Endogenous brain erythropoietin is a potent sex-specific respiratory stimulant in adult and newborn mice

Orlane Ballot, Vincent Joseph, and Jorge Soliz

Centre de Recherche du CHU de Québec, Pavillon St François d’Assise, Département de Pédiatrie, Faculté de Médecine, Université Laval, Québec, Quebec, Canada

Submitted 20 February 2015; accepted in final form 17 March 2015

Endogenous brain erythropoietin is a potent sex-specific respiratory stimulant in adult and newborn mice. J Appl Physiol 118: 1386–1395, 2015. First published March 19, 2015; doi:10.1152/japplphysiol.00143.2015.—We tested the hypothesis that endogenous brain Epo is a respiratory stimulant. Adult (3 mo) and newborn (10 days) male and female mice received an intracisternal (cisterna magna) injection of soluble Epo receptor (sEpoR; competes with EpoR to bind Epo; 50 μg/ml) or vehicle (0.1% BSA in PBS). Twenty-four hours after injection, we used whole body plethysmography to record minute ventilation (V˙E), tidal volume (Vt), respiratory frequency (fR), O2 consumption (VO2), and CO2 production (VCO2) under normoxia and progressive exposure to hypoxia (12-10-6% O2; 10 min each). In adult male and female mice sEpoR decreased normoxic V˙E (−25%), due to a decrease of Vt in males and fR in females. Moreover, sEpoR injection decreased the ventilatory response to 12% O2, assessed as V˙E/VO2 or V˙E/V˙CO2 in male but not in female mice. In newborn male and female mice sEpoR decreased V˙E (−37% in males, −59% in females) and Vt (−38% in males, −47% in females) in normoxia and fR in females. During hypoxia, sEpoR decreased V˙E/VO2 and V˙E/V˙CO2 in mice of both sexes. Upon extreme hypoxia (6% O2), the newborn mice treated with sEpoR showed respiratory depression, signs of asphyxia (gasping) and a high mortality rate in males and females. We concluded that endogenous brain Epo is a potent respiratory stimulant under normoxia and hypoxia in adult and newborn mice. Because sex-specific effects are different in newborn male and female, sex steroids secreted at different ages mice appear to modulate the effects of Epo on respiratory regulation in normoxia and in response to hypoxia.

newborn and adult mice; erythropoietin; soluble erythropoietin receptor; respiration; brain; hypoxia; sex dimorphism

ERYTHROPOIETIN (Epo) is a glycoprotein of 165 amino acids (34 kDa) belonging to the superfamily of type I cytokine and it is classically known to increase the numbers of circulating red blood cells in case of prolonged hypoxemia. However, Epo is also present in the brain where it is synthesized by neuronal and astrocytes (8, 16). Indeed, under hypoxic condition, the expression levels of Epo mRNA and protein are five times higher in the brain than in the kidney (5) and Epo exerts neuroprotective and prosurvival roles during ischemia, traumatic brain injury, and neonatal asphyxia (19, 29). Apart from pathological conditions, Epo modulates the central respiratory drive (39). Extensive studies performed in adult mice showed that Epo does not modulate basal ventilation but increases the hypoxic ventilatory response under conditions of severe hypoxia (6% O2) (39). Interestingly, in transgenic mice, the effects of systemic and central Epo overexpression on the hypoxic ventilatory response are sex-specific (more important in females than in males; 40), suggesting an important positive interaction between circulating sex steroids and Epo on respiratory control. On the contrary, during exposure to chronic hypoxia, estradiol secretion in female mice suppresses the effects of Epo on ventilation (11). However, since the animals used in these studies have a central and peripheral overexpression of Epo (with a concomitant increase of the hematocrit level), it is not easy to discriminate whether endogenous Epo acts directly on the central nervous system to modulate the translation of the inputs from the peripheral chemoreceptors (the main sensors of arterial oxygen pressure) to the respiratory network in the medulla.

Recent data obtained on the in vitro preparation of the isolated brain stem spinal cord preparation of newborn mice showed that the soluble Epo receptor (sEpoR, a competitive antagonist of Epo) changes the response to hypoxia, suggesting that endogenous synthesis of Epo might stimulate respiration, at least in response to hypoxia, in neonates by a direct effect on the central respiratory network (17). In the present study we used mice to ask whether similar effects of endogenous Epo are present in vivo, and if the effects of Epo are age- and sex-specific. We used adult (3 mo) and newborn (10 days) male and female C57Bl/6 mice for injection of sEpoR in the central nervous system (via the cisterna magna). Twenty-four hours after the injection, we recorded respiratory parameters in normoxic conditions and in response to different levels of hypoxia by whole body plethysmography. Our results show that under normoxia in adult mice sEpoR decreased tidal volume and minute ventilation in males, while it decreased respiratory frequency and minute ventilation in females. sEpoR also decreased the hypoxic ventilatory response only in males. In P10 mice, sEpoR decreased tidal volume, minute ventilation, and hypoxic ventilatory response both in males and females, but it decreased respiratory frequency (in normoxia) only in females. In addition, a large proportion of male and female newborn that received the injection of sEpoR had signs of respiratory depression and asphyxia (gasping) during severe hypoxia, leading to a respiratory arrest and death. Our results show that endogenous brain Epo is necessary in adult and in newborn to maintain the respiratory activity in normoxia and in response to hypoxia. Different sex-specific effects appearing in adult and P10 mice might be due to the different timing of sex steroid secretion between male and female mice during perinatal development and at adulthood. These results suggest that endogenous Epo might have important roles in respiratory disorders at adulthood and in neonates.

