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Blood oxidative stress and post-exercise recovery are unaffected byhypobaric and hypoxic environments

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ABSTRACT

Hypobaria and hypoxia exert independent effects on oxidative stress during exercise, while combined effectson the post-exercise recovery period remain unclear.Accordingly, this study examined the recovery period during lab-simulated hypoxic and hypobaric conditions following exercise-induced oxidative stress. Participants (n=13) performed 60-minutes of cycling (70% watts max) in a normobaric normoxic environment followed by a four-hour recovery under three conditions; 1000m normobaric normoxia (NN, 675mmHg), 4400m normobaric hypoxia (NH, 675mmHg), or 4400m hypobaric hypoxia (HH, 440mmHg). Blood samples collected at Pre, Post, 2-Hours (2-HR), and 4-Hours (4-HR) post-exercise were analyzed fora potential increase in biochemical modifications of proteins(protein carbonyls, PC; 3-nitrotyrosines, 3NT) lipids (lipid hydroperoxides, LOOH; 8-isoprostanes, 8-ISO), and antioxidant capacity (FRAP, TEAC). Gene transcripts (EPAS, HMOX1, SOD2, NFE2L2) were quantified by qRT-PCR from muscle biopsies taken Pre and Post exercise. Hypoxia and hypobaria had no effect throughout recovery. Post-exercise TEAC ($p=0.041$), FRAP ($p=0.013$), and 8-ISO ($p=0.044$) increased, while PC ($p=0.002$) and 3-NT ($p=0.032$) were decreased. LOOH was lower in Post ($p=0.018$) NH trial samples. Exercise-dependent increases occurred in NFE2L2 ($p=0.003$), HMXO1 ($p<0.001$), SOD2 ($p=0.046$), and EPAS ($p=0.038$). Exercise recovery under conditions of NH and HH did not impact blood oxidative stress or redox-sensitive gene transcripts.

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KEYWORDS
Antioxidants; free radicals; reactive oxygen species

Introduction

Acute exercise is known to cause perturbations in redox balance and commonly results in a transient blood oxidative stress response in both laboratory and field-based settings (Gomez-Cabrera, Domenech, Viña et al., 2008; Hudson et al., 2008; J.C. Quindry et al., 2003; R.J. Bloomer et al., 2005). Although chronic, long-term oxidative stress can negatively impact physical health, previous research suggests temporary, acute changes in redox balance that occur during exercise and the post-exercise recovery period could be necessary to develop exercise adaptations (Gomez-Cabrera, Domenech, Viña et al., 2008). Oxidative stress results from both exercise stimuli and various environmental conditions (Gomez-Cabrera, Domenech, Romagnoli et al., 2008; Miller et al., 2013; Ristow et al., 2009; Sinha, Ray et al., 2009). Even under resting conditions, high altitude can alter an oxidative stress response in ways that are magnified during exercise (Dosek et al., 2007; Møller et al., 2001; Sinha, Singh et al., 2009). Further, the acute oxidative stress can be blunted following exercise under normobaric normoxic conditions when the post-exercise period when recovery occurs at altitude (Ballmann et al., 2014; McGinnis et al., 2014; Peters et al., 2016). Previous investigations have produced discrepant findings, with some results showing a blunting of oxidative stress and others showing no effect of environmental conditions, which reveals that responses to exercise and environmental stressors are more dynamic than previously thought (J. Quindry et al., 2016). With many recreationally active individuals self-selecting into exercise under a host of variable environmental conditions, it is important to gain new insights into the effects of hypoxia and hypobaria on blood oxidative stress responses during the subsequent recovery period. Prior work indicates that the experimental parameters of a particular study are essential in delineating the independent and combined effects of environmental hypoxia and hypobaria during the post-exercise recovery period. Moreover, the variations in the environment can influence oxidative stress biomarkers during and following an acute bout of exercise (Ballmann et al., 2014; McGinnis et al., 2014; Miller et al., 2013; Peters et al., 2016).

Given the need to control these experimental variables, many research groups interested in this topic have opted for investigations in tightly controlled laboratory settings. Case in point, previous field-based research suggests that acute exercise under hypoxic conditions can result in an enhanced oxidative stress response as compared to normoxic exercise (Miller et al., 2013). However, conclusions from these studies are complicated by the independent observations that hypoxic exercise elevates the relative intensity of a given workload, and may therefore reflect the fact that higher intensity exercise is merely associated with magnified oxidative stress responses (Alessio et al., 1988, 2000; J.C. Quindry et al., 2003; Wagner, 2000). Thus, while field-based studies have paved the way for altitude-based studies of work physiology, well-controlled laboratory studies were needed to delineate the independent effects of hypoxia

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on the post-exercise recovery period. For example, recent laboratory studies demonstrate that exercise recovery occurring under altitude-simulated hypoxic conditions blunt the oxidative stress response (Ballmann et al., 2014). However, a gap in knowledge remains in regard to the independent impact of hypobaric conditions on blood oxidative stress during the post-exercise recovery period. Therefore, the purpose of the current study was to examine the independent effects of hypobaria and hypoxia on post-exercise blood oxidative responses during the recovery period. A strategic panel of blood oxidative stress biomarkers was selected to quantify lipid damage (lipid hydroperoxides, LOOH; 8-isopsotane, 8-ISO), protein damage (protein carbonyls, PC; 3-nitrotyrosine, 3-NT), and antioxidative status (Trolox equivalent antioxidant capacity, TEAC; Ferric reducing ability of plasma, FRAP). Additionally, the redox-sensitive gene transcripts EPAS, HMOX1, SOD2, and NFE2L2 were examined from *vastus lateralis* biopsies in order to examine potential redox changes within muscle tissue. We hypothesized that both hypoxic and hypobaric conditions would result in a blunting of the oxidative stress response during the post-exercise recovery period.

