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Hemoglobin mass and intravascular volume kinetics during and after exposure to 3,454-m altitude

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Siebenmann C, Cathomen A, Hug M, Keiser S, Lundby AK, Hilty MP, Goetze JP, Rasmussen P, Lundby C. Hemoglobin mass and intravascular volume kinetics during and after exposure to 3,454 m altitude. J Appl Physiol 119: 1194–1201, 2015. First published March 6, 2015; doi:10.1152/japplphysiol.01121.2014. —High altitude (HA) exposure facilitates a rapid contraction of plasma volume (PV) and a slower occurring expansion of hemoglobin mass (Hbmass). The kinetics of the Hbmass expansion has never been examined by multiple repeated measurements, and this was our primary study aim. The second aim was to investigate the mechanisms mediating the PV contraction. Nine healthy, normally trained sea-level (SL) residents (8 males, 1 female) sojourned for 28 days at 3,454 m. Hbmass was measured and PV was estimated by carbon monoxide rebreathing at SL, on every 4th day at HA, and 1 and 2 wk upon return to SL. Four repeated measurements, and this was our primary study aim. The second aim was to investigate the mechanisms mediating the PV contraction. Nine healthy, normally trained sea-level (SL) residents (8 males, 1 female) sojourned for 28 days at 3,454 m. Hbmass was measured and PV was estimated by carbon monoxide rebreathing at SL, on every 4th day at HA, and 1 and 2 wk upon return to SL. Four weeks of delay and reached a maximal rate of 4.04 ± 1.02 g/day after 14.9 ± 5.2 days. The probability for Hbmass to plateau increased steeply after 20–24 days. Upon return to SL, Hbmass decayed by −0.246 ± 2.3 g/day, reaching values similar to baseline after 2 wk. PV, aldosterone concentration, and renin activity were reduced at HA (P < 0.001) while the total circulating protein mass remained unaffected. In summary, the Hbmass responses to HA exposure followed a sigmoidal pattern with a delayed onset and a plateau at c. 3 wk. The decay rate of Hbmass upon descent to SL did not indicate major changes in the rate of erythropoiesis. Moreover, our data support that PV contraction at HA is regulated by the renin-angiotensin-aldosterone axis and not by changes in oncopotic pressure.

blood; erythropoietin; hypoxia; oxygen; plasma volume

THE GREATEST CHALLENGE THAT humans face at high altitude (HA) is a reduction in arterial oxyhemoglobin saturation (SaO2). At the onset of HA exposure arterial O2 content (CaO2) decreases in parallel with SaO2 and an increased cardiac output is required for the preservation of systemic O2 delivery (9). However, as exposure extends a progressive hemoconcentration reverts CaO2 to normal or higher levels (37). Initially, the hemoconcentration is the consequence of a decline in plasma volume (PV) that develops over the first hours/days (47). The magnitude hereof is related to the severity of altitude with

<0.3 l of PV loss expected at 2,000 m and 0.6–0.9 l at 3,500 m (2). With acclimatization, an expansion of total hemoglobin mass (Hbmass) progressively contributes to the hemoconcentration. In previous HA studies the rate of this Hbmass expansion was on average ~17 g/wk but this constitutes only a rough estimate since the rate and final increment depend on the severity of altitude (39). Interestingly, the rate tended to be higher in studies where HA exposure was longer, suggesting that Hbmass expansion follows a sigmoidal rather than linear pattern. The frequency of measurements in previous HA studies has, however, been too low to assess this.

The main objective with the present study was to generate a comprehensive account of the kinetics underlying the HA-induced expansion of Hbmass. Nine sea-level (SL) residents were exposed to 3,454-m altitude for 4 wk, which is expected to induce a robust increase in Hbmass (39). Hbmass was determined on every 4th day by a modified carbon monoxide (CO) rebreathing method. Further CO rebreathings were conducted 1 and 2 wk upon return to SL to establish the decay rate of Hbmass.

