Increased serum erythropoietin but not red cell production after 4 wk of intermittent hypobaric hypoxia (4,000–5,500 m)

Christopher J. Gore,1 Ferran A. Rodríguez,2,3 Martin J. Truijens,2,4 Nathan E. Townsend,5 James Stray-Gundersen,2 and Benjamin D. Levine2

1Australian Institute of Sport, Canberra, Australia; 2Institute for Exercise and Environmental Medicine, Presbyterian Hospital of Dallas and University of Texas Southwestern Medical Center at Dallas, Dallas, Texas; 3Institut Nacional d’Educació Física de Catalunya, Universitat de Barcelona, Barcelona, Spain; 4Faculty of Human Movement Sciences, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands; and 5New South Wales Institute of Sport, Sydney, Australia

Submitted 21 March 2006; accepted in final form 16 June 2006

Increased serum erythropoietin but not red cell production after 4 wk of intermittent hypobaric hypoxia (4,000–5,500 m). J Appl Physiol 101: 1386–1393, 2006. First published June 22, 2006; doi:10.1152/japplphysiol.00342.2006.—This study tested the hypothesis that athletes exposed to 4 wk of intermittent hypobaric hypoxia exposure (3 h/day, 5 days/wk at 4,000–5,500 m) or double-blind placebo increase their red blood cell volume (RCV) and hemoglobin mass (Hbmass) secondary to an increase in erythropoietin (EPO). Twenty-three collegiate level athletes were measured before (Pre) and after (Post) the intervention for RCV via Evans blue (EB) dye and in duplicate for Hbmass using CO rebreathing. Hematological indexes including EPO, soluble transferrin receptor, and reticulocyte parameters were measured on 8–10 occasions spanning the intervention. The subjects were randomly divided among hypobaric hypoxia (Hypo, n = 11) and normoxic (Norm, n = 12) groups. Apart from doubling EPO concentration 3 h after hypoxia there was no increase in any of the measures for either Hypo or Norm groups. The mean change in RCV from Pre to Post for the Hypo group was 2.3% (95% confidence limits = −4.8 to 9.5%) and for the Norm group was −0.2% (−5.7 to 5.3%). The corresponding changes in Hbmass were 1.0% (−1.3 to 3.3%) for Hypo and −0.3% (−2.6 to 3.1%) for Norm. There was good agreement between blood volume (BV) from EB and CO: EB BV = 1.03 × CO BV + 142, r² = 0.85, P < 0.0001. Overall, evidence from four independent techniques (RCV, Hbmass, reticulocyte parameters, and soluble transferrin receptor) suggests that intermittent hypobaric hypoxia exposure did not accelerate erythropoiesis despite the increase in serum EPO.

Address for reprint requests and other correspondence: C. J. Gore, Physiology Dept., Australian Institute of Sport, Leverrier Crescent, Bruce, ACT 2617, Australia or Exercise Physiology Laboratory, School of Education, Flinders University, GPO Box 2100, Adelaide, SA 5001, Australia (e-mail: chris.gore@ausport.gov.au).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
MATERIALS AND METHODS

Subjects. Twenty-seven well-trained athletes [15 swimmers (9 men, 6 women) and 12 runners (7 men, 5 women)] were recruited from high school, collegiate, and Masters teams. Two of the male swimmers and two of the male runners completed duplicate tests at baseline but did not commence the intervention (see Study design), leaving a final sample size of \( n = 23 \). Their characteristics are provided in Table 1. All subjects were sea-level residents and trained at least daily for 10 mo before the study and continued to train throughout the 10-wk study, which was conducted in Dallas during May to July 2003. Each subject provided their voluntary written, informed consent to participate in protocols approved by the Institutional Review Boards of the University of Texas Southwestern Medical Center and Presbyterian Hospital of Dallas.

