Impaired ventilatory acclimatization to hypoxia in female mice

overexpressing erythropoietin: unexpected deleterious effect of estradiol

in carotid bodies

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ABSTRACT

Apart from enhancing the production of red blood cells, erythropoietin (Epo) alters the ventilatory response when oxygen supply is reduced. We recently demonstrated that Epo's beneficial effect on the ventilatory response to acute hypoxia is sex-dependent, with female mice being better able to cope with reduced oxygenation. In the present work we hypothesized that ventilatory acclimatization to chronic hypoxia (VAH) in transgenic female mice (Tg6) harboring high levels of Epo in the brain and blood will also be improved compared to wilde type (WT) animals. Surprisingly, VAH was blunted in Tg6 female mice. To define whether this phenomenon had a central (brainstem respiratory centers) and/or peripheral (carotid bodies) origin, a bilateral transection of carotid sinus nerve (chemodenervation) was performed. This procedure allowed the analysis of the central response in the absence of carotid body information. Interestingly, chemodenervation restored the VAH in Tg6 mice, suggesting that carotid bodies were responsible for the blunted response. Coherently with this observation, the sensitivity to oxygen alteration in arterial blood (Dejour test) after chronic hypoxia was lower in transgenic carotid bodies compared to the WT control. As blunted VAH occurred in female but not male transgenic mice, the involvement of sex female steroids was obvious. Indeed, measurement of sexual female hormones revealed that the estradiol serum level was 4 times higher in transgenic mice Tg6 than in WT animals. While ovariectomy decreased VAH in WT females, this treatment restored VAH in Tg6 female mice. In line with this observation, injections of estradiol in ovariectomized Tg6 females dramatically reduced the VAH. We concluded that during chronic hypoxia, estradiol in carotid bodies suppresses the Epo-mediated elevation of ventilation. Considering the increased
application of recombinant Epo for a variety of disorders, our data implies the need to take the
patient’s hormonal status into consideration.

(284 words)
INTRODUCTION

Erythropoietin (Epo) fulfills many more functions than “just” elevation of red blood cell numbers. An increasing number of publications, including our own contributions, show that Epo is a multifunctional cytokine that apart from liver and kidney tissue is expressed in a variety of tissue types, including brain tissue (9, 19, 34). In addition, we demonstrated that in response to environmental hypoxic conditions Epo plays a dual physiological role in re-establishing the optimal availability of oxygen to the tissue: On the one hand, Epo in plasma accelerates erythropoiesis, increasing the number of red blood cells, and thereby enlarging the blood’s oxygen carrying capacity (11, 54). On the other hand, circulating Epo is able to bind the Epo receptor present in the carotid body’s glomus cell and cerebrally produced Epo to its receptor found on respiratory neurons in the brainstem, both cell types being able to increase the hypoxic ventilatory response (20, 50, 51). Alongside these observations, we discovered the expression of Epo receptors (EpoR) in the respiratory areas of the brain and in carotid body glomus cells (51). Furthermore, in agreement with several studies showing that women and female animals cope better than men and male animals when exposed to an environment of decreased oxygen partial pressure (25, 26, 28, 45), we recently demonstrated that Epo exerts a sex-dependent beneficial effect on hypoxic ventilation. By using our transgenic mouse line (termed Tg6) that shows increased Epo levels in brain and plasma, the latter leading to hematocrit values up to 80% (49), we observed that compared to males, female mice show an increased ventilatory response when exposed to acute hypoxia (53). Similar data was obtained from male and female volunteers. These observations are of clinical importance because women are known to be less susceptible to a number of hypoxia-associated syndromes at either sea level (emphysema, chronic bronchitis, cystic fibrosis,
neonatal asphyxia, infant respiratory distress syndrome, sudden infant death syndrome) or at high altitude (excessive erythrocytosis, leading to chronic mountain sickness).

In our most recent work we hypothesized that augmented levels of Epo in female mice will also improve ventilatory acclimatization to chronic hypoxia (VAH). Surprisingly, we observed the opposite: we found that VAH, defined as a progressive time-dependent increase of baseline ventilation that compensates the decrease of alveolar oxygen pressure, was blunted in Tg6 female mice. Note that in contrast, transgenic Tg6 male animals showed normal VAH (52). These results may explain why in a hypoxic environment, ovariectomy (39) or menopause (31) are both followed by an increase in plasma Epo. Moreover, keeping in mind that the administration of high-doses of rhEpo to adult humans (12, 32, 40) and preterm infants (16) are becoming more and more frequent, our results alerts about the importance of evaluating the hormonal status of the patients.