Address for reprint requests and other correspondence: J. Soliz, Faculté de Médecine, Université Laval, Centre de Recherche, HSFA, D0-717, 10, rue de l’Espinay, Québec, Canada, G1L 3L5 (e-mail: jorge.soliz@crchuq.ulaval.ca).
MATERIALS AND METHODS

Animals

Certified pathogen-free C57Bl6 mice were purchased from Charles River Laboratories (Saint-Constant, QC, Canada). Mice were housed and bred in our animal facility at the SFA hospital (at constant temperature and humidity) under a 12:12-h light cycle. Adult mice were left undisturbed in their cages for at least 1 wk before being used for the experiments. For newborn mice, one male was housed with 2 females for 1 wk, and then the females were isolated until the day of delivery (counted as postnatal day 0). Commercial diet and water were provided ad libitum. We used a total of 40 adults (3 mo old; 10 for each sex and group) and 70 newborns (control: 18 females, 18 males; sEpoR: 17 females, 17 males) mice. All experiments were approved by the local committee for animal care, in accordance with the Canadian Council on Animal Care in science.

Intracisternal Injection (ICI)

All animals were weighed, anesthetized with 2% isoflurane, and placed on a heating pad. Buprenorphine (0.08 ml in 0.1 ml of 0.9% NaCl) was administered subcutaneously for post-surgery analgesia. The dorsal base of the skull was shaved and cleaned, the mouse was placed on the stereotactic apparatus, and the flow of isoflurane was reduced to 1–1.5%. A minor incision was made at the occipital region of the skull. Subcutaneous tissue and neck muscles were separated through the midline. Then the posterior part of body was descended until forming an angle of 135° with the head, a position in which the cisterna magna is clearly visible (4). A Hamilton syringe was inserted into the cisterna magna, and we injected 30 μl (in adults) or 10 μl (in newborn) of sEpoR (50 μg/ml; E0643 Sigma-Aldrich, Canada) or control solution (0.1% BSA in PBS). The injection was performed over 2 min, and the syringe was left 1 additional minute in the cisterna magna before being withdrawn. The wound was closed (Vetbond tissue adhesive, 3M), and the mouse was kept under oxygenation (30% O2 in N2O) until its recovery. Adult mice were isolated in a cage, while newborn mice were placed back with the mother and the rest of the litter.

Respiratory Recordings Using Whole Body Plethysmography

Setup details. We used whole body plethysmography to record respiratory frequency (fR), tidal volume (VT), and minute ventilation (Ve = fR × VT). For adult mice we used a 600-ml chamber (Emka, Emka Technologies, Paris, France) continuously supplied with fresh air (around 180 ml/min) at room temperature. Newborn mice were placed in a 50-ml plethysmograph chamber (Buxco/DSI, St Paul, MN) continuously supplied with fresh air at 80–90 ml/min and heated at 34°C with a temperature control system (TCAT-2, Physitemp, Clifton, NJ). A differential pressure transducer (Emka Technologies) was connected between the recording chamber and the built-in reference chamber. A single injection of 100 μl (newborn mice) or 500 μl (adult) of air inside the chamber was used for calibration of the flow trace. A subsampling pump was used to draw (~50–75 ml/min) a sample of outflowing air for analysis of respiratory gases. In the outflowing line water pressure and CO2 levels were measured with dedicated gas analyzers (respectively: RH 300, Sable Systems International, Las Vegas, NV; and CD-3A-AEI Technologies, Pittsburgh, PA). Oxygen levels in the inflowing and outflowing gas lines were measured with a dual-channel O2 analyzer (S3-AII, AEI Technologies). All signals were acquired and recorded on a computer using the Spike 2 software (Cambridge Electronic Design, Cambridge, UK) and used offline to calculate respiratory frequency (fR), tidal volume (VT), minute ventilation (Ve = fR × VT), CO2 production (VCO2), and O2 consumption rate (VO2). Body weight was measured routinely after experiments to express the tidal volume in milliliters per 100 g. Body temperature was measured at the beginning and at the end of the experiment with a rectal thermocouple probe for adult mice and orally in newborn (Physitemp).