Materials and methods

Participants

Informed consent for this investigation was obtained via the Institutional Review Board (IRB) and the United States Army Medical Research and Development Command (USAMRMC) Human Research Protections Office (HRPO). Participants granted written consent prior to study initiation. Recreationally trained male (n = 8) and female (n = 5) participants between the ages of 18 and 40 years old were recruited for this investigation. Inclusion criteria required a VO_{2peak} of at least 35 $ml \cdot kg^{-1} \cdot min^{-1}$ to ensure the participants would be capable of completing the exercise within the study. Participants with a history of acute mountain sickness or those with a known risk factor for coronary artery disease were excluded from the study as determined by the physical activity readiness questionnaire (PAR-Q). Females taking oral contraceptives or those without a regular menstrual cycle within the previous eight months were excluded due to the potentially confounding effects on hormonal status (Tiidus, 2000). Participant characteristics are presented in Table 1.

Table 1. Participant characteristics and exercise performance data.

Characteristics	Combined
Participants (*n*)	13
Age (years)	24 ± 4
Height (cm)	177 ± 12
Body mass (kg)	72.2 ± 13.3
Percent fat (%)	15.8 ± 8.1
Fat Free Mass (kg)	60.8 ± 12.6
Fat Mass (kg)	11.4 ± 6.0
Exercise performance	
$VO_{2\ max}$ (L/min)	3.8 ± 0.8
$VO_{2\ max}$ ($ml \cdot kg^{-1} min^{-1}$)	52.0 ± 6.4
Watt max (W)	290.5 ± 68.5
Data are presented as means ± SD	

Study design

The investigation began with a baseline testing session to collect participant characteristic data, including body composition and VO_{2peak}. The subsequent laboratory visits included three exercise-recovery trials where recovery parameters from an identical exercise protocol were modified according to simulated conditions of hypoxia and hypobaria. The experimental design is presented in Figure 1.

Baseline testing

Prior to baseline testing, participants were asked to fast for a duration of three hours. Participant characteristic data included the recording of height (Seca 213 Stadiometer, United Kingdom), weight (Befour PS-660 ST Digital Scale, Saukville, WI), body composition, and VO_{2max} assessments. Body composition was quantified via hydrostatic weighing using an electronic load cell-based system (Exertech, Dresbach, MN). Residual lung volume estimates were determined and final body density values from underwater weighing were converted to percent body fat using the Siri equation (Siri, 1961). Maximal oxygen uptake (VO_{2max}) was quantified during a graded exercise cycling protocol on an electronically braked Velotron cycle ergometer (RacerMate, Seattle, WA). Starting at a workload of 95 watts, participants increased exercise intensity by 35 watts every 3-minutes until volitional fatigue was achieved. The highest oxygen uptake value obtained from 15-s intervals was used to determine VO_{2max}. The maximum achieved workload (watts max) of each participant was calculated from the time completed in the last stage, divided by the total stage duration (3-min), multiplied by 35 watts, and added to the watts of the last completed stage (Shute et al., 2020). Expired gases were quantified in 15-s intervals throughout the exercise test using a flow- and gas-calibrated metabolic cart (Parvomedics TrueOne 2400, Sandy, UT).

Exercise-recovery trials

Each participant completed three exercise recovery trials in a randomized, counter-balanced fashion. Exercise recovery trials were separated by 7 days in order to allow for biopsy recovery and to eliminate between-trial acclimation. Subjects reported to the laboratory in the morning following an eight-hour fast. Each exercise trial consisted of a 60-minute cycle ergometer challenge at 70% watts max. Participants were provided with water during the exercise trials *ad libitum*. The amount of water consumed during the first exercise trial was replicated for the two subsequent trials. Upon completion of the exercise portion of each trial, a four-hour recovery period occurred within a (32″x7′) cylindrical altitude chamber (Engineering Innovations, LLC, Littleton, CO). Hypobaria was then combined with either normoxia or hypoxia (Tescor, Warminster, PA) to create the recovery scenarios. A short break from the chamber was incorporated at the 2-h midpoint of each recovery trial due to biological need. The three experimental recovery conditions simulated the following environmental altitudes with respect to barometric pressure as delineated below:

