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Differential effects of ventilatory stimulation by sex hormones and almitrine on hypoxic erythrocytosis

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Abstract In the absence of pulmonary disease, hypoventilation is considered to be the primary cause of Chronic Mountain Sickness, and there is some reason to believe that chronic administration of respiratory analeptics could be useful for treatment of this disease. The present study was intended to define comparatively the influence of two potent ventilatory stimulants, namely a combination of progesterone and estrogen and the pharmacological agent almitrine, on catecholaminergic structures implicated in the chemoreflex pathway and on hypoxia-induced polycythemia. Three groups of young male rats born and living at high altitude (3 600 m) were examined: untreated animals (n = 25), rats given ovarian steroids (progesterone plus 17β-estradiol, n = 25) or almitrine (n = 25) for 6 weeks until sacrifice. Ovarian steroids or almitrine had pronounced neurochemical effects on the afferent chemoreflex circuitry. Both treatments inhibited norepinephrine (NE) and dopamine (DA) turnover in the carotid body, but central processing of chemosensory inputs differed between the two respiratory drugs. Ovarian steroids inhibited noradrenergic activity in the projection area of the chemosensory nerve fibers within the caudal portion (A_{2C}) of the nucleus tractus solitarius (NTS). In contrast, almitrine stimulated neurochemical activity of other brainstem noradrenergic cell groups involved in cardiorespiratory control, i.e., the rostral portion (A_{2R}) of the NTS, the nucleus reticularis lateralis (A_1), the nucleus olivaris superior (A_5) and the locus ceruleus (A_6). Although both treatments increased chemoreflex drive and ventilation, only sex hormones decreased erythropoietin (EPO) levels and the degree of polycythemia. These results suggest that stimulation of ventilation through activation of peripheral arterial chemoreceptors activation alone is not sufficient for reducing EPO levels and polycythemia. The better efficiency of female sex hormone treatment as compared to almitrine could be related either to the central effects of progesterone and estrogen and/or to the impact of these hormones on erythropoiesis at the kidney/bone marrow level.

Key words Carotid body · Dopamine · Erythropoietin · Noradrenergic cell groups · Norepinephrine · Polycythemia · Sympathetic outflow · Ventilation

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Introduction

The increase in hematocrit (Ht), in hemoglobin concentration ([Hb]) and in red blood cell count (RBC) are some of the first [28] and some of the best known (see review in [19]) adaptations to altitude hypoxia. Nevertheless, Monge [18] reported several incidences of people native to regions of high altitude who developed excessive erythrocytosis in the absence of cardiopulmonary disease. Accepting the fact that these dwellers had already adapted to living at high altitude, Monge and Leon-Vélarde [19] reasoned that they had lost their adaptation to the hypoxic environment (Chronic Mountain Sickness) and the name of Monge's disease was later given to the clinical entity. Indication that excessive polycythemia is the main factor in this pathology is given by the significant improvement of pulmonary gas exchange by phlebotomy [6] or hemodilution [30].

Alveolar hypoventilation has been assumed to be the primary cause of excessive polycythemia [12, 18] and some authors [11, 29] have proposed to treat the pathology with respiratory stimulant drugs. Thus, progesterone, which is known to be a potent and chronically effective ventilatory stimulant [5, 27], has been shown to decrease Ht [11]. On the other hand, almitrine, which stimulates arterial chemoreceptors specifically [13, 23], was also found to reduce Ht, although to a lesser extent [29]. However, in both studies [11, 29], the precise

mechanisms involved in the effectiveness of the drug therapy were not elucidated, but could be linked either to a central or a peripheral action of the drugs on ventilation.