Recordings and signal analysis. After the animal familiarized with the new environment (about 2 h for adult and 40 min for newborn) basal ventilation was recorded at 21% O2 for 10–20 min. Acute hypoxia was achieved by adding a predetermined flow of N2 to reach the desired O2 level (10, 12, and 6% O2 each maintained for 10 min). During baseline recordings, and for each level of hypoxia, the sub-sampling pump was derived to the inflowing gas line for 2–3 min. This procedure was used to verify that the two channel of the O2 analyzer read similar values in the inflowing and outflowing gas lines, and to record values of inflowing CO2 and H2O for calculation of metabolic rate (see below).

Respiratory frequency and inspiratory volume were calculated breath by breath using a custom script in Spike 2; then values of baseline ventilation were determined on the portions of the recording during which the breathing pattern was stable and regular, with the lowest respiratory frequency. During hypoxic exposure we selected portions of stable and regular breathing pattern during the last 3 min for each O2 level. For metabolic rate, we used the portions of the recordings where CO2 in the outflowing gas line displayed the lowest value.

The portion of the calibrated flow trace corresponding to inspiration was integrated by the software, and the corresponding volume was corrected by using the standard equation described for whole body plethysmography (1). VO2 and VCO2 were calculated as follows:

\[
V_O_2 = \text{Flow}_i \left[ F_{O_2} (1 - F_{O_2} - F_{CO_2} - F_{H_2O}) / (1 - F_{O_2} - F_{CO_2} - F_{H_2O}) \right]
\]

and

\[
V_{CO_2} = \text{Flow}_i \left[ (1 - F_{O_2} - F_{CO_2} - F_{H_2O}) / (1 - F_{O_2} - F_{CO_2} - F_{H_2O}) \right] \]

where Flowi is the flow rate of gas measured in the inflowing line, and Fi and Fe are the fractions of the corresponding gas measured in the inflowing and outflowing lines, respectively. This equation allows the correction for the changes of gas composition in the inflowing and outflowing gas lines due to the activity of the animal, and the day-to-day variability of CO2 and H2O in ambient air (21). VO2, VCO2, and VCO2 values were used to report minute ventilation as a function of O2 consumption (Ve/VO2) and CO2 production rates (Ve/VCO2), a reliable way to estimate hypoxic ventilatory response in rodents (24).

Statistical Analysis

The animals were divided in 4 groups (control males, control females, sEpoR Males, sEpoR Females), and differences between groups were evaluated with a one-way ANOVA followed by a Fisher’s LSD post hoc analysis when the ANOVA gave significant results. During hypoxic exposure, we used a two-way ANOVA for repeated measurement using groups as the independent variable and hypoxia levels as the repeated variable. If the ANOVA gave significant effects for groups, hypoxia, or significant groups by hypoxia interaction, we used a Fisher’s LSD post hoc analysis to test the effects of groups at each level of hypoxia, or the effects of hypoxia within each group.

In newborn mice, we tested differences between groups for survival upon exposure to hypoxia (10 and 6% O2) by using a contingency table (the outcome was either survival or death), a Fisher’s exact test, and we calculated the odds ratio of death upon hypoxic exposure (see last paragraph in RESULTS). All analysis and graphs were done with the GraphPad prism 6.0 software (La Jolla, CA). The reported values are means ± SE. Differences were considered significant at P < 0.05.
values for ANOVA are reported in the text, and \( P \) values for post hoc analysis are presented in the figures.

**RESULTS**

**In Adult mice sEpoR Decreases Tidal Volume in Males and Respiratory Frequency in Females**

In mice that received the ICI injection there was a decrease of body weight (\(-3.2 \pm 0.6 \text{ g in males and } -2.7 \pm 0.1 \text{ g in females}\)) significantly greater than in mice that received the saline injection (ANOVA \( P \) value = 0.0003; Table 1). At the beginning of the respiratory recording, rectal temperature was similar between groups.

In comparison to control animals, the basal minute ventilation decreased by \(-25\%\) (\( P \) value for ANOVA = 0.003) in male and female mice that received the injection of sEpoR (Fig. 1A). This decrease of basal ventilation was due to a decreased \( V_T \) in males (\(-20\%; \) Fig. 1B), and a decreased \( f_R \) in females (\(-30\%; \) Fig. 1C). For metabolic rate (\( V_O^2 \) and \( V_C^2 \)) there was no significant difference between groups (\( P \) value for ANOVA = 0.2 for \( V_O^2 \) and 0.6 for \( V_C^2 \)). \( V_E/V_O^2 \) was significantly decreased by sEpoR in male but not in female mice (Fig. 1F), but there was no significant effect of groups for \( V_E/V_C^2 \) (Fig. 1G).