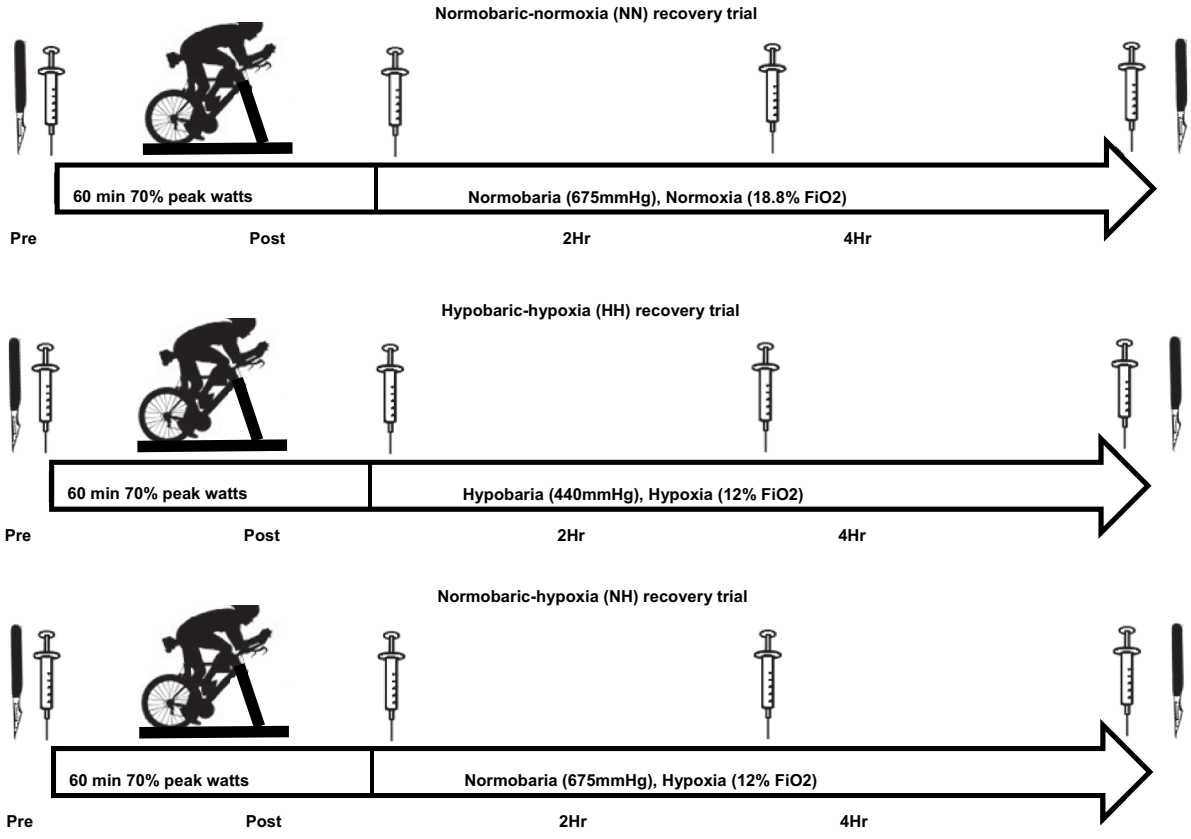

Figure 1. Experimental design. Following characterization of height, weight, body composition (hydrostatic weighing), participants completed three laboratory-based exercise recovery trials in a randomized, counter-balanced fashion. All exercise trials involved an identical 60-minute bout of cycle ergometry at 70% of peak watts. The experimental recovery conditions included three simulated environmental conditions: Normobaric-normoxia (NN), base elevation (975 m) atmospheric conditions, normobaria (675 mmHg) and normoxia (18.8% FiO_2, room air inspiratory fraction); Hypobaric-hypoxia (HH), full high elevation simulation (4,420 m) atmospheric conditions, hypobaria (440 mmHg) and hypoxia (12% FiO_2); or Normobaric-hypoxia (NH); partial high elevation simulation (4,420 m) atmospheric conditions; normobaria (675 mmHg) and hypoxia (12% FiO_2).

- Normobaric-normoxia (NN); Base elevation (975 m) atmospheric conditions; normobaria (675 mmHg) and normoxia (18.8% FiO_2, room air inspiratory fraction).
- Hypobaric-hypoxia (HH); Full high elevation simulation (4,420 m) atmospheric conditions; hypobaria (440 mmHg) and hypoxia (12% FiO_2).
- Normobaric-hypoxia (NH); Partial high elevation simulation (4,420 m) atmospheric conditions; normobaria (675 mmHg) and hypoxia (12% FiO_2).

Exercise-recovery oxygen saturation and heart rate

Tissue oxygen saturation and heart rate were recorded before and during exercise as well as at 60-minute intervals throughout the recovery period using finger pulse oximetry (Nonin WristOx2 3150, Plymouth, MN).

Phlebotomy and blood plasma collection

For each of the exercise-recovery trials, whole blood samples were collected from the antecubital vein with sodium heparinized vacutainers. Venipuncture was performed pre- (Pre), post- (Post), two (2-HR), and four (4-HR), hours post exercise. Blood samples were centrifuged at 1000 x g for 15-minutes at 4°C, and the plasma fraction was aliquoted and stored immediately at −80°C until subsequent biochemical assay for oxidative damage and antioxidant biomarkers.

Exercise-recovery trial muscle biopsies

Muscle biopsies were obtained before and after each exercise recovery trial from the *vastus lateralis*. Muscle samples were extracted with the aid of suction using a 5 mm Bergstrom percutaneous biopsy needle. Left and right legs used for biopsy were chosen randomly and counter-balanced between trials. Incision sites were cleaned and three to four mL of 1% lidocaine was injected subcutaneously and around the muscle fascia prior to incision. Next, a small incision was created through the skin and muscle fascia, followed immediately by the biopsy process. Post recovery biopsies were extracted from a separate incision ~ 2 cm proximal to pre-exercise biopsies. Newly obtained muscle tissue samples were quickly cleaned of surface blood, connective tissue, and fat prior to submersion in All-protect (Qiagen, Hilden, North Rhine-Westphalia, Germany). Samples were then stored at −30°C until assay.

Biochemical oxidative stress panel

Blood oxidative stress was quantified using a comprehensive panel that included markers of oxidative damage and

antioxidant content. Oxidative damage markers included two lipid-based markers, lipid hydroperoxides (LOOH) and 8-Isoprostanes (8-ISO), and two protein-based markers, protein carbonyls (PC) and nitrotyrosines (3-NT). Measures of plasma antioxidant capacity included Trolox equivalent antioxidant capacity (TEAC) and Ferric reducing antioxidant potential (FRAP). Individual blood plasma aliquots were subjected to a single freeze-thaw cycle to preserve sample viability for the various redox-sensitive markers. Plasma aliquots were kept on ice in a dark environment during assay to prevent confounding final results with environmental redox alterations.

Plasma antioxidant capacity

Trolox equivalent antioxidant capacity was performed using a quantifiable colourimetric reaction whereby plasma antioxidants scavenge exogenous introduction of 2, 2′ azinobis 3-ethyl-benzothiazoline-6-sulphonic acid (ABTS) radical anions. The formulation of the assay work solution began with 50 mM Glycine buffer and a 2.5 mM Trolox solution. Then, glycine peroxidase was added to 50 mM glycine buffer, followed by the addition of ABTS solution and 22 mM H_2O_2, in the final work solution. Final calculated TEAC values were derived from unknown plasma samples using the water-soluble vitamin E analogue Trolox as a standard reference (Erel, 2004). The FRAP assay was performed using a colourimetric reaction, which quantifies an exogenous ferric-to-ferrous tripyridyltriazine (TPTZ) reduction by antioxidants present within plasma. Within the FRAP assay, reduction of TPTZ is proportional to blood plasma antioxidant capacity and was quantified by absorbance spectroscopy at 593 nm according to established methodology (Benzie & Strain, 1996).