In the present study, we postulated that the effectiveness of the ovarian steroids and of almitrine at reducing polycythemia could be related to some changes in the neuroactivity of catecholaminergic structures involved in the ventilatory adaptation to hypoxia [4]. Indeed, we [24] have recently provided evidence for a significant effect of gender and castration on dopamine (DA) and norepinephrine (NE) metabolism in the carotid body and in some noradrenergic cell groups of the brainstem located in the nucleus tractus solitarius (NTS; A_2 group), the nucleus reticularis lateralis (A_1 group), the nucleus olivaris superior (A_5 group), and the locus ceruleus (A_6 group). These brainstem catecholaminergic neurons are target sites for sex steroid hormones [8] and the close anatomical interrelations between steroid receptors and catecholaminergic neurons are thought to play a significant role in the regulation of a number of physiological processes [8]. On the other hand, it has been shown that almitrine has an effect on catecholaminergic activity in the rat carotid body [23] and on respiratory neurons of the NTS [14].

In order to provide further insight into the peripheral and central effects of ventilatory stimulants on polycythemia, we evaluated the impact of chronic administration (6 week) of female sex hormones (progesterone + estradiol) and of almitrine on the chemoreceptor pathway involved in ventilatory control during exposure to high altitude. For this purpose, we used an animal model (rat) that has been shown to represent a valuable model for human-like ventilatory adaptation to chronic hypoxia [20]. We determined the long-lasting neurochemical drug-induced modifications in specific catecholaminergic structures: these include the carotid chemoreceptors and their central projections to the brainstem [26].

Materials and methods

Animals

The study was carried out at the Instituto Boliviano de Biologia de Altura (La Paz, Bolivia – mean altitude = 3 600 m). Sprague-Dawley rats, born and reared at high altitude, were housed in a climatized room (24 ± 1°C) with a 12-h light-dark cycle and allowed free access to food and water. All experiments were carried out in accordance with the ethical principles laid down by the French (Ministère de l'Agriculture) and EEC Council Directives for care of laboratory animals.

Newborn male rats (n = 75) were submitted to the experimental protocol. They were reared in La Paz without any treatment until 6 weeks of age. At this age, 25 rats were injected daily for 6 weeks with NaCl 0.9% (male control). Another 25 rats were implanted with 3-week release hormone pellets (Innovative Research of America, Toledo, Ohio, USA). The first implant (20 mg of progesterone and 0.5 mg of 17β-estradiol) took place at 6 weeks of age and was repeated at 9 weeks with pellets containing 32.5 mg progesterone and 0.6 mg 17β-estradiol. Another group of rats (n = 25) was injected daily for 6 weeks with almitrine (1 mg·kg^{-1}).

Measurement of ventilatory paramaters

These measurements took place during the 6th week of treatment. A 3-l whole-body plethysmograph chamber was used to measure ventilation according to Bartlett and Tenney [2]. Tidal volume (V_T), respiratory frequency (f) and minute ventilation ($\dot{V}_E$) were determined. Briefly, the unrestrained rat was placed in the plethysmograph chamber and breathed room air for at least 15 min to become accustomed to remaining quietly in the chamber. Fresh air was continuously supplied through the chamber at a rate of 2 000 ml·min^{-1}. Once the animal was in a quiet and awake condition, the inlet and outlet ports of the chamber were closed for 20–30 s and the pressure changes in the box recorded. This procedure was repeated three times and respiratory parameters are reported as the mean of those three measurements. The temperature in the chamber was monitored and maintained within the thermoneutral range (22–26°C). The pressure change within the chamber reflecting V_T was measured with a high-gain differential pressure transducer (Validyne BP45-14). Calibration volumes of 0.2 to 0.5 ml air were introduced before and after measurement of ventilation.

Ventilatory response to O_2 changes

The steady-state ventilation response of the rat to O_2 was measured while it was breathing gas mixtures containing a low (7–8%) or a high (90–95%) O_2 concentration for 5 min. The ventilatory response to O_2 was evaluated as the change (Δ) in ventilatory parameters between hypoxia and hyperoxia.

