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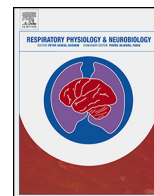
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Carbamylated erythropoietin enhances mice ventilatory responses to changes in O₂ but not CO₂ levels



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ABSTRACT

Erythropoietin (EPO) has beneficial tissue-protective effects in several diseases but erythrocytosis may cause deleterious effects in EPO-treated patients. Thus carbamylated-EPO (C-EPO) and other derivatives retaining tissue-protective but lacking bone marrow-stimulating actions have been developed. Although EPO modulates ventilatory responses, the effects of C-EPO on ventilation have not been investigated. Here, basal breathing and respiratory chemoreflexes were measured by plethysmography after acute and chronic treatments with recombinant human C-EPO (rhC-EPO; 15,000 IU/kg during 5 days) or saline (control group). Hematocrit, plasma and brainstem rhC-EPO levels were also quantified. Chronic rhC-EPO significantly elevated tissue rhC-EPO levels but not hematocrit. None of the drug regimen altered basal ventilation (normoxia). Chronic but not acute rhC-EPO enhanced hyperoxic ventilatory depression, and sustained the hypoxic ventilatory response mainly via a reduction of the roll-off phase. By contrast, rhC-EPO did not blunt the ventilatory response to hypercapnia. Thus, chronic C-EPO may be a promising therapy to improve breathing during hypoxia while minimizing adverse effects on cardiovascular function.

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1. Introduction

Erythropoietin (EPO) plays a major role in oxygen (O₂) homeostasis in mammals. This hormone is mainly synthesized in fetal liver and adult kidneys, and its basal level is upregulated when the arterial PO₂ decreases below normal levels (Beck et al., 1991; Haase, 2013; Jelkmann, 1992). Higher plasma EPO levels stimulate red blood cells production, thereby increasing the blood O₂ carrying capacity. Accordingly, the use of recombinant human EPO (rhEPO) in patients with severe anemia and/or chronic kidney disease showed a reduced requirement for blood transfusions and improved quality-of-life assessments (Canadian Erythropoietin Study Group, 1990; Eschbach et al., 1989; Kato, 2010; Pfeffer et al., 2009). Besides its erythropoietic effect, EPO has tissue-protective properties in a broad range of pathologies including hypoxia and ischemia-induced brain damages (Chateauvieux et al., 2011; Jelkmann, 2007; Kumral et al., 2011; Sanchis-Gomar et al.,

2013; Siren et al., 2009). These neuroprotective effects are mediated by endogenous EPO (i.e. produced by neurons and astrocytes) or exogenous rhEPO acting on EPO-receptors (EPO-R) that are extensively present in brain (Marti et al., 1996). However, the doses of rhEPO required to obtain such tissue-protective effects stimulate erythropoiesis and increase the hematocrit, leading to a subsequent procoagulant status and elevated blood pressure (Marti, 2004; Velly et al., 2010). Serious side effects of chronic rhEPO include hypertension, polycythemia, myocardial infarction, stroke and seizures, platelet activation and increased thromboembolic risk (Banach et al., 2010; Macdougall et al., 2012; Ponikowski et al., 2007; Shin et al., 2012; van Veldhuisen et al., 2007). This prompted for the development of new generation of EPO-R agonist drugs to preserve tissue-protective properties and avoid erythropoiesis (Siren et al., 2009). Carbamylated EPO (C-EPO) is one of the promising derivatives that may provide better outcome in clinical trials (Choi et al., 2014; Leconte et al., 2011; Leist et al., 2004).

In addition to its erythropoietic and tissue-protective effects, EPO is thought to interact with peripheral and central chemoreceptors to increase ventilation in response to hypoxia (Gassmann and Soliz, 2009; Khemiri et al., 2012; Soliz, 2013; Soliz et al., 2005). EPO may play a role in the central regulation of breathing as EPO injections in the adult rat brain increase minute ventilation (Yalcin et al., 2007). Also, the hypoxic ventilatory response is enhanced in

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transgenic mice with chronic cerebral rhEPO overexpression compared to wildtype animals (Caravagna et al., 2015). Similarly, bath application of rhEPO prior to recording of fictive breathing prevented hypoxic depression in brainstem preparations from mice neonates (Khemiri et al., 2012). Therefore, chronic rhEPO therapy was suggested as a reliable approach for treating respiratory disorders (Caravagna et al., 2015; Soliz, 2013). However, we have recently shown that polycythemia alone or increased rhEPO levels in plasma and brain blunts the hypercapnic ventilatory response (Menuet et al., 2016). This potentially deleterious effect on breathing may represent another limitation for prescription of chronic rhEPO treatment in patients. Thus, ideal drug therapy to compensate for the lack of O₂ at multiple levels should involve molecules retaining beneficial respiratory-related and tissue-protective effects independent of erythrocyte production. Although the effects of C-EPO on the neural control of breathing have not been investigated, this molecule could fulfill these criteria. Indeed, in 2004, Leist and collaborators demonstrated that carbamylation of EPO, which transforms lysine residues into homocitrullines and alters the protein conformation, maintained the tissue-protective effects of EPO and eliminated its hematopoietic effects (Leist et al., 2004). In the last years, several studies confirmed these observations and proved the neuroprotective effects of C-EPO in numerous CNS diseases (Chen et al., 2015). Therefore, this study was designed to measure ventilation by plethysmography after acute and chronic treatments with rhC-EPO, and to evaluate drug effects in normoxia, hyperoxia, hypoxia and hypercapnia. We also compared plasma and brain rhC-EPO levels as well as hematocrit in saline or rhC-EPO treated mice.

2. Material and methods

2.1. Ethics statements

Adult C57BL6 male mice (n=20; Janvier, France) from 90 to 120 postnatal days were used in this study. Animals were housed under pathogen-free conditions, had unlimited access to food and water, and were maintained on a reversed 12:12 h light/dark cycle in a temperature-controlled room (25 °C ± 2 °C) for 1 week prior to experimental manipulation. The experimental procedures were carried out in accordance with French national legislation (JO 87-848, European Communities Council Directive 2010/63/EU, 74) and local ethics committee "Direction Départementale de la Protection des Populations", with permit number 13-227 delivered to CG.

2.2. Preparation and identification of rhC-EPO

RhC-EPO was prepared from rhEPO (Eprex, Janssen Cilag, France) as previously described (Leist et al., 2004). Briefly, one volume of rhEPO (1 mg/ml) was mixed with one volume of 1 M Na-borate (pH 8.8) and recrystallized KOCN was added to a final concentration of 1 M. The mixture was incubated at 37 °C for 24 h. Samples were immediately dialyzed against Mill-Q water and subsequently against 20 mM Sodium citrate in 0.1 M NaCl, pH 6.0. After dialysis, the samples were concentrated using Centricon with pore size of 3 kDa (Amicon). The protein content was determined with a Bradford dosage. Efficient carbamylation of rhEPO was confirmed by testing the resistance of native and carbamylated rhEPO to proteolysis by Lys-C proteinase (Leist et al., 2004). Briefly, 10 µg of rhEPO or rhC-EPO were incubated with 0.4 µg of Lys-C proteinase at 37 °C for 20 h. Laemmli buffer was then added and samples were boiled for 5 min. The digestion products were finally separated by SDS-PAGE electrophoresis on 15% acrylamide gel and proteins were visualized by silver staining.

2.3. Drug injections

Mice were divided into 2 groups (10 animals per group). Mice were injected *i.p.* with sterile NaCl (0.9%) or rhC-EPO at 15,000 IU/kg diluted in final volume 100 µl of NaCl. This dose of rhC-EPO has been chosen based on both previous studies on neuroprotection (Banks et al., 2004; Ehrenreich et al., 2004; Xenocostas et al., 2005) and our preliminary plethysmography experiments suggesting central effects of EPO. Injections were made in the evening during five consecutive days.

2.4. Hematocrit measurements

After recordings of ventilation on days 1 and 5, blood samples (100 µl) were obtained after a small incision was made at the tip of the tail. The samples were collected in heparinized plastic capillaries and hematocrit (Htc) was measured by centrifugation method (5000 g for 5 min at 4 °C).

2.5. Determination of ventilation by plethysmography

Ventilation of conscious animals was recorded using non-invasive whole-body barometric plethysmography (EMKA Technologies, Paris, France), as previously described (Menuet et al., 2016). Two days before recordings, the mice were habituated to the plethysmograph chamber to reduce stress. On the day of the experiment, mice were weighted and rectal temperature was measured before and after ventilatory measurement. We used a constant flow plethysmograph (EMKA Technologies, Paris, France) with 200-ml animal chamber ventilated with air (600 ml/min). The various gas mixtures corresponding to normoxia, hyperoxia, hypoxia and hypercapnia were delivered using a programmable gas mixer (GSM3, CWE, USA). Spirograms were stored and analyzed (Spike 2; Cambridge Electronic Design, Cambridge, UK) to calculate the mean respiratory frequency (Rf, cycles/min), tidal volume normalized by the body weight (V_T, ml/100 g) and minute ventilation normalized by the body weight (V_E, ml/min/100 g, with V_E = Rf × V_T). Volume calibrations were performed by injecting small amount (100 µl) of air in the recording chambers, and these measurements were used to calculate V_T (Drorbaugh and Fenn, 1955; Menuet et al., 2016).

