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Research article

Recombinant human erythropoietin offers neuroprotection through inducing endogenous erythropoietin receptor and neuroglobin in a neonatal rat model of periventricular white matter damage☆

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HIGHLIGHTS

- EPOR was upregulated after rh-EPO treatment in a neonatal rat model of PWMD.
- Ngb was upregulated after rh-EPO treatment in a neonatal rat model of PWMD.
- EPOR and Ngb exhibit different expression patterns.
- EPOR and Ngb act as important, coordinated neuroprotectors in the brain.

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ABSTRACT

Recombinant human erythropoietin (rh-EPO) has been reported to have protective effects against brain injury. The purpose of this study was to evaluate the levels of erythropoietin receptor (EPOR) and neuroglobin (Ngb) in a neonatal rat model of periventricular white matter damage (PWMD), and to identify the relationship between the two proteins. On postnatal day 3 (P3), rats underwent permanent ligation of the right common carotid artery followed by 6% O_2 for 4 h (HI) or sham operation and normoxic exposure (sham). Immediately after HI, rats received a single intraperitoneal injection of rh-EPO (5 U/g) or saline. We assessed the expression level of Ngb and EPOR on postnatal days 5, 7, 10 and 14. EPOR in the HI rats was initially increased as compared to the sham rats at P5. Subsequently, EPOR expression decreased, but was maintained at a higher level than in sham rats from P7 to P14. In rh-EPO treated rats, the increase in EPOR was greater than in HI rats at P5. However, EPOR levels decreased sharply from P7 to P14. In HI rats, Ngb was increased compared to the sham rats from P5 to P14. Ngb levels were further upregulated after rh-EPO administration from P5 to P10 compared to HI rats. However, this upregulation decreased at P14. In conclusion, this study shows that EPOR and Ngb were upregulated, and both of them act as important coordinated neuroprotectors in rh-EPO treatment of PWMD. However, the two proteins exhibit different expression patterns.

1. Introduction

Brain injury in premature infants, particularly infants with very low birth weight (VLBW), is of enormous importance to public health because of the large number of these infants that survive with serious neurodevelopmental disabilities [1–3]. Periventricular white matter damage (PWMD), also termed periventricular leukomalacia, is the dominant neurological lesion in preterm infants who survive brain injury [4,5]. In China, approximately 1.5 million premature infants are born annually, with a 5% incidence of PWMD [6]. Moreover, the proportion of premature infants has increased gradually over the past decade. The enormous burden of neurological disability from premature birth is expected to continue. Therefore, specific targeted neuroprotective strategies are necessary.

It is believed that erythropoietin (EPO) may be a potential neuroprotective agent. Studies of central nervous system (CNS) ischemia and reperfusion in a variety of animal models have defined EPO's

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robust neuroprotective properties in the brain [7–9]. Results of studies on injured prenatal brains have shown that neonatal rh-EPO administration is beneficial to myelination [9], rescues neural cells, and induces lasting histological and functional improvements [10]. In addition, a retrospective cohort study on premature infants found a dose–response relationship between rh-EPO treatment and improved Mental Developmental Index scores [11]. At present, the precise mechanism of this neuroprotection remains to be determined. Furthermore, its effects on white-matter formation are of specific interest because of the association of premature birth and white matter injury.

A number of mechanisms are involved in the neuroprotective effects of EPO, such as activation of specific protein kinases, inhibition of reactive oxygen species and glutamate overproduction [12], modulation of neurotransmission, attenuation of apoptosis [13], and stimulation of angiogenesis [14]. Many cells besides erythroid progenitors express the EPO receptor (EPOR), including cells in the brain. EPOR activation can trigger different signaling pathways. The expression of EPOR is regulated after brain injury. Neuroglobin (Ngb) was the third type of globin discovered after myoglobin and hemoglobin, first reported by German scientists Burmester et al. [15] in 2000. Ngb is located primarily in the brain and may play an essential role in oxygen homeostasis in neural tissue [16]. Ngb supports hormone therapy applications for protection against neurodegenerative diseases [17]. Similarly to myoglobin and hemoglobin, Ngb binds oxygen with high affinity, suggesting a possible role in oxygen sensing, transport and storage. Therefore, it is considered a neuroprotective protein both in vitro and in vivo [18,19].

The purpose of the current study was to evaluate the levels of EPOR and Ngb in a neonatal rat model of PWMD, and to identify the relationship between the two proteins.

