l sample (70 ng RNA/ μ l) were prepared using the Qiagility automated pipetting system (Qiagen). Each reaction was amplified in a thermal cycler (Rotorgene Q, Qiagen) and involved reverse transcription lasting 10 min at 50°C and a transcriptase inactivation and initial denaturation phase lasting 5 min at 95°C. The PCR reaction then followed with a denaturation step lasting 10 s at 95°C and a primer annealing and extension stage lasting 30 s at 60°C repeated for 40 cycles. Fluorescence was measured following each cycle as a result of the incorporation of SYBR Green dye into the amplified PCR product. Melt curves (50 to 95°C; Ramp protocol, 5-s stages) were analyzed for each reaction to ensure only the single gene of interest was amplified.

The relative quantification of mRNA expression for each sample was assessed by determining the ratio between the cycle threshold (C_T) value of the target mRNA and the C_T values for β_2 -microglobulin. Fold change in relative mRNA expression was calculated using the $2^{-\Delta\Delta C_T}$ method (57). Because HSP72 and HSP90 α protein concentrations were not measured and thermotolerance was not tested, the superior experimental trial to confer HSP72- and HSP90 α -mediated thermotolerance cannot be determined. However, measurement of Hsp72 mRNA and Hsp90 α mRNA responses provides an indication that the heat shock response has been activated and, therefore, suggests the experimental trial most likely to confer HSP72- and HSP90 α -mediated thermotolerance.

Statistical Analysis

Central tendency and dispersion are reported as the mean and standard deviation for normally distributed data and as the median and interquartile range for nonnormally distributed data. Statistical analysis was completed using linear mixed models for repeated measures (IBM SPSS 19.0, Chicago, IL). The best fitting covariance structure was identified by minimizing the Hurvich and Tsai's criterion (20). Changes in Hsp72 and Hsp90 α mRNA are presented as a fold change from basal in accordance with previous literature (52). Where significant F ratios for main and interaction effects occurred, post hoc comparisons were made with Bonferroni-adjusted P values. Pearson correlation coefficients were determined to elucidate relationships

Table 2. *Primer Sequences*

Gene	NCBI Accession Number	Primer	Sequence (5' $\rightarrow$ 3')	Amplicon Length
β_2 -Microglobulin (β_2 -M)	NM_004048	Forward	CCGTGTGAACCATGTGACT	91
		Reverse	TGCGGCATCTTCAAACCT	
Hsp72	NM_005345	Forward	CGCAACGTGCTCATCTTTGA	198
		Reverse	TGCTTGTCTGGCTGATGT	
Hsp90 α (variant 1 and variant 2)	NM_001017963 & NM_005348	Forward	AAACTGCGCTCCTGTCTTCT	180
		Reverse	TGCGTGATGTGTGCTCATCT	

NCBI, National Center for Biotechnology Information.

Fig. 2. Perceived muscle soreness (visual analog scale of pain, VAS) mean $\pm$ SD immediately before, immediately postexercise, 3 h postexercise, and 24 h postexercise. ^AVAS increased significantly ($P < 0.005$) during downhill running compared with flat running. ^BVAS increased significantly ($P < 0.001$) in hot compared with temperate conditions.

between exercising T_{re} , VAS, QT, Hsp72, and Hsp90 α mRNA. Correlation strength was determined via Cohen's threshold (11). Statistical significance was accepted at $P < 0.05$ (two-tailed).

RESULTS

Perceived Muscle Soreness Response

VAS (Fig. 2) increased following downhill running immediately after exercise, 3 h postexercise, as well as 24 h postexercise ($P < 0.001$), and during flat running immediately postexercise and 24 h postexercise ($P < 0.006$) compared with basal. Increased VAS was observed following downhill running compared with flat running immediately postexercise, 3 h postexercise, and 24 h postexercise ($P < 0.005$). Increased VAS was also observed following hot ($P < 0.001$) and temperate ($P < 0.016$) conditions immediately postexercise, 3 h postexercise, and 24 h postexercise compared with basal and in hot compared with temperate conditions immediately postexercise ($F = 6.2$; $P = 0.020$). The interaction between environmental condition, treadmill gradient, and time had no effect on VAS ($P > 0.05$). Therefore, both downhill running and hot conditions increased VAS, with downhill running appearing to cause the largest increase.

