

**RAPID ALTERATIONS IN HEMOGLOBIN MASS WITH HIGH ALTITUDE
ACCLIMATIZATION/DE-ACCLIMATIZATION AND FOLLOWING
SIMULATED MICROGRAVITY**

by

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B.A., University of Colorado Boulder, 2009

A thesis submitted to the
Faculty of the Graduate School of the
University of Colorado in partial fulfillment
of the requirement for the degree of
Doctor of Philosophy
Department of Integrative Physiology

2016

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Rapid Alterations in Hemoglobin Mass with High Altitude Acclimatization/De-Acclimatization
and Following Simulated Microgravity

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IRB protocol # 11-0512, 14-0291

ABSTRACT

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Rapid Alterations in Hemoglobin Mass with High Altitude Acclimatization/De-Acclimatization and Following Simulated Microgravity

Thesis directed by Associate Professor William C. Byrnes

The oxygen-binding protein hemoglobin in red blood cells (RBCs) is an essential link for oxygen transport between the lungs and metabolically active tissues. Precise regulation of RBC production and destruction is important, as having too much or too little hemoglobin can have deleterious consequences. Humans undergo hematological adaptations with environmental stressors such as high altitude ascent/descent and (simulated) microgravity. However, the vast majority of studies examining adaptations to these perturbations have assessed hemoglobin concentration ([Hb]), which is influenced both by the total mass of hemoglobin in circulation (Hbmass) and plasma volume. Plasma volume changes rapidly with ascent to/descent from high altitude and with (simulated) microgravity and therefore insight into alterations in Hbmass cannot be obtained from [Hb] alone.

The early time course and mechanisms of Hbmass adaptations to high altitude and (simulated) microgravity remain unresolved. The recent development of the optimized carbon monoxide rebreathing method has enabled the routine assessment of Hbmass and the aim of this dissertation was to employ this method to examine early changes in Hbmass with high altitude acclimatization/de-acclimatization and following 4-day head-down tilt bed rest (HDTBR, a well-established microgravity analog).

We studied lowlanders at sea level and following ascent to/descent from 5260m. Large increases in Hbmass were observed within 7 and 16 days of ascent to 5260m, and the altitude-

induced Hbmass adaptation was completely lost within 7 days following descent to low altitude. Our data suggest that the rapid reduction in Hbmass was mediated by increased RBC destruction.

Hbmass was increased immediately following 4-day HDTBR before falling below baseline 5 days after return to normal living conditions. The transient increase in Hbmass may be related to decreased RBC destruction but it is also possible that spleen contraction following HDTBR contributed to this adaptation. Our data suggest that the decrease in Hbmass was mediated by decreased RBC production, not increased RBC destruction.

Overall, results from this dissertation provide novel insight into the time course of alterations in Hbmass with acclimatization to/de-acclimatization from high altitude and following simulated microgravity. These findings indicate the need to further examine mechanisms of altered RBC production/destruction that underlie these adaptations.

ACKNOWLEDGEMENTS

I would like to start by thanking Dr. Bill Byrnes—words cannot fully express my appreciation for everything you do. Thanks to Dr. Walter Schmidt- you have contributed so much to these studies and I have learned so much from your approach to science, collaboration, and friendship. Many thanks to Dr. Robert Mazzeo, Dr. Douglas Seals, and Dr. Kenneth Wright Jr. for serving as members of my comprehensive exam and dissertation committees.

Thanks to Jesse Goodrich, Lewis Kane, Dave Caha and Pablo Callejas for their assistance with the chronic CO study. Thanks to Dr. Michael Brothers for your support of the project and for making my first experience in research a fantastic one.

Thanks to the principal investigators of the AltitudeOmics study—Dr. Robert Roach, Dr. Andrew Subudhi, Dr. Andrew Lovering, and Dr. Colleen Julian— as well as the many other members of the AltitudeOmics team that made the study such an exciting, fun, and fruitful endeavor. A special thanks to Dr. Nadine Wachsmuth—it was an honor to be a part of the AltitudeOmics Hbmass team with you.

Thanks to the numerous people who made the head-down tilt bed rest study possible including Dr. Kenneth Wright Jr., Jesse Goodrich, Ellen Stothard, Madeline Jones, Connor Wall, Sewan Kim, Pablo Callejas, Rachel Gioscia-Ryan, Sean Fitzsimmons, Oliver Knauer, Andres Barandiaran, Enkh-tsogt Sainbayar, Steven Sares, Ruth Sanchez, Molly Guerin, Emily Marbas, Erin Radtke, Sophia Jaeckel, Hannah Ritchie, Sarah Morton, and Gayle Wright.

Thanks to all the members of the Applied Exercise Science Lab for discussions and friendships. Thanks to Dr. Rodger Kram for all the wisdom you share at the AESL journal club.

Finally, thanks to Rachel Gioscia-Ryan for making invaluable, behind the scenes contributions to all of my dissertation studies.

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CHAPTER I

INTRODUCTION

The oxygen-binding protein hemoglobin in red blood cells (RBCs) is an essential link for oxygen transport between the lungs and metabolically active tissues. Precise regulation of circulating levels of hemoglobin is critical, as having either too much or too little hemoglobin can have deleterious consequences. Healthy humans undergo dramatic hematological adaptations with environment stressors such as high altitude ascent/descent and microgravity. However, the vast majority of studies examining hematological adaptations to these perturbations have examined hemoglobin concentration ([Hb]), which is influenced both by the total mass of hemoglobin in circulation (Hbmass) and plasma volume. Importantly, changes in Hbmass cannot be inferred from alterations in [Hb]. Historically, measurement of Hbmass could not be performed routinely due to technical and logistical considerations. However, the development of the optimized carbon monoxide rebreathing method (Schmidt and Prommer 2005) has enabled the routine assessment of Hbmass and the goal of this dissertation was to use this method to examine rapid alterations in Hbmass in healthy humans with high altitude acclimatization/de-acclimatization and following simulated microgravity.

The time course of alterations in Hbmass during the first weeks of continuous high altitude exposure remains controversial. Early studies of lowlanders acclimatizing to high altitudes for longer than 4 weeks consistently demonstrated increases in Hbmass and red cell volume (RCV) (Pugh et al. 1964, Reynafarje et al. 1959) but there have been conflicting results regarding the time course of adaptation within the first 3 weeks (Wolfel et al. 1991, Reeves et al. 2001, Sawka et al. 1996, Grover et al. 1998, Garvican et al. 2012, Wachsmuth et al. 2013a, Wachsmuth et al. 2013b, Siebenmann et al. 2015). Gains in hemoglobin mass with adaptation to

high altitude are eventually lost following descent to low altitude, but the time course and mechanism(s) of this adaptation also remain controversial (Merino 1950, Huff et al. 1951, Reynafarje et al. 1959, Rice et al. 2001, Prommer et al. 2010, Garvican et al. 2012, Bonne et al. 2014, Gough et al. 2012a, Siebenmann et al. 2015, Wachsmuth et al. 2013a, Wachsmuth et al. 2013b). To our knowledge, no studies have examined the early time course of changes in Hbmass with ascent to/descent from altitudes greater than 5000m, where the hypoxic stimulus is dramatically increased. Thus, in Chapter III, we examined the early time course of changes in Hbmass with acclimatization to and de-acclimatization from 5260m in healthy humans.

It has long been known that reductions in Hbmass occur in astronauts (Leach and Johnson 1984, Udden et al. 1995, Alfrey et al. 1996a). The mechanisms influencing this reduction remain unresolved, but it has been proposed that an increased destruction of young RBCs mediates this adaptation (Alfrey et al. 1996a, Alfrey et al. 1996b, Alfrey et al. 1997). However, neocytolysis has not been definitively demonstrated in humans. The proposed triggers of neocytolysis during spaceflight can be recapitulated with short-term head-down tilt bed rest, a well-established ground-based model of microgravity (Kakurin et al. 1976, Nixon et al. 1979). Given that neocytolysis is proposed to occur within the first 4 days of spaceflight (Alfrey et al. 1996b), reductions in Hbmass might be expected to occur over a similar time window following head-down tilt bed rest (HDTBR). Thus, in Chapter IV, we examined changes in Hbmass in healthy humans following 4-day HDTBR.

CO rebreathing has become a valuable tool for assessing Hbmass, and for researchers employing CO rebreathing, it is important to know the potential effects resulting from CO administration. It is well established that acute inhalation of low to moderate doses of CO impairs maximal oxygen uptake and physical performance (Pirnay et al. 1971, Ekblom and Huot

1972, Vogel et al. 1972, Horvath et al. 1975, Schmidt and Prommer 2005, Morse et al. 2008). Given the clear performance-impairing effects of acute, low-dose CO inhalation, the possibility that low-dose CO administration may induce beneficial adaptations may seem counterintuitive. However, recent studies have shown that CO is an important physiological signaling molecule and that intermittent, low-dose CO administration for 5 days can induce beneficial adaptations in skeletal muscle in healthy humans (Rhodes et al. 2009, Pecorella et al. 2015). Critically, the increase in carboxyhemoglobin (HbCO%) employed in CO rebreathing methods to assess Hbmass is similar to or greater than the increase in HbCO% in studies reporting adaptive responses in skeletal muscle (Pecorella et al. 2015, Rhodes et al. 2009). Thus, it is important to know whether the methodology used to assess Hbmass might alter physiological parameters. We recently showed that acute low-dose CO inhalation does not alter submaximal exercise energetics but leads to transient increases in heart rate, blood [lactate], and rating of perceived exertion during submaximal exercise (Kane et al. 2016). Given that CO rebreathing is often performed repeatedly as part of longitudinal investigations, it is also important to examine potential effects resulting from repeated CO administration. Thus, in Chapter II, we examined the effects of ten days of intermittent, low-dose CO inhalation on Hbmass, aerobic performance predictors, and severe-intensity exercise tolerance in healthy humans.

CHAPTER II

Ten days of intermittent, low-dose carbon monoxide inhalation does not significantly alter hemoglobin mass, aerobic performance predictors, or peak-power exercise tolerance

ABSTRACT

Carbon monoxide (CO) rebreathing procedures are used to assess hemoglobin mass (Hbmass) but recent evidence suggests that CO is a signaling molecule that may alter physiological functions. We examined the effects of 10 days of intermittent, low-dose CO inhalation on Hbmass, aerobic performance predictors, and peak-power exercise tolerance. Eighteen recreationally-active men were randomized to either CO or placebo inhalation groups in a single-blind, pre-post parallel-groups trial. Primary outcomes were assessed before and after an intervention period during which subjects inhaled 1.2 ml kg⁻¹ CO or placebo (room air) once per day on 10 days over a 12-day period. Cycling tests were performed >16 hours following CO inhalation to exclude acute effects of CO exposure. CO inhalation elevated carboxyhemoglobin by 4.4±0.4% following each exposure, whereas there was no alteration in the placebo group. Compared to placebo, chronic CO inhalation did not significantly alter Hbmass (p=0.99), peak oxygen uptake (p=0.59), peak power output (p=0.10), submaximal oxygen uptake (p=0.91), submaximal RER (p=0.22), lactate threshold (p=0.65), or peak-power exercise tolerance (p=0.60). Exposure to CO at doses similar to those used in CO rebreathing methods on 10 days over a 12-day period does not significantly alter Hbmass, aerobic performance predictors, or peak-power exercise tolerance.

INTRODUCTION

Carbon monoxide (CO) rebreathing methods have been employed to assess total hemoglobin mass (Hbmass) for over 100 years and the development of the optimized CO rebreathing method (Schmidt and Prommer 2005) has enabled the routine assessment of Hbmass. For researchers employing CO rebreathing, it is important to consider any physiological effects resulting from CO administration. Acute CO inhalation using CO rebreathing procedures leads to a transient reduction in peak oxygen uptake by ~3% (Schmidt and Prommer 2005) and transient increases in heart rate, perceived exertion, and blood lactate during submaximal exercise (Kane et al. 2016). Therefore it is recommended that CO rebreathing not be performed within the 12 hours prior to endurance competition (Schmidt and Prommer 2005). Apart from these well-known, performance-impairing effects of acute CO inhalation, there is a rapidly growing body of literature demonstrating that CO is a key endogenous signaling molecule and that low doses of exogenous CO may induce beneficial/therapeutic adaptations (Mottetlini and Otterbein 2010). Indeed, Rhodes et al. (2009) found that just 5 days of intermittent CO exposure (breathing 100 ppm CO for 1 hour, once per day, resulting in an acute elevation in carboxyhemoglobin of ~2.0%) resulted in increases in several mRNA and protein markers of mitochondrial biogenesis and an increase in myosin heavy chain I (MHC I) protein expression in skeletal muscle in healthy humans. Pecorella et al. (2015) recently reported that a higher dose of intermittent CO exposure (5 days of breathing 200 ppm CO for 1 hour, once per day, resulting in an acute elevation in carboxyhemoglobin of ~4.0%) resulted in numerous adaptive responses in skeletal muscle including increases in mitochondrial DNA copy number, citrate synthase protein expression, mitochondrial volume density, GLUT-4 protein expression, myoglobin protein expression, and capillary density in healthy humans. The increase in carboxyhemoglobin

achieved during Hbmass assessment is similar to or greater than those achieved in these studies reporting beneficial adaptations in skeletal muscle from low-dose CO inhalation (Rhodes et al. 2009, Pecorella et al. 2015) and repeated Hbmass assessment over short-term periods is common in some fields of exercise physiology and sports medicine. For example, CO rebreathing has been performed in previous studies on up to 7 consecutive days (Garvican et al. 2010b) and 42 times over 100 days (Eastwood et al. 2008). Thus, it is important to know whether repeated administration of low-dose CO as part of CO rebreathing procedures may influence outcomes assessed in studies employing CO rebreathing to measure Hbmass. Therefore, we examined the influence of intermittent, low-dose CO inhalation on 10 days over a 12-day period on Hbmass, aerobic performance predictors, and peak-power exercise tolerance.

METHODS

Subjects

This study was approved by the University of Colorado Boulder Institutional Review Board and all subjects provided written, informed consent prior to participation. All procedures conformed to the standards described by Harriss and Atkinson (2015). The laboratory used in this study is located at moderate altitude (1625 m) and therefore subjects were required to have permanent residence at a similar elevation for at least 8 weeks prior to enrollment. They were required to be engaged in recreational activity at least once per week but not engaged in structured aerobic exercise training for more than 30 minutes per day, 5 days per week. All subjects were instructed to maintain their physical activity patterns throughout the duration of the study. To avoid potential confounding effects of previous CO exposure, subjects were required to have abstained from any smoking activities for at least 3 months, have smoked less than 100 cigarettes in their lifetime, and not work in an occupation associated with elevated CO exposure

(vehicle-related occupations, chemical processing, etc.). Subjects were required to abstain from donating blood or travelling to low altitude for at least 8 weeks prior to enrollment and throughout the duration of the study. A total of 20 subjects volunteered for this study; two subjects were withdrawn by study staff for failure to comply with study procedures. Select subject characteristics of the 18 men who completed the study were age: 24 ± 3 years, height: 179 ± 7 cm, body mass: 76 ± 12 kg, body mass index: 24 ± 5 kg m⁻²; peak oxygen uptake: 49 ± 7 ml kg⁻¹ min⁻¹.

Experimental Design

We employed a randomized, single-blind, pre-post parallel-groups trial. Subjects were randomized to the CO (n= 9) or placebo (n = 9) group using a random number generator and were required to come to the laboratory on 20 occasions over a 4-week period. Baseline testing included 5 visits: a graded exercise test (GXT), duplicate measures of Hbmass, and duplicate measures of peak-power exercise tolerance. The intervention consisted of 10 visits over a 12-day period; during each intervention visit, subjects inhaled 1.2 ml kg⁻¹ body mass CO gas or placebo (room air). Following the intervention, subjects repeated the initial 5 testing visits (duplicate measures of time to exhaustion at the peak power output achieved during the baseline GXT, duplicate measures of Hbmass, and a GXT). A schematic of the study design is shown in Figure 2-1. All cycling tests were conducted at the same time of day ± 1 hour, separated by a minimum of 2 days, and were at least 16 hours after any previous CO inhalation.

Methodology

Baseline Serum Ferritin

At the first visit, a 5 mL blood sample was collected from a peripheral arm vein for the measurement of serum ferritin by fluorescence enzyme immunoassay (TOSOH AIA-360, Japan).

Mean ferritin at baseline was $72 \pm 36 \text{ ng mL}^{-1}$, and no subjects were classified as iron deficient (ferritin $< 16 \text{ ng mL}^{-1}$; Sinclair and Hinton 2005).

Graded Exercise Test (GXT)

Subjects adjusted the bicycle ergometer (Lode Excalibur, Netherlands) seat and handlebar positions for comfort with the help/input of investigators; each subject's positions were replicated for all subsequent cycling sessions. After being seated on the bike for 5 minutes, a baseline blood sample ($\sim 50 \mu\text{L}$) for blood [lactate] determination (YSI 2300, USA) was collected from a fingertip. Subjects then began the GXT at an initial workload of 50 W and power was increased in 30 W steps every 4 minutes until subjects reached volitional exhaustion. Ventilatory and metabolic parameters were assessed using computerized indirect calorimetry (Parvomedics True One 2400, USA). Prior to each test, gas analyzers were calibrated using a primary standard in the physiological range (16.06% O_2 and 4.139% CO_2) and the pneumotach was calibrated at flow rates between ~ 75 and 275 L min^{-1} using a 3-L calibration syringe (Hans Rudolph, USA). Submaximal rates of ventilation, oxygen uptake, and carbon dioxide exhalation were assessed every 15 seconds but only the data collected during the last 2 minutes of each stage, representing steady-state conditions, were included in the final analysis. During the last minute of each stage, a $\sim 50 \mu\text{L}$ blood sample was collected from the fingertip for blood [lactate] determination. Lactate threshold (LT) was determined using the 1 mM above baseline method (Coyle et al. 1983). Submaximal oxygen uptake and respiratory exchange ratio (RER) were determined as pooled values from each subject's completed stages below the LT. The number of stages included in these analyses was consistent within-subject for baseline and post-intervention testing but differed between subjects due to differences in the number of stages before the LT was exceeded. The Pearson correlation coefficient between power and oxygen uptake exceeded

0.99 for all tests (mean $r = 0.997 \pm 0.002$) and respiratory exchange ratios were below 1.0 for all submaximal workloads examined. Peak oxygen uptake was determined as the highest 30-second average. Post-intervention peak oxygen uptake was excluded from one subject in the placebo group after he reported at the completion of the test that he did not give a maximal effort. Peak power output (PPO) during the GXT was determined as the power output during the penultimate stage plus the fractional completion of the final stage multiplied by 30 W (De Pauw et al. 2013).

Hbmass

Hbmass was assessed on two occasions before and after the intervention using the optimized CO rebreathing method (Prommer and Schmidt 2007, Schmidt and Prommer 2005). A dose of 1.2 mL kg^{-1} body mass CO was administered to subjects and rebreathed along with 3 L of 100% oxygen for 2 minutes. Capillary blood samples were collected from a pre-warmed finger before and 7 minutes after CO administration and HbCO% (OSM3 hemoximeter, Radiometer, Denmark) was analyzed in sextuplicate. Portable CO detectors (Pac7000, Draeger, Germany) were used to monitor potential leaks during the rebreathing procedure; 6 of 72 tests were excluded due to detection of a CO leak. Hbmass results represent the mean of duplicate measures unless a leak was detected during one of the tests, in which case the result from the one valid test was used. In total, less than 10 mL of whole blood were collected from each subject throughout the study, amounting to less than 2 g Hbmass.

