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ORIGINAL ARTICLE

Daily hypoxia increases basal monocyte HSP72 expression in healthy human subjects

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Abstract Heat shock protein 72 (HSP72) performs vital roles within the body at rest and during periods of stress. In vitro, research demonstrates HSP72 induction in response to hypoxia. Recently, in vivo, an acute hypoxic exposure (75 min at 2,980 m) was sufficient to induce significant increases in monocyte expressed HSP72 (*mHSP72*) and a marker of oxidative stress in healthy human subjects. The purpose of the current study was to identify the impact of 10 consecutive days of hypoxic exposures (75 min at 2,980 m) on *mHSP72* and erythropoietin (EPO) expression, markers of oxidative stress, and maximal oxygen consumption in graded incremental aerobic exercise. Eight male subjects were exposed to daily normobaric hypoxic exposures for 75 min at 2,980 m for 10 consecutive days, commencing and ceasing at 0930 and 1045, respectively. This stressor was sufficient to induce significant increases in *mHSP72*, which was significantly elevated from day 2 of the hypoxic exposures until 48 h post-final exposure. Notably, this increase had an initial rapid (30% day on day compared to baseline) and final slow phase (16% day on day compared to baseline) of expression. The authors postulate that 7-day hypoxic exposure in this manner would be sufficient to induce near maximum hypoxia-mediated basal *mHSP72* expression. Elevated levels of *mHSP72* are associated with acquired thermotolerance and provide cross tolerance to non-related stressors in vivo, the protocol used here may provide a useful tool for elevating *mHSP72* in vivo. Aside from these major findings, significant transient daily elevations were seen in a marker of oxidative stress, alongside sustained increases in EPO expression. However, no physiologically significant changes were seen in maximal oxygen consumption or time to exhaustion.

Keywords Stress protein · Oxidative stress · EPO · TBARS

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Introduction

The heat shock protein 70 (HSP70) family of stress proteins, particularly the inducible isoform heat shock protein 72 (HSP72), play a significant role in maintaining protein homeostasis (Hartl 1996). Protein-specific functions include de novo folding, refolding and degradation (Fink 1999). Other physiological roles of HSP72 include behaviour as a molecular chaperone (Morimoto 1998) and an ability to act as an anti-apoptotic mediator in programmed cell death (Garrido et al. 2001). Additionally, high cellular concentrations both in vitro (Garramone et al. 1994) and in vivo (Sandstrom et al. 2008) are associated with acquired thermotolerance and can confer cellular tolerance to non-related stressors (Kiang et al. 1996). These physiological and biochemical influences have ensured research in HSP72 has grown significantly, particularly elucidation of its role in exercise performance (Locke and Noble 1995) and in exercise heat acclimation strategies (McClung et al. 2008). This highly conserved and inducible member of the stress protein family demonstrates increases in concentration under diverse physiological stressors (Kregel 2002), including ischemia (Richard et al. 1996), hyperthermia (Ritossa 1962), oxidative stress

(Kukreja et al. 1994), exercise (Locke and Noble 1995), hypothermia (Yang et al. 1996) and hypoxia (Taylor et al. 2010b), amongst others. Basal monocyte expressed HSP72 (*mHSP72*) follows a diurnal quadratic trend in expression at rest (Sandstrom et al. 2009), which has been shown to be reproducible in healthy human subjects on three separate days (Taylor et al. 2010a). Recently, an acute hypoxic exposure (75 min at 2,980 m) was shown to induce significant changes in *mHSP72* expression and markers of oxidative stress 30 min post-hypoxia, in 12 healthy male subjects compared to their basal values (Taylor et al. 2010b).

Modern altitude training (live high train low) has been shown to improve sea level athletic performance in carefully controlled studies (Levine and Stray-Gundersen 1997). Current hypoxic training practises have sought to use intermittent hypoxic training (IHT), often utilising hypoxicator machines, to generate the necessary hypoxic load without the cost of commuting to and living at elevated altitudes (Hamlin and Hellemans 2007). Specific to athletic training and performance, administration of intermittent hypoxia during physical activity and at rest has been utilised in attempts to augment athletic performance, with varying efficacy (Levine 2002). The terminology used for IHT was often confusing, and has recently been addressed (Bartsch et al. 2007). In its truest form, intermittent hypoxic exposures last for approximately 60–90 min and involves repeated switches between normoxic and hypoxic air (Bartsch et al. 2007). Continuous hypoxic exposures of 1–4 h, at various elevations, ensure altitude sickness does not present and are referred to as prolonged hypoxic exposures, with hypoxic periods in excess of 4 h representing chronic hypoxic exposures (Levine 2002). Researchers have utilised intermittent hypoxic exposures in the treatment of clinical disorders (hypertension, Parkinson's disease, etc.), altitude pre-acclimation and for sporting performance (Serebrovskaya 2002).

