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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).

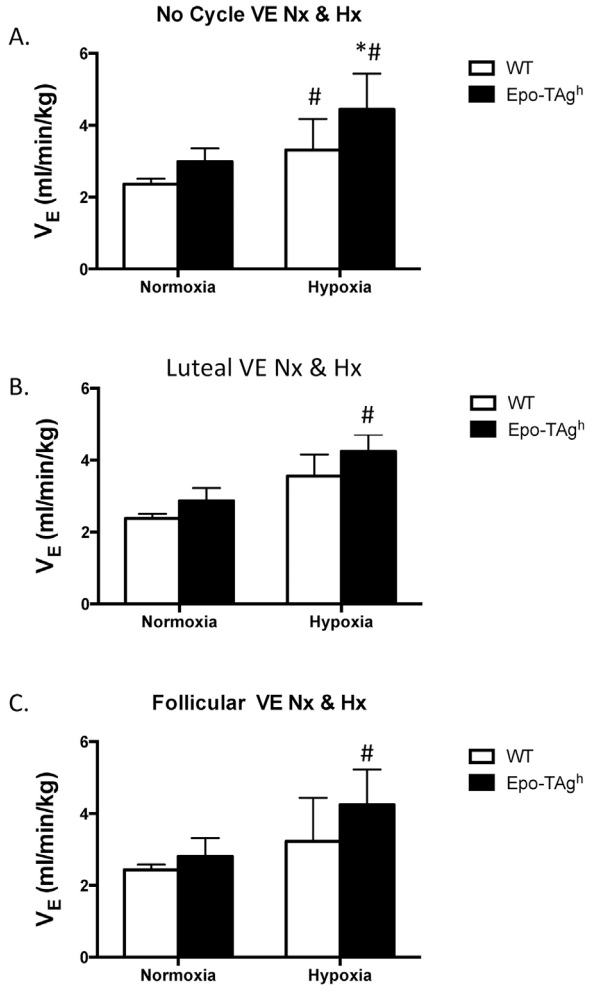

Fig. 1. Increased hypoxic ventilation in Epo-TAg^h female is estrous cycle-dependent. Minute ventilation ($\dot{V}_E$) at basal normoxic (Nx) and hypoxic (Hx) conditions in WT (n = 12) and Epo-TAg^h (n = 12) female mice when the phases of the estrous cycle was not taken in consideration (A), and when data was regrouped in the luteal (B) and follicular (C) phases. All values are mean ± SD. *p < 0.05, Epo-TAg^h *vs.* WT at normoxia or at hypoxia. # p < 0.05, Hypoxia *vs* normoxia in Epo-TAg^h and WT mice.

3.2. Progesterone inhibition according to phase of estrous cycle

To determine if progesterone was involved in modulating minute ventilation, female WT and Epo-TAg^h mice were treated with mifepristone for 12 consecutive days. The ANOVA has shown a significant main effect of drug on V_T (Table 1) and $\dot{V}O_2$ but not on $\dot{V}_E$ (Table 1, Fig. 3) in follicular and luteal phase. Moreover, we observed an interaction effect between mice strain and drug on V_T, f_R and $\dot{V}O_2$ (Table 1, Fig. 3). Globally, mifepristone increase the basal metabolism and potentiated the V_T response to hypoxia (Table 1) similarly in both phase of estrous cycle. These results suggest that progesterone has no specific interaction with Epo-deficiency but could modify the metabolism and the ventilatory pattern in female mice in response to normoxic or hypoxic environment.