An additional aim was to elaborate on the mechanisms underlying the reduction in PV. This is widely attributed to augmented diuretic fluid loss (22), although reduced water intake may play a role, particularly in settings where potable water is not readily available (20). In support, suppression of the renin-angiotensin-aldosterone axis has repeatedly been observed at HA (38, 52, 62). Furthermore, the synthesis of atrial natriuretic peptide (ANP) is accelerated in acute hypoxia (26, 57), although this seems to revert to or even below normal levels as exposure extends (1, 38). Finally, hypoxic stimulation of peripheral chemoreceptors may reduce reabsorption of sodium in the kidneys through neuronal pathways and thus reinforce diuresis (20, 53). On the other hand, it has been proposed that PV contraction relates to oncotically driven fluid shifts from the intravascular compartment secondary to transvascular leakage of plasma proteins (48). An increased capillary permeability for albumin has indeed been reported at HA (18), although not supported by all (28). To determine the role of different mechanisms we monitored plasma renin activity, aldosterone, and ANP concentrations, as well as total circulating protein mass (TCP) throughout the HA sojourn.

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Subjects first breathed room air for 10 min. For the next 4 min, they breathed pure O2 from a Douglas bag to eliminate N2 from the airways. They were then switched by a sliding valve to a rebreathing circuit that had previously been flushed with pure O2. The circuit included a partially O2 filled 3-liter rebreathing bag as counterlung and a soda lime container for CO2 absorption. Consumed O2 was replaced through a one-way valve connected to an O2 cylinder. After subject comfort was confirmed, 1.5 ml of venous blood was collected and analyzed in quadruplicate for the fraction of carboxyhemoglobin (%HbCO) and [Hb] (ABL800; Radiometer, Copenhagen, Denmark). Hematocrit was measured by the micro-method (4 min at 13,500 rpm), also in quadruplicates. A dose of 99.997% chemically pure CO was then filled into a custom-made syringe with a mechanically limited filling volume. Three different volumes were available (80, 110, and 130 ml), and the one that was closest to 1.5 ml/kg body weight was used. The resulting CO dose at SL was 1.43 ± 0.09 ml/kg. At HA the syringe volumes were increased to 110, 160, and 180 ml to account for the reduced barometric pressure, resulting in a CO dose of 2.06 ± 0.14 ml/kg. The CO was injected as a single bolus into the rebreathing circuit. After 10 min of rebreathing, a second 1.5-ml blood sample was obtained and analyzed. Subjects then performed a complete exhalation where after the rebreathing circuit was closed and the subjects were disconnected. The volume of gas remaining in the rebreathing bag was quantified by extraction with a 3-liter calibration pump (Cosmed, Rome, Italy) and the CO content was determined (Monoxor III; Bacharach, New Kensington, PA). Together with the previously measured dead space of the rebreathing circuit (980 ml) and the subjects’ predicted residual volume (49) this allowed the calculation of the number of CO molecules that had not been absorbed. This was subtracted from the number of administered CO molecules to calculate the number of absorbed CO molecules (%HbCO) and [Hb] (ABL800; Radiometer, Copenhagen, Denmark). %HbCO is the change in %HbCO between the first and second blood sample (5). Red blood cell volume (RCV = Hbmass × hematocrit/[Hb]), blood volume (BV = RCV × 100/hematocrit), and PV (= BV − RCV) were derived. All tests were performed by the same operators. The typical measurement error for Hbmass was calculated from the duplicate measurements at SL as standard deviation of difference scores/√2.

To evaluate the distribution of CO over the intravascular space we inserted an additional catheter into a suitable vein at the dorsum of the foot during one of the measurements at HA (n = 8) and assessed the difference in %HbCO between blood collected from the foot and the antecubital vein after the CO rebreathing.

Venous blood analyses. Plasma protein concentrations were determined with a Pierce BCA (bicinchoninic acid) Protein Assay Kit (PierceTM Reagens No. 23225; Thermo Fisher Scientific, Rockford, IL) and osmolality by freezing point determination (Roebling Osmometer, Soulit, Switzerland) in serum obtained from venous blood. TPC was calculated as the product of plasma protein concentration measured on HA3, HA9, and HA28 and PV measured on HA4, HA8, and HA28. The erythropoietin (EPO) concentration in plasma was determined by means of an ELISA kit (Human Erythropoetin Quantikine IVD ELISA Kit; R&D Systems, Minneapolis, MN). Reticulocyte concentration was determined in venous blood collected in EDTA tubes (Sysmex XT-2100i; Sysmex Europe, Norderstedt, Germany). Plasma aldosterone concentration was assessed according to the manufacturer also by means of an ELISA kit (Aldosterone ELISA Kit-Monoclonal Item No. 10004377; Cayman Chemicals, Ann Arbor, MI) and renin activity by a fluorometric assay kit (BioVision, Milpitas, CA). ProANP was measured with a commercial mid-regional assay (MR-proANP) on a Kryptor Plus platform (Thermo-Fisher); this assay performance was previously compared with a processing-independent method (21). We chose to measure MR-proANP, as the bioactive ANP is labile in samples and may thus be affected by the sampling procedure used in the present study (16). All parameters were determined in duplicate by researchers who were blinded to-
wards the intervention, and the results represent the average of these duplicates.

