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Graded hypoxia and blood oxidative stress during exercise recovery

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Abstract
Altitude exposure and exercise elicit oxidative stress in blood; however, exercise recovery at 5000 m attenuates oxidative stress. The purpose was to determine the altitude threshold at which blood oxidative stress is blunted during exercise recovery. Twelve males 18–28 years performed four-cycle ergometry bouts (60 min, 70% VO_{2max}, at 975 m). In a randomised counterbalanced crossover design, participants recovered 6 h at 0, 1667, 3333 and 5000 m in a normobaric hypoxia chamber (recovery altitudes were simulated by using a computerised system in an environmental chamber by lowering the partial pressure of oxygen to match that of the respective altitude). Oxygen saturation was monitored throughout exercise recovery. Blood samples obtained pre-, post-, 1 h post- and 5 h post-exercise were assayed for ferric-reducing antioxidant plasma, Trolox equivalent antioxidant capacity, uric acid, lipid hydroperoxides and protein carbonyls. Muscle biopsies obtained pre and 6 h were analysed by real-time polymerase chain reaction to quantify expression of hemeoxgenase 1, superoxide dismutase 2 and nuclear factor (euthyroid-derived 2)-like factor. Pulse oximetry data were similar during exercise, but decreased for the three highest recovery elevations (0 m = 0%, 1667 m = −3%; 3333 m = −7%; 5000 m = −17%). A time-dependent oxidative stress occurred following exercise for all variables, but the two highest recovery altitudes partially attenuated the lipid hydroperoxide response (0 m = +135%, 1667 m = +251%, 3333 m = +99%; 5000 m = +108%). Data may indicate an altitude threshold between 1667 and 3333 m, above which the oxidative stress response is blunted during exercise recovery.

Keywords: *altitude, reactive oxygen species, exercise, oxidative stress*

Introduction

Participation in acute exercise results in redox perturbations and transient oxidative stress (Gomez-Cabrera, Domenech, & Vina, 2008; Hudson et al., 2008; Quindry, Stone, King, & Broeder, 2003). While historically counter-intuitive, oxidative stress due to exercise is now recognised as a stimulus for exercise-induced adaptations (Ristow & Schmeisser, 2011; Ristow & Zarse, 2010). Scientific quantification of oxidative stress in applied exercise studies typically includes various blood biomarkers of antioxidant status and oxidative damage (Pacifici & Davies, 1991; Powers & Jackson, 2008). Given the transient time course for observing oxidative stress responses to exercise, many of these observations occur in recovery from exercise. Adding to the dynamics of redox changes to acute exercise is the compartmental exchange from muscle origin to outcomes in blood (Little, Safdar, Benton, & Wright, 2011; Nikolaidis & Jamurtas, 2009; Nikolaidis et al., 2013; Powers & Jackson, 2008; Powers, Smuder, Kavazis, & Hudson, 2010). Prior evidence clearly indicates that the magnitude of oxidative stress is often proportional to exercise intensity or duration (Alessio, Goldfarb, & Cutler, 1988; Quindry et al., 2003). Recent work by Ballmann et al. (2014) and McGinnis et al. (2014) indicates that the post-exercise environment during recovery also impacts post-exercise oxidative stress responses. Studies from multiple labs reveal that environmental factors, including hypoxia, influence exercise-induced oxidative stress responses (Ballmann et al., 2014; Dosek, Ohno, Acs, Taylor, & Radak, 2007; Miller et al., 2012; Quindry et al., 2013).

In order to provide better scientific control to previously field-based exercise and hypoxia studies,

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in the current methodological approach altitude chambers were used to simulate hypoxia in normobaric environments (Miller et al., 2013; Radak et al., 1997; Sinha, Dutta, Singh, & Ray, 2010; Taylor et al., 2011). Findings from these previous studies indicate that altitude-induced hypoxia during exercise is a direct mediator of oxidative stress (McGinnis et al., 2014; Miller et al., 2012). In a recent and related study, findings demonstrated that exercise performed at 975 m followed by hypoxic recovery at 5000 m attenuated the post-exercise blood oxidative stress responses and blunted post-exercise adaptations in redox-sensitive transcripts in skeletal muscle (Ballmann et al., 2014). Based on this collective understanding, it appears that while oxidative stress is dependent upon the work performed during the exercise bout, recovery environment exerts an independent influence. There is a rationale to suspect that the recovery environment, if experienced at high altitude, may mitigate redox-sensitive exercise adaptations. In application, this understanding may hold implications for recreational hikers or possibly warfighters and others for whom exercise and recovery occur at elevation. Currently, it is unknown what recovery elevation threshold elicits a blunting in the post-exercise oxidative stress response.

Based on this rationale, the purpose of the current investigation was to quantify the blood oxidative stress to normoxic exercise followed by recovery at various post-exercise elevations. A randomised counterbalanced crossover repeated measures study design was employed to examine a panel of oxidative stress biomarkers before and after four identical exercise bouts and the respective recovery environments at simulated altitudes. In addition, muscle biopsies were obtained and redox-sensitive transcript values were quantified from these tissues to give insight into the post-exercise adaptive stimulus. Based on prior findings, it was hypothesised that oxidative stress responses would be attenuated in a threshold-dependent fashion during hypoxic exercise recovery as compared to normoxic exercise recovery.

Materials and methods

Participants

Physically active males (n = 12) between 18 and 28 years of age (48.4 ± 13.1 VO_{2max}; 24.1 ± 3.7; height 185.0 ± 3.5 cm; body mass 84.4 ± 3.8 kg) were recruited from the University of Montana community to take part in the current study. The University of Montana's Institutional Review Board approved the study in accordance with Declaration of Helsinki. Each participant also completed a physical activity readiness questionnaire to determine their physical activity readiness.