Mice were initially exposed to normoxia (21% O₂ balance N₂) and basal ventilation (V_E, Rf and V_T) was recorded for at least thirty minutes. On days 1 and 5, animals were exposed to hypercapnia (21% O₂, 5% CO₂ balance N₂), hyperoxia (100% O₂), and hypoxia (10% O₂ balance N₂). Ventilation was recorded for 5 min during the hypercapnic (HCVR) and hyperoxic (HxVR) challenges, and for 20 min in hypoxia to study the early phase (from 0 to 5 min) and the late phase (from 15 to 20 min) of the hypoxic ventilatory response (HVR) (Maxova and Vizek, 2001; Powell et al., 1998). Each challenge was separated by a recovery period of at least 20 min in normoxia, during which basal ventilation was reassessed for 5 min before the next challenge. Only periods of breathing without body movements were studied. In some cases, animals were too agitated during a particular recording period and corresponding data were excluded from the analysis.

2.6. ELISA assay

On the last experimental day (Day 5), brainstem and plasma were collected to determine rhC-EPO concentrations. After deep anesthesia of the mice with isoflurane (5% in air), blood samples were obtained by cardiac puncture and collected in heparinized tubes. They were immediately centrifuged (760g for 15 min at 4 °C) and 100 µl of plasma were collected. After transcardiac perfusion with heparinized PBS, brainstem was dissected and removed. Plasma and brainstem samples were immediately frozen in liq-

uid nitrogen, and stored at -80°C until further use. The analysis of rhC-EPO levels in brainstem required the homogenization of the tissues. Briefly, for each group, 3 samples were prepared by pooling together brainstem from 3 mice in FastPrep@Lysing matrix D tubes (from MP), the tubes being maintained on ice. Ice-cold lysis buffer (150 μl , 0.9% NaCl with protease inhibitor cocktail from Sigma Aldrich) was then added to the tubes and tissues were homogenized using lysis homogenizer (40 s homogenization). Tubes were centrifuged to remove cell debris (12,000g for 20 min at 4°C) and clear supernatants were collected and analyzed. Concentrations of rhC-Epo in plasma and tissue homogenates were measured using ELISA kit (from R&D systems, Minneapolis, MN) according to manufacturer's instructions (Menuet et al., 2016). The sensitivity of the assay was ~ 1.0 mIU/mL. Results are expressed as mIU per gram of tissue (mIU/g) for the brainstem, and as mIU/mL for the plasma.

2.7. Statistical analysis

All statistical tests were performed using GraphpadPrism 6 (Graphpad software, LaJolla, California, USA), and values expressed as mean (SD). Results of the ELISA assay were compared using unpaired *t* tests. Hematocrit, body weight, rectal temperature, basal ventilation and values of delta V_E were compared using a two-way ANOVA with treatment and time effects as main factors. Raw HxVR, HVR and HCVR were compared using two-way repeated measures ANOVA with treatment and FiO_2 or FiCO_2 effects as main factors. When appropriate, post-hoc protected least significant difference of Fisher (PLSD) tests were used. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Validation of rhEPO carbamylation

After chemical modification of rhEPO to rhC-EPO, rhEPO and rhC-EPO were digested by Lys-C and analyzed by SDS-PAGE. After digestion by Lys-C, rhEpo was entirely degraded so that no bands of rhEPO could be observed. By contrast, rhC-EPO resisted proteolysis since no degradation peptides could be detected, confirming the efficient carbamylation of rhEPO (Fig. 1).

3.2. Acute or chronic rhC-EPO did not increase hematocrit

Results of hematocrit (Htc) measurements in mice after acute (Day 1) and chronic (Day 5) treatments with NaCl or rhC-EPO are shown in Table 1. None of the statistical comparisons revealed significant differences. Thus, control and rhC-EPO groups had similar hematocrit after acute and chronic injections.

Table 3

Comparisons of body weight and rectal temperature with 2-way repeated measures ANOVA analysis. Data are expressed as mean (SD).

Time	Groups	Weight (g)	Temperature ($^{\circ}\text{C}$)	Time effect	Treatment effect	Interaction
Day 0	Control (n = 8)	23.7 (1.08)	37.6 (0.86)	Statistics for body weight: F = 19.50 p < 0.001	F = 0.056 p = 0.816	F = 0.054 p = 0.947
	rhC-EPO (n = 9)	23.5 (1.74)	37.7 (0.88)			
Day 1	Control (n = 8)	24.0 (1.23)	37.5 (0.74)	Statistics for temperature: F = 2.371 p = 0.110	F = 0.702 p = 0.415	F = 0.795 p = 0.461
	rhC-EPO (n = 9)	23.8 (1.86)	37.9 (0.66)			
Day 5	Control (n = 8)	24.6 (1.10)	37.1 (0.66)			
	rhC-EPO (n = 9)	24.4 (1.91)	37.3 (0.87)			

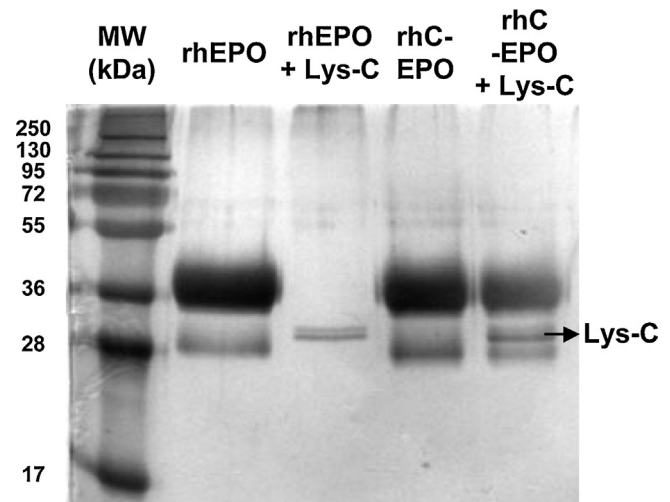


Fig. 1. Identification of rhC-EPO. The efficiency of carbamylation of rhEPO into rhC-EPO was confirmed by SDS-PAGE after digestion with the endoproteinase Lys-C and visualization by silver staining. Lane 1: molecular-weight-markers (MW). Lane 2: rhEPO without Lys-C digestion. Lane 3: rhEpo with Lys-C digestion. Lane 4: rhC-EPO without Lys-C digestion. Lane 5: rhC-EPO with Lys-C digestion. Note the lack of degradation of rhC-EPO after incubation with Lys-C.

Table 1

Comparisons of hematocrit levels with 2-way repeated measures ANOVA analysis. Data are expressed as mean (SD).

Time	Groups	Hematocrit	Timeeffect	Treatment effect	Interaction
Day 1	Control (n = 7)	41.14 (3.01)	F = 0.727	F = 3.310	F = 0.040
	rhC-EPO (n = 7)	43.41 (1.66)	P = 0.411	P = 0.094	P = 0.845
Day 5	Control (n = 7)	39.89 (2.93)			
	rhC-EPO (n = 7)	42.64 (5.07)			

Table 2

Comparisons of plasma and brainstem rhC-EPO concentrations on day 5 with unpaired *t*-test.

Parameter	Groups	Mean (SD)	t-Values
Plasma concentration	Control (n = 5)	29.55 (31.46)	-8.266
	rhC-EPO (n = 5)	1009*** (263.1)	
Brainstem concentration	Control (n = 3)	0.008 (0.002)	-3.166
	rhC-EPO (n = 3)	0.019* (0.005)	

3.3. Chronic injections increased rhC-EPO levels in plasma and brainstem

Tissue levels of rhC-EPO were measured in control and rhC-EPO groups of mice on day 5. Results indicated that chronic drug injections significantly elevated rhC-EPO concentrations in plasma and brainstem compared to control values (Table 2). Therefore, peripherally administrated rhC-EPO partially crossed the blood-brain barrier and reached the brainstem.