Implications for anti-doping testing. To assess the effects of hematological changes in an anti-doping context, the venous reticulocyte concentrations and [Hb] determined at SL and HA were entered into the hematological module of the Athlete Biological Passport (ABP) Software.

Statistics. The overall increase and decay rates of Hbmass were determined by mixed model linear regression analysis with subject as random factor. The maximal rate of the Hbmass increase was derived determined by mixed model linear regression analysis with subject as random factor. The maximal rate of the Hbmass increase was derived from the steepest slope obtained over a rolling 12-day window, i.e., three subsequent data points. Survival analyses were applied to assess the probabilities for Hbmass to start increasing and to reach a plateau after a given time at HA. The onset was defined as an increase larger than the typical measurement error for Hbmass (1.49%, see RESULTS). A plateau was considered reached on a given measurement day when the measurements from the remaining days at HA averaged lower than the measurement on that day.

All other variables were compared with baseline by repeated-measures ANOVA. Least square means differences with Dunnett’s correction were used for the post hoc analysis.

Results in the text and tables represent means ± SD unless stated otherwise. In the figures data are presented as means ± SE for better clarity unless stated otherwise. A P value < 0.05 was considered statistically significant.

RESULTS

Evaluation of the CO rebreathing protocol. The typical measurement error for Hbmass was 1.49%. The volume of CO that remained unabsorbed after the rebreathing period ranged from 0.8 to 5.06% of the applied dose (median 1.66%). In venous blood collected from the dorsum of the foot after the CO rebreathing %HbCO was 8.53 ± 1.62% while it was 9.23 ± 1.48% in the antecubital vein (P = 0.023).

Body weight. Body weight measured before CO rebreathing was at no time point significantly different from the 75.1 ± 11.3 kg determined at SL. On HA4 and HA8, body weight was 74.6 ± 11.1 kg (P = 0.51 vs. SL) and 74.4 ± 11.0 (P = 0.29), respectively, whereas from HA12 to HA28 it remained between 74.8 and 75.0 kg (P > 0.9). On RSL7 and RSL14 body weight was 75.8 ± 9.8 kg (P = 0.45) and 76.0 ± 10.1 kg (P = 0.25), respectively.

Erythropoietic response. Plasma EPO concentration was increased by 146 ± 64% on HA3 and remained elevated on HA9 (91.6 ± 53.5%) and HA28 (33.5 ± 55.5%, P < 0.001 for all, Table 2). Upon descent to SL it returned to baseline levels within 1 day (P = 0.98) and remained unchanged on RSL7 and RSL14 (P > 0.99). The reticulocyte concentration in plasma was increased in each measurement obtained at HA (P < 0.001, Table 2). On RSL1 it still tended to be elevated (P = 0.12), whereas it was similar to baseline levels on RSL7 (P = 0.99) and RSL14 (P = 0.86).

Figure 1 illustrates the mean absolute (Fig. 1A) and individual relative (Fig. 1B) changes in Hbmass. Throughout the HA sojourn Hbmass increased at an average rate of 1.82 ± 0.81 g/day (P < 0.001) and was elevated by 42.3 ± 21.7 g (5.26 ± 0.98%) on HA28 (P < 0.001). The individual final increases ranged from 2.5 to 11.1% and were not correlated to individual Hbmass at SL (r = -0.09, P = 0.82), the individual increases in EPO on HA3 (r = -0.088, P = 0.8), or the individual EPO responses expressed as average over all measurements at HA (r = 0.55, P = 0.13). Upon return to SL Hbmass decayed at a rate of -2.46 ± 2.31 g/day and was similar to baseline on RSL14 (P = 0.9). Figure 1C illustrates %HbCO measured during the CO rebreathings before the inhalation of CO, which served as a marker for the rate of erythrolysis (11). %HbCO remained similar to SL throughout the study except on RSL7 where it was increased by 45.4 ± 37.0% (P < 0.001).