Determination of the catecholamine content and catecholamine turnover

At 12 weeks of age, under halothane anesthesia, blood was withdrawn from the abdominal aorta until exsanguination. The blood was collected into chilled, heparinized tubes and Ht was measured using a microtechnique method. [Hb] was determined using a kit (525A-Sigma, Sigma, St. Quentin Fallavier, France), RBC was evaluated using standard Thoma pipettes and Hayem's solution as the diluting fluid. The remaining blood was centrifuged for plasma re-collection. Plasma samples were kept at –80°C until erythropoietin (EPO) determination (see later). The carotid bodies and the brain were rapidly removed, frozen in liquid nitrogen and stored at –80°C. The brainstem was cut into serial frontal slices 480 µm in thickness. The noradrenergic cell groups A_1, A_2, A_5 and A_6 were punched out according to the dissection procedure described by Soulier et al. [26]. Tissue samples were placed in 100 µl of 0.4 M (punches) or of 0.1 M (carotid bodies) perchloric acid containing 2.7 mM ethylenediaminetetraacetic acid (EDTA). In punch samples the excess perchloric acid was removed by addition of 8 µl of 6.4 M potassium formiate to the supernatant. Catecholamines were assayed by high-performance liquid chromatography coupled with electrochemical detection [26].

α-Methyl-*para*-tyrosine (α-MPT, Sigma), injected twice intraperitoneally at a dose of 250 mg·kg^{-1}, 4 and 2 h before sacrifice, allowed determination of catecholamine turnover by blockade of catecholamine biosynthesis [26]. Each experimental group (n = 25) was divided into two subgroups, one (n = 13) receiving α-MPT and the other (n = 12) receiving the corresponding volume of vehicle alone (0.9% NaCl), and the catecholamine (DA and NE in the carotid body and NE in the brainstem cell groups) content in these groups was measured. After injection of α-MPT, the level of catecholamine decreased exponentially. After semi-exponential linear regression, the rate of the decrease in the catecholamine content was determined and was then multiplied by the mean amine content of saline-treated rats in order to obtain the turnover rate [26].

Plasma EPO was determined by radioimmunoassay (RIA) with a standard kit (bioMérieux, France).

Table 1 Norepinephrine (*NE)* and dopamine (*DA*) contents in the carotid body, and in noradrenergic brainstem cell groups in male control rats, males treated with progesterone + estrogen, and males treated with almitrine. Results are means ±SE from 12 animals in each group. A_{2C}, A_{2R}, A_1, A_5 and A_6: see Materials and methods

Site	Catechol amine	Male control	Male (progesterone +estrogen)	Male almitrine
Carotid Body (pmol/structure)				
	[NE]	423±47	127±10*	312±50**
	[DA]	482±32	241±16*	574±67**
Noradrenergic brainstem cell groups (pmol/structure)				
A_{2C}	[NE]	8.40±0.95	10.41±1.42	7.75±0.95
A_{2R}	[NE]	19.53±1.36	21.78±1.95	24.79±1.48
A_1	[NE]	10.47±0.47	9.70±1.07	9.82±0.41
A_5	[NE]	8.46±0.71	14.38±3.20	8.64±0.71
A_6	[NE]	16.80±2.36	15.56±2.43	18.52±2.07

* Significantly different from male control ($p<0.05$)
** Significantly different from male + progesterone and estrogen ($p<0.05$)

Statistical analysis

Values are presented as means ± SE. For statistical comparisons of group means a two-way ANOVA was used followed by a post-hoc test (protected least significant difference of Fisher). The level of significance was set at 5%. The turnover rates of catecholamines were compared using Dunnett's test for the comparisons of several means to the corresponding values for one set of control conditions.

Results

Effects of sex hormones and almitrine treatment on catecholamine metabolism in the carotid bodies and their central projections (Table 1, Fig. 1)

The rate of dopamine turnover ($[DA]_{TO}$) in the carotid bodies was lower in the rats treated with progesterone and estrogen (–67%) while the rate of norepinephrine turnover ($[NE]_{TO}$) was blunted (Fig. 1). $[NE]_{TO}$ was also diminished in the A_2 cell group of treated males (–48% and –82% in the caudal and rostral subsets, respectively) but remained unchanged in A_1 and A_5 cell groups and in the locus ceruleus (group A_6). The changes in catecholamine turnover were associated with marked reductions in NE (–70%) and DA (–50%) contents in the carotid body (Table 1). The catecholamine stores in other structures studied, i.e., the brainstem noradrenergic cell groups, were not significantly altered.