Plasma oxidative damage

Plasma LOOH were quantified using the ferrous oxidation-xylenol orange assay. Plasma samples were incubated in the presence or absence of a reducing agent and incubated with a colourimetric work solution containing ferrous ammonium sulphate, butylated hydroxytolene, and xylenol orange. During the assay reaction, oxidized ferrous ions oxidize the ferrous sensitive dye contained in xylenol orange to form a quantifiable complex that was measured via absorbance spectroscopy at 595 nm. Final LOOH concentrations in unknown blood plasma samples were derived using a cumene hydroperoxide standard reaction (Nourooz-Zadeh, 1999). Blood plasma 8-ISO concentrations were quantified by a commercially available specific immunoassay enzyme (EIA) (Cayman Chemical, Ann Arbour, MI) and assay procedures were performed according to manufacturer instructions (Montuschi et al., 1999).

Prior to quantification of oxidative damage to plasma proteins, sample protein concentrations were determined using the spectrophotometric Bradford method (Bradford, 1976). Samples were then normalized for either PC or 3-NT assays. PC determination in blood plasma samples was performed by a commercially available ELISA kit according to manufacturer instructions (Biocell Corporation Ltd, Papatoetoe, NZ) (Buss et al., 1997). Samples were diluted based on anticipated ranges of 4–35 mg protein/ml. Final concentrations were calculated based on spectrophotometric absorbance readings at 450 nm. 3-NT content in blood plasma samples was determined via commercial ELISA and assayed according to manufacturer instructions (Cell Biolabs INC, San Diego, CA). Similar to PC, final 3-NT concentration were determined from spectrophotometric readings at 450 nm (Mercuri et al., 2001).

Muscle sample preparation and qRT-PCR

Skeletal muscle (11.5 ± 1.7 mg) samples were processed in 500 µL of Trizol (Invitrogen, Carlsbad, CA) using an electric blender homogenizer (Bullet Blender, Next Advance, Inc, Averill Park, NY) in 1.5 mL Red RINO tubes prefilled with RNase-free ceramic beads (Next Advance, Inc, Averill Park, NY). Homogenate was centrifuged at 12,000 g for 15-min and the aqueous phase isolated and incubated overnight at −20°C. Centrifugation was used to create a pellet, followed by resuspension in ethanol, and a 5-minute centrifugation at 7,500 g. Ethanol was removed, the pellet dried, and re-dissolved in 30 µL of RNase-free water. RNA concentrations were quantified using a nano-spectrophotometer (nano-drop ND-2000, Thermo Scientific, Wilmington, DE). Average RNA yields were 165.9 ± 12.1 $ng{\cdot}\mu l^{-1}$ and average absorbance ratio at 260:280 exceeded 1.90 as a means of determining RNA purity. RNA sample integrity (RNA integrity = 8.0 ± 0.1) was assessed using a 2100 Agilent Bioanalyzer and Agilent RNA 6000 Kit (Agilent Technologies, Santa Clara, CA). First-strand cDNA synthesis was achieved using the Superscript IV-first strand synthesis system for RT-PCR kit (Invitrogen, Carlsbad, CA) and the resulting cDNA diluted with RNase-free water to achieve a final cDNA concentration of 0.5 µg/µL for resultant PCR reactions.

qRT-PCR reactions contained 0.5 µL of probe/primer mix (PrimeTime qPCR assay Integrated DNA Technologies, Coralville, IA), 5 µL qPCR Master Mix (Integrated DNA Technologies, Coralville, IA), and 4.5 µL sample cDNA. PCR was performed in triplicate on a Startagene mx3005p PCR system (Agilent Technologies) using a 2-step protocol (1–5 second cycle at 95°C, 50–20 second cycles at 60°C). Redox-sensitive transcripts included endothelial PAS domain protein-1 (EPAS-1), hemeoxygenase-1 (HMOX1), superoxide dismutase-2 (SOD2), and nuclear factor erythroid-derived 2-like 2 (NFE2L2). mRNA quantification was completed using the $2^{-\Delta\Delta CT}$ method (Livak&Schmittgen. 2001). The geometric mean of five housekeeping genes: beta-actin (ACTB), beta-2-microglobulin (B2M), cyclophilin (CYC), ribosomal protein S18 (RPS-18), and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) were used as a reference for each participant. This reference gene combination was determined by established NormFinder software (Andersen, et al. 2004). qRT-PCR probe and primer sequences are presented in Table 2.

Statistical analysis

Blood plasma variables and redox-sensitive gene transcripts responses were examined between NN, HH, and NH trials using repeated-measures ANOVAs (trial x time) with a Bonferroni correction. Where violations of sphericity occurred, the Greenhouse-Geisser correction was used to adjust the degrees of freedom. In the event that an interaction effect occurred, a Tukey's Post Hoc test was used to identify differences between time points and trials. All statistical procedures were performed using the

Table 2. Probe and primer sequences for reference genes and genes of interest.