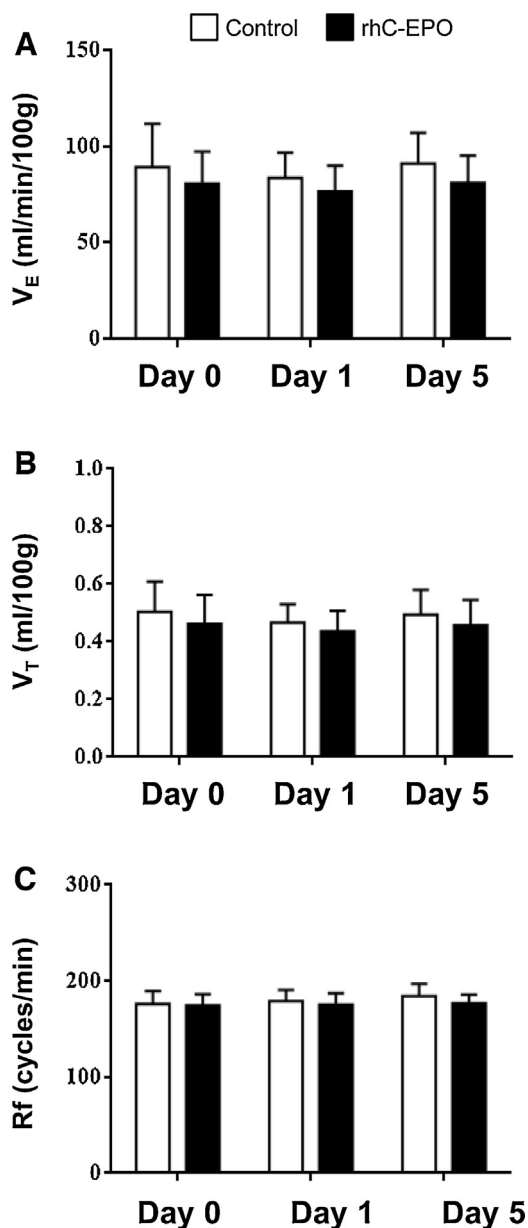


Fig. 2. Measurements of respiratory parameters in normoxia (basal ventilation) before treatment (Day 0), after acute (Day 1) and chronic (Day 5) drug injections. **A:** Minute ventilation (V_E). **B:** Tidal volume (V_T). **C:** Respiratory frequency (Rf). Values are expressed as mean (SD). Basal ventilation did not change significantly between the two groups of mice over the experimental protocol.

3.4. rhC-EPO did not alter body weight and rectal temperature

Body weight and rectal temperature were measured on days 0, 1 and 5. Statistical results showed a significant main time effect for body weight only (Table 3). Post-hoc comparisons, however, did not reveal significant within-group differences ($P > 0.05$ for all comparisons). Thus, the two groups of mice had similar body weight and rectal temperature over the experimental protocol.

3.5. Basal ventilation was not affected by rhC-EPO

Basal ventilatory parameters (V_E , V_T and Rf in normoxia) were measured before treatment (Day 0), as well as after acute (Day 1) and chronic (Day 5) drug injections in control ($n=8$) and rhC-EPO treated ($n=9$) mice (Fig. 2). All statistical group comparisons yielded non-significant results (data not shown). Therefore, control

and rhC-EPO groups of mice had similar basal ventilation over the experimental protocol.

3.6. Chronic but not acute rhC-EPO increased the hyperoxic ventilatory depression

Ventilatory responses to hyperoxia (HxVR, 100% O_2 for 5 min) were measured in control and rhC-EPO groups of mice after acute and chronic drug injections. Original traces from plethysmography are shown in Figs. 3 a–c and 4 a–c. In both groups, hyperoxia significantly decreased V_E , this ventilatory depression being mainly due to a decrease in Rf (Fig. 3d). However, there was no treatment \times FiO_2 interaction for V_E , V_T and Rf after acute injection (Day 1; Table 4). Thus, acute rhC-EPO treatment had no effect on HxVR.

After chronic drug injections (Day 5), significant treatment \times FiO_2 interactions were found for V_E and V_T denoting greater decreases in both parameters during hyperoxia in mice treated with rhC-EPO (Fig. 4d, Table 4). The magnitude of ventilatory depression (or Delta V_E) was also compared to further examine the effects of treatments over time (Fig. 5). This confirmed a lack of significant effect on day 1 ($P=0.332$), and a significant decrease ($P < 0.01$) in Delta V_E in rhC-EPO treated mice compared to control group on day 5. Thus, the hyperoxic ventilatory depression increased after chronic treatment with rhC-EPO.

3.7. Chronic but not acute rhC-EPO enhanced the hypoxic ventilatory response

The effects of rhC-EPO on the hypoxic ventilatory response (HVR, 10% O_2 for 20 min) were measured after acute (Day 1, Fig. 6) and chronic (Day 5, Fig. 7) treatments. Representative traces from plethysmography are shown in Figs. 6a–d and 7a–d, respectively. Data comparisons with ANOVA analyses are detailed in Table 5.

On day 1, both groups had significantly elevated V_E in the early phase of HVR compared to basal ventilation (significant main FiO_2 effect). The late phase of HVR was characterized by a significant ventilatory depression in the two groups of mice (i.e. a significant decrease in V_E compared to the early phase of HVR), mainly explained by decreases in Rf and/or V_T (Fig. 6e, Table 5). No treatment \times FiO_2 interaction was found (Table 5). Thus, acute treatment with rhC-EPO had no effect on the HVR. On day 5, however, a significant treatment \times FiO_2 interaction was found for V_E (Table 5). The two groups had an elevated V_E in both the early and late phases of HVR compared to basal ventilation, due to elevated V_T and Rf. However, only control mice had a clear ventilatory depression in the late phase of HVR whereas no difference in respiratory parameters could be detected between the early and late phases of HVR in rhC-EPO treated mice (Fig. 7e, Table 5). To further examine the effects of treatments over time, we also compared the magnitude of the responses as Delta V_E on days 1 and 5 (Fig. 8). This confirmed a lack of significant effects on day 1 for the magnitude of the early, late and roll-off phases of HVR ($P > 0.05$ for all comparisons), and significant increases ($P < 0.05$) in Delta V_E for the late and roll-off phases of HVR in rhC-EPO compared to control mice on day 5. Thus, rhC-EPO enhanced the late phase of HVR and reduced hypoxic ventilatory depression.

3.8. rhC-EPO did not alter ventilatory response to hypercapnia

Ventilatory parameters recorded during hypercapnia after acute and chronic rhC-EPO injections are illustrated in Figs. 9 and 10, respectively. In the two groups of mice, statistical comparisons revealed significantly elevated V_E , V_T and Rf in hypercapnia compared to basal condition (main $FiCO_2$ effect) after both acute and chronic treatments. However, in both cases, there was no treatment effect and no treatment \times $FiCO_2$ interaction (Table 6). Therefore,