MATERIALS & METHODS

Transgenic animals

The Epo-overexpressing transgenic mouse line was generated by a microinjection of human Epo cDNA driven by the human platelet-derived growth factor (PDGF) B-chain promoter into the pronuclei of fertilized oocytes derived from B6C3 hybrid mice (49). One of the resulting transgenic mouse line TgN(PDGFBEPO)321ZbZ (Tg6) showed increased Epo levels in the brain (12 fold compared to WT) and plasma (26 fold compared to WT), accompanied by almost double the hematocrit value (22, 59). The Tg6 line was back-crossed to C57BL/6 mice for even 20 generations by mating hemizygous
males to WT C57Bl/6 females. Half of the offspring was hemizygous for the transgene while the other half was WT and thus used as control animals. All live experiments were performed using 3 to 4 months old mice and in accordance with the Swiss animal protection laws and institutional guidelines.

**Exposure to chronic hypoxia**

Mice were placed in a hypoxic workstation (Invivo2 1000, Ruskinn, UK) with free access to food and water. The oxygen content of the hypoxic workstation was gradually decreased from room air to 10% O₂ within one hour. Chronic hypoxia exposure lasted three days as previously described (5, 23, 27, 33, 50). Subsequently, mice were returned to room air and placed in a whole-body flow-through plethysmograph device (see below) to perform respiratory measurements.

**Respiratory recordings after three day exposure to chronic hypoxia**

Respiration in unrestrained female animals was monitored by a whole-body flow-through plethysmograph (EMKA Technologies, France). Mice were placed in a 600-ml chamber continuously flushed with air at 0.7-0.8 l/min using flow restrictors. Calibration was performed by giving an injection of 1ml of air and the signal was amplified and recorded using respiratory acquisition software (IOX data acquisition and analysis, EMKA Technologies, France). Ventilation (\( \dot{V}_{E} \)) was calculated as the product of tidal volume (VT) and respiratory rate (fR) and normalized to 100g of body weight (e.g. ml/min/100g). Ventilatory responses in normoxia and hypoxia were performed in unrestrained unanesthetized mice. As soon as the animal was familiarized with the plethysmographic chamber
(about 1h), basal ventilation was recorded at 21% O₂. Respiratory recordings were achieved by flushing air balanced in N₂. The fraction of inspiratory O₂ (FIO₂) in the chamber was gradually decreased from 21% to 10% O₂ over a period of 15 minutes. Ventilatory measurements at 10% O₂ were performed for 20 minutes. Then the concentration of oxygen in the chamber was gradually reduced to 6% over the following 15 minutes and recordings were performed for 20 minutes in the new hypoxic environment. Body weight was measured routinely after experiments undertaken to express VT in ml per 100g in BTPS (body temperature and pressure, saturated) conditions as described previously (26, 51), the fR was defined as respirations per minute (resp/min). Body temperature was measured in normoxia and following the hypoxia protocol described above, using a rectal thermocouple (Fluke Corporation, USA). Oxygen consumption (\( \dot{V}O₂ \), ml SPTD/min/100g; STPD = standard temperature and pressure in dry air) and CO₂ production (\( \dot{V}CO₂ \), ml SPTD/min/100g) were measured in normoxia and hypoxia (10% and 6% O₂) with an open-circuit system. Each mouse was placed in a chamber where a steady 2 l/min flow of air was maintained. The fraction of O₂ and CO₂ at the inflow and the outflow of the chamber were measured using O₂ and CO₂ analyzers (Qubit Systems Inc., Kingston, Ontario).

**Dejour Test**

After a three day exposure to chronic hypoxia, the Dejour test (7) was performed on anesthetized mice (Uretane; 1.2 g/kg, i.p.; Sigma, USA). Two minutes after the onset of anesthesia, the animals showed regular ventilation and normal respiratory frequency. The baseline ventilation was then recorded at
21% O₂ over a period of 20 sec. The plethysmograph chamber was then quickly saturated with 100% O₂ and the decrease in ventilation was recorded during the following 20 sec. The magnitude of the transient ventilatory decline was assessed as the subtraction of ventilation at 100% O₂ minus the baseline value.