In rats treated with almitrine, the $[NE]_{TO}$ and $[DA]_{TO}$ were blunted in the carotid bodies (Fig. 1). No significant decrease in $[NE]_{TO}$ could be detected in the caudal A_2 subset, whereas the rostral A_2 portion was stimulated by almitrine (+112%). Other brainstem noradrenergic cell groups displayed moderate but significant increases in $[NE]_{TO}$ (A_1: +27%, A_5: +31%, and A_6: +50%) (Fig. 1). Almitrine failed to alter significantly the cate-

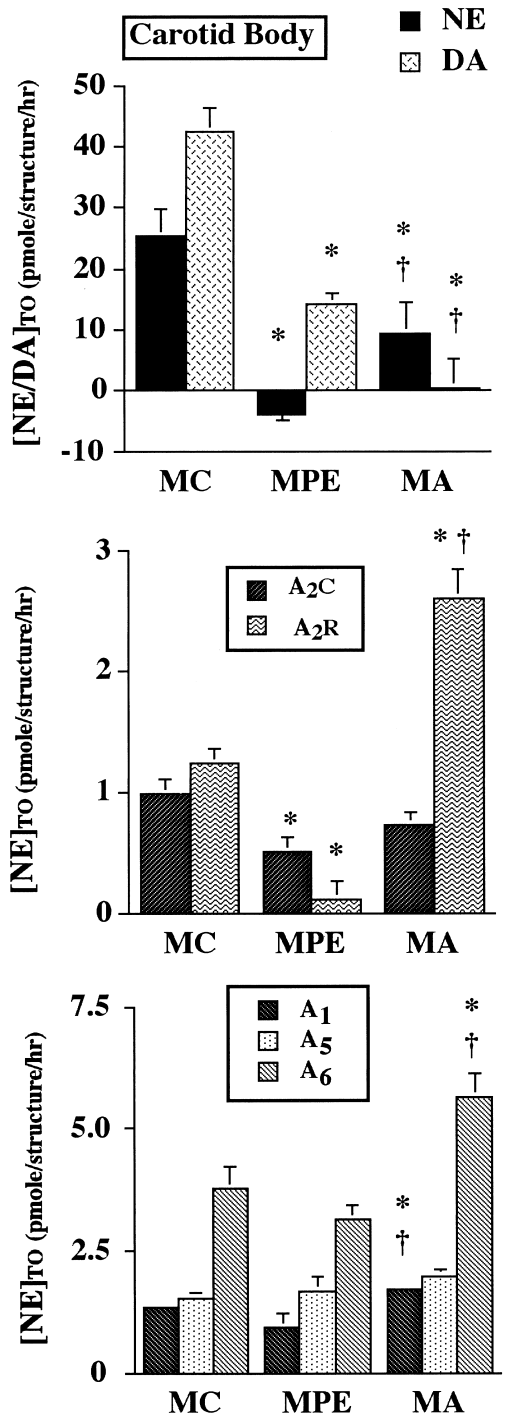

Fig. 1 Norepinephrine ($[NE]_{TO}$) and dopamine ($[DA]_{TO}$) turnover rate in carotid body, and the noradrenergic brainstem cell groups in male controls (*MC*), males treated with progesterone + estrogen (*MPE*), and males treated with almitrine (*MA*). Results are means ± SE from 12 animals in each group. A_{2C}, A_{2R}, A_1, A_5, and A_6: see Materials and methods. * Significantly different from male control ($P<0.05$). † Significantly different from male + estrogen and progesterone ($P<0.05$)

Table 2 Ventilatory parameters measured at altitude in rats breathing either ambient air (PIO_2 = 95 torror or 12.7 κPa), or a hypoxic (PIO_2 = 36 torr or 4.8 κPa) or a hyperoxic (PIO_2 = 386 torr or 51.4 κPa) gas mixture. Values are means±SE. ($\dot{V}_E$ Ventilatory output; V_T tidal volume; f breathing rate)