Peak-power exercise tolerance

Peak-power exercise tolerance was assessed in duplicate before and after the intervention as time to exhaustion (TTE) at the PPO achieved during the baseline GXT. Subjects were asked to avoid strenuous exercise for 48 hours preceding each test, abstain from food and caffeine for at least 2 hours before each test, and replicate their diet for the 24 hours preceding each cycling

test. All tests were completed on the Lode Excalibur Sport in the cadence-independent mode. Subjects underwent a standardized 10-minute warm-up at 1 W kg^{-1} body mass followed by a square-wave transition to PPO. Subjects were asked to pedal for as long as possible; if cadence fell below 40 RPM, they were given 5 seconds to increase cadence. The test ended when subjects could not maintain a cadence of at least 40 RPM or when they reached volitional exhaustion. No verbal encouragement or time feedback was provided during these tests. Three of 72 tests were excluded due to protocol deviations (2 diet- and 1 activity- related). There was no significant difference between the first and second (baseline) tests ($p = 0.50$) or between the third and fourth (post-intervention) tests ($p = 0.95$). We examined both best and mean TTE result before and after the intervention in statistical analyses.

Intervention

Subjects reported to the lab for the intervention visits on 10 days over a 12-day period. To accommodate participants' schedules, each subject was allowed to choose 2 off-days during the 12-day period. A capillary blood sample was collected from a finger before and 7 minutes after CO/placebo administration and HbCO% (OSM3 hemoximeter, Radiometer, Denmark) was analyzed in duplicate. CO/placebo administration was performed as described previously (Kane et al. 2016). Briefly, subjects inhaled 1.2 mL kg^{-1} body mass of CO or placebo (room air) directly from a pre-calibrated syringe. Subjects then continued to inhale room air through a small opening in their lips until they inhaled completely and held their breath for 30 seconds.

Statistical Analysis

Unpaired t-tests were performed to examine between-group differences at baseline and in response to the intervention. All p-values for primary outcomes are explicitly stated in Table 2-1 and the level of significance was set at $p < 0.05$. Additionally, we analyzed data using a

magnitude-based inferences approach (Batterham and Hopkins 2006). Changes in primary outcomes between the placebo and CO groups were compared using a spreadsheet for pre-post parallel-group trials (Hopkins 2006). Differences in pre-post changes between groups were assessed in relation to a smallest worthwhile change (SWC) of 0.2 times the between-subject SD in primary outcomes at baseline (Hopkins 2006). Effects were classified as positive, negative, or trivial (smaller than SWC) based on the magnitude of change in relation to the SWC, with qualitative descriptors of probabilities as follows: 25-75%, possibly; 75%-95%, likely; 95-99.5%, very likely; >99.5%, almost certainly (Hopkins 2006). Error of measurement is reported based on pre-post differences observed in the placebo group (Hopkins 2006). Data are presented as mean $\pm$ SD unless otherwise noted.

RESULTS

There were no significant between-group differences in anthropometric characteristics (height, body mass, or BMI) at baseline. There was no significant between-group difference in the change in body mass in response to the intervention (CO—Pre: 72.2 $\pm$ 9.8 kg vs. Post: 72.5 $\pm$ 9.4 kg; placebo—Pre: 78.9 $\pm$ 14.0 kg vs. Post: 78.8 $\pm$ 13.4 kg; $p = 0.50$). CO inhalation elevated HbCO% by 4.4 $\pm$ 0.4% at minute 7 following CO inhalation, whereas there was no elevation in HbCO% in the placebo group (0.0 $\pm$ 0.0%).

Individual and group mean data for the primary outcomes are shown in Figure 2-2 and Table 2-1. There were no significant between-group differences at baseline or in response to the intervention for Hbmass (CO—Pre: 916 $\pm$ 110 g vs. Post: 916 $\pm$ 105 g; placebo—Pre: 891 $\pm$ 117 g vs. Post: 896 $\pm$ 123 g). There were no significant between-group differences at baseline or in response to the intervention for peak oxygen uptake (CO—Pre: 3.68 $\pm$ 0.39 L min⁻¹ vs. Post: 3.84 $\pm$ 0.44 L min⁻¹; placebo—Pre: 3.73 $\pm$ 0.54 L min⁻¹ vs. Post: 3.84 $\pm$ 0.54 L min⁻¹), peak power output (CO—

Pre: 247 ± 32 W vs. Post: 252 ± 31 W; placebo—Pre: 275 ± 47 W vs. Post: 270 ± 50 W), lactate threshold (CO—Pre: 162 ± 28 W vs. Post: 160 ± 26 W; placebo—Pre: 180 ± 49 W vs. Post: 175 ± 50 W), submaximal oxygen uptake (CO—Pre: 1.59 ± 0.22 L min⁻¹ vs. Post: 1.63 ± 0.22 L min⁻¹; placebo—Pre: 1.67 ± 0.32 L min⁻¹ vs. Post: 1.71 ± 0.32 L min⁻¹), or submaximal respiratory exchange ratio (CO—Pre: 0.87 ± 0.04 vs. Post: 0.88 ± 0.05 ; placebo—Pre: 0.89 ± 0.04 vs. Post: 0.87 ± 0.04). There were no significant between-group differences at baseline or in response to the intervention for peak-power exercise tolerance for either the best (CO—Pre: 502 ± 89 s vs. Post: 492 ± 81 s; placebo—Pre: 489 ± 57 s vs. Post: 495 ± 85 s) or mean performance (CO—Pre: 477 ± 86 s vs. Post: 475 ± 79 s; placebo—Pre: 457 ± 62 s vs. Post: 465 ± 73 s). Using a magnitude-based inferences approach, there were no likely differences between the CO and placebo groups in response to the intervention for any primary outcomes (Table 2-1).

Parameter	%Change CO-PLA	Error of measurement	Unpaired t-tests		Magnitude Based Inferences			
	%Δ (90%CL)	%TE (90%CL)	P _{Baseline}	P _{Change}	Positive	Trivial	Negative	Qualitative Inference
Hbmass	-0.3% (-1.9-1.2%)	1.2% (0.9-2.1%)	0.66	0.99	0	98	2	Very likely trivial
VO ₂ peak	1.1% (-2.9-5.3%)	3.3% (2.3-6.1%)	0.99	0.59	31	62	7	Unclear
PPO	4.4% (0.9-8.7%)	4.1% (2.9-7.5%)	0.28	0.10	64	36	0	Possibly Positive
LT	1.8% (-4.5-8.4%)	2.8% (2.0-4.8%)	0.37	0.65	16	80	4	Likely Trivial
Submax VO ₂	-0.2% (-3.9-3.6%)	3.3% (2.3-5.6%)	0.58	0.91	5	88	7	Likely Trivial
Submax RER	2.4% (-1.0-6.0%)	1.8% (1.3-3.0%)	0.37	0.22	74	22	4	Possibly Positive
Best TTE	-2.3% (-18.9-8.4%)	10.2% (7.2-18.1%)	0.72	0.60	16	32	52	Unclear
Mean TTE	-1.3% (-10.6-9.0%)	10.4% (7.3-18.4%)	0.58	0.72	19	39	42	Unclear

Table 1. Effects of 10 days chronic, intermittent CO inhalation versus placebo on Hbmass, aerobic performance predictors, and peak-power exercise tolerance. For the magnitude-based inferences, numbers represent the percent probability that the effect of CO versus placebo was positive, negative, or trivial compared to the smallest worthwhile change (0.2 times between-subject SD at baseline). CO, Carbon monoxide; PLA, placebo; Hbmass, hemoglobin mass; $\dot{V}O_2$ peak, peak oxygen uptake; PPO, peak power output achieved during graded exercise testing; LT, lactate threshold (1mM above baseline); Submax $\dot{V}O_2$, pooled oxygen consumption at stages below LT (the number of stages included differed between subjects but was consistent within-subject); Submax RER, pooled respiratory exchange ratio at stages below LT (the number of stages included differed between subjects but was consistent within-subject); TTE, time to exhaustion at PPO achieved during baseline graded exercise test; P_{Baseline}, p-value for CO versus PLA at baseline; P_{Change}, p-value for comparison of pre-post change in CO versus PLA.

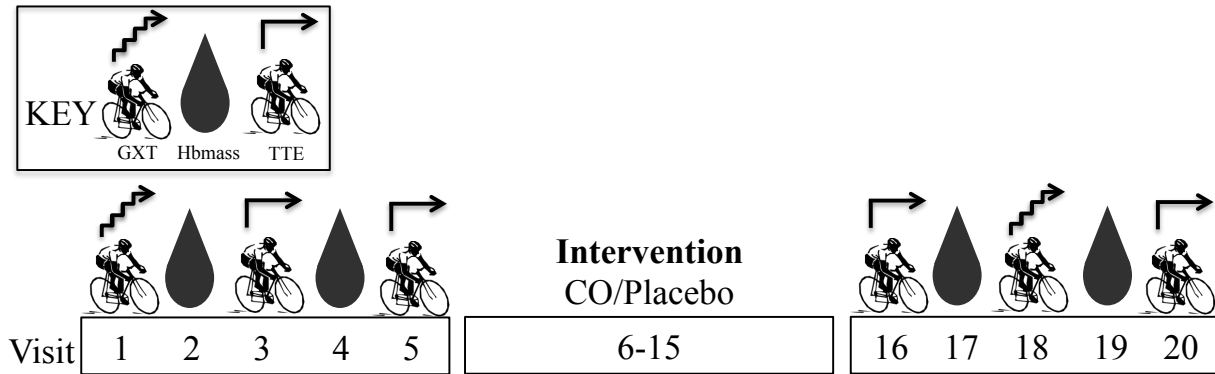


Figure 2-1. Schematic of Study Design. GXT, Graded exercise test; Hbmass, Hemoglobin mass; TTE, Time to exhaustion at pre-intervention peak power output. During the intervention, subjects inhaled 1.2 ml kg^{-1} body mass CO or placebo (room air) once per day on 10 days over a 12-day period.

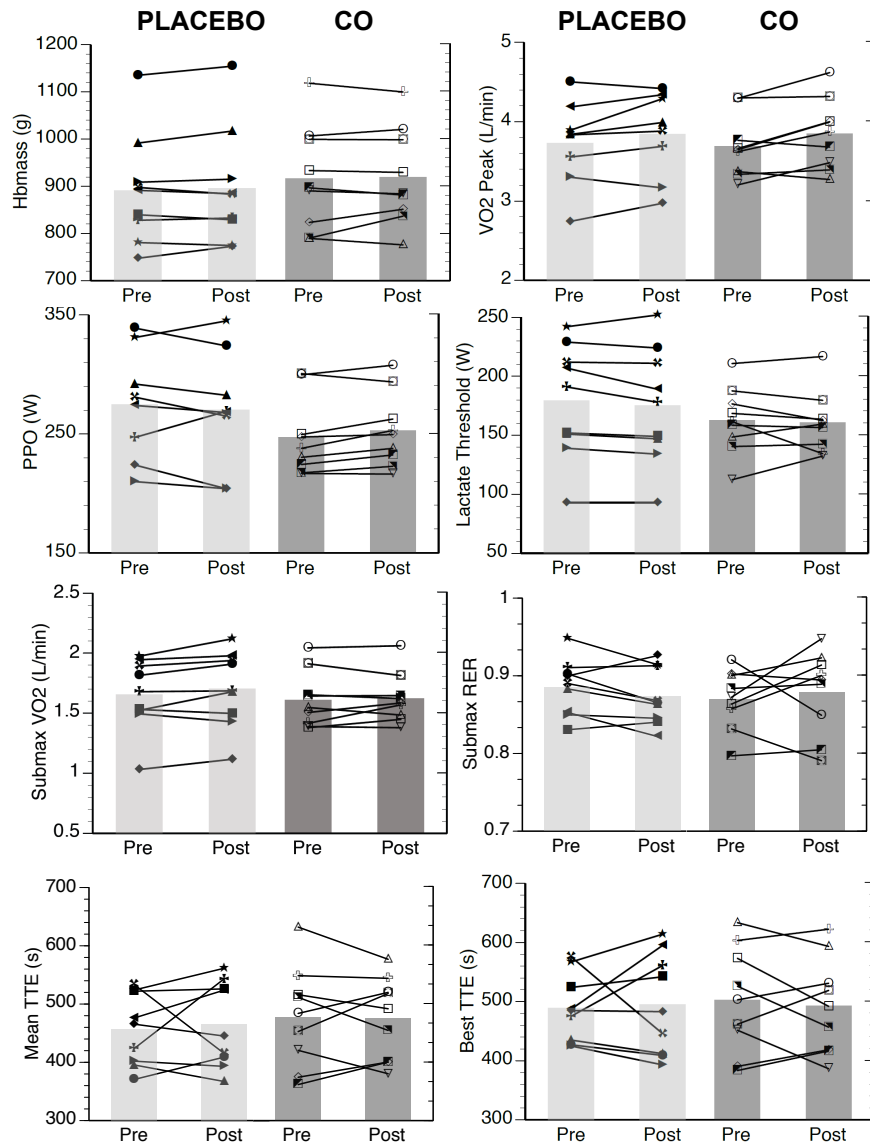


Figure 2-2. Influence of 10 days intermittent CO inhalation versus placebo on Hbmass, aerobic performance predictors, and peak-power exercise tolerance. For the placebo group, the group means are represented as light grey bars and individual subjects are represented with closed symbols. For the CO group, the group means are represented as dark grey bars and individual subjects are represented with open symbols. There were no significant differences between groups at baseline or in response to the intervention (see Table 2-1 for exact p-values). CO, Carbon monoxide; PLA, placebo; Hbmass, hemoglobin mass; $\dot{V}O_{2\text{peak}}$, peak oxygen uptake; PPO, peak power output achieved during graded exercise testing; LT, lactate threshold (1mM above baseline); Submax $\dot{V}O_2$, pooled oxygen consumption at stages below LT (the number of stages included differed between subjects but was consistent within-subject); Submax RER, pooled respiratory exchange ratio at stages below LT (the number of stages included differed between subjects but was consistent within-subject); TTE, time to exhaustion at PPO achieved during baseline graded exercise test.

DISCUSSION

The development of the optimized CO rebreathing method has enabled the routine assessment of Hbmass. For researchers employing CO rebreathing, it is important to consider any physiological effects resulting from the administration of CO. This study was motivated by recent evidence suggesting potential beneficial adaptations resulting from low-dose CO inhalation (Motterlini and Otterbein 2010). Rhodes et al. (2009) reported augmentation of several markers of mitochondrial biogenesis and an increase in MHC I protein in healthy humans following just 5 days of exposure to CO at a lower dose than we employed in the present study. More recently, Pecorella et al. (2015) reported numerous adaptive responses in skeletal muscle following just 5 days of exposure to a dose similar to that employed in the present study including increases in mitochondrial DNA copy number, citrate synthase protein expression, mitochondrial volume density, GLUT-4 protein expression, myoglobin protein expression, and capillary density. Peak oxygen uptake was not significantly altered in these studies (Rhodes et al. 2009, Pecorella et al. 2015), but it could be argued that these skeletal muscle adaptations are expected to have a larger effect on submaximal exercise responses and exercise tolerance than on peak oxygen uptake (Holloszy and Coyle 1984). We did not take muscle biopsy samples in the current investigation and therefore cannot provide any direct evidence for or against skeletal muscle adaptation in our study. However, we provide the first data describing submaximal exercise responses and exercise tolerance following chronic, intermittent, low-dose CO inhalation. Our results demonstrate that if repeated, low-dose CO exposure did induce skeletal muscle adaptation, this was not sufficient to significantly improve PPO, peak oxygen uptake, LT, submaximal oxygen uptake, submaximal RER (an index of substrate utilization), or peak-power exercise tolerance. These findings are important for researchers employing CO rebreathing to

assess Hbmass, as they demonstrate that repeated administration of low-doses of CO on up to 10 days does not significantly alter these exercise parameters.

Our data demonstrate that there was no significant effect of 10 days of intermittent CO inhalation on Hbmass. Acute inhalation of a sufficient dose of CO increases erythropoietin levels (Eckardt et al. 1990, Jelkmann and Seidl 1987), suggesting the potential for CO inhalation to induce hematological adaptations. We did not measure erythropoietin responses in the current investigation; however, given the previously reported half-life of HbCO% following CO rebreathing of 132 ± 27 minutes (Schmidt and Prommer 2005), any elevation in erythropoietin would be expected to be transient. Previous studies employing intermittent hypoxia once per day for <8 hours demonstrate that transient, intermittent elevations in erythropoietin are not sufficient to augment Hbmass (Abellán et al. 2005, Gore et al. 2006, Wilber et al. 2007) and therefore our finding that inhalation of CO once per day was insufficient to alter Hbmass is not surprising.

Our study was designed to simulate the effects of frequent assessment of Hbmass over a 2-week period, not to optimize potential beneficial effects of CO. Consequently, we tested the influence of a single dose/duration paradigm. Nevertheless, the number of CO exposures in our study doubled those in previous studies reporting skeletal muscle adaptations and the elevation in HbCO% in our study was similar to or greater than those employed in these previous investigations (Rhodes et al. 2009, Pecorella et al. 2015). Therefore, our findings indicate that low-dose CO inhalation may not translate to improvements in exercise responses in healthy humans. However, future studies examining potential beneficial adaptations resulting from alternative low-dose CO exposure paradigms are warranted.

CONCLUSIONS

Recent studies have demonstrated that CO is a key signaling molecule and suggest that low-dose CO inhalation may induce beneficial adaptations. This study provides important evidence for researchers employing CO rebreathing methods to assess Hbmass that exposure to low-dose CO on up to 10 days over a 12-day period does not significantly alter Hbmass, aerobic performance predictors, or peak-power exercise tolerance. Our findings do not preclude the possibility that alternative CO exposure paradigms may induce beneficial adaptations in humans.

ACKNOWLEDGEMENTS

This study was supported by a Beverly Sears Graduate Student Grant from the University of Colorado Boulder and a Student Research Award from the Rocky Mountain Chapter of the American College of Sports Medicine. The authors thank the study volunteers for their participation and David Caha, Pablo Callejas, and Rachel Gioscia-Ryan for study assistance. Additionally, the authors thank the staff of the Human Performance Lab at the United States Air Force Academy for providing some of the equipment used in the study.

CHAPTER III

AltitudeOmics: rapid hemoglobin mass alterations with early acclimatization to and de-acclimatization from 5260 m in healthy humans

Ryan BJ, Wachsmuth NB, Schmidt WF, Byrnes WC, Julian CG, Lovering AT, Subudhi AW, Roach RC (2014) PLoS ONE 9(10): e108788.

ABSTRACT

It is classically thought that increases in hemoglobin mass (Hbmass) take several weeks to develop upon ascent to high altitude and are lost gradually following descent. However, the early time course of these erythropoietic adaptations has not been thoroughly investigated and data are lacking at elevations greater than 5000m, where the hypoxic stimulus is dramatically increased. As part of the AltitudeOmics project, we examined Hbmass in healthy men and women at sea level (SL) and 5260m following 1, 7, and 16 days of high altitude exposure (ALT1/ALT7/ALT16). Subjects were also studied upon return to 5260m following descent to 1525m for either 7 or 21 days. Compared to SL, absolute Hbmass was not different at ALT1 but increased by $3.7 \pm 5.8\%$ (mean $\pm$ SD; $n = 20$; $p < 0.01$) at ALT7 and $7.6 \pm 6.6\%$ ($n = 21$; $p < 0.001$) at ALT16. Following descent to 1525m, Hbmass was reduced compared to ALT16 ($-6.0 \pm 3.7\%$; $n = 20$; $p = 0.001$) and not different compared to SL, with no difference in the loss in Hbmass between groups that descended for 7 ($-6.3 \pm 3.0\%$; $n = 13$) versus 21 days (-5.7 ± 5.0 ; $n = 7$). The loss in Hbmass following 7 days at 1525m was correlated with an increase in serum ferritin ($r = -0.64$; $n = 13$; $p < 0.05$), suggesting increased red blood cell destruction. Our novel findings demonstrate that Hbmass increases within 7 days of ascent to 5260m but that the altitude-induced Hbmass adaptation is lost within 7 days of descent to 1525m. The rapid time course of these adaptations contrasts with the classical dogma, suggesting the need to further examine mechanisms responsible for Hbmass adaptations in response to severe hypoxia.