Study design. As part of a multicenter international collaboration, a variety of measures not reported here were conducted on these subjects; four studies addressed the effect of IHE on performance (48a), economy (58b), hypoxic ventilatory response (58a), and autonomic function (56a). The overall study design is summarized in Fig. 1. After completing duplicate baseline measures for most variables, subjects were matched for sex, performance level, and training history and then assigned to either 4 wk of hypobaric hypoxia (Hypo; simulated altitude of 4,000 –5,500 m) or normobaric normoxia (Norm; 0 –500 m) in a randomized, double-blind, placebo-controlled trial. Carbon monoxide rebreathing was conducted twice before (Pre) and twice after (Post) treatment, whereas Evans blue dye (EB) was administered once before and once after hypoxic or placebo intervention because of limited dye availability. Venous blood was drawn eight times during the 10-wk study (twice before, once per week during, and twice after) and analyzed for hemoglobin concentration ([Hb]), hematocrit (Hct), percent reticulocytes (%Ret), mean cell volume (MCV), mean cell volume of reticulocytes (MCVr), total hemoglobin content of reticulocytes (RetHb = number of reticulocytes × per cell Hb content of reticulocytes), soluble transferrin receptor concentration (sTfr), and erythropoietin concentration (EPO). The samples taken each week during the period of hypoxia or placebo were taken just before entering the hypobaric chamber, \( 20 \) h after the previous hypoxic exposure. Venous blood was also drawn twice (week 2, week 4) at \( 3 \) h after chamber exposure and assayed for EPO. Two weeks before and \( 1 \) wk after the intervention, venous blood was assayed for ferritin concentration. During this interval, all subjects received oral iron supplementation (Feo-Sol, 9 mg elemental iron/ml) with the dose (ranging from 5–15 ml, 1–3 times/day) adjusted based on their preintervention plasma ferritin concentration.

Hypobaric hypoxia or placebo. A hypobaric chamber (Perry Baromedical, West Palm Beach, FL), located at the Institute for Exercise and Environmental Medicine, Dallas, TX was utilized for the experiment, and its air refresh rate was calculated to keep the inspired CO\(_2\) fraction \( \leq 0.2\% \). This chamber has three separate locks that can be operated independently and controlled for simulated altitude and rate of ascent or descent. Four chamber runs per day were scheduled to accommodate the living and training schedules of all subjects, Table 1. Subject characteristics

<table>
<thead>
<tr>
<th>Group</th>
<th>( n )</th>
<th>Age, yr (SD)</th>
<th>Height, cm (SD)</th>
<th>Mass, kg (SD)</th>
<th>Blood Volume, ml/kg (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypo</td>
<td>11 (5 men, 6 women)</td>
<td>2.6 (7.0) [16.3–35.4]</td>
<td>171.6 (8.2) [160.0–185.5]</td>
<td>66.6 (12.15) [48.3–90.8]</td>
<td>84.8 (11.7) [72.1–106.9]</td>
</tr>
<tr>
<td>Norm</td>
<td>12 (6 men, 6 women)</td>
<td>23.2 (9.3) [16.2–48.3]</td>
<td>174.9 (9.9) [162.6–193.0]</td>
<td>67.3 (12.05) [51.3–96.8]</td>
<td>85.0 (8.7) [71.5–99.2]</td>
</tr>
<tr>
<td>Swimmers</td>
<td>13 (7 men, 6 women)</td>
<td>20.6 (8.8)</td>
<td>178.0 (8.4)</td>
<td>73.0 (11.7)</td>
<td>81.8 (8.5)</td>
</tr>
<tr>
<td>Runners</td>
<td>10 (5 men, 5 women)</td>
<td>25.8 (6.2)</td>
<td>167.2 (5.9)</td>
<td>59.1 (6.2)</td>
<td>89.0 (10.8)</td>
</tr>
<tr>
<td>Men</td>
<td>12</td>
<td>22.5 (8.9)</td>
<td>178.1 (9.1)</td>
<td>70.3 (12.0)</td>
<td>88.1 (10.4)</td>
</tr>
<tr>
<td>Women</td>
<td>11</td>
<td>23.2 (7.4)</td>
<td>168.1 (5.6)</td>
<td>63.3 (11.0)</td>
<td>81.5 (8.7)</td>
</tr>
</tbody>
</table>

Values are mean (SD) and range in brackets. Hypo, hypobaric hypoxia (4,000–5,500 m); Norm, normoxia (0–500 m). Blood volume was estimated from Evans blue before hypoxia or placebo.

Fig. 1. Testing schedule and simulated altitude exposure of the hypobaric hypoxia (Hypo, \( n = 12 \)) and normoxic (Norm, \( n = 11 \)) groups with treatment administered in a double-blind placebo fashion. The Hypo group was exposed to 4,000–5,500 m 3 h/day for 5 days/wk whereas the Norm group was exposed to 0–500 m of simulated altitude. Pre and Post, respectively, indicate measures before and after treatment with hypoxia or normoxia for 4 wk. Chronology of the 2 Pre and 2 Post measures is indicated with sequential numbers.
divided into two treatment and placebo interventions each day in separate locks. Each chamber exposure, regardless of altitude, included a 10-min ascent and 10-min descent within the 3-h period. For the Norm group, the first 10 min of exposure involved multiple pressure changes as follows: minute 1.5, 1,800 m; minute 2.5, 900 m; minute 3, 3,700 m; minute 6, 2,500 m; minute 7, 3,300 m; minute 10, 500 m, where it remained for the duration of the exposure. United States Air Force standard atmospheric tables were used to derive the barometric pressure for each altitude (59).