Despite extensive previous research, at present repeated daily prolonged hypoxic exposures at rest (PHE_R), and the impact of such exposures on *mHSP72* expression and markers of oxidative stress in vivo, have not been documented, in healthy human subjects. The aims of this present study were to identify the impact of 10 days daily PHE_R on *mHSP72*, with potential links to oxidative stress and erythropoietin (EPO) expression also investigated. High endogenous levels of HSP72 have been postulated to provide protection against the biochemical rigours of aerobic exercise (Locke and Noble 1995; Morton et al. 2009), with daily PHE_R known to elicit no (Katayama et al. 2004), marginal (Katayama et al. 2003) or mixed (Rodriguez et al. 2007) augmentation of sea level exercise performance. Therefore, any observed changes in *mHSP72*, oxidative stress and EPO expression were to be viewed in light of potential alterations in physiological measures of maximal exercise performance $\left(\dot{V}O_{2\,max}\right)$. Additionally, attempts were made to establish a non-thermal non-exercise based protocol to increase basal *mHSP72* expression in vivo.

Methods

Subjects

Eight healthy recreationally active male subjects (mean ± SD age 20.2 ± 4.4 years, height 172.1 ± 13.9 cm, body mass 71.1 ± 8.0 kg and physical activity 5.3 ± 1.8 h $week^{-1}$) volunteered to participate in the study. Caffeine (Lu et al. 2008) and glutamine (Singleton et al. 2004) consumption were barred from all meals and beverages for 24 h prior to the commencement of the study and throughout the 18-day testing period. Subjects were requested to abstain from prolonged thermal exposures (baths, saunas, steam rooms, tanning devices, etc.), vigorous physical activity and alcohol for 7 days prior to and during the extended 18-day study period. Compliance was monitored via a questionnaire, administered before and during the intervention period with adherence 100%. Smokers were excluded from the study as it has been shown that smoking may induce HSP72 expression as a consequence of oxidative stress (Anbarasi et al. 2006). Alterations in core temperature have been shown to impact upon *mHSP72* (Sandstrom et al. 2009), however, the same hypoxic exposure under identical environmental conditions (temperature-controlled laboratory) demonstrated no significant changes in the core temperature of subjects throughout the 12-h study period (Taylor et al. 2010b). Additionally, the efforts made by Taylor et al. (2010a, b) were repeated to ensure *mHSP72* values were not unduly affected by factors aside from the hypoxic exposure. The protocol was approved by the Sport, Health and Exercise Science Departmental Human Ethics Committee and all subjects signed informed consent following the principles outlined in the Declaration of Helsinki.

Blood sampling and experimental protocol

The hypoxic exposure consisted of 75 min at a simulated altitude of 2,980 m, which equates to an oxygen concentration of 14.5% at a barometric pressure of 775 mmHg, with the intervention commencing and ceasing at 0930 and 1045, respectively. This hypoxic protocol has been used previously (Taylor et al. 2010b). The exposure was administered daily for ten consecutive days. Hypoxia was generated and delivered via a hypoxicator (HYP123 hypoxicator, Hypoxico, New York, USA) which utilises oxygen filtration to generate the necessary