INTRODUCTION

Precise regulation of erythropoiesis is critical, as both anemia and excessive polycythemia have detrimental effects on physiological function. Hypoxia is a potent stimulator of erythropoiesis and erythropoietin (EPO) increases within hours of hypobaric hypoxia (Eckhardt et al. 1989). However, it is classically thought that elevations in total hemoglobin mass (Hbmass) and red cell volume (RCV) during high altitude acclimatization require several weeks to occur (Grover and Bärtsch 2001, Sawka et al. 2000). This delayed increase fits with patterns observed with exogenous EPO administration in healthy humans, where Hbmass/RCV have been consistently reported to remain unchanged within the first 12 days of treatment (Durussel et al. 2013, Robach et al. 2009, Olsen et al. 2011). Although previous studies examining erythropoietic adaptations in lowlanders adapting to altitudes greater than 4000m for periods longer than 4 weeks have consistently reported increases in Hbmass/RCV (Pugh et al. 1964, Reynafarje et al. 1959), the time course of erythropoietic adaptation during early (i.e., first 1-3 weeks) high altitude acclimatization is less clear. For example, whereas some studies have found unchanged RCV following 2-3 weeks at 4300m (Grover et al. 1998, Sawka et al. 1996), others have found moderate increases at this same elevation over a similar time course (Reeves et al. 2001, Wolfel et al. 1991). More recently, studies examining the early time course of changes in Hbmass at lower altitudes (2000m-3600m) have reported small (2-3%) but significant increases in Hbmass following 11-13 days (Garvican et al. 2012, Wachsmuth et al. 2013a, Wachsmuth et al. 2013b). Data from these studies conflict with the classical dogma that at least 3-4 weeks are required for increases in Hbmass to be observed, but this remains a matter of considerable debate (Gore et al. 2013, Rasmussen et al. 2013). Further investigation of early erythropoietic adaptations to high altitude is warranted and, importantly, data are lacking at elevations greater than 5000m where

the hypoxic stimulus for erythropoiesis is dramatically increased (Milledge and Cotes 1985, Richalet et al. 1994).

Gains in Hbmass/RCV obtained during high altitude acclimatization are eventually lost following descent to low altitude, but the time course of this de-acclimatization also remains unclear. Based upon the traditional kinetics of red blood cell production and destruction, large reductions in Hbmass/RCV are expected to take multiple weeks to occur. Recent altitude training studies conducted at elevations between 2000m-3600m have reported full or partial retention of altitude-induced gains in Hbmass for 2-3 weeks following descent to sea level (Gore et al. 2013, Wachsmuth et al. 2013a, Wachsmuth et al. 2013b), with Hbmass eventually returning to baseline sea level values (Gore et al. 2013, Wachsmuth et al. 2013b). Hbmass/RCV have also been reported to remain elevated for several weeks following cessation of EPO treatment (Durussel et al. 2013, Lundby et al. 2008, Olsen et al. 2011). These studies suggest that elevations in Hbmass induced with short-term environmental or pharmacologic perturbation decay gradually over several weeks. In contrast, a study of polycythemic high altitude natives from 4380m reported a rapid loss in RCV of ~7% within the first week of descent to sea level; this rapid loss in RCV was coupled with increases in several markers of red blood cell destruction (Rice et al. 2001). Although this study suggested that an increased rate of red blood cell destruction may cause a rapid reduction in Hbmass following high altitude descent, the high altitude natives studied were severely polycythemic and therefore these results may not extend to subjects with less marked polycythemia. As with studies examining changes in Hbmass during the early phase of acclimatization to altitudes greater than 5000m, we are unaware of any studies examining the early time course of loss in Hbmass following descent from altitudes greater than 5000m.

AltitudeOmics was designed as a large collaborative research project examining early high altitude acclimatization/de-acclimatization in multiple physiological systems (Subudhi et al. 2014). As a result of this overall project design, we had the unique opportunity to examine the early time course of erythropoietic adaptations with ascent to and descent from 5260m in healthy humans. Because rapid changes in plasma volume (PV) occur within the first days of high altitude ascent (i.e., Imoberdorf et al. 2001, Loeppky et al. 2004, Poulsen et al. 1998, Robach et al. 2000, Robach et al. 2002) and descent (Robach et al. 2000, Robach et al. 2002), early alterations in hemoglobin concentration ([Hb]) or hematocrit (Hct) do not necessarily reflect changes in Hbmass/RCV. Therefore, we measured Hbmass and blood volume compartments in lowlanders at sea level, on 3 occasions at 5260m during 16 days of high altitude exposure, and upon initial return to 5260m following descent to low altitude (1525m) for either 7 or 21 days.

METHODS

Study Design

A detailed description of the overall AltitudeOmics study design and subject characteristics is reported elsewhere (Subudhi et al. 2014). The study was approved by the Institutional Review Boards of the Universities of Colorado and Oregon, and by the Human Research Protection Office of the U.S. Department of Defense. Subjects were informed about the possible risks and discomforts involved before giving written and verbal consent to participate.

The data reported here are novel with the exception of the basic characteristics of the 21 AltitudeOmics subjects (12 males, 9 females; age: 21 ± 1 years; height: 176 ± 8 cm; body mass: 70 ± 9 kg) that have been reported previously (Subudhi et al. 2014). Subjects were studied near sea level (130m; Eugene, OR, USA) and on 4 occasions at 5260m (Mt. Chacaltaya, Bolivia). Hbmass/BV compartments were measured in duplicate at sea level (SL) during baseline testing,

with the mean of 2 tests used as the SL value. Nine to ten weeks after SL testing, subjects were flown via commercial aircraft to El Alto, Bolivia (4050m) and then immediately driven to 1525m (Coroico, Bolivia), where they stayed for 2 days. Subjects were then driven to 5260m on ALT1 and Hbmass/BV parameters were assessed after 9-13 hours at this elevation. Subjects spent days ALT2-ALT4 at 3800m (La Paz, Bolivia), with a short visit to 5260m on ALT4, before returning to 5260m on ALT5. Subjects remained at 5260m from ALT5 to ALT17, and Hbmass and BV compartments were assessed on ALT7 and ALT16. On ALT17, subjects descended to 1525m for either 7 (POST7; n = 14) or 21 days (POST21; n = 7). Subjects were transported back to 5260m on POST7/POST21 and Hbmass/BV measurements were taken following 9-13 hours at this elevation.

Serum ferritin was assessed in all subjects 2-3 weeks prior to baseline testing. All male subjects had initial ferritin levels greater than 20 ng mL⁻¹ and none received iron supplementation during the study. Women with initial ferritin levels less than 20 ng mL⁻¹ (n = 7) were directed to take oral iron supplements (325 mg ferrous sulfate, 2-3 times daily) for 2-3 weeks prior to baseline testing and until departure to high altitude. One subject ceased supplementation prior to departure to high altitude due to gastrointestinal complaints. No subjects received iron supplementation following departure from SL. The decision not to provide iron supplementation during high altitude acclimatization/de-acclimatization was made based on potential confounding influences (Smith et al. 2008, Talbot et al. 2011) of iron supplementation on other physiological responses that were assessed as part of the overall AltitudeOmics project.

Subjects participated in many studies as part of the AltitudeOmics project and some involved blood sampling. At SL, Hbmass/BV assessments were performed prior to any other blood sampling. At high altitude, Hbmass/BV parameters were measured following other blood

sampling. The estimated volume of blood withdrawn for sampling at each altitude time point was as follows—ALT1: 212 ± 81 mL; ALT7: 64 ± 26 mL; ALT16: 191 ± 10 mL; POST7/21: 147 ± 46 mL. To examine the effect of blood sampling on Hbmass measured at ALT1, we compared our measured Hbmass values at ALT1 and SL and found that the mean values were not significantly different (see Results). Additionally, when we examined Hbmass across all time points using measured or adjusted (for estimated Hbmass withdrawn due to blood sampling), the statistical significance of our findings remained unaltered. Therefore, we have chosen to report the measured Hbmass values without adjusting for blood withdrawn for sampling but address the magnitude of Hbmass lost due to sampling in the discussion.

Analytical Methods

Hbmass and BV Parameters

Hbmass was measured using the optimized carbon monoxide (CO) rebreathing method (Prommer and Schmidt 2007, Schmidt and Prommer 2005) with minor modifications. Following at least 20 minutes of seated rest, a venous (v) blood sample (~ 2 mL) was obtained from an antecubital vein and used for determination of v[Hb] (OSM3 hemoximeter, Radiometer, Denmark) and vHct (microcentrifugation). The OSM3 was calibrated for [Hb] at regular intervals according to the manufacturers' recommendations. v[Hb] and vHct were analyzed in triplicate. Arterialized capillary blood samples (200 μ L) were obtained from a hyperemic earlobe and measured for baseline carboxyhemoglobin (HbCO%) in sextuplicate on the OSM3. End-tidal [CO] was measured using a portable CO detector (Draeger Pac 7000, Draeger, Germany). Subsequently, a bolus of 99.9% CO was administered to subjects from a calibrated syringe into a custom-built spirometer (Spico-CO Respirations-Applikator, Blood Tec, Germany) and rebreathed for 2 minutes along with 3 to 5 L of 100% oxygen.

The volume of CO administered to subjects was chosen to induce a ~5-6% increase in HbCO%. The volume of CO administered was increased at high altitude based on the reduced barometric pressure to obtain similar $\Delta\text{HbCO}\%$ in tests at sea level and high altitude. However, the largest volume of CO administered was 135 mL (maximal volume of calibrated syringe). The mean $\Delta\text{HbCO}\%$ following the rebreathing procedure was $5.4 \pm 0.8\%$.

Potential CO leaks from the subject or rebreathing apparatus were monitored throughout the rebreathing procedure using 2 portable CO detectors. Due to the effects of CO leaks on measurement error of Hbmass (Ryan et al. 2011), any test in which a CO leak was detected was excluded (total of 6 tests). End-tidal [CO] was measured 4 minutes following initial CO inhalation. Arterialized capillary blood samples (100 μL) were obtained at minutes 6 and 8 following CO inhalation and analyzed in triplicate, with the mean of minute 6 and 8 taken as post-rebreathing HbCO%. The amount of CO remaining in the spirometer was measured using a calibrated syringe and a portable CO detector (Draeger Pac 7000, Draeger, Germany). All data were compiled and used to calculate Hbmass according to previously published formulas (Prommer and Schmidt 2007, Schmidt and Prommer 2005).

The altitude in the present study was higher than any previous studies employing the optimized CO rebreathing method. To address a potential issue due to differences in oxygen saturation (Hütler et al. 2001) between sea level and high altitude testing conditions, the following minor modification was made. For tests at sea level, subjects breathed a hyperoxic gas mixture (50.5% O_2 , balance nitrogen, $\text{P}_{\text{I}}\text{O}_2 \approx 360$) for 10 minutes prior to baseline blood sampling and throughout the rest of the procedures, with the exception of the 2-minute CO rebreathing procedure, where 100% O_2 was rebreathed along with CO. At high altitude ($\text{P}_{\text{B}} = 408$ mmHg), subjects breathed 100% O_2 ($\text{P}_{\text{I}}\text{O}_2 \approx 360$) for 10 minutes prior to the baseline blood

sampling and throughout the rest of the procedures. Hyperoxia was provided at both sea level and high altitude to establish similarly high oxygen saturation levels during the Hbmass procedure in both environments in order to eliminate the influence of oxygen saturation differences on the analytical determination of HbCO% on the OSM3 hemoximeter (Hütler et al. 2001).

A single OSM3 unit was used for all HbCO% measurements during the study. To determine any potential confounding effects of the international transport on the OSM3 hemoximeter or high altitude *per se* on the measurement of HbCO%, we performed a quality control analysis using in-house arterial blood samples that we had obtained prior to this study at 2 levels of HbCO% (high and low HbCO%). These control samples were run in sextuplicate on 5 days during sea level testing and the mean $\Delta\text{HbCO}\%$ between days was $6.84 \pm 0.05\%$; control samples were kept frozen at $-80\text{ }^{\circ}\text{C}$ until transport—they remained stored on ice during transport and were analyzed in sextuplicate on 2 days within the first week at high altitude. The mean $\Delta\text{HbCO}\%$ between these days was $6.80 \pm 0.07\%$. Thus, the difference in $\Delta\text{HbCO}\%$ between sea level and high altitude was well within the intra-analyzer variability of $\Delta\text{HbCO}\%$ using OSM3 hemoximeters (Gough et al. 2011), indicating that neither high altitude nor the international transport of the OSM3 had any confounding effects on the measurement of $\Delta\text{HbCO}\%$ that is critical to Hbmass determination.

Measurement errors of Hbmass and BV parameters were calculated from duplicate baseline measurements ($n = 19$) according to Hopkins (2000). Measurement error for Hbmass was 1.5% (95%CI: 1.1-2.3%)—with duplicate Hbmass tests within a 1-week period, measurement error reflects primarily analytical error as the biological variation over this time frame has been shown to be minimal (Eastwood et al. 2008, Prommer et al. 2008).

RCV, BV, and PV were derived from Hbmass, v[Hb], and vHct as follows:

- 1) $RCV = Hbmass \times MCHC^{-1} \times 100^{-1}$
- 2) $BV = RCV \times 100 \times Hct^{-1} \times 0.91^{-1}$
- 3) $PV = BV - RCV$

Hct was multiplied by 0.96 to account for trapped plasma; the constant of 0.91 was included in the BV calculation to correct for the ratio of body hematocrit to peripheral hematocrit (Chaplin et al. 1953). Measurement errors for RCV, PV, and BV were 2.2% (95%CI: 1.6-3.2%), 4.9% (95%CI: 3.6-7.3%), and 3.4% (95%CI: 2.5-5.0%), respectively. For BV compartments, the measurement error is influenced by analytical error of Hbmass, [Hb], and Hct as well as biological variation in PV and total BV.

Serum Ferritin and EPO

Whole blood samples were collected following 30 min of rest from a catheter placed in an antecubital vein (Subudhi et al. 2014). The ALT1 ferritin sample was taken after ~2 hours at 5260m whereas the ALT1 EPO sample was taken after ~10 hours at 5260m. Samples were drawn into 10 mL syringes and immediately transferred into serum collection vacutainers (BD, Franklin Lakes, NJ, USA). These vacutainers were inverted 5 times and then allowed to sit for 30 – 60 minutes at room temperature to allow for proper clotting. Tubes were spun for 20 minutes at 800 relative centrifugal force at room temperature. Once separated, serum was stored on ice for 10 minutes before being stored in either a -80 °C freezer (Eugene), or in a charged nitrogen vapor shipper (Bolivia). Frozen serum samples were transported in charged nitrogen vapor shippers, and then stored at -80 °C until analysis. Serum ferritin was assessed via nephelometry (within-run coefficient of variation (CV): 7%; between-run CV: 5%; Siemens BNII Nephelometer, Erlangen, Germany). Serum EPO was assessed in duplicate using a

Quantikine IVD Human Epo ELISA kit (intra-assay CV: 4%; inter-assay CV: 6%; R&D Systems, Minneapolis, MN, USA).

Missing Data

As mentioned above, 6 Hbmass tests were excluded due to CO leaks. We also missed Hbmass tests due to logistical difficulties (6 tests) and subject discomfort prior to Hbmass testing (1 test). Some v[Hb] or vHct samples (total of 16 tests) were missed for logistical reasons and difficulties with obtaining or processing venous samples; in the case of missing v[Hb] or vHct, data were also excluded from the analysis of changes in BV parameters. The majority of missing tests for Hbmass and BV occurred at ALT1. Ferritin values were missing from 3 tests and EPO values were missing from 8 tests.

Statistics

Statistical analyses were performed using Statistical Package for the Social Sciences (version 20, SPSS Inc., Chicago, IL, USA) and Microsoft Excel 2008 (Redmond, WA, USA). We performed linear mixed model statistical analyses to examine our outcome variables across acclimatization using the Mixed procedure in SPSS. A major advantage of linear mixed model statistical analyses is that missing values do not result in casewise deletion of other longitudinal measurements, as is required with repeated-measures analysis of variance. Time (SL, ALT1, ALT7, ALT16), sex, and a time x sex interaction were included in the linear mixed models as fixed factors. Time comparisons were made with SL as the reference. Separate paired-tests were performed to compare ALT7 and ALT16. No adjustments were made for multiple testing. Due to largely reduced subject numbers at ALT1 for Hbmass/BV parameters, we could not be certain that the missing data at ALT1 met the missing-at-random requirement of the linear mixed model. Therefore, we performed paired t-tests to examine changes between SL and ALT1. To examine

differences at POST7/POST21 compared to SL and ALT16, we used linear mixed models with time (SL, ALT16, POST), group (POST7, POST21), and a time x group interaction included as fixed factors. The POST timepoint includes POST7 and POST21 measurements, with differences between the groups descending for 7 versus 21 days assessed by comparing the effect of group. Time comparisons were made with POST as the reference—therefore, data from SL and ALT16 were excluded from these analyses for subjects missing the POST7/POST21 time point. Due to the reduced number of female subjects included at POST21 ($n = 2$), sex was not included in these models. We performed simple linear regressions to examine relationships between variables. For all analyses, statistical significance was accepted when $p \leq 0.05$. Data are presented throughout the paper as mean $\pm$ SD unless otherwise noted.

RESULTS

Subject characteristics and ferritin status during high altitude acclimatization and de-acclimatization

A detailed description of subject characteristics is presented elsewhere (Subudhi et al. 2014)—briefly, body mass was reduced by 2.6 ± 1.6 kg after 16 days of high altitude exposure. Serum ferritin levels are presented in Table 3-1. Ferritin levels were lower in women compared to men. At ALT1, all men had ferritin above 20 ng mL^{-1} , whereas 4 of 8 women had ferritin levels below this value. Ferritin levels decreased from ALT1 to ALT16 in both men ($-68 \pm 16\%$) and women ($-65 \pm 26\%$) and increased following descent from high altitude in both men ($+189 \pm 196\%$) and women ($+184 \pm 283\%$).

Hematological adaptations during 16 days of high altitude acclimatization

EPO increased from a baseline level of $8.3 \pm 5.0 \text{ IU L}^{-1}$ by 4.9 ± 2.8 fold at ALT1 ($n = 16$), 8.3 ± 8.7 fold at ALT7 ($n = 18$), and 2.5 ± 1.6 fold at ALT16 ($n = 21$); all $p < 0.05$ compared

to SL). There were no significant correlations between the increases in EPO at ALT1, ALT7, or ALT16 and changes in Hbmass. Data comparing SL and ALT1 for subjects with Hbmass/BV measurements at both time points are presented separately in Table 3-2 so that the effect of acute altitude on Hbmass and BV parameters can be distinguished from inter-individual variation. We found a non-significant 11 g loss in absolute Hbmass at ALT1 compared to SL ($p = 0.206$); there was also a trend for relative Hbmass to be slightly (0.3 g kg^{-1}) reduced at ALT1 compared to SL ($p = 0.056$). A small decrease in Hbmass was expected, as Hbmass was assessed after the required blood sampling for other protocol procedures on ALT1. $v[\text{Hb}]$, $v\text{Hct}$, and BV compartments were not significantly different at ALT1 compared to SL.

Table 3-3 presents data on hematological parameters at SL, ALT7, and ALT16. Compared to SL, absolute Hbmass was increased at ALT7 ($+3.7 \pm 5.8\%$; $n = 20$; $p < 0.01$) and ALT16 ($+7.6 \pm 6.6\%$; $n = 21$; $p < 0.001$), with the gain larger at ALT16 compared to ALT7. The increase in absolute Hbmass was larger in men compared to women at ALT16. Relative Hbmass was increased compared to SL at ALT7 and ALT16; relative Hbmass was greater at ALT16 compared to ALT7 and the increases were greater in men than women at both ALT7 and ALT16. Due to the lower absolute and relative Hbmass levels in women compared to men, we also examined the percent change in absolute Hbmass from SL and found no significant difference between men and women (Figure 3-1A). At ALT16, Hbmass was elevated compared to SL in all 12 men and 7 out of 9 women. There was no significant correlation between ferritin level upon initial exposure to altitude and the percent change in absolute Hbmass at ALT16 (Figure 3-1B).

