Eur Respir J 2011; 37: 880–887
DOI: 10.1183/09031936.00156009

Effects of intermittent hypoxia on erythropoietin, soluble erythropoietin receptor and ventilation in humans

J.V. Brugniaux*,#,¶,+, V. Pialoux*,¶,+, G.E. Foster*,¶, C.T.C. Duggan*,¶, M. Eliasziw¶,§,ƒ,, P.J. Hanly#,¶,## and M.J. Poulin*,#,¶,+,§,¶¶**

ABSTRACT: Erythropoietin (EPO) and soluble EPO receptors (sEPOR) have been proposed to play a central role in the ventilatory acclimatisation to continuous hypoxia in mice.

In this study, we demonstrated for the first time in humans (n=9) that sEPOR is downregulated upon daytime exposure to 4 days of intermittent hypoxia (IH; 6 h·day^{-1}, cycles of 2 min of hypoxia followed by 2 min of reoxygenation; peak end-tidal oxygen tension (P_{ET,O_2}) 88 Torr, nadir P_{ET,O_2} 45 Torr), thereby allowing EPO concentration to rise. We also determined the strength of the association between these haematological adaptations and alterations in the acute hypoxic ventilatory response (AHVR).

We observed a nadir in sEPOR on day 2 (-70%), concomitant with the peak in EPO concentration (+50%). Following exposure to IH, tidal volume (V_T) increased, respiratory frequency remained unchanged, and minute ventilation (V'_E) was increased. There was a negative correlation between EPO and sEPOR (r= -0.261; p=0.05), and between sEPOR and V_T (r= -0.331; p=0.02). EPO was positively correlated with V'_E (r=0.458; p=0.001).

In conclusion, the downregulation of sEPOR by IH modulates the subsequent EPO response. Furthermore, the alterations in AHVR and breathing pattern following IH appear to be mediated, at least in part, by the increase in EPO.

KEYWORDS: Erythropoietin, hypoxic ventilatory response, soluble erythropoietin receptor

In humans, the increase in erythropoietin (EPO) concentration in response to hypoxia depends upon the duration and severity of hypoxia [1], with noticeable increases in the plasma concentration of EPO after short exposures to hypoxia (altitude ~4,000 m for ~84 min) [2], reaching peak levels after ~48 h [3]. Nocturnal hypoxia (12–14 h·night^{-1}) has also been shown to stimulate EPO secretion, but the elevation of EPO concentration is not sustained [4]. Intermittent hypoxia (IH), as experienced by patients with obstructive sleep apnoea (OSA), has not consistently been shown to be associated with increased concentration of EPO. Some studies reported no systematic changes in EPO [5–7], while in contrast, elevated levels of EPO, persisting during daytime, have already been reported in OSA patients [8]. The mechanisms underlying this discrepancy have not been elucidated yet, but one could argue that factors such as different levels of severity of OSA or comorbidities often associated with OSA may play a role.

From a clinical perspective, EPO has been used for two decades to treat chronic anaemia and is routinely used in both the prevention and/or correction of anaemia associated with renal failure [9]. More recently, extensive research on the mechanisms of action of EPO led to the discovery of potential new roles for this protein. Based on animal experiments, it has been proposed that EPO plays a role in the ventilatory acclimatisation to hypoxia (VAH) and modulates the neural control of breathing [10]. In wild-type mice, a systemic injection of recombinant human EPO (rhEPO) leads to binding of EPO to EPO receptors (EPOR) in the carotid bodies, which is believed to increase the hypoxic ventilatory response (HVR). The breathing pattern of transgenic mice overexpressing EPO in the brain [10] and those overexpressing EPO in the brain and in the blood [11] has also been studied. Thus, at least in experimental animals, EPO appears to influence the central control of breathing and to modulate HVR. A recent experiment by the same

This article has supplementary material available from www.erj.ersjournals.com

AFFILIATIONS
*Dept of Physiology and Pharmacology,
#Hotchkiss Brain Institute,
¶Faculty of Medicine,
+Faculty of Kinesiology,
§Dept of Clinical Neurosciences,
ƒDept of Community Health Sciences,
**Depts of Oncology,
##Dept of Medicine, and
¶¶Libin Cardiovascular Institute of Alberta, University of Calgary, Calgary, Canada.

CORRESPONDENCE
M.J. Poulin
Dept of Physiology and Pharmacology, Faculty of Medicine
University of Calgary
Calgary
Alberta
T2N 4N1
Canada
E-mail: poulin@ucalgary.ca

Received:
Oct 03 2009
Accepted after revision:
Aug 01 2010
First published online:
Oct 14 2010

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

group [12] showed there is a sex dimorphism, with female subjects exhibiting a greater increase in ventilation than their male counterparts after a single injection of EPO at the systemic level (5,000 U rhEPO). However, very little is known in healthy humans about the effect of endogenous secretion of EPO under conditions of sustained or intermittent hypoxia, especially with respect to short bouts of hypoxia.

The effect of EPO is related to its binding on the EPOR, which is present in several anatomical locations, including the carotid bodies, the pre-Bötzinger complex and the nucleus tractus solitarii [10]. A soluble form of the EPOR (sEPOR), corresponding to the extracellular domain of the EPOR, has been identified in several tissues [13], including human plasma [14]. Soluble EPOR competes with the EPOR to bind EPO, thereby limiting the ability of EPO to bind EPOR [15]. Recently, SOLIZ *et al.* [16] demonstrated in mice that downregulation of sEPOR in the brain, induced by 3 days of sustained hypoxia (10% O_2), is necessary to allow the VAH to unfold. Furthermore, the authors showed that the infusion of sEPOR directly in the brain not only blunted VAH, but also downregulated the cerebral expression of both EPO and EPOR. This is consistent with the hypothesis that the underlying mechanism whereby EPO influences the control of breathing is determined by a balance between increased EPO and decreased sEPOR.