Baseline testing

Per cent body fat was determined using hydrodensitometry. Underwater weights were obtained using a digital scale (Exertech, Dresbach, MN). Participants repeated trails until three hydrostatic weight values within 100 g were obtained. Underwater weights were corrected for estimates of residual lung volume (residual lung volume = (0.0115 * age) + (0.019 * height) − 2.24). The relationship between hydrostatic weight and dry land weight was used to calculate body volume and converted to the per cent fat using the Siri equation (BF = (4.95/ρ – 4.50) * 100) (Siri, 1993).

Participants completed a peak maximal aerobic power test on an electronically braked cycle ergometer (Velotron, RacerMate Inc., Seattle, WA) at the laboratory altitude (975 m) to quantify peak aerobic fitness. The initial workload of 95 W was increased incrementally every 3 min (35 W/stage) until participants achieved volitional fatigue. Gas expiration was collected during exercise and analysed in 15 s intervals using a gas analyser (ParvoMedics, Inc., East Sandy, UT). Subsequent steady-state workloads were determined by the power output associated with VO_{2peak} values (W_{max}).

Steady-state exercise trials

Participants were instructed to abstain from physical exercise 24 h before each trial commenced. Additionally, participants reported to the lab having completed a 12 h overnight fast, where they were instructed to abstain from any alcohol (caffeine was allowed but not on the morning of the trial). Participants were instructed to hydrate *ad libitum* and to be consistently hydrated for all study trials. To ensure compliance, participants completed a 2-day exercise log and a 1-day dietary record, which were replicated prior to all steady-state exercise sessions. For each exercise trial, participants completed four 1 h steady-state exercise sessions at a work rate equivalent to 70% VO_{2max} on a cycle ergometer (Velotron, RacerMate Inc., Seattle, WA). Upon cessation of each exercise bout, participants recovered for 6 h at a randomised simulated altitude chamber of 0, 1667, 3333 or 5000 m (recovery altitudes were simulated by using a computerised system in an environmental chamber by altering the partial pressure of oxygen to match that of the respective altitude). Participants remained in the altitude chamber for the entire duration of the observed recovery period. Independent of hypoxia, the environmental chamber was set at 23°C and 40% relative humidity

for all recovery periods (Tescor, Inc., Warminster, PA). Oxygen saturations were monitored via pulse oximetry (Nonin Onyx Finger Pulse Oximeter, Nonin Medical Inc., Plymouth, USA) by spot check measurements throughout the exercise trial and during the 6 h recovery. The measurements were taken at baseline; 45 min post-exercise, and every hour during the 6 h recovery period. Participants consumed 600 ml of water during the 1 h of exercise and 600 ml during the 5 h post-exercise recovery. The participants were also allowed to consume a Clif Bar. Clif Bar choices were either white chocolate macadamia (the white chocolate macadamia nut bar contained 260 calories per bar and consists of 100% vitamin E, 30% vitamin A, 11% total fat, 16% dietary fibre, 90% vitamin A, 14% total carbohydrate and 18% protein) or chocolate chip (the chocolate chip bar contained 240 calories per bar and consists of 100% vitamin E, 30% vitamin A, 8% total fat, 30%, 15% total carbohydrate and 18% protein) at 0 h of recovery. Participants ate the same flavour bar for all four trials. The study design is illustrated in Figure 1.

Blood samples

Blood samples were collected pre-, post-, 1 h, 5 h post-exercise from the antecubital vein with sodium heparinised vacutainers (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 1000 × *g* for 15 min at 4°C. Plasma was aliquoted and stored immediately at −80°C until subsequent biochemical analysis of oxidative damage and antioxidant biomarkers. Individual aliquots were assayed within a few months of collection and were subject to a single freeze-thaw. In an effort to preserve sample viability upon thawing, plasma aliquots were kept on ice and in the dark to prevent redox alterations.

Muscle biopsies and tissue storage

Genes of interest were measured using quantitative real-time polymerase chain reaction measured pre-exercise and at the 6 h recovery time point. A total of 8 (two samples per trial × four trials) skeletal muscle biopsies (four from each leg) were obtained across the four trials by trained researchers working under

