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Original Research Article

Erythropoietin exerts neuroprotective effect against hypoxic-ischemic brain injury in neonatal mice by activating AMPK pathway and upregulating UCP2

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Abstract

Purpose: *To investigate the neuroprotective effect of erythropoietin (EPO) against hypoxic-ischemic brain injury in neonatal mice, and the underlying mechanism(s) of action.*
Methods: *Sixty neonatal mice were assigned to 3 groups: sham, model and EPO intervention groups, each with 20 mice. Western blot assay was used to determine changes in the protein expressions of AMPK, UCP2 and KLF2. Fluorescence intensity of reactive oxygen species (ROS) was evaluated with flow cytometry.*
Results: *The protein expressions of AMPK and KLF2 in the cerebral tissues of EPO intervention mice were significantly up-regulated, relative to model mice ($p < 0.05$), while UCP2 protein level was also significantly higher in EPO intervention mice than in model mice ($p < 0.05$). Mean fluorescence intensity of ROS was significantly higher in the cerebral cortex of model mice than in sham mice, but it was down-regulated in the cerebral cortex of mice in EPO intervention group, relative to the model group ($p < 0.05$).*
Conclusion: *EPO exerts neuroprotective effect by activating AMPK pathway, up-regulating protein expression of UCP2, inhibiting production of mitochondrial ROS, and reducing oxidative stress in brain tissue. Thus, EPO has potentials for use in clinical practice as a neuroprotective agent.*

Keywords: *Hypoxic-ischemic brain injury, Erythropoietin, Adenylate-activated protein kinase, Uncoupling protein 2, Oxidative stress*

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INTRODUCTION

Hypoxic-ischemic brain injury is a common brain tissue injury caused by asphyxia and hypoxia in perinatal newborns. It is caused by fetal intra-uterine distress due to wrapping of umbilical cord around the neck of unborn baby, abnormal amniotic fluid, and abnormal delivery [1]. If the degree of cerebral hypoxia exceeds the compensatory capacity of the body, it will lead to permanent impairment of neurological function or intellectual impairment, and in severe cases, it may result in death [2]. Studies have demonstrated that hypoxic and ischemic brain injury is a disorder of energy metabolism. When ischemic and hypoxic brain injury occurs,

pathological changes such as excitotoxicity, oxidative stress and cell apoptosis after hypoxia and ischemia lead to nerve cell death and nerve dysfunction [3]. Therefore, it is important to protect patients with hypoxia and ischemic brain injury against neuronal cell apoptosis, and to treat and prevent neuronal nerve dysfunction.

Erythropoietin (EPO) is a glycoprotein hormone secreted by the renal cortex. It enhances the proliferation, differentiation and maturation of red blood cells, and it promotes angiogenesis, reduces cell apoptosis, protects nerves, and it exerts anti-inflammatory and antioxidant effects [4]. Recent studies have shown that adenylate-activated protein kinase (AMPK) or adenine ribonucleotide (AMP)-dependent protein kinase, is a key sensor of cell energy homeostasis. It is an important kinase involved in coordination of metabolism and energy balance. On activation, AMPK enhances the expression of uncoupling protein-2 (UCP2), and inhibits the generation of oxygen free radicals (ROS), thereby inhibiting oxidative stress-induced damage [5].

This study was intended to provide a reference on choice of a clinical treatment method through investigation of the neuroprotective influence of EPO against hypoxic-ischemic cerebral lesion in newborn mouse model, and the involvement of ROS generation and AMPK pathway in the process.

EXPERIMENTAL

Animals and reagents

Sixty full-term, male neonatal C57BL/6 mice were purchased from Beijing Vitonhua Experimental Animal Technology Co. Ltd, while ROS fluorescence detection kit was produced by Abcam Biotechnology, UK. The other reagents were EPO Injection (Shenyang Sansheng Pharmaceutical Co. Ltd), AMPK, KLF2, UCP2 and β-actin antibody (Abcam Biotechnology Limited, UK); and BCA protein concentration determination kit, sheep immunohistochemical anti-rabbit secondary antibody and gel electrophoresis preparation kit (Shanghai Yuntian Company).

Ethical issues

The study received approval (no. XMU202202) from the Animal Ethics Authority of The 2nd Affiliated Hospital of Xi'an Medical University, and was conducted in line with NIH guidelines [6].