Gene symbol	Gene accession #	Probe	Primer 1	Primer 2
EPAS-1	NM_001430	/56-FAM/AGA ACT TGT GCA CCA A/3IABkFQ	CTT TGC GAG CAT CCG GTA	AGC CTA TGA ATT CTA CCA TGC G
HMOX1	NM_002133	/56-FAM/TTC AAG CTG/ZEN/GTG ATG GCC TCC C/3IABkFQ/	TCC TTG TTG CGC TCA ATC TC	AGA ATG CTG AGT TCA TGA GGA A
SOD2	NM_000636	/56-FAM/TGG CTT CCA/ZEN/GCA ACT CCC CTT T/3IABkFQ/	CGT CAG CTT CTC CTT AAA CTT G	GAC AAA CCT CAG CCC TAA CG
NFE2L2	NM_006164	/56-FAM/CTA CTC CCA/ZEN/GGT TGC CCA CAT TCC/3IABkFQ/	GCA GTC ATC AAA GTA CAA AGC A	ACA TCC AGT CAG AAA CCA GTG

Redox-sensitive gene transcripts were quantified by qRT-PCR from *vastus lateralis* muscle biopsy samples; Endothelial PAS domain protein-1 (EPAS-1), Hemeoxygenase-1 (HMOX1), Superoxide Dismutase-2 (SOD2), and Nuclear Factor Erythroid-derived 2-like 2 (NFE2L2).

Statistical Package for Social Sciences software (SPSS) Version 24.0 (Chicago, IL). All values are presented as means ± standard deviation (SD). Significance was set at $p \leq 0.05$ *a priori*.

Results

Exercise trials

No main effect of trial was observed in HR ($p = 0.387$); however, a significant time effect occurred ($p < 0.001$). HR was significantly elevated during EX ($p < 0.001$) and remained elevated at POST ($p < 0.001$).

Oxygen saturation during recovery

Exercise-recovery oxygen saturation data are presented in Figure 2. A significant effect for trial ($p < 0.001$), time ($p < 0.001$), and interaction ($p = 0.037$) occurred. Due to the observed interaction effect, a Tukey's Post Hoc test was utilized to further analyse the interaction effect. Oxygen saturation was significantly lower in the NH trial when compared to the NN trial at 1-HR, 2-HR, 3-HR, and 4-HR ($p < 0.005$). Additionally, oxygen saturation was significantly lower in the HH trial when compared to the NN trial at 1-HR, 2-HR, 3-HR, and 4-HR ($p < 0.005$).

Plasma antioxidant status

Mean responses for plasma antioxidant status assessed through the measurement of TEAC and FRAP are presented in Figure 3(a and b). A significant time effect ($p = 0.012$) was present for TEAC values, with an increase at POST ($p = 0.011$) and a return to baseline at 2-HR ($p = 0.288$) and 4-HR ($p = 0.149$). Similar to TEAC, FRAP demonstrated a significant time effect ($p = 0.001$), with an increase at POST ($p = 0.006$), but statistical analyses indicated a returned to baseline values by 2-HR and 4-HR ($p > 0.99$). No differences between trials were observed in TEAC ($p = 0.093$) or FRAP ($p = 0.050$), which indicated that increases in antioxidant markers were related to the bout of exercise.

Biomarkers for plasma oxidative damage

Biomarkers for oxidative damage as quantified by LOOH and 8-ISO are presented in Figure 3(c and d). Analysis of LOOH findings indicated a main effect of trial ($p = 0.005$) and time ($p = 0.019$); however, no trial x time interaction was observed ($p = 0.064$). At 2-HR, LOOH was significantly higher than the POST time point ($p = 0.011$). It is plausible that the observed main effect was not caused by the environmental conditions considering the values were also elevated at PRE, before exercise and any environmental condition was introduced. 8-ISO was unaffected by trial ($p = 0.263$), but a time effect was observed ($p = 0.001$). 8-ISO was significantly increased at 2-HR ($p = 0.044$), indicating an exercise-induced elevation.

Protein modification biomarkers PC and 3-NT are presented in Figure 3(e and f). Statistical analysis indicated there was no effect of trial on PC ($p = 0.368$); however, there was a main effect of time ($p < 0.001$). Exercise resulted in a significant

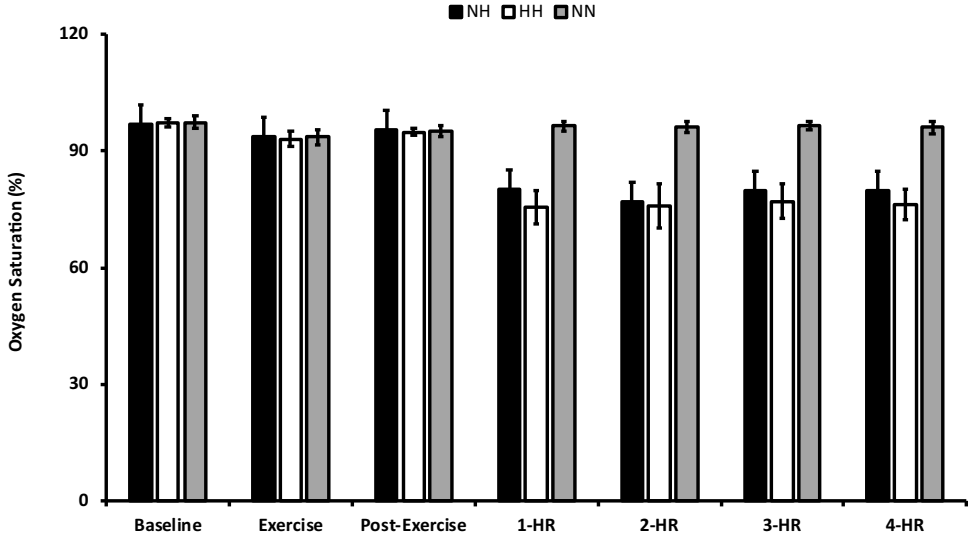

Figure 2. Data are presented as means ± SD. * significantly different from PRE. □significantly different from NN trial.