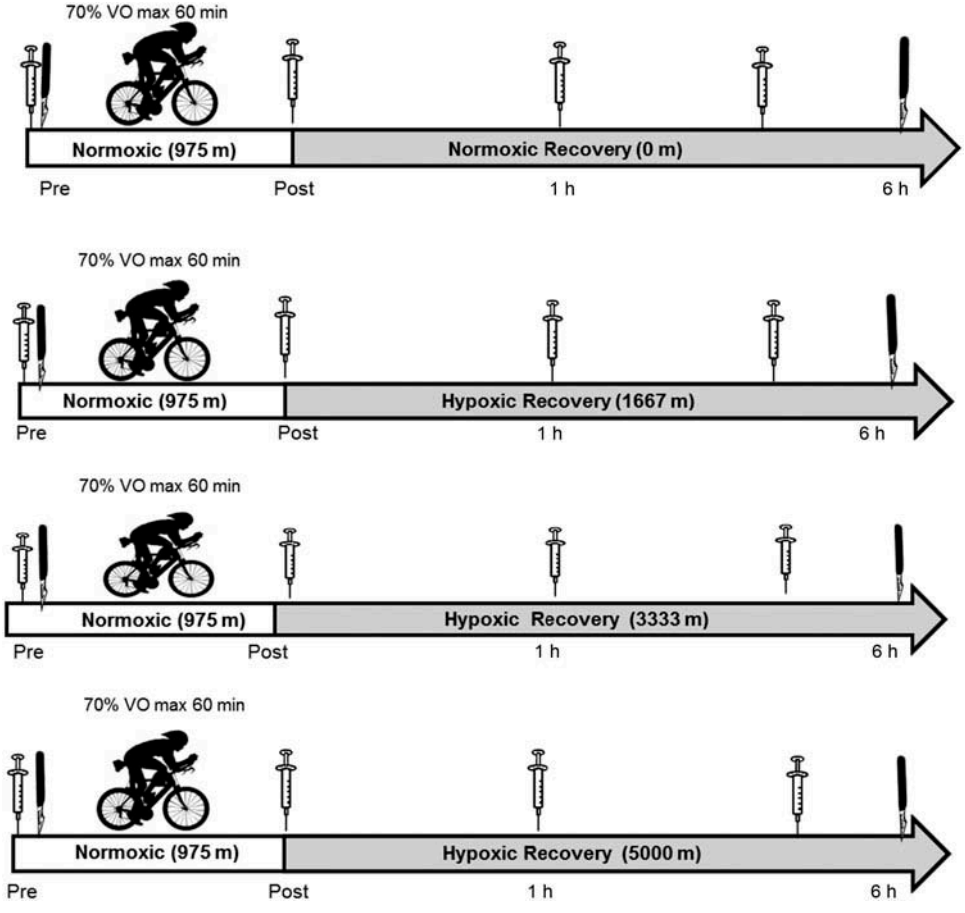

Figure 1. Study design. Participants performed in identical 60 min interval cycle ergometer exercise session at normoxic conditions (975 m altitude) indicated by the *open arrow*. In a randomised counterbalanced crossover design, participants recovered for 5 h at 0, 1667, 3333 and 5000 m (normobaric hypoxia chamber) indicated by a *shaded arrow*. Blood samples were obtained pre-, post-, 1 h post- and 5 h post-exercise. Muscle biopsies were obtained from the vastus lateralis at pre- and 6 h time points.

the supervision of a study physician as approved by the University of Montana's Institutional Review Board. Samples were obtained under a common recovery conditions in terms of fluid and food intake. Based on these study controls leading up and during the trials, the lone change variable was the ambient exercise recovery condition in the environmental chamber. Muscle biopsy samples were taken from the vastus lateralis muscle using a percutaneous needle pre- and 6 h post for each exercise trial. The area was treated with local anaesthesia (1% lidocaine) through subcutaneous and intramuscular injections prior to incision. Following anaesthesia, a small incision (approximately 0.25 in.) was performed and 50–100 mg of tissue was obtained. Incisions were closed with a single suture, supported with Steri-Strip, and covered with sterile adhesive bandage. Muscle tissue samples were immersed in ribonucleic acid later stabilisation solution (Life Technologies, Grand Island, NY) and stored at −80°C until further analysis.

Biochemical assays for oxidative stress

A biochemical assay panel was performed to quantify blood oxidative stress during each exercise recovery trial. To measure total and non-enzymatic antioxidant capacity, ferric-reducing ability of plasma and Trolox equivalent antioxidant capacity assays were performed. The ferric-reducing ability of plasma assay utilises a colorimetric reaction of ferric to ferrous tripyridyltriazine reduction by plasma antioxidants at an acidic pH. The reduction of tripyridyltriazine is proportional to blood plasma antioxidant capacity and was quantified by absorbance spectroscopy at 593 nm (Benzie & Strain, 1996). The Trolox equivalent antioxidant capacity assay measures present antioxidants scavenging of 2,2′ azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) radical anions, thus quenching a quantifiable colorimetric reaction. Calculated Trolox equivalent antioxidant capacity values for each sample were based on standard reactions with calculated values compared to the water-soluble vitamin E analogue Trolox (Erel, 2004). The uric acid (UA) assay was used to examine the catalytic activity of peroxidase the generated H_2O_2. Measurements of H_2O_2 were determined by peroxidase catalysed oxidation of chromogenic and fluorigenic substrates or by catalyse-mediated conversion of alcohols to aldehydes, which were measured spectrophotometrically using a reaction mixture containing 3-methyl-benzothiazoline-2-one hydrazone and 3-dimethylaminobenzoic acid. Final plasma UA values were determined by comparison with internal standard responses (Kovar, El Bolkiny, Rink, & Hamid, 1990).

To quantify the oxidative damage in blood plasma, protein carbonyls and lipid hydroperoxides were measured. For protein carbonyls, plasma sample protein concentrations were analysed via absorbance spectroscopy according to the methods of Bradford (1976). Plasma samples were diluted to 4 mg · ml^{-1} accordingly and protein carbonyls values determined by a commercially available ELISA (Biocell Corporation Ltd., Papatoetoe, New Zealand) according to the manufacturer's directions (Buss, Chan, Sluis, Domigan, & Winterbourn, 1997). To quantify plasma lipid hydroperoxides, the ferrous oxidation-xylenol orange assay was implemented where oxidised ferrous ions react with the ferrous-sensitive dye contained in xylenol orange forming a complex that is quantified through absorbance spectroscopy at a wavelength of 595 nm (Nourooz-Zadeh, 1999). Calculated adjustments for post-exercise plasma volume shifts were performed for all plasma variables according to established methods (Dill & Costill, 1974).