**Ovarectomy and chemodenervation**

Ovariectomy was performed via a bilateral dorsal incision when animals were anesthetized using a mixture of gases (4% halothane, 70% N₂O, and O₂). During the surgery body temperature was maintained at 37°C using a temperature-controlled heating pad. Two weeks after recovery, animals were exposed to chronic hypoxia (Jorge: % of O₂!!!) and then ventilation was assessed as described above. Another group of ovariectomized females was used for ventilatory measurements performed subsequent to estradiol replacement. Two weeks after ovariectomy animals were injected with estradiol, 0.008 mg i.p./day, (26) for 7 days (4 days in normoxia and 3 during exposure to chronic hypoxia). After estradiol treatment, ventilatory measurements were performed as described above.

Carotid sinus nerve transection (chemodenervation) was performed as described (48, 51). In brief, anesthesia was induced with a gas mixture (4% halothane, 70% N₂O, and O₂) and maintained by reducing the inspired halothane concentration to 1-1.5%. Body temperature was maintained at 37°C using a temperature-controlled heating pad. To prevent any functional regeneration of chemosensory fibers, the carotid sinus nerves were removed completely from the cranial pole of the carotid body until reaching the branch of the glosopharyngeal nerve. The wound was carefully closed and disinfected with 10% polividone iodine (Betadine, Asta Medica, Merignac, France). After two weeks recovery,
chemodenervated mice were exposed to chronic hypoxia. Sham-operated mice underwent the same procedure but the carotid sinus nerve was left intact.

**Quantitation of glomic cells in WT and Tg6 carotid bodies**

Wild type and transgenic female mice were anaesthetized with a mixture of gases (4% halothane, 70% N₂O, and O₂) and the carotid bifurcations were removed, post-fixed and cryo-protected as previously described (1, 24). Standard immunohistochemistry protocol for tyrosine hydroxylase staining (TH: rate limiting enzyme for catecholamine synthesis, used as specific marker of chemosensitive cells) followed by hematoxylin/eosin counterstaining was carried-out on serial slices (10 μm thick) from the carotid bifurcation as previously described (24). We then performed morphological analysis of the slices of carotid body, measuring the area (μm²) of the carotid body on each slice and the area of the TH positive (TH+) tissue (i.e. glomic, or chemosensitive tissue) on each slice. The sum of each specific area was then combined to calculate the volume (μm³) of the carotid body and glomic tissue.

**Detection of Epo binding sites in carotid bodies**

Wild type and transgenic female mice were anaesthetized with a mixture of gases (4% halothane, 70% N₂O, and O₂) and maintained by reducing the inspired halothane concentration to 1-1.5%. After perfusing with a phosphate buffer (0.1m, pH 7.4), the carotid bodies of the animals were removed and incubated for 2 hours in the phosphate buffer solution which contained a high concentration of rhEpo (1000 U/ml). Subsequently, the carotid bodies were fixed (4% paraformaldehyde, 24 hours), cryoprotected (30% sucrose-phosphate solution, 48 hours), and frozen in OCT. Specimens were...
serially sectioned at 8 μm, washed in phosphate-buffered saline (PBS), exposed for 1 hour to 1.5 normal goat serum, and incubated for 24 hours with a tyrosine hydroxylase antibody (NB300-173, Novus Biologicals, Inc, Littleton, CO, USA; 1:500). Next, the sections were exposed to an rhEpo antibody (MAB2871, R&D Systems; 1:100 in 3% of goat serum) for an additional 24 hours. Finally, samples were incubated for 2 hours with corresponding secondary antibodies (Cy3 goat anti rabbit, Jackson Immunoresearch, 1:200; Cy5 goat anti mouse, Jackson Immunoresearch, 1:200). DAPI (10 mM; 1:5000) was used for visualization of the nucleus of cells, and MOWIOL mounting media (DABCO, Sigma, D2522) was used for mounting the coverslips.

Quantification of sex hormones in plasma

The diestrus period was determined by performing a series of vaginal smears. When in diestrus, mice were anesthetized with an i.p. injection of a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). Blood samples were drawn by cardiac puncture into heparinized tubes and plasma was collected after samples were centrifuged at 15,000 rpm. Progesterone and estradiol concentration in the plasma was determined using the 125I-progesterone- and 125I-estradiol-based radioimmunoassay (RIA) (Coat-a-count, DPC, USA). The lower detection of our RIA was 4 U/l, the intrassay/interassay variances <2% and <6%.

Statistical analysis

Analysis was performed using Statview software (Abacus Concepts, Berkeley, CA). The reported values are means ± SD. For simple measurements, data was analyzed by one-way ANOVA followed
by a post-hoc PLSD (Protected Least Significant Difference of Fisher) test. For the ventilatory
responses to hypoxia, data was analyzed using two-way ANOVA for repeated measurements. The
differences were considered to be significant at p<0.05.

