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Enhanced erythropoietin expression in the brainstem of newborn rats at high altitude

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ABSTRACT

In addition to its role in elevating red blood cell number, erythropoietin (Epo) exerts protective functions against acute and delayed degenerative diseases of the brain. Moreover, we have recently demonstrated that endogenously synthesized Epo and soluble Epo receptor (a negative regulator of Epo binding to the Epo receptor) in the central nervous system play a crucial role in facilitating the ventilatory response and acclimatization to hypoxia. Here we hypothesized that cerebral Epo in the brainstem is implicated in the process that allows cardiorespiratory acclimatization to high altitude hypoxia during the postnatal period. Thus, we evaluated the postnatal ontogeny of cerebral Epo concentration of Sprague-Dawley rats living and reproducing at high altitude for longer than 19 years (3600 m in La Paz, Bolivia). Our results show that postnatal Epo concentration in high-altitude rats is higher in the brainstem than in the forebrain. Moreover, although Epo concentration in the forebrain of high-altitude rats is similar to sea-level controls, Epo level in the brainstem is surprisingly 2-fold higher in high-altitude rats than in sea-level controls. These findings strongly suggest that brainstem Epo plays an important role in tolerance to high altitude hypoxia after birth. From a clinical perspective, a better understanding of the role of Epo in the postnatal development of cardiorespiratory responses in neonates exposed to acute or chronic hypoxia might be useful.

Originally discovered as a "blood hormone", and widely administered to anemic patients, erythropoietin (Epo) is nowadays recognized as one of the most promising biopharmaceuticals in the treatment of a wide variety of acute and chronic diseases. During the last two decades, animal and human studies have revealed that Epo has protective functions against hypoxic, ischemic, and traumatic brain injuries, as well as delayed degenerative diseases such as Alzheimer's, Parkinson's, epilepsy, and multiple sclerosis (reviewed in [17]). Interestingly, most reports on non-erythropoietic Epo functions point towards a protective effect of this cytokine against various types of injuries. In addition, some physiological functions of Epo have also been identified. For instance, during physiological hypoxia (such as that occurring at high altitudes), pulmonary and arterial O_2 contents are elevated by the neural respiratory response, which leads to increased minute ventilation, thereby increasing tissue oxygenation, and by renal-derived Epo, which activates erythropoiesis in the bone marrow, thus increasing the blood O_2 carrying capacity. In addition, we previously observed in adult transgenic mice that both increased levels of renal-derived Epo in blood and cerebral brain-derived Epo (i.e. Epo synthesized in neurons and astrocytes) augment the ventilatory response to physiological O_2 deprivation [18,19,21]. We also showed that Epo receptors are expressed in the brainstem and in the carotid bodies of adult mice, controlling respiration in both central and peripheral respiratory networks. More specifically, the Epo receptor is expressed in the main respiratory areas of the brainstem, such as the pre-Bötzinger complex and the nucleus tractus solitarii [18]. During the postnatal period, the expression of cerebral Epo has been reported to decrease with time in mammals [6,11,13].

These observations raise a number of questions regarding the role of Epo in the neuronal control of ventilation at sea level, but also in tolerance and acclimatization to hypoxia at high altitude. Indeed, although these results imply that cerebral Epo plays a crucial role in the regulation of O_2 homeostasis, the impact of cerebral Epo on the ability of newborns to acclimatize to high altitude hypoxia has not yet been examined. Several studies have revealed that in

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order to survive otherwise lethal reductions in tissue oxygenation, humans and animals can mobilize a suit of genetic and physiological defenses, under the control of hypoxia-inducible factor (HIF) gene, one of the main O_2-sensing signal transducers [2,24]. As hypoxia is the major regulator of Epo production, and since Epo is one of the main target genes activated by HIF, we tested the hypothesis that brain-derived Epo in the brainstem is implicated in the process that allows cardiorespiratory acclimatization to high altitude hypoxia during the postnatal period. To address this question, the present project was initiated at the Bolivian High Altitude Biology Institute (Instituto Boliviano de Biologia de la Altura, IBBA), which is located at 3600 m altitude in La Paz, Bolivia. Epo concentration was assessed in the brainstem of Sprague-Dawley rats which have been adapted to the IBBA's altitude over several generations, and which are considered as reliable models to study long-term response to hypoxia in mammals.