Figure 1A suggests that the increase in Hbmass at HA followed a sigmoidal pattern and this is supported by the results of the survival analyses (Fig. 2). The onset of the increase (Fig. 2A) varied between individuals but required >4 days in the majority of subjects and up to 12 days in one subject (although this particular subject only narrowly missed the 1.49% cut-off on HA8). Furthermore, there was a marked increase in the probability for Hbmass to reach a plateau after 20–24 days compared with HA12 or HA16 (P < 0.05, Fig. 2B). Finally, the highest rate of Hbmass increase, which occurred after 14.9 ± 5.2 days, was 4.04 ± 1.02 g/day and thus considerably larger than the overall rate.

Figure 1A furthermore suggests that the decay of Hbmass upon return to SL also followed a sigmoidal pattern. The measurement frequency was, however, too low to confirm this statistically.

Intravascular volumes, hematocrit, and [Hb]. On HA4 PV (Fig. 3A) was decreased by 371 ± 234 ml (10.8 ± 6.4%, P < 0.001) and it remained reduced throughout the entire HA sojourn (P < 0.001). On RSL7 PV still tended to be reduced (P = 0.18), whereas it had returned to baseline levels on RSL14 (P = 0.97). As a result, and despite an expansion of RCV, BV was reduced at HA, although this did not quite reach statistical significance on HA20 (P = 0.07). On RSL7 BV was restored to baseline levels (P = 0.91).

Hematocrit and [Hb] were increased (P < 0.001) throughout HA exposure (Fig. 3B). On RSL7 hematocrit still tended (P = 0.07) to be and [Hb] was still elevated (P = 0.02), whereas both were normalized to baseline values on RSL14 (P = 1.0 for hematocrit and P = 0.87 for [Hb]).

Arterial oxygenation. SaO2 was 97.2 ± 1.9% at SL, 88.5 ± 5.3% after arrival at HA, i.e., in acute hypoxia (P < 0.001 vs. SL), 89.5 ± 2.6% on HA3 (P < 0.001), and 93.1 ± 1.4 on HA22 (P = 0.016). As a result, CaO2 decreased from 190 ± 11 ml/l at SL to 172 ± 8 ml/l in acute hypoxia (P < 0.001). On HA3 CaO2 was restored to 187 ± 17 ml/l (P = 0.6 vs. SL), which was related to ~85% to the reduction in PV and to ~15% to the slight increase in SaO2 compared with acute hypoxia. On HA22 CaO2 was increased to 203 ± 12 ml/l (P = 0.004) and the increase compared with acute hypoxia was.

Table 2. Concentrations of EPO and reticulocytes in plasma

<table>
<thead>
<tr>
<th></th>
<th>SL</th>
<th>HA3</th>
<th>HA9</th>
<th>HA28</th>
<th>RSL1</th>
<th>RSL7</th>
<th>RSL14</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPO, U/l</td>
<td>13.8 ± 5.2</td>
<td>31.7 ± 7.1*</td>
<td>24.5 ± 5.1*</td>
<td>18.5 ± 4.1*</td>
<td>14.6 ± 4.9</td>
<td>14.3 ± 4.0</td>
<td>13.9 ± 5.4</td>
</tr>
<tr>
<td>Reticulocytes, ×10^9/l</td>
<td>33.9 ± 21.9</td>
<td>62.2 ± 17.7*</td>
<td>73.9 ± 18.4*</td>
<td>60.7 ± 14.0*</td>
<td>46.5 ± 8.5</td>
<td>31.1 ± 10.1</td>
<td>28.7 ± 7.4</td>
</tr>
</tbody>
</table>

Values are means ± SD. SL, sea level; HA, days at 3,454 m altitude; RSL, days after descent to SL; EPO, erythropoietin. *P < 0.05 vs. SL.
related to ~30% to the elevation in $S_{aO_2}$, to ~55% to the reduction in PV, and to ~15% to the increase in Hbmass.