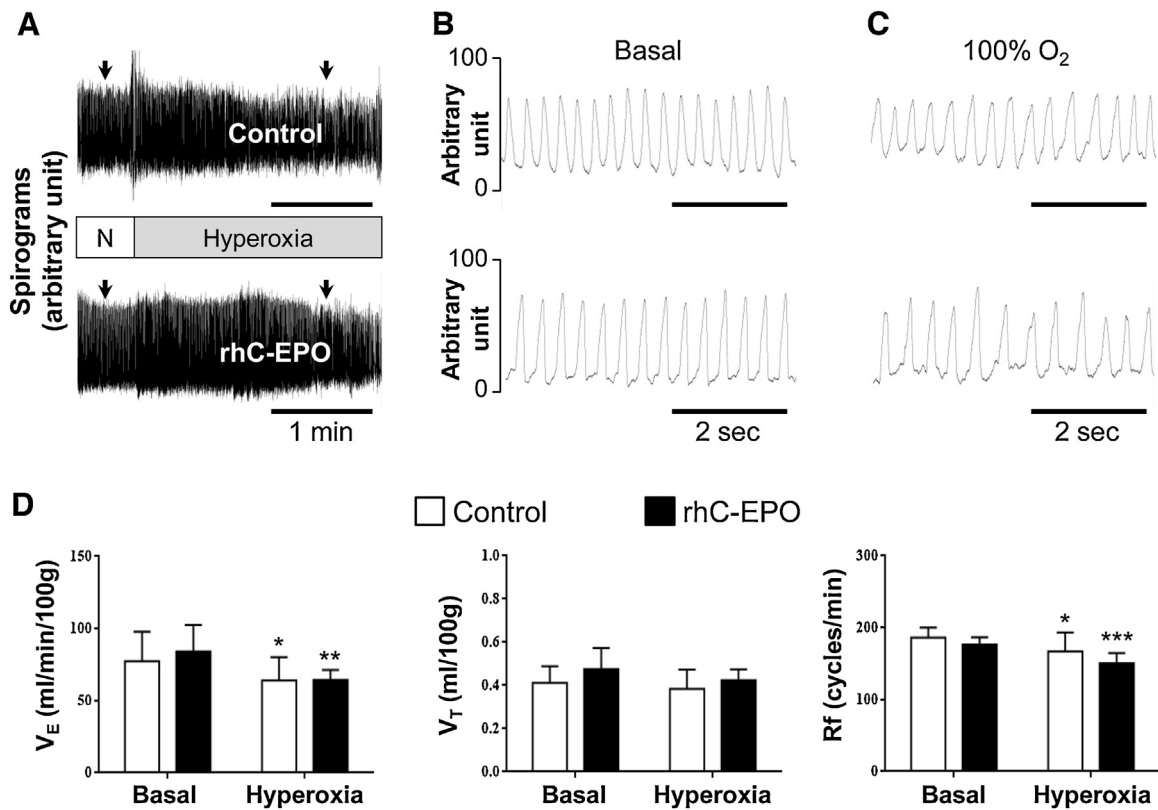


Fig. 3. Changes in ventilation in response to hyperoxia after acute drug injections (Day 1). **A:** Original tracings from plethysmography. **B-C:** Enlargements of the spirogram at periods indicated by arrows in **A**. **B:** basal ventilation (basal) under normoxia (N). **C:** Ventilatory response to hyperoxia (100% O₂). Vertical scale bars correspond to one arbitrary unit. **D:** Measurements of minute ventilation (V_E), tidal volume (V_T), and respiratory frequency (Rf). Values are expressed as mean (SD). *, **, ***: P < 0.05, P < 0.01 and P < 0.001 post-hoc differences in hyperoxia vs basal ventilation. Note the similar ventilatory depression in response to hyperoxia in the two groups of mice, as evidenced by ANOVA analyses detailed in Table 4.

Table 4
Comparisons of ventilatory parameters in hyperoxia after acute (Day 1) and chronic (Day 5) treatments with 2-way repeated measures ANOVA analysis.

Time	Parameter	Groups	FiO ₂ effect	Treatment effect	Interaction
Day 1	V _E (Basal, Hyperoxia)	Control (n = 7) rhC-EPO (n = 8)	F = 26.53 P < 0.001	F = 0.203 P = 0.660	F = 1.015 P = 0.332
	V _T (Basal, Hyperoxia)		F = 3.921 P = 0.069	F = 2.027 P = 0.178	F = 0.402 P = 0.537
	Rf (Basal, Hyperoxia)		F = 36.91 P < 0.001	F = 2.793 P = 0.119	F = 0.854 P = 0.372
Day 5	V _E (Basal, Hyperoxia)	Control (n = 8) rhC-EPO (n = 9)	F = 95.27 P < 0.001	F = 0.068 P = 0.797	F = 9.001 P = 0.009
	V _T (Basal, Hyperoxia)		F = 46.05 P < 0.001	F = 0.479 P = 0.500	F = 6.452 P = 0.026
	Rf (Basal, Hyperoxia)		F = 18.98 P < 0.001	F = 0.955 P = 0.344	F = 0.874 P = 0.365

Table 5
Comparisons of ventilatory parameters in hypoxia after acute (Day 1) and chronic (Day 5) treatments with 2-way repeated measures ANOVA analysis.

Time	Parameter	Groups	FiO ₂ effect	Treatment effect	Interaction
Day 1	V _E (Basal, Early and Late hypoxia)	Control (n = 8) rhC-EPO (n = 9)	F = 46.64 P < 0.001	F = 0.268 P = 0.613	F = 0.787 P = 0.464
	V _T (Basal, Early and Late hypoxia)		F = 41.18 P < 0.001	F = 1.641 P = 0.220	F = 1.515 P = 0.236
	Rf (Basal, Early and Late hypoxia)		F = 55.14 P < 0.001	F = 1.046 P = 0.323	F = 0.294 P = 0.747
Day 5	V _E (Basal, Early and Late hypoxia)	Control (n = 7) rhC-EPO (n = 7)	F = 30.23 P < 0.001	F = 2.818 P = 0.119	F = 5.353 P = 0.012
	V _T (Basal, Early and Late hypoxia)		F = 30.69 P < 0.001	F = 6.935 P = 0.022	F = 3.827 P = 0.036
	Rf (Basal, Early and Late hypoxia)		F = 44.30 P < 0.001	F = 0.424 P = 0.527	F = 4.045 P = 0.031

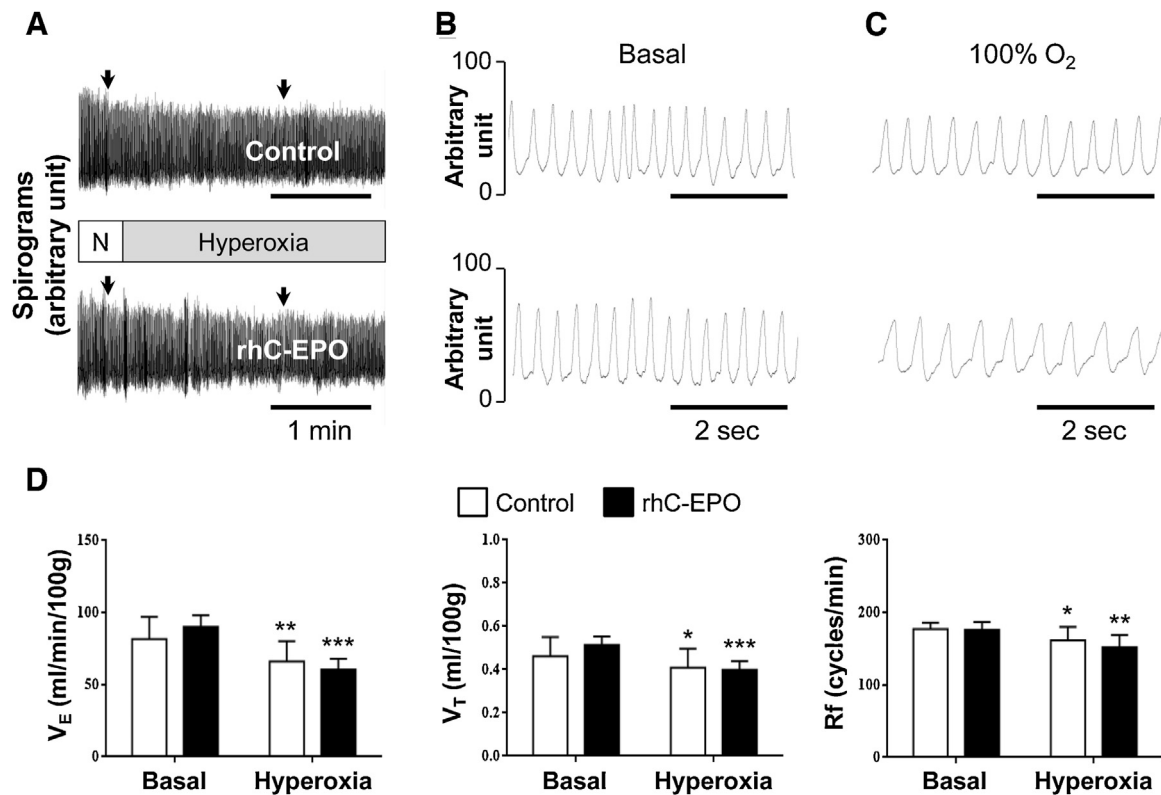


Fig. 4. Changes in ventilation in response to hyperoxia after chronic drug injections (Day 5). **A:** Original tracings from plethysmography. **B-C:** Enlargements of the spirogram at periods indicated by arrows in **A**. **B:** basal ventilation (basal) under normoxia (N). **C:** Ventilatory response to hyperoxia (100% O₂). Vertical scale bars correspond to one arbitrary unit. **D:** Measurements of minute ventilation (V_E), tidal volume (V_T), and respiratory frequency (Rf). Values are expressed as mean (SD). *, **, ***: $P < 0.05$, $P < 0.01$ and $P < 0.001$ post-hoc differences in hyperoxia vs basal ventilation. Ventilatory depression in response to hyperoxia was more pronounced in rhC-EPO mice, as evidenced by ANOVA analyses detailed in [Table 4](#).

Table 6

Comparisons of ventilatory parameters in hypercapnia after acute (Day 1) and chronic (Day 5) treatments with 2-way repeated measures ANOVA analysis.

Time	Parameter	Groups	FiCO ₂ effect	Treatment effect	Interaction
Day 1	V_E (Basal, Hypercapnia)	Control (n=8) rhC-EPO (n=9)	F = 57.39 P < 0.001	F = 0.199 P = 0.662	F = 0.088 P = 0.771
	V_T (Basal, Hypercapnia)		F = 73.02 P < 0.001	F = 0.060 P = 0.810	F = 0.471 P = 0.503
	Rf (Basal, Hypercapnia)		F = 84.57 P < 0.001	F = 1.638 P = 0.220	F = 1.375 P = 0.259
Day 5	V_E (Basal, Hypercapnia)	Control (n=7) rhC-EPO (n=7)	F = 96.51 P < 0.001	F = 0.034 P = 0.855	F = 0.297 P = 0.593
	V_T (Basal, Hypercapnia)		F = 125.0 P < 0.001	F = 0.002 P = 0.967	F = 0.188 P = 0.670
	Rf (Basal, Hypercapnia)		F = 123.6 P < 0.001	F = 0.106 P = 0.749	F = 1.266 P = 0.277

control and rhC-EPO treated mice had similar patterns of ventilation in hypercapnia over the entire experimental protocol.