EPO is now a major area of research regarding its protective effect against ischaemia in the heart. Although it is not clear that chronic IH induces an increase in EPO in patients with OSA, the expected change is an increase in chemosensitivity and, thus, greater breathing instability. The present study is of great clinical interest since it will clearly help in understanding crucial clinical issues such as this one.

While it has been proposed that EPO (at nonphysiological concentrations) controls ventilation both centrally and peripherally, there is no clear evidence whether sEPOR expression in the plasma plays a similar role in this regulation loop as it does in the brain. Furthermore, very little is known about this regulation loop in humans. Therefore, we hypothesised that healthy humans would exhibit downregulation of plasma sEPOR and upregulation of EPO during exposure to 4 days of IH mimicking OSA in waking healthy subjects. Furthermore, we hypothesised that the IH-mediated changes in the expression of EPO and sEPOR would be associated with changes in breathing pattern (tidal volume (V_T) and respiratory frequency (f_R)) and the VAH.

METHODS

The present work is part of a larger study using an experimental human model of IH mimicking the type of IH experienced by patients with moderate OSA (but during daytime, *i.e.* while the participants are awake) [17, 18]. In these experimental studies, we aimed to investigate the effects of IH on the vasculature (both cardio- and cerebrovascular) and on control of breathing. The present study falls into the latter group, along with another part dealing with the relationship between oxidative stress and HVR. Our group recently published two articles, involving the same subjects and protocol, on both the cardiovascular and cerebrovascular regulation following exposure to IH [17] and the role of oxidative stress in the acute hypoxic ventilatory response (AHVR) induced by IH (*i.e.* hypoxaemia and reoxygenation cycles) [18].

Ethics approval

This study of EPO, sEPOR and breathing patterns was undertaken in conjunction with a study examining the cerebrovascular and cardiovascular responses to IH in humans [17]. The protocol was approved by the Conjoint Health Research Ethics Board at the University of Calgary (Calgary, Canada) and conformed to the Declaration of Helsinki. Written informed consent was obtained from each subject prior to participation in the study.

Subjects

Nine healthy male volunteers were recruited to participate in the study. They were mean$\pm$SD 29$\pm$5 yrs of age, and had a height and weight of 173.8$\pm$9.5 cm and 76.2$\pm$10.6 kg, respectively. The average body mass index was 25.1$\pm$1.4 kg·m^{-2}. None of the volunteers smoked cigarettes and none had a history of cardiovascular, cerebrovascular or respiratory diseases. All subjects were residents of Calgary (altitude 1,100 m) for $\geqslant$1 yr prior to enrollment.

Protocol

The experiments were conducted in our laboratory, located at 1,103 m above sea level, and the average barometric pressure during the study days was 663$\pm$1 mmHg. This protocol has been described previously [17]. Briefly, subjects attended the laboratory on seven different days over a 13-day period. The first two days (days -4 and 0) served as control days, during which subjects were exposed to 6 h sham IH. On the following four consecutive days (days 1–4), subjects were exposed to 6 h IH. 4 days after the last day of exposure (day 8), subjects returned to the laboratory for a follow-up assessment. Daily exposures to either sham IH or IH were conducted during daytime and the subjects were instructed not to sleep. At the end of each experimental day (days -4, 0, 1–4 and 8), a graded hypoxic test was performed to assess the breathing pattern. This test was performed ~30 min after the end of the daily exposure to either sham IH or IH, and at the same time of day on day 8.

Blood samples were taken on days -4, 0, 1, 2, 4 and 8, ~15 min after the subjects exited the chamber to assess the serum concentrations of EPO and the plasma concentration of sEPOR. Ventilatory parameters (minute ventilation (V'_E), V_T, f_R and AHVR) were evaluated on days -4, 0, 1, 2, 3, 4 and 8.

Procedures

Full details of the ventilatory measurements are provided in the online supplementary material.

IH exposure

During the 4 days of IH (days 1–4), subjects were exposed to 6 h of continuous cycles of 2 min hypoxia (nadir end-tidal oxygen tension (P_{ET,O_2}) 45.0 mmHg) and 2 min normoxia (peak P_{ET,O_2} 88.0 mmHg). Further details of this protocol are published elsewhere [17].

Blood samples and analysis

Blood sample collection is described in the online supplementary material. Both serum concentration of EPO and plasma

concentration of sEPOR were determined by ELISA. Briefly, EPO concentration was determined using an ELISA kit (R&D Systems, Minneapolis, MN, USA), while we used slightly modified version of the method described by WESTPHAL *et al.* [14] to assess sEPOR. A detailed description of these methodologies can be found in the online supplementary material.