\( Hb_{mass} \) was estimated by a modified version of a CO-rebreathing technique (10) with a total rebreathing volume of 3.1 liters. After an initial priming dose of 99.9% CO (15 ml for women and 20 ml for men) rebreathed for 10 min, blood was sampled from an antecubital vein via a cannula (20-G, Medex, Carlsbad, CA) into a capped 5-ml glass syringe prerinsed with liquid heparin (1,000 IU/ml). A second dose (1.25 ml/kg for women and 1.5 ml/kg for men) of 99.9% CO was then rebreathed for 10 min, after which a second blood sample was obtained while the athlete remained on the rebreathing circuit. When a subject was connected to the rebreathing system, medical-grade O\(_2\) was bleed in continuously at a rate equivalent to its consumption: ~200–350 ml/min.

Each blood sample was stored on ice until assayed for percent carboxyhemoglobin (%HbCO) and hemoglobin concentration (Hb) by using a diode-array spectrophotometer (OSM3 hemoximeter, Radiometer Medical, Copenhagen, Denmark). Samples were analyzed repeatedly until five values for %COHb were obtained within 0.1% of each other, which was usually achieved within six replicates. The OSM3 hemoximeter was calibrated according to manufacturer’s specifications for Hb concentration (using 25190 calibrating solution, Radiometer) immediately before the Pre and Post data-collection periods. The change in %HbCO (\( \Delta %HbCO = \text{difference between first and second measures} \)) was used to calculate the Hb\(_{mass}\) through which the CO had distributed using the method of Burge and Skinner (10); that is, Hb\(_{mass}\) (g) = (volume CO\(_{added}\) \* pH\(_{1.34}\) ml CO per g Hb)/\( \Delta %HbCO \), where the volume of CO\(_{added}\) is multiplied by 0.978 to correct for the amount of CO remaining in the rebreathing circuit after 10 min (10). The same mercury barometer (Princo, Southampton, PA) and mercury-in-glass thermometer (Baxter Scientific, Deerfield, IL) were used for all tests to determine the ambient conditions to enable the transformation of the CO gas volume to standard temperature and pressure. The percent typical error (TE; see Statistics) based on the first two trials for \( n = 27 \) was 2.0% (95% confidence limits = 1.6–2.7%) for a group mean of 823 g or absolute TE of 17 g. When Hb\(_{mass}\) was measured over a longer period of time (i.e., from Pre1 to Post1, where sequential numbers indicate the chronology of the 2 Pre and 2 Post measures similar to the time period of measurement for EB-derived volumes), the TE was increased to ~3.8% (95% CL 3.0–5.5%).

Hb\(_{mass}\) calculated as described above is not volumetric and therefore does not depend on determination of [Hb] or Hct (20). However, to allow direct comparison to EB-derived RCV, Hb\(_{mass}\) was divided by [Hb] to allow estimation of BV via CO (BV\(_{CO}\)). [Hb] was the mean of the six values determined on the OSM3 hemoximeter for the last blood sample; that is, after 30 min of semirecumbent rest. Plasma volume, BV, and RCV. The plasma volume (PV) of the fasted subjects was determined by the EB technique (39). PV was measured subsequent to CO rebreathing because EB interferes with the spectrophotometric measurement of HbCO (40).

Even though subjects were semirecumbent throughout the 30 min required to measure Hb\(_{mass}\), a further 20 min of fully supine rest preceded the PV procedure. An initial blood sample was then taken to provide a plasma blank and for determination of Hct and other hematological indexes. Immediately thereafter, ~2.5 ml of EB were injected intravenously and blood was resampled ~10, 20, and 30 min later. The exact dye volume was determined as initial minus final syringe mass determined on a 0.1-mg resolution balance (Mettler-Toledo GmbH AE100, Columbus, OH). After centrifugation, PV was calculated from time zero extrapolation of the regression derived from the three postinjection absorbances at 620 and 740 nm (Hewlett-Packard model 8452A diode array spectrophotometer). Hct was measured in duplicate with a microcapillary centrifuge, and BV via EB (BV\(_{EB}\)) was calculated as PV/(1 – Hct), with a 0.96 correction for plasma trapping associated with a spun Hct and 0.92 for peripheral sampling. RCV was then calculated as BV – PV. Optical density of the batch of EB used in this experiment was measured directly at 620 nm from diluted (1,000 times) EB obtained from two different ampoules as the average of three repetitions from each sample. The mean optical density (0.39625) \( \times \) the dilution ratio was then multiplied by the specific extinction of EB to determine the actual initial dye concentration. The %TE values using the Pre vs. Post data for PV, RCV, and BV\(_{EB} \) were 7.2 (95% CL = 5.8–10.8%), 6.7 (95% CL = 5.3–10.0%), and 6.0% (95% CL = 4.7–8.8%), respectively.