Establishment of mouse model of hypoxic-ischemic brain injury

The 60 neonatal mice were assigned to 3 groups: sham surgery, model, and EPO intervention groups, each having 20 newborn mice. All mice were anesthetized using ether inhalation. Thereafter, the mice were fixed on a surgical plate, and a median incision was made in the neck of each mouse. The left common carotid artery was separated, ligated with double lines, and the wound was stitched up immediately. Then, a neonatal mouse model of hypoxic-ischemic brain injury was established by placing the mice in a 37 ^{o}C water bath in an environment with a mixture of 8 % O_2 + 92 % N_2 at an input rate of 1.0 to 2.0 L/min for 2 h. The newborn mice in the sham group were stitched immediately after the neck incision without hypoxic-ischemic treatment. The EPO intervention mice received EPO injection at a dose of 5,000 IU/kg immediately after the establishment of the hypoxic-ischemic model, while model mice and sham group were injected with isotropic saline.

Nerve function scores

Mice in the various groups were tested for fine-tuning of motor ability, location perception, and proprioception. The speed of a one-set rotating rod was accelerated from 4 to 40 rpm in 120 sec, and mice were placed on the rotating rod. The duration of time during which each mouse remained balanced on the rotating rod was recorded.

Material extraction and sample preparation

Mice in each group were sacrificed, and the left-side brain tissue was removed from the open brain cavity. Half of the brain tissue was preserved frozen at -80 ^{o}C prior to analysis, while the other half was fixed overnight in 4 % paraformaldehyde solution for histological analysis and H&E staining.

Histomorphological examination of brain tissue

After paraffin sections were subjected to H&E staining and sealing, changes in neonatal brain tissue morphology in the 3 groups were examined under a light microscope.

Levels of oxidative stress indices in brain and lung tissue

Each excised brain tissue was placed in an EP tube containing 1 mL of PBS buffer and the

tissue was subjected to fragmentation using an ophthalmic shear. The resultant cell suspension was filtered into flow tubes and stained with DCFDA, followed by incubation at 37 ^{0}C for 30 min and exposure to probes. Then, ROS fluorescence intensity analysis was done with a flow cytometer.

Assay of protein expressions using Western blotting

Total protein was extracted from each lung tissue using RIPA buffer. The supernatant from a 10 % homogenate was obtained by centrifugation, and the protein concentration was determined using the BCA method. Then, equal amounts of protein were resolved using SDS-polyacrylamide gel electrophoresis, followed by transfer to PDVF membranes. The membranes were blocked with non-fat milk solution and incubated overnight at 4 ^{o}C with relevant primary antibodies, followed by incubation at room temperature with HRP-linked secondary antibodies for 2 h. The protein bands were subjected to enhanced chemiluminescence, and the results were analyzed using Bio-Rad image laboratory software.

Statistical analysis

Data analysis was done using SPSS 20.0. Measurement data are presented as mean ± SD. Comparison amongst groups was done with ANOVA, while LSD test or Tamhane test for pairwise test was used for comparison between groups. Values of $p < 0.05$ were taken as indicative of statistically significant differences.

RESULTS

Morphological changes in brain tissues of mice

Results from H&E staining showed that mice in the sham operation group did not have infarction. The brain tissues were all red, the nerve cells were well arranged, the nucleoli were prominent, and the nuclei were round and faintly stained. In model group, there were obvious white infarct areas in the hippocampus, striatum, cerebral cortex and other areas. Moreover, cortical histological stratification was absent, and the structure was disorganized, with loose empty network, degeneration and necrosis of nerve cells. In effect, the cell morphology was obviously abnormal. At high magnification, the nuclei were abnormally hyperchromatic and pyknotic, and the axons and dendrites were not identifiable. In the EPO intervention group, there was fewer number of white infarcted areas in the brain tissue, and the histological structure of the cerebral cortex was basically normal, with clear stratification and rare cavities. There was full neuronal morphology, with basically normal neuronal structure, and the nucleoli were clear. The structure of Nishii, axons and dendrites were distinguishable under magnification.

Effect of EPO on neural function recovery in mice with hypoxic-ischemic brain injury

The neurological function score of the model mice was markedly lower than that of sham mice. However, neurological function score was markedly higher in EPO intervention mice than in model mice ($p < 0.05$). These results are shown in Table 1.