4. Discussion

This study examined the effects of rhC-EPO on mice ventilation and other physiological parameters. As expected, chronic rhC-EPO injections did not stimulate erythropoiesis despite an increased plasma rhC-EPO concentration. Results demonstrated that 1/acute or chronic drug treatment did not change basal breathing in normoxic condition, 2/chronic but not acute rhC-EPO treatment enhanced the hyperoxic ventilatory response, and sustained ventilation in response to hypoxia by reducing the hypoxic ventilatory depression (the roll-off phase of the HVR), and 3/none of the drug

regimen altered ventilatory response to hypercapnia. We discuss the relevance of these findings with available literature, including the effects of EPO on neural control of breathing.

4.1. Methodological considerations

Drug was injected at 15,000 IU/kg using an intra-peritoneal route. In humans, such a dose and route of rhEPO administration allow the drug to cross the blood-brain barrier and reach the cerebral parenchyma ([Rasmussen et al., 1985](#); [Xenocostas et al., 2005](#)). This is in agreement with our ELISA measurements showing increased levels of rhC-EPO in mice brainstem tissue after chronic treatment. Besides, drug levels were higher in plasma than in brainstem, consistent with previous observations demonstrating that

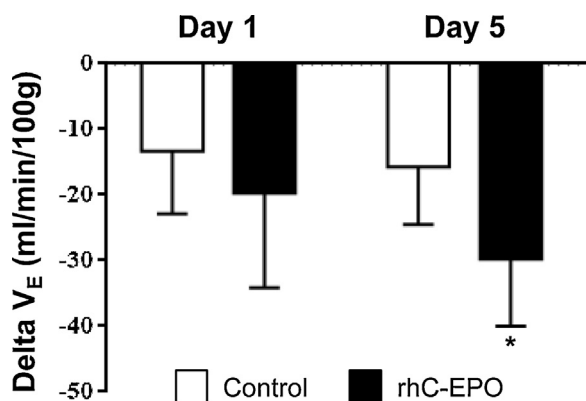


Fig. 5. Differences in minute ventilation (ΔV_E) between normoxia and hyperoxia after acute (Day 1) and chronic (Day 5) drug injections. Values are expressed as mean (SD). *: $P < 0.05$ vs control group. Chronic but not acute rhC-EPO injections enhanced the ventilatory depression seen in hyperoxia.

only a fraction of circulating rhEPO could reach the cerebrospinal fluid (Brines and Cerami, 2005). Leist and collaborators suggested that rhEPO and rhC-EPO share similar pharmacokinetics (Leist et al.,

2004). Since rhC-EPO tissue concentration was measured at only one time point (Day 5) in the present study, our data cannot confirm this suggestion. Low drug levels were also detected in tissues from control animals. These levels likely correspond to endogenous mouse EPO as a high degree of sequence homology exists between human and murine EPO (Wen et al., 1993). The lack of significant effects of chronic rhC-EPO treatment on body weight and core body temperature in our study is consistent with another observation in mice (Fantacci et al., 2006). This is particularly important for validation of our ventilatory measurements with plethysmography, as changes in body temperature (e.g. when mice are exposed to hypoxia) may confound interpretation of results.

4.2. Lack of acute rhC-EPO treatment on ventilation

Acute rhC-EPO has no effect on ventilation in normoxia, hyperoxia, hypoxia or hypercapnia. This is in agreement with a previous report showing a similar lack of drug effect on both basal ventilation and in response to moderate hypoxia (10% O₂) 2 h after acute injection of 2000 IU/kg rhEPO (Soliz et al., 2005). Although drug concentration in tissues was not measured on day 1, plasma levels of rhEPO and rhC-EPO remained elevated for 24 h after a single drug

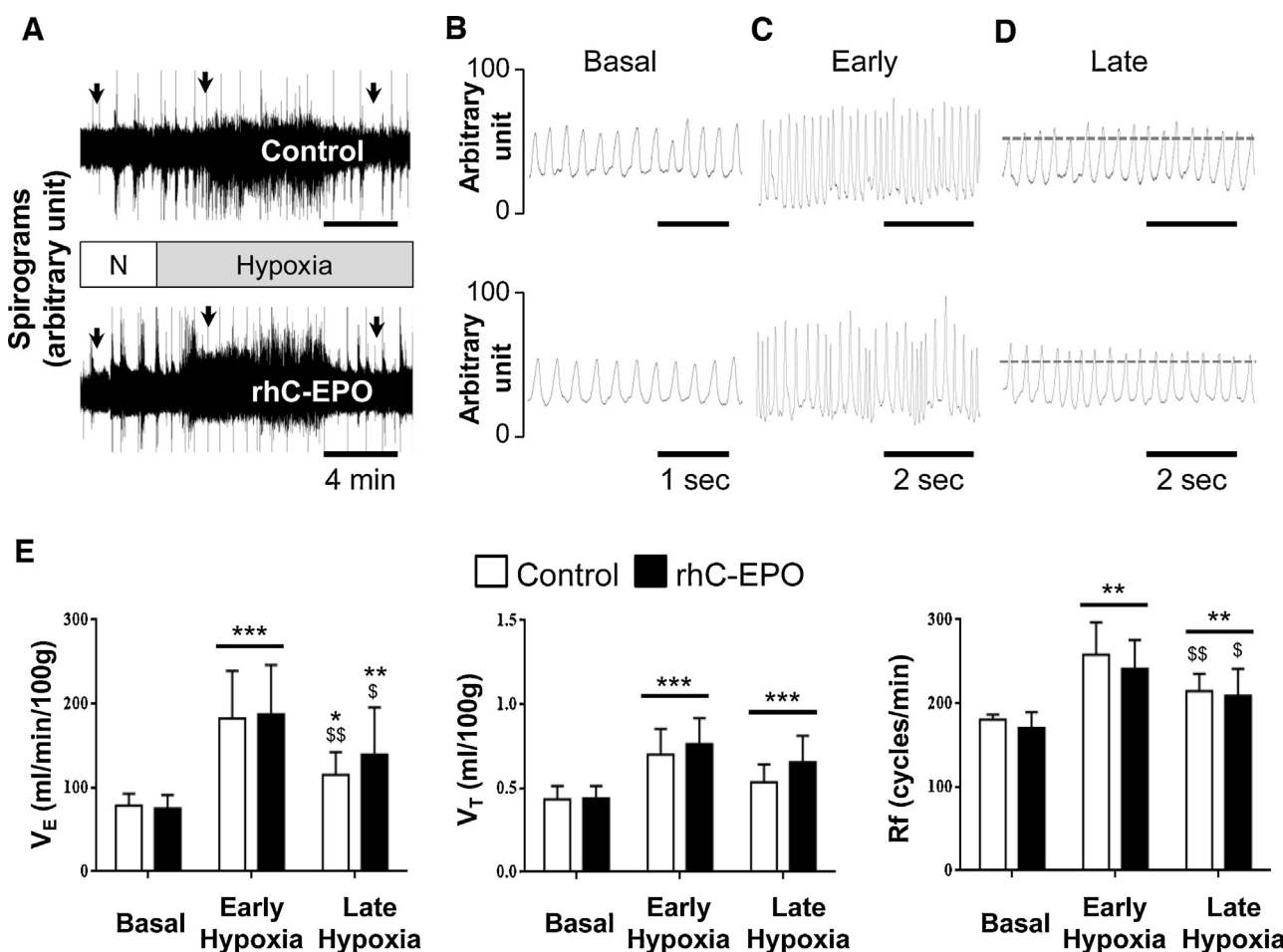


Fig. 6. Changes in ventilation in response to hypoxia (10% O₂) after acute drug injections (Day 1). A: Original tracings from plethysmography. B-D: Enlargements of the spirogram at periods indicated by arrows in A. B: basal ventilation (basal) under normoxia (N). C-D: Early and late phases of the hypoxic ventilatory response, respectively. Vertical scale bars correspond to one arbitrary unit. E: Measurements of minute ventilation (V_E), tidal volume (V_T), and respiratory frequency (Rf). Values are expressed as mean (SD). *, **, ***: $P < 0.05$, $P < 0.01$ and $P < 0.001$ post-hoc differences in hypoxia vs basal ventilation. \$, \$\$, \$\$\$: $P < 0.05$, $P < 0.01$ and $P < 0.001$ post-hoc differences in late vs early hypoxia. Both groups had similar increases in ventilation in the early phase of hypoxia followed by a significant decline (or ventilatory depression) in the late phase of hypoxia (see also Table 5).