**In Adult Mice sEpoR Decreases Hypoxic Ventilatory Response in Males but not in Females**

Under progressive exposure to hypoxia (12%, 10%, and 6% \( O_2 \)), minute ventilation increased in all groups of mice (\( P < 0.0001; \) Fig. 2); however, male and female mice injected with sEpoR maintained a lower value of minute ventilation compared with control mice at all levels of hypoxia (\( P = 0.0005 \) for group; Fig. 2, A and B). For both sexes, this effect was due to a reduced \( f_R \) (\( P = 0.0002 \)). Indeed, while \( f_R \) increased during hypoxic exposure in control mice, it remained at the baseline level in male and female mice that received the sEpoR (\( P \) value for group \( \times \) hypoxia = 0.002; Fig. 2, C and D). However, in mice injected with sEpoR, \( V_T \) was significantly higher than the baseline value at all levels of hypoxic exposure (\( P \) value for group \( \times \) hypoxia = 0.026; Fig. 2, E and F), while in control animals \( V_T \) increased only in female mice at 6% \( O_2 \).

Metabolic rate declined during progressive exposure to hypoxia in all groups of mice (\( P < 0.0001 \) for \( V_O^2 \) and \( V_C^2 \); Fig. 3, A and D). For \( V_C^2 \), there was a significant effect of group (\( P = 0.02 \)); at 12% \( O_2 \) \( V_C^2 \) was lower in female mice injected with sEpoR compared with control animals (Fig. 3D). \( V_E/V_O^2 \) and \( V_E/V_C^2 \) increased in hypoxia in all groups (\( P < 0.0001 \) for both values; Fig. 3, E–H), with significant effects of group (\( P = 0.009 \) for \( V_E/V_O^2 \) and \( P = 0.04 \) for \( V_E/V_C^2 \)). At 12% \( O_2 \), the values of \( V_E/V_O^2 \) and \( V_E/V_C^2 \) were lower in male mice that received the injection of sEpoR compared with controls (Fig. 3, E and G). At 12% \( O_2 \), both \( V_E/V_O^2 \) and \( V_E/V_C^2 \) were higher in control males compared with control females (Fig. 3, E–H).

**In Newborn Mice sEpoR Decreases Tidal Volume in Males and Females and Respiratory Frequency in Females but not in Males**

As reported for adult, newborn mice that received the ICI sEpoR injection exhibited a decrease of body weight (\(-0.53 \pm

---

**Table 1. Body weight on the days of surgery and recording (24 h later), difference between the two values (weight loss), and rectal temperature on the day of recording in adult male and female mice that received an intracisternal injection of saline (Cont) or sEpoR**

<table>
<thead>
<tr>
<th></th>
<th>Cont (( n = 10 ))</th>
<th>sEpoR (( n = 10 ))</th>
<th>Cont (( n = 10 ))</th>
<th>sEpoR (( n = 10 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight at surgery, g</td>
<td>23.6 ± 0.5</td>
<td>25.6 ± 0.6**</td>
<td>20.1 ± 0.5††††</td>
<td>21.1 ± 0.2††††</td>
</tr>
<tr>
<td>Body weight at recording, g</td>
<td>22.5 ± 0.6</td>
<td>22.4 ± 0.4</td>
<td>19.2 ± 0.5††††</td>
<td>18.4 ± 0.3††††</td>
</tr>
<tr>
<td>Weight loss, g</td>
<td>0.8 ± 0.5</td>
<td>-3.2 ± 0.6***</td>
<td>-0.9 ± 0.5</td>
<td>-2.7 ± 0.1**</td>
</tr>
<tr>
<td>Rectal temperature at recording, °C</td>
<td>36.3 ± 0.3</td>
<td>36.1 ± 0.2</td>
<td>36.7 ± 0.3</td>
<td>36.8 ± 0.1</td>
</tr>
</tbody>
</table>

All values are means ± SE; \( n = \) no. of animals for each group. ** and ***: \( P < 0.01 \) and \( P < 0.001 \), sEpoR vs. control (Cont). † and †††: \( P < 0.01 \) and \( P < 0.001 \), females vs. males.

