Heat acclimation attenuates physiological strain and the HSP72, but not HSP90α, mRNA response to acute normobaric hypoxia

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1Centre for Sport and Exercise Science and Medicine (SESAME), Environmental Extremes Laboratory, University of Brighton, Welkin Human Performance Laboratories, Eastbourne, United Kingdom; 2English Institute of Sport, EIS Performance Centre, Loughborough University, Loughborough, United Kingdom; and 3Muscle Cellular and Molecular Physiology (MCMP) and Applied Sport and Exercise Science (ASEP) Research Groups, Department of Sport Science and Physical Activity, Institute of Sport and Physical Activity Research (ISPAR), University of Bedfordshire, Bedfordshire, United Kingdom

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Gibson OR, Turner G, Tuttle JA, Taylor L, Watt PW, Maxwell NS. Heat acclimation attenuates physiological strain and the HSP72, but not HSP90α, mRNA response to acute normobaric hypoxia. J Appl Physiol 119: 889–899, 2015. First published July 23, 2015; doi:10.1152/japplphysiol.00332.2015.—Heat acclimation (HA) attenuates physiological strain in hot conditions via phenotypic and cellular adaptation. The aim of this study was to determine whether HA reduced physiological strain, and heat shock protein (HSP) 72 and HSP90α mRNA responses in acute normobaric hypoxia. Sixteen male participants completed ten 90-min sessions of isothermic HA (40°C/40% relative humidity) or exercise training [control (CON); 20°C/40% relative humidity]. HA or CON were preceded (HYP1) and proceeded (HYP2) by a 30-min normobaric hypoxic exposure [inspired O2 fraction = 0.12; 10-min rest, 10-min cycling at 40% peak O2 uptake (VO2peak), 10-min cycling at 65% VO2peak]. HA induced greater rectal temperatures, sweat rate, and heart rates (HR) than CON during the training sessions. HA, but not CON, reduced resting rectal temperatures and resting HR and increased sweat rate and plasma volume. Hemoglobin mass did not change following HA nor CON. HSP72 and HSP90α mRNA increased in response to each HA session, but did not change with CON. HR during HYP2 was lower and O2 saturation higher at 65% VO2peak following HA, but not CON. O2 uptake/HR was greater at rest and 65% VO2peak in HYP2 following HA, but was unchanged after CON. At rest, the respiratory exchange ratio was reduced during HYP2 following HA, but not CON. The increase in HSP72 mRNA during HYP1 did not occur in HYP2 following HA. In CON, HSP72 mRNA expression was unchanged during HYP1 and HYP2. In HA and CON, increases in HSP90α mRNA during HYP1 were maintained in HYP2. HA reduces physiological strain, and the transcription of HSP72, but not HSP90α mRNA in acute normobaric hypoxia.

altitude; cardiovascular; cross-acclimation; cross-tolerance; heat stress; plasma volume

HYPOXIA INCREASES PHYSIOLOGICAL strain both at rest and during exercise (6), with impairment of exercise performance (72), notably during exercise where aerobic metabolism predominates (3). The physiological advantages and disadvantages of repeated hypoxic/altitude exposures for attenuating the negative effects of hypoxia (2) have been summarized in numerous review articles (20, 46). Altitude/hypoxic training methods are varied, with synergistic interactions between simulated and terrestrial, resting or exercise, and continuous and intermittent exposures, each eliciting different magnitudes of adaptation (46). Irrespective of precise application, hypoxic training requires lengthy durations of exposure over prolonged, repeated periods (typically 14–28 days) for meaningful adaptation (27). Heat acclimation (HA), and acclimatization interventions, carried out by repeated exercise in hot conditions (58), reproducibly reduce physiological strain in hot and cooler conditions (32, 33, 39). Recent reviews support a novel adaptive pathway whereby HA may reduce physiological strain in hypoxia (14, 56, 73). Mechanistic pathways can be subdivided into cross-acclimation, whereby HA attenuates physiological strain (73) and cross-tolerance, whereby cellular responses to HA provide cytoprotection during hypoxia (14). Acute physiological responses to hypoxia (2) can be used as criteria for validating heat-induced cross-acclimation. HA reduces glycolysis and metabolic rates during exercise (34), with plasma volume (PV) expansion (39, 50) and improved myocardial efficiency (38) preserving cardiac output and skeletal muscle blood flow. Muscle oxygenation is also sustained by HA-induced maintenance of central blood volume (BV) via reductions in the core/skin temperature gradient (58) and enhanced evaporative heat loss (51). Improved temperature and hematological regulation facilitate a leftward shift in the oxyhemoglobin saturation curve (37). HA induces expedient and beneficial adaptations within 5–14 daily sessions, demonstrating a greater efficiency of adaptation compared with altitude/hypoxic interventions (23).

