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Full Length Research Paper

Protective effect of erythropoietin on spinal ischemia-reperfusion injury

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Our study aimed at investigating the protective effect of erythropoietin (EPO) on the spinal ischemia/reperfusion (I/R) injury in a murine model and exploring the potential mechanism. Adult male mice were subjected to spinal IR injury. The aortic arch and proximal left sub-clavianarteries were obstructed by clamping for 5 min and the animals were monitored for 48 h. The neurological function of hind limb was assessed every 12 h. In the experimental group rats (n=7) were treated with EPO 4 h pre-operation. In the IR group (n=7), no EPO treatment was administered, while in the sham group, no arteries were clamped (n=6). Forty-eight hours after IR injury, thoraco-lumbar spinal cord was collected for histological analysis. The results showed that in the experimental group, the neurological function was significantly protected. Two days after injury, the rats which did not receive treatment were used for analysis. The neurological function of hind limb improved in three rats, but was not evaluated after injury. Histological examination showed that EPO treatment markedly compromised the damage to neurons. The study concluded that EPO can protect the spinal cord against IR injuries and preserve neuron phenotype.

Key words: Erythropoietin, Spinal cord, ischemia-reperfusion injury, protective effect, mechanism.

INTRODUCTION

Erythropoietin (EPO) is an endogenous glycoprotein of 34 kDa and known to have the ability to promote hematopoiesis (Jelkmann, 2004). Under the hypoxic conditions in numerous tissues, hypoxia-inducible factor 1, alpha subunit (HIF1α, basic helix-loop-helix transcription factor) is stabilized resulting in increase of EPO expression and secretion (Webb et al., 2009). The up-regulation of EPO then lead to the hematopoiesis but cellular protection is absent (Joyeux-Faure, 2007). In animal models, EPO has been found to exert neuroprotective effect on brain and spinal cord injury including ischemia/reperfusion injury (Hasselblatt et al., 2006). Especially, in animal myocardial infarction model, Meta analysis showed EPO has been demonstrated to reduce the infarct ratio and improve the myocardial function (Minnerup et al., 2009).

The mechanism underlying the neuroprotective effect of EPO on brain and spinal injury is largely unclear. There is evidence showing that this protective effect is independent of the hematopoietic ability of EPO (Wang et al., 2007). The protective effect of EPO on IR injury of other solid organs may be related to the positive feedback of EPO leading to HIF1α up-regulation, which was confirmed in renal IR injury (Imamura et al., 2007). The protective effect of HIF1α up-regulation has been confirmed in mild and moderate cerebral ischemia, but not in spinal ischemia (Shi, 2009). Thus, we hypothesize that EPO may also exert neuroprotective effect on spinal IR injury through up-regulating HIF1α expression. The present study aimed to evaluate the neuroprotective effect of EPO in rat spinal IR injury and explore the potential mechanism.

MATERIALS AND METHODS

Animals and surgical procedures

The adult male Sprague Dawley rats aged 20 weeks were

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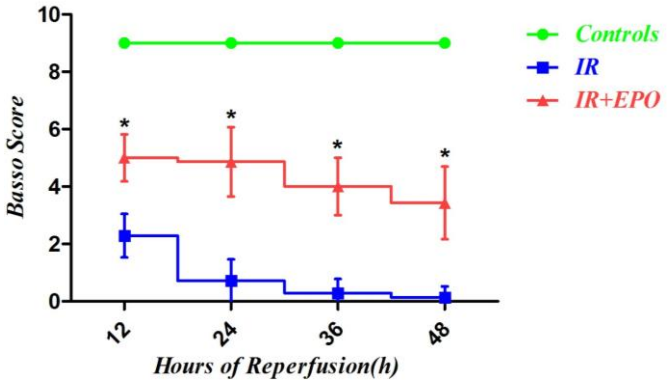

Figure 1. Functional neurologic outcomes after aortic cross clamping. Note: Neurological function of rats in spinal cord IR group and sham group: 9: normal; 0, complete paralysis. The neurological function of hind limb was significantly improved at different time points after EPO treatment. *, P<0.05.

anesthetized with 2% isoflurane (Pure Chemistry Scientific Inc. USA) followed by thoracotomy and the aortic arch was exposed. The left common carotid artery and left subclavian artery were obstructed by clamping for 5 min. The color Doppler ultrasonography (Moor Instruments Ltd, Axminster, UK) was performed to confirm that the blood flow of distal aorta was absent and that of femoral artery reduced by 90%. Before surgery, heparin was administered intra-peritoneally at 400 IU/kg. Animals in the treatment group (n=7) received a 12-μg/kg dose of mouse recombinant erythropoietin (Sigma-Aldrich Co, St Louis, MO USA) intraperitoneally 4 h before operation. In the sham group (n=6), the aortic arch was exposed but clamping was not performed. In the IR group (n=7), rats received arterial obstruction and treated with normal saline before surgery. At 50 h after reperfusion, animals were sacrificed and the spinal cord was collected.