Statistical analysis

Except for the anthropometric data and data presented in the online supplementary material, all data are presented as mean (95% CI). Two out of the 54 blood samples were not included in the statistical analysis. In one subject, the blood data were not collected on day 1 due to technical difficulties. In another volunteer, the concentration of sEPOR on day 8 was more than five-fold higher (13.2 SD) than the mean and, as such, it was treated as an outlier and removed from further analyses. This datapoint was treated as an outlier on the basis that it fell outside of the $\pm$2-SD boundary. Ventilatory and haematological data for the two control days, *i.e.* days -4 and 0, were not statistically different and, therefore, these two baselines were combined for further analysis and are termed ''baseline'' (Bsl) for the remainder of this article. The data for the ventilatory components (V'_E, V_T and f_R) and blood markers (EPO and sEPOR) were analysed using a one-way repeated-measure ANOVA. A Dunnett–Hsu test was then applied to identify significant differences between means. Linear regression analysis (with corresponding correlation coefficients (r)) was used to analyse the overall relationship between the changes in blood markers (EPO and sEPOR) and ventilatory components (V'_E, V_T and f_R). Generalised linear modelling (GLM; with corresponding multiple correlation coefficients (mR)) was used to analyse the relationship between the changes in blood markers and ventilatory components across the days of study. A likelihood ratio test was used to assess whether the relationships significantly differed from each other depending upon the day of study. A mixed-model regression analysis with an autoregression correlation structure was used to describe the relationship between V'_E and time while adjusting for a subjects' level of EPO at each time-point as a time-varying covariate. Statistics were performed using the analytical software SPSS (version 16.0; SPSS Inc., Chicago, IL, USA) and with SAS (version 9.2; SAS Institute Inc., Cary, NC, USA). The level of significance was set at $p \leqslant 0.05$.

RESULTS

Resting measurements

Resting ventilatory data are outlined in online supplementary table 1.

Effects of exposure to IH on the components of ventilation

Figure 1 illustrates changes in the components of minute ventilation measured during isocapnic hypoxia. We observed an increase in V_T over time ($p=0.001$) with a plateau between days 1 and 4 (+20% *versus* Bsl), followed by a return to Bsl level 4 days after the end of the exposure (*i.e.* day 8). Conversely, f_R did not change significantly throughout the study ($p=0.11$). Collectively, these changes resulted in an increase in total ventilation over time ($p=0.004$), with every data point being higher than Bsl, except day 8. Similar to V_T, we observed that V'_E reached a plateau at day 1, which persisted during the exposure to IH before returning to Bsl level 4 days after the completion of exposure. More detailed results for AHVR are presented elsewhere [18], and a brief overview is provided in the online supplementary material.

Effects of IH on the secretion of EPO

Figure 2a illustrates that our model of IH stimulated the secretion of EPO over time ($p=0.04$). The peak in EPO secretion appeared on day 2 (+50% *versus* Bsl; $p=0.003$). On day 4, the stimulation of EPO remained significantly higher

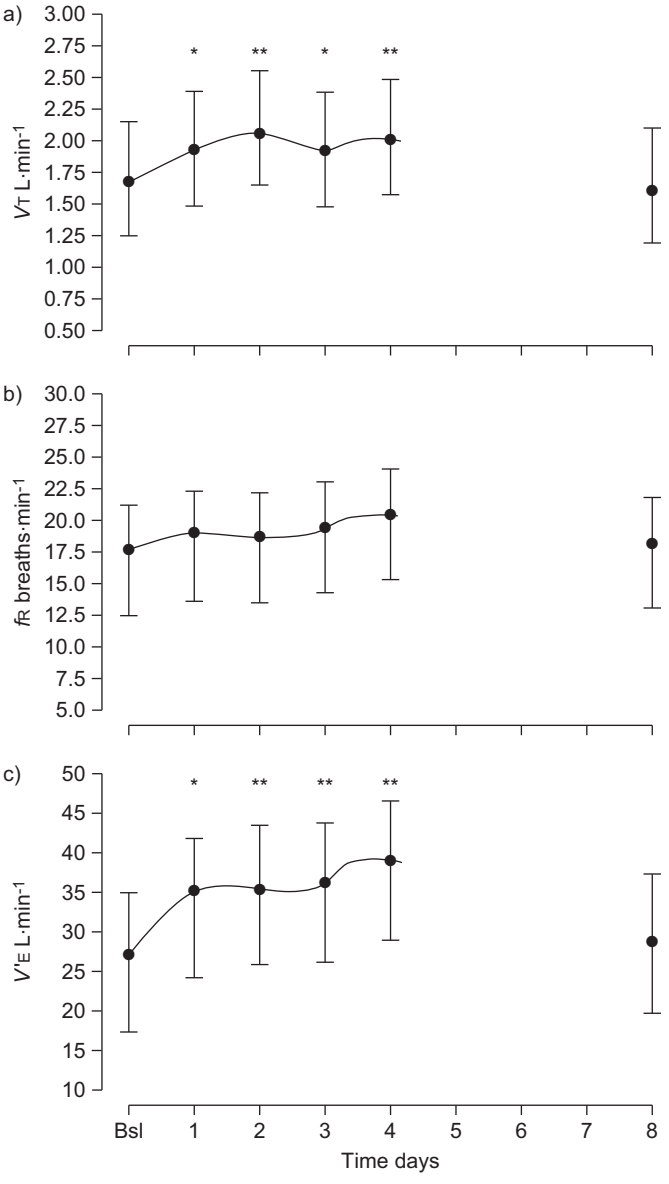

FIGURE 1. Alterations in ventilation over 4 days of intermittent hypoxia. The changes in a) tidal volume (V_T), b) respiratory frequency (f_R) and c) minute ventilation (V'_E) during an acute hypoxic test (end-tidal oxygen tension 45.0 mmHg and end-tidal carbon dioxide tension 1.5 mmHg above resting) are shown. V_T increased from day 1 to 4 compared with baseline (Bsl), while f_R did not change. As a result, V'_E increased during the hypoxic exposure. Data are presented as means with whiskers representing 95% CI for the nine participants. *: $p \leqslant 0.05$ *versus* Bsl; **: $p \leqslant 0.01$ *versus* Bsl.

than baseline level (+36% *versus* Bsl; p=0.02) and, thereafter, the EPO concentration returned to Bsl values by day 8.