Cross-tolerance has been defined as single or repeated sublethal exposures to a stressor, eliciting a positive adaptive effect to a subsequent exposure to a different stressor (35). The cellular pathway for this shares commonality with those seen within in vivo thermotolerance (47). In this model, cellular thermotolerance accompanies the induction of phenotypic adaptations associated with HA (43, 45). Thermotolerance confers cytoprotection against subsequent thermal exposure (45, 74), principally by changes in heat shock proteins (31). Heat shock proteins facilitate important cellular processes as protein chaperones (19) and anti-apoptotic mediators (1). In particular, increases in the inducible proteins heat shock protein (HSP) A1A (HSP72) and HSPC1 (HSP90α) mitigate pathophysiological responses to endogenously stressful stimuli. Both HSP72 and HSP90α augment proportionally to increased cellular stress (increased cellular temperature) in response to ex vivo heat shock (45) and have been implicated as important modulators of the adaptive cellular/molecular response to hypoxia.
(56, 65, 66); this suggests a shared signaling pathway. Both HSP72 and HSP90α mRNA and protein responses have been used as a marker for identifying the magnitude of stimuli required to initiate the in vivo stress response (45). However, not all of the HSP72 mRNA transcripts are translated to HSP72 protein increase within peripheral blood mononuclear cells following exercise heat stress in humans (44). Basal heat shock protein measurement provides the optimal indication of the acquired capacity to mitigate disruption to cellular homeostasis due to known increases with acclimation (45). The delayed responsiveness of the protein response (16, 17), compared with the within-session heat shock protein mRNA response (25, 44), emphasizes the benefits of the gene transcript as a primary indicator of the magnitude of the stress stimuli and necessity to signal protein transcription should the stimuli be maintained or repeated. Consequently, the mRNA transcription is appropriate to determine whether the HSP72 and HSP90α responses have been attenuated or mitigated, either in response to reductions in physiological strain, or increased basal protein, ultimately highlighting whether cross-tolerance may have been conferred.

HA has been evidenced in improving oxygen saturation and heart rates (HRs) during hypoxic exercise performance (28), with HA also mitigating increases in HSP72 protein in hypoxia, due largely to increased basal concentrations of HSP72 (37). These data support the existence of cross-acclimation/tolerance (37); however, mechanisms for this interaction are presently unknown (39, 45). The aim of this experiment was to determine whether HA would reduce physiological strain and the HSP72 and HSP90α mRNA responses to an acute hypoxic exposure (at rest and at various exercise intensities) compared with exercise training matched for intensity and duration in temperate conditions. It was hypothesized that HA would reduce physiological strain in hypoxia via cardiovascular and thermoregulatory adaptations and that the heat shock protein response to hypoxia would be reduced following HA.

MATERIALS AND METHODS

Participants. Sixteen healthy men, who completed various forms of exercise training between three and six times per week, were assigned to matched groups to perform 10 days of isothermic HA [age 22.5 ± 3.5 yr, nude body mass (NBM) 74.6 ± 7.9 kg, body surface area 1.95 ± 0.13 m², peak oxygen uptake (VO₂ peak) 4.32 ± 0.68 l/min, 58.5 ml·kg⁻¹·min⁻¹], or act as a normothermic exercise control (CON; age 26.0 ± 1.9 yr, NBM 74.6 ± 0.62 l/min, 56.6 ml·kg⁻¹·min⁻¹). Confounding environmental and pharmacological variables were all controlled in line with previous work in the field (24, 25). Urine osmolality was used to confirm hydration in accordance with established guidelines before each experimental/training session [<700 mosmol/kgH₂O (57)]. This experimental control was not violated for any participant for any experimental/training session. All protocols, procedures, and methods were approved by the institutional ethics committee, with participants completing medical questionnaires and written, informed consent following the principles outlined by the Declaration of Helsinki, as revised in 2013.

Preliminary testing. Before assessment of VO₂ peak, anthropometric data were collected with NBM measured using digital scales, precise to 0.01 kg (GFK 150, Adam Equipment, Danbury, CT). VO₂ peak (l/min) was determined from an incremental test on a cycle ergometer, which was used for all subsequent trials (Monark e724, Monark AB, Varberg, Sweden), at a starting intensity of 80 W, increasing by 24 W/min, in temperate laboratory conditions [20°C, 40% relative humidity (RH)] at sea level (inspired O₂ fraction = 0.2093). VO₂ peak was defined as the highest average VO₂ obtained in any 30-s period, with VO₂ peak more appropriately describing the end point of the test due to an absence of VO₂ plateau in all participants. The confirmation of VO₂ peak was made via the attainment of a HR within 10 beats/min of age-predicted maximum, and respiratory exchange ratio (RER) >1.1 in all participants.Expired metabolic gas was measured using breath-by-breath online gas analysis (MetaLyser 3B, Cortex, Leipzig, Germany). HR was recorded continually during all experimental/training sessions by telemetry (PolyMed Electro Oyo, Kempele, Finland).

Hematological measures. Twenty-four hours before hypoxic exposures, hemoglobin mass (Hbmass; g) was measured. Hbmass, BV (ml), and PV (ml) were determined in accordance with the optimized carbon monoxide (CO)-rebreathing method (59). Participants were seated for 20 min, before being connected to a closed glass spirometer, allowing inspiration of a CO bolus of 1.0 ml/kg (68), followed by 2 min rebreathing of a 3.5-liter O₂ bolus. Before and 4 min after CO rebreathing, participants completely exhaled to residual volume into a CO gas meter (Pac 7000, Drager, Pittsburgh, PA). CO volume not remaining within the body was calculated from the remainder of CO in the spirometer, and exhaled CO was measured immediately after the spirometer was disconnected from the participant (68). Fingertip capillary samples, for determination of carboxyhemoglobin concentration (%HbCO) were taken immediately before the rebreathing procedure and at 6 and 8 min after the CO bolus was administered. Blood samples were measured immediately in triplicate (69), using anABL80 CO-OX FlexOXFlex hemoximeter (Radiometer, Copenhagen, Denmark). Hbmass was calculated from the mean change in %HbCO before and after rebreathing CO (68). At the relevant intervals within the optimized CO-rebreathing method, hemoglobin concentration (g/dl) was collected from fingertips in duplicate using a microcuvette and analyzed using a B-Hemoglobin Photometer (Hemocue, Angelholm, Sweden), and hematocrit (%) was collected in triplicate (~50 µl) with glass capillary tubes and analyzed following centrifugation at 14,000 rpm for 3 min (Haemotospin 1300 Centrifuge, Haskewly & Sons, West Sussex, UK) (69). The experimenter typical error of measurement for total Hbmass before commencing this experiment was ±1.98% (± 17.0 g).