Like VAS, quadriceps tenderness (QT; Fig. 3) increased during downhill running at 3 h ($P = 0.001$) and 24 h postexercise ($P < 0.002$) compared with basal. However, during flat running, QT only increased 24 h postexercise ($P = 0.010$) compared with basal. Environmental temperature and all other interactions had no effect on QT ($P > 0.05$). Therefore, downhill running was the main factor that induced QT.

Physiological Responses

Exercising T_{re} (Fig. 4) increased during downhill running compared with flat running between 10 and 30 min ($P < 0.006$). There was also a trend for exercising T_{re} to be increased in hot ($38.9 \pm 0.4^\circ\text{C}$) compared with temperate conditions ($38.6 \pm 0.5^\circ\text{C}$) at 30 min ($F = 4.0$; $P = 0.068$). Increases at 30 min during downhill running ($39.0 \pm 0.4^\circ\text{C}$) compared with flat running ($38.5 \pm 0.4^\circ\text{C}$; 0.5°C) were

Fig. 3. Quadriceps tenderness (QT) means $\pm$ SD immediately before, immediately postexercise, 3 h postexercise, and 24 h postexercise. *QT increased significantly ($P = 0.010$) during flat running compared with basal. **QT increased significantly ($P < 0.002$) during downhill running compared with basal. ^AQT increased significantly ($P < 0.001$) during downhill running compared with flat running.

greater than during hot compared with temperate environmental conditions (0.3°C), indicating downhill running had a greater effect on exercising T_{re} than hot environmental temperatures. Exercising T_{re} ($39.2 \pm 0.2^\circ\text{C}$) increased at 30 min of HOT_DOWN; however, the interaction between environmental condition, treadmill gradient, and time did not reach significance ($P > 0.05$). Heart rate (Table 3) increased over time ($F = 318.0$; $P < 0.001$). No other main effect or interaction reached significance ($P > 0.05$), indicating HR did not differ between treadmill gradients or environmental conditions; however, there was a tendency ($F = 4.3$; $P = 0.056$) for HR to be increased during hot (162 ± 36 beats $\cdot\text{min}^{-1}$) compared with temperate conditions (153 ± 33 beats $\cdot\text{min}^{-1}$).

Oxygen uptake ($F = 7.4$; $P < 0.001$; Table 3) and B[La] ($F = 30.0$; $P < 0.001$; Table 3) increased over time; however, no other main effect or interaction reached significance ($P > 0.05$). Participants exercised at an average % $\dot{V}O_{2\text{max}}$ of $76.0 \pm 6.4\%$ during temperate flat trials, $76.1 \pm 9.0\%$ during temperate downhill trials, $76.3 \pm 4.2\%$ during hot flat trials, and $76.5 \pm 5.1\%$ during hot downhill trials.

Fig. 4. Rectal temperature (T_{re}) means $\pm$ SD during each experimental trial. ^A T_{re} increased significantly ($P < 0.010$) during downhill running compared with flat running.