Effects of IH on the plasma sEPOR concentration

During normoxia, the concentration of sEPOR was similar to what has been proposed by WESTPHAL *et al.* [14]. After exposure to IH, we observed a decline that started on day 1 (-43% *versus* Bsl; p=0.007), with a nadir on day 2 (-70% *versus* Bsl; p<0.001). Thereafter, sEPOR concentration returned towards Bsl on day 4 (-46% *versus* Bsl; p=0.002) and remained at the same level 4 days after the end of the exposure, *i.e.* on day 8 (fig. 2b). We also studied the EPO/sEPOR ratio; we observed a similar biphasic evolution similar to EPO but not significant (p=0.124; data not shown). As discussed later, we observed ''responders'' and ''nonresponders'', which may, at least partly, explain this result.

Relationships between EPO and sEPOR, ventilatory components, and hypoxic sensitivities

We observed an overall inverse relationship between sEPOR and EPO (r=0.26; p=0.05; fig. 3a) while, according to the GLM model, the relationship between EPO and sEPOR depended upon study day but was not significant (r=0.41; p=0.77; fig. 3b). There was an inverse relationship only on days 1 and 2, and no relationship at Bsl, and days 4 and 8.

There was a relatively strong overall positive correlation between EPO and V'_E during the AHVR test (r=0.46; p=0.001; data not shown). However, the results from the mixed model regression analysis, whereby the relationship between ventilation and time was adjusted for EPO at each time-point, suggests that EPO has very limited effect on V'_E, as measured during the AHVR (fig. 4). This is evident, as the unadjusted and adjusted means are close to each other.

Overall, we also observed that the alterations in sEPOR correlated with those in V_T (r=0.33; p=0.02; data not shown).

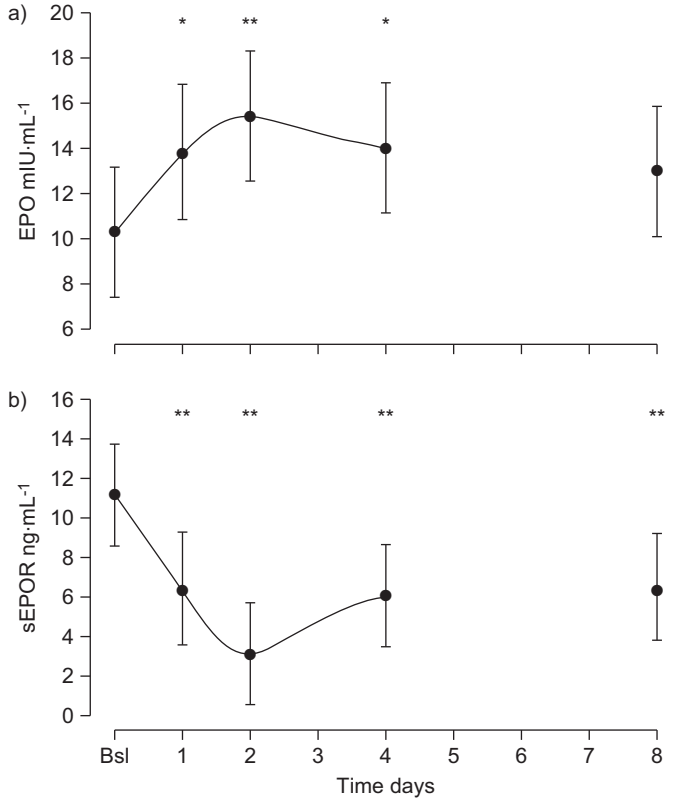

FIGURE 2. Serum a) erythropoietin (EPO) and b) soluble EPO receptor (sEPOR) over 4 days of intermittent hypoxia. The changes in EPO and sEPOR over time are shown. Note the increase in EPO concentration, starting on day 1 with a peak on day 2. Thereafter, EPO concentration decreased towards the baseline (Bsl) level but remained higher on day 4 compared to Bsl. 4 days after the end of hypoxic exposure, EPO concentration did not differ from Bsl. sEPOR decreased during the exposure to hypoxia (days 1–4), with a nadir on day 2 compared with Bsl. After 4 days of recovery from hypoxia, sEPOR concentration was still lower than Bsl. Data are presented as means with whiskers representing 95% CI for the nine participants. Data are missing for one subject on day 1 (for both EPO and sEPOR) and for another subject on day 8 (for sEPOR only). *: p≤0.05 *versus* Bsl; **: p≤0.01 *versus* Bsl.

DISCUSSION

Main findings

Four novel findings emerge from this study of IH in healthy human volunteers. First, we found a substantial increase in V'_E due to higher V_T as early as after 1 day of exposure to IH, which persisted over the 4 days of exposure (accompanied by a progressive increase in AHVR; see the online supplementary