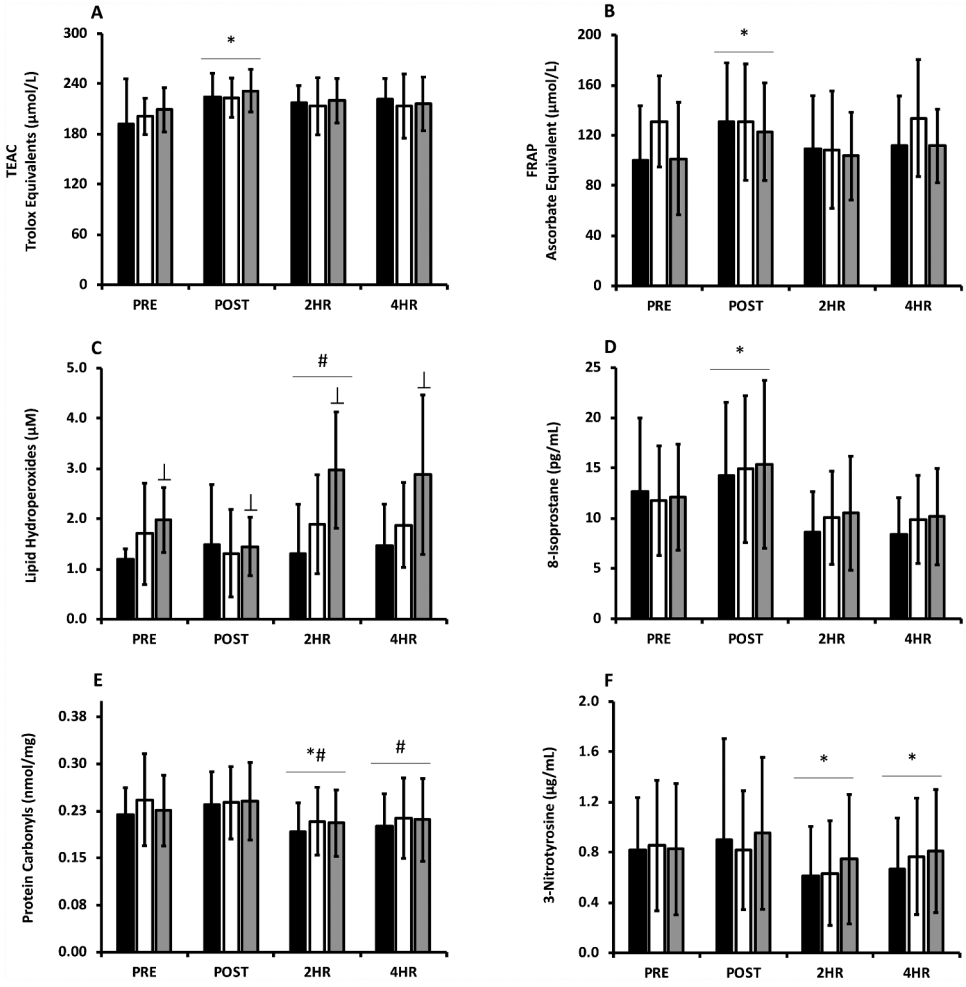

Figure 3. (a) Trolox equivalent antioxidant capacity values are expressed as Trolox equivalent antioxidant capacity equivalents (µmol/L) for normobaria normoxia (*black bars*), hypobaria hypoxia *(white bars)*, and normobaria hypoxia *(shaded bars)*. (b) Ferric-reducing ability of plasma values are expressed as ascorbate in equivalents (µmol/L) for normobaria normoxia (*solid black bars*), hypobaria hypoxia *(solid white bars)*, and normobaria hypoxia *(shaded bars)*. (c) Plasma lipid hydroperoxides are expressed as lipid hydroperoxide equivalents (µM) for normobaria normoxia (*black bars*), hypobaria hypoxia (*white bars*), and normobaria hypoxia (*shaded bars*). (d) Plasma 8-Isoprostanes values are expressed in standard comparison to 8-isoprostanes protein content (pg/ml), for normobaria normoxia (*black bars*), hypobaria hypoxia (*white bars*), and normobaria hypoxia (*shaded bars*). (e) Plasma Protein Carbonyl values are expressed in standard comparison to Protein Carbonyl equivalents (µmol/mg prot) for normobaria normoxia (*black bars*), hypobaria hypoxia (*white bars*), and normobaria normoxia (*shaded bars*). (f) Plasma Nitrotyrosine values are expressed in standard comparison to Nitrotyrosine protein content (µg/ml) for normobaria normoxia (*black bars*), hypobaria hypoxia (*white bars*), and normobaria hypoxia (*shaded bars*). Data are expressed means ± SD. *significantly different from PRE; # significantly different from POST; □ significantly different from normobaria normoxia recovery.

decrease of PC at 2-HR ($p = 0.002$) when compared to pre-exercise values. For 3-NT, there was no main effect of trial ($p = 0.222$), but a significant time effect occurred ($p = 0.006$). A mean decrease of 3-NT occurred at the 2-HR time point ($p = 0.001$).

Gene expression

Gene expression of NFE2L2, HMOX1, SOD2, and EPAS measured at PRE and 4-HR are presented in Figure 4. There was no main effect of trial in NFE2L2 ($p = 0.509$); however, a main effect of time was observed ($p = 0.003$). A significant increase in NFE2L2 occurred at 4-HR ($p = 0.003$), indicating an exercise-induced increase. Similar to NFE2L2, no effect of trial occurred in HMOX1 ($p = 0.519$), but a time effect was observed ($p < 0.001$). HMOX1 was significantly elevated at 4-HR ($p < 0.001$) following the bout of exercise. No main effect of trial was observed in SOD2 ($p = 0.691$), but a time effect occurred ($p = 0.046$) with a significant increase above pre-exercise values at 4-HR ($p = 0.046$). Finally, there was no main effect of trial in EPAS ($p = 0.282$); however, a time effect occurred ($p = 0.038$) with a significant elevation at 4-HR ($p = 0.038$).