Hypoxic exposures. Hypoxic exposures were performed 24 h before commencing the first session of HA or CON (HYP1) and 24 h following the final HA or CON training session (HYP2). Participants performed a 30-min normobaric hypoxic exposure adapted from Lunt et al. (40). After entering normobaric hypoxic conditions (inspired O₂ fraction = 0.12; 18°C, 40% RH) achieved using a purposed built nitrogen-enriched chamber (Altitude Centre, London), participants immediately rested in a supine position for a period of 10 min. Supine rest was followed by two bouts of exercise, where participants first cycled at a workload corresponding to 40% of normoxic VO₂ peak for a period of 10 min (HA = 102 ± 27 W, CON = 104 ± 26 W) and then immediately proceeded to exercise at a workload corresponding to 65% of normoxic VO₂ peak (HA = 201 ± 41 W, CON = 192 ± 37 W) for a further 10 min. During rest and exercise, HR, oxygen uptake (VO₂; l/min), carbon dioxide production (VCO₂; l/min), ventilation (VE; l/min), RER, and peripheral arterial oxygen saturation (SpO₂; %) estimated using a fingertip pulse oximeter (Nonin 2500, Nonin Medical) were recorded continuously, with the final 5 min of measures used for analysis. Before entry, and following every 10 min, participants reported rating of perceived exertion (RPE) and Lake Louise Questionnaire (LLQ) symptoms. Metabolic parameters (VO₂, VCO₂, and VE) was measured using online breath-by-breath analysis.

HA/exercise protocols. Each HA or CON training session was conducted at the same time of day (0700–1000) to control for effects of daily variation in exercise performance (12) and heat shock protein expression (67) inside a purpose-built environmental chamber (WATFLOW control system; TISS, Hampshire, UK). Temperature and hu-
midity were controlled using automated computer feedback (WatFlow control system; TISS, Hampshire, UK). On arrival to the laboratory, participants provided a midflow urine sample for assessment of hydration. Towel-dried NBM was measured before and after the trials, with no fluid consumption permitted between measurements. Sweat rate (SR; l/h) was estimated using the change in NBM from the pre- to postexercise periods. Participants inserted a rectal thermistor (Henley's Medical Supplies, Welwyn Garden City, UK; Meter logger model 401, Yellow Springs Instruments, Yellow Springs, MO) 10 cm past the anal sphincter to measure rectal temperature (Trec) and affixed a HR monitor to the chest. Following a 10-min seated stabilization period in temperate laboratory conditions, at sea level, resting measures [Trec, HR, RPE, and thermal sensation (TSS)] were recorded, and participants immediately entered the environmental chamber (40.2 ± 0.4°C, 41.0 ± 6.4% RH) and mounted a cycle ergometer. Participants allocated to the HA group performed ten 90-min sessions involving a combination of cycling exercise and rest in accordance with established isometric HA protocols (25, 26). HA participants initially exercised, at a workload corresponding to 65% VO2 peak, with the workload adjusted to match the total work output (W), RPE, and TSS were recorded every 5 min with adjustments in power (including the cessation of exercise) only made following each completed 5-min period. In compliance with ethical approval, HA was terminated if a subject attained a Trec of 39.7°C (zero incidences); data describing the prescription, physiological, and perceptual responses to HA and CON are contained in Table 1.

Blood sampling and RNA extraction. Venous blood samples were taken immediately before and after HYP1 and HYP2, and before and after the 1st (day 1) and 10th day (day 10) of HA or CON with RNA extraction performed using a validated method (9). Briefly, blood samples were drawn from the antecubital vein into 6-ml EDTA tubes (Greiner BIO-one, Stonehouse, UK). Venous blood (1 ml) was pipetted into 10 ml of 1 in 10 red blood cell lysis solution (10× Red Blood Cell Lysis Solution, Milltenyi Biotech, Bisley, UK). Samples were incubated for 15 min at room temperature before isolation via centrifugation at 400 g for 5 min. Because of belonephobia, one participant from HA was excluded from blood sampling and mRNA analyses. 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, Waltham, MA).

One step reverse transcription-quantitative polymerase chain reaction. HSP72 and HSP90α relative mRNA expression was quantified using reverse transcription-quantitative polymerase chain reac-

<table>
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<th>Table 1. Summary of metabolic and physiological data recorded throughout rest and exercise of 10 sessions of heat acclimation or control</th>
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<td>Exercising duration, min</td>
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<td>Total work done, kJ</td>
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<td>Mean Trec, °C</td>
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<td>Duration Trec ≥38.5°C, min</td>
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<td>Rate of Trec increase, °C/h</td>
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<td>Mean RPE</td>
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<td>Mean TSS</td>
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Values are means ± SD; n, no. of subjects. HA, heat acclimation; CON, control; Trec, rectal temperature; HR, heart rate; RPE, rating of perceived exertion; TSS, thermal sensation. *Significant difference from CON (P < 0.05)
tion. Primers β2-microglobulin [National Center for Biotechnology Information (NCBI) accession number: NM_004048; forward: CCGTGTGACCATGTGTACT, reverse: TGCCGGCATCTTCAACACT], HSP72 (NCBI accession number: NM_005345; forward: CGAAGCTT-GTACCTTCTTGGA, reverse: TGCCGTGTCTGCTGTTGAG), and HSP90α [NCBI accession numbers: NM_001017963 (variant 1) and NM_005348 (variant 2); forward: AACTGCCCTGCTGTTCT, reverse: TGCCGTGATGTGCTGCATCT] were designed using primer design software (Primer Quest and OligoAnalyzer-Integrated DNA Technologies, Coralville, IA) (70). Relative quantification of mRNA expression for each sample was assessed by determining the ratio between the cycling threshold (CT) value of the target mRNA and β2-microglobulin CT values. Fold change in relative mRNA expression was calculated using the 2-ΔΔCT method.