Hematological indexes. Erythrocyte and reticulocyte parameters were analyzed with an ADVIA 120 hematology analyzer (Bayer Corporation, Tarrytown, NY), which performs flow cytometric measurements (8). All analyses were completed within 8 h of collection. The ADVIA was calibrated against appropriate reference materials and controlled daily using Bayer ADVIA TESTpoint hematolgy low, normal, and high controls and Bayer ADVIA TESTpoint reticulocyte low and high controls. The %TE (and 95% CLs) from the two Pre measures for the parameters reported in our results were as follows: Hct 4.0% (3.2–5.9%), [Hb] 3.7% (2.9–5.3%), %Ret 19.5% (16.3–31.8%), MCV 0.5% (0.4–0.7%); MCVr 1.2% (0.9–1.7%); and RetHb 21.6% (19.0–33.4%).

All sera were separated and aliquoted into cryotubes, stored at \(-20^\circ C\) or \(-80^\circ C\), packed on dry ice, and then shipped to Australia for analysis. The EPO and sTfR concentrations were determined by use of an automated solid-phase chemiluminescent immunoassay (Diagnostic Products, Los Angeles, CA) and an automated immunonephelometric assay (Dade Behring), respectively. The automated immunoassays for EPO and sTfR were controlled by three and two levels of controls, respectively. We have previously reported on the calibration procedures as well as the within- and between-assay coefficients of variation in our hands (56). Ferritin concentration was measured using a Hitachi 911 biochemistry analyzer (Roche Diagnostics, Tarrytown, NY), which performs flow cytometric measurements (8). All analyses were completed within 8 h of collection. The ADVIA was calibrated against appropriate reference materials and controlled daily using Bayer ADVIA TESTpoint hematolgy low, normal, and high controls and Bayer ADVIA TESTpoint reticulocyte low and high controls. The %TE (and 95% CLs) from the two Pre measures for the parameters reported in our results were as follows: Hct 4.0% (3.2–5.9%), [Hb] 3.7% (2.9–5.3%), %Ret 19.5% (16.3–31.8%), MCV 0.5% (0.4–0.7%); MCVr 1.2% (0.9–1.7%); and RetHb 21.6% (19.0–33.4%).

Statistics. All data are expressed as means and standard deviations (SD), with most analyses completed by using Statistica (version 6.0, Tulsa, OK). For BV and Hb\(_{mass}\), repeated-measures ANOVA was used to compare groups (Hypo vs. Norm) by time (duplicate or single measures Pre and Post). Repeated-measures ANOVA for group by time was also used for the blood and serum parameters that were measured on eight or ten occasions. After significant main effects or interactions, Tukey’s post hoc tests were used to identify differences between cell means. Linear regression was used to examine relationships between variables, and TE (= SD of difference scores/\( \sqrt{2} \)) was used to quantify reliability expressed in both absolute units or as a percentage of the mean. Slopes and intercepts were compared with the line of identity by use of GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).

Two of the 23 subjects (both from the Norm group) were unable to complete the POST2 measure of Hb\(_{mass}\). To avoid casewise deletion in the associated ANOVAs, substitution of the mean of their other values was used for their fourth value. Mean substitution was not used for TE calculations.

RESULTS

Hb\(_{mass}\). Hb\(_{mass}\) did not increase significantly in response to hypoxia or placebo (Fig. 2). When the data of the duplicate Pre and Post trials were averaged, the means (SD) for the Hypo...
group were 789 (188) and 794 (181) g before and after hypoxia, respectively. The corresponding values for the Norm group were 818 (188) and 819 (181) g. The overall mean change in Hb_mass for the Hypo group was 1.0% (95% CL from 1.3 to 3.3%) and for the Norm group was 0.3% (95% CL from 2.6 to 3.1%) when calculated from the individual percent changes from Pre to Post.