hypoxic load. Subjects remained within the temperature-controlled laboratory (mean ± SD: WBGT 21.2 ± 0.2°C, humidity 47 ± 4%) during the exposure. Subjects did not sleep within the laboratory, but within a nearby University-owned accommodation block for the night prior to and during all days of the study. Subjects gained 10 ± 1.8 h sleep prior to study commencement and repeated this sleep cycle on all study nights (10.5 ± 1.8 h). Blood samples were taken immediately pre- and post-hypoxic exposure on days 1, 2, 3, 4, 5 and 10 for analysis of *mHSP72* and TBARS with EPO-specific blood samples taken on day 1, 2, 3, and 10. Sampling was limited to specific days as blood collection on all experimental days would have involved 24 separate venipunctures with pilot testing demonstrating significant volunteer discomfort under this regime. Additionally, pilot work demonstrated that EPO expression peaked after 2/3 days of hypoxic exposures and remained relatively unchanged until cessation of the hypoxic protocol. Hypoxia-mediated *mHSP72* expression demonstrated a biphasic response, with TBARS illustrating a consistent diurnal transitory response. In light of this pilot work (*mHSP72*, TBARS and EPO) sampling frequency was designed to limit subject discomfort whilst gaining maximum insight into the expression kinetics of the parameters investigated (*mHSP72*, EPO and TBARS). Blood samples were also drawn 24 h pre (baseline levels) and 48 h post 10-day intervention period. Blood samples were drawn immediately prior (0925–0930) and post (1045–1050) hypoxic exposure on each hypoxic day, for control sampling and 48 h post-hypoxic intervention period. This consistent timing of blood sample collection was important to account for diurnal and circadian (Sandstrom et al. 2009; Taylor et al. 2010a) variations in basal *mHSP72*, especially as basal values can dictate the magnitude of stressor-mediated changes in HSP72, both in vivo (Gjovaag and Dahl 2006) and in vitro (Vince et al. 2010). Post-hypoxic blood sampling was conducted immediately post-intervention, unlike previous work (Taylor et al. 2010b), to ascertain if the *mHSP72* response is instantaneous post-hypoxia. Blood samples were drawn from the antecubital vein into potassium EDTA Vacuette tubes (Vacuette®, Greiner BIO-one, UK). It has been shown (Fortes and Whitham 2009) repeated venipuncture elicits no stress-induced changes in HSP72 in comparison to repeated cannulation blood draws. Heart rate and oxygen saturation were recorded every 5 min during the hypoxic exposure via a finger pulse oximeter (Nonin 9550 Onyx II Finger Pulse Oximeter, Nonin Medical Inc, Plymouth, USA). Methods, procedures and restrictions replicated those used previously to gain serial data for *mHSP72* (Sandstrom et al. 2009; Taylor et al. 2010a, b) and other physiological markers of homeostasis (Madden et al. 2008; Vince et al. 2009). Samples were processed immediately for measurements of *mHSP72*. The $\dot{V}O_{2\,max}$ tests were conducted (at sea level) 8 days before and 48 h after the 10-day exposure period, to ensure the undue influence of physical activity (Morton et al. 2006) was not seen in *mHSP72* pre, during and post-hypoxic programme. Additionally, subjects had been familiarised with the $\dot{V}O_{2\,max}$ protocol and equipment to negate possible test order effects. The continuous incremental treadmill $\dot{V}O_{2\,max}$ protocol utilised started at a speed of 8 k h^{-1} which increased by 1 k h^{-1} per minute until volitional fatigue of the subject. Before study commencement subjects completed a food diary, with a standardised daily food regime agreed upon between subjects and research organisers based on this. Compliance was monitored via this food diary and was at 100%.

Monocyte HSP72 assay

The choice, justification and measurement of *mHSP72* has previously been discussed in detail (Sandstrom et al. 2009; Taylor et al. 2010a, b). An IgG1 isotype and concentration matched FITC conjugated negative control was used to asses non-specific binding. Briefly, cells obtained after red cell lysis were fixed and permeabilised (AbD Serotec, UK) and a negative control (FITC, AbD Serotec, UK) or anti-HSP72 antibody (SPA-810, Assay Designs, USA) was added to a final concentration of 100 μg/ml, this was used to label 1×10^6 cells according to the manufacturer's instructions and then incubated for 30 min in the dark. Samples were then analysed on a BDFACSCalibur (BD Biosciences) by flow cytometry with monocytes gated by forward/side scatter properties and further discriminated by CD14 expression. Mean fluorescence intensity (MFI) was then calculated using CELLQuest software (BD Biosciences) with a total of 50,000 cells counted.