4. Discussion

In the present study we investigate in female mice the impact of EPO deficiency as well as the possible roles of estrus cycle and progesterone on ventilatory control in normoxic and hypoxic conditions. Here we showed that Epo-TAg^h female mice had increased hypoxic ventilation and a tendency to increased normoxic ventilation, similar to what was reported in a previous study. However, we found no effect of estrus cycle on basal normoxic and hypoxic ventilation in WT or Epo-

Table 1
Ventilatory and metabolic parameters in WT and Epo-TAgh mice. Minute ventilation ($\dot{V}_E$, ml/min/g), respiratory frequency (f_R, cycle/min), tidal volume (V_T, µg/1), oxygen consumption ($\dot{V}O_2$, ml/min/g) and ventilatory equivalent ratio ($\dot{V}_E/\dot{V}O_2$, ml/min/g) in WT and Epo-TAgh mice during normoxia and hypoxia, in both phases of estrous cycle, before and after Mifepristone treatment. All values are mean ± SD. #p < 0.05 normoxia vs hypoxia; *p < 0.05, WT *vs.* Epo-TAg^h, $p < 0.05 untreated vs Mifepristone. In italics, main effect obtained with MANOVA.

		WT			Epo-Tagh			
		Follicular		Luteal	Follicular		Luteal	
		Control	Mifepristone	Control	Mifepristone	Control	Mifepristone	Control	Mifepristone
VE	Nx	2.43 ± 0.15	2.45 ± 0.37	2.38 ± 0.13	2.48 ± 0.28	2.81 ± 0.51	3.07 ± 0.53	2.87 ± 0.35	3.05 ± 0.31
	Hx	3.22 ± 1.21	4.29 ± 1.02#	3.56 ± 0.60	4.13 ± 1.03#	4.24 ± 0.98#	4.69 ± 0.78#	4.24 ± 0.47#	4.77 ± 0.87#
Fr	Nx	276 ± 21	232 ± 17	271 ± 10	236 ± 10	245 ± 28	266 ± 27	240 ± 32	267 ± 19
	Hx	307 ± 37	311 ± 22#	319 ± 10#	309 ± 17#	342 ± 23#	327 ± 17#	345 ± 27#	332 ± 24#
VT	Nx	8.52 ± 0.94	10.53 ± 1.22	8.78 ± 0.48	10.55 ± 1.18	11.45 ± 1.35	11.53 ± 0.99	11.97 ± 0.71	11.53 ± 0.99
	Hx	9.82 ± 2.48	13.65 ± 2.55$	11.12 ± 1.66	13.30 ± 2.68	12.30 ± 2.10	14.28 ± 1.63	13.32 ± 2.12	14.28 ± 1.63
VO2	Nx	0.065 ± 0.006	0.092 ± 0.003$	0.083 ± 0.008	0.096 ± 0.005	0.072 ± 0.013	0.089 ± 0.005$	0.072 ± 0.013	0.086 ± 0.009$
	Hx	0.024 ± 0.005#	0.033 ± 0.004#	0.030 ± 0.006#	0.036 ± 0.003#	0.025 ± 0.004#	0.032 ± 0.004#	0.027 ± 0.006#	0.033 ± 0.004#
VE/VO2	Nx	36.05 ± 5.38	26.61 ± 3.38	28.14 ± 1.32	26.01 ± 3.01	40.25 ± 11.60	34.37 ± 5.40	40.80 ± 8.46	35.41 ± 2.21
	Hx	128.52 ± 39.18#	128.44 ± 26.53#	106.54 ± 19.95#	114.19 ± 23.25#	178.58 ± 37.26#*	145.97 ± 23.89#$	172.79 ± 34.67#*	146.50 ± 29.63#
		V_T and VO_2 main effect Control vs Mifepristone: p < 0.0005		*V_T and VO_2 main effect Control vs Mifepristone: p < 0.05*		*V_T and VO_2Control vs Mifepristone: p < 0.0005*		*V_T and VO_2 Control vs Mifepristone: p < 0.05*	