$v[\text{Hb}]$ and $v\text{Hct}$ were increased at ALT7 and ALT16 compared to SL, with no significant differences between ALT7 and ALT16. Men had larger increases in $v[\text{Hb}]$ and $v\text{Hct}$ at ALT16 compared to women. Absolute and relative PV were reduced at ALT7 and ALT16 compared to

SL, with no significant difference between ALT7 and ALT16. The reduction in absolute PV at ALT16 was greater in men compared to women, but no significant difference was detected in the change in relative PV. Absolute BV was reduced at ALT7 and ALT16 compared to SL, with no significant difference between ALT7 and ALT16 or in the change in BV between men and women. Relative BV was not significantly different from SL at ALT7 or ALT16, but there was a trend ($p = 0.057$) for women to have a greater reduction in relative BV compared to men at ALT7. Relative BV was greater at ALT16 compared to ALT7. Changes in absolute and relative RCV mirrored changes in Hbmass.

Hematological adaptations following descent to low altitude

Table 3-4 presents hematological parameters for subjects with complete measurements at SL, ALT16, and POST7/POST21. For all hematological parameters, there were no significant differences in responses between POST7 and POST21 groups or any significant group x time interactions. Absolute ($-6.0 \pm 3.7\%$) and relative ($-6.8 \pm 4.3\%$) Hbmass declined following high altitude descent—absolute Hbmass at the POST7/POST21 measurement was not significantly different from SL ($+0.8 \pm 4.5\%$), but relative Hbmass was slightly elevated compared to SL ($+3.2 \pm 5.5\%$). Figure 3-2A shows the percent changes in absolute Hbmass from SL at ALT16 and POST7/POST21. A similar pattern was observed for RCV, with absolute and relative RCV values reduced at POST7/POST21 compared to ALT16. Absolute and relative PV were increased at POST7/POST21 compared to ALT16 and not significantly different from SL. Absolute and relative BV at POST7/POST21 were not significantly different from ALT16 or SL. $v[Hb]$ and $vHct$ were reduced at POST7/POST21 compared to ALT16 and not significantly different compared to SL.

The gain in Hbmass from SL to ALT16 was correlated with the reduction in Hbmass from ALT16 to POST7/POST21 (Figure 3-2B; $r = -0.77$; $n = 20$; $p = 0.00006$). The reduction in Hbmass from ALT16 to POST7 was correlated with an increase in serum ferritin (Figure 3-2C; $r = -0.64$; $n = 13$; $p = 0.02$).

	Time				Significant effects	
	SL	ALT1	ALT16	POST	Sex	Time
M	63.2 ± 29.0 (12)	66.8 ± 42.2 (11)	25.8 ± 24.0 (12)	52.3 ± 44.0 (11)	M > W	ALT16 < SL
W	28.9 ± 15.5 (9)	19.7 ± 10.9 (8)	7.5 ± 5.2 (9)	19.6 ± 21.3 (9)		ALT16 < ALT1 ALT16 < POST

Table 3-1. Serum ferritin during high altitude acclimatization and de-acclimatization.

Data are presented as mean ± SD (ng ml⁻¹) with the number of subjects indicated in parentheses. The POST measurement took place upon initial return to 5260m following descent to 1525m for 7 days (7W, 7M) or 21 days (2W, 4M) duration. Linear mixed model statistical analyses were performed to examine the effects of sex and time with SL as the reference. Paired t-tests were performed to compare ALT16 with ALT1 and POST. Effects were accepted as significant when $p \leq 0.05$.

	SL	ALT1	Percent Change
Hbmass (g)	723 ± 175 (6,6)	711 ± 173 (6,6)	-1.6 ± 4.4%
Rel Hbmass (g kg ⁻¹)	10.1 ± 1.4 (6,6)	9.8 ± 1.4 (6,6)	-2.7 ± 4.4%
v[Hb] (g dL ⁻¹)	13.9 ± 1.0 (8,4)	14.3 ± 1.5 (8,4)	2.5 ± 6.7%
vHct (%)	42.6 ± 2.6 (8,4)	42.6 ± 4.5 (8,4)	-0.3 ± 6.5%
BV (ml)	6224 ± 848 (5,3)	6130 ± 517 (5,3)	-0.8 ± 7.5%
Rel BV (ml kg ⁻¹)	83.2 ± 6.6 (5,3)	81.9 ± 7.3 (5,3)	-1.4 ± 7.0%
PV (ml)	3907 ± 416 (5,3)	3866 ± 276 (5,3)	-0.2 ± 11.5%
Rel PV (ml kg ⁻¹)	52.4 ± 4.7 (5,3)	51.9 ± 7.3 (5,3)	-0.9 ± 10.9%
RCV (ml)	2317 ± 442 (5,3)	2264 ± 417 (5,3)	-2.1 ± 5.0%
Rel RCV (ml kg ⁻¹)	30.8 ± 3.0 (5,3)	29.9 ± 3.3 (5,3)	-2.6 ± 5.6%

Table 3-2. Hematological parameters at sea level and the first day of exposure to 5260m.

Data are presented as mean ± SD with the number of subjects (M,W) indicated in parentheses. This table only includes data for subjects with measures at both time points so that the effect of acute altitude on Hbmass and BV parameters can be distinguished from the inter-individual variation. Paired t-tests were performed for each parameter and none of the differences were statistically significant (all $p > 0.05$).

	Time			Significant Effects		
	SL	ALT7	ALT16	Sex	Time	Interaction
Hbmass (g)						
M	905 ± 95 (12)	950 ± 110 (12)	989 ± 110 (12)	M > W	ALT7 > SL ALT16 > SL ALT16 > ALT7	$\Delta M > \Delta W$ ALT16
W	559 ± 62 (9)	567 ± 76 (8)	590 ± 91 (9)			
Rel Hbmass (g kg⁻¹)						
M	11.9 ± 1.4 (12)	13.0 ± 1.7 (12)	13.8 ± 1.6 (12)	M > W	ALT7 > SL ALT16 > SL ALT16 > ALT7	$\Delta M > \Delta W$ ALT7 $\Delta M > \Delta W$ ALT16
W	9.1 ± 0.8 (9)	9.2 ± 1.0 (8)	9.7 ± 1.2 (9)			
v[Hb] (g dL⁻¹)						
M	14.6 ± 0.6 (12)	16.2 ± 1.1 (12)	16.9 ± 0.6 (12)	M > W	ALT7 > SL ALT16 > SL	$\Delta M > \Delta W$ ALT16
W	12.4 ± 0.8 (9)	13.7 ± 1.1 (8)	13.4 ± 1.0 (8)			
vHct (%)						
M	44.2 ± 1.4 (12)	48.5 ± 3.0 (12)	50.6 ± 2.2 (12)	M > W	ALT7 > SL ALT16 > SL	$\Delta M > \Delta W$ ALT16
W	39.1 ± 2.3 (9)	42.4 ± 2.8 (8)	42.1 ± 3.3 (8)			
BV (ml)						
M	6813 ± 667 (12)	6434 ± 687 (12)	6420 ± 588 (12)	M > W	ALT7 < SL ALT16 < SL	
W	4974 ± 565 (9)	4592 ± 730 (7)	4821 ± 756 (8)			
Rel BV (ml kg⁻¹)						
M	89.9 ± 9.3 (12)	88.0 ± 8.9 (12)	89.5 ± 8.0 (12)	M > W	ALT16 > ALT7	
W	80.9 ± 7.1 (9)	74.2 ± 7.8 (7)	78.7 ± 9.8 (8)			
PV (ml)						
M	4190 ± 412 (12)	3713 ± 473 (12)	3580 ± 304 (12)	M > W	ALT7 < SL ALT16 < SL	$\Delta M > \Delta W$ ALT16
W	3287 ± 417 (9)	2921 ± 502 (7)	3047 ± 499 (8)			
Rel PV (ml kg⁻¹)						
M	55.3 ± 5.5 (12)	50.7 ± 5.5 (12)	49.9 ± 3.8 (12)		ALT7 < SL ALT16 < SL	
W	53.4 ± 5.6 (9)	47.2 ± 5.5 (7)	49.8 ± 6.9 (8)			
RCV (ml)						
M	2623 ± 279 (12)	2721 ± 298 (12)	2839 ± 331 (12)	M > W	ALT7 > SL ALT16 > SL ALT16 > ALT7	
W	1687 ± 188 (9)	1671 ± 247 (7)	1773 ± 300 (8)			
Rel RCV (ml kg⁻¹)						
M	34.6 ± 4.0 (12)	37.3 ± 4.6 (12)	39.6 ± 4.8 (12)	M > W	ALT7 > SL ALT16 > SL ALT16 > ALT7	$\Delta M > \Delta W$ ALT7 $\Delta M > \Delta W$ ALT16
W	27.4 ± 2.3 (9)	27.1 ± 2.8 (7)	28.9 ± 4.0 (8)			

Table 3-3. Hematological adaptations during 16 days high altitude acclimatization in healthy men and women.

Data are presented as mean ± SD with the number of subjects indicated in parentheses. Linear mixed model statistical analyses were performed to examine the effects of sex, time (with SL as the reference) and a sex x time interaction. Paired t-tests were performed to compare ALT7 with ALT16. Effects were accepted as significant when $p \leq 0.05$.

	Time			Significant Effects
	SL	ALT16	POST	
Hbmass (g)				
POST7	726 ± 172 (6,7)	785 ± 194 (6,7)	734 ± 173 (6,7)	POST < ALT16
POST21	802 ± 245 (5,2)	865 ± 296 (5,2)	810 ± 266 (5,2)	
Rel Hbmass (g kg⁻¹)				
POST7	10.2 ± 1.4 (6,7)	11.3 ± 1.9 (6,7)	10.5 ± 1.6 (6,7)	POST < ALT16 POST > SL
POST21	11.7 ± 2.4 (5,2)	13.3 ± 3.3 (5,2)	12.3 ± 2.9 (5,2)	
v[Hb] (g dL⁻¹)				
POST7	13.6 ± 1.2 (5,4)	15.4 ± 1.6 (5,4)	13.8 ± 1.5 (5,4)	POST < ALT16
POST21	14.2 ± 1.1 (5,2)	16.0 ± 2.1 (5,2)	14.7 ± 1.8 (5,2)	
vHct (%)				
POST7	42.3 ± 2.9 (5,4)	47.0 ± 4.3 (5,4)	42.0 ± 4.1 (5,4)	POST < ALT16
POST21	43.1 ± 2.9 (5,2)	48.5 ± 6.1 (5,2)	44.4 ± 4.1 (5,2)	
BV (ml)				
POST7	6017 ± 907 (4,4)	5694 ± 926 (4,4)	5965 ± 825 (4,4)	
POST21	6125 ± 1569 (5,2)	5820 ± 1476 (5,2)	5914 ± 1445 (5,2)	
Rel BV (ml kg⁻¹)				
POST7	83.6 ± 5.2 (4,4)	81.4 ± 7.1 (4,4)	85.0 ± 6.0 (4,4)	
POST21	90.1 ± 13.2 (5,2)	90.2 ± 12.5 (5,2)	90.9 ± 12.0 (5,2)	
PV (ml)				
POST7	3813 ± 435 (4,4)	3363 ± 403 (4,4)	3775 ± 423 (4,4)	POST > ALT16
POST21	3799 ± 885 (5,2)	3300 ± 664 (5,2)	3587 ± 749 (5,2)	
Rel PV (ml kg⁻¹)				
POST7	53.2 ± 3.1 (4,4)	48.3 ± 3.0 (4,4)	54.0 ± 4.4 (4,4)	POST > ALT16
POST21	56.1 ± 6.9 (5,2)	51.5 ± 4.0 (5,2)	55.4 ± 5.3 (5,2)	
RCV (ml)				
POST7	2204 ± 485 (4,4)	2331 ± 556 (4,4)	2190 ± 480 (4,4)	POST < ALT16
POST21	2326 ± 695 (5,2)	2520 ± 847 (5,2)	2326 ± 711 (5,2)	
Rel RCV (ml kg⁻¹)				
POST7	30.4 ± 3.6 (4,4)	33.2 ± 5.3 (4,4)	31.0 ± 4.2 (4,4)	POST < ALT16
POST21	34.0 ± 6.7 (5,2)	38.7 ± 9.4 (5,2)	35.5 ± 7.3 (5,2)	

Table 3-4. Hematological adaptations following descent from high altitude to low altitude.

Data are presented as mean ± SD with the number of subjects (M,W) indicated in parentheses. Subjects were studied at sea level, at 5260m after 16 days high altitude acclimatization, and upon initial return to 5260m after descent to 1525m for 7 (POST7) or 21 (POST21) days. Linear mixed model statistical analyses were performed to examine the effects of time (with POST as the reference), group (POST7 versus POST21) and a time x group interaction. Effects were accepted as significant when $p \leq 0.05$. There were no significant effects of group or any significant group x time interactions (all $p > 0.05$).

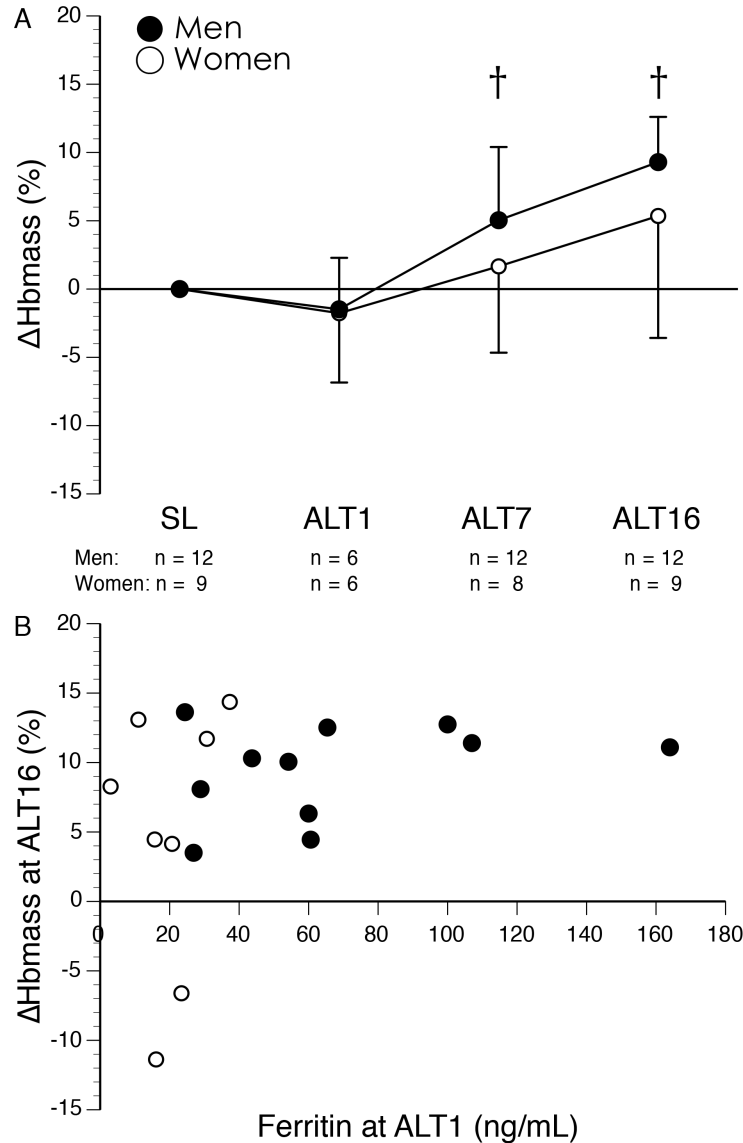


Figure 3-1. Hemoglobin mass in men and women during 16 days high altitude acclimatization.

A) Time course of changes in absolute Hbmass. Data are presented as mean $\pm$ SD, with the number of men and women tested at each time indicated below the x-axis. †Significantly different from sea level ($p < 0.05$; main effect of time). The percent changes were not significantly different between men and women ($p > 0.05$). B) Relationship between serum ferritin level upon arrival at high altitude and the percent change in absolute Hbmass following 16 days at high altitude. Two subjects had missing ferritin data at ALT1 and their Hbmass data were excluded from this graph. There was no correlation between initial ferritin level upon arrival at altitude and the percent change in absolute Hbmass during high altitude acclimatization ($r = 0.33$; $n = 19$; $p = 0.16$).

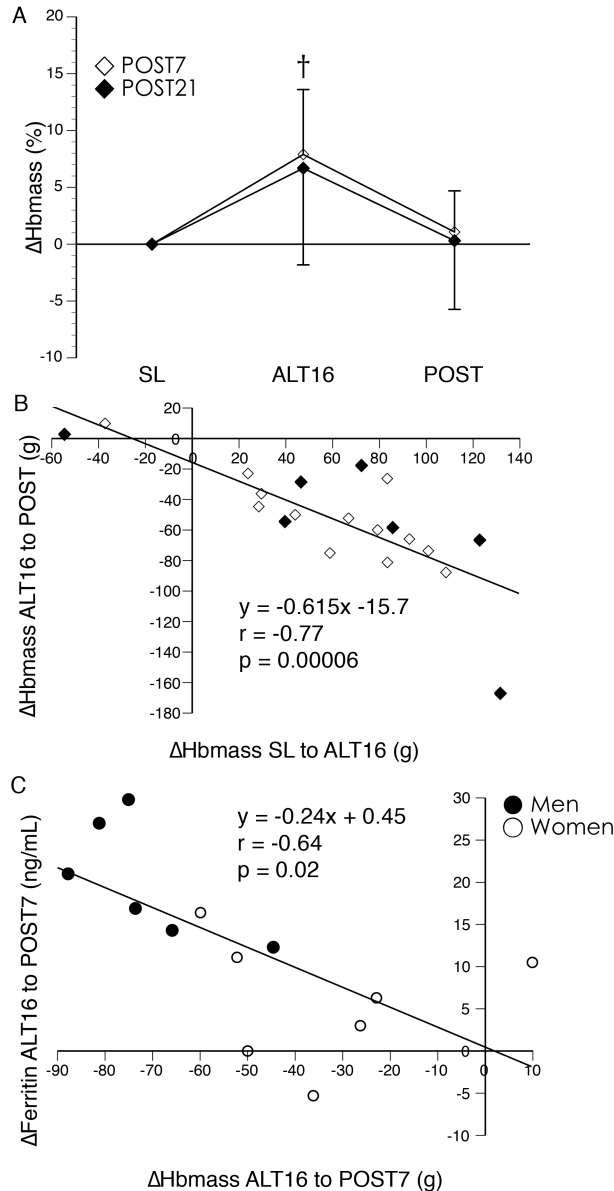


Figure 3-2. Change in hemoglobin mass following descent from high altitude to low altitude.

Subjects were tested at high altitude at the end of a 16 day acclimatization period and upon return to high altitude after descent to low altitude (1525m) for either 7 (POST7; $n = 13$) or 21 days (POST21; $n = 7$). Data are presented as mean $\pm$ SD. A) Changes in Hbmass. † Significantly different from POST ($p < 0.05$; main effect of time). There were no significant differences between the POST7 and POST21 groups or between POST and SL ($p > 0.05$). B) Relationship between changes in Hbmass following 16 days high altitude acclimatization and changes in Hbmass following descent to low altitude. C) Relationship between changes in Hbmass and changes in serum ferritin following descent to low altitude for 7 days.