---

**Fig. 1. Effects of sEpoR on ventilatory and metabolic parameters in normoxia in adult male and female mice. Minute ventilation (\( V_T \); A), tidal volume (\( V_E \); B), frequency (\( f_R \); C), oxygen consumption (\( V_O^2 \); D), carbon dioxide production (\( V_C^2 \); E), \( V_E/V_O^2 \) (F), and \( V_E/V_C^2 \) (G) in male and female adult mice recorded in normoxia 24 h after intracisternal injection of saline (Cont) or sEpoR. All values are means ± SE. *, **: \( P < 0.05 \) and \( P < 0.01 \), sEpoR vs. Cont. †, †††: \( P < 0.05 \) and \( P < 0.01 \), females vs. males.**
In Newborn Male and Female Mice sEpoR Decreases Hypoxic Ventilatory Response

Under progressive exposure to hypoxia (12% and 10%), minute ventilation increased ($P$ value for hypoxia < 0.0001; Fig. 5, A and B). However, newborn male and female mice injected with sEpoR showed a lower value of minute ventilation compared with control mice at 12% and 10% of hypoxia ($P$ value for group < 0.0001; Fig. 5, A and B). This effect was due to a reduced $V_T$ in males (Fig. 5E) and females (Fig. 5F).

Metabolic rate decreased significantly during exposure to 12% and 10% oxygen in all groups of mice (for $V_O2$ and $V_C02$; Fig. 6, A–D). In male and female mice that received the injection of sEpoR $V_e/V_O2$ was lower at 12% $O_2$ (Fig. 6, E and F), and $V_e/V_C02$ was lower at 12% and 10% $O_2$ (Fig. 6, G and H).

During the exposure to severe hypoxia (6% $O_2$), the newborn mice that received the injection of sEpoR showed signs of respiratory depression and asphyxia (gassing); this pattern occurred during the first minutes of exposure, and was gener-

0.07 g in males and $-0.49 \pm 0.08$ g in females) significantly greater than mice that received the saline injection (ANOVA $P$ value < 0.0001; Table 2). At the beginning of the respiratory recording, body temperature (taken orally) was similar between groups.

In comparison to control animals, basal minute ventilation decreased after sEpoR injection by 37% in males and by 59% in female ($P$ value for ANOVA < 0.0001; Fig. 4A). In mice that received the sEpoR injection, basal minute ventilation was lower in females ($162 \pm 13$ ml/min$^{-1}$·100 g$^{-1}$) than in males ($227 \pm 15$ ml/min$^{-1}$·100 g$^{-1}$; $P = 0.006$). In sEpoR-injected mice $V_T$ decreased both in males and females (Fig. 4B), and $V_T$ was lower in females ($0.72 \pm 0.05$ ml/100 g) than in males ($1.04 \pm 0.10$ ml/100 g; $P = 0.008$), $f_k$ decreased only in female mice after sEpoR injection (Fig. 4C). $V_O2$ was lower in male mice treated with sEpoR than in control mice, while $V_C02$ was similar (Fig. 4, D and E). $V_e/V_O2$ was lower in female mice treated with sEpoR compared with control mice ($P$ value for ANOVA = 0.008; Fig. 4F), and $V_e/V_C02$ was lower in male and female pups treated with sEpoR compared with controls (Fig. 4G).

Fig. 2. Effects of sEpoR on ventilatory response to hypoxia in adult male and female mice. $V_e$, $f_k$, and $V_T$, in male (A, C, E) and female (B, D, F) adult mice recorded under different levels of hypoxia 24 h after intracisternal injection of saline (Cont) or sEpoR. All values are means ± SE. *: $p < 0.05$, $< 0.01$, and $< 0.001$, sEpoR vs. Cont. **, ***: $p < 0.05$, $< 0.01$, and $< 0.001$ vs. 21% $O_2$. †: males vs. females.

Fig. 3. Effects of sEpoR on metabolic and ventilatory responses to hypoxia in adult male and female mice. $V_O2$, $V_C02$, $V_e/V_O2$, and $V_e/V_C02$ in male (A, C, E, G) and female (B, D, F, H) adult mice recorded under different levels of hypoxia 24 h after intracisternal injection of saline (Cont) or sEpoR. All values are means ± SE. *: $p < 0.05$, $< 0.01$, and $< 0.001$, sEpoR vs. Cont. †: $p < 0.05$ and $< 0.01$, females vs. males.
Metabolic Variables

Table 2. Body weight on the days of surgery and recording (24 h later), difference between the two values (weight loss), and rectal temperature on the day of recording in newborn male and female mice that received an intracisternal injection of saline (Cont) or sEpoR

<table>
<thead>
<tr>
<th></th>
<th>Males (n = 18)</th>
<th>sEpoR (n = 18)</th>
<th>Females (n = 17)</th>
<th>sEpoR (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight at surgery, g</td>
<td>5.16 ± 0.20</td>
<td>5.12 ± 0.16</td>
<td>5.35 ± 0.15</td>
<td>5.50 ± 0.17</td>
</tr>
<tr>
<td>Body weight at recording, g</td>
<td>5.00 ± 0.22</td>
<td>4.59 ± 0.16</td>
<td>5.23 ± 0.14</td>
<td>5.01 ± 0.17</td>
</tr>
<tr>
<td>Weight loss, g</td>
<td>-0.16 ± 0.05</td>
<td>-0.53 ± 0.07***</td>
<td>-0.12 ± 0.08</td>
<td>-0.49 ± 0.08***</td>
</tr>
<tr>
<td>Rectal temperature at recording, °C</td>
<td>34.2 ± 0.3</td>
<td>33.5 ± 0.4</td>
<td>34.0 ± 0.3</td>
<td>34.4 ± 0.3</td>
</tr>
</tbody>
</table>