Group	Parameter	P_IO_2		
		12,7 κPa (95 torr)	4.8 κPa (36 torr)	51.4 κPa (386 torr)
Male control				
	$\dot{V}_E$ (ml · min.kg$^{0.75}$)	457.1±25.5	565.9±25.6	479.8±24.9
	V_T (ml · kg$^{0.75}$)	3.85±0.25	4.77±0.24	4.62±0.34
	f (min^{-1})	120.7±4.4	119.8±4.8	106.0±3.5
Male + progesterone + estrogen				
	$\dot{V}_E$(ml · min.kg$^{0.75}$)	556.4±13.2*	792.8±39.0*	605.0±39.1*
	V_T(ml · kg$^{0.75}$)	4.80±0.21*	6.24±0.43*	5.95±0.46*
	f (min^{-1})	118.1±3.9	130.8±4.8	105.9±5.5
Male + Almitrine				
	$\dot{V}_E$(ml · min.kg$^{0.75}$)	548.0±22.2*	759.2±32.4*	596.2±35.7*
	V_T(ml · kg$^{0.75}$)	4.27±0.21	5.89±0.30*	5.18±0.35
	f (min^{-1})	132.0±5.8**	131.3±6.4	117.3±5.6

*: Significantly different from male control (P<0.05)

Table 3 Hematological parameters in male control rats, males treated with progesterone + estrogen, and males treated with almitrine. Results are means±SE. Number of animals in *parentheses*. (*Ht* haematocrit, [*Hb*] haemoglobin concentration, *RBC* red blood cell count, *EPO* plasma erythropoietin level)

Parameter	Male control	Male (progesterone + estrogen)	Male almitrine
Ht (%)	59.1±1.0 (24)	45.5±0.5 (25)*	59.7±0.8 (25)**
[Hb] (g.dl^{-1})	18.4±0.4 (24)	14.9±0.2 (25)*	18.1±0.2 (25)**
RBC (10^{-6})	11.37±0.55 (24)	6.41±0.15 (25)*	9.44±0.25 (23)**
EPO (mU.ml^{-1})	10.4±2.0 (12)	4.3±0.7 (12)*	9.9±1.2 (12)**

* Significantly different from male control (p<0.05).
** Significantly different from male + progesterone and estrogen (p<0.05)

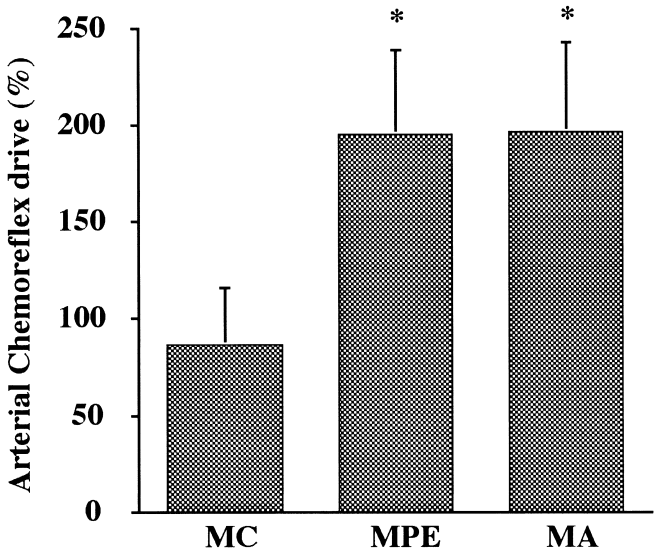

Fig. 2 Arterial O_2 chemoreflex drive in male control rats (*MC*), in males treated with progesterone + estradiol (*MPE*) and in males treated with almitrine (*MA*). Results are means ± SE. Arterial O_2 chemoreflex drive was determined as the change (in%) in minute ventilation from switching from a hyperoxic (PIO_2 = 386 torr or 51.4 kPa) to a hypoxic (PIO_2 = 36 torr or 4.8 kPa) gas mixture. * Significantly different from male control (P<0.05)

cholamine stores in the carotid body, and in the brainstem noradrenergic cell groups (Table 1).

Ventilatory and hematological changes (Tables 2 and 3)

In male rats, chronic progesterone plus estrogen treatment increased ventilatory output, which was brought about by a significant increase in V_T. Similarly, prolonged administration of almitrine improved ventilation but, in this case, it was linked to an increase in f (Table 2).