Transcript analysis from skeletal muscle

In total, 8–20 mg portions of the vastus lateralis skeletal muscle, obtained from post-trial skeletal muscle biopsies, were homogenised in Trizol (Invitrogen, Carlsbad, CA, Cat#15596-018). Samples were homogenised (Tissue Tearor, Biosped Products Inc., Bartlesville, OK) and messenger ribonucleic acid was purified using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol using the additional deoxyribonuclease digestion step (ribonuclease-free deoxyribonuclease set, Qiagen, Valencia, CA). Ribonucleic acid was quantified using a nano-spectrophotometer (nano-drop 2000C, Wilmington, DE). Average ribonucleic acid yield were 274 ± 108 ng · μl^{-1} and the average absorbance ratio at 260:280 was 1.9 ± 0.10, which indicated high-purity ribonucleic acid. The integrity of ribonucleic acid was measured using an Agilent 2100 bioanalyzer using ribonucleic acid nano chips (Agilent Technologies Inc., Santa Clara, CA). The result for the average integrity number was 7.8 ± 0.56, which indicated intact ribonucleic acid. First-strand complementary deoxyribonucleic acid synthesis was achieved using Superscript III-first-strand synthesis system for real-time polymerase chain reaction kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Real-time polymerase chain reaction was performed using 500 nM primers (RPS18:TCC ATC CTT TAC ATC CTT CTG TC; superoxide dismutase 2: CGT CAG CTT CTC CTT AAA CTT g; Hemeoxgenase 1: TCC TTG TTG CGC TCA ATC TC; nuclear factor (euthyroid-derived 2)-like factor: GCA GTC ATC AAA GTA CAA AGC A), 250 nM probe (PrimeTimeqPCR assay, Integrated DNA Technologies), Brillant III Ultra-Fast quantitative real-time polymerase chain reaction master mix

(Agilent Technologies Inc., Santa Clara, CA), Stratagene Mx3005P real-time polymerase chain reaction detection system (Agilent Technologies Inc., Santa Clara, CA) using a two-step protocol (one cycle at 95°C for 3 min, followed by 40 cycles at 95°C for 5 s and 60°C for 20 s). Quantification of messenger ribonucleic acid for the genes of interest were calculated using the $2^{-\Delta\Delta CT}$ method and stability of the housekeeping genes was determined using the $2^{-\Delta CT}$ (Schmittgen & Livak, 2008). Additionally, four housekeeping genes were analysed (β-actin, cyclophilin, RPS18 and GAPDH) and the most stable gene (RPS18) between trials was used to normalise genes of interest.

Statistical analysis

Statistical analysis was conducted using SPSS 20 (IBM, New York City). A factorial (trial [4] × time [4]) repeated measures analysis of variance was used to test for treatment differences for the key dependent variables. Given the repeated measures study design, tests of sphericity were employed for all key dependent oxidative stress and gene expression variables to confirm no violations of sphericity occurred in the current study. Where appropriate, main effects and interaction effects were examined using a Tukey post hoc. Significance was set at $P \leq 0.05$ *a priori*. Data are presented as mean ± *s*.

Results

Participant characteristics and performance data

Participants' physical characteristics and performance data are presented in Table I. Recruited participants exhibited an average body fat of 15.8% and average aerobic capacity of 48.4 ml · kg^{-1} · min^{-1}.

Steady-state exercise and recovery

Pulse oximetry data recorded during exercise and exercise recovery are presented in Figure 2. Across the four trials, oxygen saturation during exercise was unchanged ($P = 0.864$), but decreased during recovery in an altitude-dependent fashion ($P \leq 0.001$) (95% confidence interval (95% CI) [91.3,92.5]) for the three highest simulated elevations (average % change: 0 m 0%, 1667 m −3%, 3333 m −7% and 5000 m −17%).

Table I. Participant characteristics and performance data.

Characteristics	
Participants (*n*)	12
Age (years)	24.1 ± 3.7
Height (cm)	185.0 ± 3.5
Body mass (kg)	84.4 ± 13.2
Body Mass index (kg · m^{-2})	25.3 ± 3.8
Per cent fat	15.8 ± .10
Exercise performance	
VO_{2peak} (ml · kg^{-1} · min^{-1})	48.4 ± 13.1
Max watts (W_{max})	288.4 ± 48.7

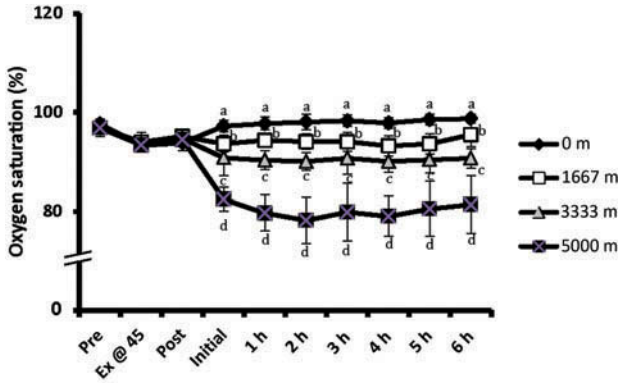

Figure 2. Data are per cent oxygen saturation mean ± *s*. Finger pulse oximetry at respective time points was used to measure oxygen saturation; *black triangles* represent 0 m recovery, *open squares* represent 1667 m, *shaded triangles* are representative of 3333 m and *black squares* represent 5000 m above sea level. Data reveal during recovery trial-dependent differences in blood oxygen saturation ($P < 0.05$).

Plasma antioxidant capacity

Plasma antioxidant capacity assessed by UA, Trolox equivalent antioxidant capacity and ferric-reducing ability of plasma assays are presented in Figure 3A–C, respectively. The respective coefficient of variation was 2.1% for UA, 3.4% for Trolox equivalent antioxidant capacity and for 3.6% ferric-reducing ability of plasma. For UA data, a main effect for time ($P < 0.001$) (95% CI [175.2, 248.7]) was present, indicating significant elevations in the 1 h and 5 h recovery time points as compared to pre- and post-exercise. Trolox equivalent antioxidant capacity analyses also revealed a time main effect ($P < 0.001$) (95% CI [183.3, 207.8]), but not trial ($P = 0.341$), whereby significant differences were observed between pre and all recovery time points. Ferric-reducing ability of plasma results was similar to UA and Trolox equivalent antioxidant capacity in that a time main effect ($P < 0.001$) (95% CI [547.0, 653.2]) was present and all plasma ferric-reducing ability of plasma values were significantly higher than pre.