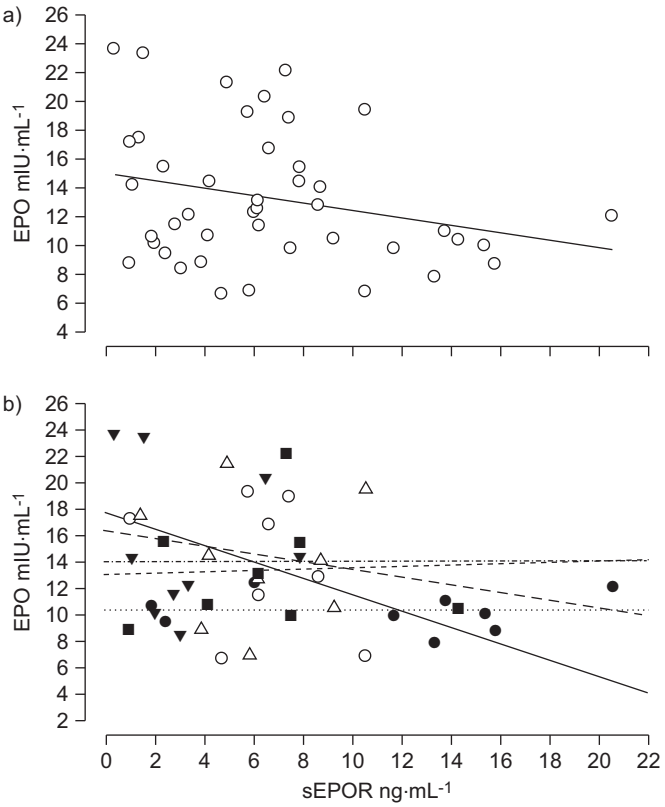

FIGURE 3. Correlation between erythropoietin (EPO) and soluble EPO receptor (sEPOR). a) Overall (r=0.26; p=0.05) and b) daily (mR=0.41; p=0.77) relationship between EPO and sEPOR (n=9). All individual data are shown except for one subject whose data for EPO and sEPOR were missing for day 1 and another subject whose data for sEPOR were missing for day 8. a) ○ and —: all data points. b) ● and ·····: baseline; ○ and —: day 1; ▼ and – – –: day 2; △ and -·-·-: day 4; ■ and ---: day 8.

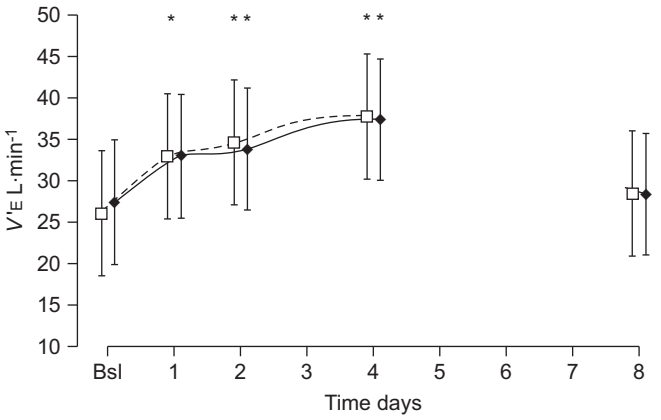

FIGURE 4. Mixed model regression analysis for the relationship between minute ventilation (V'_E) and time. This figure shows the changes in V'_E over the course of the experiment as presented in figure 1 (unadjusted; □) and adjusted for erythropoietin (EPO; ◆). No data are presented for day 3, as no blood sample was taken on that day. It appears that a systemic increase in EPO had very limited effect on the alterations in V'_E. Data are presented as means with whiskers representing 95% CI for the nine participants. *: $p \leqslant 0.05$ *versus* Bsl; **: $p \leqslant 0.01$ *versus* Bsl for both unadjusted an adjusted for EPO regression analyses.

material and [18]). Secondly, we demonstrated in humans that plasma sEPOR concentration is decreased during exposure to IH. Thirdly, we observed an increase in EPO concentration concomitant with the decrease in sEPOR. Finally, moderately strong associations were observed between EPO and V'_E, and between sEPOR and V_T, suggesting that both EPO and sEPOR may play a role in the increase in ventilation that is observed after daytime exposure to IH.

Alterations in breathing pattern at rest and in response to acute isocapnic hypoxia following exposure to IH

Signs of VAH we observed (*i.e.* increase in V_T with little change in f_R; fig. 1) are consistent with the changes in AHVR that are mediated by chronic exposure to sustained hypoxia (for review, see [19]). However, exposure to chronic IH, such as that experienced in patients with OSA, is a more complex paradigm [20]. While some studies have shown an increase in the ventilatory response to acute hypoxia with IH [21], others have not [22], and some have found a blunted ventilatory response to hypoxia in OSA patients [23]. In our model of IH, we observed an increase in V_T, V'_E and AHVR (see online supplementary material for an overview of AHVR results and [18] for a more detailed presentation). This is consistent with another study using a model of OSA in healthy subjects [24]. TAMISIER *et al.* [24] used a slightly different model from ours, insofar as the subjects were exposed during sleep and reoxygenated for 15 s every 2 min (*versus* 2 min of deoxygenation and 2 min of reoxygenation in the present study). Moreover, their subjects were exposed to $\leqslant$28 days of IH. They did not measure ventilatory acclimatisation during the first 4 days of exposure as we did, but they provide very useful information on the longer-term acclimatisation. They actually observed a progressive rise in hypoxic chemosensitivity after 7 and 14 days of exposure [24]. A number of factors may explain the discrepancies between these results using models of OSA and previous studies involving patients with OSA patients. For instance, it has been proposed that age, duration and severity of disease may play a role in the alterations in AHVR observed in patients with OSA [20]. In our study, subjects were healthy young males and no such confounding factors were implicated.

Interestingly, while we observed an increase in both V_T and V'_E as early as after 1 day of exposure, we did not observe a further increase during the following 3 days of exposure to IH. This is most likely due to the relatively short total time spent in hypoxia in the present study. In the study by TAMISIER *et al.* [24], it is likely that the increase in HVR they reported was mainly due to an increase in V_T. The fact that their subjects spent more time daily (9 *versus* 6 h IH) for a longer period of time (14 or 28 *versus* 4 days) suggests incomplete VAH in the present study.