**Statistical analysis.** A priori power analysis for key HA-dependent variables selecting conventional α (0.05) and β (0.20) levels observed that eight participants were required in each experimental group. Before statistical analysis, all outcome variables were checked for normality using Kolmogorov-Smirnov and sphericity tests, using the Greenhouse Geisser method before further analysis. Protocol-specific and physiological data for HA/CON were compared using independent samples T-tests. Two-way mixed-design ANOVA was performed to determine differences between HA and CON and day 1/pre and day 10/post. Two-way mixed-design ANOVA was performed to determine differences between HA and CON, as well as HYP1 with HYP2; thus rest, 40% V̇O₂peak, and 65% V̇O₂peak conditions within each HYP were analyzed independently from one another. Three-way mixed-design ANOVA was performed on the HSP72 and HSP90α mRNA data to determine differences between pre- and postvalue (repeated measures – within subjects) on different days (repeated measures – within subjects) from the two interventions (between subjects). Adjusted Bonferroni comparisons were used as post hoc analyses for all ANOVA. Effect sizes [Cohen’s d (small = 0.2, medium = 0.5, large = 0.8) or partial r² (np²); small = 0.01, medium = 0.06, large = 0.13] were calculated to analyze the magnitude and trends with data. All data are reported as means ± SD. For all analysis, two-tailed significance was accepted at P < 0.05.

**RESULTS**

**HA/exercise interventions.** HA and CON were successfully matched for exercising duration (t = 0.635; P = < 0.001; d = 0.34), work done (t = -0.168; P = 0.869; d = 0.09), and session intensity (t = -0.355; P = 0.728; d = 0.19) (Table 1).

Differences were observed for mean Trec (t = 9.138; P < 0.001; d = 4.88), rate Trec increase (t = 6.876; P < 0.001; d = 3.68), duration Trec ≥38.5°C (t = 14.106; P < 0.001; d = 7.54), between HA and CON interventions, with mean Trec different between HA and CON (t = 55.619; P < 0.001; np² = 0.799) (Fig. 1). Additionally, SR (t = 7.254; P < 0.001; d = 3.88), mean HR (t = 3.444; P = 0.004; d = 1.84), mean RPE (t = 2.918; P = 0.011; d = 1.56), and mean TSS (t = 8.394; P < 0.001; d = 4.49) were greater in HA compared with CON interventions (Table 1).

**Adaptation to HA.** An interaction effect was observed between HA and CON and day 1 and day 10 for resting Trec (f = 11.507; P = 0.004; np² = 0.451), resting HR (f = 20.579; P < 0.001; np² = 0.595), SR (f = 7.146; P = 0.018; np² = 0.338), PV (f = 23.501; P < 0.001; np² = 0.627), and BV (f = 25.582; P < 0.001; np² = 0.646) in HA, but not CON (Table 2). SR was greater on day 1 and day 10 in HA than CON (P < 0.001), but resting Trec (P = 0.007) and resting HR (P = 0.03) were lower on day 10 in HA compared with CON (Table 2). No difference was observed between days (f = 0.275; P = 0.608; np² = 0.019) or days × groups (t = 0.237; P = 0.634; np² = 0.017) for Hbmax (Table 2).

**HSP72 mRNA and HSP90α mRNA during HA/CON.** An interaction effect was observed for HSP72 mRNA (f = 20.428; P = 0.001; np² = 0.611) and HSP90α mRNA (f = 10.282; P = 0.007; np² = 0.422). No difference was observed between HA or CON before day 1 or day 10 (P = 0.396 and P = 0.180), but a difference was observed post-HA compared with CON (P = 0.004 and P = 0.012). HSP72 mRNA and HSP90α mRNA increased pre- to post-HA (P < 0.001 and P < 0.001) in HA, but not CON (P < 0.051) and P = 0.394).

**Hypoxic tolerance tests.** At rest in hypoxia, there was an interaction effect between groups and HYP1 and HYP2 for V̇O₂/HR (f = 6.852; P = 0.020; np² = 0.329) and RER (f = 7.078; P = 0.041; np² = 0.266). In HYP2, at rest differences occurred following HA for V̇O₂/HR (P = 0.039; Fig. 2) and RER (P = 0.045; Fig. 3), but not CON (P > 0.05). No difference was observed in HR (f = 0.820; P = 0.381; np² = 0.055) or SpO₂ (f = 2.123; P = 0.167; np² = 0.132) at rest in hypoxia (Fig. 2).

When exercising at 40% V̇O₂peak in hypoxia, no differences were observed within the group × HYP comparison for HR (f = 1.575; P = 0.230; np² = 0.101), SpO₂ (f = 0.000; P = 1.000; np² = 0.000), V̇O₂/HR (f = 2.651; P = 0.126; np² = 0.126), or RER (f = 0.047; P = 0.831; np² = 0.003) (Fig. 2). When exercising at 65% V̇O₂peak in hypoxia, differences were observed for HR (f = 4.751; P = 0.047; np² = 0.253), SpO₂ (f = 5.616; P = 0.033; np² = 0.286), and V̇O₂/HR (f = 10.584; P = 0.006; np² = 0.431) within the group × HYP comparison. In HYP2, at 65% V̇O₂peak, differences occurred following HA for HR (P = 0.001), SpO₂ (P = 0.006), and V̇O₂/HR (P = 0.006), but not CON (P > 0.05); see Fig. 2. No difference was observed in RER (f = 0.248; P = 0.626; np² = 0.017) when exercising at 65% V̇O₂peak (Fig. 2).