Discussion

The purpose of this investigation was to examine the independent and collective effects of hypobaria and hypoxia on post-exercise oxidative stress responses. These outcomes were quantified utilizing a panel of oxidative damage markers, antioxidant capacity/potential, and redox-sensitive gene transcripts collected in a controlled laboratory setting. In order to assess the effects of the environmental variables on post-exercise oxidative stress, normobaric hypoxia and hypobaric hypoxia were manipulated during exercise recovery. The results of the current investigation demonstrated no effect of hypobaria or hypoxia on markers of oxidative stress (LOOH, 8-ISO,

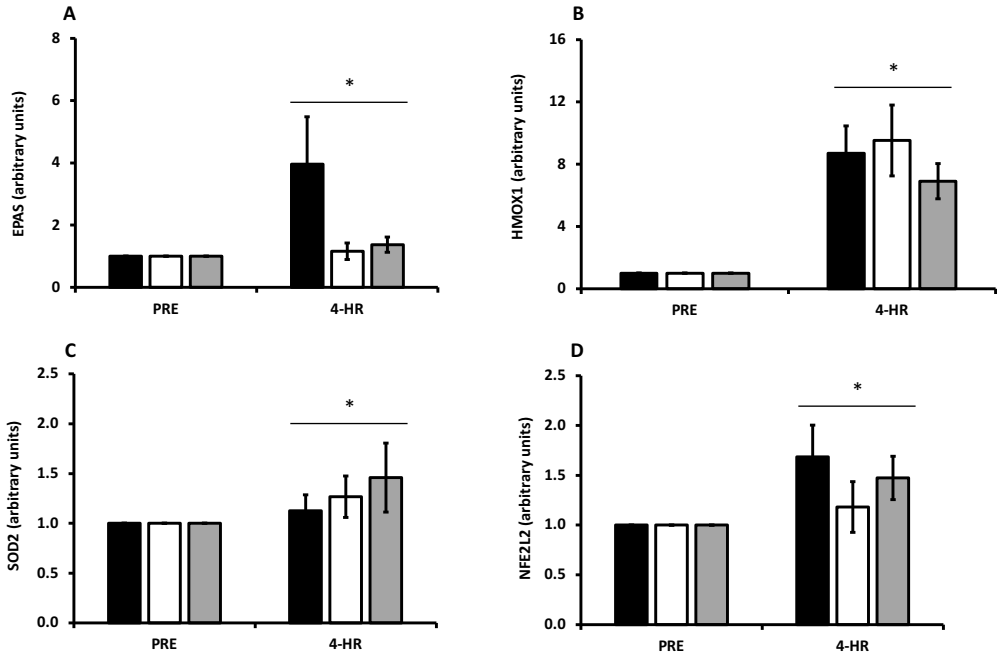

Figure 4. Data are presented as means ± SD. (a) EPAS (b) HMOX1 (c) SOD2 (d) NFE2L2; *black bars* represent normobaria normoxia, *white bars* represent hypobaria hypoxia, and *shaded bars* represent normobaria hypoxia; * significantly different from PRE.

PC, 3-NT) or antioxidant capacity/potential (TEAC, FRAP) during post-exercise recovery. Of note, exercise elicited an increase two-hours after exercise in LOOH and 8-ISO, but these responses were not impacted by alterations in the hypoxic or hypobaric recovery conditions. In further support, redox-sensitive transcripts (EPAS, HMOX1, SOD2, NFE2L2) were elevated in the post-exercise muscle biopsy samples, but variations in the hypoxic or hypobaric recovery conditions did not have an intervening influence. These findings are contrary to our working hypothesis that hypoxia and hypobaria would blunt the post-exercise oxidative stress response, and they are discussed subsequently.

Antioxidant status

Antioxidant status was assessed through the quantification of antioxidant capacity (TEAC) and antioxidant potential (FRAP). Within the current investigation, both TEAC and FRAP were unaffected by recovery under both hypobaric and hypoxic conditions. Our findings are in conflict with prior work, in which Ballmann et al. demonstrated that recovery in a hypoxic environment mitigated the exercise-dependent increase of FRAP and TEAC that typically occurs in a normoxic environment (Ballmann et al., 2014). The exercise bout selected within the aforementioned study was longer in duration (90-minutes) and consisted of intervals that peaked at a much higher exercise intensity (80% VO_{2max}). In further support, Peters et al. examined the effects of hypoxia on recovery of blood oxidative stress following one-hour of cycling at 70% VO_{2max} (Peters et al., 2016). An exercise-induced elevation of TEAC and FRAP was observed, but there were no differences between recovery in a hypoxic environment (1667 m, 3333 m, or 5000 m) when compared to normoxia (Peters et al., 2016). Although an exercise-induced increase in TEAC and FRAP was present within the current study, the magnitude of the oxidative stress response was modest and may not have been sufficient to observe changes among the environmental conditions. Importantly, previous research has demonstrated a post-exercise drop in FRAP, with no observed changes in TEAC, following one-hour of moderate-intensity cycling exercise (60% VO_{2peak}) under normoxic and hypoxic conditions; thus, biomarkers of antioxidant status are not always elevated in response to a bout of exercise (McGinnis et al., 2014).