RESULTS

Basal normoxic ventilation after ventilatory acclimatization is blocked in Tg6 Epo-overexpressing
female mice (Tg6)

The progressive increase in minute ventilation over days is defined as ventilatory acclimatization to
hypoxia (VAH) (46). Considering that mice achieve VAH within 3 days (33, 42), we evaluated basal
ventilation (\(\dot{V}E\)), respiratory frequency (fR) and tidal volume (VT), before and after acclimatization to
chronic hypoxia (10% O_2 for 3 days; Fig. 1a). Before acclimatization, basal normoxic \(\dot{V}E\) of Tg6
female mice (that show constitutively elevated levels of Epo in the brain and plasma), was significantly
higher than corresponding WT animals, as a result of higher VT (Fig. 1b,d before). However, while WT
mice acclimatized to chronic hypoxia and thus showed large increase in normoxic ventilatory
parameters (\(\dot{V}E\), fR and VT) (Fig. 1b,c,d), Tg6 animals unexpectedly showed a blunted VAH, as
demonstrated by significantly decreased normoxic ventilation after three days of exposure to hypoxia
(Fig. 1b). This blunted response was due to a dramatic reduction in the fR (Fig. 1c), rather than VT
(Fig. 1d).

Ventilatory response to acute hypoxia following acclimatization is blunted in Tg6 Epo-overexpressing
female mice
Ventilatory acclimatization to chronic hypoxia is also manifested in an increased response to subsequent acute hypoxia (27). Accordingly, acute hypoxic ventilatory response before and after the acclimatization period (10% O₂ for 3 days) was compared (Fig. 2). In control mice acute hypoxic ventilation was significantly increased (both at 10% and 6% O₂), whereas in Tg6 mice it was completely abolished (Fig. 2a). In agreement with the model of VAH proposed by Powell (Fig. 2b), control WT mice gradually increased minute ventilation from normoxia to acute hypoxia before acclimatization, and from hypoxia before acclimatization to acute hypoxia after acclimatization. In constrast, this effect was inhibited in Tg6 mice (Fig. 2c). Detailed comparison after acclimatization shows that the lower ventilatory response to 10% and 6% O₂ after acclimatization of transgenic Tg6 mice (Fig. 2d), was due to diminished fR and VT (Fig 2e,f). In parallel to this, under conditions of our experiment, the evaluation of metabolic parameters after acclimatization (10% O₂ for 3 days) showed similar body temperature, oxygen consumption (\( V\dot{O}_2 \)) and carbon dioxide production (\( V\dot{CO}_2 \)) in WT and Tg6 female animals (Fig. 2g,h,i). Thus, the change in metabolic drive cannot account for the observed changes in ventilation and breathing pattern after chronic exposure to hypoxia in transgenic Tg6 animals.

Chemodenerved WT and Tg6 female mice show similar ventilatory acclimatization to hypoxia

The bilateral transection of carotid body sinus nerves (chemodenervation) is a common approach to studying the ventilatory response of brainstem respiratory centers in the absence of signals coming from the carotid bodies (44, 51). Chemodenerved WT and Tg6 mice were exposed to chronic hypoxia (10% O₂ for 3 days). Following this period, basal ventilation (21% O₂) and ventilatory response to
acute hypoxia (at 10% and 6% O\textsubscript{2}) were measured. Interestingly, neither minute ventilation (\textit{VE}) nor the response to acute hypoxia was different between the two chemodenervated groups (Fig. 3a).

Furthermore, no differences in ventilatory pattern (f\textsubscript{R} and VT) were observed between chemodenervated WT and Tg6 female mice (Fig. 3b,c). These data suggest that the blunted VAH observed in intact Tg6 female animals originates at the periphery, most probably in the carotid body cells.

**Epo binds the carotid body glomus cells**

The carotid body plays a critical role in ventilatory acclimatization to hypoxia (47), thus we hypothesized that the systemic overexpression of Epo in Tg6 female mice is responsible for the observed blunting of ventilatory acclimatization. Previously, we demonstrated that Epo receptors (EpoR) are present in the mouse carotid body cells (51). However, as the specificity of EpoR antibodies is currently debated (14), we evaluated the presence of Epo-binding sites in the carotid body cells of our WT mice. To this end, we incubated carotid bodies with rhEpo and subsequently added anti-Epo antibodies to the preparation. The double staining for Epo and tyrosine hydroxylase (TH), the latter representing a reliable marker of chemosensitive cells, reveled that Epo binding overlaps with TH immunoreactivity. Thus the fact that Epo binds to a specific site strongly confirms the presence of EpoR in the peripheral chemoreceptors (Fig. 4).