The high-altitude rats are descendants of a Sprague-Dawley lineage originally purchased from Iffa-Credo (L'Arbresle, France) and implanted in the IBBA since 1992. The IBBA is located in La Paz (Bolivia), at the altitude of 3600 m, where the mean barometric pressure (P_b) is 495–500 mmHg [10]. Accordingly, it is estimated that during this period, 35–40 generations of rats were continuously bred in La Paz. High-altitude rats at postnatal ages of P0, P3, P7, P10, P15, P21 and P30 were used (6 males and 6 females for each age group). At sea level, Sprague-Dawley rats (Charles River Canada, St-Constant, QC, Canada) reared in Québec (QC, Canada) (altitude of 175 m; mean P_b = 750 mmHg) were used at P0 (6 pups: 3 males and 3 females from 4 different litters of >12 pups/litter). All animals were housed and bred under similar conditions in Québec and La Paz. The animals had free access to standard chow and water and were maintained in a room with controlled humidity and temperature under a 12:12-h light–dark cycle. All experiments were carried out in accordance with the guidelines detailed by the Canadian Council on Animal Care.

Animals at P0 and P3 were cryoanesthetized (i.e. by total immersion in ice for 4–5 min [5]), whereas the older animals (P7, P10, P15, P21, and P30) were anesthetized by intraperitoneal injection of sodium pentobarbital (0.6 mg/kg of body weight). Once the animals had lost palmar reflexes, blood samples were drawn by cardiac puncture into heparinized tubes. For each sample, the hematocrit (Ht%) was measured after microcentrifugation and hemoglobin (Hb) concentration was determined by using the Hemocue field spectrophotometer (Hemocue AB, Ängelholm, Sweden).

The forebrain and brainstem of each animal were harvested following perfusion of the animal with saline (0.9% NaCl). Tissues were snap-frozen in liquid nitrogen and stored at −80 °C until their shipping on dry ice to Québec. Forebrain and brainstem proteins were then individually extracted using a published protocol [12] with slight modifications. Briefly, tissues were put in 2 ml of protein extraction buffer (150 mM NaCl, 10 mM EDTA, and 50 mM Tris at pH 7.4) containing Protease Inhibitor Cocktail Set III at 1:200 (Calbiochem) and homogenized with a Polytron during 30 s. Proteins were extracted in the supernatant obtained following centrifugation of the homogenates at 4 °C for 40 min at 14,000 × *g*. Protein contents were determined by the bicinchoninic acid method using the Micro BCA Protein Assay Reagent kit (Pierce Biotechnology), with bovine serum albumin as standard.

Total Epo concentration in the forebrain and brainstem of each animal was determined with an ^{125}I-Epo-based radioimmunoassay (RIA; Amersham, Zurich, Switzerland), as described previously [12]. The lower detection limit of the Epo RIA was 4 U/L, and the intra-assay/inter-assay variances were <2% and <6%, respectively.

All results were expressed as means ± standard error of the mean (SEM). Experimental groups were compared with 2-way analysis of variances (ANOVAs) by considering 2 factors, namely