**Table 2. Comparison of day 1 and day 10 (Trec, HR, and sweat rate) and pre- and postintervention data (plasma volume, Hbmax, blood volume) for heat acclimation and control groups**

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<tr>
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<th>Day 1/Pre</th>
<th>Day 10/Post</th>
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<td>HA</td>
<td>CON</td>
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<tr>
<td>Resting Trec, °C</td>
<td>36.97 ± 0.25</td>
<td>36.99 ± 0.32</td>
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<tr>
<td>Resting HR, beats/min</td>
<td>74 ± 13</td>
<td>68 ± 14</td>
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<tr>
<td>Sweat rate, l/h</td>
<td>1.13 ± 0.28*</td>
<td>0.45 ± 0.20</td>
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<tr>
<td>Hbmax, g/kg</td>
<td>869 ± 92</td>
<td>865 ± 110</td>
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<tr>
<td>Plasma volume, ml</td>
<td>2.981 ± 335</td>
<td>3.142 ± 530</td>
</tr>
<tr>
<td>Blood volume, ml</td>
<td>5.627 ± 501</td>
<td>5.686 ± 847</td>
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Values are means ± SD; n, no. of subjects. *Significant difference from CON within day (P < 0.05). †Significant difference from day 1 within group (P < 0.05).

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No differences ($P > 0.05$) were observed between HYP1 and HYP2 trials, at rest, 40% $\dot{V}O_2$peak, or 65% $\dot{V}O_2$peak during HA or CON for $\dot{V}O_2$, $V_e$, breathing frequency, RPE, or LLQ (Table 3).

HSP72 mRNA and HSP90α mRNA during hypoxic tolerance tests. HSP72 mRNA increased during HYP1 ($f = 17.005; P = 0.001, \eta_p^2 = 0.567$). In the HA group, an increase in HSP72 mRNA was observed following HYP1 ($P = 0.006$), but not HYP2 ($P = 0.440$). This was supported by the observation that HSP72 mRNA was greater post-HYP1 compared with HYP2 ($P = 0.021$). No changes in the pattern of HSP72 mRNA expression were observed in CON. HSP90α mRNA increased before to after HYP1 and HYP2.

Fig. 2. Heart rate (HR; top), oxygen saturation ($SpO_2$; middle), and oxygen pulse [$O_2$ uptake ($\dot{V}O_2$)/HR; bottom] during rest and while exercising at 40% normoxic peak $O_2$ uptake ($\dot{V}O_2$peak) and 65% $\dot{V}O_2$peak in hypoxia [inspired fraction of $O_2$ ($fio2$) = 0.12] before (HYP1; open bars) and after (HYP2; solid bars) HA (left; $n = 8$) or CON (right; $n = 8$). Values are means ± SD. *Significant difference from HYP1 within condition ($P < 0.05$).
The authors (39) reported similar absolute changes in $T_{\text{rec}}$ ($-0.5^\circ C$; our data $= -0.49^\circ C$), HR ($-15$ beats/min; our data $= -18$ beats/min), and SR ($+0.4$ l/h; our data $= +0.4$ l/h). Additionally, over the same number of HA sessions, we observed a larger expansion of PV ($+6.5$%; our data $= +15$%). Despite a cascade of mechanisms well attributed to PV expansion, including increased vascular filling to support cardiovascular stability, increased specific heat capacity of blood, and attenuated skin blood flow responses, the observable magnitude of these adaptive responses may be finite, or demonstrate an exponential decay beyond moderate levels of hypervolemia (58). These responses are agreeable with the consensus that isothermic protocols controlling hyperthermia to a core temperature of at least $38.5^\circ C$ should be implemented to optimize adaptations (53), due to maintenance of the endogenous thermal stimuli for adaptation (50). Increased core temperature (Fig. 1), leading to elevated and sustained sweating, is the fundamental potentiating stimulus initiating phenotypic responses known as HA (54). Consequently, in HA, increased mean $T_{\text{rec}}$ ($+0.8^\circ C$), and the duration that $T_{\text{rec}}$ exceeded the isothermic threshold of $38.5^\circ C$ (47 min) (18), induced greater adaptation than the normothermic training of CON (Table 1). Greater heat dissipation through evaporation in hot conditions was evidenced by threefold elevation in SRs in HA compared with CON (51). Increased heat storage in HA is the stimuli for observed increases in HR, RPE, and TSS for the same exercise prescription as CON (21). HA increased BV ($+500$ ml) compared to CON ($+170$ ml).

**DISCUSSION**

This experiment observed that HA reduced physiological strain and the HSP72 mRNA response to an acute hypoxic exposure combining rest and exercise. The adaptation pathway was likely mediated in part by PV expansion, which improved $V_{\text{O}_2}/$HR at rest and exercise in hypoxia, as well as attenuating HR responses and preservation of $Sp_O_2$ during exercise in hypoxia. Resting RER was reduced after HA, an observation not true of CON, suggesting greater fat oxidation at rest in hypoxia. Hypoxia postintervention. At a cellular level, HA mitigated the group-specific HSP72 mRNA increase, but not the HSP90 mRNA response to hypoxia. The HSP90 mRNA response also increased comparably to HA before and after CON; however, no increase in HSP72 mRNA was observed in either trial in this group.

