SUPPLEMENTAL MATERIAL

Intermittent hypoxia increases arterial blood pressure in humans through a renin-angiotensin-system dependent mechanism.

Supplemental Methods

Subjects

We studied ten healthy male volunteers who met the inclusion/exclusion criteria for the study. Subjects were included if they were between 19 and 45 years, were free from cardiovascular and respiratory disease and, at the time of study, had resided in Calgary (elevation 1,100m) for at least one year. Subjects were excluded if they had a prior diagnosis or history of cardio-pulmonary and renal disease (urinary protein excretion >150mg per 24hrs; estimated glomerular filtration rate <60 ml min⁻¹ 1.73m⁻²), obesity (BMI>35 kg m⁻²), sleep apnea (RDI>10 events hr⁻¹, confirmed by home cardio-pulmonary monitoring during sleep), smoking within the past year, hypertension (systolic > 140 mmHg; diastolic > 90 mmHg), and a high fasting blood glucose level (≥7 mmol) during the initial screening visit. These criteria helped to confirm that all subjects were healthy and did not have medical conditions that could alter cardio-respiratory function and RAS activity. Females were not studied because previous research has shown that female rats may be protected from the hypertension and tachycardia induced by IH. Furthermore, fluctuations in estrogen throughout the menstrual cycle may affect the RAS response to IH.

Experimental Protocols

Subjects attended the Laboratory of Human Cerebrovascular Physiology at the University of Calgary on four separate occasions to complete one screening session and three experimental protocols described below (in print Figure 1). Each protocol was separated by a minimum of one week, occurred at the same time of day, and were randomized in a single blind fashion. The LOSARTAN and PLACEBO protocols occurred in a double blind, randomized, crossover design. For each of the three experimental protocols subjects were asked to control their diet. On each testing day and the three days leading up to the experimental session, subjects were instructed to consume the same foods at similar times of day. This information was recorded on a dietary diary. On the morning of each experimental session, subjects provided a urine sample to estimate sodium balance using the method described by Tanaka et al. to ensure a similar salt state on each study day. Subjects were also provided with an automatic blood pressure monitoring device (model HEM-775, Omron Healthcare, Burlington, ON, Canada) and instructed to measure their blood pressure three times a day (morning, afternoon, and evening) over the three days leading up to each experimental day. Subjects were asked to make three blood pressure measures at each time of day, and the average of these three values was taken as their resting blood pressure.

Screening Session

During the screening session subjects were able to view the experimental set-up, ask the investigators questions, and provide written informed consent. Subjects completed a short questionnaire to assess their inclusion/exclusion criteria as described above. In addition, subjects provided a fasting venous blood sample taken from the antecubital vein to assess the blood glucose level. Further, 5 mL of venous blood was collected into one serum separator tube, centrifuged and the serum aliquoted into 1.5 mL microcentrifuge tubes and analyzed for serum creatinine for estimation of glomerular filtration rate. Proteinuria was estimated by urinalysis. Subject characteristics were also measured during this time (i.e. age, height, weight). Finally, all subjects were instructed how to set-up and perform an unattended, continuous, overnight, home cardiopulmonary monitoring study (Remmers Sleep Recorder Model 4.2, Saga Tech Electronic, Calgary, AB, Canada). This device consists of an oximeter to record oxyhemoglobin saturation and heart rate variability, a pressure transducer to record nasal airflow, a microphone to record snoring, and a body position sensor. The oximeter provides the data for an automated scoring algorithm, which calculates the respiratory disturbance index (RDI) based on the number of episodes of oxyhemoglobin desaturation greater than 4% divided by the duration of recording. The raw data were reviewed for each subject by an experienced sleep medicine physician.
(P.J.H.). The Remmers recorder has been validated by comparison to attended polysomnography.6,7

SHAM IH Protocol

A time control study was completed to ensure that any observed changes were due to exposure to IH and not to diurnal variation. Subjects reported to the laboratory at 8:00am. The protocol began with baseline cardiovascular, cerebrovascular, and respiratory measurements. Following baseline measurements, the cardiovascular, cerebrovascular, and respiratory responses to acute isocapnic hypoxia were conducted before and after exposure to 6 hours of SHAM IH or normoxia. The experimental set-up was identical to that used to administer IH. Fifteen minutes following the afternoon acute hypoxia measurements, a period of recovery measurements was made. A typical experimental day lasted approximately 8 – 8.5 hours.

PLACEBO IH Protocol

PLACEBO was identical to SHAM except that placebo tablets were taken for 4 consecutive days with the last placebo tablet being taken approximately one hour prior to the beginning of baseline measurements. In addition, IH was administered rather than SHAM IH.

LOSARTAN IH Protocol

LOSARTAN was identical to PLACEBO except that subjects were pre-treated with once daily doses of the AT1R antagonist, losartan, for 4 consecutive days. The dose of losartan was 25mg on day 1, 50mg on day 2, and 100mg on days 3 and 4. The last tablet was taken approximately one hour prior to baseline measurements on the experimental day.