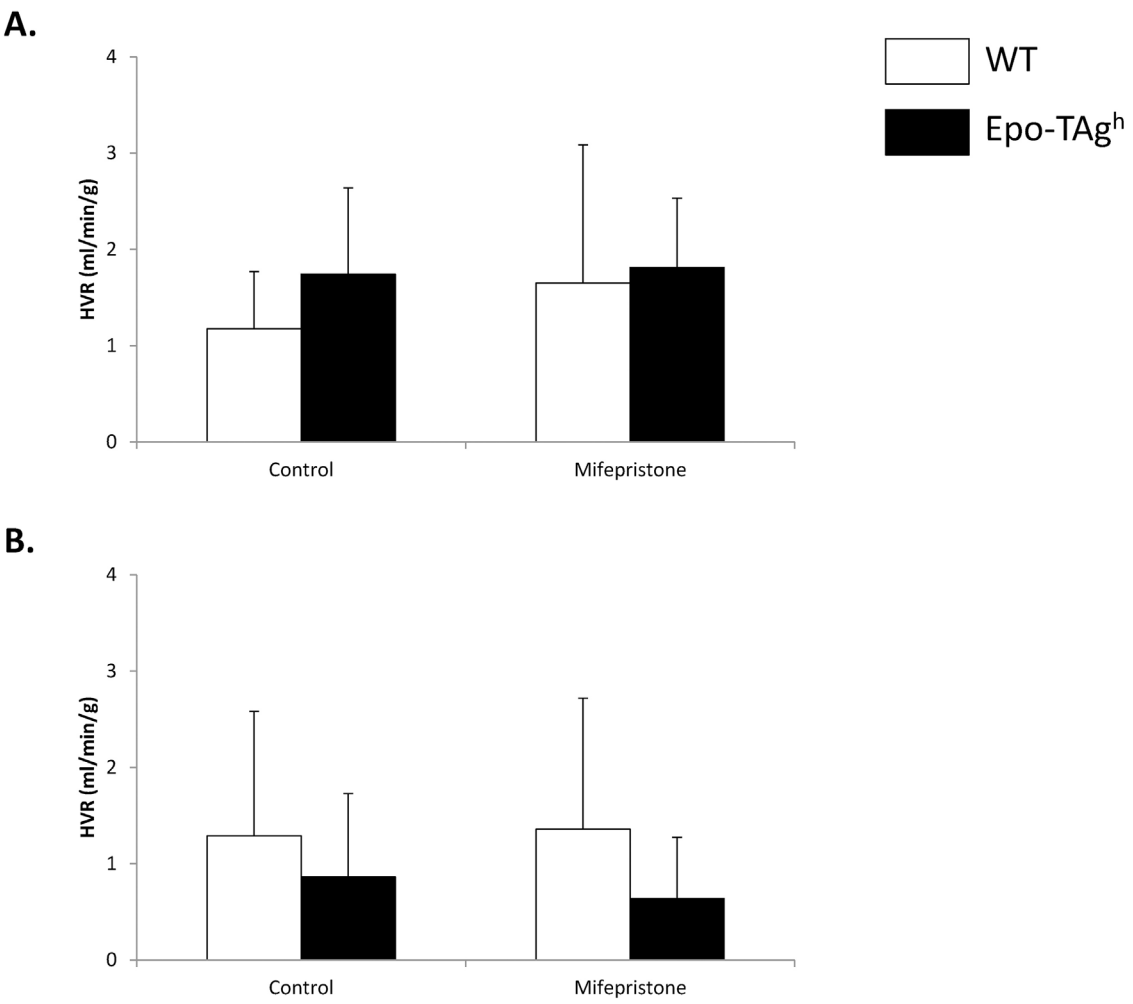

Fig. 2. The hypoxic ventilatory response is not modified by estrous cycle or progesterone inhibition. Hypoxic ventilatory response (HVR; $\dot{V}_E$ at 8% O_2 − $\dot{V}_E$ at 21% O_2) recorded in luteal (A) and follicular (B) phase before and after mifepristone treatment in WT (n = 6) and Epo-TAg^h (n = 6) female mice. All values are mean ± SD.