Table 1: Comparison of neural function scores among the three mouse groups (n = 20)

Group	Neurological function score
Sham	290.74±22.64
Model	177.66±19.82[a]
EPO	267.38±21.20[b]
F	157.823
P-value	0.0000

[a,b]$P < 0.05$: [a]vs sham; [b]vs model

AMPK and KLF2 levels in mouse brain tissues

Protein expression levels of AMPK and KLF2 were significantly lower in model mice than in sham mice, but were markedly higher in EPO intervention group than in model group ($p < 0.05$). These results are shown in Table 2.

Table 2: Protein expression levels of AMPK and KLF2 in mouse brain tissues (n = 20)

Group	AMPK	KLF2
Sham	0.68±0.20	0.83±0.35
Model	0.40±0.18[a]	0.59±0.17[a]
EPO	0.62±0.24[b]	0.76±0.23[b]
F	10.031	4.474
P-value	0	0.016

[a,b]$P < 0.05$: [a]vs sham; [b]vs model

Protein expression of UCP2 in mice

There was significantly lower protein expression of UCP2 in the model group than in the sham group, but UCP2 protein expression was significantly higher in the EPO intervention group than in the model group ($p < 0.05$; Table 3).

Oxidative stress

The mean fluorescence intensity of ROS was markedly lower in model mice than in sham mice, but it was markedly lower in EPO intervention group than in model mice ($p < 0.05$; Table 4).

Table 3: Protein expression levels of UCP2 in mice (n = 20)

Group	UCP2 protein expression
Sham	0.74±0.23
Model	0.38±0.12[a]
EPO	0.62±0.19[b]
F	19.497
P-value	0

[a,b]*P* < 0.05: [a]vs sham; [b]vs model

Table 4: Mean fluorescence intensity of ROS in the cerebral cortex of mice (n = 20)

Group	Fluorescence intensity of ROS
Sham	11.57±1.13
Model	15.81±1.23[a]
EPO	13.64±1.15[b]
F	65.587
P-value	0.000

[a,b]*P* < 0.05: [a]vs sham; [b]vs model

DISCUSSION

Hypoxic-ischemic brain injury is seen frequently in the neonatal department in clinics. It is a brain damage-associated disease caused by neonatal perinatal hypoxic-ischemia and decreased cerebral blood flow due to a combination of various perinatal factors [7]. If the disease is not timely treated, it may damage nerve function in the affected children, resulting in neonatal mental defects which may threaten the life of the newborn. Therefore, the use of effective treatment measures to enhance regeneration and repair of neurons in children will lead to positive prognosis and prevention of neonatal disability in the affected patients.

The emergence of hypoxic-ischemic brain injury provokes the initiation of a series of cascade reactions such as cell apoptosis, inflammatory response, disorders in energy metabolism, and oxidative stress [8]. In babies, EPO is produced mainly in the liver, but in adults, the glycoprotein hormone is produced in the kidney. Research has shown that EPO, an endogenous cytokine produced in response to hypoxia, protects the nervous system by reducing brain histopathological lesions [9]. Under hypoxic conditions, EPO increases the production of ROS. However, due to the influence of tissue metabolism, the endogenous amount of synthesized EPO is not sufficient to fully compensate for the degree of tissue cell hypoxia, thereby underscoring the need for exogenous ROS [10].

In this study, H&E staining results showed that mice in the sham operation group had no infarction, and the brain tissues were all red. The nerve cells were well arranged, the nucleoli were obvious, and the nuclei were round and faintly-stained. In model group, there were obvious white infarct areas in hippocampus, striatum, cerebral cortex and other areas, the cortical stratification was absent, and the structure was disorganized, with loose empty network, degeneration and necrosis of nerve cells, and abnormal cell morphology. At high magnification, the nuclei were abnormally hyperchromatic and pyknotic, and the axons and dendrites were not identifiable.

In the EPO intervention group, there was a fewer number of white infarcted areas in the brain tissue, and the histological structure of the cerebral cortex was basically normal, with clear stratification and rare cavities. The morphology of neurons was full, the structure was basically normal, and the nucleoli were clear. The structure of Nishii, axons and dendrites were distinguishable under magnification. These results indicate that the neonatal mouse model of hypoxic-ischemic brain injury was successfully established. In addition, the results of this study showed that the neurological function score of mice in the model group was significantly lower than that in the sham group, and the neurological function score of mice in the EPO intervention group was significantly higher than that in the model group. These results indicate that EPO alleviated pathological damage in hypoxic ischemic brain tissue and protected the nervous system. In addition, it was found that the neural function score in model mice was significantly lower than that in sham mice, but it was markedly higher in EPO intervention mice than in model mice. This suggests that EPO reduced pathological injury in hypoxic-ischemic brain tissues and protected the nervous system.

