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Erythropoietin in the *Locus coeruleus* attenuates the ventilatory response to CO_2 in rats

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ABSTRACT

The *Locus coeruleus* (LC) is a pontine area that contributes to the CO_2/pH chemosensitivity. LC cells express erythropoietin (Epo) receptors (EpoR), and Epo in the brainstem is a potent normoxic and hypoxic respiratory stimulant. However, a recent study showed that the intra-cisternal injection (ICI) of Epo antagonist does not alter the hypercapnic ventilatory response in mice. As ICI leads to a widespread dispersal of the product throughout the brainstem, in this work we evaluated the specific impact of Epo in the LC-mediated ventilatory response to CO_2 (by whole body plethysmography) in juvenile male Wistar rats. Normocapnic and hypercapnic ventilation were evaluated before and after unilateral microinjection of Epo (1 ng/100 nL) into the LC. To evaluate the long-term effect of Epo, the HcVR was re-evaluated 24 h later. Our results show that Epo attenuates the hypercapnic ventilation. We conclude that Epo in the LC tunes the hypercapnia-induced hyperpnea.

1. Introduction

The central respiratory chemosensitivity requires sensory cells that detect changes in PCO_2 and/or pH (Kumar et al., 2015). The Locus Coeruleus (LC) is considered a central CO_2/pH chemoreceptor site in mammals (Gargaglioni et al., 2010; Santin and Hartzler, 2013; Taxini et al., 2013). More than 80% of LC neurons are chemosensitive and respond to hypercapnia with an increased firing rate (Filosa et al., 2002; Oyamada et al., 1998; Pineda and Aghajanian, 1997). Furthermore, the lesion of LC neuroadrenergic neurons leads to a large decrease in the response to CO_2 (Biancardi et al., 2008). These facts evidence that the LC nucleus exert a profound effect on the response to hypercapnic ventilation.

Erythropoietin (Epo) and its receptor (EpoR) are widely distributed in the mammalian brain (Rabie and Marti, 2008). EpoR is expressed in neurons, oligodendrocytes, glial cells, and in brain vascular endothelial cells (Brines et al., 2000). Moreover, studies in our group revealed that EpoR is extensively expressed in the brainstem cells, including the LC noradrenergic neurons (Soliz et al., 2005). Although Epo stimulates the neural control of ventilation during hypoxia (Ballot et al., 2015a; Caravagna et al., 2014, 2015; Caravagna and Soliz, 2015; Khemiri et al., 2011), we reported recently that central chemosensitivity to CO_2 is not altered by blocking the cerebral Epo. Specifically, we showed that the soluble EpoR (sEpoR, the natural antagonist of Epo), injected in the brainstem region of adult mice via the cisterna Magna (intracisternal injection, ICI) did not alter the ventilation under normocapnia and hypercapnia (Ballot et al., 2015b). Since a central ICI of sEpoR might activate excitatory and inhibitory areas associated with the ventilatory control, it precludes discriminating the precise impact of Epo in specific respiratory nuclei. In fact, hypoxic studies performed in transgenic mice overexpressing Epo only in the brain showed unaltered, increased, and decreased noradrenaline (NE) content in catecholaminergic cell groups A6, A5 and A2C2 respectively (Soliz et al., 2005). Accordingly, in this study, we tested the hypothesis that Epo modulates the respiratory stimulation elicited by the LC during activation of CO_2 chemoreceptor. This hypothesis is also supported by the fact that apart from affecting the hypercapnic ventilation, the LC is also implicated in several brain functions (such as the control of pain, stress and wakefulness, (de Carvalho et al., 2014; Samuels and Szabadi, 2008)), in which Epo is also involved (reviewed in (Wang et al., 2014)). To test this hypothesis, we evaluated the hypercapnic ventilatory response in male rats after receiving a unilateral microinjection of Epo in the LC. Our

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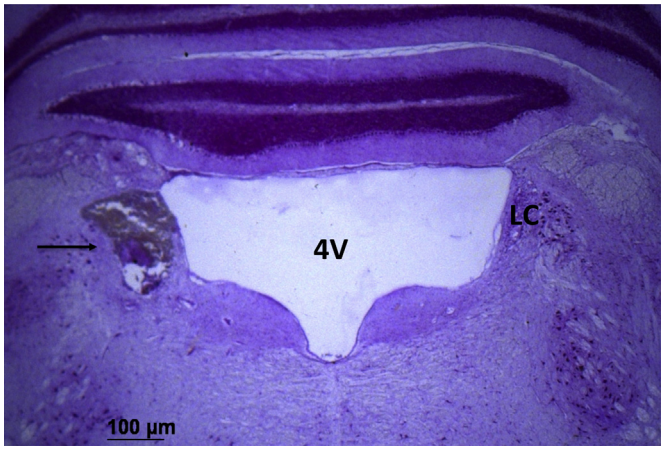

Fig. 1. Representative photomicrograph of unilateral microinjection into the *Locus Coeruleus* (LC) of rat. A black arrow indicates a typical intra-LC microinjection. 4 V: fourth ventricle.

results show that Epo in the LC area of rats produces an attenuation of the ventilatory response to CO_2. These results suggest that Epo is involved in the LC regulation of the control of breathing.