deficient mice. Additional experiments using progesterone antagonist mifepristone suggested that progesterone does not account in the modulation of the ventilatory and metabolic parameters specifically in Epo deficient mice despite its expression is known to be higher in luteal phase of the estrus cycle. However, progesterone could influence metabolism and respiratory pattern. As the expression level of progesterone at follicular phase is lower compared to luteal phase, in line with the results obtained at luteal phase, these data suggest that the setting of normoxic and hypoxic ventilation in Epo-TAg^h mice was due to the Epo deficiency, rather than progesterone

4.1. Epo-deficiency led to a stimulation of ventilation in normoxic and hypoxic conditions depending of estrus cycle

Insufficient Epo production in adults is mostly due to the damage of Epo-producing cells or the suppression of Epo production by inflammatory cytokines (Bunn, 2013). Patients with such pathologies usually develop anemia, a condition in which the organism does not have enough amounts of red blood cells to provide efficient tissue oxygenation. In this context, we used our Epo-deficient transgenic mouse line (Epo-TAg^h) to investigate the impact of Epo deficiency and/or anemia in the neural control of ventilation. Previous research showed that in male mice, basal ventilation and HVR were similar in WT and Epo-TAg^h male mice (Pichon et al., 2016; Voituron et al., 2014). However, we found profound alteration in the expression levels of several genes or proteins that are involved in the regulation of oxygen metabolisms in the brain. Specifically, we observed that the level of the hypoxia inducible factor (HIF), as well as its target genes VEGF, Gluco-transporter-1 (GLUT-1), and endothelial and neural NOS were significantly augmented under basal condition (El Hasnaoui-Saadani et al., 2009). In fact, in line with these results it was reported that HIF-1 regulates the shift to anaerobic metabolism by inducing the expression of glycolytic enzymes and glucose transporters (Schonenberger and Kovacs, 2015; Ziello et al., 2007). Furthermore, results showed an interaction between hypoxia and Epo-deficiency on $\dot{V}_E/\dot{V}O_2$ in female mice. Indeed, $\dot{V}_E/\dot{V}O_2$ was significantly higher in Epo-deficient female mice. This result indicates that Epo-TAg^h mice could present a lower ventilatory efficiency when expose to hypoxia as compare to WT mice. This result is probably linked to the severe anemia and to the decrease in oxygen transport capacity observed in Epo-TAg^h mice (Pichon et al., 2016), which conduce to larger ventilation and probably to a lower aerobic capacity.

Motivated by results of several reports showing that Epo contributes to the ventilatory sex-dimorphism in mice (Ballot et al., 2015; Gassmann et al., 2009; Soliz et al., 2009), and humans (Soliz et al., 2009), we wanted to investigate the role of Epo deficiency in Epo-TAg^h female mice and the possible effects of estrus cycle. Contrary to our previous observations in male mice, our results show that ventilation in hypoxic conditions was increased in Epo-TAg^h animals.