Table 3. *Physiological and perceptual responses*

		TEMP _{FLAT}		TEMP _{DOWN}		HOT _{FLAT}		HOT _{DOWN}	
B[La], mmol/l	Basal	0.9 ± 0.2		0.9 ± 0.3		0.9 ± 0.2		0.9 ± 0.2	
	Immediately post	1.4 ± 0.5 ^A		1.5 ± 0.7 ^A		2.1 ± 1.2 ^A		1.7 ± 0.4 ^A	
HR, beats·min ⁻¹	0 min	5 min	10 min	15 min	20 min	25 min	30 min		
	TEMP _{FLAT}	78.6 ± 8.4	152.6 ± 14.0	157.8 ± 13.8	161.6 ± 14.3	163.6 ± 15.2	165.9 ± 14.9	167.7 ± 15.0	
	TEMP _{DOWN}	83.0 ± 11.4	155.1 ± 14.3	162.8 ± 14.7	167.3 ± 15.7	171.4 ± 15.7	175.0 ± 17.0	180.1 ± 17.1	
	HOT _{FLAT}	83.4 ± 5.1 ^B	157.1 ± 17.4 ^B	166.7 ± 18.4 ^B	169.9 ± 18.9 ^B	174.0 ± 18.1 ^B	178.6 ± 18.0 ^B	182.2 ± 18.3 ^B	
V _{O₂} , ml·kg ⁻¹ ·min ⁻¹	0 min	5 min	10 min	15 min	20 min	25 min	30 min		
	TEMP _{FLAT}	5.5 ± 0.8	40.4 ± 3.3	41.1 ± 3.0	41.6 ± 3.1	42.6 ± 2.7	43.2 ± 2.8	43.6 ± 3.0	
	TEMP _{DOWN}	5.5 ± 0.7	39.6 ± 1.0	40.1 ± 0.7	41.5 ± 1.0	42.5 ± 1.7	43.2 ± 1.3	44.4 ± 1.5	
	HOT _{FLAT}	5.9 ± 0.7	41.1 ± 3.0	42.0 ± 3.8	42.7 ± 3.7	42.9 ± 3.5	42.6 ± 4.0	42.4 ± 5.0	
RPE	0 min	5 min	10 min	15 min	20 min	25 min	30 min		
	TEMP _{FLAT}	6.0 ± 0.0	10.0 ± 0.9	11.1 ± 1.6	12.3 ± 1.4	12.6 ± 1.3	13.1 ± 1.0	13.7 ± 1.3	
	TEMP _{DOWN}	6.0 ± 0.0	11.9 ± 1.6	13.1 ± 1.4 ^D	14.9 ± 1.6 ^D	15.6 ± 2.0 ^D	16.4 ± 1.7 ^D	16.7 ± 1.8 ^D	
	HOT _{FLAT}	6.0 ± 0.0	11.6 ± 1.8	12.7 ± 1.4 ^C	13.6 ± 1.5 ^C	14.7 ± 1.2 ^C	16.0 ± 1.3 ^C	16.8 ± 1.1 ^C	
TS	0 min	5 min	10 min	15 min	20 min	25 min	30 min		
	TEMP _{FLAT}	4.0 ± 0.0	4.2 ± 0.3	4.4 ± 0.4	4.7 ± 0.5	4.9 ± 0.7	5.1 ± 0.7	5.4 ± 0.7	
	TEMP _{DOWN}	4.0 ± 0.0	4.3 ± 0.4	4.5 ± 0.5 ^C	5.1 ± 0.5 ^C	5.4 ± 0.6 ^C	5.9 ± 0.7 ^C	6.1 ± 0.8 ^C	
	HOT _{FLAT}	4.0 ± 0.0	4.7 ± 0.4	5.4 ± 0.4	5.6 ± 0.6	6.1 ± 0.5	6.7 ± 0.4	7.1 ± 0.6	
	HOT _{DOWN}	4.0 ± 0.0	4.6 ± 0.4	5.5 ± 0.5 ^C	6.4 ± 0.4 ^C	6.6 ± 0.4 ^C	7.2 ± 0.4 ^C	7.8 ± 0.3 ^C	

Values are expressed as means ± SD. ^AIncreased immediately post compared to basal as a main effect. ^BTrend to increase in hot compared to temperate conditions; $P = 0.056$. ^CIncreased in hot compared to temperate conditions between 10–30 min; $P < 0.05$. ^DIncreased during downhill running compared to flat running between 10–30 min; $P < 0.05$.