HA and CON were successfully matched for the prescribed training parameters (duration, absolute intensity, and work done; Table 1) with the equality of these training parameters giving confidence that adaptations were induced by the increased physiological/thermal strain of the hot environment of HA, compared with the temperate conditions of CON (Table 1). The elegant experimental design of Lorenzo et al. (39) is most closely representative of ours. In agreement with previous data (26), the magnitude of adaptation induced by our isothermic HA regimen is at least equal to that observed by their fixed-intensity HA regimen (39), which improved physiological responses and exercise performance in hot and cool conditions. The authors (39) reported similar absolute changes in $T_{\text{rec}}$ ($-0.5^\circ C$; our data $= -0.49^\circ C$), HR ($-15$ beats/min; our data $= -18$ beats/min), and SR ($+0.4$ l/h; our data $= +0.4$ l/h). Additionally, over the same number of HA sessions, we observed a larger expansion of PV ($+6.5$%; our data $= +15$%). Despite a cascade of mechanisms well attributed to PV expansion, including increased vascular filling to support cardiovascular stability, increased specific heat capacity of blood, and attenuated skin blood flow responses, the observable magnitude of these adaptive responses may be finite, or demonstrate an exponential decay beyond moderate levels of hypervolemia (58). These responses are agreeable with the consensus that isothermic protocols controlling hyperthermia to a core temperature of at least $38.5^\circ C$ should be implemented to optimize adaptations (53), due to maintenance of the endogenous thermal stimuli for adaptation (50). Increased core temperature (Fig. 1), leading to elevated and sustained sweating, is the fundamental potentiating stimulus initiating phenotypic responses known as HA (54). Consequently, in HA, increased mean $T_{\text{rec}}$ ($+0.8^\circ C$), and the duration that $T_{\text{rec}}$ exceeded the isothermic threshold of $38.5^\circ C$ (47 min) (18), induced greater adaptation than the normothermic training of CON (Table 1). Greater heat dissipation through evaporation in hot conditions was evidenced by threefold elevation in SRs in HA compared with CON (51). Increased heat storage in HA is the stimuli for observed increases in HR, RPE, and TSS for the same exercise prescription as CON (21). HA increased BV ($+500$ ml) compared to CON ($+170$ ml).

**Table 3. Comparison of physiological and perceptual data at rest and while exercising at 40% normoxic $V_{\text{O}_2_{\text{peak}}}$ and 65% $V_{\text{O}_2_{\text{peak}}}$ in hypoxia ($F_{\text{O}_2} = 0.12$) before (HYP1) and after (HYP2) heat acclimation or normothermic exercise**

<table>
<thead>
<tr>
<th></th>
<th>HYP1-Rest</th>
<th>HYP2-Rest</th>
<th>HYP1-40%</th>
<th>HYP2-40%</th>
<th>HYP1-65%</th>
<th>HYP2-65%</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{O}_2}$, l/min</td>
<td>$0.34 \pm 0.06$</td>
<td>$0.34 \pm 0.05$</td>
<td>$0.35 \pm 0.05$</td>
<td>$0.31 \pm 0.02$</td>
<td>$1.82 \pm 0.32$</td>
<td>$1.98 \pm 0.44$</td>
</tr>
<tr>
<td>$V_{\text{E}}$, l/min</td>
<td>$10.5 \pm 2.3$</td>
<td>$10.4 \pm 1.8$</td>
<td>$10.2 \pm 1.4$</td>
<td>$9.9 \pm 0.9$</td>
<td>$54.0 \pm 12.5$</td>
<td>$62.0 \pm 16.3$</td>
</tr>
<tr>
<td>BF, breaths/min</td>
<td>$13 \pm 3$</td>
<td>$14 \pm 2$</td>
<td>$14 \pm 3$</td>
<td>$15 \pm 1$</td>
<td>$25 \pm 4$</td>
<td>$29 \pm 6$</td>
</tr>
<tr>
<td>RPE</td>
<td>$6.0 \pm 0.0$</td>
<td>$6.0 \pm 0.0$</td>
<td>$6.0 \pm 0.0$</td>
<td>$6.0 \pm 0.0$</td>
<td>$9.4 \pm 1.9$</td>
<td>$12.3 \pm 1.8$</td>
</tr>
<tr>
<td>LLQ</td>
<td>$0.0 \pm 0.0$</td>
<td>$0.0 \pm 0.0$</td>
<td>$0.0 \pm 0.0$</td>
<td>$0.0 \pm 0.0$</td>
<td>$0.1 \pm 0.4$</td>
<td>$0.3 \pm 0.5$</td>
</tr>
</tbody>
</table>

Values are means $\pm$ SD; $n = 8$ subjects for HA and CON groups. $V_{\text{O}_2}$, $O_2$ uptake; $V_{\text{E}}$, ventilation; BF, breathing frequency; LLQ, Lake Louise Questionnaire.
heat loss, thus closely controlling $T_{rec}$ at 38.5°C, rather than exercise, which matches heat production to evaporative cooling methods (50) for HA are the most probable causes for the reduction in HR during exercise. Implementation of nonthermal, O2-sensing pathways are most important for increased plasma albumin and the renin-angiotensin-aldosterone system (50). Large PV has been proposed as maladaptive due to hemodilution (14), where maintenance of cardiac output may be potentially confounded by a reduced relative O2-carrying capacity of blood. Improved $SpO_2$ (+3%) following HA suggests that a 15% increase in PV is beneficial in hypoxia, even if optimal PV expansion is currently unknown. Maintenance of $SpO_2$ following HA (Fig. 2) occurs as a reduction in HR and blood viscosity affords a greater erythrocyte alveolar transit time, facilitating more complete resaturation within the pulmonary system (11). This is important in hypoxia, and for more well-trained individuals, due to a greater reduction in $SpO_2$, resulting from a typically larger cardiac output and reduced pulmonary gas exchange at higher exercise intensities (52).