Measurement of oxidative stress and erythropoietin

An EDTA Vacuette tube (Vacuette®, Greiner BIO-one, UK) filled with venous blood was mixed and then centrifuged at 1,500 × *g* for 10 min, with the resulting EDTA plasma removed and stored at −80°C. This plasma was, at a later date, analysed for lipid peroxidation. Lipid peroxidation was measured utilising a commercially available thiobarbituric acid reactive substances (TBARS) kit (ZeptoMetrix, USA) according with the manufacturer's instructions. Results are expressed as malondialdehyde equivalents. Measurement of EPO was obtained by chemiluminescent immunoassay (Immulite 1000 EPO Kit and Immulite 1000 analyser; Siemens DPC, USA) as per manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using PASW statistics 17 software (SPSS Inc., Chicago, IL). Normality was checked using Q–Q plots and deemed plausible in each instance. Polynomial contrasts were used to identify trends in *mHSP72*, EPO and TBARs concentrations, oxyhaemoglobin saturation and heart rate across time, both within days (pre- to post-intervention) and across the 10-day intervention period. In the event of a significant F statistic, Fisher's least significance test was used to locate significant paired differences. The *mHSP72* expression was expressed as a percentage of the first measurement, which is in accordance with previous literature (Morton et al. 2007; Taylor et al. 2010a, 2010b). Paired t tests were used to compare the $\dot{V}O_{2\,max}$ values, maximal heart rate (HR_{max}) and time to exhaustion before and after the 10-day intervention period. Two-tailed statistical significance was accepted as $p < 0.05$.

Results

There were no significant changes observed over the 10-day hypoxic exposure intervention period for absolute $\dot{V}O_{2\,max}$ ($t = 2.3$, $p = 0.065$) or heart rate max (HR_{max}) ($t = 1.6$, $p = 0.16$), however, there was a very small significant increase in time to exhaustion for the incremental test ($t = 3.9$, $p = 0.008$, $\omega^2 = 0.50$) (Table 1), though this is likely physiologically negligible.

A significant fifth order polynomial trend was observed for EPO concentration over the 10 days ($F = 34.5$, $p = 0.001$, partial $\eta^2 = 0.85$), characterised by a dramatic 39% increase in EPO concentration the day after the first hypoxic exposure ($p = 0.001$), followed by a relative plateau over the rest of the hypoxic exposure period, and then a dramatic reduction immediately post-intervention (Fig. 1). There was no significant within-day effect ($F = 0.01$, $p = 0.92$) and no significant within-day, between-day interaction ($F = 3.2$, $p = 0.12$).

Table 1 Mean (SD) $\dot{V}O_{2\,max}$, maximal heart rate (HR_{max}) and time to exhaustion observed during the incremental test before and after the 10-day hypoxic exposure intervention period

Variable	Before	After
$\dot{V}O_{2\,max}$ (ml/kg/min)	50.1 (6.5)	50.8 (6.6)
$\dot{V}O_{2\,max}$ (ml/min)	3,930 (769)	3,948 (776)
HR_{max} (beats/min)	191 (10)	195 (6)
Time to exhaustion (min)	11.8 (1.5)	12.1 (1.6)*

* Significantly different from before the 10 day intervention period ($p = 0.008$)

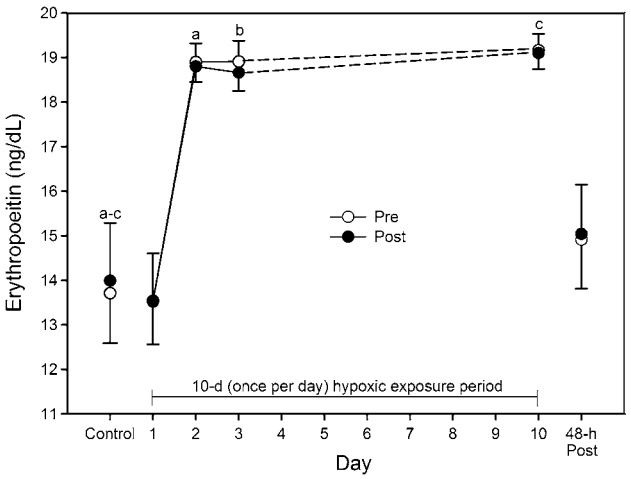

Fig. 1 Mean (SEM) erythropoietin concentration immediately before (*Pre*) and after (*Post*) hypoxic exposure over the 10-day intervention period. Control values, where no hypoxic exposure occurred, are also shown for before (*Control*) and after (*48-h Post*) the intervention period. *Like letters above error bars* represent significant differences in mean erythropoietin concentration across time ($p < 0.05$)