Perceptual Response

Rate of perceived exertion (Table 3) increased during hot compared with temperate conditions ($F = 12.3$; $P = 0.002$), in downhill running compared with flat running ($F = 34.8$; $P < 0.001$), and over time ($F = 171.6$; $P < 0.001$). RPE also increased in hot compared with temperate conditions at 10 min ($P = 0.045$), between 20 and 30 min ($P < 0.002$), and during downhill running compared with flat running at 10 min ($P = 0.003$) and between 15 and 30 min ($P < 0.001$).

Thermal sensation (Table 3) increased during hot compared with temperate conditions ($F = 45.1$; $P < 0.001$), in downhill running compared with flat running ($F = 9.0$; $P = 0.004$), and over time ($F = 95.1$; $P < 0.001$) as main effects. Thermal sensation also increased within hot compared with temperate conditions between 10 min and 30 min of exercise ($P < 0.001$). However, no other interactions had any effect on RPE or TS ($P > 0.05$). Therefore, both RPE and TS were greater during downhill running and within hot conditions.

Leukocyte Hsp72 mRNA and Hsp90α mRNA Responses

Leukocyte Hsp72 mRNA (Fig. 5) increased over time with increases observed immediately postexercise and 3 h postexercise ($P < 0.001$). Leukocyte Hsp72 mRNA also increased following downhill running ($P < 0.002$), flat running ($P < 0.015$), and within hot ($P < 0.001$) and temperate environmental conditions ($P < 0.020$) immediately postexercise and 3 h postexercise compared with basal. These leukocyte Hsp72 mRNA increases were greater following downhill running (1.9 ± 0.9 -fold) compared with flat running (1.3 ± 0.4 -fold; $F = 15.2$, $P = 0.001$) and in hot (1.9 ± 0.6 -fold) compared with temperate (1.1 ± 0.5 -fold) environmental conditions ($F = 11.7$; $P = 0.003$) immediately postexercise. Leukocyte Hsp72 mRNA returned to basal levels at 24 h ($P > 0.05$). Therefore,

downhill running and hot environmental conditions were the best stimuli to induce leukocyte Hsp72 mRNA. Leukocyte Hsp72 mRNA increased immediately post HOT_{DOWN} (0.8 ± 0.2 -fold to 2.1 ± 1.2 -fold); however, the interaction between environmental condition, treadmill gradient, and time did not reach significance ($P > 0.05$). Significant correlations were observed between Hsp72 mRNA and peak exercising T_{re} ($r = 0.625$; $P < 0.001$, large), VAS ($r = 0.402$; $P = 0.042$, moderate) and QT ($r = 0.436$; $P = 0.026$, moderate). This suggests that exercising T_{re} and DOMS may have a role in the Hsp72 mRNA increases observed.

Leukocyte Hsp90α mRNA (Fig. 6) increased following downhill running ($P < 0.002$) and in hot conditions ($P < 0.006$) immediately postexercise and 3 h postexercise compared with basal. Leukocyte Hsp90α mRNA also increased immediately postexercise following flat running ($P = 0.008$) and in temperate conditions ($P = 0.049$). Leukocyte Hsp90α mRNA increases were greater following downhill running (1.4 ± 0.8 -fold) compared with flat running immediately postexercise (0.9 ± 0.6 -fold; $F = 13.2$; $P = 0.002$), 3 h postexercise ($F = 6.0$; $P = 0.025$), and 24 h postexercise ($F = 6.7$; $P = 0.019$). Leukocyte Hsp90α mRNA also increased immediately postexercise within hot (1.6 ± 1.0 -fold) compared with temperate (0.9 ± 0.6 -fold) conditions ($F = 12.4$; $P = 0.003$). Therefore, downhill running and hot conditions were the best stimuli to induce leukocyte Hsp90α mRNA. Leukocyte Hsp90α mRNA increased following HOT_{DOWN} (0.7 ± 0.2 -fold to 1.7 ± 1.0 -fold); however, the interaction between environmental condition, treadmill gradient, and time did not reach significance ($P > 0.05$). Significant correlations were observed between Hsp90α mRNA and peak exercising T_{re} ($r = 0.706$; $P < 0.001$, large), VAS ($r = 0.453$; $P = 0.02$, moderate), and QT ($r = 0.436$; $P = 0.026$, moderate). This