All values are means ± SE; n = no. of animals for each group. ***P < 0.001, sEpoR vs. Cont.

ally not observed in control animals (Fig. 7). After a few minutes, breathing movements were no longer apparent, the recording was immediately stopped, and the chamber opened, but the animals were not able to recover a normal breathing pattern. In a few cases (4 animals in total, 1 for each group and sex), this pattern was present at 10% O₂. This resulted in a very high mortality rate in males (13 of 17 animals) and in females (10 of 17) mice that received the injection of sEpoR (Fig. 7). Accordingly the physiological data from 6% were not presented in Figs. 5 and 6. In control mice 4 males (of 18) died upon exposure to hypoxia (1 at 10% O₂, 3 at 6% O₂), and 3 (of 18) females died (1 at 10% O₂, 2 at 6% O₂). If the death and survival rate number are pooled for 10% and 6% O₂, they are: 7.1 with a 95% CI of 1.5–34.4 (P<0.001), sex interaction = 0.0006). Finally, the effect of sEpoR on respiratory frequency was more pronounced in females than in males both in P10 and adults, and in adult females the effect of sEpoR injection was more pronounced than in P10 females.

**DISCUSSION**

In the present study we injected sEpoR in the cisterna magna of adult and newborn mice to determine whether endogenous Epo contributes to the control of ventilation in normoxia and in response to hypoxia (hypoxic ventilatory response: HVR). Our results clearly indicate that Epo is a critical factor for respiratory regulation, and in newborn mice it seems to play a key role allowing survival under extreme hypoxia. In addition, some sex-specific effects of sEpoR injection appeared only in adults (reduced V̇ₜ in normoxia and reduced HVR), while other effects appeared both in newborn and in adults (reduced fR in normoxia; see Fig. 8).

These results raise two intriguing points: 1) at some point of postnatal development, mice become resistant to the respiratory depression and asphyxia in severe hypoxia induced by sEpoR injection; and 2) female mice lose the sEpoR response of decreased tidal volume and HVR between P10 and adulthood. So far we do not have specific elements to understand when exactly, and what are the underlying mechanisms, that explain this intriguing developmental phenomenon. On the other hand, the age-specific pattern of differences between

**Fig. 4.** Effects of sEpoR on ventilatory and metabolic parameters in normoxia in newborn male and female mice. V̇ₑ (A), V̇ₜ (B), fR (C), V̇O₂ (D), V̇CO₂ (E), V̇ₑ/V̇O₂ (F), and V̇ₑ/V̇CO₂ (G) in male and female newborn mice recorded in normoxia 24 h after intracisternal injection of saline (Cont) or sEpoR. All values are means ± SE. *, **, ****: P < 0.05, < 0.01, and < 0.0001, sEpoR vs. Cont. †, ††, †††: P < 0.05, < 0.01, and P < 0.001, females vs. males.
Endogenous Epo Stimulates Ventilation

Fig. 5. Effects of sEpoR on ventilatory response to hypoxia in newborn male and female mice. \( V_E, f_E, \) and \( V_T \), in male (A, C, E) and female (B, D, F) newborn mice recorded under different levels of hypoxia 24 h after intracisternal injection of saline (Cont) or sEpoR. All values are means ± SE. *: Cont vs sEpoR, °: vs. 21% O2, †: F vs. M.

Males and females strongly suggests that there is a tight interaction between Epo and sex steroids secreted by the gonads of adult mice (as already shown; 11, 39, 40), but also between Epo and sex steroids secreted around birth in males (6). This second point is discussed in more detail below.

**Methodological Considerations**

Injection in the cisterna magna (or intracisternal injection; ICI) is a well-described technique (4, 10, 20, 30, 36), and puncture at this level is classically used to collect cerebrospinal fluid in rodents. While intracerebroventricular (ICV) injection cannot be performed in newborn animals due to the fragility of the cranial bone, ICI injection is fast and easy to use at any age. Moreover, since the cisterna magna is in direct contact with the brain stem, this is an approach of choice for studies on respiratory regulation.

Like the other type I cytokine receptor, the Epo receptor (EpoR) is synthesized as a transmembrane functional and as a soluble form (sEpoR) by alternative splicing of the EpoR mRNA. sEpoR is secreted into the extracellular medium of several tissues (37, 45) and competes with the functional EpoR, thus playing the role of an endogenous antagonist. In the brain stem-spinal cord preparations, the samples were incubated in 3 \( \mu \)g of sEpoR for 1 h (17). Based on these different studies and on the present results, it might be postulated that the dose injected was sufficient to obtain a reliable antagonization of the endogenous Epo in the brain stem region.