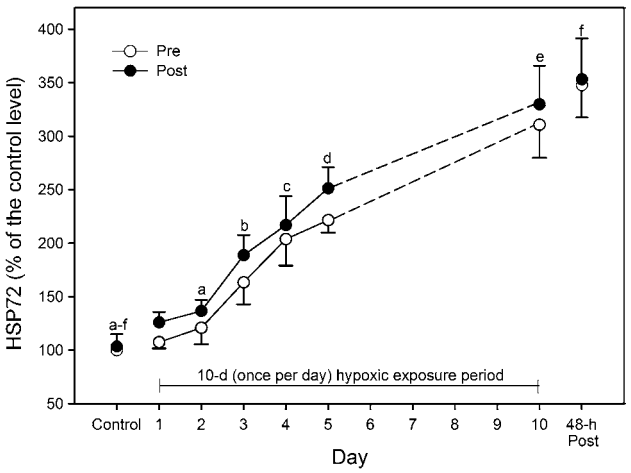

Fig. 2 Mean (SEM) HSP72 expression immediately before (*Pre*) and after (*Post*) hypoxic exposure over the 10-day intervention period. Control values, where no hypoxic exposure occurred, are also shown for before (*Control*) and after (*48-h Post*) the intervention period. The HSP72 is expressed as a percentage of the first measurement (control level). *Like letters above error bars* represent significant differences in mean HSP72 across time ($p < 0.05$)

A significant linear trend was observed for *mHSP72* expression, where *mHSP72* expression increased throughout the 10-day experimental period ($F = 73.2$, $p < 0.001$, partial $\eta^2 = 0.92$) (Fig. 2). The *mHSP72* expression increased, on average, by 30% per day between days 0 and 5, and by 16% per day between days 5 and 10. Within each day, *mHSP72* expression was consistently higher after hypoxic exposure ($F = 6.2$, $p = 0.047$, partial $\eta^2 = 0.51$). However, no significant within-day, between-day interaction was observed ($F = 0.4$, $p = 0.53$).

There were significant main effects for day ($F = 9.0$, $p = 0.024$, partial $\eta^2 = 0.60$) and time (157.4, $p < 0.001$, partial $\eta^2 = 0.96$) for TBARS concentration, however, the within-day, between-day interaction displayed a significant quadratic-by-linear trend ($F = 11.2$, $p = 0.015$, partial $\eta^2 = 0.65$). The TBARS concentration increased significantly by around 20% in response to each hypoxic exposure (Fig. 3), whereas no differences were observed in the control days, pre- and post-hypoxic intervention.

The change in oxyhaemoglobin saturation within each 75 min hypoxic exposure period exhibited a significant quadratic trend ($F = 3{,}678.4$, $p < 0.001$, partial $\eta^2 = 0.998$). Oxyhaemoglobin saturation dropped from 99% to around 90–91% 10 min after hypoxic exposure, remained depressed for the remainder of the hypoxic exposure, and returned to baseline 10 min after exposure was terminated (Fig. 4). A within-day, between-day linear-by-quartic interaction effect also was observed for oxyhaemoglobin saturation ($F = 33.1$, $p = 0.001$, partial $\eta^2 = 0.83$).

Heart rate exhibited no significant trend over the 10-day intervention period ($F = 2.3$, $p = 0.18$), however, a significant quartic trend was observed across time within each day ($F = 397.4$, $p < 0.001$, partial $\eta^2 = 0.98$) (Fig. 5). The mean heart rate was 70 beats/min 10 min before the hypoxic exposure, significantly increased to 83 beats/min within 5 min of exposure ($p < 0.001$), significantly decreased to 80 beats/min 5 min later ($p = 0.013$), and remained relatively constant for the remainder of the exposure, before returning to baseline 10 min after exposure ($p < 0.001$). A within-day, between-day linear-by-quartic interaction effect also was observed for heart rate ($F = 8.6$, $p = 0.022$, partial $\eta^2 = 0.55$).