Neurological evaluation

The neurological function of hind limb was evaluated by using the 9 grade Basso scoring system (0, complete paralysis, 9, normal) (Basso et al., 2006). Evaluation was carried out at 12, 24, 36 and 48 h after reperfusion.

Histological examination

The spinal cord from T10 to L3 was collected and fixed in 10% formaldehyde for 24 h followed by embedding in paraffin, sectioning and hematoxylin and eosin staining (HE or H and E staining). The number of viable neurons was counted.

Western blot assay

The spinal cord was collected and stored at -80°C and lysed in ethylenediamine tetraacetic acid buffer (Roche Diagnostics, IN USA) followed by determination of protein concentration with a kit (Thermo Fisher Scientific Inc, Rock-ford IL USA). The equal content of protein was subjected to SDS-PAGE (Bio-Rad Laboratories, Inc, Hercules, CA USA) and then transferred onto nitrocellulose membrane which was blocked in 5% non-fat milk for 1 h. Then, the membrane was treated with rat anti-human HIF1α primary antibody (1:500) at 4°C overnight. After washing in tromethamine thrice (5 min for each), the membrane was incubated with horseradish peroxidase conjugated secondary antibody (1:5000) at room temperature for 1 h. After washing in tromethamine, visualization was performed with chemiluminescence kit. The gel image system (National Institutes of Health, Bethesda, MD USA) was employed to determine the optical density.

Statistical analysis

SPSS for Windows Ver.11.5 (SPSS Inc., IL, USA) was applied for statistical analysis, and differences were identified as statistically significant when P <0.05. Analysis of variance was used for statistical analysis. Fisher post hoc was performed to evaluate the neurological function. Neurological function was compared among three groups at different time points. Student's t-test was employed to compare the optical density.

RESULTS

Immediately after recovering from anesthesia, the neurological function deficit was noted. In the IR group, the improvement of neurological function recovered significantly in the EPO treated rats. In the subsequent 48 h, the neurological function in EPO treated animals was improved further. In the IR group, the compromised neurological function was observed at different time points (Figure 1). The un-treated rats developed paralysis progressively. On the contrary, 43% of rats receiving EPO treatment recovered completely. Two days later, this protective effect was uncertain.

Histological examination showed the number of viable neurons in the EPO treated rats was markedly reduced when compared with sham group. At 50 h after reperfusion, the spinal cord from T9 to L3 were collected and stained with H&E. The number of viable anterior horn motor cells in the EPO group and sham group was markedly higher than that in the IR group. However, the number of viable neurons in the IR group was lower than that in the EPO group, which suggests EPO treatment can increase the viable neurons. In addition, the number of vacuoles in the EPO group and IR group was increased, but the EPO treatment reduced the vacuoles when compared with IR group. Moreover, pyknosis was less observed in the EPO group and the structure of nucleus largely preserved. These findings suggest the cellular viability is improved. In the IR group, the inflammation was more obvious than that in the EPO group and sham group. Of note, there was significant difference in the HIF1α expression among three groups before 48 h, and difference was absent at 48 h after injury (Figure 2).

DISCUSSION

Our results showed EPO could exert protective effect of spinal injury following aortic surgery. In previous different models, studies have demonstrated the protective effect