2. Materials and methods

2.1. Animals

Pregnant Sprague-Dawley (SD) rats were obtained from Nanjing Medical University of China and allowed to deliver. All animals were treated appropriately according to the Guide for the Care and Use of Laboratory Animals (National Research Council). Experimental processes were approved by the Southeast University Animal Experimentation Committee. Littermates were randomized into three groups: the drug treatment group (HI with rh-EPO administration [HI-EPO group]), the control group (HI with saline administration [HI group]) and the sham group (without HI). Each group was further divided into 13 subgroups (6–8 rats in each subgroup).

2.2. Neonatal rat model of cerebral unilateral hypoxia-ischemia and drug administration

As in our previous study [14], postnatal day 3 (P3) rats underwent permanent ligation of the right common carotid artery after anesthetization with ether. The pups were returned to the home cage for 2 h, and then exposed to hypoxia (94% N_2 + 6% O_2) for 4 h by being placed in a sealed chamber partially submersed in a 37 °C water bath. In sham-operated rats, the same surgery was performed without ligation and exposure to hypoxia. Immediately after hypoxia, rats in the drug treatment group received a single intraperitoneal injection of rh-EPO (5 U/g; Sunshine Pharmaceutical Co., Ltd., Shenyang, China). Others received the same volume of saline.

2.3. Brain tissue preparation

Rats were sacrificed with a lethal dose of pentobarbital (>50 mg/kg i.p.) when they were 5, 7, 10 and 14 days old. For hematoxylin and eosin (HE) staining and immunohistochemistry, brain tissues (3 mm either side of the optic chiasm in the coronal plane) were removed after decapitation and fixed in 4% paraformaldehyde overnight at 4 °C. Then, for cryoprotection, the brain tissue was transferred sequentially to 30% sucrose in 0.1 MPBS until sank. The brain tissue was then embedded in O.C.T. (Zhongshan Biotechnology Co., Ltd., Beijing, China) and stored at −80 °C. For western blots and qRT-PCR, the right brains were rapidly harvested, snap frozen in liquid nitrogen and kept at −80 °C.

2.4. HE staining and immunohistochemistry

Consecutive frozen brain sections were used for HE staining and immunohistochemistry. The HE stained sections were examined under the microscope for any alteration in histopathology. For immunohistochemistry, slides were incubated with primary antibody: anti-EPOR (1:200; SC-697, Santa Cruz, USA) or anti-Ngb (1:200; SC-22001, Santa Cruz) overnight at 4 °C, and then treated for 2 h at room temperature with the appropriate secondary antibody (Boster Biotechnology Co., Ltd., Wuhan, China) for EPOR and Ngb. The substrate 3,3′-diaminobenzidine (DAB, Sigma) was added for <5 min. Slides were examined with a computer-assisted Olympus CK2 microscope. Five sections from each rat were taken of the right white matter region. Cells in the white matter were counted (per ×400) in three areas by two blinded independent observers.

2.5. Western blotting

Complete Mini Protease Inhibitor Cocktail Tablet (Roche) and phosphatase inhibitor PMSF (10 mM) were added to inhibit proteases and phosphatases. Protein concentration was determined by the Bradford protein assay. Homogenate proteins (50 μg) were heated for 10 min at 90 °C and then loaded onto 10% SDS-PAGE. The protein was transferred electrophoretically onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk, and subsequently incubated overnight at 4 °C with primary anti-EPOR polyclonal antibody (1:500, SC-697, Santa Cruz). Specific protein was detected with secondary antibody (1:5000, BA1003, Boster Biotechnology Co., Ltd.), and were visualized by ECL reagents (Pierce, USA). The optical density of GAPDH was used as a loading control. Densitometry analysis was performed with Image-J software.

2.6. Real-time fluorescence quantitative PCR (qRT-PCR)

The expression of Ngb in the brain was determined by qRT-PCR. Briefly, total RNA was extracted from the right brain using Trizol (Invitrogen, CA, USA), according to the manufacturer's specifications. Approximately 1 μg of total RNA was used for the generation of complementary DNA (cDNA) using the PrimeScript® RT Reagent Kit with gDNA Eraser (Takara, Japan). The reverse transcription protocol was 15 min at 37 °C, and 5 s at 85 °C. The amplification protocol included an initial cDNA denaturation of 2 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 40 s at 60 °C. PCR of β-actin was used as an internal control for the amount of input RNA. Primer pairs used for amplification were as follows: Ngb, forward 5′-AGGTTATGCTTGTGATTGATGC-3′, reverse 5′-AGAGCAGGGACTCACCTACTGT-3′; β-actin, forward 5′-CTGAACCCTAAGGCCAACC-3′, reverse 5′-AGCGCGTAACCCTCATAGAT-3′. qRT-PCR was carried out using