Biomarkers for plasma oxidative stress

Biomarkers for oxidative damage measured by lipid hydroperoxides and protein carbonyls are presented in Figure 4A and B, respectively. The respective

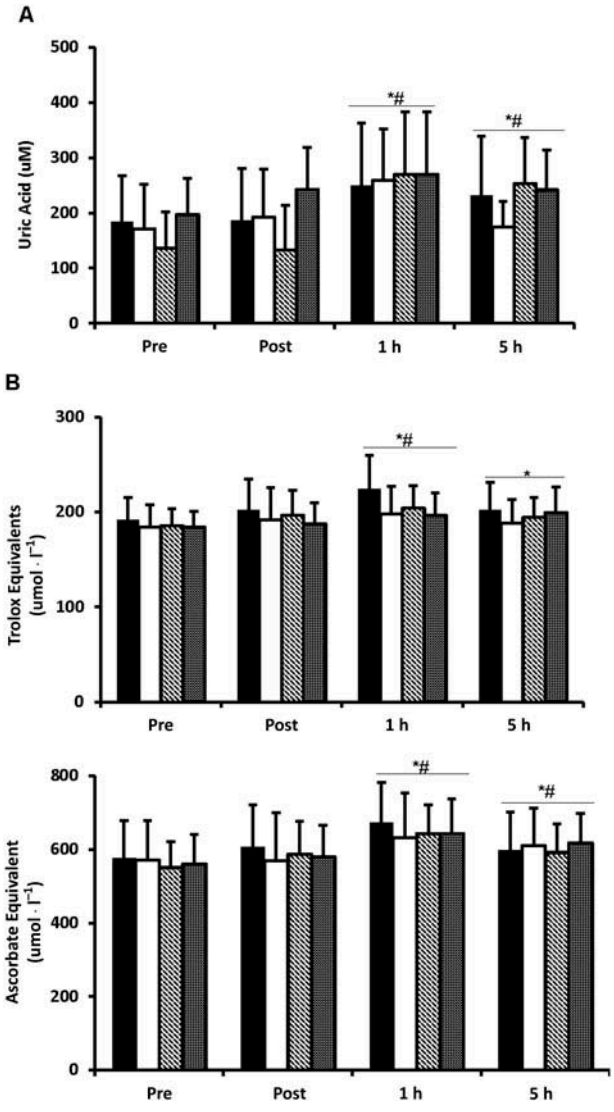

Figure 3. Data are mean ± *s*. (A) UA values are expressed as UA equivalents (μM). (B) Trolox equivalent antioxidant capacity values are expressed as Trolox equivalent antioxidant capacity equivalents (μmol · l^{-1}). (C) Ferric-reducing ability of plasma values is expressed as ascorbate in equivalents (μmol · l^{-1}); *solid black bars* represent 0 m recovery, open *bars* 1667 m, open *stripped bars* are representative of 3333 m and *shaded bars* represent 5000 m above sea level; *significantly different from pre; #significantly different from post.

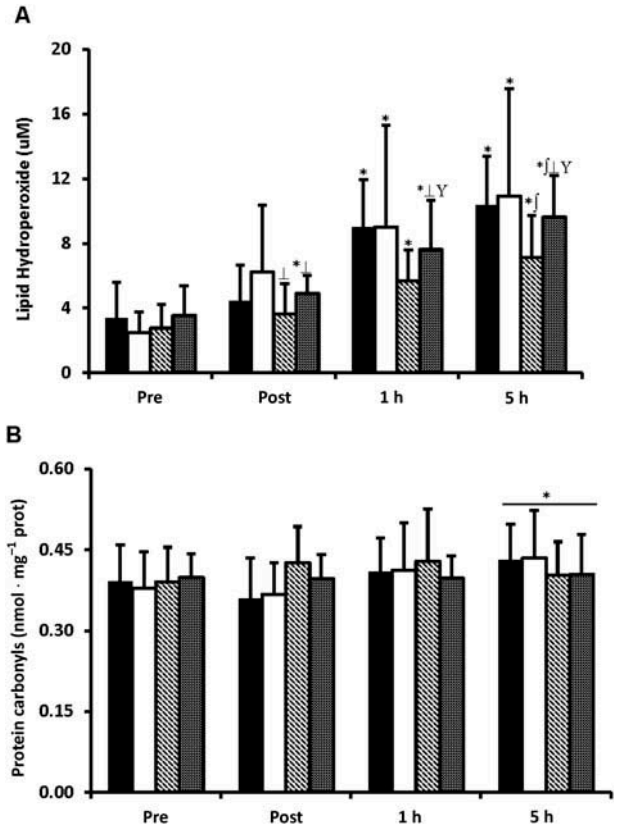

Figure 4. (A) Lipid hydroperoxide values are expressed as lipid hydroperoxide equivalents (μM), *solid black bars* represent 0 m recovery, open *bars* 1667 m, open *stripped bars* are representative of 3333 m and *shaded bars* represent 5000 m above sea level. (B) Protein carbonyl values are expressed in standard comparison to protein carbonyl equivalents (μM), *solid black bars* represent 0 m recovery, *open bars* 1667 m, *open stripped bars* are representative of 3333 m and *shaded bars* represent 5000 m above sea level; *significantly different from respective pre; ∫significantly different from 0 m; ⊥significantly different from 1667 m; Ysignificantly different from 3333 m.