Markers of protein damage

When examining biomarkers of protein damage, the current investigation demonstrated a mean decrease in both PC and 3-NT following exercise, with no differences occurring between normoxic, hypoxic, or hypobaric recovery trials. Previous research examined cycling exercise at 70% VO_{2peak}, which elicited an elevation in PC concentrations that returned to baseline levels within one-hour after the cessation of exercise (R. Bloomer et al., 2007). When considering the combined effects of exercise and altitude, Peters et al., who conducted a well-controlled, laboratory-based study, observed an increase in PC five-hours after a 60-minute bout of cycle ergometry at 70% VO_{2max}; however, PCs were unaffected by the hypoxic recovery when compared to normoxic recovery (Peters et al., 2016). Within the current study, the sampling time points did not extend to five-hours post-exercise; thus, it is possible that the elevation in PCs was not captured. Although an investigation examining the effects of hypoxic conditions following a cycling bout at 60% of VO_{2peak} demonstrated a drop PC similar to the findings of the current study, following exercise and unchanged by the hypoxic recovery conditions (McGinnis et al., 2014). It is possible that observed decrease was due to the spontaneous oxidation of proteins, and subsequent clearance (Wada et al., 2018). These data are in support of the working theory that

the exercise stimulus must be sufficient to produce an elevation in markers of protein damage; thus, it is not possible to draw clear conclusions on the effects of the environmental recovery conditions. For instance, previous work has demonstrated that hypoxic recovery environments blunt the necessary oxidative stress response to exercise (Ballmann et al., 2014). However, if a sufficient exercise-dependent increase is not observed, then it is not possible to determine if the hypoxic or hypobaric conditions resulted in mitigation of the exercise-induced oxidative stress.

Markers of lipid damage

The current investigation demonstrated no effect of hypoxic or hypobaric recovery on LOOH, a marker of lipid damage. In response to exercise, LOOH were elevated at 2-HR, when compared to the immediate post-exercise values. Previous research on the LOOH response to hypoxic exercise has been inconclusive (Ballmann et al., 2014; McGinnis et al., 2014; Peters et al., 2016). An investigation conducted by Peters et al. demonstrated a significant exercise-dependent elevation in LOOH as well as differences in the oxidative stress response when recovery occurred under hypoxic conditions (Peters et al., 2016). Following a one-hour bout of cycling exercise at 70% VO_{2max}, the magnitude of elevation in LOOH during the recovery period was greatest in the normoxic environment, with an elevation of lesser magnitude occurring at 3333 m. These data suggest that LOOH elevation may be blunted under hypoxic conditions (Peters et al., 2016). In contrast, previous work by Ballmann et al. exhibited an exercise-induced decrease in LOOH immediately after 90-minutes of interval exercise, with no differences in the recovery period when comparing normoxic and hypoxic conditions (Ballmann et al., 2014). Additionally, LOOH become elevated following higher intensity exercise in both normoxic and hypoxic recovery environments (McGinnis et al., 2014). Evidence supports the sensitivity of lipid peroxidation to both intensity and volume of exercise (Hudson et al., 2008). Therefore, it is likely the divergent responses can be explained by the selected exercise stimuli of the previous studies.

Redox-sensitive gene transcripts

In order to understand the effects of hypobaric and/or hypoxic conditions on the recovery of blood oxidative stress following exercise, four redox-sensitive gene transcripts were examined before and four-hours following exercise. Redox-sensitive gene transcripts selected for the current investigation include EPAS, HMOX1, SOD2, and NFE2L2. These data collectively demonstrated an exercise-induced increase in all four redox-sensitive gene transcripts at the 4-HR time point, indicating the exercise-stimulus was sufficient to elicit a modest blood oxidative stress response. Previous literature indicates that HMOX1, SOD2, NFE2L2 increase with exercise (Baghaiee et al., 2016; Essig et al., 1997; Muthusamy et al., 2012; Safdar et al., 2010; Taylor et al., 2012). Importantly, none of the redox-sensitive gene transcripts were affected by the environmental recovery conditions. Previous work had examined the effect of both normobaria hypoxia and hypobaria hypoxia on gene transcripts of mitochondrial biogenesis, which is known to be upregulated with exercise (Ross et al., 2019). The findings demonstrated no differences in mitochondrial-related gene expression with normobaria hypoxia or hypobaria hypoxia (Ross et al., 2019; Slivka et al., 2014). Similar to the findings of the current study, Peters et al. found no differences among NFE2L2 or SOD2 during the exercise recovery period under three different hypoxic conditions (1667 m, 3333 m, 5000 m) (Peters et al., 2016). Of note, it has been suggested that exercise in hypobaric and hypoxic may not result in hypoxia at the level of the tissues (Angeli et al., 2019). However, previous research has determined the exercise-induced response of NFE2L2 and SOD2 can be blunted when recovery occurs under hypoxic conditions (Ballmann et al., 2014). When examining HMOX1, our findings are in agreement with those who have shown no effect of hypoxia during post-exercise recovery (Ballmann et al., 2014; Peters et al., 2016).

Conclusion

Although the current study was carefully designed and implemented, it is not without its limitations. For instance, the four-hour recovery period in which the participants were exposed to the experimental conditions was interrupted by a brief break after two hours. This aspect of the study design was included in order to consistently meet the anticipated need for a biological break from the altitude chamber. Additionally, the findings of the study would have benefitted from an additional trial to examine hypobaric normoxic condition, to further isolate the effects of hypobaria on post-exercise recovery. Based upon prior observations that hypoxic recovery blunted the post-exercise oxidative stress response, the current study was undertaken to determine whether this outcome could be attributed to hypobaria, hypoxia, or the combination of both. Under these constraints, where post-exercise hypoxia and hypobaria were examined within a controlled laboratory setting, it was determined that there were no observed changes in exercise-induced oxidative stress responses under the conditions of normobaric normoxia, normobaric hypoxia, and hypobaric hypoxia. Thus, these environmental factors were not as impactful as hypothesized. However, we confirmed that the exercise challenge imposed a modest oxidative stress response, as indicated by elevations in markers of antioxidant capacity/potential, lipid damage, and redox-sensitive gene transcripts. Based on the comparison of these findings with previous work (Ballmann et al., 2014; McGinnis et al., 2014; Miller et al., 2013; Peters et al., 2016; Slivka et al., 2014), a more intensive and/or longer duration exercise bout could have elicited a more robust oxidative stress response. It is possible that achieving a certain threshold of oxidative stress is necessary to identify an environmentally dependent blunting of the oxidative stress response to exercise.

Disclosure statement

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