Fig. 5. A: leukocyte Hsp72 mRNA median $\pm$ interquartile range immediately before exercise, immediately postexercise, and 3 and 24 h postexercise. Data pooled to enable comparisons between all individuals who exercised in temperate conditions to those who exercised in hot conditions, and between all individuals who completed flat running trials to those who completed downhill running trials (i.e., comparison of main effects). B: individual leukocyte Hsp72 mRNA response to temperate flat (20°C, 50% RH, 1% gradient), C: temperate downhill (20°C, 50% RH, -10% gradient), D: hot flat (30°C, 50% RH, 1% gradient), E: hot downhill (30°C, 50% RH, -10% gradient). Data are presented as fold change from basal. *Significant increase compared with basal ($P < 0.05$). ^ASignificant increase ($P = 0.001$) during downhill running compared with flat running. ^BSignificant increase ($P = 0.003$) in hot group compared with the temperate group.

suggests exercising T_{re} and DOMS may have a role in the Hsp90 α mRNA increases observed.

DISCUSSION

The current study demonstrated that observed increases in Hsp72 and Hsp90 α mRNA were treadmill gradient and environmental temperature-dependent with downhill running and hot conditions being the biggest stimuli.

Leukocyte Hsp72 mRNA

Exercising T_{re} increased during downhill running compared with flat running and showed a trend to be increased within hot compared with temperate conditions. Further, a significant positive correlation was observed between peak exercising T_{re} and Hsp72 mRNA immediately postexercise, suggesting exercising T_{re} increases were an important stimuli for Hsp72 mRNA induction. This is in agreement with previous literature in which an elevated exercising T_{re} led to increased Hsp72

mRNA transcription within peripheral blood mononuclear cells (PBMCs) (18, 38) and lymphocytes (40), probably via increased protein denaturation (40), activating heat shock factor-1 (HSF-1) (49). Activation of a distinct immune response by each leukocyte subset occurs following exercise and exercise heat stress via the release of factors into the systemic circulation (67–68). This immune response includes activation of an oxidative burst within neutrophils via stimulation by ligands, including extracellular HSP72 and noradrenaline (24). Increased oxidative stress has also been observed within lymphocytes (40, 60) following exercise. These prooxidant events could increase protein denaturation-activating transcription of Hsp72 and Hsp90 α mRNA. Caution should be taken with these conclusions, as other studies demonstrate an attenuated oxidative stress response within neutrophils (53) and monocytes (42) following high-intensity exercise (53), likely via the effects of large adrenaline (64) and cortisol (50) increases.

Fig. 6. A: leukocyte Hsp90 α mRNA median $\pm$ interquartile range immediately before exercise, immediately postexercise, and 3 and 24 h postexercise. Data pooled to enable comparisons between all individuals who exercised in temperate conditions to those who exercised in hot conditions, and between all individuals who completed flat running trials to those who completed downhill running trials (i.e., comparison of main effects). B: individual leukocyte Hsp90 α mRNA response to temperate flat (20°C, 50% RH, 1% gradient). C: temperate downhill (20°C, 50% RH, -10% gradient). D: hot flat (30°C, 50% RH, 1% gradient). E: hot downhill (30°C, 50% RH, -10% gradient). Data are presented as fold change from basal. *Significant increase ($P < 0.05$) from basal. ^ASignificant increase ($P < 0.05$) during downhill running compared with flat running. ^BSignificant increase ($P < 0.05$) within hot compared with temperate conditions.