4.2. Progesterone is not involved in the increased ventilation observed in Epo-TAg^h mice

In adult mice, long-term progesterone treatment increase minute

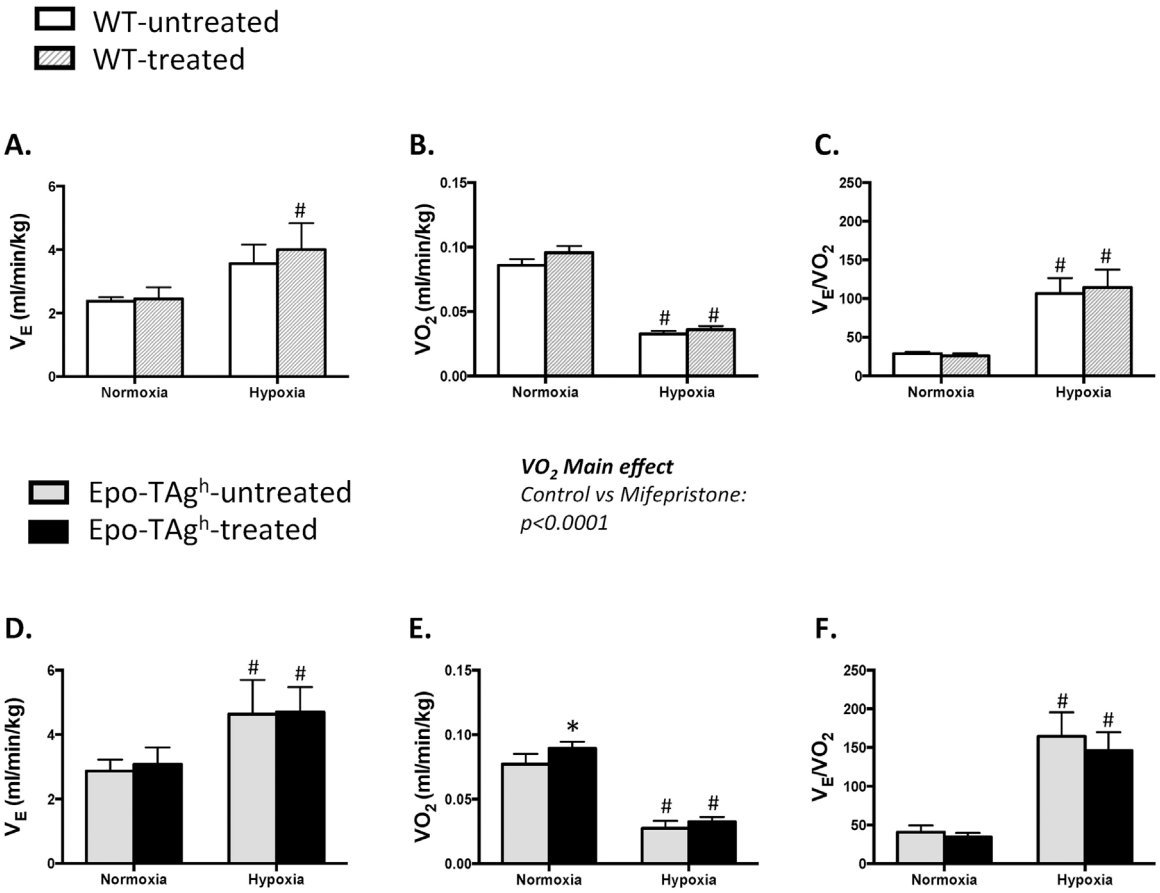

Fig. 3. Progesterone inhibition does not alter ventilation in WT and Epo-TAg^h mice. Minute-ventilation ($\dot{V}_E$; A-D), oxygen consumption ($\dot{V}O_2$; B-E) and ventilatory equivalent ratio ($\dot{V}_E/\dot{V}O_2$; C-F) in WT (n = 6) and Epo-TAg^h (n = 6) female mice in luteal phase, recorded under normoxic and hypoxic conditions. All values are mean ± SD. $^{\#}p < 0.05$ normoxia *vs* hypoxia; *p < 0.05, untreated *vs*. Mifepristone in normoxia or in hypoxia. In italics, main effect obtained with MANOVA.

ventilation and metabolic rate during sleep (Marcouiller et al., 2014) and the nuclear and membrane progesterone receptors are involved in respiratory control under normoxia or in response to hypoxia (Boukari et al., 2016; Marcouiller et al., 2014). However, our results showed that when differentiating luteal and follicular phases, hypoxic ventilation and HVR were not significantly different in Epo-TAg^h or WT mice. Recent studies suggest that HVR is a sex-dependent mechanism (Joseph et al., 2000; Joseph et al., 2002). Indeed, HVR is increased during pregnancy (Moore et al., 1987) and female circulating hormones are able to enhance hypoxic sensitivity (Gassmann et al., 2009). Taken together, these conflicting results suggest no interaction between Epo-deficiency and progesterone in the implementation of ventilatory response to hypoxia in female mice. As such, to elucidate the implication of progesterone in our results, we performed experiments in which animals were treated with mifepristone. Interestingly, our results showed that progesterone receptor blockade with mifepristone did neither alter the normoxic nor hypoxic ventilatory output in WT and Epo-TAg^h female mice. These results strongly suggest that progesterone is not involved in the increased ventilation observed in Epo-TAg^h mice, and point out that Epo deficiency and/or anemia should be the main factor(s) leading to such effects. As such, our results showed that the neural network controlling ventilation seems to be affected in female animals with Epo deficiency and/or anemia, while no such effect was currently reported in males (Pichon et al., 2016). More studies are required to determine until what extent the molecular restructuration observed in male, are present in Epo deficient female mice. It is important to note here that estradiol has a crucial implication on the expression and activity of key molecules regulating the oxygen metabolism, such HIF-1 and VEGF both in target tissues (Kazi and Koos, 2007) as in the brain (Habib et al., 2014; Mukundan et al., 2004a,b; Shin et al., 2015; Zheng et al., 2013).