The reduction in $T_{rec}$ and increased SR (Table 2) following HA has a dual role in facilitating enhanced heat balance. Reduced $T_{rec}$ mediates a greater spectrum for temperature increase, while increased SR is facilitated by an earlier sweat onset, even when accounting for decreased $T_{rec}$ (55). Within HYP1/HYP2, the heat stress was moderate (10, 39) and would appear compensable (8), thus reduced $T_{rec}$ following HA as a mechanism for prolonging permissible physiological strain and exercise performance in temperate hypoxia is not fundamental. Instead, reduced $T_{rec}$ during exercise in hypoxia causes a leftward shift in the oxyhemoglobin dissociation curve, signifying the potential for enhanced O2 saturation (73). This thermoregulatory adaptation is relevant in hypoxia vs. normoxia as O2 utilization is more greatly compromised. Preservation of $SpO_2$, observed at 65% $V_{O2peak}$ alone is likely a result of the increased demand for O2 at the muscle at this higher intensity (63). Interestingly, improved physiological response to matched exercise did not augment a reduction in the RPE or LLQ in hypoxia (Table 3), as previously observed regarding TSS in the heat (26). It should be noted that there is potential for the reduction in physiological strain in HYP2 to be a reflection of a reduced relative exercise intensity, as HA has been shown to increase maximum $V_{O2}$ in both cool and hot conditions (39). No data exist stating HA improves $V_{O2peak}$ or maximum $V_{O2}$ in hypoxia; however, a post-HA $V_{O2peak}$ test in the present study would have been able to determine that this is likely to have occurred. On the basis of this notion, it should be observed that cross-acclimation was effective using a model testing an absolute workload equal before and after intervention, which may accurately reflect occupational or military populations completing a fixed task. However, it is unknown whether the reduction in physiological strain would have also been observed if workload were derived from the relative exercise intensity of a postexercise $V_{O2peak}$ test. This perspective is analogous to exercise performance within a given intensity domain.

Despite no change in our data, hypothetically a sufficient dose of HA could increase $Hb_{mass}$ (60), via the induction of hypoxia-inducible factor (HIF)-1α (42), as is well established of altitude exposure (27). Trends for increases in erythrocyte volume have been observed following 5-day interventions similar to HA (4.1 ± 0.9%) (22). Conversely, and in agreement with our data, training for 10 days in 30°C at 610 m elicited no change in erythrocyte volume (+0.4 ± 0.6 ml/kg), whereas training at the same temperature at 2,000 m elicited significant gain (+1.9 ± 0.4 ml/kg). This suggests long established nonthermal, O2-sensing pathways are most important for in-

pared with CON (Table 2). No change in $Hb_{mass}$ indicates HA-induced hypervolemia was a response to increases in extracellular fluid, with increases in PV (+446 ml) approximate to the absolute change in BV, reaffirming this as a primary adaptation to heat (61), and an established mechanism for the reduction in HR during exercise. Implementation of isometric methods (50) for HA are the most probable causes for greater PV expansion (+15%) compared with others utilizing similar participants, protocol length, and environmental conditions [6.5% (39), 9.0% (50), and 11.1% (7)]. It remains to be experimentally elucidated whether maintaining lower intensity exercise, which matches heat production to evaporative heat loss, thus closely controlling $T_{rec}$ at 38.5°C, rather than implementing passive rest following $T_{rec}$ exceeding the target of 38.5°C, would augment even more favorable adaptations resulting from higher SRs and elevated cardiovascular response. As such, despite a large magnitude of adaptation observed within this experiment, this is a potential limitation of the implemented experimental design. With no change in gross efficiency as indicated by similar $V_{O2}$, the $V_{O2}/HR$ ratio becomes more efficient after HA (Fig. 2). Hyphoponization from increased sweating (HA = 2.9% NBM/session, CON = 1.0% NBM/session) and the sustained endogenous stimuli (increased $T_{rec}$) of isothermic HA (26) increases PV expansion via increased plasma albumin and the renin-angiotensin-aldosterone system.
creasing Hbmass (27, 60). Our interventions did not increase Hbmass; thus they did not, or cannot, induce sufficient heat strain and/or training load to stimulate erythropoiesis (27). This disparity from comparable research suggests more data are required to elucidate whether HA can effectively induce changes in Hbmass.

The metabolic response to altitude is a preferential shift toward glycolysis (48); as such, the reduction in RER following HA, an indicator of substrate utilization, was an unexpected observation in hypoxia, although a reduction in metabolism has been observed in response to heat (30). Absent of changes in VE and breathing frequency (Table 3), the RER reduction at rest during HYP2 (following HA; Fig. 3) appears to be a metabolic response, rather than an artifact of hyperventilation between trials (5). The present data cannot determine whether hypoxia-induced hyperventilation from normoxia occurred or was reduced following HA. HIF-1 alters metabolism at altitude (48), the typical response being an initial increase in glycolysis on acute exposure, followed by a reduction with acclimatization/acclimation. HIF-1 is known to increase following HA (42, 62); thus increases may accelerate the desensitization, or inhibit the immediate shift in substrate metabolism (49). Another theory implicates HSP72 as having a therapeutic role in glycogen regulation among other metabolic disorders, e.g., type 2 diabetes and obesity (29). Transgenic mice overexpressing HSP72 evidence increased fatty acid oxidation and reduced mitochondrial dysfunction, alongside increased VO2 and exercise capacity (29). At present, these mechanisms are speculative; however, responses within our data warrant further investigation to authenticate or refute this observation.