During downhill running, DOMS was increased, suggesting EIMD was present (23). Muscle damage induced following isokinetic eccentric contractions (52) and downhill running (63) increases Hsp72 mRNA postexercise within the human vastus lateralis (VL). This increase is greater during muscle damaging eccentrically biased exercise compared with non-damaging concentrically biased exercise (66). Despite characterization of the Hsp72 mRNA response to EIMD in the VL, no data exist on the Hsp72 mRNA response within leukocytes. Further, the precise mechanism linking EIMD and leukocyte Hsp72 mRNA is unclear. A Toll-like receptor 4 (TLR4)-mediated cellular stress response occurs within leukocytes following muscle-damaging exercise (19). Signaling pathways induced by TLR activation increase oxidative stress via NADPH oxidase activation (4), increasing protein denaturation and activation of JNK and p38 MAPK signaling (28). These changes are known to induce Hsp72 mRNA transcription via HSF-1 activation. Damage-associated molecular patterns (DAMPs), including extracellular HSPs, endogenous nucleic acids, circulating cell-free DNA, high-mobility group box -1 and liposaccharide activate this TLR-mediated leukocyte stress

response (25, 29, 47). However, little evidence exists for DAMPs being released from skeletal muscle following muscle damage in humans. IL-6 infusion increases Hsp72 mRNA in the VL at rest (16). Whether IL-6 has the same effect on leukocytes following exercise heat stress has not been established. Further, IL-6 is only released from skeletal muscle after 120 min of exercise (59). Therefore, it is unlikely that factors released from skeletal muscle following muscle damage induced Hsp72 mRNA within the current study design. A higher rate of ATP breakdown occurs when exercise of the same steady-state velocity is completed within a hot environment compared with the same trial within a temperate environment (15). Therefore, a higher relative exercise intensity is required to sustain the same running velocity in hot conditions. Consequently, this increased metabolic strain could partially account for Hsp72 mRNA increases within hot environmental conditions as the HSP72 protein response is intensity-dependent both within skeletal muscle (34) and leukocytes (38). This is not surprising as protein denaturation, the key cellular change driving Hsp72 mRNA transcription, is exercise intensity (31) and metabolic strain-dependent (7).

Although the current study demonstrated that Hsp72 mRNA increases following downhill running and exercise within a hot environment, the combination of the two stressors HOT_{DOWN} did not induce any further increases. Upregulation of Hsp72 mRNA appears to be largely exercising T_{re} -dependent within the current study design. However, exercising T_{re} did not increase any further during HOT_{DOWN} and, therefore, the lack of any additional physiological stimuli could partially explain the observed responses. The variability of the individual Hsp72 mRNA responses could also explain why a significant increase was not found. Indeed, despite Hsp72 mRNA increasing within all participants who completed downhill running and exercise in hot environments, these increases were highly variable (0.2–4.2 fold). Typically, the HSP72 response to exercise is highly variable (45). However, the use of two experimental groups would have compounded this variability (36) due to the different thermoregulatory and metabolic responses between individuals.

Previous research demonstrated that HSP72 increases were sustained for 24 h following exercise heat stress despite Hsp72 mRNA expression returning to baseline (18). Therefore, both downhill running and exercise in a hot environment could elevate basal HSP72 concentrations for up to 24 h postexercise. However, the current study cannot suggest that downhill running or exercise in hot environments can translate this signal into HSP72-mediated thermotolerance within leukocytes because increased Hsp72 mRNA expression is not necessarily reflective of functional steady-state HSP72 content (38, 66). Any HSP72 increases that were potentially induced by downhill running or exercise in a hot environment would not have had a confounding effect on the Hsp72 and Hsp90 α mRNA responses, as HSP72 increases are sustained for less than 7 days within leukocyte subsets (monocytes) (18, 32).

