Effects of Intermittent Hypoxia on Epo, Soluble Epo Receptor and Ventilation in Humans

Julien V. Brugniaux, Vincent Pialoux, Glen E. Foster, Cailean T.C. Duggan, Misha Eliaziw, Patrick J. Hanly and Marc J. Poulin

Online Data Supplement
METHODS

Procedures

Resting Measurements and Acute Hypoxic Ventilatory Response (AHVR). Prior to the acute hypoxic test, resting values for $\text{PETO}_2$ and $\text{PETCO}_2$ ($\text{PETO}_{2-\text{RA}}$ and $\text{PETCO}_{2-\text{RA}}$, respectively) were recorded for 10 min while subjects were breathing room air and resting in a semi-seated position. The respiratory gases were sampled continuously at a rate of 20 ml min$^{-1}$ using a catheter held on a mouthpiece and analyzed by mass spectrometry (AMIS 2000, Innovision, Odense, Denmark). A computer sampled the resulting values for both $\text{PO}_2$ and $\text{PCO}_2$ every 10ms, and $\text{PETO}_2$ and $\text{PETCO}_2$ were identified and recorded breath-by-breath by a computer and dedicated software (Chamber v2.43, University Laboratory of Physiology, Oxford, UK).

A test to assess AHVR followed the resting measurements. A detailed description of the methodology used to assess AHVR can be found elsewhere [1]. Briefly, this test started with a 7.5 min isocapnic eucapnic baseline, during which end-tidal $\text{PO}_2$ ($\text{PETO}_{2-\text{ISO}}$) and $\text{PCO}_2$ ($\text{PETCO}_{2-\text{ISO}}$) were held constant at 88mmHg and +1.5mmHg above $\text{PETCO}_{2-\text{RA}}$, respectively. Thereafter, $\text{PETO}_2$ was decreased in six 90-sec steps (75.2, 64.0, 57.0, 52.0, 48.2, 45.0 mmHg) while $\text{PETCO}_2$ remained constant. The level of the six descending steps in $\text{PETO}_2$ were calculated to provide an equal decrease in $\text{SaO}_2$, as described by Severinghaus [2]. The duration of each step (i.e. 90 sec) was determined to ensure the ventilatory response to be essentially mediated by the carotid bodies [3]. Accurate control of end-tidal gases was performed using the technique of dynamic end-tidal forcing (BreatheM V2.40, University Laboratory of Physiology, Oxford, UK). This technique has already been described [4]. Tidal volume and $f_R$ were subsequently determined during off-line analysis from the ventilatory records by the BreatheM software as the average for
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the last 30-sec of each PETO₂ step. The data for VT and fR and total minute ventilation (VE) during the last hypoxic step, i.e., PETO₂ = 45.0 mmHg and PETCO₂ = 1.5 mmHg above resting values, are presented here.

Blood samples and analysis. Blood samples were collected from the antecubital vein on Days -4, 0, 1, 2, 4 and 8. Blood was collected in three 5 ml ethylenediaminetetraacetic acid (EDTA) tubes and one serum separator tube (SST) tube. The plasma was obtained by centrifugation of the samples at 1000g for 10 min at 4°C from the EDTA tubes. Plasma was separated into several aliquots and frozen at -80°C. The serum from the SST tube was obtained by coagulation at room temperature for 30 min and then centrifugation of the samples at 1000g for 10 min at 4°C. Serum was separated into aliquots and frozen at -80°C.

The serum concentrations of Epo (mIU/ml) were determined by Enzyme-Linked Immunosorbent Assay (ELISA) technique using human Epo Quantikine IVD (R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions and measured on a microplate reader (Fluostar OPTIMA, BMG Labtech, Durham, NC, USA). The Quantikine IVD Epo ELISA is based on the double-antibody sandwich method.