coefficient of variation was 7.7% for lipid hydroperoxides and for 5.3% protein carbonyls. Lipid peroxidases exhibited significant time ($P = 0.038$) and trial ($P < 0.001$) main effects with values increasing in all recovery time points. Notably, post hoc analyses revealed that the rise in plasma lipid hydroperoxides was more dramatic in the 0 m recovery climates as compared to 3333 m ($P = 0.011$) and 5000 m ($P = 0.039$). In fact, the mean per cent increase (combined post, 1 h and 5 h) in lipid hydroperoxides was 0 m + 135%, 1667 m + 251% versus 3333 m + 99% and 5000 m + 108%. Analysis of protein carbonyls assay results indicated a significant time ($P = 0.038$) but not trial ($P = 0.909$) effect. Time comparisons revealed a pre-5 h difference only ($P = 0.031$).

Gene expression and quantitative polymerase chain reaction (QPCR)

Mean responses for hemeoxgenase 1, nuclear factor (euthyroid-derived 2)-like factor and superoxide dismutase 2 are presented in Table II. Neither time ($P = 0.187$) nor trial ($P = 0.211$) effects were observed for hemeoxgenase 1. In similar fashion, neither time ($P = 0.631$) nor trial ($P = 0.565$) main effects were statistically significant for nuclear factor (euthyroid-derived 2)-like factor. Superoxide dismutase 2 transcript levels approached, but did not achieve, significance for time ($P = 0.070$). Trial main effects for superoxide dismutase 2 were not significant ($P = 0.146$).

Table II. Gene expression results.

	0 m	1667 m	3333 m	5000 m	Time main effect	Trial main effect
Hemeoxgenase 1	0.98 ± 0.65	1.25 ± 2.8	0.94 ± 0.75	1.03 ± 0.78	$P = 0.18$	$P = 0.21$
Nuclear factor (euthyroid-derived 2)-like factor	1.23 ± 1.30	1.07 ± 0.64	1.08 ± 0.50	0.82 ± 0.30	$P = 0.63$	$P = 0.63$
Superoxide dismutase 2	1.08 ± 0.60	2.47 ± 2.9	1.28 ± 0.77	1.13 ± 0.74	$P = 0.07$	$P = 0.15$

Discussion

The key finding from this study is based on plasma lipid hydroperoxide outcomes and suggests an attenuation of the exercise-induced oxidative stress response during recovery occurs at an altitude threshold between 1667 and 3333 m. As presented in Figure 4A, plasma lipid hydroperoxide values at the 3333 and 5000 m were respectively −26% (1 h) and −19% (5 h) as compared to the corresponding 0 and 1667 m recoveries. The results from this study extend prior investigations (Ballmann et al., 2014; McGinnis et al., 2014) to reveal that recovery at a relatively modest altitude will blunt some exercise-induced oxidative stress responses as observed with blood plasma biomarkers. Prior understanding is based on the findings from a series of strategically designed studies, which indicate in aggregate that the exercise-induced increase blood oxidative stress is blunted during recovery in simulated altitude environments. Prior study designs were designed to control for exercise intensity and workload, indicating that ambient environment recovery conditions exert independent effects on post-exercise oxidative stress responses (Ballmann et al., 2014; McGinnis et al., 2014). The general altitude threshold, at which this observance occurs, however, is unknown and serves as the impetus for the current investigation. As a collective body of work, these findings raise important insights regarding exercise, oxidative stress and adaptations during recovery. Novel findings from the current study may apply to recreational hikers or possibly warfighters who ascend mountains and recover at elevation. Although the presented conclusions are based on speculation, the current understanding is that acute adaptive responses may prove particularly important in environmental extremes where maintenance of fitness and health is of vital importance. The potential implications of these findings are detailed subsequently.

Markers of oxidative damage

Current study findings reveal a time-dependent increase in plasma lipid hydroperoxides following the four exercise challenges throughout the 6 h recovery sampling time window (Figure 4A). However, results also revealed attenuation in lipid hydroperoxides response at the two highest simulated altitudes of 3333 (average −1.7 μm as compared to 0 and 1667 m) and 5000 m (average −1.6 μm as compared to 0 and 1667 m). This key finding can be interpreted to suggest that an altitude threshold occurs between 1667 and 3333 m that results in a blunting of oxidative stress during post-exercise recovery as determined by plasma lipid hydroperoxides. The results agree with previous findings from an investigation with closely related exercise and environmental study design facets (Ballmann et al., 2014), in addition to prior observations of elevated oxidative stress when exercise is performed at altitude (Dosek et al., 2007). Although the findings suggest that there is a response that occurs at the two highest altitudes, which may infer an altitude threshold, the relationship between altitude and the magnitude of the response remains inconclusive. Historically, the quantitative estimation of protein hydroperoxides has presented some difficulties (Gay & Gebicki, 2003). Over the last decade, improvements have been made in which the ferrous oxidation or lipid hydroperoxide method provided a convenient assay when measuring both lipid and protein hydroperoxide content in samples (Gay & Gebicki, 2003). In lieu of these findings, lipid hydroperoxides were used as an oxidative stress measure opposed to thiobarbituric acid reactive substances because of its relative methodological strength (Gay & Gebicki, 2003).

