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Increased ventilation in female erythropoietin-deficient mouse line is not progesterone and estrous stage-dependent

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ABSTRACT

Previous studies suggest that chronic erythropoietin (Epo) deficiency in male mice does not alter normoxic/hypoxic ventilation. As effects of Epo are sex specific and as progesterone could be a respiratory stimulant, we evaluated the impact of Epo deficiency and its possible interaction with progesterone in ventilatory control in female mice during estrous cycle phases. Compared to wild type (WT) animals, Epo-TAg^{h} female mice exhibited higher ventilation in hypoxia. However, when data were separated into luteal and follicular phases of the estrous cycle, basal ventilation and hypoxic ventilation were not different in both mice strains. As progesterone is known to be a potent respiratory stimulant, additional experiments were performed to elucidate its role. Interestingly, after mifepristone treatment, HVR was not modified in WT and Epo-TAg^{h} mice, showing that the ventilatory stimulation observed in females was not directly mediated by progesterone. We conclude that Epo-TAg^{h} female mice show no estrous stage-dependent increase of ventilatory control and progesterone independent response to hypoxia.

1. Introduction

Erythropoietin (Epo) is a kidney-secreted glycoprotein that plays an essential role in erythropoiesis (Bunn, 2013). Under hypoxic/hypoxemic conditions, Epo is crucial for improving tissue oxygenation and arterial oxygen carrying capacity by enhancing red blood cells synthesis in bone marrow (Koulnis et al., 2014). In parallel with erythropoiesis, hypoxia also activates neural respiratory areas (central and peripheral) to increase minute ventilation and thereby contribute to tissue oxygenation (Ivy and Scott, 2015). Epo and its receptors (EpoR) are present both in the respiratory areas of the brainstem and in carotid bodies (Soliz et al., 2005). Accordingly, using transgenic mice overexpressing Epo in brain only or WT animals treated with a specific antagonist of Epo (the soluble Epo receptor; sEpoR), we demonstrated that Epo is a crucial regulator of basal ventilation and also strongly contributes to the hypoxic ventilatory response (HVR) (Soliz, 2013). Moreover, we also reported that the respiratory effects of Epo are sex-specific, larger in females, suggesting an important positive interaction between sex steroids hormones and Epo on respiratory control (Soliz et al., 2012). However, erythropoiesis does not seem affected by menstrual cycle phase at high altitude in human (Reeves et al., 2001).

In line with these investigations, we performed experiments using our transgenic Epo deficient mouse line (Epo-TAg^{h}). Interestingly, our results in male animals did not show altered basal minute ventilation and HVR (Pichon et al., 2016; Voituron et al., 2014). As these animals are exposed to chronic anemia (a condition in which the organism does not have enough amount of red blood cells to provide tissue oxygenation), we hypothesized that they should display some physiological and molecular responses that could improve tissue oxygenation. In fact, we found that Epo-TAg^{h} mice show a cardiac adaptation in order to limit consequences of anemia (El Hasnaoui-Saadani et al., 2013) and up-regulates the basal expression of genes controlling oxygen metabolism such as HIF, VEGF, GLUT-1, EpoR, NOSi, NOSe at brain level (El Hasnaoui-Saadani et al., 2009; Voituron et al., 2014).

Considering that both the expression of Epo in plasma, as well as the impact of Epo on the neural control of ventilation is sex-specific (Iturri et al., 2017), in this work we investigated the impact of Epo deficiency on basal normoxic ventilation and HVR of female Epo-TAg^{h} mice.

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Moreover, keeping in mind that progesterone is a potent respiratory stimulant and it's expression is stronger at the luteal than the follicular phase, it's tempting to suggest a positive interaction between progesterone and Epo deficiency. In this context, our aim was to explore the interaction between Epo deficiency and progesterone in the setting and modulation of ventilation in normoxic and hypoxic conditions. As progesterone concentration change according to estrous phase, we analyzed ventilatory variables depending on these phases. Our results showed that Epo-TAg^h female mice show increased ventilation in hypoxic conditions. Such increase however is not differently modulated through the estrous cycle, and moreover, our experiments with mifepristone (progesterone receptor antagonist) suggest that this effect is independent from the respiratory effects of progesterone.

2. Materials and methods

2.1. Ethical approval

Experimental protocols were approved by the Ethics Committee for Animal Experiment Charles Darwin (Ce5/2011/05 and APAFIS #8192 2016110716039730 v5), done in accordance with the European Communities Council Directive of September 22, 2010 (2010/63/EU) for animal care, and conducted in accordance with the French legislation for animal care.

2.2. Animals

All experiments were performed in-house bred wild type (WT, n = 6) and Epo-deficient (Epo-TAg^h; n = 6) adult female littermate mice (≈10–12 weeks) from an hybrid Bl6/CBA strain. Epo-TAg^h mice present a targeted disruption in the 5′ untranslated region of the Epo gene (Pichon et al., 2016) that reduces the whole body Epo expression. This leads to a reduced plasmatic (around 120 pg/ml in WT *vs* 50 pg/ml in Epo-TAgh) and brain (around 0.40 pg/mg of total protein in WT *vs* 0.10 pg/mg of total protein in Epo-TAg^h) concentration of Epo (El Hasnaouis 2009-AJP-regul Integr Compr Physiol 296), low hematocrit (> 50% in WT *vs* < 21% in Epo-TAg^h) and low haemoglobin concentration (around 17 g/dl in WT *vs* 7 g/dl in Epo-TAg^h). Mean body weight was 27 ± 5 g for WT mice and 25 ± 4 g for Epo-TAg^h mice respectively. All animals were housed in a 12 h/12 h light/dark cycles at an ambient temperature of 20–22 °C and had ad libitum access to water and food. Ventilatory and metabolic variables were measured in normoxia and during 5 min of hypoxic (8% O_2) challenge.

