Brain & Development 36 (2014) 864–869

BRAIN & DEVELOPMENT
Official Journal of the Japanese Society of Child Neurology

www.elsevier.com/locate/braindev

Original article

Erythropoietin improves brain development in short-term hypoxia in rat embryo cultures ☆

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Received 1 October 2013; received in revised form 7 January 2014; accepted 14 January 2014

Abstract

Background: Hypoxic ischemic encephalopathy continues to be a significant cause of death and disability worldwide. Erythropoietin (EPO) has the potential to lessen neurologic sequelae due to hypoxia–ischemia. *Methods:* The in vitro effects of EPO on total embryonic development and brain VEGF receptor (VEGFR) expressions were investigated in 50 rat embryos at 9.5 days of gestation that were cultured in whole rat serum (WRS). According to the study protocol, the embryos were divided into two groups. The first group is comprised hypoxia, 100 and 50 U/ml EPO after hypoxia groups. Group 2 comprised control (WRS) and WRS + EPO. After 48-h culture, the embryos from each group were harvested to be analyzed according to a morphological scoring system and also genetically to measure brain VEGFR expression. *Results:* The mean morphological scores for the embryos grown in control, WRS + EPO, hypoxia, and in the presence of 100 and 50 U/ml EPO in hypoxic medium were 55.30 ± 7.22, 52.10 ± 5.27, 23.0 ± 4.60, 36.20 ± 5.07, and 19.70 ± 5.07, respectively. Expressions of VEGFR-1, -2, -3 were significantly elevated in the 100 U/ml EPO and WRS + EPO groups compared to the hypoxia group ($p < 0.05$). *Conclusions:* These results support the conclusion that (1) VEGFR-1, -2, -3 may increase with EPO treatment in hypoxic conditions, (2) VEGF and EPO may be part of a self-regulated physiological protection mechanism to prevent neuronal injury including in utero neural tube defects.

Keywords: Brain development; Embryo cultures; Erythropoietin; Hypoxia

1. Introduction

Erythropoietin (EPO) is a primary regulator of erythropoiesis and is fundamentally produced by the kidney in adults and by hepatocytes in the fetus in response to hypoxia [1,2]. Besides its established function in erythropoiesis, EPO is currently also appreciated for its neuroprotective effects [3–6]. Increasing evidence suggests that EPO signaling may also play a role in stimulating the development of multiple organisms [3]. Vascular endothelial growth factor (VEGF), a hypoxia-inducible endothelial cell mitogen, has been characterized as a potent vascular permeability factor and a critical factor in vasculo- and angiogenesis [7].

It has been shown that both VEGF and EPO exert neuroprotective effects in both in vitro and in vivo

Abbreviations: EPO, erythropoietin; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptors; WRS, whole rat serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; VYS, visceral yolk sac; ED, embryonic days.

☆ This study was supported by the Research Foundation of Erciyes University.

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http://dx.doi.org/10.1016/j.braindev.2014.01.005

experimental models [8], probably through the common oxygen-sensing pathway stimulated by hypoxia. However, to date, the exact molecular mechanisms by which EPO might mediate its actions in the central nervous system, and the possible implications for VEGF in this regard are not well understood [9].

Originally developed for studying basic mechanisms in developmental biology, the post-implantation embryo culture method has been extended and applied to the testing of chemicals and pathologic condition in the prenatal period [10]. Examination of rat conceptuses from dams on pregnancy days 9.5–11.5 led to the selection of 17 morphological features for use in the system. Up to six developmental stages of each feature were defined and assigned scores of 0–5 [11]. Using this system, it is possible to test growth factors (EPO) on in vitro embryonic development during the early organogenesis period (between 9.5 and 11.5 days).

The aim of the present study was to investigate the effects of EPO on hypoxia-induced growth retardation and embryo brain development during the organogenesis period and to determine whether VEGF has an effect on the action mechanism of EPO.

2. Materials and methods

All protocols were approved by the Animal Care and Use Committee (Ethics Committee) of Erciyes University. The Wistar rats (Rattus norvegicus) used in this study were obtained from Erciyes University's Experimental and Research Center. Two sets of experiments were carried out. First, the culture of rat embryos with different gassing concentrations was evaluated, and second, the culture of rat embryos in rat serum with increasing concentrations of EPO was studied; VEGFR expressions were measured in all rat embryo brains.

2.1. Serum for whole-embryo culture (whole-rat serum; WRS)

The rat serum was collected by puncture of the abdominal aorta of ether-anesthetized adult rats and was heat inactivated for 30 min at 56 °C. The heat-inactivated serum was stored at 4 °C if used within 48 h, or at −80 °C for up to 2 weeks. Frozen serum was thawed on the day of use. WRS was used in control groups.

2.2. Rat embryo culture

Whole embryo culture (WEC) technique has been developed in 1950's by New and his colleagues, and applied for developmental biology [10]. Although development and growth of mammalian embryos are critically dependent on the function of the placenta, WEC technique allows us to culture mouse and rat embryos *ex vivo* condition during limited periods corresponding to midgestation stages during embryonic day E8.5–E14.5 in the rat [11]. In WEC, we can directly target desired areas of embryos using fine glass capillaries because embryos can be manipulated under the microscope. Therefore, rodent WEC is very useful technique when we want to study dynamic developmental processes of postimplanted mammalian embryos.

The five pregnant rats were humanely killed by ether overdose at 9.5 days of gestation, and the embryos (approximately 10 embryos from each pregnant rat) were removed from the mother by the explantation procedure described by New (1978) [10].

Using a dissection microscope, the decidual mass was split to expose the conceptus, which was gently teased free and immediately immersed in Hank's balanced salt solution. Four embryos were placed in each culture bottle of 4 ml volume, and the bottles were placed on a roller incubator at 37 °C. WRS was used in the control groups. Fifty embryos were used in total: 10 for 0 h controls, 10 for controls in which EPO (100 U/mL) was administrated in control cultures without hypoxia, 10 for 24 h hypoxia, and 20 for EPO supplementation (50 and 100 U/mL). The control group embryos were cultured in WRS and WRS + EPO (100 U/mL). Different gas concentrations were tested to obtain hypoxia. At the end of the procedures, all embryos were gassed for 1 min in the culture bottles using a mixture of 5% O_2, 5% CO_2, and 90% N_2 and then incubated at 37 °C in an incubator. During incubation, the culture bottles were continuously rotated at 30 rpm and re-gassed after 24 h. Embryos were gassed for 1 min with 20% O_2, 5% CO_2, and 75% N_2 in the control (only WRS) and WRS + EPO groups, and with 5% O_2, 5% CO_2, and 90% N_2 in the hypoxia group after 24 h. Embryos were gassed for 1 min with 40% O_2, 5% CO_2, and 55% N_2 in all groups at hour 44, 4 h before the morphological evaluation. The experimental groups were cultured in hypoxic medium, 50 and 100 U/mL EPO per bottle after hypoxia, for 24 h. In order to assess the effect of EPO on total embryonic growth and brain development, the embryos were cultured in different concentrations of EPO (50–100 U/mL) to produce the appropriate concentration required for the embryos to develop [12].

2.3. Morphologic evaluation

It is possible to compare the embryonic development of control and experimental groups morphologically on day 11.5 using a morphologic scoring system [10]. A morphological scoring system takes into account the growth and differentiation of different embryological features, including the appearance of yolk sac circulation and diameter (vasculogenesis and morphology), crown-rump length (growth), allantois, embryo turning, neural tube closure, branchial bar number, maxillary processes, mandibular processes, heart, optic, otic, and