Determinants of PV. TCP (Fig. 4A) was unchanged ($P > 0.99$) on HA3 and HA9 but tended to be reduced on HA28 ($P = 0.07$), whereas plasma protein concentration (Fig. 4A) was increased on HA3 ($P = 0.007$) and HA9 ($P = 0.001$) but not on HA28 ($P = 0.22$) or on any day after descent ($P > 0.8$ for each).

Plasma osmolality (Fig. 4B) remained similar to the baseline value both at HA ($P > 0.7$ for each) and upon return to SL ($P > 0.7$ on RSL1 and RSL14, $P = 0.16$ on RSL7).

Renin activity and aldosterone concentration (Fig. 4C) were reduced ($P < 0.001$) on each measurement time point at HA but returned to baseline levels within 1 day after descent ($P > 0.95$).

Plasma proANP concentration tended to be reduced on HA3 ($P = 0.09$) and was thereafter reduced on all HA time points (Fig. 4D). On RSL1 proANP was still reduced ($P = 0.014$) but returned to levels similar to baseline on RSL7 ($P = 0.44$).

Athlete Biological Passport. Out of 63 plasma samples, 5 exceeded the 99% specificity for [Hb] (and 2 out of 9 sequential analyses), 6 exceeded the 99% specificity for the “Off-score” (2 of 9 sequential analyses), and 1 the 99% specificity for the reticulocyte concentration (2 of 9 sequential analyses). The number of values exceeding the 99% specificity ranged from 0 in 4 of the participants to 8 in 1 participant.

DISCUSSION

Four weeks at 3,454 m increased Hbmass of lowlanders by $5.26 \pm 0.98\%$. The individual increases were initiated after a delay of up to 12 days whereas after 20–24 days Hb mass plateaued in the majority of subjects. The fastest expansion rate was $4.04 \pm 1.02$ g/day and occurred after ~15 days. Upon
return to SL Hb_mass decayed by $-2.46 \pm 2.31$ g/day. The HA-induced loss in PV occurred simultaneous with a decreased plasma renin activity and aldosterone concentration but not with a reduction in TCP.

The overall rate of Hb_mass expansion was $\sim 25\%$ lower than the average rate of previous HA studies (39), which is not surprising since these studies have included a wide variety of HA exposure protocols. The final magnitude of the expansion is in line with the prediction that a 5% increase in Hb_mass occurs after 17–41 days at 3,500 m (39). This broad time span may reflect the large interindividual variability that we observed. As the erythropoietic response to injected EPO is dose dependent (30), this interindividual variability may relate to the even larger interindividual variability in the EPO response to HA (15). However, we observed no correlation between the individual EPO and Hb_mass responses, probably because circulating EPO represents the balance between EPO synthesis and degradation and thus is only an indirect marker of the erythropoietic stimulus. Variations in EPO sensitivity may also affect the individual Hb_mass response to HA (43), but its determinants are poorly explored. EPO sensitivity may be decreased by iron insufficiency but this was probably not prevalent in our subject group which contained only one female, did not perform excessive exercise, and maintained a normal diet. A high initial Hb_mass may also blunt the erythropoietic response to HA (39, 45). The absence of a correlation between baseline Hb_mass and the increase at HA in the present study was likely related to the narrow range of baseline Hb_mass.

Fig. 3. Changes in intravascular volumes (A) and the resulting hematocrit and Hb concentration in venous blood ([Hb], B) during and after exposure to 3,454 m. The gray area highlights the results obtained after return to SL. Data points represent means $\pm$ SE. *$P < 0.05$ vs. SL.

Fig. 4. Determinants of plasma volume (PV) during and after exposure to 3,454 m. The gray area highlights the results obtained after return to SL. A: total circulating protein (TCP) mass. B: plasma osmolality. C: renin activity. D: proANP concentration. Data points represent means $\pm$ SE. *$P < 0.05$ vs. SL.
The repeated determinations confirmed that the Hb\textsubscript{mass} expansion followed a sigmoidal pattern. The initiating stimulus for erythropoiesis commences in the peritubular cells of the renal cortex where a decrease in tissue PO\textsubscript{2} stimulates the transcription of EPO (25, 32). Since plasma EPO concentration increases within the first hours of HA exposure (10), this step does not significantly delay the expansion of Hb\textsubscript{mass}. EPO then binds to specific receptors on erythroid progenitor cells in the bone marrow and promotes their survival, proliferation, and differentiation into reticulocytes, resulting in an increased abundance of reticulocytes after \textasciitilde 2 days (61). The maturation into erythrocytes requires another \textasciitilde 4 days (4) and since reticulocytes lack EPO receptors (19) this likely remains unchanged at HA. Together, these periods can explain the delayed onset of the Hb\textsubscript{mass} expansion.