**Cerebral Epo is a Powerful Endogenous Respiratory Stimulant**

While numerous studies describe the respiratory stimulant effect of Epo, most of them were conducted on transgenic animal models with constitutive overexpression of Epo (11, 37, 39, 40), and only a limited number of studies addressed the respiratory effect of endogenous synthesized Epo in the CNS. We showed recently that incubation of the isolated brain stem-spinal cord preparations with sEpoR depresses neuronal respiratory activity in response to hypoxic exposure (17). However this experimental model can only be used in newborn rodents up to 4 days of age, when the respiratory control system is not fully mature. Furthermore, the relevance of this preparation for in vivo respiratory regulation is not straightforward and translation to in vivo preparations remains a necessary step to fully understand the multiple regulations of the respiratory control system.

We previously reported in adult mice that endogenous Epo production contributes to respiratory regulation in vivo (38), but in this earlier study we addressed the contribution of endogenous Epo on the ventilatory acclimatization to chronic hypoxia in mice, without questioning its implication under normoxic conditions and in response to acute hypoxia. Nonetheless, the results of this study showed that after 3 days of exposure to 10% O2 and concomitant ICV infusion of sEpoR in the lateral ventricle, minute ventilation and tidal volume were reduced by about 30–35%, an effect that was only slightly more important than the effects reported in the present study under normoxic conditions (sEpoR infusion reduced minute ventilation by 26% and tidal volume by 20% in males). It is tempting to compare directly the results of these two studies and conclude that Epo affects the respiratory regulation in normoxia, the ventilatory response to acute hypoxia, and the ventilatory acclimatization to chronic hypoxia. However, since the dose, timing, and site of injection of sEpoR are different in these two studies, this comparison might be done with caution.

In adult transgenic mice overexpressing Epo in brain only (Tg21; 39) the basal ventilation and the HVR at 10% O2 are not affected, but the HVR at 6% O2 is higher. At the time, we postulated that Epo stimulates ventilation only under severe conditions of hypoxia. The present work clearly demonstrates however that Epo stimulates ventilation in normoxia, but also under moderate (and severe) hypoxia. Indeed, the activity of Epo depends on the available number of Epo receptor and on the concentration of sEpoR (44). Since in Tg21 mice the level of EpoR is normal (46), it is tempting to suggest that EpoR in these transgenic mice should be almost saturated, and that the functional consequences of Epo overexpression are limited, appearing only under extreme conditions of hypoxia. In the
present work, it might be suggested that the administration of sEpoR should bind a substantial proportion of free Epo, therefore displacing Epo binding from the full-length functional receptor, and reducing the activity of the Epo signaling pathways.

Effect of Epo on the Respiratory Control System are Sex Dependent in Adults

In previous work performed in adult transgenic and wild-type mice we showed that the effects of Epo on respiratory response to acute hypoxia are sex-specific (more pronounced in females; 11, 12, 40). These data suggested an intricate interaction between Epo and sex steroids for the modulation of the respiratory control system. As an example it is intriguing to report that long-term Epo therapy in hemodialyzed patients decreases levels of FSH and LH and increases plasma levels of estradiol and testosterone (18). On the same line of evidence plasma estradiol levels are higher in female mice that overexpress Epo and have higher hematocrit level (11). It is also well known that the sex steroids modulate the erythropoietic functions of Epo: in human and animals under normoxic and hypoxic conditions the level of hemoglobin and hematocrit is lower in females than in males (2, 22, 32). This effect is partly due to the inhibiting effect of estradiol on the expression of Epo in females (26), and the stimulating effect of testosterone on the expression of Epo in men (3, 7, 13), male rats (31), and male mice (9). Combined with our present data, these results suggest that sex steroids regulate the expression and function of Epo in the central nervous system, and that these effects might explain the sex-specific effects induced by sEpoR injection on respiratory regulation in adult mice.