2.3. Ventilatory and metabolic variables analysis

In non-anesthetized and unrestrained mice, breathing variables were recorded by whole-body plethysmography (Bartlett and Tenney, 1970; Voituron et al., 2014). Briefly, mice were placed in a recording chamber (200 ml). A differential pressure transducer (model DP 45-18, Validyne Engineering Northridge, CA, USA) measured pressure fluctuations within the recording chamber, relative to a reference chamber of the same volume. The differential pressure transduced signals were recorded by Spike 2 data analysis system (CED, Cambridge UK). To avoid stress effects on ventilatory variables, mice were habituated in the recording chamber two or three days before the experiments (30 min–1 h/days). To evaluate the acute ventilatory response to hypoxia, air was replaced by an hypoxic gas mix (O_2 8%, CO_2 0%, balanced N_2) for 5 min. Only periods of breathing without body movements were analyzed. We evaluated respiratory frequency (f_R, in cycles per min, c min^{-1}), tidal volume (V_T, µl) normalized as the ratio of V_T divided by body weight (V_T, µl g^{-1}) and minute ventilation ($\dot{V}_E$, ml g^{-1} min^{-1}).

To estimate O_2 consumption ($\dot{V}O_2$, ml g^{-1} min^{-1}; atmospheric temperature and pressure in dry air) in normoxia and hypoxia, an open-circuit system with gas analyzers was used. Briefly, mice were placed in a chamber with a steady flow of air (0.5 l/min). Fractions of O_2 at the inflow and outflow of the chamber were measured by an O_2 analyzer (FC-10, Sable system, Las Vegas, USA). The air was dried before entering in the analyzers. $\dot{V}O_2$ was calculated as previously described (Marcouiller et al., 2014) according to the following formula and was normalized by body weight:

$$\dot{V}O_2 = \text{flow} \times \frac{[(F_iO_2 - F_eO_2) - F_eO_2 \times (F_eCO_2 - F_iCO_2)]}{(1 - F_eO_2)}$$

where F_i and F_e are the fraction of O_2 and CO_2 in the inflowing and outflowing lines respectively. $\dot{V}_E$ and $\dot{V}O2$ values were used to report ventilatory equivalent to oxygen ($\dot{V}_E/\dot{V}O_2$).

2.4. Mifepristone treatment

The estrous cycle is divided in 4 phases, which are distinguished by the characterization of vaginal cytology and changes in hormone levels (Byers et al., 2012). The proestrous and estrous, which constitute the follicular stage and the metestrous and diestrous, which constitute the luteal stage. Stage of estrous cycle was determined by visual observation (Byers et al., 2012) and ventilatory variables were recorded in each mouse during follicular and luteal phases. After that, some female mice (WT, n = 6; Epo-TAg^h, n = 6) received daily oral gavage (10 µl/g) with the progesterone antagonist receptor Mifepristone (Sigma-Aldrich; 40 µg/g/day) for 12 consecutive days and ventilatory parameters were evaluated during luteal stage and follicular stage. Sham animals (WT, n = 5; Epo-Tag^h, n = 7) were force-fed with corn oil to see if force-feeding had and effect on ventilatory parameters.

2.5. Statistical analysis

Values are presented as mean ± standard deviation (SD). D'Agostino-Pearson omnibus normality test was realized to assess the distribution of the data. Three-way ANOVA, followed by Tukey comparisons test, was used to assess the respective effect of strain (WT vs Epo-TAg^h), environment (NX vs HX) and estrus cycle (luteal vs follicular) on respiratory parameters. Then another ANOVA was performed to assess the effect of drug (vehicle vs mifepristone) in the two strains of mice (WT vs Epo-TAg^h) in both environments (NX vs HX). All analyses were performed with the Graph Pad − Prism software (Graph Pad software, La Jolla, CA, USA). Differences were considered significant when $p < 0.05$.

3. Results

3.1. Increased ventilation in Epo-TAg^h female is not estrus cycle-dependent

First of all, sham experiments showed that the act of force-feeding does not modify basal ventilatory values in WT ($\dot{V}_E$: 2.36 ± 0.15 for untreated *vs* 2.07 ± 0.47 with corn oil only) and Epo-TAg^h mice ($\dot{V}_E$: 2.84 ± 0.42 for untreated *vs* 3.14 ± 0.76 with corn oil only).

First observation showed that Epo-Tag^h mice tend to hyperventilate in normoxic conditions, compared to WT mice (NS, p = 0.09; Fig. 1A). Furthermore, we observed a classical increase of ventilation after hypoxic exposure in WT and Epo-TAg^h mice (Fig. 1A). Moreover, $\dot{V}_E$ was significantly higher in Epo-TAg^h female mice in hypoxic condition compared to WT animals (Fig. 1A). When data were separated between the two estrous-cycle phases (luteal and follicular) we found that $\dot{V}_E$ was significantly increased during hypoxia in Epo-TAg^h mice compared to normoxia (Fig. 1B and C). The same main effects of hypoxia and mice strain were observed on V_T (Table 1) with no significant effect of estrus cycle. However, we observed a main effect of hypoxia and estrus cycle on $\dot{V}O_2$ and a main effect of hypoxia and an interaction effect between hypoxia and mice strain on $\dot{V}_E/\dot{V}O_2$. HVR was not different between the two strains and no significant change was observed between luteal and follicular phase (Fig. 2).