Significant elevations in protein carbonyls occurred during exercise recovery (+6% at 3333 and 5000 m as compared to 0 m) and confirm that the exercise trial elicited an oxidative stress response (Figure 4B). Given the methodological overlap between the current study and prior studies, this current finding for plasma protein carbonyls agrees with an earlier finding (Ballmann et al., 2014). In this prior study with a similar exercise recovery study design, outcome differences were observed during exercise recovery (Ballmann et al., 2014). There are some obvious applications for this data that can be contextualised during sporting events whereby repeated periods of endurance may be required within a day or over a consecutive number of days, which align with discussion mentioned elsewhere (Cobley, McGlory, Morton, & Close, 2011). Of interest in the current data set is the fact that plasma protein carbonyls were elevated independent of the simulated recovery altitude. We do not currently have a definitive

explanation for an altitude-dependent response in lipid hydroperoxides but not protein carbonyls. One possible explanation is that during concentric-dominant exercise similar to the current study, membrane-bound enzymes like xanthine oxidase and nicotinamide adenine dinucleotide phosphate oxidase promote lipid damage that is disproportionate to that of protein (Powers, Nelson, & Hudson, 2011). Previous observation demonstrates that the post-exercise rise in plasma protein carbonyl is dependent upon the total work performed (Hudson et al., 2008). In the Hudson et al. study, strength and hypertrophy squat workouts were normalised for the total amount of work performed. The study demonstrated normalised plasma protein carbonyl responses for differences in recovery time (due to different time requirements to complete the two strength protocols); the magnitude of the rise in plasma protein carbonyls was identical (Hudson et al., 2008). There are obvious methodological differences between the prior strength-based study and the current cardiovascular exercise, but both investigations controlled for workload. As such, it is tempting to speculate that in applied physiology studies like the current investigation lipid biomarkers may be more reflective of the entire exercise recovery period while protein carbonyls were mostly influenced by the more stressful exercise portion (Powers et al., 2011). Further study is needed to resolve these fundamental questions about protein versus lipid oxidative damage markers.

Plasma antioxidant capacity and exercise-induced oxidative stress

To ensure a comprehensive assessment of blood plasma redox status, plasma antioxidant capacity measures were performed to determine total antioxidant capacity and antioxidant potential using Trolox equivalent antioxidant capacity, ferric-reducing ability of plasma and UA measurements (Figure 3). These markers indicate that redox-sensitive metabolic activity continues for several hours following exercise cessation (Erel, 2004; Hudson et al., 2008; Quindry et al., 2008). It was previously noted that the post-exercise measurements of Trolox equivalent antioxidant capacity and ferric-reducing ability of plasma values were greatly influenced by plasma concentration of UA (Ballmann et al., 2014; Hudson et al., 2008; Quindry et al., 2008). Similar to the previous findings, there was a time-dependent increase in plasma UA following exercise. For all groups, there was a significant difference between normoxic recovery and hypoxic recovery. Although there is an intuitive inclination that oxidative stress equates to a lower antioxidant capacity, post-exercise increases in ferric-reducing ability of plasma and Trolox equivalent antioxidant capacity values are typically observed (Ballmann et al., 2014; Hudson et al., 2008; Quindry et al., 2008). Findings probably reflect acute increases in plasma UA values during exercise recovery (Cao & Prior, 1998) due to production of UA in fatiguing muscle that results in a compartmental shift to blood plasma in response (Quindry et al., 2008; 2003).

Gene expression

Muscle biopsies of the vastus lateralis were obtained with the intent of comparing blood oxidative stress outcomes to redox-sensitive gene transcript changes. Despite numerical differences in the current investigation (Figure 5), data were variable and did not signify any statistical differences for nuclear factor (euthyroid-derived 2)-like factor (95% CI [−0.27, 0.11]), superoxide dismutase 2 (95% CI[−0.96, −0.02]) or hemeoxgenase 1 (95% CI[−9.45, 50.6]). Current findings are in contrast to a prior study where an elevation of nuclear factor (euthyroid-derived 2)-like factor and superoxide dismutase 2 gene expression was abolished during hypoxic recovery (Ballmann et al., 2014). The expectation that there would be a trial-dependent change is based on the fact that nuclear factor (euthyroid-derived 2)-like factor is a redox-sensitive transcript that has links to over 200 cytoprotective genes that regulate cell growth, cell cycle and help to maintain homeostasis (Lewis, Mele, Hayes, & Buffenstein, 2010). Preceding research findings supported the view that increases in nuclear factor (euthyroid-derived 2)-like factor expression are acutely elevated post-exercise (Ballmann et al., 2014). Transcripts were also measured for two downstream antioxidant enzymes superoxide dismutase 2 and hemeoxgenase 1 for which changes were not statistically significant.

Differences in the gene transcripts from prior findings may be the result of the variation among study parameters, where in a previous study participants exhibited greater mean aerobic power (VO_{2max} = 54.4 ± 9.7) as compared to the current study (VO_{2max} = 48.4 ± 13.1). Moreover, the current sample was more heterogenous, with a standard deviation in functional aerobic capacity of 13 ml · kg^{-1} · min^{-1} as compared to the prior study of 9 ml · kg^{-1} · min^{-1}. As such, there is reason to believe that similar observations in a more homogenous way may have reached significance. Alternately, there are limitations inherent to application of gene transcript measures to serial biopsies as in the current study. Small-volume, hard-to-obtain samples are subject to day-to-day variability. In the current study, new baseline values were created for each trial and may have masked day-to-day differences. That is, transcript signals are very labile in response to athletic and environmental factors including altitude. While muscle biopsy applied to exercise