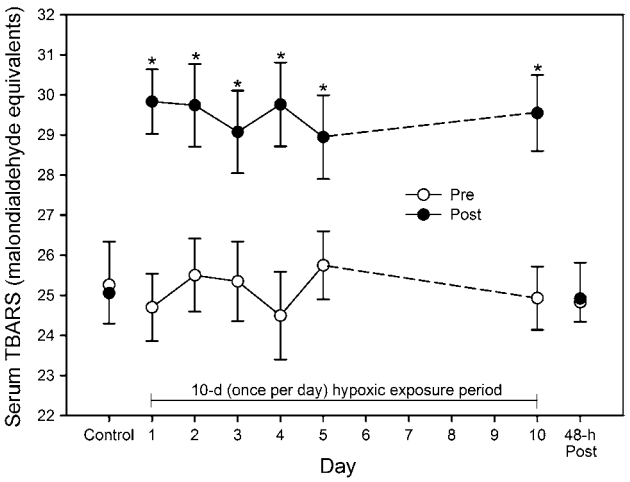

Fig. 3 Mean (SEM) serum TBARS concentration immediately before (*Pre*) and after (*Post*) hypoxic exposure over the 10-day intervention period. Control values, where no hypoxic exposure occurred, are also shown for before (*Control*) and after (*48-h Post*) the intervention period. *Significantly different from Pre ($p < 0.05$)

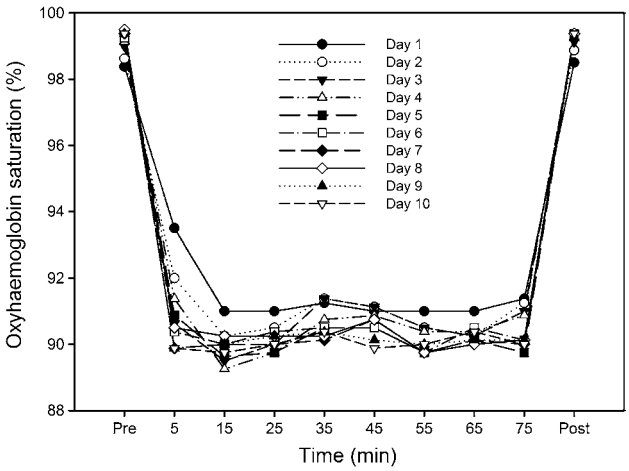

Fig. 4 Mean oxyhaemoglobin saturation before (*Pre*), during, and after (*Post*) 75 min of hypoxic exposure for each day of the 10-day intervention period. Error bars have been omitted to aid clarity

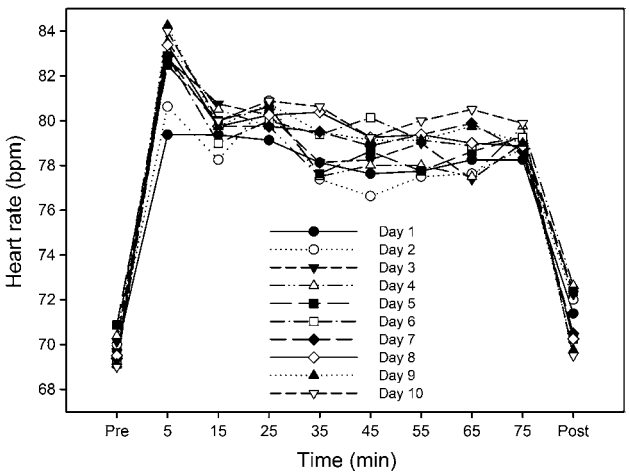

Fig. 5 Mean heart rate before (*Pre*), during, and after (*Post*) 75 min of hypoxic exposure for each day of the 10-day intervention period. Error bars have been omitted to aid clarity

Discussion

The same daily oxidative stress, as determined by TBARS reaching approximately the same value after each hypoxic exposure (Fig. 1) is caused by hypoxic air breathing (Bailey et al. 2001). This transient increase in oxidative stress appears to be a trigger for *mHSP72* synthesis, as shown elsewhere (Kukreja et al. 1994; Ahn and Thiele 2003), resulting in an increase in *mHSP72* proportional to the basal content pre-exposure and the prior day (Gjovaag and Dahl 2006; Vince et al. 2010). It can be seen that ten consecutive days of daily PHE_R are sufficient to induce significant elevations in *mHSP72* (Fig. 2). Previously, we have shown that an identical hypoxic stimulus elicits a prolonged (9 h) significant elevation in concentrations of *mHSP72* compared to control, however, these findings

