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Research report

Erythropoietin improves hypoxic-ischemic encephalopathy in neonatal rats after short-term anoxia by enhancing angiogenesis

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ABSTRACT

Erythropoietin (EPO) is important for angiogenesis after hypoxia/ischemia. In this study, we investigated whether recombinant human erythropoietin (rhEPO) can enhance angiogenesis, and promote cognitive function through vascular endothelial growth factor (VEGF)/VEGF receptor 2 (VEGFR2) signaling pathway in a rat model of hypoxic-ischemic encephalopathy (HIE). RhEPO, selective VEGFR2 inhibitor (SU5416) or vehicle was administrated by intraperitoneal injection. The assessment for cognitive function begins on day 60 after anoxia. Vascular density in hippocampus and white matter damage within corpus callosum were examined on day 28 after anoxia. The expression of erythropoietin receptor (EPOR), VEGF, rapidly accelerated fibrosarcoma 1 (Raf1), and extracellular-signal-regulated kinases 1 and 2 (ERK1/2) in hippocampus were evaluated on day 7 after anoxia. RhEPO-treated anoxia rats had better cognitive recovery, higher vascular density, and less white matter damage than in the vehicle anoxia rats. These protective effects associated with increased expression of EPOR, VEGF; and increased phosphorylation of Raf1 and ERK1/2. While this up-regulation, and changes in the histopathologic and functional outcomes were abolished by SU5416. Our data indicate that rhEPO can enhance angiogenesis, reduce white matter damage, and promote cognitive recovery through VEGF/VEGFR2 signaling pathway in anoxia rats.

1. Introduction

Hypoxic–ischemic encephalopathy (HIE), with high morbidity and mortality, is the most common disease in neonate during the perinatal period and is inevitable in some cases (H. Wu et al., 2012; Y.W. Wu et al., 2012; Kurinczuk et al., 2010). Neonatal anoxia is the main pathogenic factor of HIE (H. Wu et al., 2012; Y.W. Wu et al., 2012). To date, hypothermia is the only therapy that has been proved to be effective in the clinical care (Shea and Palanisamy, 2015). Yet, clinical trials suggest that hypothermia alone cannot reduce the mortality or neurological disability rate of neonatal HIE (Muller and Marks, 2014). Therefore, novel neuroprotective therapies are urgently needed.

Erythropoietin (EPO), mainly produced by the fetal liver and adult kidney, is a glycoprotein that primarily recognized as an essential stimulator for erythropoiesis (Rangarajan and Juul, 2014). However, a recent study revealed that EPO and its receptor (EPOR) also localize in the hippocampus, cortex, internal capsule, and midbrain in mouse (Digicaylioglu et al., 1995). Further research demonstrated that EPO mRNA can only be detected in human astrocytes, while EPOR can be found in human neurons, astrocytes, microglia and endothelial cells (Nagai et al., 2001; Trincavelli et al., 2013; Ogunshola and Bogdanova, 2013). The neuroprotective efficacy of EPO have been demonstrated in a wide variety of experimental models, including stroke (Gonzalez et al., 2013; Minnerup et al., 2009), spinal cord injury (Freitag et al., 2015), and traumatic brain injury (Bouzat et al., 2013; Xiong et al., 2010a). However, the mechanism by which EPO protects the brain is not yet completely clarified.

The pathophysiology of HIE is complex, and has not been fully understood. As has been known, neurons are sensitive to anoxic injury. Neurogenesis and angiogenesis is important for the repairment of impaired brain after HIE (Dixon et al., 2015). The role of angiogenesis is particular important in this process, for angiogenesis also plays a critical role for the development and maturation of the central nervous system (CNS) by providing metabolic nutrition (Ma and Zhang, 2015). Vascular endothelial growth factor (VEGF) is regarded as an essential factor for the regulation of angiogenesis and vasculogenesis by combining with its receptor

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(VEGFR2) and activation of a series of downstream signaling pathways (Wang et al., 2014). Studies have reported that up-regulation of VEGF/VEGFR2 signaling pathway plays an important role in EPO-mediated angiogenesis in other rodent models, such as ischemic stroke (Meng et al., 2011; Thau-Zuchman et al., 2010).

EPOR is also expressed in the brain of neonatal rats and VEGF/VEGFR2 signaling pathway plays an important role for the enhancement of post-ischemic angiogenesis (Spandou et al., 2004; Li et al., 2011). Given this background, we hypothesized that EPO may also protect the brain in experimental model of HIE by promoting angiogenesis via VEGF/VEGFR2 signaling pathway. To test this hypothesis, the VEGFR inhibitor (SU5416) was applied. We tested whether rhEPO can enhance angiogenesis, reduce white matter damage, and promote cognitive recovery through VEGF/VEGFR2 signaling pathway in anoxia rats. If this hypothesis is confirmed, it will provide a new theoretical basis for EPO application in anoxic encephalopathy.

2. Results

2.1. RhEPO administration increases body weight gain in anoxia animals

As shown in Fig. 1A, the body weight of anoxia groups did not show any significant differences on P7, P14, P21 and P28 when compared with the control group. While on P40, the body weight of anoxia groups were significantly lower than the control group ($F=15.66$, $p<0.01$; Fig. 1A). The rhEPO group showed higher body weight than other three anoxia groups, which was abolished by infusion of SU5416. Body weight on P60 showed the same tendency as on P40 ($F=132.73$, $p<0.01$; Fig. 1A). However, physical characteristics examined as we stated (eyelid opening, ear erecting and incisor exposing) did not manifest any significant differences among the 5 groups (all $p>0.05$; Fig. 1B–D).