**PV, RCV, and BVE**. PV (Fig. 2) as well as RCV and BVE (Fig. 2) did not change significantly with hypoxia or placebo with the respective latter two group-by-time ANOVA results of $F(1,21) = 0.04, P = 0.84$; and $F(1,21) = 0.61, P = 0.44$. The individual and mean data for RCV are illustrated in Fig. 2. Calculated from the individual percent changes, the overall mean change in RCV from Pre1 to Post1 for the Hypo group was 2.3% (95% CL from 4.8 to 9.5%) and for the Norm group was 0.2% (95% CL from 5.7 to 5.3%). The change in RCV was not significantly correlated with the change in Hb_mass whether expressed in absolute terms ($r^2 = 0.10, P = 0.14$) or as a percent ($r^2 = 0.08, P = 0.20$). The mean change in RCV after intermittent hypoxia or placebo for the pooled Hypo and Norm groups was 1.0% (95% CL from 3.3 to 6.1%) and that for Hb_mass was 0.6% (95% CL from 1.1 to 2.3%).

**BV comparison.** For the pooled data of both Hypo and Norm groups before and after intermittent hypoxia or placebo the mean BV_{EB} was 5,696 (1,043) ml, and BV_{CO} was 5,738 (995) ml. The Pearson correlation between methods was statistically significant ($r^2 = 0.85, P < 0.0001$; Fig. 3). Both the slope ($P = 0.90$) and the intercept ($P = 0.96$) of this regression were not different from those of the line of identity. The TE 95% confidence intervals for BV measured using these two independent techniques were overlapping: BV_{CO} = 3.1–5.8%; BV_{EB} = 4.7–8.8%.

**Hematological indexes.** Serum EPO concentration was significantly increased in the Hypo group 3 h after the hypoxic

**Fig. 2.** Individual and mean hemoglobin mass (Hb_mass; top) and red cell volume (bottom) before and after 4 wk of intermittent hypoxia or placebo (3 h/day, 5 days/wk).

**Fig. 3.** Correlation between blood volume measured via Evans blue dye and CO rebreathing. Pooled data from 23 subjects before (Pre) and after (Post) 4 wk of intermittent hypoxia or placebo.
Table 2. Hematological response to 4 wk of intermittent hypoxia or placebo

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>PRE1</th>
<th>PRE2</th>
<th>Hypoxia or placebo</th>
<th>POST1</th>
<th>POST2</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPO, IU/l</td>
<td>Hypo</td>
<td>10.6 (4.0)</td>
<td>10.5 (4.0)</td>
<td>10.5 (4.0)</td>
<td>10.6 (4.0)</td>
<td>10.5 (4.0)</td>
</tr>
<tr>
<td></td>
<td>Norm</td>
<td>10.6 (4.0)</td>
<td>10.5 (4.0)</td>
<td>10.5 (4.0)</td>
<td>10.6 (4.0)</td>
<td>10.5 (4.0)</td>
</tr>
<tr>
<td>sTfr, ng/ml</td>
<td>Hypo</td>
<td>1.7 (3.0)</td>
<td>1.7 (3.0)</td>
<td>1.7 (3.0)</td>
<td>1.7 (3.0)</td>
<td>1.7 (3.0)</td>
</tr>
<tr>
<td></td>
<td>Norm</td>
<td>1.7 (3.0)</td>
<td>1.7 (3.0)</td>
<td>1.7 (3.0)</td>
<td>1.7 (3.0)</td>
<td>1.7 (3.0)</td>
</tr>
<tr>
<td>[Hb], g/dl</td>
<td>Hypo</td>
<td>14.4 (4.1)</td>
<td>14.4 (4.1)</td>
<td>14.4 (4.1)</td>
<td>14.4 (4.1)</td>
<td>14.4 (4.1)</td>
</tr>
<tr>
<td></td>
<td>Norm</td>
<td>14.4 (4.1)</td>
<td>14.4 (4.1)</td>
<td>14.4 (4.1)</td>
<td>14.4 (4.1)</td>
<td>14.4 (4.1)</td>
</tr>
<tr>
<td>Hct, %</td>
<td>Hypo</td>
<td>42.9 (3.3)</td>
<td>42.9 (3.3)</td>
<td>42.9 (3.3)</td>
<td>42.9 (3.3)</td>
<td>42.9 (3.3)</td>
</tr>
<tr>
<td></td>
<td>Norm</td>
<td>42.9 (3.3)</td>
<td>42.9 (3.3)</td>
<td>42.9 (3.3)</td>
<td>42.9 (3.3)</td>
<td>42.9 (3.3)</td>
</tr>
<tr>
<td>MCV, fL</td>
<td>Hypo</td>
<td>88.7 (4.2)</td>
<td>88.7 (4.2)</td>
<td>88.7 (4.2)</td>
<td>88.7 (4.2)</td>
<td>88.7 (4.2)</td>
</tr>
<tr>
<td></td>
<td>Norm</td>
<td>88.7 (4.2)</td>
<td>88.7 (4.2)</td>
<td>88.7 (4.2)</td>
<td>88.7 (4.2)</td>
<td>88.7 (4.2)</td>
</tr>
<tr>
<td>MCVr, fL</td>
<td>Hypo</td>
<td>107.8 (4.3)</td>
<td>107.8 (4.3)</td>
<td>107.8 (4.3)</td>
<td>107.8 (4.3)</td>
<td>107.8 (4.3)</td>
</tr>
<tr>
<td></td>
<td>Norm</td>
<td>107.8 (4.3)</td>
<td>107.8 (4.3)</td>
<td>107.8 (4.3)</td>
<td>107.8 (4.3)</td>
<td>107.8 (4.3)</td>
</tr>
<tr>
<td>Ferritin, ng/ml</td>
<td>Hypo</td>
<td>79.7 (48.6)</td>
<td>79.7 (48.6)</td>
<td>79.7 (48.6)</td>
<td>79.7 (48.6)</td>
<td>79.7 (48.6)</td>
</tr>
<tr>
<td></td>
<td>Norm</td>
<td>94.9 (88.7)</td>
<td>94.9 (88.7)</td>
<td>94.9 (88.7)</td>
<td>94.9 (88.7)</td>
<td>94.9 (88.7)</td>
</tr>
</tbody>
</table>