**Impaired carotid body response to hyperoxia in Tg6 female mice after VAH**
As the magnitude of the transient ventilatory decline in response to brief hyperoxic (100% O₂) exposure is used as an index of carotid body sensitivity (7), the so-called Dejour-test was performed on both WT and Tg6 female mice, following exposure to chronic hypoxia (10% O₂ for 3 days). Our results showed that the ventilatory decline in Tg6 mice was significantly less than in the control WT mice (Fig. 5a). This diminished response of the carotid bodies to oxygen changes in arterial blood in Tg6 animals was due to a reduced fR, rather than VT (Fig. 5b,c). Taken together, these sets of data strongly suggest that the ineffectiveness of Tg6 female mice in achieving ventilatory acclimatization after chronic hypoxia is due to the blunted sensitivity of the carotid bodies.

Enhanced carotid body hypertrophy in Tg6 female mice after VAH

Because carotid bodies undergo hypertrophy during chronic exposure to hypoxia (36, 41, 43), we suspected that transgenic carotid bodies, associated with blunted sensitivity, might show impaired hypoxia-dependent growth. In contrast, however, we observed that carotid bodies were greater in size in Tg6 mice than in the WT ones after 3 days of chronic hypoxia (fig. 5d,e). This result suggests that the hypoxic stimulus that induces the carotid body’s growth is higher in transgenic mice than in the control animals, thereby most probably compensating for its decreased sensitivity to hypoxia.

Increased plasma estradiol in Tg6 female mice

Knowing that VAH occurs in Tg6 males (52), we suspected that female sexual hormones are implicated in the blunted VAH observed in Tg6 female mice after exposure to chronic hypoxia.
Accordingly, we evaluated the levels of sex hormones in the plasma of WT and Tg6 females. The plasma concentration of progesterone was similar between groups (WT: 15.1±0.3 nmol/l; Tg6: 12.8±2.4 nmol/l). In contrast however, we found that the level of estradiol in Tg6 females was four times larger than in WT animals (WT: 3.0±0.7 pg/ml; Tg6: 12.3±3.2 pg/ml). Keeping in mind that estradiol decreases the expression of HIF-1 (2, 10, 38, 55), and considering that HIF-1 in carotid body cells is crucial in achieving VAH (27), these observations suggest that estradiol eliminates the carotid body’s sensitivity under chronic hypoxia, thereby blunting the VAH of transgenic Tg6 females.

Ovariectomy of Tg6 female mice restores adequate ventilatory acclimatization

Taking into account that progesterone and estradiol are mainly produced in the ovaries of non-pregnant females, we ovariectomized WT and Tg6 female mice. After one week of recovery, ovariectomized mice were exposed to chronic hypoxia (10% O2 for 3 days). Subsequently, ventilatory acclimatization was evaluated by measuring basal ventilation (21% O2) and ventilatory response to acute hypoxia (at 10% and 6% O2). Compared to sham-operated animals, ovariectomy in WT mice previously exposed to chronic hypoxia decreased the basal ventilation and the acute ventilatory response to 10% oxygen (Fig. 6a,b,c). In striking contrast, when compared to sham operated transgenic mice, ovariectomy restored the VAH of Tg6 females, resulting in dramatically increased basal ventilation and acute ventilatory response to 10% and 6% O2 (Fig. 6d). The remarkable increase in Tg6 ventilation was due to a significant increase in both the fR and VT (Fig. 6e,f).

Estradiol replacement in ovariectomized Tg6 female mice blunts VAH
In view of the fact that the Dejour test and ovariectomy suggested that estradiol in carotid body cells is responsible for blunted VAH in Tg6 mice, we evaluated the ventilatory acclimatization to chronic hypoxia in ovariectomized WT and Tg6 mice after estradiol replacement therapy (8 μg/day for seven consecutive days). Estradiol replacement did not affect basal ventilation or acute ventilatory response to hypoxia (at 10% and 6% O2) in ovariectomized WT mice compared with ovariectomized untreated WT mice (Fig 7a,b,c). In contrast, estradiol replacement eliminated VAH in ovariectomized Tg6 females (Fig 7d,e,f). Taken together, these data strongly suggest that the blunted ventilatory acclimatization to chronic hypoxia in Tg6 female mice was due to a hitherto unknown interaction between estradiol and Epo in carotid body cells.