Observations that HSP72 mRNA increases (+2.5-fold) are supported by our data (+2.0 ± 1.0-fold), which also support similar sustained increases in HSP90α mRNA (+2.4 ± 1.5-fold) (25). It is notable that these data are supportive of others observing no daily change in resting HSP72 or HSP90α mRNA (25, 44, 70); thus, following an initial stress response, the removal of the stress stimuli is sufficient to remove the necessity for transcription back to basal quantities within 24 h. It is this observation that reaffirms that the HSP72 mRNA response to exercise is a potential marker of acclimation; however, resting levels do not provide sufficient discrimination (44), as basal intracellular
HSP72 protein do (44, 45). These data highlight that isothermic HA could provide a stimulus for increases in HSP72 and HSP90α protein at both the onset and culmination of the regimen (Fig. 4). Endogenous physiological and cellular strain induced by CON was insufficient to induce the respective gene transcripts for HSP72 (+0.5 ± 0.2-fold) and HSP90α (+0.4 ± 0.1-fold), likely due to the failure to induce sufficient changes in Trec or oxidative stress. Thus this exercise prescription is unlikely to increase basal protein within the cell, a requirement of cross-tolerance.

Increased HSP72 and HSP90α mRNA in hypoxia highlights the sensitivity of each gene to both exercise and endogenous environmental stimuli (temperature and oxidative stress induced by hypoxia). Increased gene expression in HYP1 suggests our protocol was effective at providing potentiating stimuli for a heat shock protein response, albeit with smaller expression than the HA sessions, suggesting inferior endogenous stimuli for transcription. Reductions in HSP72 mRNA in HYP2 following HA evidences either reduced physiological strain during HYP2 (cross-acclimation) or an HA-induced increase in intracellular HSP72 (41) (cross-tolerance), mitigating requirements for further gene transcription. Neither of these mechanistic pathways is true of HSP90α mRNA, which shares a similar response between HYP1 (+0.3 ± 0.4-fold) and HYP2 (+0.4 ± 0.4-fold) (Fig. 5). A longer hypoxic exposure may have elicited a greater magnitude of heat shock protein mRNA responses, especially for participants in CON, who demonstrated no increase in HSP72 mRNA. This prolonged protocol may have also further enhanced observable differences in HSP72 mRNA before and after HA. Maintenance of increased HSP90α in HYP2 may relate to a specific role of the protein within recovery and adaptation to cellular stress, i.e., control of cellular signaling cascades (64), recovery of global protein synthesis (13), and coordination of cellular repair (15). Hence continued gene transcription may be required. Relative exercise intensity, which is known to change under different environmental conditions, affects the metabolic strain and molecular responses (4, 36). Accumulation of HSP72 and HSP90 protein occurs with HA (45); however, basal HSP90α has been demonstrated as lower than HSP72 (45). Within HYP1/HYP2, the metabolic strain likely induced protein denaturation, activating the heat shock protein response via heat shock factor-1. However, it is plausible that basal HSP72 was sufficient to cope with the hypoxic stress post-HA (4); thus transcription was mitigated. However, basal HSP90α protein remained lower than necessary; thus HYP2 induced further mRNA transcription to a similar extent as observed in HYP1. As previously observed (70), the present study cannot suggest that our intervention can translate the mRNA signal into HSP72- and HSP90α-mediated thermotolerance or hypoxic cross-tolerance within leukocytes (31), because increased mRNA expression is not necessarily reflective of functional steady-state basal protein content, which may or may not be the most important component for observing cross-tolerance (71). Although it is unknown whether HA induced HSP72 and HSP90α protein accumulation, it has previously been stated that observed mRNA increases provide an indication that the heat shock response has been activated, potentiating protein translation (70).

The present data suggest the existence of pathways for transferring adaptations to HA to other environments (varying temperature/oxygen availability), although future experiments should determine whether the attenuated responses are specific to thermal stimuli alone, or the combined exercise-heat stress training stimuli that our intervention applied. With likely increases in aerobic capacity (39), the absolute workload model implemented may have also elicited different responses than a relative intensity model based on postacclimation aerobic capacity; thus this is a limitation of the experiment, which should be considered relevant to future research in the area. Data pertaining to cross-acclimation within this experiment are clear, particularly when cardiovascular stress compromises the individual in a particular environment (14, 73). The true thermal adaptation might be of greater relevance with regard to the heat shock protein response, which is known as responsive to different relative exercise intensities (4, 36). Benefits of increased intracellular HSP72 and HSP90α have been recently reviewed (14, 56, 73); however, cross-tolerance pathways cannot be fully confirmed given the lack of protein data presently available. Implications for HA-induced changes in HSP72 and HSP90α could be confirmed by measuring HSP72 and HSP90α protein pre- and postintervention and then applying hypoxic shock/stress within ex vivo and in vivo models. Further research is required to determine the benefits of cross-acclimation and cross-tolerance across a spectrum of simulated and actual altitudes and workloads within those environments.

Conclusion. HA is an effective intervention for reducing physiological strain associated with acute normobaric hypoxia, primarily through HA-derived PV expansion improving cardiovascular efficiency, which reaffirms a cross-acclimation mechanism. Normothermic training failed to reduce physiological strain or alter the HSP72 and HSP90α mRNA response to hypoxia. The HSP72 mRNA increase pre-HA was attenuated following acute normobaric hypoxia following HA, giving efficacy to cross-tolerance pathways at a cellular/molecular level; however, no changes in HSP90α mRNA were observed. These data suggest hyperthermia as a viable potentiating stimulus for the cross-adaptive mechanisms.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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