Alterations in EPO and sEPOR following exposure to IH

In the present study, we report an increase in EPO concentration after the first day of exposure to IH, corresponding to 3 h of moderate hypoxia. CAHAN *et al.* [8] reported increased serum EPO concentration in OSA patients exposed to IH for several years, while others did not [5–7]. These diverging results may be explained by different degrees of severity in the patients studied and/or by confounding factors often associated with OSA. Adaptive changes in OSA patients might also explain the discrepancy; however this point remains somewhat speculative.

The general consensus is that EPO concentration reaches a peak after 2 days of exposure to chronic hypoxia [3]. We observed a similar pattern (*i.e.* a peak of EPO after day 2), but this was induced by a shorter period because of our pattern of exposure. Unfortunately, our data do not provide clear evidence of the underlying mechanisms.

This is the first human study in which plasma sEPOR concentration was measured following exposure to IH. We provide evidence that sEPOR is decreased following exposure to IH. Our findings are consistent with those reported by SOLIZ *et al.* [16] in mice, despite different hypoxic exposure paradigms (shorter duration and severity of hypoxia). Given that we observed a significant decrease after day 1 and a nadir on day 2, it appears that the sEPOR response is rapid and very sensitive to hypoxia. Therefore, the decrease reported by SOLIZ *et al.* [16] at the end of 3 days exposure to hypoxia may have underestimated the magnitude of this reduction. On day 8, sEPOR concentration was still lower than Bsl values. This observation might relate to the residual increase in AHVR that is occasionally observed following IH [25], but this explanation is not consistent with the present results. Therefore, this aspect of the sEPOR response warrants further study.

Another interesting observation is the large interindividual variability in the absolute sEPOR levels despite the global trend towards a decrease with a nadir on day 2. In fact, two out of nine subjects did not exhibit any decrease between Bsl and day 2 measurements, and these two ''nonresponders'' exhibited particularly low normoxic baseline levels of sEPOR compared with the other subjects. Similar to what is known for EPO, we observed groups of ''responders'' and ''nonresponders'' in the sEPOR response to IH. The dose of hypoxia to which our subjects were exposed was mild compared to the only other available study [16], and our subjects may have

responded differently to sustained hypoxia. Notably, one of these two subjects exhibited very limited changes in ventilation throughout the study.

In their animal model, SOLIZ *et al.* [16] proposed that the increase of EPO in the brain appears first, and then directly stimulates ventilation, while the decrease in sEPOR occurs later and maintains increased EPO availability. However, our data do not support this hypothesis, at least at the systemic level, since we found the patterns of EPO and sEPOR responses matched closely (but in opposite directions) exhibiting a peak and nadir, respectively, on day 2. Moreover, the negative relationship we observed between EPO and sEPOR on days 1 and 2 (fig. 3) seems to further corroborate this hypothesis despite the lack of significance according to the global GLM. Hence, in the circulating plasma, it seems that both EPO and sEPOR are finely tuned, with the release of EPO from the kidney [9] occurring at the same time as the decrease in circulating sEPOR concentration in order to maximise EPO bioavailability.

Interaction between alterations in Epo and both breathing pattern and HVR during IH

SOLIZ *et al.* [10, 11] worked on lines of mice over-expressing EPO either in the brain only (Tg21) [10] and in the brain and blood (Tg6) [11]. The authors concluded that central EPO overexpression alters the breathing pattern towards an increase in f_R, while systemic EPO overexpression modulates the response of the carotid bodies. These two studies concluded that EPO has the ability to stimulate ventilation. However, a recent study reported the effect of EPO injection in humans [12] and they observed a large difference in the ventilatory response between males and females, the latter being the only one positively affected by the single injection of 5,000 U rhEPO. GASSMANN *et al.* [26] recently pooled the results of their previous studies [10–12] and generated a schematic summarising the effect of EPO in light of the sex dimorphism [26]. Thosee authors proposed that the effects of increased EPO in the brain are not subject to sex dimorphism, while increased plasma EPO (either after a single injection or in their transgenic mice line) does induce sex dimorphism. In the present experiment, only males were studied and our results are consistent with the existing literature insofar as we did not observe a clear effect of EPO on V'_E (fig. 4). We therefore confirm that systemic increase in EPO has a very limited effect on ventilation, at least in adult males.

However, sustained elevation in plasma EPO concentration seems to have stimulating effects on the carotid bodies insofar as we observed an overall correlation between EPO and HVR over the entire study ($r=0.475$; $p=0.001$; data not shown). Unfortunately, based on recent literature (see above), the mechanisms underlying this observation remain unclear.

Because of the sustained elevation in serum EPO concentration we observed (fig. 2a) one might speculate that EPO could eventually cross the blood–brain barrier, a mechanism already demonstrated in rabbits [27, 28]. Similarly, astrocytes [29, 30] and neurons [30] of the hypoxic brain have also been shown to release EPO, both contributing to increasing its central concentration. Altogether, these mechanisms could ultimately lead to an increase in the central level of EPO, therefore gaining the ability to stimulate ventilation, but we acknowledge this remains speculative at this stage.

Interaction between alterations in sEPOR and breathing pattern during IH

Because of its strong affinity for EPO, sEPOR competes with EPOR to bind on EPO [15]. As a consequence, its decrease upon exposure to hypoxia allows more EPO to bind the EPOR and, in turn, to account for VAH [16]. Hence, sEPOR plays a central role in this process. In the present study, we report that sEPOR in the plasma is actually decreased during exposure to IH. Furthermore, we observed an association between sEPOR and V_T (data not shown). This result is consistent with recent reports in mice showing that constant infusion of sEPOR during 3 days of exposure to sustained hypoxia (10% O_2) not only maintained the sEPOR at its pre-exposure concentration but also abolished the ventilatory response to a subsequent AHVR test [16]. However, we acknowledge this relationship represents one of the involved parameters and, most likely, shows an indirect link between sEPOR and the ventilation centres.