DISCUSSION

We have previously demonstrated that Epo increases the ventilatory response to acute and chronic hypoxia in male mice (20, 50-52). Moreover, coherent with the fact that women and female animals cope better than men and male animals when exposed to a hypoxic environment, we demonstrated that Epo is a critical factor that enhances the ventilatory response to acute hypoxia (53). Accordingly, in our present work we expected that Epo in female mice would also enhance ventilatory acclimatization to chronic hypoxia. To test this notion we used female transgenic mice (Tg6) showing constitutively high levels of human Epo in the brain (26-fold/WT) and plasma (12-fold/WT) (49, 57, 58). Surprisingly, our results demonstrate that (i) ventilatory acclimatization is prevented in Tg6 female animals; (ii) it is the carotid bodies, rather than the central respiratory centers in the brainstem, that
confer this unexpected response; and (iii) high levels of circulating estradiol blunts ventilatory acclimatization to chronic hypoxia in Tg6 female mice.

Several possibilities can account for the absence of an enhanced hypoxic ventilatory response in transgenic mice. However, as the body temperature and changes in $\dot{V}O_2$ or $\dot{V}CO_2$ are similar in WT and transgenic females after chronic hypoxia, alteration in the metabolic rate can be regarded as separate from the observed changes in ventilatory acclimatization. On the other hand, several lines of evidence suggest that sensitization of carotid body chemoreceptors is critical for developing VAH (6, 8, 18). Alternatively, impaired processing of chemoreceptor inputs in the central nervous system and/or depressed respiratory motor output might also contribute to the absence of VAH (33). Considering this, we performed a bilateral transection of the carotid sinus nerves (chemodenervation), disconnecting the brain from its main peripheral sensor, to assess the impact of brain Epo on ventilatory acclimatization in the absence of a relay of carotid body information. Interestingly, under this condition, both, basal ventilation and hypoxic ventilatory response following VAH (10% $O_2$ for 3 days) were similar in female WT and Tg6 transgenic chemodenerved animals. These results strongly suggest that transgenic carotid bodies are responsible for the eliminated VAH. This effect was not related to an impairment of the carotid body hypertrophia under chronic hypoxia. On the contrary, carotid body and glomic tissue size in the carotid body of Tg6 female mice was slightly higher than in WT. We interpret this result as a compensatory response, that obviously is not sufficient to restore full functionality. Therefore, the absence of VAH in Tg6 mice might be due to a lack of an increase in
hypoxic sensitization rather than structural alterations in transgenic carotid bodies. These results are also in line with the observation that VAH does not occur in animals that do not have functioning carotid bodies, and that central oxygen sensitivity is not important for VAH (8).

On the other hand, in previous work performed on Tg6 males we showed that Epo in carotid bodies stimulates hypoxic ventilatory response (51) and ventilatory acclimatization to chronic hypoxia (53). As VAH only occurs in male Tg6 mice and not in female ones, we obviously suspected that female sex hormones might be involved in this effect. Indeed, we found that the levels of estradiol, but not progesterone, was 4 times higher in the plasma of Tg6 mice than in WT females. Why do Tg6 female mice have elevated estradiol levels? Interestingly, it was previously demonstrated that the expression of estradiol and renal Epo synthesis are closely related (38, 39). More specifically, it was observed that plasma Epo increases in ovariectomized rats exposed to hypoxia (after 8h at 12% O₂), but the level of Epo is significantly decreased after estradiol replacement (20 µg during 7 days) (39). Moreover, it was shown that the level of estradiol is higher in women living at high altitude than those that live at sea level (15). These observations might point towards a reciprocal feedback loop between Epo and estradiol by which an increase in Epo drives an increase in estradiol, and high levels of estradiol reduce Epo up-regulation in kidney tissue. Likewise, it is interesting to note that under hypoxia high concentrations of circulating estradiol specifically reduce the erythropoietic response. In consequence, it might be hypothesized that in Tg6 female mice estradiol is increased in order to decrease the erythropoietic response, however as the Epo level is genetically clamped its expression cannot be reduced and estradiol level remains elevated.
How might the high estradiol level impair the VAH of Tg6 female mice? We speculate that under oxygen deprivation, estradiol decreases the expression of hypoxia-inducible genes such as HIF-1 (2, 10, 37, 55). More specifically it was demonstrated that estradiol in Hep 3B cells attenuates the hypoxic induction of Epo via the down-regulation of HIF-1 (37). In parallel it was demonstrated that mice partially deficient in HIF-1 (HIF-1+/-) have a defective carotid body function and an impaired VAH (27). We suggest that the high level of circulating estradiol in Tg6 female mice impairs the HIF-1 expression/function in carotid bodies. In turn, impaired HIF-1 blunts the chemoreceptor sensitization when mice are exposed to chronic hypoxia. This explanation is in line with the results of chemodenervation and ovariectomy, showing that a high level of estradiol in carotid bodies is responsible for the loss of VAH in Epo overexpressing transgenic females. We are currently conducting electrophysiological and cell culture studies of carotid bodies to investigate the interaction of estradiol, Epo and HIF-1 in reaction to acute and chronic hypoxic stimulus.