The ventilatory stimulation provided by the drugs persisted when oxygen availability was either decreased (partial pressure of inspired O_2, PIO_2 = 36 torr or 4.8 kPa) or increased (PIO_2 = 386 torr or 51.4 kPa). The O_2 chemoreflex drive, as assessed by the increment in $\dot{V}_E$ produced by the fall in PIO_2 from 51.4 to 4.8 kPa (386 to 36 torr), was enhanced to a similar extent by administration either of female sex hormones or of almitrine (Fig. 2).

Nevertheless, the improvement of ventilation and O_2 chemoreflex drive provided by the drugs did not result in a similar effect on hematological status. Thus, Ht, [Hb], RBC, and the plasma EPO level were significantly re-

duced by treatment with both progesterone and estrogen. By contrast, Ht, [Hb], RBC, and EPO remained unchanged after treatment with almitrine (Table 3).

Discussion

Some life-long residents at high altitude have Ht, [Hb], and RBC levels that are well above the normal range for those expected at the altitude [18, 19]. The characteristic of this disease (chronic mountain sickness, or Monge's disease) is that these patients had a complete recovery after relocation to sea level but the symptoms reappear on return to high altitude. However, many patients want to remain at high altitude for family or economic reasons. In these cases, phlebotomy is beneficial: it improves pulmonary gas exchanges [6] and lowers the excessive erythrocytosis [30].

An alternative to phlebotomy is the long term use of respiratory stimulants. Thus, Kryger et al. [11] have reported success with medroxyprogesterone acetate, which has been claimed to act through an increased ventilation. In the present study we chose to administer estrogen and progesterone simultaneously because progesterone receptors are induced by treatment with estrogen and an elevation both of hormone levels and the number of progesterone receptors is required to stimulate ventilation in the ovariectomized rat [5]. This potentiation of ventilatory effects of progesterone by estrogen treatment was recently confirmed by Hannhart et al. [7] using the cat and by Tatsumi et al. [27] using the rat. However, the exact site of ventilatory stimulation by progesterone remains unclear. Indeed, previous studies have hypothesized that the acute effects of progesterone involve central sites [3]. Recently, however, Hannhart et al. [7] clearly showed that female sex hormones have a combination of central and peripheral (carotid body) sites of action, such that administration of both hormones together has a more consistent stimulatory effect on ventilatory responsiveness to hypoxia compared with that of either hormone alone.

The mechanisms by which female hormones affect the peripheral and central respiratory system are not readily apparent but could be linked to modulation of neurotransmitter metabolism in the chemoafferent circuitry. Recently, we have shown that $[NE]_{TO}$ and $[DA]_{TO}$ in the carotid body and in noradrenergic cell groups of the brainstem (A_1, A_2, A_5) are higher in female than in male rats; this catecholaminergic hyperactivity being severely reduced by early removal of endogenous steroids by castration [24]. In the present experiment, we found that prolonged administration of a combination of progesterone and estradiol in male rats resulted in a significant decrease in the NE and DA contents in the carotid body (Table 1) and drastically reduced the turnover rate of these amines both at the peripheral (carotid body) and the central (noradrenergic cell groups) level (Fig. 1). It is likely that the difference between endogenous and exogenous female steroids on the catecholaminergic activity of the chemoafferent pathway is due to the relative levels of the hormones. Indeed, progesterone and estradiol are able to modify the activity and expression of the rate-limiting enzyme of catecholamine biosynthesis tyrosine hydroxylase (TH), but the effects are variable according to both the region examined and the hormone [1, 15, 22]. Thus, estradiol inhibits TH or its gene expression in the tuberoinfundibular dopaminergic neuronal activity [22], but activates the hypothalamic noradrenergic pathway [1, 25]. In both cases progesterone interacts with estradiol to reverse the estrogen effects. In particular, progesterone antagonizes the estradiol-induced increase in $[NE]_{TO}$ within the hypothalamus [25]. The present data showing an inhibitory influence of progesterone combined with estrogen on A_2 noradrenergic neurons are consistent with this finding, since A_2 neurons provide one major noradrenergic innervation of the hypothalamus. These neurochemical changes in the chemoafferent circuitry were associated with an increase in ventilation (Table 2) and with an enhanced responsiveness to hypoxia (Fig. 2). In addition, it has been shown previously that simultaneous administration of progesterone and estradiol increases arterial O_2 tension [27] and it is likely that this improved oxygenation is partly responsible for the decreased level of plasma EPO and for the attenuation of altitude erythrocytosis (Table 3). On the other hand, it has been shown that estrogens can impair EPO production [17] and inhibit the action of EPO on the differentiation and maturation of erythrocytic progenitor cells [10].

