Erythropoietin reduces hippocampus injury in neonatal rats with hypoxic ischemic brain damage via targeting matrix metalloproteinase-2

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Abstract. – OBJECTIVE: Erythropoietin (EPO), as a type of the tissue-protective cytokines, is a 30.4 kDa hematopoietic glycoprotein. The purpose of this study was to explore the neuroprotective effects of EPO on the neonatal hypoxic-ischemic-induced hippocampus injury and the MMP-2 expression.

MATERIALS AND METHODS: Neonatal Sprague-Dawley (SD) rats were randomly divided into an untreated group (control) and two hypoxia-ischemia (HI) groups treated with saline control or EPO. Hippocampi were harvested at various times after return to normoxia (6 h, 24 h, 3 days and 7 days post-HI) for analyses of infarct areas and expression using histology, Western blot and reverse transcriptase-polymerase chain reaction (RT-PCR).

RESULTS: EPO injections reduced the infarction and loss of brain tissue. HI group exhibited an enhanced MMP-2 positive staining compared to controls at 24 h, 3 and 7 days post-HI by immunohistochemistry. These results were confirmed by Western blot analysis of MMP-2 expression at 7 days post-HI. Levels of MMP-2 mRNA in the injured hippocampi increased significantly at 24 h and 7 days post-HI. In particular, the EPO treatment further significantly enhanced this increase.

CONCLUSIONS: EPO protected hypoxic-ischemic-induced neonatal brain damage by up-regulating the MMP-2 expression. Hence, systemic EPO may have potential utility for the treatment of HI injury in human newborns.

Key Words: Erythropoietin, Hippocampus, Hypoxia-ischemia brain, Matrix metalloproteinase 2.

Introduction

In all causes of neonatal death and neurological injury, hypoxic-ischemic (HI) damage still remains the major cause. Due to severe limitations of hypothermia therapy, there are not effective therapies so far to reduce brain damage in infants.

In recent years, several studies have shown that the erythropoietin (EPO) has neuroprotective activities on hypoxic-ischemic brain damage (HIBD). EPO is functioning in decreasing the apoptosis, reactive oxygen species, glutamate excitotoxicity and inflammation, and then stimulating the neurogenesis and angiogenesis.

Mounting evidence has shown that the metalloprotein kinases (MMPs), especially the MMP-2 are dramatically increased in the adult brain after cerebral ischemia. Moreover, MMP-2 shows an increased activity during the HIBD in the immature brain and contributes to the protective role in the brain injury. On the other hand, MMP-2 seems to be correlated with the protective effects of EPO on focal cerebral ischemia.

The aim of this paper was to determine the role of EPO in HIBD induced MMP-2 expression, and to elucidate the role of EPO/MMP-2 after neonatal hypoxia-ischemia.

Materials and Methods

Animal Preparation

7-day-old (P7) Sprague-Dawley rats of each gender with weight of 8-15 g were purchased from the Animal Research Center of Taishan Medical College (Taian, Shandong, China). This study was approved by the Animal Ethics Committee of the Animal Center of Central Hospital Taian City (Taian, Shandong, China).

Experimental Neonatal Hypoxia-Ischemia

Cerebral hypoxia-ischemia was induced by a unilateral permanent ligation of the common carotid artery; then systemic hypoxia was induced. Besides,
rat pups were anesthetized with sodium with 4% pentobarbital (40 mg/kg, i.p.). After the anesthesia, the midline neck was incised in rat pups and then the left common carotid artery was separated and cut off. The Sham operated animals experienced the same surgical procedure without the ligation of the carotid artery. After the surgery, the rat pups were sent to their primary home cage to recover for 1 h. Hypoxia was then caused by subjecting the animals in a special chamber (37 °C) with humidified nitrogen mixture of 8% oxygen for 2 h. The age-matched sham-operated animals were the controls. Pups which underwent hypoxia-ischemia were randomly assigned to the control group (vehicle treatment) and the EPO group. EPO group was injected intraperitoneally with recombinant human erythropoietin (rhEPO, Beijing Sihuan Biopharmaceutical Co., Ltd., Beijing, China) at a dose of 5000 U/kg. Control group was injected intraperitoneally with an equal volume of saline. Animals were sacrificed at specific time points (6 h, 24 h, 3 days and 7 days) after the injury (12 rats per group and time point).