What is the role of progesterone in this process? Early studies have shown that progesterone and estradiol have the complementary effect of acting as respiratory stimulants (3, 21, 28). Moreover, our previous studies shown that concomitant administration of progesterone and estradiol increases normoxic and hypoxic ventilation by reducing the dopaminergic drive in carotid bodies (26). However it is clear that progesterone is the most important factor in enhancing ventilation and the sensitivity of the carotid body to hypoxia (3, 21, 28, 30, 56), and estradiol increases the progesterone receptor synthesis, thus further supporting the effect of progesterone (29, 35). Interestingly however, our results
highlight the fact that progesterone and estradiol have different functions in the ventilatory control and response to hypoxia. Similar observations were noted in newborn rats exposed to hypoxia, in which the contrasting effects of estradiol and progesterone in the control of ventilation were observed (29).

These data are especially important to consider in light of the traditional physiological concept that progesterone and estradiol have similar properties and effects on ventilation.

Perspectives and Significances

The most important physiological strategies for coping with hypoxia are the increase in ventilation (to compensate for the decrease in alveolar oxygen pressure), and the induction of erythropoiesis (to enlarge the capacity of the blood to carry oxygen). However, because excessive erythrocytosis may have a pathological impact, prioritizing the ventilatory strategy may provide a better functional adaptation to hypoxia. It may be that case that women that ventilate more but have a lower erythrocytic response than men, are less susceptible to a number of hypoxia-associated syndromes at sea level and at high altitude (4, 25, 26, 28, 45). In line with this observation, estradiol and progesterone have a double role to play during hypoxia, to stimulate the ventilatory drive in carotid bodies (progesterone) (26) and to reduce the number of red blood cells in the plasma (estradiol) (17, 31, 39). Accordingly, it was found that when estradiol is significantly reduced in plasma, which occurs with ovariectomy or menopause, Epo is up-regulated (31, 39), and the subsequent administration of estradiol to ovariectomized rats decreases the Epo level in the plasma (39). In agreement with these observations, our current data show however that a simultaneous over-expression of estradiol and Epo under hypoxia eliminates the ventilatory response to chronic hypoxia. This observation is of...
particular clinical importance because the administration of high-doses of rhEpo to adult and infants (12, 13, 16, 32, 40) is becoming more frequent. Accordingly, our results highlight the importance of evaluating the hormonal status of the patient.

In conclusion, our results support our previous findings showing that Epo exerts a sex-dependent impact on the ventilatory response to acute and chronic hypoxia. Moreover, we provide convincing evidence that when the level of Epo in plasma is elevated, a subsequent increase of circulating estradiol impairs the ventilatory acclimatization to hypoxia, most probably due to the impairment of the carotid body chemoreception. These results have potential clinical implications in respiratory responses evoked by environmental (i.e. high altitude) and pathological conditions.

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**FIGURE LEGENDS**

**Figure 1.** Blunted basal normoxic ventilation after acclimatization to hypoxia in female Tg6 mice.

a) Schematic diagram showing the sequential steps of the experimental protocol. Normoxic minute ventilation was evaluated before and after chronic hypoxia (3 days at 10% O2), the latter leading to ventilatory acclimatization to hypoxia (VAH) (b,c,d). Before and after acclimatization, mice were kept at
normoxic conditions and basal minute ventilation (\(\dot{V}E\)), respiratory frequency (fR) and tidal volume (VT) were determined. *p<0.01, ***p<0.001; n= 9 animals per group.