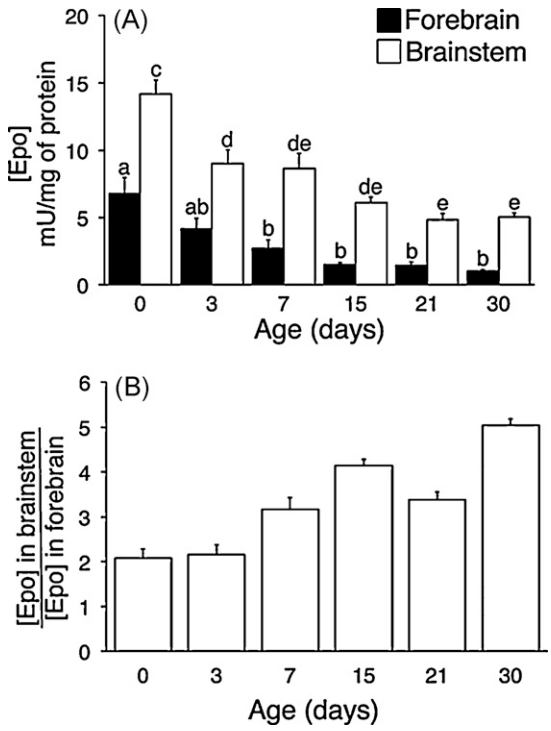

Fig. 1. Erythropoietin (Epo) concentration in the forebrain and brainstem of rats permanently living at high altitude. Epo concentration was assessed by RIA in12 high-altitude rats for each age group considered (6 males and 6 females), and the results are reported as the mean ± SEM. (A) Postnatal ontogeny between birth and 1 month of age. Groups sharing a common-letter label are not significantly different ($p < 0.05$). (B) (Epo concentration in the brainstem/Epo concentration in the forebrain) ratio between birth and 1 month of age.

altitude and postnatal age. Scheffe post hoc test was performed when the ANOVA reached significance. All statistical analyses were carried out using the Statview software. The threshold for statistical significance (α) was fixed at 0.05 for all statistical tests.

Since the postnatal evolution of resting ventilatory rate of male and female high-altitude rats has been previously reported to be sexually dimorphic at P15 [10], we assessed whether Epo concentration in the brain exhibits differences between males and females during postnatal development in these rats. No significant sex-related differences in Epo concentration were observed in the forebrain (1.0 ± 0.2 to 6.2 ± 1.3 mU Epo/mg protein in males and 1.0 ± 0.2 to 7.5 ± 2.2 mU Epo/mg protein in females) nor in the brainstem (5.3 ± 0.3 to 13.3 ± 1.3 mU Epo/mg of protein in males and 4.8 ± 0.5 to 15.1 ± 1.7 mU Epo/mg of protein in females) of high-altitude rats at any of the ages considered (i.e. from P0 to P30). Accordingly, results obtained from male and female samples at each age investigated were pooled for all data reported thereafter.

Epo concentration in both forebrain and brainstem decreased with time, showing a peak of expression at P0 and reaching a plateau around P15 (Fig. 1(A)). Moreover, the Epo concentration was significantly higher in the brainstem than in the forebrain for each age group. Interestingly, the difference in Epo concentration between brainstem and forebrain increased from P0 (~2-fold difference) to P30 (~5-fold difference) (Fig. 1(B)).

We also documented the body weight, hemoglobin concentration, and hematocrit of high-altitude rats from the day of birth (P0) to P30 (Table 1). Hemoglobin concentration in high-altitude rats was relatively stable from birth to P21, with a significant increase occurring at P30. Hematocrit was also stable from birth to P15, and then significantly increased at P21 and P30. Of note is the sexual dimorphism found for the hematocrit of rats at P30, with males