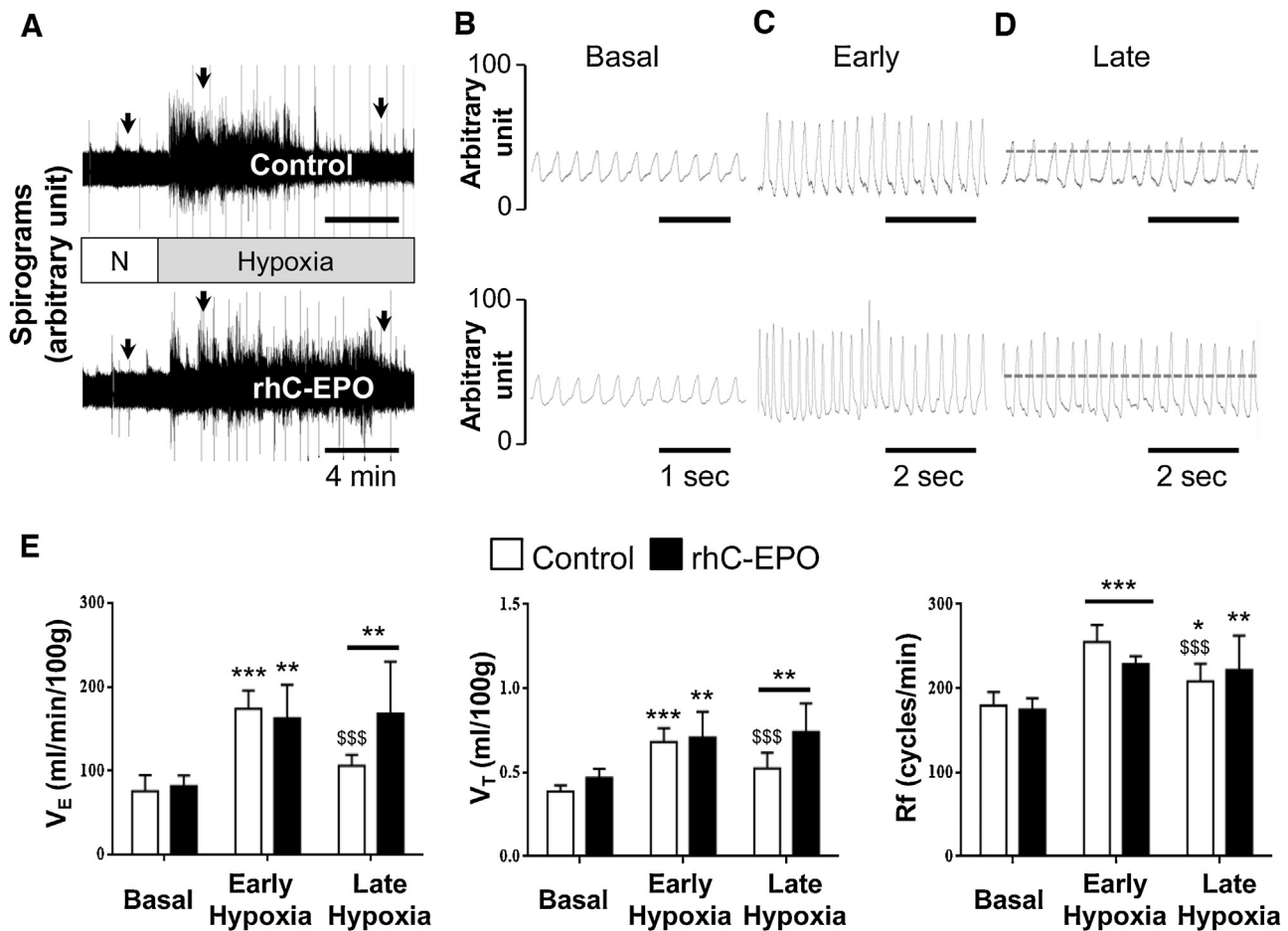


Fig. 7. Changes in ventilation in response to hypoxia (10% O_2) after chronic drug injections (Day 1). **A:** Original tracings from plethysmography. **B–D:** Enlargements of the spirogram at periods indicated by arrows in **A**. **B:** basal ventilation (basal) under normoxia (N). **C–D:** Early and late phases of the hypoxic ventilatory response, respectively. Vertical scale bars correspond to one arbitrary unit. **E:** Measurements of minute ventilation (V_E), tidal volume (V_T), and respiratory frequency (Rf). Values are expressed as mean (SD). *, **, ***: $P < 0.05$, $P < 0.01$ and $P < 0.001$ post-hoc differences in hypoxia vs basal ventilation. \$, \$\$, \$\$\$: $P < 0.05$, $P < 0.01$ and $P < 0.001$ post-hoc differences in late vs early hypoxia. Note the lack of ventilation depression in the late phase of hypoxia in rhC-EPO mice (see also Table 5).

injection at similar doses in rats (Leist et al., 2004). Also, acute injection with a higher dose of rhEPO (77,000 IU/kg) in mice resulted in elevated rhEPO levels in both plasma and brain up to 48 h post-injection (Schuler et al., 2012). Thus, our results reinforce the idea that stimulation of EPO receptors after acute injection of rhC-EPO does not exert significant effects on basal ventilation, nor on respiratory chemoreflexes, despite elevated drug levels in tissues.

4.3. Lack of chronic effects of rhC-EPO on basal ventilation

Chronic treatment with rhC-EPO did not change ventilatory parameters measured in normoxia. Similar findings have been reported in transgenic mice with chronic overexpression of rhEPO in both plasma and brain (Tg6 mice) or in brain only (Tg21 mice), as well as in wildtype mice after chronic rhEPO injections (Menuet et al., 2016; Soliz et al., 2007; Soliz et al., 2005). The present results in adult mice also corroborated previous observations in newborns, demonstrating no change in fictive respiratory activity after preincubation of isolated brainstem with rhEPO (Khemiri et al., 2012). Thus our data suggest that, similarly to rhEPO, chronic treatment with rhC-EPO does not alter neuronal mechanisms involved in basal breathing.

4.4. rhC-EPO enhanced ventilatory responses to changes in O_2 levels

It is widely accepted that changes in ventilation after exposure of animals to 100% O_2 , the so-called Dejour's test (Dejours, 1962), is due to the silencing of carotid bodies. Thus, this test can be used as an indirect measure of carotid bodies' sensitivity to O_2 (Ramanantsoa et al., 2006; Soliz et al., 2007). Our results obtained in mice injected with rhC-EPO over five consecutive days showed greater ventilatory depression in hyperoxia than control mice. A similar finding was reported in Tg6 mice with an overexpression of rhEPO in both plasma and brain (Soliz et al., 2007). This suggests that chronic treatment with one or another drug increased carotid bodies' O_2 sensitivity. Interestingly, since a normal hematocrit was observed in rhC-EPO treated mice whereas chronic rh-EPO is known to induce polycythemia (Menuet et al., 2016), it is unlikely that distinct blood carrying O_2 capacities contribute to changes in hypoxic breathing patterns.

Chronic rhC-EPO treatment did not change the early phase (first 5 min) of the HVR. At first glance, these results are surprising since 1/the initial increase in ventilation in response to hypoxia is mainly attributed to carotid bodies (Powell et al., 1998; Teppema and Dahan, 2010), and 2/enhancement of the HxVR after chronic rhC-EPO suggested an increase in carotid bodies' O_2 sensitivity (see