**Figure 2. Blunted acute hypoxic ventilatory response in Tg6 female mice subsequent to acclimatization.**

Acute hypoxic ventilation was evaluated after acclimatization (3 days at 10% O\(_2\)) in female WT and Tg6 mice (a,b,c). Hypoxia was achieved with a gradual reduction of FIO\(_2\) (black triangle on the x-axis); from 21% to 10% O\(_2\) (over 15 min) and from 10% to 6% O\(_2\) (over 15 min). Hypoxic ventilatory response was evaluated during 20 min to 10% and at 6% O\(_2\) (a,b,c). Metabolic parameters; body temperature (d), \(\dot{V}O_2\) (e), and \(\dot{V}CO_2\) (f) were also determined in WT and Tg6 female mice after chronic hypoxia. *p<0.001. n= 8-9 animals per group.

**Figure 3. Ventilatory acclimatization to hypoxia is similar in chemodenerved WT and Tg6 females.**

\(\dot{V}E\) response in normoxia and acute hypoxia after acclimatization to chronic hypoxia (3 days at 10% O\(_2\)). Hypoxia was achieved with a gradual reduction of FIO\(_2\) (black triangle); from 21% to 10% O\(_2\) (over 15 min) and from 10% to 6% O\(_2\) (over 15 min). The hypoxic ventilatory response was evaluated over a period of 20 min to 10% and at 6% O\(_2\) (a,b,c). n= 7-8 animals per group.

**Figure 4. Epo binds carotid body cells**
Identification of carotid body cells in the carotid bifurcation of WT mice was achieved by immunodetection of tyrosine hydroxylase (TH). Recombinant human Epo was added to the carotid bodies and binding of rhEpo was verified by using anti-Epo antibodies. Co-localization of TH expression and Epo binding was determined by using serial sections of 8 μm thickness. Nuclei were made visible by DAPI.

Figure 5. Sensitivity and morphology of WT and Tg6 carotid bodies after VAH.

Ventilation (\(\dot{V}E\)), fR and VT declined (transition from 21% to 100% O2) in response to hyperoxic testing (Dejour test) (a,b,c). The number of cells in function to glomic volume (d) and body volume (e) was evaluated in WT and Tg6 carotid bodies before and after ventilatory acclimatization to chronic hypoxia. Data are means ± SD for n= 7-8 animals per group. *p<0.01.

Figure 6. Ventilatory acclimatization in ovariectomized (OVX) WT and Tg6 females

\(\dot{V}E\) response in normoxia and acute hypoxia after acclimatization to chronic hypoxia (3 days at 10% O2) was evaluated in OVX WT (a,b,c) and Tg6 (d,e,f) female mice. Hypoxia was achieved with a gradual reduction of FIO2 (black triangle); from 21% to 10% O2 (over 15 min) and from 10% to 6% O2 (over 15 min). Hypoxic ventilatory response was evaluated over a period of 20 min to 10% and at 6% O2. *p<0.001; n= 8 animals per group.
Figure 7. Estradiol resubstitution in ovariectomized (OVX) WT and Tg6 females

VE response in normoxia and acute hypoxia after acclimatization to chronic hypoxia (3 days at 10% O2) was evaluated in OVX WT (a,b,c) and Tg6 (d,e,f) female mice i.p. injected with estradiol (0.005 mg i.p./day) or vehicle during 7 days (4 days in normoxia and 3 during exposure to chronic hypoxia).

Hypoxia was achieved with a gradual reduction of FIO2 (black triangle); from 21% to 10% O2 (over 15 min) and from 10% to 6% O2 (over 15 min). Hypoxic ventilatory response was evaluated over a period of 20 min to 10% and at 6% O2 (a,b,c). *p<0.001; n= 5-6 animals per group.
Figure 1

a) 3 days exposure to chronic hypoxia

b) Changes in VE (mL/min/100g) before and after acclimatization

c) Changes in fR (resps/min) before and after acclimatization

d) Changes in VT (mL) before and after acclimatization
Figure 4

- **DAPI**
  - control
  - anti-TH
  - anti-Epo
  - merged
Figure 5

a) Ventilation

b) Resp Frequency

c) Tidal Volume

d) number of cells/gonad volume

before after

e) number of cells/carotid body volume

before after

*
Figure 6

**WT females**

a) 

b) 

c) 

**Tg6 females**

d) 

e) 

f)
Figure 7

WT females

(a) Exposure time (min) 1 5 10 15 20 1 5 10 15 20

(b) Exposure time (min) 1 5 10 15 20 1 5 10 15 20

(c) Exposure time (min) 1 5 10 15 20 1 5 10 15 20

Tg6 females

d) Exposure time (min) 1 5 10 15 20 1 5 10 15 20

(e) Exposure time (min) 1 5 10 15 20 1 5 10 15 20

(f) Exposure time (min) 1 5 10 15 20 1 5 10 15 20