To determine the plasma sEpoR concentration (ng/ml), we used the ELISA method previously described by Westphal et al. [5] with the exception that the secondary antibody differed from the original technique. We used the goat anti-mouse IgG horseradish peroxidase from Cayman Chemicals (Ann Arbor, MI, USA). This method is an indirect, non-competitive ELISA using a commercially available recombinant human sEpoR (R&D System Minneapolis, MN, USA) as a
standard. Since sEpoR is assessed directly from human plasma, no protein extraction was undertaken in this method. Therefore, Epo/sEpoR complexes are not altered and the antibody binds to both free and bound sEpoR. As a consequence, the observed changes reflect alterations in total sEpoR (i.e. sEpoR alone and Epo/sEpoR complex). Briefly, this method consists of coating the microplate with the recombinant human sEpoR or the plasma at 4°C overnight. After carefully washing the wells 8 times with PBS, non-specific binding sites were blocked with 5% fat free powder milk in PBS at room temperature for 5 hours. The blocking solution was again carefully removed and the wells were washed 5 times with PBS (only the first washing steps differed by the addition of 0.05% of Tween 20 (Sigma-Aldrich, Oakville, ON, Canada) to the solution). A human anti-erythropoietin receptor monoclonal antibody (hEpo-R-MoAb), mh2er/16.5.1, (mh2er/16.5.1, Wyeth Research, Cambridge, Mass., USA) recognizing the extracellular domain of the human EpoR [6] was added and incubated at room temperature for 1 hour. After washing 5 times with PBS, a goat anti-mouse IgG horseradish peroxidase antibody (Cayman Chemicals, Ann Arbor, MI, USA) was added and incubated at room temperature for 1 hour. Following this, the wells were washed 5 times with PBS. Then a substrate solution containing tetramethylbenzidine (Sigma-Aldrich, Oakville, ON, Canada) was added to every well, followed by incubation in the dark for 30 minutes. The reaction was then blocked by the addition of 1 ml 2 N sulfuric acid and the absorbance measured in a plate reader at 450 nm (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA).
RESULTS

Resting measurements

Daily measurements of end-tidal PCO$_2$ (*i.e.* PET$_{CO_2}$-RA) during room-air breathing showed a significant decrease over time (P < 0.01). Compared with values at Bsl, every data point during the 4 days of IH was lower while PET$_{O_2}$-RA was not significantly altered (P = 0.06) (Table 1).

Similar to the findings for PET$_{O_2}$-RA, PET$_{O_2}$-ISO was unchanged but there was a small but significant decrease in PET$_{CO_2}$-ISO (P ≤ 0.01) throughout the exposure to IH. Every daily measurement, except Day 8, was lower than Bsl. There were no changes in the ventilatory data at Bsl (*i.e.*, $V_E$-ISO, $f_R$-ISO and $V_T$-ISO) (Table 1).

Acute hypoxic ventilatory response (AHVR)

As presented in the main manuscript (see Results section), we observed a significant increase in $V_T$ over time (P<0.01), but no change in $f_R$ over the four days of exposure to IH compared to baseline. Compared to baseline, AHVR was also increased throughout the four days of exposure to IH (*i.e.* from Day 1 to Day 4). However, AHVR was returned to baseline 4 days after the end of the exposure (*i.e.* on Day 8) (Table 2). More detailed information about AHVR can be found in another paper from our group involving the same procedures and subjects [7].
REFERENCES


TABLES

Table 1. Resting measurements.

Footnote: Room air end-tidal O\textsubscript{2} (P\textsubscript{ET\textsubscript{O\textsubscript{2}-RA}}) and CO\textsubscript{2} (P\textsubscript{ET\textsubscript{CO\textsubscript{2}-RA}}), isocapnic eucapnia end-tidal O\textsubscript{2} (P\textsubscript{ET\textsubscript{O\textsubscript{2}-ISO}}) and CO\textsubscript{2} (P\textsubscript{ET\textsubscript{CO\textsubscript{2}-ISO}}), tidal volume (V\textsubscript{T-ISO}), breathing frequency (f\textsubscript{R-ISO}) and ventilation (V\textsubscript{E-ISO}) data for the baseline measurements (Bsl), during 4 days of IH (Day 1-4) and the recovery day (Day 8) are displayed. The values are means ± SD for 9 subjects. ** P ≤ 0.01 vs. Bsl.