2.2. RhEPO ameliorates histological deficits in anoxia rat brain

Histopathological assement revealed that anoxia caused evident brain injury on day 7 after anoxia. The pathological scores of each group are as the followings: vehicle group (1.80 ± 0.23), rhEPO group (0.75 ± 0.19), SU5416+rhEPO group (1.67 ± 0.13), SU5416 group (1.75 ± 0.19). There are significantly difference among the 4 groups ($F=399.38$, $p<0.05$).

Cresyl violet staining revealed that damaged neurons were more commonly seen in the CA 1 area of hippocampus of anoxia groups than the control group on day 7 after anoxia ($F=1664.76$, $p<0.01$; arrows in Fig. 2A). Quantification analysis showed that more viable neurons presented in the hippocampus of the rhEPO group ($84.14\%\pm4.26\%$, $^{*}p<0.05$) than in the vehicle group ($53.17\%\pm3.40\%$), which was significantly decreased by SU5416 ($53.74\%\pm3.94\%$, $^{\$}p<0.05$; Fig. 2B). These data suggested that rhEPO treatment significantly alleviated neuronal damage in CA 1 area after HIE.

Furthermore, to study the role of rhEPO on white matter damage, sections from the rats in each group were stained with Luxol fast blue on day 28 after anoxia. Normal myelin sheath appeared blue, while demyelination of the white matter appeared white (Fig. 3A). Quantitative analysis revealed that rhEPO ($78.44\%\pm1.57\%$) significantly reduced the loss of LFB-stained myelin when compared with vehicle group ($67.70\%\pm1.66\%$), which was reversed by SU5416 injection ($67.46\%\pm1.18\%$, $F=2469.90$, $p<0.01$; Fig. 3B). Taken together, these results demonstrate that rhEPO administration may ameliorate the histological deficits in anoxia rat brain via VEGFR2 signaling.

2.3. RhEPO improves cognitive function in anoxia rats

Rats that suffered from anoxia exhibited poor performance in Morris Water Maze test compared to the control group. As shown in Fig. 4A, the control group exhibited a better memory than all anoxia groups ($F=239.87$, $p<0.01$). Further analysis indicated that rats in rhEPO group had better performance in this task than

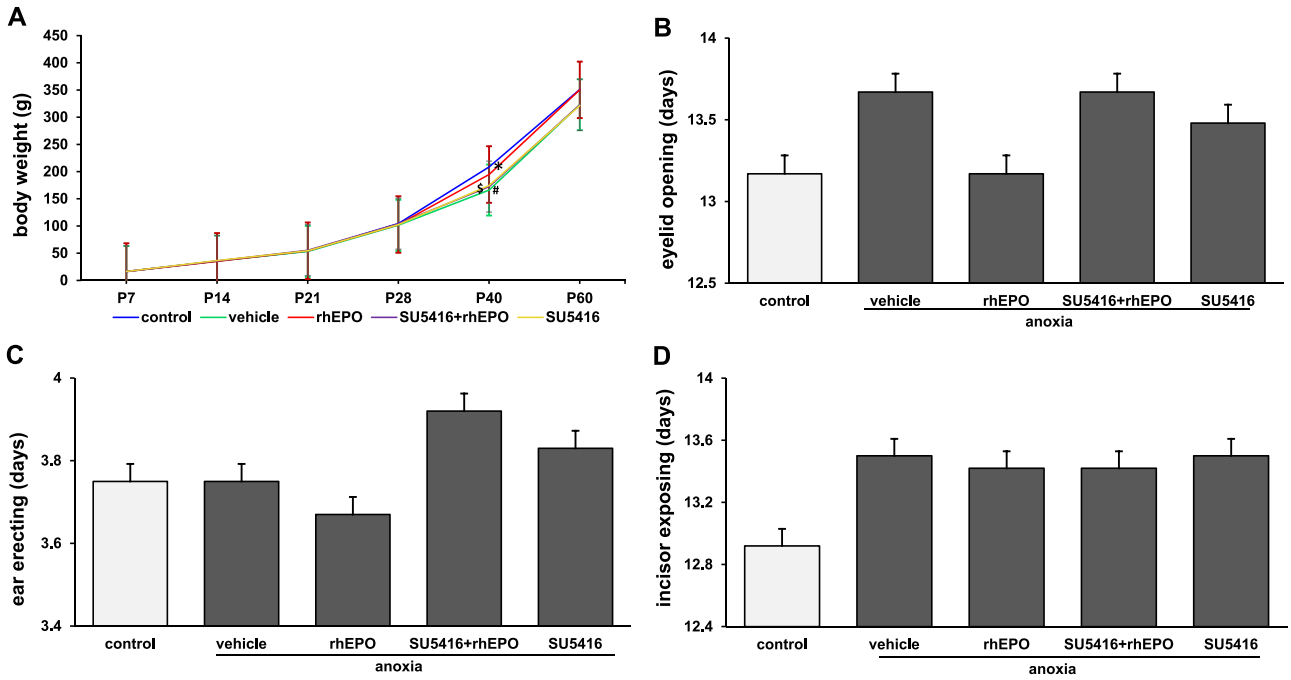

Fig. 1. Effect of rhEPO on general development. A, Postnatal evolution of body weight, $^{\#}p<0.01$ vs. control group, $^{*}p<0.01$ vs. vehicle group, and $^{\$}p<0.01$ vs. rhEPO group. B, C, and D, The time of eyelid opening, ear erecting, and incisor exposing of each group. There was no statistical significance among all groups (all $p>0.05$). Values are expressed as mean ± S.D, n=12/group.