2. Material and methods

2.1. Animals

Experiments were performed on male Wistar rats with 6 to 7 weeks of age (average weight of 325 g). The animals had free access to water and food and were housed in a temperature-controlled chamber at 24–26 °C (ALE 9902001; Alesco, Monte Mor, SP, Brazil), with a 12:12 h light/dark cycle (lights on at 6:30 a.m.). All experiments were performed between 9:00 am to 5 pm. Animal care was carried out in compliance with National Council for the Control of Animal Experimentation (CONCEA-MCT-Brazil) guidelines and was approved by Faculty of Agricultural and Veterinary Sciences and Animal Care and Use Committee (CEUA-FCAV-UNESP-Jaboticabal campus; Protocol n° 007094/13).

2.2. Surgery

Animals were anaesthetized by the intraperitoneal administration of ketamine (100 mg/kg; Union National Pharmaceutical Chemistry S/A, Embu-Guaçu, SP, Brazil) and xylazine (10 mg/kg; Laboratories Calier S/A Barcelona, Spain).

The head and a portion of the abdomen were shaved, and the skin was sterilized with betadine solution and alcohol. Rats were fixed to a Kopf stereotaxic frame and implanted with a stainless steel guide cannula. The guide cannula (0.7 mm o.d. and 15 mm in length) was implanted unilaterally 1 mm above the LC region (distance from lambda: anterior: −3.4 mm; lateral: −1.2 mm; and dorsal: −5.8 mm deep from the skull and inclination of vertical stereotaxic bar at 15°), according to Paxinos and Watson atlas (Paxinos and Watson, 1998). The cannula was attached to the bone with stainless steel screws and acrylic cement. A tight fitting styled was kept inside the guide cannula to prevent occlusion. Following the surgery, animals received two doses of enrofloxacin (10 mg kg^{-1}, intramuscular) and flunixin meglumine (2.5 mg kg^{-1}, subcutaneous) to prevent infection and post-surgical discomfort respectively. The surgical procedures were performed over a period of approximately 40 min. Experiments were initiated six days after surgery.

A day before the experiments a temperature datalogger (SubCue, Calgary, AB, Canada) was implanted in the abdominal cavity through a midline laparotomy for body temperature (Tb°) measurements. The datalogger was programmed to acquire data every 5 min.

2.3. Drug and gas mixture

Recombinant human Epo (rhEpo = 1 ng/100 nL; half-life of 4–6 h after administration; CilagAG, Switzerland) was dissolved in artificial cerebral spinal fluid (aCSF; in mM: 14.61 NaCl; 4.03 $NaHCO_3$, 0.45 KCl, 0.3 $MgSO_4$, 0.34 KH_2PO_4, 3.6 glucose; 0.56 $CaCl_2$). The gas mixtures used in this study were room air (normocapnia) and a hypercapnic gas mixture (7% CO_2, 21% O_2, balance N_2; White Martins Gases Industriais Ltda, Sertãozinho, SP, Brazil). The percentage of CO_2 was chosen based on previous studies (Biancardi et al., 2008).

2.4. Determination of the respiratory recording

Detailed description of whole body plethysmography technique has been previously reported (Vicente et al., 2016). In short, all signals were acquired and recorded on a computer using the data analysis software Acknowledge (v. 4.2.3 data acquisition system, Biopac Systems), and used offline to calculate tidal volume (V_T), respiratory frequency (f_R), and minute ventilation (V. e = V_T x f_R). A volume calibration was performed for each experiment by injecting 1 mL of air into the animal chamber.

2.5. Microinjection of Epo and vehicle

Detailed description of microinjection protocol into the LC has been previously reported (Taxini et al., 2013). In short, microinjections were performed after six days recovery from cannula implantation. A Hamilton syringe (of 5-µL volume; Reno, NV, USA) was prefilled with Epo or aCSF and then connected to PE-10 tubing and a thin needle injector (33 gauge). Next, the needle injector was inserted into the LC. All microinjections were made with a volume of 100 nL and were performed over a period of 30 s, with 30 additional sec allowed to elapse before the injection needle was removed from the guide cannula to avoid reflux. All injections were performed using a microinjector machine (model 310, Stoelting Co., IL, USA).

2.6. Experimental protocol

Each animal was individually placed in a plexiglass chamber (5 L) maintained at 25 °C and allowed to move freely while the chamber was flushed with humidified room air. After the animals remained calm for ∼30 min, baseline measurements of V. e and Tb° were recorded. Subsequently, rats received a microinjection of vehicle (aCSF) or Epo (1 ng/100 nL) into the LC. Then the V. e was measured at 5, 10, 15, 20 and 30 min after injection under room-air or the exposure to hypercapnia. Finally, minute ventilation was evaluated after 30 min of recovery to gas exposure. To test the long-term effect of the Epo microinjection, the HcVR was re-evaluated for a second time, 24 h later.

2.7. Statistical analysis

Values are reported as means ± SEM. The variances in the Tb° and ventilatory responses to hypercapnia among the groups were analyzed by two- way ANOVA followed by Tukey's test for *post hoc* comparisons. The significance level was set to $P < 0.05$. The statistical analysis was performed using computer software (SIGMA STAT; Systat Software, Point Richmond, CA, USA).