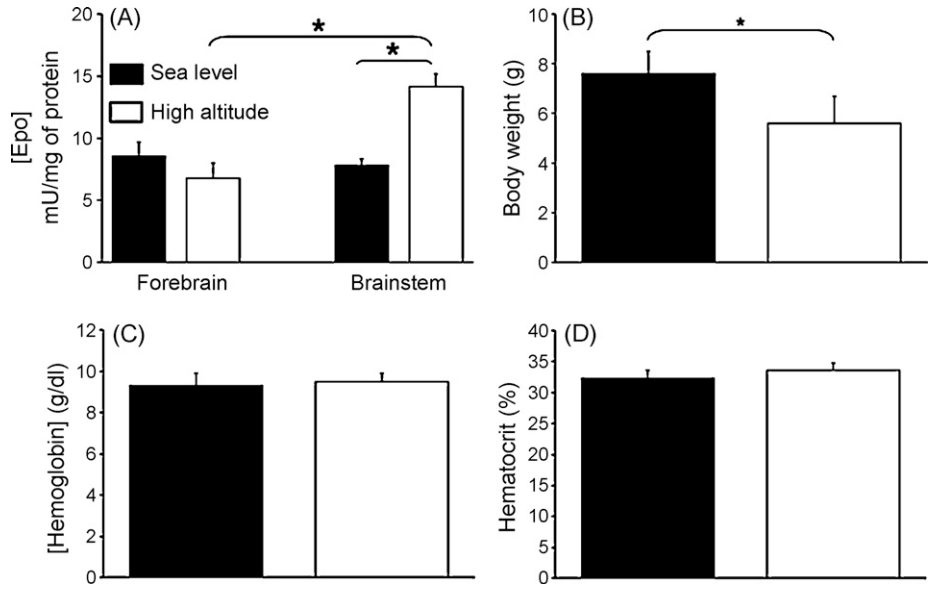

Fig. 2. Comparison between neonatal rats from sea level and high altitude at time of birth (P0). Measurements were performed on 12 high-altitude rats and 6 sea-level rats. (A) Erythropoietin (Epo) concentration in forebrain and brainstem, (B) body weight, (C) hemoglobin concentration, and (D) hematocrit percentage are reported as the mean ± SEM. Significant differences ($p < 0.05$) are represented by an asterisk (*) between two groups sharing a bracket.

showing a significantly higher hematocrit than females (52.6 ± 0.3% vs. 47.5 ± 0.6%).

Since the highest expression level of postnatal cerebral Epo could be observed on the first day of life, and because the difference between the expression of forebrain and brainstem Epo was already significant at this stage, we compared high-altitude and sea-level rats at P0 (Fig. 2). In the forebrain, Epo concentration was not significantly different between high-altitude and sea-level rats (Fig. 2(A)). Moreover, no differences in Epo concentration were observed between the forebrain and brainstem of sea-level rats. Surprisingly, however, Epo concentration was 2-fold higher in high-altitude brainstems than in high-altitude forebrains as well as sea-level brainstems. It is noteworthy that compared to sea-level rats, the body weight of high-altitude rats was significantly lower at P0 (Fig. 2(B)). However, no differences were found in hemoglobin concentration and hematocrit between sea-level and high-altitude rats at that age (Fig. 2(C) and (D), respectively).

Hypoxia is defined as a relative deficiency in O_2 availability/delivery for maintaining adequate physiological O_2 tension, and thus results from an imbalance between demand and supply of O_2 [9]. At high altitudes, physiological hypoxia occurs upon drops in environmental O_2 partial pressure (pO_2). As such, tolerance to hypoxia is the ability to develop a complement of genetic and physiological defenses for surviving to otherwise lethal reductions in tissue oxygenation [7]. Several mammalian species, including humans, have developed a capacity to adapt to a high-altitude environment. Indeed, humans reside in the Andes since ~10,000–12,000 years B.C. [3,4] and today, 140 million people live permanently at more than 2500 m above sea level [16].

To perform this study, we have used a strain of Sprague-Dawley rats implanted in La Paz (Bolivia; 3600 m) in 1992. A recent study determined that this rat strain possesses some physiological characteristics similar to those of chronic mountain sickness patients (i.e. hypoventilation, relative increased erythrocytosis, cardiac hypertension and decreased lifespan) (Lumbroso et al., personal communication). This is not surprising considering that no rat species is known to have naturally conquered high-altitude environments [1,25]. In addition, we observed that, compared to sea-level controls, body weight is significantly decreased in high-altitude rats at birth. In previous work, we had also determined that, starting at P15, basal ventilation in these animals is significantly higher than in sea-level controls [10]. Moreover, we had found a sexually dimorphic ventilation (at P15 and from P35 henceforth) in that basal ventilation is higher in females than in males [10]. Thus, this rat strain is clearly a valuable animal model to study the acclimatization process to high altitude. Since animals were originally purchased from distinct suppliers (high altitude: Iffa-Credo in France; sea level: Charles River in Canada) one potential limitation in our experimental protocol might be that inherent genetic differences could exist between Iffa-Credo and Charles River strains. Although both rat groups are *bona fide* Sprague-Dawley rats, differences in genetic backgrounds might account for the reported differences in cerebral Epo concentrations. Quite remarkably, however, as mentioned above, previous studies using only Iffa-Credo animals reported marked physiological differences in the cardiorespiratory system of high-altitude and sea-level rats [10]. Thus, although the possible contribution of genetic variations cannot be excluded, the latter evidence supports the hypothesis that the observed differences in Epo concentrations mainly result from altitudinal effects. Although we had previously reported several physiological parameters characteristic of these rats, this is the first time that a direct target of the O_2-sensingsystem in the brain such as Epo, is investigated.