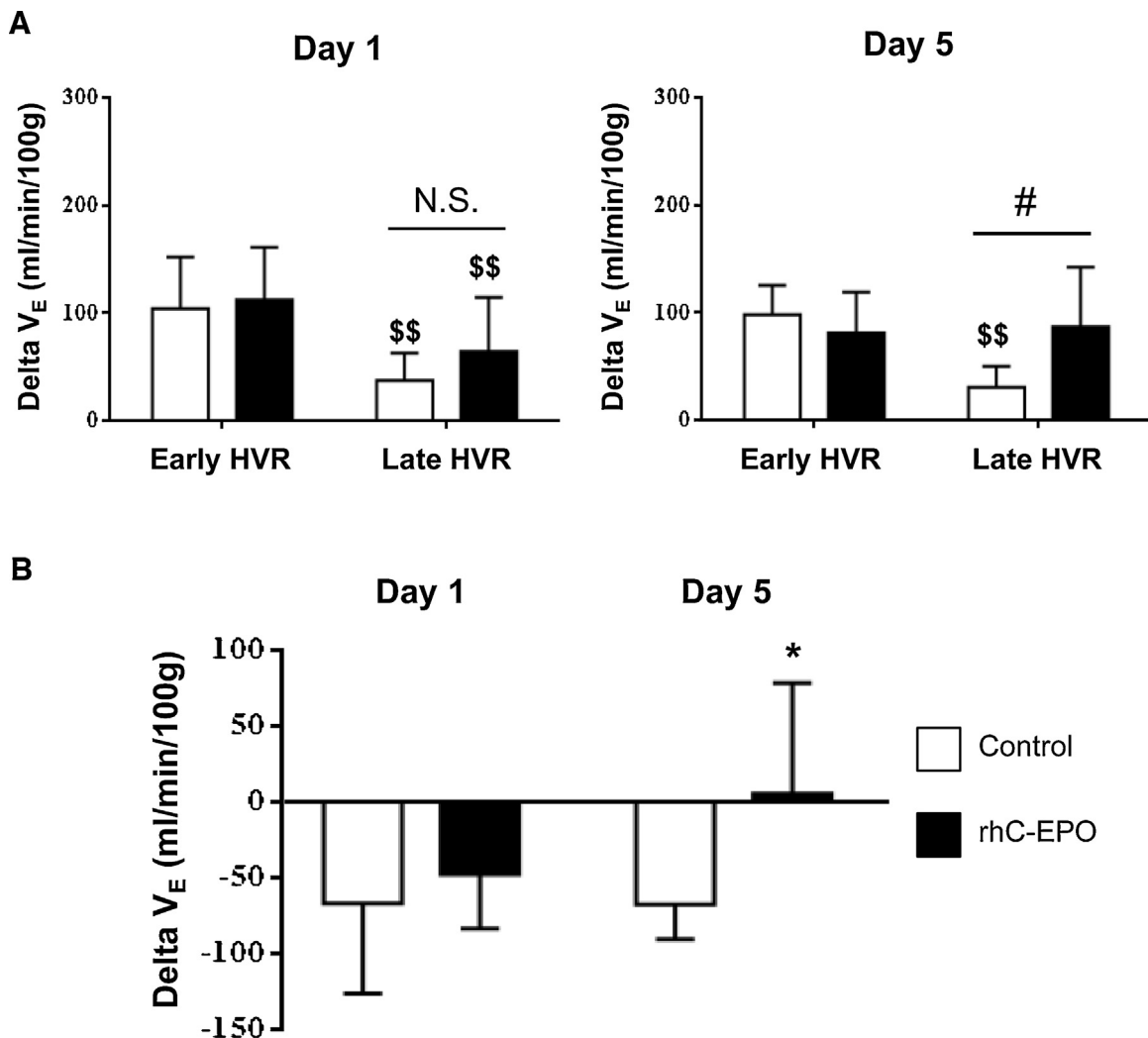


Fig. 8. Differences in minute ventilation (Delta V_E) between normoxia and hypoxia in control and rhC-EPO mice on days 1 and 5. **A:** Magnitudes of the early and late phases of hypoxia. **B:** Magnitude of the roll-off phase. Values are expressed as mean (SD). *: $P < 0.05$ vs control group on day 5. ##: $P < 0.01$ within group differences in late vs early hypoxia. #: $P < 0.05$ between groups difference in late hypoxia. N.S.: not statistically significant. Chronic but not acute rhC-EPO injections decreased the hypoxic ventilatory depression.

above). Interestingly, rhEPO also enhances carotid bodies' O_2 sensitivity in male mice but does not alter the early phase of HVR in response to moderate hypoxia (10% O_2) (Soliz et al., 2005). In the latter study, however, changes in patterns of ventilation attributable to a role of EPO on carotid body cells could be demonstrated only after a severe hypoxic challenge (6% O_2) in both Tg21 mice with chronic rhEPO overexpression and wildtype mice receiving an acute injection of rhEPO (2000 IU/kg, 2 h before hypoxia). Therefore, it is possible that the early phase of HVR did not increase in our study due to insufficient changes in arterial PO_2 between normoxia (21% O_2) and moderate hypoxia (10% O_2). By contrast, drastic changes in arterial PO_2 occurred between normoxia and hyperoxia (100% O_2) so that the resulting firing alteration of peripheral chemosensitive fibers may be sufficient to enhance the HxVR.

Another striking similarity between the effects of rhC-EPO and rhEPO is the reduction of ventilatory depression in the late phase (last 5 min) of the HVR (Maxova and Vizek, 2001; Powell et al., 1998), thereby enhancing ventilation in response to low O_2 levels. Hypoxic ventilatory depression, also called the roll-off phase, is very likely attributable to a central inhibitory effect of hypoxia although the precise mechanisms are still debated (Gestreau et al., 2010; Maxova and Vizek, 2001). Transgenic Tg21 mice with a con-

stitutive cerebral overexpression of rhEPO also have a sustained ventilation in the late phase of HVR at a similar level of hypoxia (10% O_2) (Soliz et al., 2005). Also, Tg21 mice display a sustained hypoxic response after bilateral transection of carotid sinus nerve which is correlated with alterations in catecholamine synthesis in both medulla and pons (Soliz et al., 2007; Soliz et al., 2005). Thus, a simple interpretation of our results is that rhC-EPO may play a modulatory role on central structures involved in the control of breathing during hypoxia. The elevated brainstem rhC-EPO concentration found after chronic drug injection (present results), and the wide expression of EPO-R in brainstem nuclei involved in respiratory control (Soliz et al., 2005) are in line with this hypothesis.

To further study central chemoreception, we also measured changes in ventilation in response to hypercapnia after chronic rhC-EPO or saline injections. Results revealed similar increase in ventilatory parameters in both mice groups, suggesting a lack of effect of carbamylated EPO on central chemoreceptors detecting changes in CO_2 /pH, such as neurons of the retrotrapezoid nucleus (Guyenet and Bayliss, 2015; Guyenet et al., 2010; Wang et al., 2013). Thus, our study pointed to a major difference between the ventilatory effects of rhEPO and rhC-EPO since chronic rhEPO treatment blunts the HCVR (Menuet et al., 2016). Consequently, it is unlikely