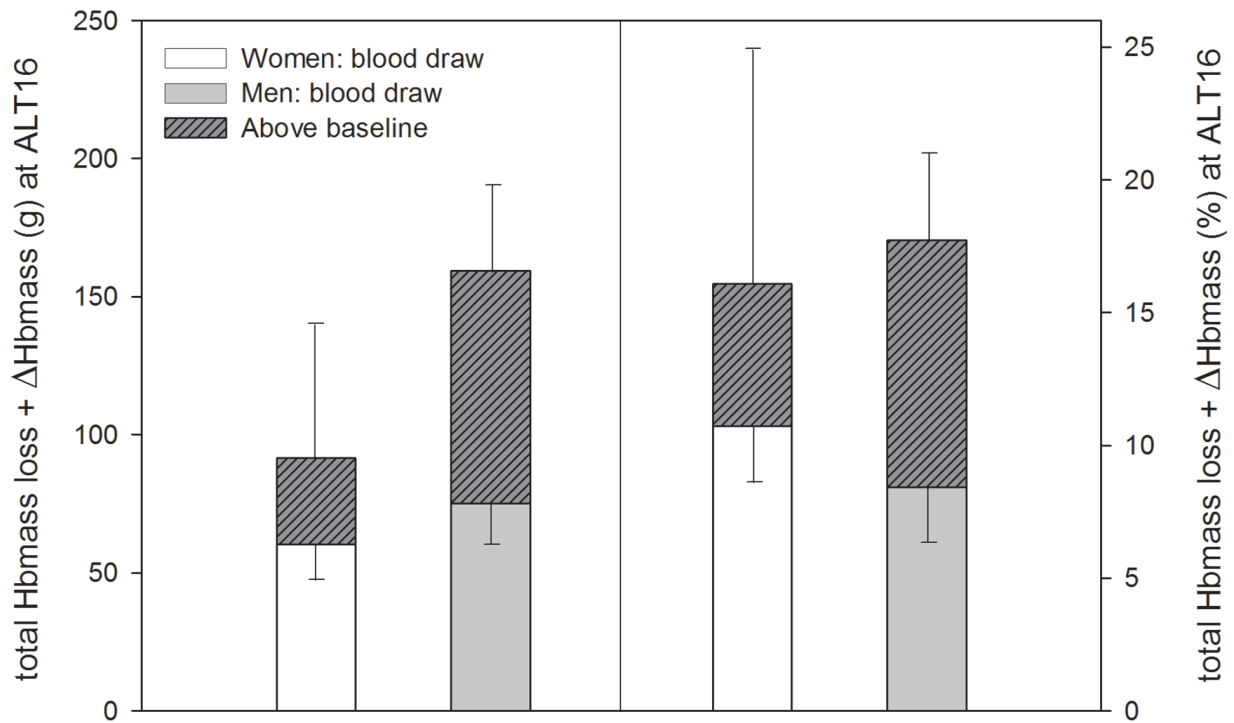


Figure 3-3. Estimate of the increase in hemoglobin mass produced during 16 days high altitude acclimatization determined from the measured Hbmass increase above sea level baseline plus the calculated Hbmass loss due to blood sampling.

Panel A represents the absolute increase in Hbmass (g) produced. Panel B represents the percent increase in Hbmass produced. Data are presented as mean $\pm$ SD. Upward SD bars represent the SD of the increase in Hbmass measured above baseline and the downward SD bars represent the SD of calculated Hbmass loss due to blood sampling.

DISCUSSION

This study provides the first data on early Hbmass alterations in healthy humans with ascent to and descent from altitudes greater than 5000m. We found an increase in Hbmass at ALT7 and a further augmentation by ALT16. However, the altitude-induced gain in Hbmass was remarkably short-lived, as descent to low altitude resulted in a reduction in Hbmass to baseline values within 7 days. The correlation between the loss in Hbmass and increase in serum ferritin following descent to low altitude suggests that this rapid reduction in Hbmass was mediated by increased red blood cell destruction. Overall, this study demonstrates the capacity for rapid alterations in Hbmass with ascent to and descent from high altitude and suggests the need to further examine mechanisms of erythropoietic adaptations to severe hypoxia.

Increase in Hbmass during high altitude acclimatization

The veracity of our finding of swift alterations in Hbmass is predicated on the validity and sensitivity of our methodological approach for measuring Hbmass. We have several reasons to believe our measurements were robust and that our findings were not the result of analytical error or artifact. First, CO rebreathing methods have been shown to have low measurement error compared to other methodological approaches for assessing the red cell compartment (Gore et al. 2005) and we achieved a measurement error of 1.5% from duplicate baseline tests in the present study. At ALT7 and ALT16, the mean increases in Hbmass we observed were 2-5 times greater than our measurement error. Second, we performed quality-control analysis for $\Delta\text{HbCO}\%$ both at SL and high altitude and found near-identical results (Gough et al. 2011). Third, the ALT7 and ALT16 measures, at which Hbmass was elevated compared to SL, took place at the same location and with the same equipment and personnel as the POST7 and POST21 measures, at which Hbmass had returned to SL values following a 7 or 21 day de-acclimatization period.

Therefore, a spurious inflation of Hbmass only at ALT7 and ALT16 seems unlikely. Taken together, we are confident that our findings reflect true physiological alterations in Hbmass with early ascent to and descent from high altitude and were not caused by normal biological variation or analytical error.

The speed of change in Hbmass observed in our study conflicts with the classical dogma that increases in Hbmass during high altitude acclimatization take at least 3-4 weeks to develop (Grover and Bärtsch 2001, Sawka et al. 2000). Recent studies at moderate altitude (2000-3600m) suggested that erythropoietic adaptations may be swifter than previously thought (Garvican et al. 2012, Wachsmuth et al. 2013a, Wachsmuth et al. 2013b, Gore et al. 2013), but the speed and magnitude of increase in Hbmass we observed at ALT7 and ALT16 exceed previous findings. The magnitude of change we observed is particularly striking given that some Hbmass was lost due to sampling (estimated loss of 29 ± 12 g at ALT1, 10 ± 4 g at ALT7, and 30 ± 4 g at ALT16). We report the Hbmass values that we measured without adjusting for the blood lost due to sampling—this underestimates the total increase in the amount of hemoglobin produced at high altitude (estimate presented in Figure 3-3), which includes the Hbmass change we measured above our SL baseline values plus the Hbmass lost due to blood sampling on ALT1, ALT7 and ALT16 combined (calculated as 69 ± 16 g; $n = 21$). Our findings raise the intriguing question of what mechanism enables this rapid and robust increase in Hbmass in response to severe hypoxia.

The regulation of red cell production is known to be largely influenced by EPO (Jelkmann 2011). EPO peaks within the first 2-3 days of altitude exposure before beginning to fall towards baseline (Abbrecht and Littell 1972, Robach et al. 2004) and it has been suggested that the rapid return of EPO to baseline levels with continued altitude exposure should reduce the magnitude of the erythropoietic stimulus compared to exogenous EPO treatment (Sawka et al.

2000). However, single measurements of circulating EPO do not adequately reflect the complex kinetics of EPO secretion over days and weeks, and it is unclear if the fall in circulating EPO with continued altitude exposure results from a decrease in EPO expression or is related to an increased rate of clearance from circulation (Jelkmann 2011). It is noteworthy that much of the RCV expansion in lowlanders ascending to 4540m for a 12-month period occurred after 1-2 months (Reynafarje et al. 1959), a time at which EPO would be expected to have returned to baseline (Abbrecht and Littell 1972, Robach et al. 2004). Importantly, our finding of an increase in Hbmass within 7 days of ascent to high altitude is in stark contrast to studies involving exogenous EPO administration, where Hbmass has been consistently reported to remain unchanged within the first 12 days of treatment (Durussel et al. 2013, Olsen et al. 2011, Robach et al. 2009) despite continuous elevation of circulating EPO above baseline (Robach et al. 2009). In comparing high altitude ascent with EPO treatment, it is important to consider that the distinct stimuli of high altitude residence *versus* pharmacological EPO administration are markedly different. Whereas the elevation in EPO with severe hypoxia is secondary to hypoxia-inducible factor (HIF) signaling (Haase 2013), the provision of exogenous EPO bypasses broad HIF activation. Differences between these conditions are reflected in divergent responses in other pathways affected by HIF signaling that influence erythropoietic adaptations such as iron mobilization (Haase 2013, Robach et al. 2007, Robach et al. 2009). Ultimately, we cannot provide mechanistic data from our study explaining the swifter increase in Hbmass in our subjects compared to previous studies at lower elevations or involving exogenous EPO administration, and we are not suggesting that EPO is not a key player in augmenting erythropoiesis in response to severe hypoxia. Rather, the more rapid increase in Hbmass in

severe hypoxia compared to exogenous EPO treatment suggests that mechanisms in addition to augmentation of EPO may play an important role in the rapid erythropoietic response.

To our knowledge, we are the first to compare changes in Hbmass/RCV in men and women at identical time points and under similar experimental conditions at altitudes greater than 4000m. We found that the percent increase in absolute Hbmass following 16 days at high altitude was not significantly different between men and women. Some previous cross-sectional studies of moderate altitude residents have suggested that erythropoietic responses may be lower in females compared to males (Böning et al. 2001, Böning et al. 2004), and it has been suggested that the ventilatory-stimulating effects of the female sex hormones play a key role (Christancho et al. 2007). However, Reeves et al. (2001) found no effect of menstrual phase on ventilatory or erythropoietic adaptations in healthy women acclimatizing to 4300m despite large differences in sex hormone levels between subjects in the luteal versus follicular phases. Arterial oxygen pressure and saturation did not differ between men and women at ALT1 and therefore the impact of ventilatory effects on potential sex differences in erythropoietic responses to high altitude would be minimal in our study.

Our finding that the percent change in absolute Hbmass did not differ between men and women is particularly striking given the low ferritin levels of our female subjects upon arrival at high altitude. Although subjects with low ferritin during baseline testing were directed to take oral iron supplements up until departure for high altitude, several women arrived at high altitude with low ferritin levels, and based on previous work at moderate altitude (Stray-Gundersen et al. 1991), one might expect that the low iron stores would prevent an increase in Hbmass. In contrast, most (7 out of 9) of the women increased Hbmass in response to high altitude exposure. However, while all 12 men had increases in Hbmass following 16 days high altitude

acclimatization, 2 women failed to increase Hbmass; indeed, these 2 women had reductions in Hbmass that were similar to the calculated amount of Hbmass withdrawn from these subjects for blood sampling at altitude. We examined the EPO response of these 2 individual subjects and found that their increases in EPO with high altitude exposure were above the group median at ALT1, ALT7, and ALT16, suggesting that the failure to increase Hbmass was not caused by a lack of EPO upregulation. As can be observed in Figure 3-1B, these 2 women were not distinguished by particularly low iron stores upon arrival at high altitude. Indeed, our individual data demonstrate the capability to increase Hbmass despite low ferritin levels upon initial arrival at high altitude. The subject with the lowest ferritin (3 ng mL^{-1}) upon initial high altitude exposure had a relatively large (8.3%) increase in Hbmass.

It might be questioned whether the level of storage iron indicated by these low ferritin values would be sufficient to enable a large increase in Hbmass. However, previous work has demonstrated increases in intestinal iron absorption at high altitude (Reynafarje et al. 1959, Mairbäurl et al. 1990, Goetze et al. 2013) and it is possible that dietary iron intake (not measured in the current study) provided sufficient iron for increasing hemoglobin production. Additionally, recent data suggest that a decrease in skeletal muscle iron content during the first week at high altitude may increase iron available for erythropoiesis (Robach et al. 2007). Admittedly, because we did not measure dietary iron intake or iron-related proteins in skeletal muscle, the role of these mechanisms in allowing increased erythropoiesis in our subjects with low ferritin is purely speculative. However, these other studies highlight the complexity of iron homeostasis at high altitude and suggest that the iron required for increasing erythropoiesis may have been obtained from increased intestinal absorption or mobilization of iron from skeletal muscle stores. Further studies involving Hbmass assessments coupled with more comprehensive assessments of iron

homeostasis are needed to more robustly determine the relationship between iron availability and erythropoiesis at high altitude. Our data suggest that low initial iron stores do not requisitely prevent high altitude-induced erythropoiesis; however, we stress that we cannot determine from our data whether low iron stores limited the magnitude of increase in Hbmass in some subjects.

Decrease in Hbmass following descent from high altitude to low altitude

We found that the Hbmass gained during high altitude acclimatization was quickly lost following descent to 1525m. Hbmass had returned to SL baseline in our subjects who descended to low altitude for 7 days, and there was no further decrement in the group who descended for 21 days. To our knowledge, we are the first to report a complete loss of altitude-induced Hbmass adaptation within 7 days; the speed of this de-acclimatization response contrasts with previous studies, in which Hbmass has been reported to remain elevated above baseline for multiple weeks following descent to SL (Böning et al. 1997, Gore et al. 2013, Wachsmuth et al. 2013a, Wachsmuth et al. 2013b). Although a rapid loss in RCV following high altitude descent has been previously reported by Rice et al. (2001), there are several aspects of our study that make our findings unique. We studied lowlanders following just 16 days of high altitude acclimatization whereas Rice et al. (2001) studied polycythemic high altitude natives. This was reflected by dramatic differences in the degree of polycythemia obtained at high altitude (mean [Hb] of 23.4 g dL⁻¹ in the high altitude natives versus 15.5 g dL⁻¹ at ALT16 in our subjects). Indeed, the majority of subjects in the study of Rice et al. (2001) met the criteria for excessive erythrocytosis ([Hb] ≥ 21 in men or ≥ 19 in women; León-Velarde et al. 2005) whereas none of our subjects came within 2 g dL⁻¹ of this criterion at ALT16. Therefore, our results show that the development of excessive polycythemia is not required for high altitude descent to induce a rapid loss in Hbmass.

Based on the kinetics of red blood cell turnover ($\sim 0.83\%$ of circulating cells destroyed per day; Finch et al. 1977) and the delayed influence of changes in EPO on red blood cell production (Jelkmann et al. 2011, Rice et al. 2001), the large reduction in Hbmass we observed within 7 days is unlikely to be explained by a reduction in red blood cell production. The correlation between the loss in Hbmass and increase in serum ferritin from ALT16 to POST7 suggests an increase in red blood cell destruction, as the iron contained in destroyed red blood cells is transferred to iron stores (Rice et al. 2001). It is possible that neocytolysis, the selective destruction of a population of young red blood cells (Alfrey et al. 1996a, Alfrey et al. 1997, Rice and Alfrey 2005), may have been the mechanism of this rapid loss in Hbmass. However, the strength of the evidence for neocytolysis has recently been questioned (Risso et al. 2014). We did not measure markers of red blood cell production or examine red blood cell age distributions during high altitude acclimatization and de-acclimatization and therefore cannot provide direct evidence in support of, or against a role for, neocytolysis.

Our finding of a rapid loss in Hbmass following descent from high altitude contrasts with patterns observed following cessation of exogenous EPO administration (Durussel et al. 2013, Lundby et al. 2008, Olsen et al. 2011), despite the fact that these studies induced similar or larger elevations in Hbmass and [Hb]/Hct compared to our observations. Although it was hypothesized that cessation of exogenous EPO therapy would induce neocytolysis and lead to a rapid reduction in Hbmass (Rice and Alfrey 2005, Rice et al. 2001), recent studies provide compelling evidence that this is not the case, with Hbmass/RCV consistently reported to remain unchanged for 2 weeks following treatment cessation before beginning to fall gradually back to baseline (Durussel et al. 2013, Lundby et al. 2008, Olsen et al. 2011). The stimulus for increased red blood cell production with high altitude acclimatization is hypobaric hypoxia, whereas EPO

treatment elevates Hbmass in the absence of systemic hypoxia. Although this suggests that the production of red blood cells under conditions of hypoxia may influence the retention of Hbmass adaptations, it is important to note that rapid reductions in Hbmass have also been reported with spaceflight (Alfey et al. 1996a) and dehydration-induced rapid weight loss (Reljic et al. 2013) and neither of these situations involve systemic hypoxia. Further work is required to clearly establish the mechanism(s) of rapid loss of Hbmass in healthy humans.

What are the implications of the rapid loss in Hbmass following descent to low altitude on acclimatization status upon return to high altitude? The reduction in oxygen carrying capacity might be expected to impair submaximal endurance performance upon return to 5260m; however, despite large reductions in absolute and relative Hbmass from ALT16 to POST7, the improvement in 3.2 km run time-trial performance from ALT1 to ALT16 with acclimatization was fully maintained at POST7 (Subudhi et al. 2014). This calls into question the importance of the altitude-induced Hbmass adaptation for submaximal endurance performance at high altitude. Previous studies examining the effects of artificial Hbmass alterations with erythrocyte infusion (Pandolf et al. 1998, Young et al. 1996), recombinant EPO treatment (Robach et al. 2008), and isovolemic hemodilution (Calbet et al. 2002) have failed to observe alterations in maximal oxygen uptake or endurance performance at altitudes greater than 4300m. Our results extend these findings by showing that the loss in Hbmass accompanying descent to low altitude does not result in impaired submaximal endurance performance upon return to high altitude. However, previous work suggests that there may be a threshold altitude above which alterations in Hbmass have minimal effects on maximal oxygen uptake (Robach et al. 2008), and we stress that our finding of maintained endurance performance despite significant loss of Hbmass may not apply to performances at less severe altitudes.

Changes in blood volume compartments during high altitude acclimatization and de-acclimatization

In support of previous high altitude studies (Reviewed in Sawka et al. 2000), we observed large reductions in absolute and relative PV at ALT7 and ALT16. However, we did not detect a significant reduction in PV in the first 9-13 hours of initial exposure to 5260m and the PV measured 9-13 hours after return to 5260m following descent to low altitude was not different from SL. Some studies have found reductions in PV within the first hours of high altitude exposure (Sawka et al. 1996, Grover et al. 1998) but other studies have failed to detect changes within this time window (Imoberdorf et al. 2001, Loeppky et al. 2004). Differences between studies are likely influenced by several factors including hydration status, exercise prior to PV assessments, acute mountain sickness, and the methodology used to assess PV. PV returned to SL values following descent to low altitude. Previous work has indicated that the recovery of PV following high altitude descent occurs within 2 days and is influenced by changes in fluid-regulating hormones including renin, aldosterone, and vasopressin (Robach et al. 2000, Robach et al. 2002), so our finding of a recovered PV following 1 and 3 weeks at low altitude is not surprising. As expected, changes in RCV paralleled the changes we observed in Hbmass. Compared to SL, relative BV was unchanged by high altitude acclimatization and de-acclimatization, at least at the time points we assessed—relative BV was influenced both by alterations in the absolute sizes of the BV compartments and small changes in body mass (Subudhi et al. 2014) during high altitude acclimatization and de-acclimatization. In our subjects, reductions in relative PV during high altitude acclimatization were offset by an augmentation of relative RCV. The opposite response occurred following descent to low altitude, with the diminution in relative RCV offset by an enlargement of relative PV.

Limitations

There are some potential limitations to the current study that should be considered. The overall experimental design lacked a separate lowlander control group that was studied over time in the absence of altitude exposure. However, Hbmass has been consistently shown to be stable over time in subjects at SL (Eastwood et al. 2008, Prommer et al. 2008) and we took several steps to ensure that the SL and high altitude measurements were comparable, as detailed above. Additionally, because examining responses to acute hypoxia on ALT1 was a key component of the overall AltitudeOmics study design, many steps were taken to minimize the subjects' exposure to hypoxia prior to ALT1 (Subudhi et al. 2014). During the travel period prior to ALT1 (including flight time), subjects spent less than 20 hours exposed to hypoxia equivalent to 2000m or greater. A recent meta-analysis of changes in Hbmass with hypoxia reported gains in Hbmass of ~1% per 100 hours spent above 2000m (Gore et al. 2013). Therefore, the effect of hypoxic exposure prior to ALT1 on the Hbmass response is estimated as less than 0.2%, dramatically lower than the increases we observed at ALT7 and ALT16.

As described previously, subjects were unable to maintain their normal physical activity habits at high altitude and some detraining may have occurred during acclimatization, with some fitness restoration during the period spent at low altitude (Subudhi et al. 2014). Eastwood et al. (2012) found a 3.1% reduction in Hbmass after 30 days of detraining (~90% reduction in training volume) in triathletes at SL, but reported unchanged Hbmass at 10 and 20 days following training reduction. A potential interaction between hypoxia and detraining on changes in Hbmass with ascent to high altitude has not been previously examined. There is a very strong cross-sectional relationship between lean body mass and Hbmass at sea level (Schumacher et al. 2008) and it could be speculated that the mean loss of ~1.5 kg lean body mass between ALT1 and

ALT16 (Subudhi et al. 2014) may have reduced the erythropoietic stimulus. However, data examining a potential interaction between changes in lean body mass and Hbmass during altitude sojourn are currently lacking. Next, although subjects with low ferritin prior to baseline testing were directed to take oral iron supplements, supplementation was not directly monitored and the efficacy of supplementation in increasing serum ferritin was not determined prior to arrival at high altitude. Some subjects arrived at high altitude with low ferritin levels and it is possible that this may have limited the increase in Hbmass. However, as noted above, several subjects had robust increases in Hbmass despite low ferritin levels upon arrival.