4.3. Methodological considerations

4.3.1. Animal model

Experiments were performed on Epo-deficient mice that display a whole-body reduced Epo expression (Binley et al., 2002; Pichon et al., 2016). This model combines chronic anemia (low O_2 content) with chronic Epo deficiency (Pichon et al., 2016). Therefore, we cannot exclude that the observed effects are due to chronic anemia and/or Epo deficiency.

4.3.2. Ventilatory measurement accuracy

Ventilatory variables were evaluated by whole body plethysmography, which present some limitations in small mammals that have already largely debated (Mortola and Frappell, 1998). Indeed, results obtained on f_R were very reliable whereas those obtained with V_T was doubted because the accuracy of Drorbaugh and Fenn's equation (1955) for calculating the V_T was questioned (Epstein and Epstein, 1978; Jacky, 1980).

4.3.3. Mifepristone treatment

in order to test the effect of gavage on the measured variables, sham experiments were performed in a previous unpublished series of experiments where we treated animals by gavage with only corn oil. As no effects was observed, only treatment with Mifepristone was used in this set of experiments. Mifepristone is a mixed antagonist of progesterone and glucocorticoid receptors, which permits to this synthetic steroid compound to bind with very high affinity to progesterone and glucocorticoid receptors in rats (Schreiber et al., 1983). Furthermore,

mifepristone also binds weakly to the androgen receptor and might also have a weak anti-androgenic activity (Schreiber et al., 1983). Although mifepristone is a non-selective progesterone receptor antagonist that also antagonizes the glucocorticoid or androgen receptors, it is the only compound freely available in the market. Therefore, we could not exclude that obtained results were also due to an undefined role of glucocorticoids or androgens on HVR.

4.4. Perspectives and significances

Some of the most important physiological strategies for coping with hypoxia are the increase in ventilation and the induction of erythropoiesis to enlarge the capacity of the blood to carry oxygen. Epo is involved in both processes. However, under conditions of Epo deficiency, both the erythropoietic and the ventilatory strategies are reduced. Such abnormal situation may lead to prioritizing the up regulation of molecules controlling oxygen metabolism. In the case of female animals, estradiol could play an important role in such reorganization, thus this would allow better functional adaptation to hypoxia. In fact, anemia is recurrent during pregnancy and the physiology of female animals and women should be efficiently prepared to cope with such situation (Mayama et al., 2017; Semasaka et al., 2016). Accordingly, the results of this study open new perspectives in the comprehension of Epo deficiency-related pathologies that so far remain poorly investigated. In this context, further experiments are needed to validate these hypotheses.

In conclusion, when the level of Epo is chronically decreased, female mice did not show estrous cycle effect on basal and hypoxic ventilation. These results have potential clinical implications in respiratory responses evoked under anemic conditions and the possible negative interaction between Epo deficiency and progesterone level.

Authorship

FJ, JS, DM, AP and NV: conception and design of the study, acquisition of data, analysis and interpretation of data. JS, VJ, JPR, AP and NV: drafting the article and revising it critically for important intellectual content; FJ, JS DM, VJ, JPR, AP and NV: final approval of the version to be submitted.

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