Leukocyte Hsp90 α mRNA

Previously, investigation of the Hsp90 α mRNA response within humans was limited; however, increases were demonstrated following exercise (12) and exercise heat stress (44) within PBMCs and lymphocytes, respectively. As these responses were measured in isolation and the response following EIMD is absent from the literature, the best singular stressor or combination of exercise-related stressors to increase Hsp90 α mRNA was unclear. The current study demonstrated that downhill running and hot conditions offered the largest stimuli for increasing Hsp90 α mRNA. Induction of Hsp90 α mRNA was correlated with exercising T_{re} and DOMS; however, as previously discussed for Hsp72 mRNA, it is difficult to mechanistically link EIMD and the leukocyte stress response. Therefore, Hsp90 α mRNA increases are likely exercising T_{re} -dependent. The increased metabolic strain during exercise in hot environments and the exercise-mediated activation of the innate immune response also likely play a role. Further investigation is required to elucidate whether Hsp90 α mRNA needs to remain elevated to sustain increased basal HSP90 α concentrations and whether these increases mediate conferred thermotolerance.

Practical Applications and Future Directions

Exercise heat stress and downhill running demonstrated similar magnitude Hsp72 and Hsp90 α mRNA increases with

no additive effect of combining the two stressors (HOT_{DOWN}) observed. However, downhill running is accompanied by the negative effects of DOMS and EIMD on thermoregulation (21, 43) and exercise performance (37), which may persist for up to 24 h and 7 days, respectively. This is important as leukocyte HSP72 protein concentrations appear to return to basal levels within 48 h postexercise (18). Consequently, exercise heat stress still appears to be the superior intervention (of those tested within the current experimental design) to increase Hsp72 and Hsp90 α mRNA, at least within leukocytes and, therefore, potentially confer HSP72- and HSP90 α -mediated thermotolerance (39). Induction of HSP72 protein also occurs within monocytes following exercise within an hypoxic environment (32). Future studies are required to assess whether exercise heat stress and hypoxia (singularly and in combination) are the superior intervention to induce HSP72 and HSP90 α within an experimental design, where metabolic strain is controlled more precisely. This would provide a better understanding of the acute intervention most suited to enhancing HSP72- and HSP90 α -mediated thermotolerance within leukocytes. Consideration of the HSP72 and HSP90 α responses within skeletal muscle is also warranted due to the role of this tissue within exertional heat illness pathophysiology (56). Skeletal muscle HSP72 is induced when exercise is completed in a glycogen-depleted state (17) and following muscle-damaging exercise (52). These stressors should be tested along with exercise heat stress and exercise within hypoxic conditions. Future studies should also consider measuring whether the leukocyte Hsp72 and Hsp90 α mRNA responses are a valid surrogate measure for other more relevant tissues, such as skeletal muscle.

Summary and Conclusions

The current study demonstrated for the first time that leukocyte Hsp72 and Hsp90 α mRNA increases were treadmill gradient- and environmental temperature-dependent with downhill running and hot environmental conditions being the biggest stimuli. Exercising T_{re} appeared to be the major physiological stimuli with increased metabolic strain during hot conditions and activation of the innate immune response potentially also contributing. As downhill running was accompanied by DOMS, exercise heat stress is the most practical stressor (of those tested) to induce Hsp72 and Hsp90 α mRNA within leukocytes.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: J.A.T., P.C.C., L.T., and M.P.L. conception and design of research; J.A.T. and A.J.M. performed experiments; J.A.T., P.C.C., A.W.M., L.T., and M.P.L. analyzed data; J.A.T., P.C.C., L.T., and M.P.L. interpreted results of experiments; J.A.T. prepared figures; J.A.T. drafted manuscript; J.A.T., P.C.C., A.J.M., A.W.M., L.T., and M.P.L. edited and revised manuscript; J.A.T., P.C.C., A.J.M., A.W.M., L.T., and M.P.L. approved final version of manuscript.

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