In the present study, we have found that compared to sea level controls, Epo expression in the postnatal brainstem is enhanced in rats living at high altitude. As compared to sea-level controls, Epo expression in high-altitude forebrains is unchanged whereas

Table 1
Postnatal ontogeny of weight and hematological parameters in high altitude rats.

Age[a]	Weight (g)	Hb (g/dl)[b]	Ht (%)[c]
P0	5.6 ± 1.1	9.5 ± 0.4	33.6 ± 1.1
P3	8.9 ± 1.7	9.6 ± 0.9	32.4 ± 1.6
P7	11.3 ± 2.1	9.9 ± 0.6	33.7 ± 0.8
P15	20.8 ± 2.4	8.7 ± 0.4	32.4 ± 1.6
P21	27.4 ± 2.8	10.1 ± 0.4	39.2 ± 1.2
P30	66.5 ± 3.0	13.6 ± 0.5	50.1 ± 0.5

[a] $n = 12$ rats for each age (6 males and 6 females).
[b] Hb = hemoglobin concentration.
[c] Ht = hematocrit.

Epo expression in brainstems is doubled at P0. It is noteworthy that the highest expression of cerebral Epo after birth occurs at P0.As the brainstem contains the major circuitry controlling cardiorespiratory functions, these data suggest that Epo plays a major role in setting up neuronal control of these functions. Furthermore, we found that despite that cerebral Epo concentration decreases exponentially with time, the difference in Epo concentration between brainstem and forebrain increases from P0 to P30 (2- and 5-fold differences, respectively). Interestingly, the Epo receptor is expressed in catecholaminergic areas of the adult brainstem [18]. Furthermore, Epo is known to modulate tyrosine hydroxylase activity and catecholamine release [14,15]. Similarly, we previously reported that plasma and cerebral Epo alter catecholaminergic metabolism in adult brainstem catecholaminergic centers as well as in carotid bodies [18,20,21]. In agreement with these observations, the Sprague-Dawley rat strain living at high altitude also shows altered tyrosine hydroxylase activity in brainstem catecholaminergic centers (A2r, A2c, A5, A6) during the postnatal development, as compared to sea-level controls [10]. Taking into account that catecholamines are known to be implicated in the ventilatory response to acute and chronic hypoxia [8,22,23], it is tempting to suggest that at high altitude, brainstem Epo affects postnatal cardiorespiratory control by regulating catecholaminergic metabolism.

We also observed that blood hemoglobin level was similar in either postnatal high-altitude or sea-level rats. This result is consistent with previous observations showing that hemoglobin and hematocrit start exhibiting higher values at high altitude than at sea level at the pubertal age only [10], suggesting that plasma Epo levels should be similar in both rat populations. However, plasma Epo is produced by kidney while cerebral Epo is produced by neurons and astrocytes, thus explaining the differences in Epo expression levels between the plasma and brain compartments, as well as the disparity between blood hemoglobin variations and cerebral Epo expression. Thus, differences in Epo expression levels between high-altitude and sea-level rats can be observed in brain prior to blood.

In summary, we have demonstrated that Epo expression is dramatically increased in the brainstem, but not in the forebrain of rat shaving lived and bred at 3600 m for longer than 19 years. Regarding the specific phenotype of these animals, which apparently fail to acclimatize properly to high altitudes under normal conditions [1,25], these findings strongly suggest that cerebral Epo plays an important role in the ability to tolerate high altitude hypoxia from birth. Keeping in mind that adequate cardiorespiratory functions are crucial for surviving upon hypoxic conditions, our findings suggest that Epo may have potential clinical implications in the postnatal development of cardiorespiratory responses in neonates exposed to acute or chronic hypoxia resulting from pathological causes (e.g. neonatal hypoxia/ischemia) or due to the environmental context (e.g. high altitude).

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