Limitations

Despite its novelty we acknowledge that our study has some limitations. First, the innovative feature of the IH model we developed to simulate OSA is that it uses healthy subjects to unravel confounding factors often associated with OSA. However, there is a major difference between ''real'' OSA and our model of OSA in healthy subjects; our model is poikilocapnic hypoxia, while OSA involves hypercapnia. Unfortunately, another powerful model proposed by TAMISIER *et al.* [24] faced the same limitation. Another difference arising between our model and the IH experienced by patients with OSA is that the present study was conducted during daytime while the subjects were awake. Secondly, the GLM provides useful complementary information as to inter-day mechanisms. However, despite a sufficiently high power to detect differences in EPO and sEPOR (0.77 and >0.95, respectively) we suggest that our modest sample size may limit a full interpretation of the GLM analysis. Similarly, while this study highlights novel mechanisms supported by the existing literature, the associations we observed do not represent clear cause and effect relationships. For instance, the mechanisms relating EPO and HVR (*i.e.* central *versus* peripheral increase in EPO) warrant further research.

Summary and conclusions

In summary, our data demonstrate that sEPORs are down-regulated during daytime exposure to IH. Concomitant with the decrease in sEPOR, we observed an increase in EPO. We also confirmed that IH triggers an increase in V'_E mediated by a higher V_T, while f_R is not affected. However, we did not show a clear effect of EPO on either V'_E or the hypoxic ventilatory response, but we provide evidence of what is most likely an indirect relation between sEPOR and V_T. Taken together, these novel findings provide evidence for the role of sEPOR in the increase of ventilation during acute hypoxia following an exposure to IH. The role EPO plays is, however, not as clear. The present findings confirm the results of a recent

experiment [12] demonstrating that systemic EPO has little positive effect on HVR, especially in male subjects. Because of the sustained elevation in EPO concentration in the present study, we cannot exclude a more central and, therefore, positive effect on VAH, but this aspect clearly warrants further investigation. Therefore, it seems that the effects of an increase in Epo at the systemic level should be interpreted differently from those following the same increase at the central level. According to the present results, the involved mechanisms and their relative contribution along the acclimatisation process need further study to be fully understood.

Finally, the present study, along with another recently published study from our group on the same volunteers [18] highlighting the role of oxidative stress in the stimulation of HVR, describe two novel mechanisms working in conjunction to facilitate VAH. Indeed, multiple regression analysis revealed that, together, EPO and oxidative stress (8-hydroxy-29-deoxyguanosine; 8-OHdG) explain 40% (*i.e.* $r^2=0.40$; $p<0.001$) of the HRV responses. Additionally, both variables are significant predictors of HVR (EPO $\beta=0.49$ ($p<0.001$) and 8-OHdG $\beta=0.39$ ($p<0.001$)) and are not correlated together, suggesting that EPO and oxidative stress modulate HVR independently. These results provide insight into new potential physiological mechanisms to understand how the control of breathing is altered with IH. Moreover, the present study may provide important avenues to potential future therapeutic approaches, as sEPOR has been demonstrated to have the ability to abolish VAH and, thus, may stabilise breathing.

SUPPORT STATEMENT

This study was supported by an Establishment Grant from the Alberta Heritage Foundation for Medical Research (AHFMR; M.J. Poulin), a Grant-in-Aid from the Heart and Stroke Foundation of Alberta, NWT and Nunavut (P.J. Hanly and M.J. Poulin), a New Opportunities Funding Award from the Canadian Foundation for Innovation (M.J. Poulin), full-time studentships from the AHFMR and the Heart and Stroke Foundation of Canada (G.E. Foster), the Canadian Institutes of Health Research (CIHR) Canada Graduate Scholarship Masters' Award (C.T.C. Duggan), and the CIHR Strategic Training Fellowships in TORCH (Tomorrow's Research Cardiovascular Health Professionals) (G.E. Foster and C.T.C. Duggan), full-time fellowships from the Hotchkiss Brain Institute (J.V. Brugniaux), the AHFMR (V. Pialoux), and the Heart and Stroke Foundation of Canada Focus on Stroke programme (J.V. Brugniaux anf V. Pialoux), and an AHFMR Senior Medical Scholar Award (M.J. Poulin).

STATEMENT OF INTEREST

None declared.

ACKNOWLEDGEMENTS

We are grateful to all the volunteers for their cheerful and dedicated participation to this study. The immunological reagent (mh2er/16.5.1) was kindly supplied by Wyeth Research (Cambridge, MA, USA).