<table>
<thead>
<tr>
<th></th>
<th>Bsl</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 8</th>
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</thead>
<tbody>
<tr>
<td>P\textsubscript{ET\textsubscript{O\textsubscript{2}-RA}} (mmHg)</td>
<td>87.0 ± 1.5</td>
<td>88.0 ± 2.6</td>
<td>89.4 ± 4.1</td>
<td>89.7 ± 3.2</td>
<td>88.4 ± 2.1</td>
<td>87.4 ± 3.8</td>
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<tr>
<td>P\textsubscript{ET\textsubscript{CO\textsubscript{2}-RA}} (mmHg)</td>
<td>36.4 ± 1.6</td>
<td>35.0 ± 1.5**</td>
<td>33.9 ± 2.0**</td>
<td>33.9 ± 2.3**</td>
<td>34.2 ± 1.9**</td>
<td>35.8 ± 2.1</td>
</tr>
<tr>
<td>P\textsubscript{ET\textsubscript{O\textsubscript{2}-ISO}} (mmHg)</td>
<td>87.6 ± 1.0</td>
<td>88.0 ± 1.1</td>
<td>87.4 ± 1.0</td>
<td>88.2 ± 1.1</td>
<td>88.0 ± 0.9</td>
<td>87.8 ± 0.9</td>
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<tr>
<td>P\textsubscript{ET\textsubscript{CO\textsubscript{2}-ISO}} (mmHg)</td>
<td>37.9 ± 1.2</td>
<td>36.4 ± 1.4**</td>
<td>35.7 ± 1.7**</td>
<td>35.4 ± 1.6**</td>
<td>35.5 ± 1.9**</td>
<td>37.2 ± 1.4</td>
</tr>
<tr>
<td>V\textsubscript{E-ISO} (l/min)</td>
<td>10.5 ± 2.2</td>
<td>10.8 ± 2.4</td>
<td>10.9 ± 3.1</td>
<td>11.8 ± 1.8</td>
<td>11.9 ± 2.9</td>
<td>11.4 ± 2.1</td>
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<td>V\textsubscript{T-ISO} (l/min)</td>
<td>0.83 ± 0.39</td>
<td>0.79 ± 0.26</td>
<td>0.85 ± 0.34</td>
<td>0.85 ± 0.25</td>
<td>0.85 ± 0.29</td>
<td>0.81 ± 0.30</td>
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<tr>
<td>f\textsubscript{R-ISO} (/min)</td>
<td>14.1 ± 5.0</td>
<td>15.2 ± 4.9</td>
<td>14.3 ± 5.8</td>
<td>15.0 ± 5.6</td>
<td>15.0 ± 4.8</td>
<td>15.3 ± 4.2</td>
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</table>
Table 2. Acute hypoxic ventilator response (also see supplemental ref. [7] for more detailed results on AHVR).

Footnote: Acute hypoxic ventilator response (AHVR) data for the baseline measurements (Bsl), during 4 days of IH (Day 1-4) and the recovery day (Day 8) are displayed. The values are means ± SD. for 9 subjects. * P ≤ 0.05 vs. Bsl; ** P ≤ 0.01 vs. Bsl.

<table>
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<th>Day 1</th>
<th>Day 2</th>
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<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHVR (l/min/mmHg)</td>
<td>0.90 ± 0.4</td>
<td>1.35 ± 0.8*</td>
<td>1.46 ± 0.9**</td>
<td>1.48 ± 0.9**</td>
<td>1.59 ± 0.9**</td>
<td>1.11 ± 0.8</td>
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