Finally, the potential influence of blood loss due to sampling should be considered. Blood loss due to sampling occurs in many studies but its potential influence on hematological and other physiological outcomes is often ignored. The amount of blood removed due to sampling at ALT1 is of a magnitude that may induce a small EPO response at SL (Miller et al. 1982). It is important to consider that it takes ~5 weeks to recover Hbmass lost from a 550 mL blood donation at SL (Pottgiesser et al. 2009), whereas our subjects were able to increase Hbmass above SL baseline within 7 days despite the loss of blood due to sampling. While we cannot rule out a potential interaction between blood loss due to sampling and the hypoxic stimulus on the magnitude of the erythropoietic response, it is clear that the hypoxic stimulus drives the rapid gain in Hbmass observed at 5260m.

CONCLUSIONS

We documented the early time course of Hbmass adaptations at 5260m and found rapid increases following just 7 and 16 days of high altitude acclimatization. The altitude-induced gain in Hbmass was remarkably short-lived, as descent to low altitude resulted in a dramatic loss in Hbmass within 7 days. The loss in Hbmass was correlated with an increase in serum ferritin,

suggesting an increase in red blood cell destruction. Overall, this study demonstrates the capacity for rapid alterations in Hbmass with high altitude acclimatization and de-acclimatization in healthy men and women and suggests the need to further examine mechanisms of erythropoietic adaptations to severe hypoxia.

ACKNOWLEDGEMENTS

This study was part of a series titled "AltitudeOmics" that together represent a group of studies that explored the basic mechanisms controlling human acclimatization to hypoxia and its subsequent retention. Many people and organizations invested enormous amounts of time and resources to make AltitudeOmics a success. Foremost, the study was made possible by the tireless support, generosity and tenacity of our research subjects. AltitudeOmics principal investigators were Colleen G. Julian, Andrew T. Lovering, Andrew W. Subudhi and Robert C. Roach. A complete list of other investigators on this multinational, collaborative effort involved in development, subject management and data collection, supporting industry partners, and people and organizations in Bolivia that made AltitudeOmics possible is available elsewhere (Subudhi et al. 2014). The overall AltitudeOmics study was funded, in part, by grants from the U.S. Department of Defense (W81XWH-11-2-0040 TATRC to RCR, and W81XWH-10-2-0114 to ATL); the Cardiopulmonary & Respiratory Physiology Laboratory, University of Oregon; the Altitude Research Center and the Charles S. Houston Endowed Professorship, Department of Emergency Medicine, School of Medicine, University of Colorado Denver. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

CHAPTER IV

Hemoglobin mass alterations in healthy humans following four-day head-down tilt bed rest

Ryan BJ, Goodrich JA, Schmidt WF, Stothard ER, Wright KP, Byrnes WC (2016) Exp Physiol DOI: 10.1113/EP085665.

ABSTRACT

Rapid decreases in hemoglobin mass (Hbmass) have been reported in healthy humans following spaceflight and descent from high altitude. It has been proposed that a selective increase in the destruction of young red blood cells (RBCs) mediates these decreases but conclusive evidence demonstrating neocytolysis in humans is lacking. Based on the proposed triggers and time course of adaptation during spaceflight, we hypothesized that Hbmass would be reduced following 4 days of -6° head-down tilt bed rest (HDTBR) and that this would be associated with evidence for increased RBC destruction. We assessed Hbmass in 7 healthy, recreationally active men before (PRE), 5 hours after (POST), and 5 days after (POST5) 4 days of HDTBR. [Erythropoietin] decreased from 7.1 ± 1.8 mIU mL⁻¹ at PRE to 5.2 ± 2.8 mIU mL⁻¹ at POST (mean $\pm$ SD; $p=0.028$). Contrary to our hypothesis, Hbmass was increased from 817 ± 135 g at PRE to 849 ± 141 g at POST ($p=0.014$) before decreasing below PRE to 789 ± 139 g at POST5 ($p=0.027$). From PRE to POST, [haptoglobin] increased from 0.54 ± 0.32 g L⁻¹ to 0.68 ± 0.28 g L⁻¹ ($p=0.013$) and [bilirubin] decreased from 0.50 ± 0.24 mg dL⁻¹ to 0.32 ± 0.11 mg dL⁻¹ ($p=0.054$), suggesting that decreased RBC destruction may have contributed to the increased Hbmass. However, it is possible that spleen contraction following HDTBR also played a role in the increase in Hbmass at POST but since the transient increase in Hbmass was unexpected, we did not collect data that would provide direct evidence for or against spleen contraction. From PRE to POST5, [soluble transferrin receptor] decreased from 20.7 ± 3.9 nmol L⁻¹ to 17.1 ± 3.3 nmol L⁻¹ ($p=0.018$) but [ferritin], [haptoglobin], and [bilirubin] were not significantly altered, suggesting that the

decrease in Hbmass was mediated by decreased RBC production rather than increased RBC destruction. Peak oxygen uptake decreased by $0.31 \pm 0.16 \text{ L min}^{-1}$ from PRE to POST ($p=2E^{-4}$) but was not significantly altered at POST5 compared to PRE. Overall, these findings indicate that 4-day HDTBR does not increase RBC destruction and that re-examination of the time course and mechanisms of Hbmass alterations following short-term spaceflight and simulated microgravity is warranted.

INTRODUCTION

Changes in total hemoglobin mass (Hbmass) and red cell volume (RCV) depend on the balance between red blood cell (RBC) production and destruction. It is classically thought that the rate of RBC destruction is largely invariant in healthy humans and that changes in RBC production are slow-acting, taking weeks to affect Hbmass/RCV (Finch et al. 1977, Sawka et al. 2000). However, some investigations have reported large decreases in Hbmass/RCV within 1 week following spaceflight (Alfrey et al. 1996a), descent from high altitude (Rice et al. 2001, Ryan et al. 2014), and rapid weight loss (Reljic et al. 2013, Reljic et al. 2015), suggesting an increase in RBC destruction. Alfrey et al. (1996a) estimated RBC production and survival as well as RCV in humans participating in 9- and 14- day spaceflight missions. A large decrease in RCV was found after landing and the authors interpreted a slowed rate of decrease in ^{51}Cr specific activity during the first 4 days of spaceflight as suggesting a select destruction of young RBCs occurring within the first 4 days of spaceflight (Alfrey et al. 1996a, Alfrey et al. 1996b). They termed this phenomenon neocytolysis and suggested it was mediated by the early fall in erythropoietin concentration ([EPO]) during spaceflight (Alfrey et al. 1996a, Alfrey et al. 1996b, Alfrey et al. 1997). However, this initial evidence for neocytolysis was largely indirect, as RCV was not measured during spaceflight and no biochemical markers to support increased RBC destruction within the first four days (i.e., increased [ferritin], increased [bilirubin], or decreased [haptoglobin]) were reported (Alfrey et al. 1996a).

Alfrey and colleagues predicted that neocytolysis would occur in other situations on earth where the levels of RBCs are excessive for a new environment such as descent from altitude and following cessation of exogenous EPO treatment (Alfrey et al. 1997, Rice and Alfrey 2005). In a follow-up study of high altitude natives descending to sea level, Rice et al. (2001) found a rapid

decrease in RCV and evidence for increased RBC destruction, but evidence for select destruction of young RBCs was not conclusive. In contrast to this rapid decrease in RCV, a study of moderate altitude natives descending to sea level found Hbmass to remain stable for 2 weeks, followed by a slow decline over the next 3 weeks (Prommer et al. 2010). Studies examining changes in Hbmass in lowlanders following altitude descent after 2-4 week moderate or high altitude acclimatization have provided mixed results, with some indicating rapid decreases in Hbmass/RCV within 1 week (Garvican et al. 2012, Ryan et al. 2014) but others reporting partial or full retention of altitude-induced Hbmass/RCV adaptations for at least 7 days following descent (Böning et al. 1997, Bonne et al. 2014, Gough et al. 2012a, Siebenmann et al. 2015, Wachsmuth et al. 2013a). Interestingly, studies involving exogenous EPO administration to healthy humans have consistently reported that gains in Hbmass/RCV are maintained well beyond 1 week following cessation of EPO treatment (Durussel et al. 2013, Lundby et al. 2008, Olsen et al. 2013). Thus, although it was predicted that neocytolysis would occur with cessation of EPO administration (Alfrey et al. 1997, Rice and Alfrey 2005), rapid decreases in Hbmass/RCV have not been observed following EPO withdrawal in healthy humans. In a recent review, Risso et al. (2014) highlighted the need for further work to establish whether neocytolysis is a mechanism for rapid decreases in Hbmass/RCV in humans.

In both spaceflight and -6° head-down tilt bed rest (HDTBR), a well-established analog of microgravity, there is an initial increase in blood at the level of the chest and head (central hypervolemia), a rapid decrease in plasma volume (PV), and a decrease in [EPO] (Alfrey et al. 1996a, Gunga et al. 1996, Leach et al. 1996, Nixon et al. 1979). The reduction in PV occurs within the first day of (simulated) microgravity and changes in EPO have been reported within 2 days (Alfrey et al. 1996a, Gunga et al. 1996, Leach et al. 1996). It has been proposed that these

responses trigger neocytolysis in spaceflight (Alfrey et al. 1996a, Alfrey et al. 1996b, Alfrey et al. 1997), so a loss in Hbmass/RCV might be expected to occur over a similar time frame following HDTBR. Some studies have reported decreases in Hbmass/RCV during days 7 to 10 of HDTBR (Convertino et al. 1984, Dunn et al. 1984, Millet et al. 2001) but little data are available on changes in Hbmass/RCV following short-term HDTBR. Measuring Hbmass following rather than during HDTBR enables a more consistent comparison with data collected following spaceflight—to our knowledge, there are no published measurements of Hbmass/RCV during spaceflight. Given the suggestion based on erythrokinetic data that neocytolysis occurs within the first 4 days of spaceflight (Alfrey et al. 1996a, Alfrey et al. 1996b, Alfrey et al. 1997, Rice and Alfrey 2005), we hypothesized that Hbmass would be decreased following 4 days of HDTBR and that the decrease in Hbmass would persist for at least 5 days after return to normal living conditions. Further, we hypothesized that the decrease in Hbmass would be associated with a decrease in [EPO] and evidence for increased RBC destruction (increased [ferritin] and [bilirubin] and decreased [haptoglobin]). To provide some insight into the functional consequences of 4-day HDTBR, we also examined graded exercise responses to upright bicycle ergometer exercise. We hypothesized that peak oxygen uptake would be decreased immediately following HDTBR and remain decreased for at least 5 days after return to normal living conditions.

METHODS

Ethical Approval

This study was approved by the University of Colorado Boulder Institutional Review Board and participants provided written informed consent. All procedures conformed to the standards set by the Declaration of Helsinki.

Subjects

Participants were recruited from flyers and electronic announcements posted at the University of Colorado Boulder. Inclusion criteria were as follows: male; age 18-44; recreationally active (participate in recreational activity at least once per week); BMI > 18.5 and < 30.0; permanent residence at moderate altitude for at least 8 weeks leading into the study; no history of drug or alcohol dependence. Exclusion criteria were as follows: any acute or chronic health condition that would affect the ability to complete all protocol procedures; any clinically significant unstable medical or surgical condition within the last year; history of psychiatric, cardiovascular, respiratory, immunological, renal, neurological, cognitive, circadian rhythm, sleep, endocrine, or hematological illnesses or disorders; history of treatment with antidepressants, neuroleptic medications, or tranquilizers; history of acid reflux disease; use of medications within 1 month of the study or need of medications any time during study; any musculoskeletal disorders that may impact the ability to exercise or remain in bed; history of chronic or recurring low back pain; blood donation within the 8 weeks leading into the study; descent to low altitude for more than 3 days in the 4 weeks leading up to the study; current smoker; aerobically well-trained (participating in aerobic exercise training for more than 30 minutes per day on more than 5 days per week). Select characteristics of the 8 healthy, recreationally active men participating in the investigation were age: 21 ± 3 years, height: 180 ± 11 cm, mass: 73 ± 10 kg, peak oxygen uptake: 50 ± 6 mL kg⁻¹ min⁻¹.

Study Design

The study design consisted of an initial visit, a 7-day ambulatory at-home baseline period, a 5-day in-lab period including 4 days in -6° HDTBR, and a follow-up visit 5 days after the end of HDTBR. The 3 primary time points were PRE (prior to HDTBR), POST (the day coming out

of HDTBR), and POST5 (5 days after HDTBR). During the initial visit, we measured Hbmass and instructed the subjects to maintain a consistent 8-hour sleep schedule at their habitual bedtime during the ambulatory baseline period. Subjects lived at home during this period but adherence to the sleep schedule was monitored through the use of actigraphy, sleep logs, and call-ins to a time-stamped voice recorder at bed and wake times. Subjects were instructed to maintain their normal diet over the baseline period and caffeine, alcohol, and use of medications were proscribed. Subjects were instructed to refrain from strenuous exercise for the 48 hours preceding the in-lab period.

Subjects arrived at the laboratory for the in-lab period 5 hours prior to their habitual bedtime and slept the first night in the lab with the bed in the un-tilted (horizontal) position. Resting metabolic rate was measured upon awakening and used to determine the energy content of meals provided during the HDTBR period as $1.2 \times$ resting metabolic rate (Biolo et al. 2008). Subsequently, a venous blood sample (12-14 mL) was collected from subjects in the supine position 30 minutes after awakening, following an overnight fast. After the blood sample, subjects were provided a snack (250 kcal; 45 g carbohydrates) and rested (sitting) for 2 hours prior to a graded exercise test (GXT). Two hours after the completion of the GXT, a second baseline Hbmass measurement was performed. After the Hbmass test, at ~2PM, subjects began the HDTBR period. They remained in HDTBR for the next 90 ± 1 hours (until ~8AM on day 4). Subjects were monitored continuously and had frequent interactions with the study staff. Food was provided in the form of 3 meals and a snack each day and water was consumed ad libitum. The macronutrient ratio of food was ~55% carbohydrate, ~15% protein, and ~30% fat and the sodium content was 2.6 ± 0.5 g day⁻¹. Subjects remained in the HDT position at all times except for approximately 5 minutes per day in the seated position for defecation (Williams et al. 2003).

They were provided sanitary wipes for personal hygiene and were prohibited from standing throughout the 4-day period.

Upon awakening on the final day of HDTBR, the bed was returned to the horizontal position and a venous blood sample (12-14 mL) was collected after subjects rested supine for 30 minutes. Following the blood sample, subjects were provided a snack (250 kcal; 45 g carbohydrates) and rested for 2 hours prior to the POST GXT. During this time, they were gradually transitioned from supine to seated and then from seated to standing before being seated on the bicycle. Two hours after the completion of the GXT, the POST Hbmass measurement was performed. Following this Hbmass assessment, subjects left the lab and returned home for a 5-day period. They were asked to resume their normal activities, maintain their habitual diet and sleep schedule, and avoid strenuous exercise, caffeine, and alcohol for 48 hours prior to the final visit (POST5). They arrived at the lab 15 minutes after their habitual awakening time and a venous blood sample (12-14 mL) was collected after 30 minutes in the supine position, following an overnight fast. After the blood sample, subjects were provided a snack (250 kcal; 45 g carbohydrates) and rested for 2 hours prior to the POST5 GXT. Two hours after the completion of the GXT, the POST5 Hbmass measurement was performed. In total, less than 50 mL of whole blood were collected from each subject during the entire study.

Hematology

Collected blood samples were assessed for hemoglobin concentration ([Hb]) and hematocrit (Hct). [Hb] was assessed in triplicate using an OSM3 hemoximeter (Radiometer, Denmark) and Hct was measured in triplicate using microcentrifugation, with a correction factor of 0.96 to account for trapped plasma. Mean corpuscular hemoglobin concentration (MCHC) was calculated as $[Hb] \times 100 \times Hct^{-1}$. Blood for reticulocyte analysis was collected in a K₂EDTA

tube and analyzed using a Sysmex XN-2000 Automated Hematology Analyzer (Sysmex, Japan). Venous blood for analysis of serum [ferritin], [bilirubin], [haptoglobin], [EPO], soluble transferrin receptor ([sTfr]), and C-reactive protein ([CRP]) was collected in a serum separator tube, allowed to clot for 30 minutes at room temperature, and then centrifuged for 15 minutes. [Ferritin] was assessed using an automated immunoassay analyzer (TOSOH AIA-360, Japan). Serum for [EPO], [sTfr], [haptoglobin], [CRP], and [bilirubin] analyses was frozen at -70°C until batch analysis; [EPO], [sTfr], [haptoglobin], and [CRP] were assessed using commercially-available enzyme-linked immunoassays (R&D Systems, USA). [Bilirubin] was assessed using a commercially-available photolorimetric assay (Sigma Aldrich, USA). As [haptoglobin], [EPO], and [ferritin] can be influenced by acute phase responses (Gebay and Kushner 1999, Ramadori et al. 2010, Trey and Kushner 1995), we examined changes in these parameters both with and without the inclusion of data points where $CRP > 5 \text{ mg L}^{-1}$ (Thomas and Thomas 2002).

Hbmass and BV compartments

Hbmass was assessed using the optimized carbon monoxide (CO) rebreathing method (Prommer and Schmidt 2007, Schmidt and Prommer 2005). Briefly, a bolus of 99.5% CO (1.2 mL CO kg⁻¹ body mass) was administered to subjects and rebreathed along with 3 L of 100% O₂ for 2 minutes. Arterialized capillary blood samples were taken from a pre-warmed fingertip before and 7 minutes after the rebreathing procedure and analyzed in sextuplicate using an OSM3 hemoximeter (Radiometer, Denmark). End tidal [CO] was assessed before and 2 minutes after the rebreathing procedure using a portable CO detector (Draeger Pac7000, Germany). Hbmass was assessed in duplicate prior to the HDTBR intervention; there was no significant difference between the 2 baseline tests (test 1: $830 \pm 134 \text{ g}$, test 2: $829 \pm 127 \text{ g}$; $n = 8$; $p = 0.96$) so the mean of each subject's 2 tests was used as their baseline value. Typical error for Hbmass

determined from these duplicate measurements was 2.4%. Blood volume compartments were calculated as follows, with the factor 0.91 included in the BV calculation to account for the ratio of peripheral to total body hematocrit (Chaplin et al. 1953, Schmidt et al. 2002).

$$1) \text{ Red cell volume (RCV)} = \text{Hbmass} \times \text{Hct} \times [\text{Hb}]^{-1} \times 100^{-1}$$

$$2) \text{ Blood volume (BV)} = \text{RCV} \times 100 \times \text{Hct}^{-1} \times 0.91^{-1}$$

$$3) \text{ Plasma volume (PV)} = \text{BV} - \text{RCV}$$

The portable CO detector was also used to monitor potential CO leaks coming from the subject or spirometer. A CO leak was detected during the POST5 test for 1 subject. The test was repeated after 6 hours; repeating Hbmass tests after this time interval has been shown to provide precise and valid measurements (Naef et al. 2015).