REFERENCES

1 Ge RL, Witkowski S, Zhang Y, *et al.* Determinants of erythropoietin release in response to short-term hypobaric hypoxia. *J Appl Physiol* 2002; 92: 2361–2367.
2 Eckardt KU, Boutellier U, Kurtz A, *et al.* Rate of erythropoietin formation in humans in response to acute hypobaric hypoxia. *J Appl Physiol* 1989; 66: 1785–1788.
3 Robach P, Fulla Y, Westerterp KR, *et al.* Comparative response of EPO and soluble transferrin receptor at high altitude. *Med Sci Sports Exerc* 2004; 36: 1493–1498.
4 Brugniaux JV, Schmitt L, Robach P, *et al.* Eighteen days of ''living high, training low'' stimulate erythropoiesis and enhance aerobic performance in elite middle-distance runners. *J Appl Physiol* 2006; 100: 203–211.
5 Carlson JT, Hedner J, Fagerberg B, *et al.* Secondary polycythaemia associated with nocturnal apnoea: a relationship not mediated by erythropoietin? *J Intern Med* 1992; 231: 381–387.
6 Goldman JM, Ireland RM, Berthon-Jones M, *et al.* Erythropoietin concentrations in obstructive sleep apnoea. *Thorax* 1991; 46: 25–27.
7 Pokala P, Llanera M, Sherwood J, *et al.* Erythropoietin response in subjects with obstructive sleep apnea. *Am J Respir Crit Care Med* 1995; 151: 1862–1865.
8 Cahan C, Decker MJ, Arnold JL, *et al.* Diurnal variations in serum erythropoietin levels in healthy subjects and sleep apnea patients. *J Appl Physiol* 1992; 72: 2112–2117.
9 Jelkmann W. Control of erythropoietin gene expression and its use in medicine. *Methods Enzymol* 2007; 435: 179–197.
10 Soliz J, Joseph V, Soulage C, *et al.* Erythropoietin regulates hypoxic ventilation in mice by interacting with brainstem and carotid bodies. *J Physiol* 2005; 568: 559–571.
11 Soliz J, Soulage C, Hermann DM, *et al.* Acute and chronic exposure to hypoxia alters ventilatory pattern but not minute ventilation of mice overexpressing erythropoietin. *Am J Physiol Regul Integr Comp Physiol* 2007; 293: R1702–R1710.
12 Soliz J, Thomsen JJ, Soulage C, *et al.* Sex-dependent regulation of hypoxic ventilation in mice and humans is mediated by erythropoietin. *Am J Physiol Regul Integr Comp Physiol* 2009; 296: R1837–R1846.
13 Fujita M, Takahashi R, Liang P, *et al.* Role of alternative splicing of the rat erythropoietin receptor gene in normal and erythroleukemia cells. *Leukemia* 1997; 11: Suppl. 3, 444–445.
14 Westphal G, Braun K, Debus J. Detection and quantification of the soluble form of the human erythropoietin receptor (sEpoR) in the growth medium of tumor cell lines and in the plasma of blood samples. *Clinical and experimental medicine* 2002; 2: 45–52.
15 Baynes RD, Reddy GK, Shih YJ, *et al.* Serum form of the erythropoietin receptor identified by a sequence-specific peptide antibody. *Blood* 1993; 82: 2088–2095.
16 Soliz J, Gassmann M, Joseph V. Soluble erythropoietin receptor is present in the mouse brain and is required for the ventilatory acclimatization to hypoxia. *J Physiol* 2007; 583: 329–336.
17 Foster GE, Brugniaux JV, Pialoux V, *et al.* Cardiovascular and cerebrovascular responses to acute hypoxia following exposure to intermittent hypoxia in healthy humans. *J Physiol* 2009; 587: 3287–3299.
18 Pialoux V, Hanly PJ, Foster GE, *et al.* Effects of exposure to intermittent hypoxia on oxidative stress and acute hypoxic ventilatory response in humans. *Am J Respir Crit Care Med* 2009; 180: 1002–1009.
19 Powell FL, Milsom WK, Mitchell GS. Time domains of the hypoxic ventilatory response. *Respir Physiol* 1998; 112: 123–134.
20 Prabhakar NR, Kline DD. Ventilatory changes during intermittent hypoxia: importance of pattern and duration. *High Alt Med Biol* 2002; 3: 195–204.
21 Narkiewicz K, van de Borne PJ, Pesek CA, *et al.* Selective potentiation of peripheral chemoreflex sensitivity in obstructive sleep apnea. *Circulation* 1999; 99: 1183–1189.
22 Marcus CL. Pathophysiology of childhood obstructive sleep apnea: current concepts. *Respir Physiol* 2000; 119: 143–154.
23 Sullivan CE, Parker S, Grunstein RR, *et al.* Ventilatory control in sleep apnea: a search for brain neurochemical defects. *Prog Clin Biol Res* 1990; 345: 325–334.

24 Tamisier R, Gilmartin GS, Launois SH, *et al.* A new model of chronic intermittent hypoxia in humans: effect on ventilation, sleep, and blood pressure. *J Appl Physiol* 2009; 107: 17–24.
25 Townsend NE, Gore CJ, Hahn AG, *et al.* Living high-training low increases hypoxic ventilatory response of well-trained endurance athletes. *J Appl Physiol* 2002; 93: 1498–1505.
26 Gassmann M, Tissot van Patot M, Soliz J. The neuronal control of hypoxic ventilation: erythropoietin and sexual dimorphism. *Ann N Y Acad Sci* 2009; 1177: 151–161.
27 Grasso G, Buemi M, Alafaci C, *et al.* Beneficial effects of systemic administration of recombinant human erythropoietin in rabbits subjected to subarachnoid hemorrhage. *Proc Natl Acad Sci USA* 2002; 99: 5627–5631.
28 Brines ML, Ghezzi P, Keenan S, *et al.* Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. *Proc Natl Acad Sci USA* 2000; 97: 10526–10531.
29 Marti HH, Wenger RH, Rivas LA, *et al.* Erythropoietin gene expression in human, monkey and murine brain. *Eur J Neurosci* 1996; 8: 666–676.
30 Bernaudin M, Bellail A, Marti HH, *et al.* Neurons and astrocytes express EPO mRNA: oxygen-sensing mechanisms that involve the redox-state of the brain. *Glia* 2000; 30: 271–278.