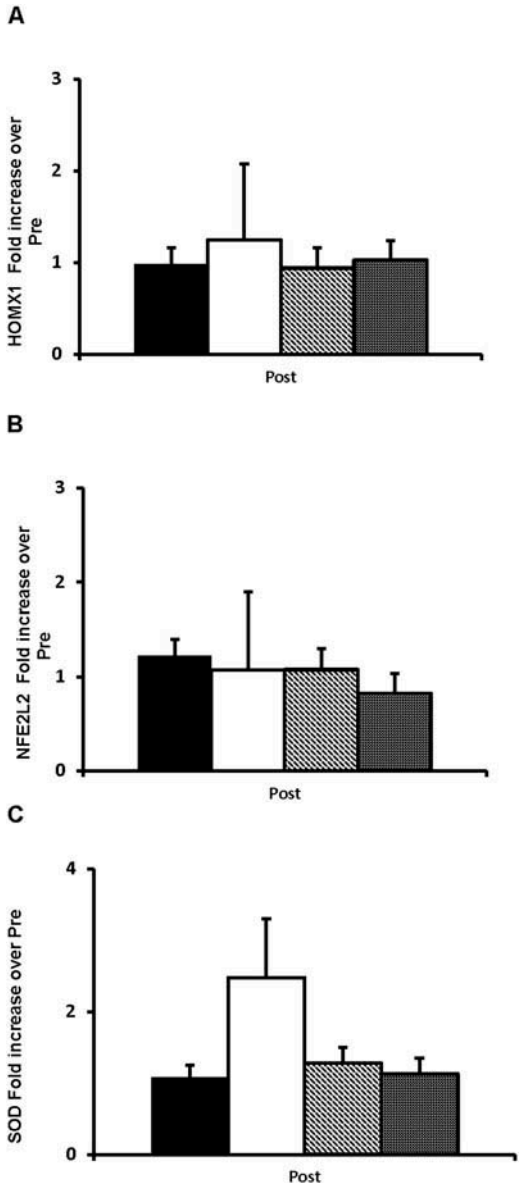

Figure 5. QPCR findings from skeletal muscle biopsies. (A) HOMX1 values. (B) NFE2L2 values. (C) SOD values. *Solid black bars* represent 0 m recovery, open *bars* 1667 m, open *stripped bars* are representative of 3333 m and *shaded bars* represent 5000 m above sea level. Data are mean ± *s* and expressed as fold increase over pre as compared to the 0 m trial.

and performance scenarios can be scientifically powerful, extreme care is needed in order to account for outcome responses due only to the intervention. Further empirical investigation is needed to resolve these methodological considerations in guiding future studies and resulting interpretations.

Study limitations

The reader should be aware of the following study limitations. First, the oxidative stress biomarkers examined currently in blood plasma are often criticised in whole-body studies due to their labile nature (Powers et al., 2010). In an effort to prevent day-to-day variability concerns, Trolox equivalent antioxidant capacity, ferric-reducing ability of plasma and UA were examined from a common "first thaw" plasma aliquot. All assays were performed within a few hours of thaw and exhibited a coefficient of variation less than 2%. Lipid hydroperoxides and protein carbonyls were assayed from dedicated plasma samples on separate days and exhibited coefficients of variation below 5%. Notably, exercise intensity was determined relative to VO_{2max}. This approach does not account for functional differences in lactate threshold, a physiological parameter that could have influenced both oxidative stress and gene transcript outcomes. On a related note, participants from the current study were derived from an academic community located at 975 m in elevation. As such, some elevation-based adaptations may have influenced outcomes in some, particularly if they frequent spent time at higher elevation in the weeks prior to participation in the current study. If correct, there is reason to believe that confounding effects of prior altitude habituation may have had the most influence on gene transcripts. Alternately, the lower partial pressure at 975 m is within the 500–2000 m altitude window considered to be minimally impactful on exercise performance (Bartsch & Saltin, 2008; Gore et al., 2013). Additionally, antioxidant supplementation was not controlled for between participants. The consumption of the Clif Bar, which contains antioxidants, may have added a confounding component to the results. Research has shown that the consumption of antioxidants does quench the reactive oxygen species production (Powers, DeRuisseau, Quindry, & Hamilton, 2004). In future studies, it would be imperative to control for such issues by looking into alternative dietary means during recovery period.

Conclusion

The current study is an important continuation of the investigations of hypoxic exercise-induced oxidative stress by Ballmann et al. (2014), McGinnis et al. (2014) and Quindry et al. (2013). Earlier investigations examined high-altitude exercise, followed by the independent influences of altitude on exercise and recovery. The current study extends upon a linear progression of laboratory investigations where experimental conditions were controlled for by simulating altitude during exercise and recovery in order to observe the effect on oxidative stress response to acute exercise. Current data confirm that exercise recovery at high altitude results in altered redox balance and blood oxidative stress

markers and indicates that the altitude threshold for this response is above 1666 m. The collective findings of the current study add to the growing body of literature focused on the influence of environmental temperature on exercise-induced oxidative stress (Gomes, Stone, & Florida-James, 2011; McAnulty et al., 2005; Mounier et al., 2009; Pialoux et al., 2010, 2009). With the understanding that exercise and environmental influence on oxidative stress, more reductionistic research approaches with serial muscle biopsies are probably needed to better understand the fundamental mechanisms responsible for redox-sensitive adaptations to exercise and how high-altitude and hypoxic environments influence these responses to acute exercise at the tissue level. Additionally, breath and urinary markers may also prove beneficial in better defining the total body sensitivity to hypoxia/altitude thresholds. Refinements in study design are also needed to better resolve the roll of exercise intensity and altitude exposure on redox changes on blood and muscle measures of oxidative stress. As final consideration, application of more directed research approaches should also work to better resolve the exact altitude threshold (between 1667 and 3333 m as investigated currently) at which redox-sensitive alterations in exercise recovery occur.

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