Graded Exercise Testing

The GXT protocol consisted of 10 minutes of seated rest and then submaximal and maximal exercise completed in the upright, seated position on an electromagnetically-braked bicycle ergometer (Lode Excalibur, Netherlands). Metabolic and cardiorespiratory parameters were assessed using a computerized indirect calorimetry system (ParvoMedics True One 2400, USA) interfaced with a heart rate monitor (Polar T31, Finland). The pneumotachometer was calibrated at flow rates between ~75 and 275 L min⁻¹ using a 3 L calibration syringe and gas analyzers were calibrated using a primary standard (O₂: 16.06%; CO₂: 4.139%). The submaximal phase began at a power output of 50 W and increased by 30 W every 3 minutes until subjects reached a rating of perceived exertion of ~16. After completion of the submaximal test, subjects were given 10 minutes of rest before commencing the maximal testing portion, which began 1-2 workloads below the final stage reached during the submaximal protocol. Subjects cycled at a

cadence of 80-90 rpm until they reached volitional exhaustion. Peak metabolic and cardiorespiratory parameters were determined as the highest 30-second averages.

Statistics

Statistical analyses were performed using Statistical Package for the Social Sciences (version 22, SPSS Inc., Chicago, IL, USA). Prior to analyses, data were assessed for normality, homogeneity of variance, and sphericity. We performed linear mixed model statistical analyses to examine changes in dependent variables across time. When a significant main effect of time was observed, post-hoc comparisons between time points were made using paired t-tests. For parameters with non-normal distributions in this sample (sTfr, peak heart rate, normalized peak oxygen uptake), we performed Friedman's test followed by Wilcoxon signed-rank post-hoc comparisons when appropriate. For all analyses, statistical significance was accepted when $p \leq 0.05$ and trends were noted when $0.05 < p < 0.10$. Data are presented throughout the paper as mean $\pm$ SD.

RESULTS

One subject was withdrawn from the protocol by study staff on day 2 of HDTBR due to flu-like symptoms. All other subjects completed the protocol and the data presented below represent the responses of these 7 subjects. The change in body mass across time was not significant (PRE: 71.4 ± 10.5 kg; POST: 70.6 ± 10.4 kg; POST5: 71.4 ± 10.4 kg; $p = 0.084$), but a trend for change in body mass was driven by a non-significant decrease in body mass from PRE to POST that had recovered by POST5.

Hemoglobin mass and blood volume

Hematological data are presented in Figure 4-1 and Table 4-1. Contrary to our hypothesis, there was a significant 32 ± 35 g increase in Hbmass from PRE to POST. However, from POST

to POST5, Hbmass was significantly decreased by 60 ± 33 g, falling 28 ± 16 g below PRE. [Hb] and Hct were significantly increased $\sim 11\%$ from PRE to POST and significantly decreased from POST to POST5, falling below PRE. PV and BV were significantly decreased from PRE to POST (PV: -463 ± 127 mL; BV: -365 ± 205 mL) but had recovered by POST5. Changes in RCV mirrored changes in Hbmass. No significant changes in MCHC were observed across time.

Markers of RBC production and destruction

Markers of RBC production and destruction are presented in Table 4-1 and Figure 4-2. [CRP] was elevated above 5 mg L^{-1} (maximal range of [CRP] assay) for 1 subject at POST and another subject at POST5, so we analyzed changes in [CRP], [EPO], [haptoglobin], and [ferritin] both with and without these data points included. No significant changes in [CRP] across time were observed. With all data points included, there was a trend for [EPO] to be decreased from PRE to POST ($p = 0.070$); [EPO] subsequently increased from POST to POST5, with no significant difference between PRE and POST5. With the 2 [EPO] data points from samples at which [CRP] was elevated above 5 mg L^{-1} excluded, [EPO] was significantly decreased from $7.1 \pm 1.8 \text{ mIU mL}^{-1}$ at PRE to $5.2 \pm 2.8 \text{ mIU mL}^{-1}$ at POST and there was a trend for EPO to be above PRE at POST5 ($p = 0.073$). [sTfr] was significantly increased from PRE to POST and then significantly decreased from POST to POST5, falling below PRE. With all data points included, [ferritin] was significantly increased from PRE to POST; there was no significant difference in [ferritin] from POST to POST5, and there was a trend for [ferritin] to be increased at POST5 compared to PRE ($p = 0.073$). After excluding the 2 data points from samples at which [CRP] was elevated above 5 mg L^{-1} , [ferritin] significantly increased from $63 \pm 38 \text{ ng mL}^{-1}$ at PRE to $77 \pm 48 \text{ ng mL}^{-1}$ at POST but there were no significant differences between PRE and POST5 or POST and POST5. With all data points included, the change in [haptoglobin] across time trended

towards significance ($p = 0.059$). After excluding the 2 data points from samples at which [CRP] was elevated above 5 mg L^{-1} , there was a significant increase in [haptoglobin] from $0.54 \pm 0.32 \text{ g L}^{-1}$ at PRE to $0.68 \pm 0.28 \text{ g L}^{-1}$ at POST but no significant difference in [haptoglobin] from POST to POST5 or from PRE to POST5. There was a trend for [bilirubin] to be decreased from $0.50 \pm 0.24 \text{ mg dL}^{-1}$ at PRE to $0.32 \pm 0.11 \text{ mg dL}^{-1}$ at POST ($p = 0.054$); [bilirubin] significantly increased from POST to POST5 and was not significantly different from PRE at POST5. There were no significant alterations in reticulocyte count across time. One subject's reticulocyte count data were missing at PRE due to a laboratory processing error.

Graded exercise testing

Graded exercise responses are presented in Table 4-2. Peak oxygen uptake was significantly decreased by $0.31 \pm 0.16 \text{ L min}^{-1}$ from PRE to POST, significantly increased by $0.36 \pm 0.16 \text{ L min}^{-1}$ from POST to POST5 and was not significantly different from PRE at POST5. Changes in peak normalized oxygen uptake followed the same pattern. There were no significant differences in peak heart rate, peak respiratory exchange ratio, peak power output, or peak ventilation over time.

	PRE	POST	POST5
Hbmass (g)	817 ± 135	849 ± 141*	789 ± 139†‡
[Hb] (g dL ⁻¹)	15.7 ± 0.4	17.4 ± 0.9*	15.1 ± 0.8†‡
Hct (%)	45.3 ± 1.8	50.4 ± 2.7*	43.4 ± 2.4†‡
MCHC (g dL ⁻¹)	34.7 ± 1.8	34.6 ± 1.1	34.9 ± 1.1
Plasma Volume (mL)	3369 ± 569	2905 ± 551*	3476 ± 594‡
Red Cell Volume (mL)	2356 ± 371	2455 ± 422*	2265 ± 425†‡
Blood Volume (mL)	5725 ± 921	5360 ± 938*	5741 ± 987‡
C-Reactive Protein (mg L ⁻¹)	0.4 ± 0.3	1.0 ± 1.8	1.0 ± 1.8
C-Reactive Protein (mg L ⁻¹) (>5 mg L ⁻¹ excluded)	0.4 ± 0.3	0.4 ± 0.3 ^a	0.3 ± 0.2 ^a
Ferritin (ng mL ⁻¹)	63 ± 38	76 ± 44*	74 ± 34¶
Ferritin (ng mL ⁻¹) (CRP >5 mg L ⁻¹ excluded)	63 ± 38	77 ± 48* ^a	73 ± 37 ^a
Haptoglobin (g L ⁻¹)	0.54 ± 0.32	0.73 ± 0.28	0.72 ± 0.38
Haptoglobin (g L ⁻¹) (CRP >5 mg L ⁻¹ excluded)	0.54 ± 0.32	0.68 ± 0.28*	0.66 ± 0.28 ^a
Bilirubin (mg dL ⁻¹)	0.50 ± 0.24	0.32 ± 0.11§	0.57 ± 0.23‡
Erythropoietin (mIU mL ⁻¹)	7.1 ± 1.8	5.3 ± 2.5§	8.4 ± 2.6‡
Erythropoietin (mIU mL ⁻¹) (CRP >5 mg L ⁻¹ excluded)	7.1 ± 1.8	5.2 ± 2.8* ^a	8.8 ± 2.6‡¶ ^a
Soluble Transferrin Receptor (nmol L ⁻¹)	20.7 ± 3.9	23.2 ± 6.7*	17.1 ± 3.3†‡
Reticulocyte Count (%)	1.33 ± 0.28 ^a	1.39 ± 0.36	1.39 ± 0.36

Table 4-1. Hematological parameters before, immediately after, and 5 days after 4-day head-down tilt bed rest.

Data are mean ± SD. Measurements occurred immediately before (PRE) 4 days of HDTBR, the day coming out of HDTBR (POST), and 5 days after HDTBR (POST5). For the POST time point, venous blood samples were collected 30 minutes after HDTBR whereas Hbmass was measured 5 hours after HDTBR. *POST significantly different than PRE ($p < 0.05$). †POST5 significantly different than PRE ($p < 0.05$). ‡POST5 significantly different than POST ($p < 0.05$). § $0.05 < p < 0.10$ POST versus PRE. ¶ $0.05 < p < 0.10$ POST5 versus PRE. Data are from $n = 7$ except when indicated by ^a $n = 6$. One subject had [CRP] > 5 mg L⁻¹ at POST and a separate subject had [CRP] > 5 mg L⁻¹ at POST5. HDTBR, head-down tilt bed rest. Hbmass, hemoglobin mass. [Hb], hemoglobin concentration. Hct, hematocrit. MCHC, mean corpuscular hemoglobin concentration. CRP, C-reactive protein.

	PRE	POST	POST5
Peak Oxygen Uptake (L min^{-1})	3.55 ± 0.59	$3.24 \pm 0.59^*$	$3.61 \pm 0.54^\ddagger$
Peak Oxygen Uptake ($\text{mL kg}^{-1} \text{ min}^{-1}$)	49.9 ± 5.9	$46.0 \pm 5.7^*$	$50.9 \pm 7.3^\ddagger$
Peak Power Output (W)	290 ± 55	285 ± 53	294 ± 44
Peak Respiratory Exchange Ratio	1.09 ± 0.02	1.09 ± 0.05	1.12 ± 0.03
Peak Heart Rate (bpm)	189 ± 7	188 ± 8	185 ± 8
Peak Ventilation ($\text{L min}^{-1} \text{ BTPS}$)	172 ± 32	155 ± 38	170 ± 28

Table 4-2. Graded exercise testing responses before, immediately after, and 5 days after 4-day head-down tilt bed rest.

Data are mean $\pm$ SD. Measurements occurred immediately before (PRE) 4 days of HDTBR, 3 hours after HDTBR (POST), and 5 days after HDTBR (POST5). *POST significantly different than PRE ($p < 0.05$). ‡ POST5 significantly different than POST ($p < 0.05$). HDTBR, head-down tilt bed rest.

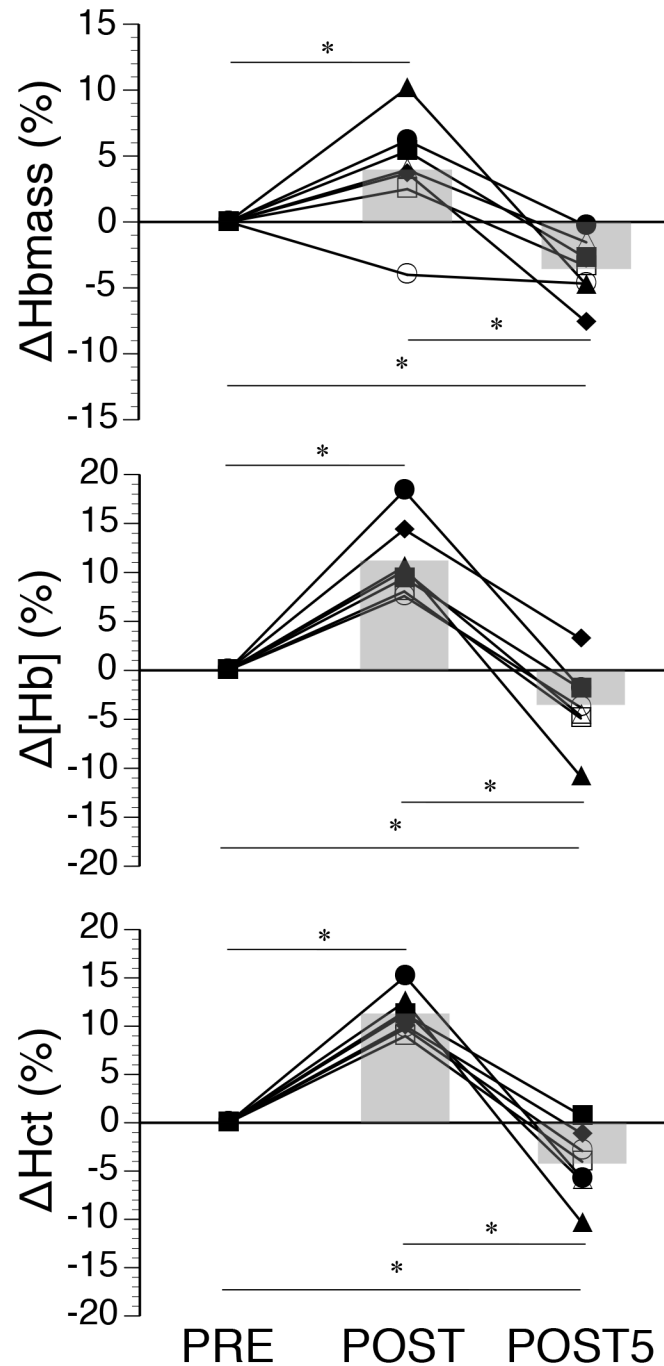


Figure 4-1. Individual changes in Hbmass, [Hb], and Hct following 4-day head-down tilt bed rest.

Data were collected before (PRE), immediately after (POST), and 5 days after (POST5) 4 days of HDTBR. For the POST time point, venous blood samples for [Hb] and Hct were collected 30 minutes after HDTBR whereas Hbmass was measured 5 hours after HDTBR. Each subject's individual data is represented by the same symbol throughout the figures and grey bars represent group means. * $p < 0.05$. HDTBR, head-down tilt bed rest. Hbmass, hemoglobin mass. [Hb], hemoglobin concentration. Hct, hematocrit.

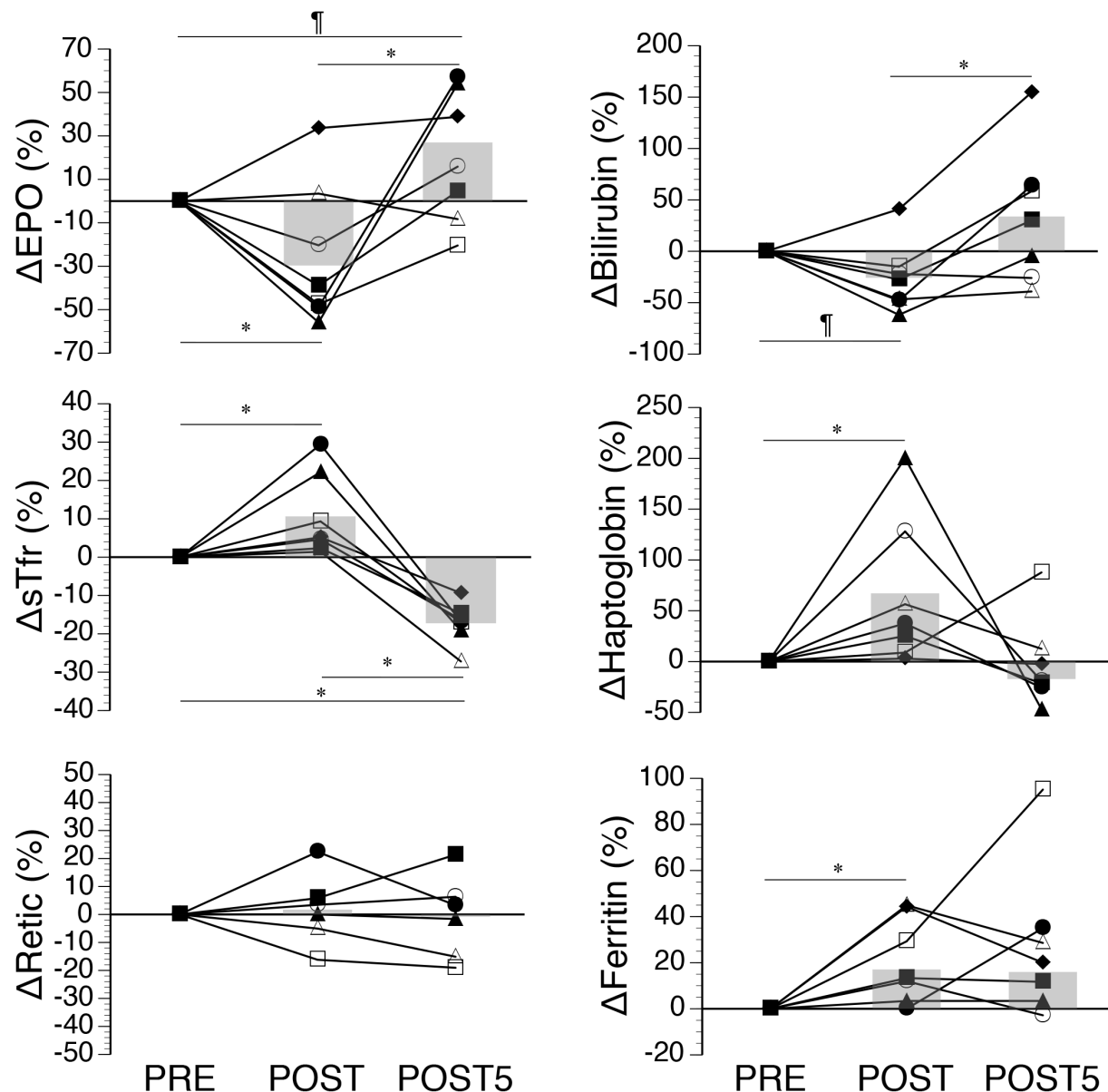


Figure 4-2. Individual changes in markers of RBC production/destruction following 4-day head-down tilt bed rest.

Data were collected before (PRE), immediately after (POST), and 5 days after (POST5) 4 days of HDTBR. Each subject's individual data is represented by the same symbol throughout the figures and grey bars represent group means. All individual data points are presented in the figures, including data points at which [C-reactive protein] was elevated above 5 mg L^{-1} . The group means and symbols of statistical significance reflect analyses of all data points for [sTfr], [bilirubin], and reticulocyte count whereas the group means and symbols of statistical significance for [EPO], [haptoglobin], and [ferritin] reflect analyses excluding data points with elevated [C-reactive protein] due to confounding influences of acute phase responses on these parameters. The data points at which [C-reactive protein] was elevated above 5 mg L^{-1} are represented by the open triangle at POST and the open square at POST5. * $p < 0.05$. ¶ $0.05 < p < 0.10$. HDTBR, head-down tilt bed rest. EPO, erythropoietin. sTfr, soluble transferrin receptor. Retic, reticulocyte count (%).

DISCUSSION

This study was conducted to examine if Hbmass would be reduced following 4-day HDTBR and if increased RBC destruction mediates this adaptation. As expected, we found a large decrease in PV, substantial increases in [Hb] and Hct, and a decrease in [EPO] immediately following HDTBR. In the model of neocytolysis, a decrease in [EPO] is hypothesized to result in increased RBC destruction and decrease in Hbmass. However, despite a decrease in [EPO] of nearly identical magnitude to that previously reported in spaceflight (Alfrey et al. 1996a), Hbmass was significantly increased the day coming out of HDTBR, and this was associated with a decrease in [bilirubin] and increase in [haptoglobin], suggesting decreased rather than increased RBC destruction. However, since Hbmass was assessed after re-ambulation and graded exercise testing, other potential factors need to be considered. Regardless of a potential confounding influence of re-ambulation and graded exercise testing, our data indicate that 4-day HDTBR does not induce an increase in RBC destruction. After a 5-day period during which subjects returned to normal living conditions, Hbmass was significantly decreased below baseline and none of the markers of RBC destruction were significantly altered compared to baseline. Together, this suggests that 4-day HDTBR resulted in a decrease in Hbmass mediated by decreased RBC production rather than increased RBC destruction.