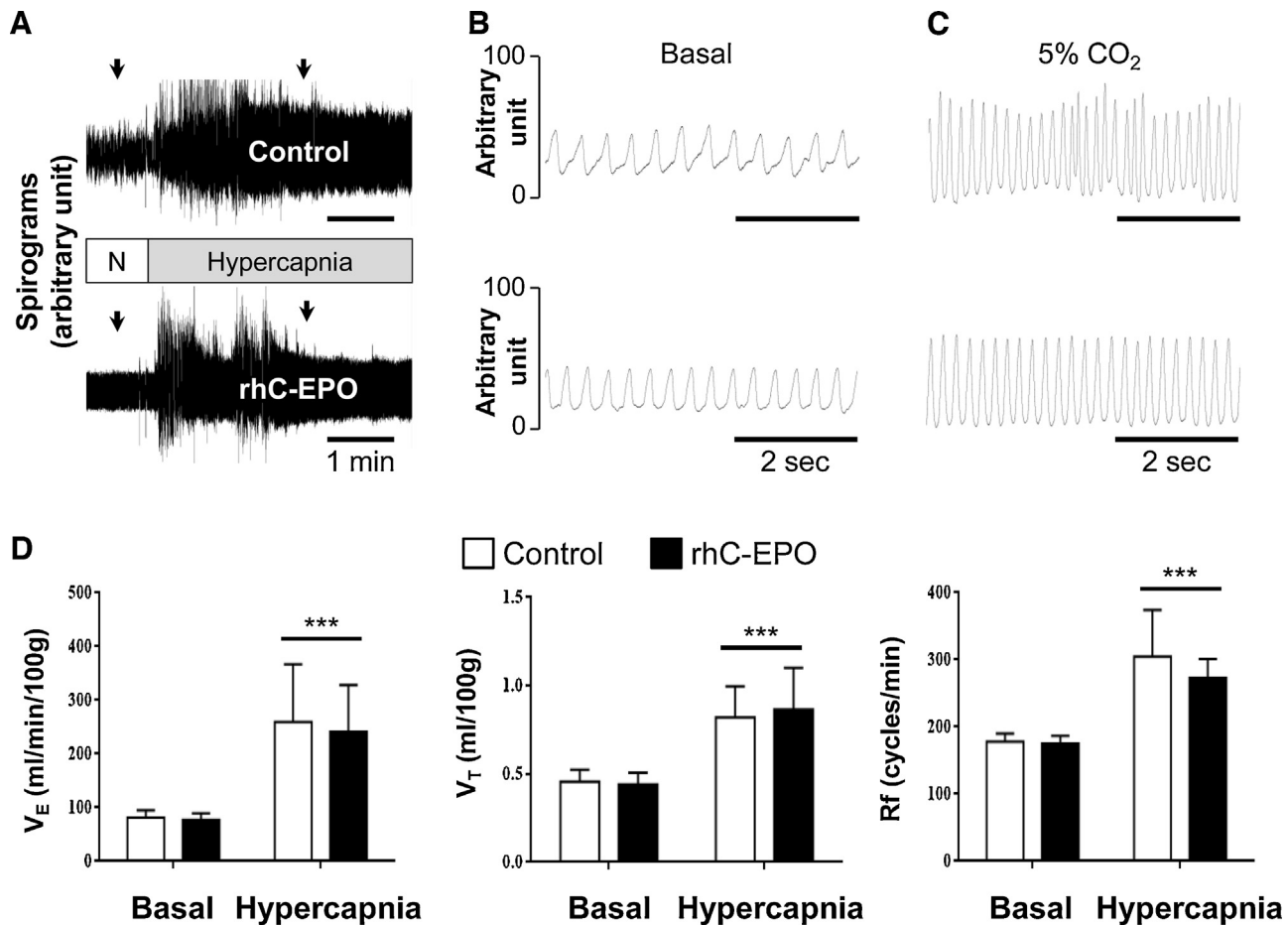


Fig. 9. Changes in ventilation in response to hypercapnia after acute drug injections (Day 1). **A:** Original tracings from plethysmography. **B-C:** Enlargements of the spirogram at periods indicated by arrows in **A**. **B:** basal ventilation (basal) under normoxia (N). **C:** Ventilatory response to hypercapnia (5% CO₂). Vertical scale bars correspond to one arbitrary unit. **D:** Measurements of minute ventilation (V_E), tidal volume (V_T), and respiratory frequency (Rf). Values are expressed as mean (SD). ***, $P < 0.001$ post-hoc differences in hypercapnia vs basal ventilation. Both groups of mice had similar increases in ventilation in response to hypercapnia (see also Table 6).

that the enhanced HVR reported herein after chronic rhC-EPO treatment could be explained by changes in central CO₂ sensitivity in these mice.

4.5. Are the heterodimeric EPO receptors responsible for the effects on ventilation?

EPO is thought to modulate respiratory activity by acting on classical homodimeric (EPO-R/EPO-R) EPO receptors (Bunn, 2013). This view is supported by expression of EPO receptors in carotid bodies and many brainstem respiratory areas (Soliz et al., 2005). However, the specificity of most antibodies directed against EPO-R has been recently challenged (Elliott et al., 2006; Elliott et al., 2014; Kirkeby et al., 2007). Here, we show that rhC-EPO, like rhEPO, altered both hyperoxic and hypoxic ventilatory responses although this drug lacks erythropoietic effect and binds heterodimeric (EPO-R/ β c-R) receptors (Brines et al., 2004; Bunn, 2013; Chamorro et al., 2013; Chateauvieux et al., 2011). Therefore, modulation of breathing in response to changes in O₂ levels may be mediated by interaction of both drugs with EPO-R/ β cR receptors. The β cR subunit is common for several cytokine receptors (D’Andrea and Gonda, 2000). It was detected in various structures of the central nervous system (Brines et al., 2004; Nadam et al., 2007; Sanchez et al., 2009), but no information is available on its localization in the brainstem, in particular in respiratory areas, nor at the level of the carotid bodies. Future studies should identify the precise mechanisms of EPO-mediated

respiratory effects, including the target cells and the identity of their EPO receptors.

4.6. Conclusions

Chronic treatment with rhC-EPO altered both hyperoxic and hypoxic ventilatory responses in the absence of polycythemia. These effects are very likely mediated by neuromodulation of peripheral and central structures contributing to ventilatory adaptation to changes in O₂ levels. Interestingly, rhC-EPO is safe and well tolerated in Humans (Boesch et al., 2014). Thus, heterodimeric EPO-R/ β c-R agonists such as C-EPO could represent good candidates for the treatment of diseases linked to respiratory network dysfunction when erythropoiesis-stimulating effect is unwanted. Since chronic rhEPO, but not rhC-EPO, blunts the hypercapnic ventilatory response and may be deleterious for breathing (Menuet et al., 2016), clinical use of rhC-EPO may provide greater efficacy than rhEPO.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

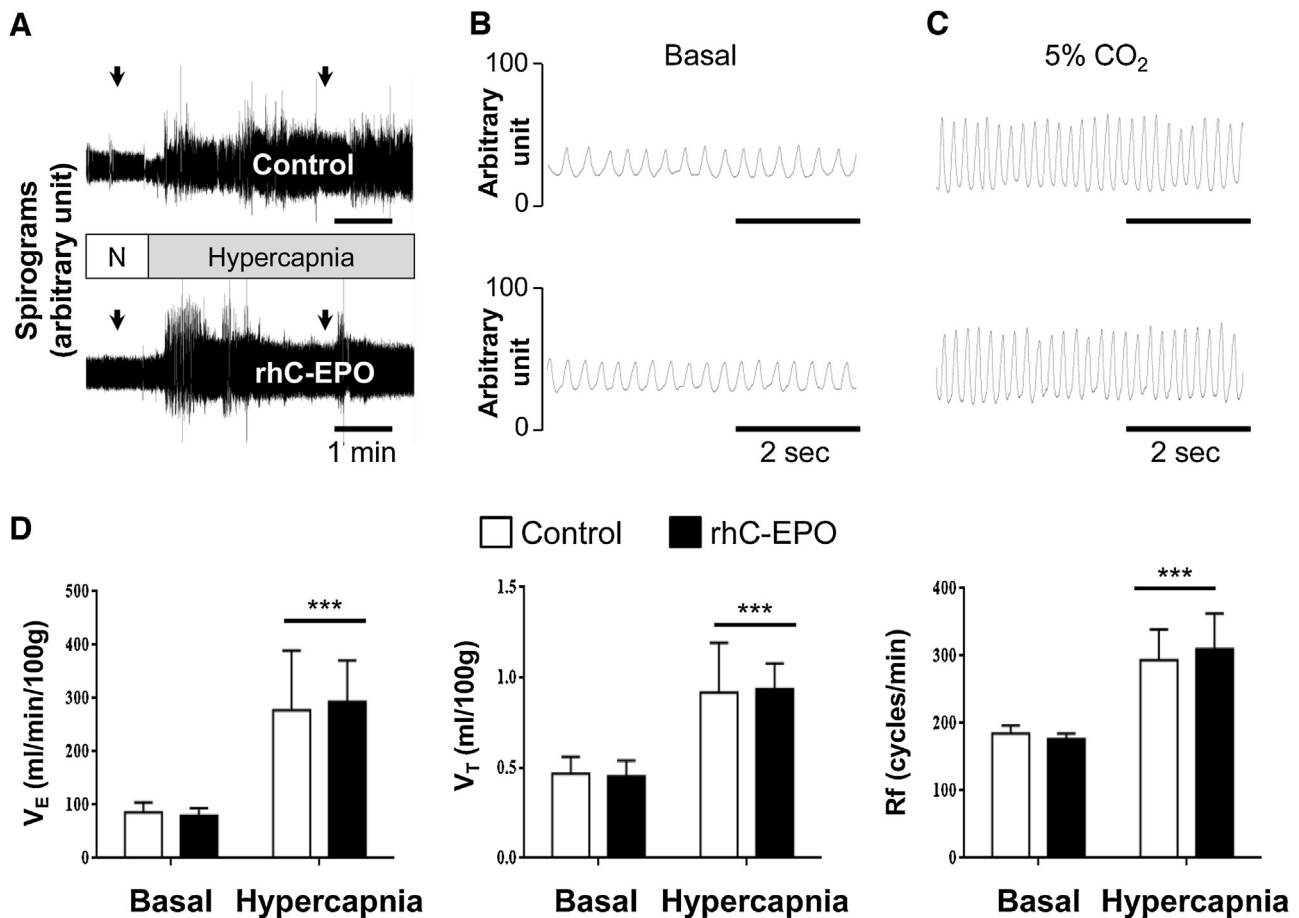


Fig. 10. Changes in ventilation in response to hypercapnia after chronic drug injections (Day 5). **A:** Original tracings from plethysmography. **B-C:** Enlargements of the spirogram at periods indicated by arrows in **A**. **B:** Basal ventilation (basal) under normoxia (N). **C:** Ventilatory response to hyperoxia (100% O₂). Vertical scale bars correspond to one arbitrary unit. **D:** Measurements of minute ventilation (V_E), tidal volume (V_T), and respiratory frequency (Rf). Values are expressed as mean (SD). ***: $P < 0.001$ post-hoc differences in hypercapnia vs basal ventilation. Chronic rhC-EPO injections did not change the ventilatory response to hypercapnia (see also Table 6).

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