It makes intuitive sense that Hb\textsubscript{mass} plateaued after \textasciitilde 3 wk since Ca\textsubscript{O}\textsubscript{2} was increased beyond SL values. The number of circulating erythrocytes depends on the balance between erythrocyte formation and lysis. As the life span of erythrocytes exceeds the duration of this study, the number of erythrocytes reaching senescence should have remained unchanged and the plateau thus probably reflected a normalization of the erythrocyte production rate. Nevertheless, although reticulocyte count decreased towards the end of the HA sojourn it was still higher than at SL. A potential explanation is that during erythropoietic stress reticulocytes are released from the bone marrow at an earlier stage, thus requiring a longer maturation period in the circulation (33, 61). Furthermore, the fraction of reticulocytes that complete the maturation into erythrocytes may be reduced (61).

It has been suggested that EPO withdrawal after return from HA induces neocytolysis, i.e., the selective lysis of young erythrocytes (41). However, although the augmented \%Hb\textsubscript{CO} on RSL7 supported accelerated erythrolysis, only 1.1 \pm 3.5% of Hb\textsubscript{mass} was lost over the first week after descent. As under homeostatic conditions 1% of erythrocytes are subjected to phagocytosis daily (19), this could have been explained by only a slightly attenuated erythrocyte production. It should, however, be mentioned that the available evidence for pronounced neocytolysis was found in subjects descending from higher altitudes, i.e., 4,380 m (42) and 5,260 m (46).

It is of note that \%Hb\textsubscript{CO} remained unchanged at HA. There is evidence that HA up regulates the expression of heme oxygenase 1, which may facilitate the degradation of heme to provide iron for erythropoiesis (7). Another product of heme degradation is CO, which may modulate the vascular response to hypoxia (29). However, although an increased \%Hb\textsubscript{CO} has been observed at HA in some animals (31, 34), our data do not support that enhanced endogenous CO formation occurs at HA in humans.

A practical aspect of this study is related to altitude training. It has been indicated that \textasciitilde 5% Hb\textsubscript{mass} expansion is required to improve SL performance (45). Since 4 wk of continuous exposure to 3,454 m expanded Hb\textsubscript{mass} of normally trained individuals by merely 5.3%, it seems unlikely that the common altitude training recommendations, i.e., to spend \textasciitilde 14 h per day at 2,500 m for 3–4 wk (6), reliably increases Hb\textsubscript{mass} to the same extent, particularly in athletes, where the erythropoietic response to HA is reduced (39, 45). Another common practice is to complete altitude training 2–3 wk before a SL competition. Based on the rapid decay of Hb\textsubscript{mass} upon return to SL, this approach appears questionable.

PV was reduced throughout the entire HA sojourn and the transient peak on HA20 was within a range that can be explained by biological and analytical variation (54). The magnitude of the reduction was smaller than predicted for this altitude (2) potentially due to the readily available potable water and the absence of strenuous exercise. PV contraction constitutes a rapid mechanism to enhance Ca\textsubscript{O}\textsubscript{2} as illustrated by the normalization of Ca\textsubscript{O}\textsubscript{2} within a few days. This did, however, not prevent the increase in Hb\textsubscript{mass} likely reflecting that renal EPO transcription is regulated by tissue PO\textsubscript{2}, which also depends on arterial PO\textsubscript{2} and blood O\textsubscript{2} affinity (24). Although the decrease in PV is traditionally attributed to diuretic fluid loss (22), it may also relate to oncotic changes following a decrease in TCP (48). The latter is, however, not supported by the present results since TCP remained unchanged and plasma protein concentration increased when the PV loss occurred. The partial normalization of plasma protein concentration on HA28 may have been the result of a declined albumin synthesis and/or accelerated degradation of albumin and IgG at HA (50, 51, 58). Conversely, the decreased plasma renin activity and aldosterone concentration at HA supports increased diuretic fluid loss that might also be reflected in the (nonsignificant) reduction in body weight on HA4 and HA8.