Values are means (SD). EPO, serum erythropoietin concentration; sTfr, soluble transferrin receptor concentration; [Hb], hemoglobin concentration; Hct, hematocrit; %Ret, percentage of reticulocytes; RetHb, total reticulocyte hemoglobin (number of reticulocytes × cell hemoglobin content of reticulocytes); MCV, mean cell volume; MCVr, mean cell volume of reticulocytes. Wk2+3h and Wk4+3h denote sampling time 3 h after a 3 h exposure to hypobaric hypoxia or placebo. *Significantly different from both (PRE1) and (PRE2), where the chronology of the 2 Pre and 2 Post measures is indicated with sequential numbers.

DISCUSSION

The major finding of this study is that 4 wk of IHE (simulated altitude of 4,000–5,500 m for 3 h/day, 5 days/wk) was insufficient to increase RCV or Hbmass. This finding was supported by the absence of any evidence of accelerated erythropoiesis from a comprehensive assessment of indirect markers such as soluble transferrin receptor or red cell indexes derived from flow cytometry. Together, these findings suggest that this dose of hypoxia is inadequate to stimulate erythropoiesis, despite significant increases (doubling) in serum EPO 3 h after each hypoxic treatment. The second main outcome is that there was good agreement between the BV determined with EB and CO rebreathing (7) and provides strong evidence that both techniques are appropriate to use for evaluation of the red cell compartment after exposure to hypobaric hypoxia given an expected change greater than the TE of the technique used.

Lack of increased erythropoiesis. The degree and extent of hypoxia to increase serum EPO is quite well characterized, and there is no doubt that exposure to just a few hours of adequate hypoxia increases production of EPO (1, 14, 30). For example, 84 min at 4,000 m was sufficient to significantly increase serum EPO (14), 90 min at 5,500 m increased serum EPO by 55% (49), and, after 3 h of continuous hypoxia, EPO was 120% of the preexposure concentration. Likewise, 2 h at 5,450 m yielded a 50% increase in serum EPO (30), and, in our Hypo group, there was a doubling of serum EPO 3 h after both the fourth and nineteenth exposures at 4,500 and 5,500 m, respectively. This robust increase in EPO in response to repeated exposure to simulated high altitude (4,000–5,500 m) for 3 h/day for 4 wk has also been reported when IHE is administered to triathletes (2). For chronic exposure to terrestrial altitude, it has been confirmed that an altitude of ~2,200 m is necessary to reach the steep part of the oxyhemoglobin dissociation curve when arterial oxygen tension falls below 65 Torr (62). More recent work makes it clear that there is substantial between-subject variation in the threshold altitude, but for most people it appears to be between 2,100 and 2,500 m (17). With a minimum altitude of 4,000 m and duration of 3 h in the present study of IHE, it is clear that we exceeded the dose of hypoxia required to increase serum EPO.