**Evaluation of Immunohistochemistry**

The number of positively stained cells was counted in each of the 5 randomly selected consecutive fields under 400-fold magnification. Considering the staining diffuseness, the stained sections areas were grouped as the following criteria: 0: no staining; 1: < 25% of the area stained; 2: 25% to 50% of the area stained, 3: 50% to 75% of the area stained, and 4: >75% of the area stained. In consideration of the staining intensity, the sections were graded as the following criteria: 0: no staining, 1: weak but detectable staining above the control level, 2: distinct staining, and 3: intense staining. Total IHC scores were acquired by addition of staining intensity and diffuseness scores. In the hippocampus samples, scores <1.5 were considered negative staining, whereas scores >1.5 were considered positive staining.

**Western Blot**

After treatment, the hippocampus was rinsed with ice-cold phosphate buffered saline (PBS) and subjected to total protein extraction with radioimmunoprecipitation assay (RIPA) lysis buffer. The protein concentrations were quantified by the bicinchoninic assay (BCA) method. 30 mg protein samples were run on 10% gels, and then transferred to the PVDF membrane. After 1 hour of blocking with the 5% skim milk, the membranes were incubated with the primary mouse anti-MMP-2 antibody (1:1000, Abcam, Cambridge, MA, USA), the rabbit anti-GAPDH antibody (1:1000, Abcam, Cambridge, MA, USA) overnight at 4 °C. After washing in tris-buffered saline and Tween 20 (TBST) for 3 times, the membranes were then incubated with a peroxidase (HRP)-labeled secondary antibody (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h. The bands were washed again, enhanced with chemiluminescence reagents and visualized with the Chemidoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA).

**Quantitative Real-time PCR**

After treatment, the hippocampus was collected to determine the expression of MMP-2 mRNA by qRT-PCR. Total RNA was extracted by the Tri-
zol RNA extraction reagent (Invitrogen, Carlsbad, CA, USA) following the protocols and quantified by spectrophotometer method. Purified RNA with equal volume was reverse transcribed (RevertAid First Strand cDNA Synthesis Kit, Thermo, K1622, Waltham, MA, USA). The cDNA product was subjected to real-time quantification on an ABI PRISM 7000 Sequence Detection System (TaKaRa Biotechnology, Dalian, Liaoning, China), with the following PCR cycling: 95 °C for 20 s, followed by 39 cycles of 95 °C for 10 s and 60 °C for 20 s, then 70 °C for 10 s. Final extension was completed from 70 °C to 95 °C at 0.4 °C/5 s. The primers were designed as follows: MMp-2: Sense: 5'-GCC CAG AGA CTG CTA TGT CC-3'; Antisense: 5'-TCG TTAG TTG GTT GTG GC-3'; GAPDH: Sense: TGG GTG TGAACC ACG AGAA-3'; Antisense: 5'-GGC ATG GAC TGTGGT CAT GA-3'. The relative expression of MMP-2 was measured by the comparative method with the formula \(2^{-\Delta\Delta Ct}\) and normalized with the corresponding GAPDH Ct values. All data presented three independent experiments.

**Statistical Analysis**

All data were expressed as the mean ± standard error of the mean (SEM), and were analyzed using a one-way analysis of variance followed by LSD (Least Significant Difference). Statistical analysis was performed using SPSS 19.0 software (IBM, Armonk, NY, USA).

**Results**

**EPO Attenuates Brain Injury in HI Rats**

Compared to the sham group, the treatment of hypoxia significantly increased the infarction and loss of brain tissue (Figure 1 A, B). Both morphological and quantitative analysis revealed that the EPO treatment dramatically down-regulated the infarction and loss of brain tissue in comparison with those in the HI group (Figure 1 C, D). These results were further confirmed by Nissl-staining in hippocampus sections. The Hypoxia-ischemia treatment significantly induced a cell loss, deformation and swelling of the neurons in HI rats, while EPO treatment partially reversed this damage (Figure 1E). All the results have demonstrated that EPO attenuates brain injury in HI rats.