Interpreting our findings compared to previous literature is complicated by differences in blood loss due to sampling between studies, which can dramatically influence the changes in Hbmass/RCV observed. For example, the amount of RCV removed due to sampling in between RCV measurements in the Udden et al. (1995) and Alfrey et al. (1996a) studies of 9- and 14- day spaceflights was ~6% and ~8% (Alfrey 1995), more than 50% of the magnitude of change in RCV reported in each study. Critically, the changes in RCV were not corrected for the loss of

blood due to sampling, but calculations of RBC survival were adjusted (Alfrey 1995, Alfrey et al. 1996a, Udden et al. 1995), complicating interpretation of the data for each parameter. Many previous HDTBR studies have not reported the magnitude of blood loss to sampling. In the current study, less than 50 mL of whole blood were collected, which equates to less than 8 g (< 1%) of the subjects' initial Hbmass, an amount far smaller than the changes in Hbmass we observed. The subsequent discussion addresses potential mechanisms that could explain our findings.

Hematological adaptations immediately following 4-day HDTBR

Although we hypothesized that Hbmass would be significantly decreased at POST, Hbmass was significantly increased by 4.0%. Given this unexpected finding, we must consider whether the increase in Hbmass could be the result of an analytical artifact related to our methodology or HDTBR *per se*. Several factors suggest that this finding was not the result of methodological artifact. There was no significant difference between the 2 baseline measures ($p = 0.96$) and we obtained a typical error of 2.4%, which is similar to the mean value of 2.2% reported in a meta-analysis of studies employing CO rebreathing (Gore et al. 2005) and only slightly higher than the 2.0% coefficient of variation in a more recent meta-analysis of studies using the optimized CO rebreathing method (Gore et al. 2013). Taking the mean of duplicate baseline measurements reduces the impact of random analytical variability. Our finding of no significant difference between the first and second baseline Hbmass tests was expected and demonstrates that there was no significant effect of performing exercise 2 hours prior to these measurements of Hbmass (only one of the two baseline measurements for each subject was completed 2 hours after exercise). We also must consider how adaptations related to HDTBR may affect the CO rebreathing method; speeding of CO mixing kinetics or increased

extravascular loss of CO to myoglobin could cause an artificial overestimation of Hbmass (Garvican et al. 2010a, Prommer and Schmidt 2007). Large increases in muscle blood flow reduce mixing time and could augment the loss of CO to myoglobin (Garvican et al. 2010a) but given the decreased BV following HDTBR, an increased muscle blood flow is unlikely. An increase in myoglobin content could increase extravascular CO loss but given the 4-day period of inactivity prior to the POST measurement, this adaptation is also unlikely. Therefore, we must consider mechanisms that would enable an increase in Hbmass within 4 days of HDTBR.

Normal rates of RBC production and destruction are each 0.83-1.0% per day (Finch et al. 1977, Franco 2009). Therefore, if the 4.0% increase in Hbmass within 4 days were due to increased RBC production, this would require a doubling of RBC production and release into circulation. This appears unlikely based both on the current understanding of the time required to increase RBC production (Hattangadi et al. 2011, Jelkmann 2011, Koury and Bondurant 1992) and on our finding that reticulocyte count remained stable. If there were a large upregulation of erythropoiesis, reticulocytes would be expected to increase and serum ferritin would be expected to decrease, as observed with both hypoxia and exogenous EPO-induced augmentation of RBC production (Goetze et al. 2013, Goodnough et al. 2000, Haase 2013, Merino 1950, Parisotto et al. 2000, Reynafarje et al. 1959).

Given that the increase in Hbmass does not appear to be explained by increased RBC production, the adaptation could reflect a decreased RBC destruction and/or the release of RBCs stored in the spleen. We hypothesized that we would observe evidence for increased RBC destruction—decreases in [haptoglobin] and increases in [ferritin] and [bilirubin]—immediately following HDTBR but instead we found an increase in [haptoglobin], decrease in [bilirubin], and increase in [ferritin] at POST. Haptoglobin binds free hemoglobin and changes in [haptoglobin]

are inversely related to alterations in RBC destruction, with increased haptoglobin indicating decreased RBC destruction (Barcellini and Fattizzo 2015). Bilirubin is produced via heme degradation and the vast majority of circulating bilirubin comes from RBC destruction; decreased [bilirubin] indicates decreased RBC destruction (Barcellini and Fattizzo 2015). [Ferritin], a marker of total body iron stores (Walters et al. 1973), increases with increased RBC destruction as iron contained in the destroyed RBCs is transferred to iron stores (Barcellini and Fattizzo 2015). This pattern of change in [haptoglobin], [bilirubin], and [ferritin] is entirely consistent with studies of short-term spaceflight (Leach et al. 1994). [Haptoglobin], [bilirubin], and [ferritin] can all increase in response to inflammatory or stress responses (Gebay and Kushner 1999, Trey and Kushner 1995) and are all primarily removed by the liver (Barcellini and Fattizzo 2015). If changes in our markers of RBC destruction were due to inflammatory/stress responses or alterations in splanchnic blood flow rather than alterations in hemolysis, they would be expected to change in a similar pattern. In contrast, ferritin and [haptoglobin] increased while [bilirubin] decreased. It is important to recognize that changes in concentrations of circulating parameters can be influenced by alterations in PV. In our study, when we accounted for the large decrease in PV at POST by calculating total circulating levels (concentrations multiplied by PV) of haptoglobin, bilirubin, and ferritin, the changes in haptoglobin and ferritin at POST were no longer significant and the decrease in bilirubin became even more magnified. These results provide no support for increased RBC destruction but suggest the possibility of a decrease in RBC destruction. Physical training increases RBC turnover (Schmidt et al. 1988) and it is possible that the removal of normal physical activity and mechanical stress on RBCs could contribute to decreased RBC destruction (Smith 1995).

However, we cannot provide any data from the present investigation that would identify potential mechanisms of decreased RBC destruction.

The possibility of decreased RBC destruction during short-term spaceflight has been ignored, likely due to the fact that Hbmass/RCV is consistently decreased following spaceflight. Alfrey and colleagues (Alfrey et al. 1996a, Udden et al. 1995) reported 11% and 13% decreases in RCV during 9- and 14- day spaceflight missions and proposed that the decrease in RCV occurred primarily within the first 4 days based on a slowed rate of decrease in ^{51}Cr specific activity (Alfrey et al. 1996a, Alfrey et al. 1996b). Assuming that the elution of ^{51}Cr from RBCs is not affected by microgravity (Alfrey et al. 1996a, Risso et al. 2014), a slowed rate of decrease in ^{51}Cr specific activity could be explained by 2 alternative possibilities. Alfrey et al. (1996a) proposed that a large removal of unlabeled cells (RBCs released after the labeling with ^{51}Cr which took place 12 or 21 days prior to spaceflight) caused the remaining labeled cells to become “less diluted by unlabeled neocytes” (Rice and Alfrey 2005). However, the alternative possibility that would slow the rate of decrease in ^{51}Cr specific activity is a transient decrease in RBC destruction. Given that RCV measurements were not performed during spaceflight, it is unknown when the decrease in RCV occurred and it is possible that despite a transient decrease in RBC destruction, a decrease in RBC production combined with significant removal of blood due to sampling (as highlighted in our discussion above) resulted in the decrease in RCV measured upon landing.

Admittedly, a near complete cessation of RBC destruction would be required to completely account for a 4% increase in Hbmass within 4 days. No previous studies have provided evidence for an increase in Hbmass/RCV during HDTBR of 5-16 days duration (Branch et al. 1998, Convertino et al. 1984, Convertino et al. 1986a, Convertino et al. 1986b,

Dunn et al. 1984, Leach and Johnson 1984, Linnarrsson et al. 2015, Millet et al. 2001, Williams and Convertino 1988) and we are the first to have measured Hbmass/RCV in the upright, seated position and after graded exercise testing following HDTBR. This unique aspect of our study design leaves open the possibility that the return to orthostasis and graded exercise testing prior to Hbmass assessment may have influenced our finding of a transient increase in Hbmass. As we note above, there was no significant effect of prior exercise on the assessment of Hbmass at baseline, and we did not expect any influence of prior exercise following HDTBR. However, the unexpected, transient increase in Hbmass at POST leads us to consider a possible interaction between prior HDTBR and prior exercise on the measurement of Hbmass. With initial return to orthostasis at POST, subjects are hypovolemic and it is possible that the combination of hypovolemia and/or other adaptations to HDTBR and prior exercise resulted in the spleen releasing RBCs to help augment BV. The human spleen stores up to ~10% of the total Hbmass/RCV and contracts in response to a variety of stimuli including exercise, apnea, and hypoxia (Shepard 2016, Stewart and McKenzie 2002). The spleen has been shown to contract in response to hypovolemia in a number of animal species (Barcroft et al. 1925, Greenway and Stark 1969, Opdyke and Ward 1973), though caution is warranted in extrapolating these results to humans given lower splenic RBC storage in humans. It has not been confirmed whether RBCs in the spleen are fully labeled 7 minutes following CO rebreathing; if not, release of RBCs from the spleen could result in an increase in the total circulating Hbmass. A previous study reported a ~3% increase in Hbmass assessed 1-3 hours following an ultra-endurance triathlon and the authors speculated that spleen contraction (not assessed) may have resulted in this increase in measured Hbmass (Gough et al. 2012b). If spleen contraction did influence our assessment of Hbmass at POST, this would indicate an increase in circulating Hbmass/RCV that may be an

important, previously unrecognized adaptation following return to orthostasis subsequent to simulated microgravity. Because this finding was completely unexpected, we did not perform any measures that would point to a role for, or against, spleen contraction and future studies are clearly required to support or refute this possibility.

Hematological adaptations 5 days after return to normal living conditions

We hypothesized that Hbmass would be decreased compared to PRE at the POST5 time point and indeed, Hbmass was decreased by 3.6%. [sTfr], a sensitive marker of erythropoietic activity (Beguin 2003), was reduced by 17% below baseline, suggesting a diminution of erythropoiesis resulting from 4-day HDTBR. However, reticulocyte count remained stable—it is possible that we lacked the temporal resolution to find significant decreases in reticulocyte count. [Bilirubin] increased from POST to POST5 but neither [haptoglobin] nor [ferritin] were significantly changed across this time period and none of these parameters were significantly different from PRE to POST5, suggesting that the decrease in Hbmass was mediated by decreased RBC production rather than increased RBC destruction. The significant decreases in Hbmass, [Hb], and Hct at POST5 all suggest that 4-day HDTBR induces decreases in these parameters that are not recovered 5 days after return to normal living conditions. Future studies with greater temporal resolution are required to provide insight into the time course of these changes after simulated microgravity exposure.

Graded exercise responses immediately after and 5 days after HDTBR

As expected, peak oxygen uptake was decreased immediately following HDTBR. Peak oxygen uptake is highly related to Hbmass/RCV and also to total BV; alterations in Hbmass/RCV affect oxygen carrying capacity, whereas alterations in BV can affect cardiac output (Schmidt and Prommer 2010). Hypovolemia and consequent reductions in stroke volume

have been identified as primary contributors to the decrease in peak oxygen uptake following short-term (simulated) microgravity (Convertino 1997, Levine et al. 1996) and the concordant pattern of change in BV and peak oxygen uptake in our study support an important role for BV reduction in mediating the decline in peak oxygen uptake. We hypothesized that peak oxygen uptake would also be decreased at POST5 due to a decrease in Hbmass but despite the ~3.6% decrease in Hbmass, peak oxygen uptake was not significantly different than PRE. Levine et al. (1996) reported that peak oxygen uptake was not significantly different from baseline values within 1 week after 9- and 14- day spaceflight, despite ~12% reductions in RCV reported in these subjects (Alfrey et al. 1996) that would not be expected to be recovered within 1 week. Although both our study (n = 7) and the study of Levine et al. (1996) (n = 6) were likely underpowered to detect small changes in peak oxygen uptake, changes in Hbmass/RCV do not appear to be major causal factors in the decline in aerobic performance following short-term HDTBR or spaceflight.

Limitations

There are some limitations to the current experimental design that should be discussed. First, our sample size of 7 subjects is relatively small. Future studies are needed to investigate changes in Hbmass and markers of RBC production/destruction following (simulated) microgravity using larger sample sizes. Next, we used a sensitive within-subjects design to measure changes from baseline, but we did not incorporate an independent control group. Importantly, Hbmass has been shown to remain stable across time in healthy human subjects in the absence of perturbations such as altitude exposure or EPO administration (Eastwood et al. 2008, Prommer et al. 2008). We are unable to separate the role of inactivity versus the HDT position on the adaptations observed. However, our goal was to determine if short-term HDTBR,

a well-established analog of microgravity, can be employed as a model for studying rapid decreases in Hbmass, and distinguishing inactivity versus HDT was not one of our aims. Next, this study included only men and future investigations are required to examine Hbmass alterations following short-term HDBTR in women. Finally, as discussed above, the transient increase in Hbmass at POST was unexpected and thus needs to be replicated. Further investigations using greater sample sizes, more comprehensive and frequent measures of RBC production/destruction, and markers of RBC survival are needed to improve our understanding of the control of Hbmass/RCV following simulated microgravity and spaceflight. In planning such studies, careful consideration of the amount of blood removed due to sampling is vital, as this can affect interpretation of changes in erythropoietic parameters. Additionally, the influence of the spleen on hematological adaptations following spaceflight and simulated microgravity warrants further inquiry.

Summary

In this study, we sought to determine if Hbmass is reduced following 4-day HDTBR and if increased RBC destruction mediates this adaptation. Our findings indicate that 4-day HDTBR does not increase RBC destruction and therefore is not a model for studying neocytolysis in humans. Our unexpected finding of a transient increase in Hbmass immediately following HDTBR raises some important questions about the mechanism(s) of this adaptation that require further investigation. Decreased RBC destruction may have contributed to the increased Hbmass but it is also possible that spleen contraction following HDTBR influenced circulating Hbmass. Since we did not collect data that would provide direct evidence for or against spleen contraction, future studies are needed to support or refute this possibility. Our data suggest that the decrease in Hbmass at POST5 resulted from decreased RBC production rather than increased RBC

destruction. Overall, our study indicates that re-examination of the time course and mechanisms of Hbmass alterations with short-term spaceflight and simulated microgravity is warranted.

FUNDING

This research was supported by the NASA Space Physiology Research Grants from the American College of Sports Medicine Foundation. Preliminary subject screening used REDCap, which is supported by NIH/NCRR Colorado CTSI Grant Number UL1 TR001082.

ACKNOWLEDGEMENTS

We thank the study volunteers for their participation. Additionally, we thank Madeline Jones, Connor Wall, Sewan Kim, Pablo Callejas, Rachel Gioscia-Ryan, Sean Fitzsimmons, Oliver Knauer, Andres Barandiaran, Enkh-tsogt Sainbayar, Steven Sares, Ruth Sanchez, Molly Guerin, Emily Marbas, Erin Radtke, Sophia Jaeckel, and Gayle Wright for study assistance.

CHAPTER V

CONCLUSIONS

As highlighted in this dissertation, the vast majority of previous research examining hemoglobin changes in response to environmental perturbations has focused on [Hb], which is influenced both by Hbmass and plasma volume. The purpose of this dissertation was to use the optimized CO rebreathing method to examine rapid alterations in Hbmass with early high altitude acclimatization/de-acclimatization and following short-term HDTBR.

Before evaluating the early time course of Hbmass alterations with ascent to/descent from high altitude and following simulated microgravity, it was important to confirm that performing CO rebreathing repeatedly does not influence Hbmass or exercise responses. We examined the influence of 10 days of intermittent, low-dose CO inhalation on Hbmass, aerobic performance predictors, and peak-power exercise tolerance and found that CO inhalation did not significantly alter any of these parameters compared to placebo.

After determining that repeated assessment of Hbmass using CO rebreathing does not influence Hbmass or exercise responses, we examined the early time course of Hbmass alterations with ascent to/descent from high altitude. Classical dogma in altitude physiology suggests that it takes 3-4 weeks for increases in Hbmass to occur. We studied lowlanders at sea level and at 5260m during the first 16 days of high altitude exposure and found large increases in Hbmass at days 7 and 16 of acclimatization. The speed and magnitude of this adaptation are greater than previously reported and these data demonstrate the capacity for humans to swiftly increase erythropoiesis in response to severe hypoxia. The altitude-induced Hbmass adaptation was lost within 7 days following descent to low altitude and our finding that the decrease in

Hbmass was significantly correlated with the increase in ferritin suggests that increased RBC destruction mediated this rapid adaptation.

Our finding of a rapid loss in Hbmass following altitude descent led us to employ an experimental model to investigate neocytolysis, a potential mechanism of increased RBC destruction proposed to enable a rapid decrease in Hbmass with altitude descent and during spaceflight. The theory of neocytolysis was developed based on data collected in astronauts and based on the proposed triggers and time course of adaptation during spaceflight, we hypothesized that four-day HDTBR, a microgravity analog, would induce a rapid decrease in Hbmass mediated by increased RBC destruction. However, Hbmass was transiently increased 5 hours following HDTBR before decreasing below baseline 5 days later. These novel findings indicate complex hematological adjustments following short-term simulated microgravity and future research is warranted to conclusively determine the mechanisms and functional implications of these adaptations. A decrease in [bilirubin] and increase in [haptoglobin] immediately following HDTBR suggest the possibility that decreased RBC destruction contributed to the increase in Hbmass. However, it is possible that spleen contraction following HDTBR also contributed to this adaptation. Five days after return to normal living conditions, [Stfr], a marker of RBC production, was decreased but none of our markers of RBC destruction were significantly altered at this time point compared to baseline. Thus, the decrease in Hbmass following 4-day HDTBR appears to be mediated by decreased RBC production, not increased RBC destruction.

Future Directions

The findings in this dissertation suggest several future research directions. Our findings that 10 days of low-dose CO inhalation did not significantly alter Hbmass, aerobic performance predictors, or peak-power exercise tolerance are critical for research and applied physiologists

employing CO rebreathing to assess Hbmass, but do not preclude the possibility that alternative CO exposure paradigms may induce beneficial adaptations in healthy humans. The understanding of the potential beneficial/therapeutic effects of low-dose CO exposure remains in its infancy, and future studies investigating potential physiological adaptations resulting from alternative CO exposure paradigms are warranted. One intriguing area for investigation, with studies currently underway, is to examine effects resulting from chronic elevation of HbCO% (i.e., by administering CO multiple times per day) over a longer number of days (i.e., 3-4 weeks). This exposure paradigm has the potential to augment erythropoiesis, which may have important practical and clinical consequences.

Our findings regarding hematological adaptations with high altitude ascent/descent raise several interesting questions for future research. Given that the rate of increase in Hbmass in our study exceeds that observed with pharmacological EPO administration, future studies should examine potential mechanisms that may, in addition to EPO upregulation, enable this rapid adaptation in response to severe hypoxia. Additionally, our findings suggest the need to further examine the role of iron bioavailability versus iron stores on hematological adaptations to hypoxia. There has been explosion of research into interactions between hypoxia, iron homeostasis, and erythropoiesis, and future altitude investigations incorporating measures of Hbmass coupled with robust measures of iron homeostasis are warranted. Finally, studies examining the mechanisms of loss in Hbmass following descent from moderate and high altitudes are warranted. While our findings strongly indicate a role for increased RBC destruction, the mechanism of increased RBC destruction remains unresolved.

Future studies are warranted to determine the mechanism of our finding of a transient increase in Hbmass 5 hours following short-term HDBTR. Our findings suggest a decrease in

RBC destruction, and future studies should attempt to replicate this finding while assessing more comprehensive measures of RBC production and destruction and RBC survival. Concomitantly, a potential role for spleen contraction following HDTBR warrants further inquiry. If spleen contraction does play a role in the transient increase in Hbmass, this may represent an important physiological response following (simulated) microgravity. We designed our HDTBR investigation to determine if 4-day HDTBR could be a model for studying neocytolysis in humans. The proposed trigger of neocytolysis, a decrease in [EPO], was recapitulated by HDTBR yet our findings provide no support for neocytolysis. Therefore, future studies are needed to determine what role, if any, neocytolysis plays in enabling rapid reductions in Hbmass in humans.

CHAPTER VI

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