Mitochondria are the regulatory centers of cell oxidative stress and energy metabolism, and they are known as the "power houses of cells". When mitochondria use oxygen for oxidative phosphorylation, a small number of electrons which leak out in the respiratory chain readily bind to oxygen and generate ROS [11]. Hypoxic-ischemic brain injury or cerebral ischemic hypoxia triggers apoptosis in mitochondria, ion imbalance, and increased levels of ROS. When ROS generation level exceeds cellular antioxidant clearance capacity, oxidative stress results, leading to mitochondrial dysfunction, DNA damage, activation of apoptotic proteases, and enhanced neuronal apoptosis [12].

When brain tissue suffers acute hypoxic-ischemic injury, endogenous protective proteins act as neuroprotective factors by fighting

oxidative stress, inhibiting apoptotic body formation, and clearing ROS [13]. There are many types of endogenous protective proteins viz uncoupling proteins, heat shock proteins, encephaloglobin and other substances which are expressed in brain tissue [14]. The UCP2 is a mitochondrial membrane protein involved in proton transport across the mitochondrial membrane. In the mitochondrial respiratory chain, UCP2 functions as an uncoupler involved regulation of ATP production and thermogenesis, apoptosis and oxidative stress response, and it has a significant neuroprotective effect against neurological diseases [15].

It has been reported that UCP2 increases the proton permeability of the inner mitochondrial membrane and reduces H^+ and ROS generation, thereby attenuating cellular oxidative stress and cell death [16]. Another study found that AMPK, the upstream protein of UCP2, is an important switch of cellular energy metabolism: elevated AMP/ATP ratio in hypoxic-ischemic brain tissue leads to AMPK activation and increased efficiency of oxidative phosphorylation, thereby further activating UCP2. The results of this study showed that the UCP2 protein expression in EPO group was significantly lower than that in the sham group, but it was significantly higher than that in the model group [17].

The mean fluorescence intensity of the ROS in mice in the model group was significantly lower than that in the sham group, and the average ROS fluorescence intensity in EPO group was significantly lower than that in the model group. These results show that in hypoxic-ischemic brain injury, the expression of UCP2, an endogenous protective factor, was increased. The increased level of UCP2 reduced the generation of ROS, and protected brain tissue from oxidative stress injury.

The serine/threonine protein kinase, AMPK is an important factor involved in regulation of cell energy homeostasis and inflammation. It is expressed in various metabolism-related organs and tissues, and it coordinates the body metabolism and energy balance [18]. An imbalance in body metabolism and energy activates AMPK which regulates the expressions of downstream malonyl-Coenzyme A and lipid synthesis genes through phosphorylation, as well as biosynthesis of fatty acids, leading to inhibition of inflammatory response and oxidative stress response, and restoration of energy balance status [19]. In addition, AMPK and UCP2 proteins have close association in mechanisms of function: AMPK promotes mitochondrial enzyme activities, increases efficiency of cellular oxidative phosphorylation, improves UCP2 expression, reduces oxidative stress response, and promotes balance in energy metabolism [20]. The results of this study showed that AMPK and KLF2 levels were significantly lower in model group than in sham group, but were significantly higher in EPO intervention group than in model group. This suggests that the brain tissue AMPK and KLF2 in neonatal mice with hypoxic-ischemic brain injury were decreased significantly, and that EPO boosted UCP2 levels by activating the AMPK pathway. It is known that EPO promotes the expression of AMPK protein, up-regulates the expression of transcription factor KLF2, increases endothelial nitric oxide production, promotes angiogenesis, and protects the nervous system from damage caused by ischemia [21]. In addition, EPO phosphorylates AMPK, increases mitochondrial enzyme activities, improves UCP2 expression level, activates mitochondrial uncoupling, reduces mitochondrial ROS production and decreases brain tissue oxidative stress, thereby exerting a neuroprotective effect [21].

CONCLUSION

This study has demonstrated that EPO exerts neuroprotective effect against hypoxic-ischemic brain injury in mice by up-regulating the expression level of UCP2 protein, inhibiting the production of mitochondrial ROS, and reducing oxidative stress in brain tissue. Therefore, EPO has a potential for clinical use as a neuroprotective agent.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Weini Bian and Ling Zhou contribute equally to this work and should be considered as co-first authors.

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