In striking contrast, we found that almitrine failed to improve the hematological status (Table 3) in spite of a similar effect on the chemoreceptor afferent pathway (Table 1), and on the O_2 chemoreflex drive (Fig. 2). Several lines of evidence indicate that almitrine acts by specifically stimulating the peripheral arterial (carotid body) chemoreceptors [13, 23]. In agreement with a previous study [23], the present data confirmed that, like the progesterone plus estrogen treatment, almitrine inhibits the catecholamine activity in the carotid body. Almitrine did not alter the catecholamine content, thus indicating that almitrine inhibited both the release and synthesis of catecholamines to a similar extent. On the other hand, the central effects of almitrine on brainstem noradrenergic cell groups involved in the regulation of breathing [4] differed from those produced by sex steroids. Indeed, almitrine produced changes in neurotransmitter metabolism in the A_1, A_{2R}, and A_6 areas but not in A_{2C}, whereas $[NE]_{TO}$ was unchanged in A_1, A_5, and A_6 groups and depressed in A_{2C} following the progesterone plus estrogen treatment. Thus, the central processing of the chemosensory inputs appeared to be different between the two respiratory drugs. It has been shown that the increased PaO_2 [23] following almitrine is not only due to an increased ventilation (Table 2, [23] but also to an enhancement of pulmonary vasoconstriction [16]. Nevertheless, the almitrine-induced increased ventilation and hypoxic ventilatory responsiveness (Table 2)

as well as the improved oxygenation [23] provided by this drug failed to reduce the plasma EPO level and erythrocytosis. It has to be mentioned, however, that the vasoconstricting effects of almitrine are not restricted to the pulmonary vasculature, but can also be observed in the kidney [9]. It can be thus hypothesized that the beneficial ventilatory effects of almitrine treatment (Fig. 1, Table 2) have been negated by the effects of this drug on renal hemodynamics. This hypothesis is, however, unlikely because Pagel et al. [21] have shown that a reduction of renal blood flow appears not to be a major stimulus for the production of EPO, even when the O_2 tension in the kidney becomes very low. It might be that improvement of the hematological status during chronic hypoxia requires either a peripheral and central stimulation of ventilation or a direct effect of the drug on erythropoiesis at the progenitor cell level. Indeed, female sex hormones stimulate ventilation both centrally [3] and at the chemoreceptor level [7], and estrogens have been shown to affect erythropoiesis at the kidney [10] and bone marrow [17] level. In contrast, the ventilatory effects of almitrine are linked exclusively to peripheral chemoreceptor stimulation [13], and we are not aware of any data concerning an effect of this drug on EPO production or erythropoiesis even though long-lasting stimulation of arterial chemoreceptors by almitrine was reported as resulting in an increased Ht [9].

In conclusion, the present study demonstrates that chronic administration of progesterone in combination with estrogen to male rats born and reared at high altitude reduces the hypoxia-induced polycythemia. This effect of ovarian steroids is associated with marked neurochemical and physiological changes in the chemoreflex function, including a decrease in the catecholaminergic activity of structures involved in the chemoafferent pathway, and an increase in the O_2 chemoreflex drive. In contrast, chronic treatment with almitrine treatment failed to counteract polycythemia despite a similar neurochemical effect in the carotid body and a pronounced stimulatory influence on ventilation. It is suggested that peripheral chemoreceptor stimulation alone is not sufficient to reduce EPO production by the kidney and to attenuate the hypoxia-induced erythrocytosis.

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