**EPO Treatment Increased the MMP-2 Expression in HI Rats**

As shown in Figure 2A, the MMP-2 positive cells were appeared to be dispersed throughout the hippocampus in sham animals. At 24 h after...
HI, a marked increase in the number and intensity of MMP-2 positive staining cells was observed in hippocampus in treatment with or without EPO animals. Quantitative analysis revealed that the treatment with HI significantly increased the MMP-2 protein levels in a time dependent manner. Furthermore, EPO treatment elevated the MMP-2 expression in HI rats at specific time points (24 h, 3 days and 7 days) after the injury (Figure 2B). These results indicated that EPO enhanced MMP-2 expression in HI rats. Increased level of MMP-2 in hippocampus after treatment with EPO for 7 days (Figure 3) further confirmed the results.

**EPO Treatment Increased mRNA Level of MMP-2**

As shown in Figure 4, the results of MMP-2 mRNA expression in hippocampus in HI rats treated with or without EPO were similar to the MMP-2 immunochemical staining.

**Discussion**

This study showed that EPO and MMP-2 may be involved in the events induced by hypoxia ischemia in the newborn brain of rat. It also demonstrated that the treatment with EPO enhanced the MMP-2 expression to HI challenge. The administration of EPO not only prevented brain injury in immature rat during HI, but also led to the increase of MMP-2 expression after injury.

MMPs contains a multigene family of zinc-dependent endopeptidases which participate in the healing, the inflammatory response and the angiogenesis of many common tissues. MMP-2, a
subfamily of MMPs, secreted by neurons, glial cells, vascular endothelial cells and neutrophils in the nervous system played an important role in pathological functions within the development of brain injury. The enhanced activity of MMP-2 was associated with the breakdown of blood-brain barrier (BBB) and neuro-inflammatory response in the early phase of brain injury after focal and global brain ischemia. Furthermore, it was proved that the MMP-2 activity is expressed in wound healing by modulating the glial scar formation, which is associated with the involvement in neural repair and spinal cord injury. It has been shown that MMP-2 was up-regulated during the brain injury after 7 days. MMP-2 is considered to have functions in promoting the repair and extension of synapse by degrading glial scar, and taking part in neuroprotection.

Another work demonstrated a significant increase in both MMP-2 activity and VEGF levels during ischemia-reperfusion injury after 7 and 14 days in rats. It suggested that the increase of MMP-2 and secretion caused by treadmill training may be involved in the increase of neuroprotection which correlated with the angiogenesis increase. The timing of MMP-2 expression changed after HIBD in this study, which is similar to the time frame of MMP-2 activation previously observed in glial scar formation and angiogenesis.

Erythropoietin (EPO) is a hematopoietic glycoprotein produced in the fetal liver and adult kidney. It has been widely used in clinical practice to treat anemia due to the beneficial effect on hematopoiesis. Some recent researches have demonstrated that the EPO receptor (EPOR) was expressed in various tissues and cells, including neurons and glial cells. EPO may exert a neuroprotective effect through the enhancement of the EPOR levels on rats during hypoxic-ischemic brain damage. Intraperitoneal administration of recombinant human erythropoietin (rhEPO) results in an attenuation of brain damage in HIBD. In addition, the angiogenic, anti-inflammatory and endothelial cell stabilizing effects of EPO, including the improvement of the neurogenesis, cognitive and behavioral performance, have been demonstrated in different groups.

**Figure 3.** mRNA expression of MMP-2 in hippocampus for different groups. (*Compared with Sham, \( p < 0.05 \); #Compared with HIBD, \( p < 0.05 \)).

**Figure 4.** Enhanced expression of MMP-2 was detected by Western blotting in hippocampus after treatment with EPO for 7 days. (*Compared with Sham, \( p < 0.05 \); #Compared with HIBD, \( p < 0.05 \)).
previously described20. EPO affects the angiogenesis and neurogenesis by activating various signaling pathways, including PI3K/Akt and ERK1/2 pathway.

Conclusions

We observed that the EPO treatment of HIBD protected hypoxic-ischemic-induced neonatal brain damage by up-regulating the MMP-2 expression. The close correlation between the MMP-2 and EPO administration provided evidence for the possible effect of the EPO against HIBD.

Conflict of interest

The authors declare no conflicts of interest.

References