were not examined in excess of 9 h post-exposure (Taylor et al. 2010b). The present study, demonstrates 24-h post-first exposure, *mHSP72* is significantly elevated, with increases compared to control evident on each subsequent day and 48 h post-final exposure (Fig. 2). Immediately after post-first hypoxic exposure, no significant increase is evident in *mHSP72*, this is likely attributable to the immediate nature of post-hypoxia blood sampling, i.e. within 5 min of hypoxia ceasing. Previously (Taylor et al. 2010b), approximately 30-min post-hypoxia, significant increases in *mHSP72* were seen. Future work could examine this period immediately post-hypoxia to elucidate the threshold in time required, post-hypoxia, for significantly elevated *mHSP72* expression to occur. Interestingly, the elevations in *mHSP72* demonstrated an initial rapid rise, 30% day on day up to day 5, whereas average increases between days 5 and 10 are approximately 50% lower at 16% day to day (Fig. 2). For example, extrapolating data from day 5 to day 7 (30% per day) would demonstrate a notable plateauing in *mHSP72*, with minimal *mHSP72* increase from this point, despite further hypoxic exposure. This initial phase (days 0–7) of hypoxia-mediated *mHSP72* expression, suggest that as a preconditioning strategy, the first week of PHE_R may be sufficient to induce close to maximum at-rest (baseline) *mHSP72* levels. The authors do not suggest that whole body cytoprotection has been initiated or conferred by these hypoxia-mediated elevations in *mHSP72*. Assumptions regarding the skeletal muscle and extracellular HSP72 (within the serum or plasma for example) content in response to an intervention such as the one utilised here requires further elucidation in tandem with measures of *mHSP72*. This would allow a greater understanding of the in vivo contribution of both extra and intracellular HSP72 to hypoxia and any conferred cellular tolerance to further non-lethal non-related stressors.

Endurance trained individuals expose themselves to repeated periods of oxidative stress as a consequence of regular aerobic training, resulting in decreased basal *mHSP72* expression (Fehrenbach et al. 2000). However, the present study demonstrates no reduction in basal *mHSP72* in response to daily elevations in oxidative stress, as measured by TBARS. Although endurance training has been shown to decrease basal levels of *mHSP72* (Fehrenbach et al. 2000), in comparison to untrained controls, individuals who are heat acclimated (HA) and thus acquire some degree of thermotolerance (ATT) display elevated basal values in *mHSP72* compared to non-HA controls (McClung et al. 2008). This elevation in intracellular HSP72 within HA individuals is present in other peripheral blood mononuclear cells (PBMC) (Fehrenbach et al. 2001) aside from monocytes and within skeletal muscle (Marshall et al. 2007). The HSP72 expression kinetics seen for ATT whereby daily hyperthermic exercise stress elicits sustained increases in *mHSP72*, compared to baseline levels (McClung et al. 2008), mirror those seen here for PHE_R (Fig. 2). Increased bioavailable HSP72 within cell cultures and animal models is implicitly linked to augmented cellular protection and survival to subsequent stressors (Garramone et al. 1994; McArdle et al. 2004). Heat stressed cells have been shown to display HSP72-dependant resistance to the necrotic effects of hypoxia (Kiang et al. 1996) with HA animals displaying resistance to the hypoxic rigours of various surgical procedures (Horowitz 2002; Arieli et al. 2003). Continued elevations in *mHSP72* (Fig. 2) may provide protection for subsequent hypoxic exposures, in the same way elevated extracellular HSP72 expression in response to hyperthermic exercise protocols provides protection to exercise in high ambient temperatures (Sandstrom et al. 2008). Significantly, decreased apoptosis is seen in animals that have attained HA upon exposure to further non-lethal non-related stressors (Horowitz et al. 2004). This ability of HA to confer tolerance to subsequent diverse stressors has led to common protective pathways being suggested as the underlining mechanism in HA-induced preconditioning (Shein et al. 2007). It has been shown that heat shock and ischemia induce similar changes in expression of various genes (Horowitz et al. 2004), including increases in HSP72 and EPO (Shein et al. 2005; Shein et al. 2007). This cross tolerance is hypothesised to stem from increases in oxidative stress providing the stimulus for hormesis to occur (Fisher-Wellman and Bloomer 2009).