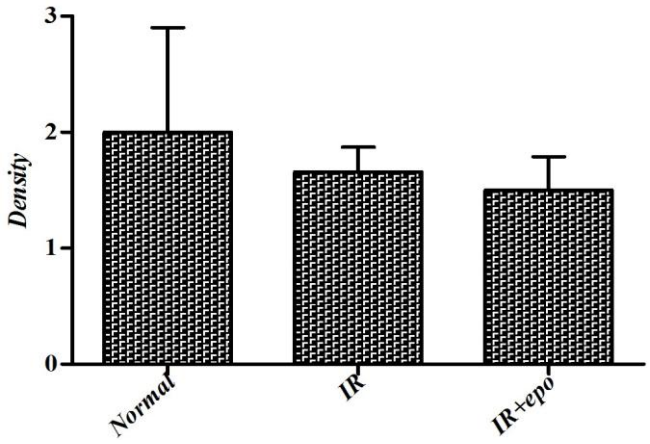

Figure 2 HIF band density/action. Note: Detection of HIF1α expression by western blot assay. Results showed the HIF1α expression in the sham group and EPO group was significantly higher than that in the IR group

of EPO on the brain injury (Wang et al., 2007; Talving et al., 2010). The neuroprotective effect of EPO has been confirmed in not only animal model but clinical trials. EPO has also been found to be beneficial for patients with amyotrophic lateral sclerosis or subarachnoid hemorrhage. In a patient with subarachnoid hemorrhage due to aneurysm, EPO reduced the local recurrence of ischemia from 40 to 7.5%. In this study, treatment of vasospasm was also performed and found to be protective, but the mechanism of neuroprotective effect of EPO was still not elucidated. The mechanism of protective effect of EPO on cerebral injury is less studied, and the knowledge on this mechanism in spinal injury is more insufficient. Some researchers postulate the anti-apoptosis, anti-inflammation, neurogenesis promotion and anti-oxidation are involved in the protective effect of EPO, but these mechanisms are not confirmed in the spinal cord injury model (Byts and Siren, 2009). EPO was found to beneficial for the spinal IR in rabbit and rat models, but the exact mechanisms are still largely unclear (Sönmez et al., 2007).

In addition, in the animal models, the diseases can be mimicked and this facilitates the investigation on molecular mechanisms. The transient ischemia may cause high metabolic rate in animals. When compared with other animal models, murine spinal vasculature system is similar to that in human (Basso et al., 2008). These findings suggest pre-treatment with EPO is beneficial for the acute and delayed spinal injury in rat spinal IR injury model. In the present study, we observed the progression of primary spinal injury into paralysis which occurs within 48 h after injury. Our results showed EPO pre-treatment significantly improved the primary injury and delayed hind limb paralysis and paraplegia. In the present study, rats with spinal IR injury which were not treated with EPO developed paraplegia. In the EPO pre-treated rats, the neurological function recovered completely. This finding provides partial evidence for the elucidation of mechanism of protective effect of EPO and its clinical application. At 48 h after injury, our results showed there was significant difference in the HIF1α protein expression. Thus, the protective effect of EPO might be mediated by the up-regulation of HIF1α. In renal IR injury model, the up-regulation of HIF1α was also confirmed at 24 and 72 h after injury. The metabolic rate is significantly different between murine and human. In the present study, significant difference in HIF1α expression was found shortly after IR injury between different groups *in vitro* and *in vivo*.

In the acute and delayed spinal IR injury, the impairment can be divided into two stages. In the primary injury, the metabolism and local ischemia may be the main contributing factors and the impairment is thought to be caused by metabolism induced injury and secondary inflammation. However, the 2-stage hypothesis does not support the above mechanism. In the hypothesis of brain injury, the primary injury may cause infiltration of inflammatory cells (microglia cells). Then, the inflammatory mediators and pro-inflammatory cells cause the secondary injury (Jin et al., 2010). Of note, is the fact that whether EPO inducing drugs can exert protective effect before and after injury is poorly understood. These findings suggest EPO may activate the protection against IR injury. Thus, EPO can be applied in patients before thoracic aortic surgery. The potential thrombosis following EPO treatment may resolve by administration of heparin. In addition, thrombosis often occurs in patients with hematocrit of >40% and this situation is rarely found in patients after surgery. Actually, the hematopoietic effect of EPO reduces the requirement for blood transfusion. In some studies, the side effects of EPO have been confirmed to be beneficial. Although the results in the present study were favorable, there were limitations in our study. Recent double-blinded, placebo-control studies showed EPO treatment increases the mortality and is detrimental for patients with acute transient ischemia. These finding may significantly reduce the clinical application of EPO. However, the mechanism underlying the injury and the clinical situation are different between animals and humans. In addition, the sample size was small in our study. This limitation may not overcome by increasing sample size.

Taken together, our results demonstrate EPO is beneficial for spinal IR injury which may be mediated by up-regulation of HIF1α expression. Our findings provide evidence for the treatment of spinal IR injury with EPO.

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