Experimental Techniques

Baseline and Recovery Measurements

Resting measurements of PETO2, PETCO2, blood pressure (MAP, SBP, DBP), heart rate, and indices of cerebral blood flow (Vp, V1WM, P, PViWM) were obtained as the subject rested quietly and comfortably for 10 minutes while breathing room air through a mouthpiece. These measures were made prior to the morning acute hypoxia measurements and again 15 minutes following the afternoon acute hypoxia measurements. Respired gases were sampled via a fine catheter at the mouth. The gas was sampled continuously at a rate of 20 ml/min and analyzed for PO2 and PCO2 by mass spectrometry (AMIS 2000, Innovision, Odense, Denmark). A computer was used to acquire values for PO2 and PCO2 every 10 ms and the values for PETO2 and PETCO2 were determined and recorded for each breath using dedicated software (Chamber V2.43, University Laboratory of Physiology, Oxford, UK). All cardiovascular parameters were acquired every 10 ms and the values for each determined beat-by-beat by specifically designed computer software (BreatheM V2.40, University Laboratory of Physiology, Oxford, UK). Baseline and recovery values were taken as a 3-minute average of breath-by-breath and beat-by-beat data during the final 3 minutes of the 10-minute baseline period.

Morning and Afternoon Acute Isocapnic Hypoxia Measurements

Following baseline measurements, the subject continued breathing through a mouthpiece with the nose occluded by a nose clip. The protocol began with a 5-minute period of isocapnic euoxia (PETO2 = 88.0 mmHg and PETCO2 = +1.5 mmHg above rest) followed by six cycles comprised of 90 seconds of isocapnic hypoxia (PETO2 = 45.0 mmHg and PETCO2 = +1.5 mmHg above rest) and 90 seconds of isocapnic euoxia. Respiratory volumes and flow were obtained by using a pneumotachograph and differential pressure transducer (RSS 100-HR, Hans Rudolf Inc., Kansas City, MO, USA). Respiratory flow direction and timing were measured by using a turbine volume transducer (VMM-400, Interface Associates, CA, USA).
Accurate control of end-tidal gases was achieved by using the technique of dynamic end-tidal forcing (BreatheM V2.40, University Laboratory of Physiology, Oxford, UK) as previously described. All measurements described above were also recorded throughout acute isocapnic hypoxia. Data from each cycle of hypoxia and euoxia were interpolated at a 1s interval, overlaid, and averaged together to create a single 3-minute cycle of euoxia and hypoxia using specifically designed software created in Matlab (V7.4.0.287, MathWorks, Inc., MA, USA).

Intermittent Hypoxia Exposure

IH consisted of 6 hours of continuous cycles of one-minute of hypoxia (nadir PETO2 = 45.0 mmHg) and one-minute of normoxia (peak PETO2 = 88.0 mmHg). IH was delivered by using a purpose-built normobaric chamber. Periods of normoxia were constituted by delivering 100% oxygen to the subject’s inspirate through a facemask (mirage NV Series 2, Resmed, New South Wales, Australia) connected to a two-way non-rebreathing valve (2600 Series, Hans Rudolph, Kansas, USA) and a 25cm long section of wide bore tubing. During periods of normoxia, oxygen flowed through the inspired circuit at a rate that provided a PETO2 = 88.0 mmHg. PETCO2 was also controlled during IH by adding 100% CO2 to the subject’s inspirate at a flow rate that maintained PETCO2 constant. Computer-controlled gas solenoid valves were used to turn the flow of oxygen and carbon dioxide on and off at 60 second intervals. During exposure to IH, respired gas was sampled from a nasal cannula and analyzed by a dual oxygen and carbon dioxide analyzer (NormocapOxy, Datex-Ohmeda, Louisville, CO, USA) for PO2 and PCO2. These values were acquired every 10ms by a computer, and PETO2 and PETCO2 were identified and recorded for each breath using a computer and dedicated software (LabChart V6.13 and Powerlab/16SP, ADInstruments, Colorado Springs, CO, USA). SaO2 was recorded continuously throughout the exposure by means of an ear probe and pulse oximetry (3900, Datex-Ohmeda, Louisville, CO, USA). The same set-up was used for sham IH except that gas in the chamber was kept at a normoxic level (i.e., PETO2 = 88 mmHg), and compressed air, instead of 100% oxygen, was administered through the wide bore tubing at 60-second intervals.

RAS measurements

Blood samples taken for the measurement of plasma renin activity (PRA) and aldosterone were drawn in the morning before the acute isocapnic hypoxia measurements and repeated in the afternoon after the acute isocapnic hypoxia measurements. The details for each collection are outlined below:

**Plasma Renin Activity (PRA):** Venous blood samples were collected into two 6.0 mL EDTA coated tubes. Collected samples were immediately centrifuged and the separated plasma was aliquoted into 1.5 mL flat top microcentrifuge tubes. The separated plasma was stored at -80°C until later assayed. PRA was assayed by radioimmunoassay techniques (RIA) and the PRA assessed by measuring the amounts of angiotensin I generated per hour.