Endurance training leads to physiological and haematological improvements in $\dot{V}O_{2\,max}$(McNicol et al. 2009). This augmentation has been linked to biologically derived reactive oxygen species (ROS) acting in a hormetic manner, thus potentially conveying tolerance to further stressors (Fisher-Wellman and Bloomer 2009). The present study does not support this hypothesis (Fisher-Wellman and Bloomer 2009); here we demonstrate daily transient increases in TBARS, yet augmentation of any of the parameters associated with increased maximal exercise performance are either unchanged or in the case of time to exhaustion physiology negligible (Table 1), these results are aligned with findings elsewhere (Katayama et al. 2003, 2004). This study does not address any hormetic influence of repeated PHE_R induced changes in oxidative stress on sub-maximal aerobic exercise performance, this avenue of research may warrant further investigation. Specifically as elevations in HSP72, as seen within the monocytes in the current study, have been postulated to provide protection to the biochemical rigours of aerobic exercise (Locke and Noble 1995; Morton et al. 2009).

Research specifically utilising PHE_R have employed a vast array of methodologies (elevations, delivery systems,

subject training status, etc.) and consequently no consensus within the literature has been reached regarding EPO expression in response to PHE_R or IHT in general (Levine 2002). A protocol similar to the one employed here (14-day, 2-h daily exposures at 4,100 m, at rest) demonstrated no significant changes in haemoglobin, EPO and reticulocytes (Lundby et al. 2005), with findings by Katayama et al. (2003) supporting these observations. However, in accordance with our data, though to a greater magnitude (55 vs. 39% increase from control in the current study), Rodriguez et al. (2000) demonstrated that a single hypoxic exposure of 90 min (5,000 m) was sufficient to induce a significant increase in serum EPO concentration. Additionally, in agreement with the current study's results (Fig. 1), immediately post-exposure, no significant change in EPO expression was demonstrated (Rodriguez et al. 2000). Rodriguez et al. (2000) only monitored EPO expression for 300-min post-exposure, where results in this current study demonstrate that elevated EPO expression in response to PHE_R is prevalent for up to 48 h post-exposure. The elevations in expression seen here mirror those seen previously for chronic exposures, whereby EPO production peaks 24-h post-hypoxic exposure (Abbrecht and Kittell 1972). Under the influence of chronic hypoxia, this zenith (24-h post-exposure) in expression dwindles to a nadir, which is still significantly elevated compared to control (Berglund et al. 2002). Results presented here (Fig. 1), utilising PHE_R rather than chronic exposures, demonstrate no reduction in this initial zenith in expression (24-h post-exposure), with no apparent nadir throughout the 10-day period. Daily PHE_R likely provides a greater stimulus for EPO response due to repeated periods of oxidative stress (Fig. 3), specifically daily reperfusion of hypoxic tissues and a resulting transient elevation in oxidative stress (Clanton 2007), in comparison to chronic exposures (Berglund et al. 2002).

Oxidative stress has been highlighted as a key signalling factor in pathways and transduction cascades for a variety of hormones, genes, cytokines and growth factors (Allen and Balin 1989; Maulik 2002). Therefore, increases in oxidative stress could be a stimulus (signalling molecule) for the elevated HSP72 expression seen during and post the 10-day PHE_R period (Allen and Balin 1989; Maulik 2002). High cellular levels of oxidative stress are known to damage membrane structures and proteins and activate pathways of apoptosis (Kulkarni et al. 2007), which are known to represent a powerful stimuli for HSP72 induction (Bienemann et al. 2008).

In summary it can be seen that daily administration of PHE_R is sufficient to induce sustained increases in *mHSP72* and EPO, with transient increases in oxidative stress also seen. These elevations in *mHSP72* display an initial fast phase in response to hypoxia (day 1–5) and a slower phase (5–10). The authors postulate that 7-day PHE_R in this manner would be sufficient to induce close to maximum at-rest hypoxia-mediated *mHSP72* expression. The resulting increase in bioavailable *mHSP72* may provide some protective influence during sub-maximal aerobic exercise yet fails in the current study to have a physiology significant impact on maximal exercise performance. These elevations in expression (*mHSP72*) are similar to those seen in exercise HA and may provide a useful tool for manipulating stress protein expression in order to gain ATT, as tentatively postulated elsewhere (Taylor et al. 2010b). The observed hypoxia-induced *mHSP72* response may benefit from investigation in other tissues and cells, for example, other PBMCs, serum, plasma and muscle tissue. Additionally, whether the hypoxia-mediated increases in *mHSP72* may provide some resistance or conferred cellular tolerance to the biochemical rigours of sub-maximal exercise may warrant further investigation.

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