Effects of Epo on the Respiratory Control System are Sex Dependent in Newborn

In newborn mice the injection of sEpoR decreased minute ventilation in normoxia, and as reported in adults, fE decreased in female mice, but not in males. In addition, and in contrast

---

Fig. 6. Effects of sEpoR on metabolic and ventilatory responses to hypoxia in newborn male and female mice. VO₂, VCO₂, VE/VO₂, and VE/VCO₂ in male (A, C, E, G) and female (B, D, F, H) newborn mice recorded under different levels of hypoxia 24 h after intracisternal injection of saline (Cont) or sEpoR. All values are means ± SE. *: * * *: P < 0.05, < 0.01, and < 0.001 sEpoR vs. Cont. °: ° ° ° °: P < 0.05, < 0.01, < 0.001, and < 0.0001 vs. 21% O₂.
with the results obtained in adults, tidal volume was decreased in males and females, the decrease of minute ventilation was more pronounced in females than in males, and HVR was reduced in males and females (see Fig. 8 for a summary of these data). Most of these results are consistent with the facts that are explained above on the role of sex-steroid hormones secreted in adults to modify the effects of Epo on respiratory regulation, and support the hypothesis that ovarian secretion in adult female might blunt the effects of Epo on respiratory regulation. Nonetheless, this does not explain that in newborn female mice, as in their adult counterparts, sEpoR has no effect on respiratory frequency.

In newborn mammals, sexual dimorphism is mainly established by secretion of testosterone in males during late fetal and early postnatal life (6, 25, 27, 28). During this developmental period, testosterone acts as a masculinizing factor for the male genital tract and for reproductive behavior, influencing the development of related anatomical elements in peripheral tissues but also in brain and spinal nuclei in males. Notably, as circulating steroids readily cross the blood-brain barrier, the perinatal testosterone affects the development of brain areas by favoring cell death or survival in distinct sexually dimorphic neural groups that are involved in male sexual behavior (sexual dimorphic nucleus of the hypothalamic preoptic area) and in the neural control of the male genital tract (spinal nucleus of the bulbocavernosus and dorsolateral nucleus in the lumbar spinal cord) (14, 33). These actions are thought to be mediated by an effect on the expression of key factors that are involved...
in neural survival and synaptogenesis, such as members of the neurotrophin family (15, 41), and proteins associated with the axonal growth cone (34, 35).

In the brain, testosterone exerts its biological function in its native chemical form by binding to the androgen receptor. A fraction of testosterone is converted to dihydrotestosterone, which also binds and activates the androgen receptor. However, an important fraction of testosterone is metabolized to 17β-estradiol by the P450 aromatase inside the cytosol of the target neurons (23, 43), and an important amount of the effect of testosterone on brain development is from the activation of the estradiol receptor (23). As explained above, both testosterone and estradiol regulate the synthesis of Epo, and our data suggest that in newborn mice sex-specific effects of Epo might be regulated by perinatal testosterone secretion.

In conclusion, our results show that intracisternal injection of sEpoR produces a profound depression of resting minute ventilation that is more important in newborn mice than in adults. Indeed, in newborn, sEpoR injection impaired survival under conditions of severe hypoxia; however, during development mice become resistant to this effect, and the mechanisms underlying this resistance would need more investigation. Furthermore, sEpoR injection also reduced the ventilatory response to moderate hypoxia, suggesting that Epo modulates the translation of the inputs of the peripheral chemoreceptors to the respiratory control system in the medulla in newborn and in adults. Finally, sex-specific effects of sEpoR injection were present in adults, but also in newborn, suggesting a complex interaction between Epo and sex steroids secreted in male and female mice at adulthood and around birth. Since it has been recently reported that Epo treatment in preterm infants reduces the need of ventilatory support (42), these findings appear to be clinically relevant, and sex-specific efficiency of Epo treatment in preterm neonates should be further investigated.

REFERENCES

We thank the Canadian Institutes of Health Research (MOP 130258) and the Star Foundation for Children’s Health Research, along with the Molly Towell Foundation, for financial support. The salary of J. Soliz is supported by the “Fonds de recherche du Quebec-Sante” (FRQ-S).

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: O.B., V.J., and J.S. conception and design of research; O.B. performed experiments; O.B. and V.J. analyzed data; O.B., V.J., and J.S. interpreted results of experiments; O.B. and V.J. prepared figures; O.B., V.J., and J.S. drafted manuscript; O.B., V.J., and J.S. edited and revised manuscript; O.B., V.J., and J.S. approved final version of manuscript.

GRANTS

We thank the Canadian Institutes of Health Research (MOP 130258) and the Star Foundation for Children’s Health Research, along with the Molly Towell Foundation, for financial support. The salary of J. Soliz is supported by the “Fonds de recherche du Quebec-Sante” (FRQ-S).

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: O.B., V.J., and J.S. conception and design of research; O.B. performed experiments; O.B. and V.J. analyzed data; O.B., V.J., and J.S. interpreted results of experiments; O.B. and V.J. prepared figures; O.B., V.J., and J.S. drafted manuscript; O.B., V.J., and J.S. edited and revised manuscript; O.B., V.J., and J.S. approved final version of manuscript.

REFERENCES


J Appl Physiol • doi:10.1152/japplphysiol.00143.2015 • www.jappl.org

Downloaded from http://jappl.physiology.org/ by 10.220.33.4 on April 1, 2017