It has been previously assumed that a sustained increase in serum EPO will lead to an obligatory increase in the total RCV and hence Hbmass (15, 26). We used two independent methods to measure the red blood cell compartment and neither yielded a significant mean change with the Hypo group. One possible explanation is that the measurement techniques were too insensitive to measure an important change. However, the mean changes in the Hypo group were just 2.3 and 1.0% for RCV
and Hb\textsubscript{mass}, respectively, which corresponds to 46 ml of red blood cells and 8 g of Hb relative to the Pre values for that group. The constancy of the red blood cell compartment in the face of IHE is supported by the lack of change in [Hb], Hct, %Ret, RetHb, and MCV\textsubscript{r} and was likely not due to inadequate iron (57) because serum ferritin tended to increase, not decrease, in the Hypo group and both groups were supplemented with iron throughout the study. Finally, sTfr did not increase in the Hypo group and sTfr is considered as a prime and sensitive marker of the erythroid mass (25). Therefore, evidence from four independent techniques (RCV, Hb\textsubscript{mass}, reticulocyte parameters, and sTfr) suggests that IHE did not accelerate erythropoiesis despite the transient increases in serum EPO.

An alternate explanation is that, despite the doubling of serum EPO, the 21 h spent in normoxia each day, as well as the weekend spent in normoxia, were sufficient to counter the transient increases induced by IHE. Serum EPO clearance and total area under the curve warrant consideration as possible mechanisms, although counterbalancing pathways for inhibition of the hypoxia response pathway also should be considered. Both the rapid destruction of hypoxia-inducible factor-1\textalpha{} (27, 60) and a marked decrease in red cell survival time (42), termed “neocytolysis” (3, 43), may compromise the ability of short-duration hypoxia to increase the red cell mass.

The total “dose” of hypoxia required to increase RCV and Hb\textsubscript{mass} is uncertain (19, 27, 33, 34). For example, it was previously reported that 31 days at 2,690 m did not increase the Hb\textsubscript{mass} of a small number of cyclists (18), though some of them were ill during the final week of their altitude sojourn, which may have inhibited their EPO response. In contrast, 4 wk at 20–22 h/day at 2,500 m was sufficient to increase RCV of a large number of runners (32), and a cross-sectional study has observed a higher Hb\textsubscript{mass} of cyclists who are life-long residents at 2,600 m (54). More recently, accelerated erythropoiesis has been confirmed in elite athletes at moderate altitudes (16, 23, 61). Thus, despite rare exceptions (18), the accumulated evidence from several research groups has confirmed that sufficient durations of moderate altitude exposure for nearly 24 h/day increases the red cell mass even in elite athletes.

But what about more intermittent exposures? It has been shown that 12–16 h/day of normobaric hypoxia for 3 (9) or 4 wk (50) closely replicates the results observed in the field studies with an increase in both Hb\textsubscript{mass} and \(\overline{V}_\text{O}_2\text{max}\). In contrast, 8–10 h/day of normobaric hypoxia (2, 500–3,000 m) for 10–21 days did not increase in Hb\textsubscript{mass} or \(\overline{V}_\text{O}_2\text{max}\) (5). We speculate that this dose of intermittent moderate hypoxia exposure, in which the duration of time spent in normoxia exceeds that spent in hypoxia, may be insufficient to initiate and sustain a robust erythropoietic response (35).

Comparison with other studies using short duration but severe hypoxic exposure. Our double-blind protocol of 5:5 min of hypoxia (10%; \text{O}_2) to normoxia for 70 min 5 times/wk recently failed to find any increase in erythropoiesis based on reticulocyte parameters or sTfr (28), which may have been due to an inadequate duration of hypoxia (1, 14, 30). On the other hand, it has been reported that 180–300 min/day of IHE (4,000–5,500 m) for 9–17 days was sufficient to increase Hct, [Hb], and red blood cell count by \textasciitilde{}10% (11) and double the percentage of reticulocytes (47). Similar increases were observed after just 90 min or IHE 3 times/wk for 3 wk (49). PV loss could explain most of these observations, and additional exercise can stimulate reticulocytes (4).