Previous studies in acute (<60 min) hypoxia have observed an increase in circulating ANP, which may contribute to diuretic fluid loss (26, 57). This is not supported by the present and previous studies reporting a reduction in (pro)ANP levels during prolonged HA exposure (1, 62). ANP levels may rise in acute hypoxia due to increased central venous and/or arterial pressure secondary to sympathoexcitation, whereas a decrease in chronic hypoxia may be related to reduced PV. Another peptide that is released by the heart and stimulates diuresis is brain natriuretic peptide (BNP), but this seems to be unaffected by both acute (56) or chronic HA exposure (12).

Although hypoxia does not increase insensible water loss significantly (59), we cannot exclude that insufficient water intake at HA contributed to the loss in PV. Nevertheless, voluntary fluid uptake in humans exposed to HA closely tracks changes in water loss (60). Furthermore, insufficient fluid intake would have led to a reduction in body weight considerably exceeding the loss in PV (8).

It has to be considered whether the Hb\textsubscript{mass} or PV response of the female subject was affected by the menstrual cycle. The normal menstrual blood loss is 35–50 ml (17), which corresponded to \textasciitilde 1.5% of her baseline Hb\textsubscript{mass}. Given the interindividual variability in the Hb\textsubscript{mass} response to HA such a blood loss in a single subject is too small to affect the overall results. In addition, the increase in Hb\textsubscript{mass} in the female subject was in close vicinity to the overall increase. PV does not differ between the follicular and luteal phases (13), and most evidence suggests that [Hb] and hematocrit remain unchanged throughout the menstrual cycle (23). Finally, removing the female subject from the statistical analysis did not affect the overall interpretation.

Although we used a tilt table to promote blood mixing, we observed differences in \%Hb\textsubscript{CO} between the foot and antecubital vein after CO rebreathing. These were, however, smaller than previously observed in seated subjects (27). It should be considered whether peripheral blood pooling could have been
increased at HA due to hypoxic vasodilation (3) as this would have led to a systematic underestimation of Hbmass. Arguing against this, on HA4, where hypoxemia was the most pronounced, the determined Hbmass was similar to SL values (0.1% higher). Another limitation of the CO rebreathing method is the binding of CO to molecules other than hemoglobin. It has been estimated that during a 10-min CO rebreathing ~1.78% of the administered CO is lost to myoglobin (14), whereas the loss to other binding sites is negligible (36). The effect of HA acclimatization on human myoglobin is controversial since 17% higher levels were found in HA natives (40), whereas a 35% reduction in myoglobin expression occurred in lowlanders exposed to 4,559 m (44). A similar increase or reduction in our subjects would have led to a 2.5-g overestimation or 5-g underestimation of the Hbmass expansion at HA. The measurement precision of the CO rebreathing was likely improved by the quantification of the amount of CO that remained in the rebreathing circuit. Usually, this is assumed to correspond to 2.2% of the administered dose (55) but we measured values ranging from 0.8 to 5.1%.

A limitation to our study is that the conclusion that diuresis and not fluid redistribution regulates the PV loss at HA is based on indirect evidence, i.e., reduced renin activity and aldosterone concentration and an unchanged TCP. The measurement of total body water and extracellular fluid could have provided direct evidence, but we did not have access to this technique. In conclusion, our results demonstrate that the Hbmass response to HA exposure follows a sigmoidal pattern. The merely ~5% increase observed after 4 wk at 3,454 m suggests that the HA exposure protocols used for altitude training may be too low to reliably induce sufficient Hbmass expansion, particularly in athletes. Furthermore, the rapid Hbmass decay after return to SL questions the paradigm that the optimal timing for competitions is 2–3 wk after altitude training. Regarding the PV loss at HA our results support a major role of the renin-angiotensin-aldosterone axis but not of changes in TCP.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: C.S., P.R., and C.L. conception and design of research; C.S., A.C., M.H., S.K., A.-K.L., M.P.H., and J.P.G. performed experiments; C.S. and P.R. analyzed data; C.S., P.R., and C.L. interpreted results of experiments; C.S. prepared figures; C.S. drafted manuscript; C.S., P.R., and C.L. edited and revised manuscript; C.S., A.C., M.H., S.K., A.-K.M.L., M.P.H., P.R., and C.L. approved final version of manuscript.

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