**Aldosterone:** Venous blood was collected into two 5.0 mL serum separator tubes. The sample was centrifuged 20 minutes after its collection, and serum was aliquoted into 1.5 mL flat top microcentrifuge tubes. The separated serum was stored at -80°C until later assayed. Aldosterone was assayed by RIA techniques.

**Plasma endothelin-1 measurements**

Venous blood samples were collected into two 6.0 mL EDTA coated tubes. Collected samples were immediately centrifuged and the separated plasma was aliquoted into 1.5 mL flat top microcentrifuge tubes. The separated plasma was stored at -80°C until later assayed. Concentrations of plasma endothelin-1 was determined using an ELISA kit from R&D Systems.
(R&D Systems, Inc. Minneapolis, MN, USA) and in following the procedures provided by the manufacturer.

Capillary Blood Samples

Capillary (i.e., arterialized) blood samples (200 µl) were taken in the morning immediately before the acute isocapnic hypoxia measurements and repeated in the afternoon following the acute isocapnic hypoxia measurements. These samples were taken from a small puncture on the finger and analyzed for acid-base status and glucose.
**Supplemental Results.**

Estimated 24hour sodium excretion: SHAM = 126 ± 7; PLACEBO = 116 ± 6; LOSARTAN = 123 ± 10 mmol day^{-1}; \( p=0.49 \)

MAP in the morning, afternoon and evening during the three days prior to each experimental day:

SHAM: Day-3 = 87 ± 2; Day-2 = 88 ± 2; Day-1 = 88 ± 2 mmHg; PLACEBO: Day-3 = 88 ± 2; Day-2 = 87 ± 2; Day-1 = 87 ± 2 mmHg; LOSARTAN: Day-3 = 87 ± 2; Day-2 = 86 ± 2; Day-1 = 85 ± 2 mmHg; \( p=0.23 \)

Arterialized blood pH: 7.42 ± 0.01 for SHAM and PLACEBO and 7.42 ± 0.00 for LOSARTAN (\( p=0.27 \)).

Bicarbonate ion concentration: 24.7 ± 0.2 for SHAM, 24.6 ± 0.3 for PLACEBO and 24.7 ± 0.2 mmol L^{-1} for LOSARTAN (\( p=0.89 \)).
Supplemental References

**Supplemental Tables**

Table S1. Indices of hypoxemia and reoxygenation.

<table>
<thead>
<tr>
<th>Variables</th>
<th>SHAM</th>
<th>PLACEBO</th>
<th>LOSARTAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>ODI, desaturations hr⁻¹</td>
<td>0.0 ± 0.0</td>
<td>28.8 ± 0.2*</td>
<td>28.7 ± 0.1*</td>
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<tr>
<td>% time SaO₂&lt;90%</td>
<td>0.6 ± 0.1</td>
<td>23.1 ± 1.9*</td>
<td>25.7 ± 1.0*</td>
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<td>% time SaO₂&lt;85%</td>
<td>0.0 ± 0.0</td>
<td>2.2 ± 0.7†</td>
<td>3.6 ± 1.2†</td>
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<tr>
<td>% time SaO₂&lt;80%</td>
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<td>0.1 ± 0.1</td>
<td>0.2 ± 0.2†</td>
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<tr>
<td>Mean Minimum SaO₂, %</td>
<td>94.5 ± 0.4</td>
<td>86.0 ± 0.4*</td>
<td>85.2 ± 0.3*</td>
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<tr>
<td>Mean Maximum SaO₂, %</td>
<td>96.4 ± 0.1</td>
<td>95.8 ± 0.2</td>
<td>96.0 ± 0.2</td>
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<tr>
<td>Mean SaO₂, %</td>
<td>95.7 ± 0.2</td>
<td>92.3 ± 0.3*</td>
<td>92.2 ± 0.2*</td>
</tr>
</tbody>
</table>

*p<0.001; †p<0.05 compared to SHAM; Definition of abbreviations: ODI = oxygen desaturation index (number of times SaO₂ decreases by greater than or equal to 4%); Mean minimum SaO₂ = the mean nadir for all SaO₂ desaturations; Mean maximum SaO₂ = the mean peak for all SaO₂ resaturations.
Supplemental Figures.

Figure S1A.
Figure S1B.
Figure S1C. Representative data traces from three different subjects (A-C) during exposure to SHAM and intermittent hypoxia (PLACEBO and LOSARTAN).

Footnote: The first and last 20 minutes of the 6-hour exposure are expanded. Breaks in the data occur during lunch and washroom breaks and if the pulse oximeter signal was lost. Definition of abbreviations: PETO2 = end-tidal partial pressure of oxygen; PETCO2 = end-tidal partial pressure of carbon dioxide; SaO2 = arterial oxyhemoglobin saturation.