A recent study by this Spanish research group (2) failed to demonstrate an increase in reticulocytes using the identical model of IHE as employed in the present investigation; instead of using the imprecise manual method of reticulocyte counting (52, 58), for the first time they used an automated flow-cytometer analyzer for this measurement. In addition, hypobaric hypoxia can stimulate the release of immature red cell forms from the bone marrow, thus increasing reticulocytes without actually accelerating erythropoiesis (21). Interestingly, the consistent rise in EPO in the fourth week compared with the second week of our study is similar to that observed in Chilean miners or European sojourners who are exposed to repetitive severe hypobaric hypoxia for many years (21) but in contrast to the widely reported gradual decrease in EPO during sustained high-altitude residence (32, 44). We suggest that this finding is one more marker of the failure to increase blood oxygen content during chronic intermittent hypobaric hypoxia and argues against a vigorous acclimatization response.

Failure to use a control group exposed to a placebo altitude was a limitation of most (11, 47, 49), but not all (2) the previous IHE studies. The present study circumvented this shortcoming and is only the second double-blind investigation to have administered hypoxia to athletes; the first was Julian and coworkers (28). The additional benefit of double baseline measures of the hematological variables and a control group against which to assess them allows for confidence in our results.

Overall, it appears that, to accelerate erythropoiesis with IHE, not only the level of EPO increase induced by the hypoxic exposure has to be considered, but also other factors such as the duration of the normoxic conditions after each IHE exposure, the training history of the athletes, and possibly the extent of training-induced neocytolysis subsequent to an increase in Hb\textsubscript{mass}. These factors may also play integrated, nonlinear roles in the increase of the erythroid mass after intermittent hypoxia as suggested elsewhere (46).

Hemoglobin mass and RCV: agreement of methods. We attained good agreement between the BV assessed via RCV and that estimated from CO rebreathing. One of the prime criticisms of the CO-rebreathing method has been that it overestimates the Hb\textsubscript{mass} because it is distributed beyond the circulation to nonblood iron porphyrin molecules such as in muscle and liver (24, 41, 53). However, CO is produced endogenously (13) and furthermore in the presence of normal or high inspired \text{O}_2, as used previously (10, 18), oxygen binds preferentially to myoglobin even in the presence of CO (12, 36). There is also evidence that CO loaded on to red blood cells via the lungs is not redistributed between erythrocytes during circulation (6). Recently it has been demonstrated that with 14 min of suprasystolic occlusion at rest the deoxy-myoglobin signal is not attenuated with a preload of 20% COHb (45). Richardson and colleagues (45) conclude that these data support Burge and Skinner’s previous contention (10) that it is not necessary to correct measures of Hb\textsubscript{mass} for loss of CO to myoglobin.

In the present experiment, the two methods agreed closely and EB tended to marginally overestimate the BV (by 3%) compared with that estimated from CO rebreathing. The 3% “difference” is likely trivial because the slope of the line for the regression between the two methods was not different from the
line of identity. Overall, the data from the present experiment demonstrate that, when carefully performed, both methods are suitable to measure BV and associated variables in athletes undergoing hypobaric hypoxia, although averaged across many studies Hbmass has better reliability than RCV derived from EB (20) and is the preferred method when changes are anticipated to be small.

In conclusion, with a double-blind protocol, 4 wk of hypobaric hypoxia equivalent to 4,000–5,500 m altitude for 3 h/day, 5 days/wk was insufficient to increase RCV or Hbmass. Evidence from four independent techniques (RCV, Hbmass, reticulocyte parameters, and stTr) confirms that IHE did not accelerate erythropoiesis despite the transient increase in serum EPO. The dissociation between an increase in serum EPO without an increase of red blood cells alludes to the requirement of a larger and more sustained, critical dose of EPO to yield a substantial effect on the red cell compartment.

ACKNOWLEDGMENTS

We acknowledge the technical support of the following staff at the Institute for Exercise and Environmental Medicine: Paul Chase, Dean Palmer, Michael Lisby, Emily Martini, Kimberly Williams, and Sarah Witkowski. We also thank Sally Wright and Graeme Allbon at the Australian Institute of Sport, both of whom ran the EPO and stTr assays, and Merill Dane from University of Texas Southwestern Medical Center, who kindly maintained the OSM3 CO-oximeter on our behalf. Finally, this study would not have been possible without the excellent cooperation of our subjects.

GRANTS

This study was supported by grants from the US Olympic Committee, the Australian Institute of Sport, Institut Nacional d’Educació Física de Catalunya-Universitat de Barcelona, Departament d’Universitats, Recerca i Societat de la Informacio (Generalitat de Catalunya), and the American College of Sports Medicine (2003 International Scholar and Visiting Scholar awards).

REFERENCES
