Erythropoietin relieves neuronal apoptosis in epilepsy rats via TGF-β/Smad signaling pathway

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INTRODUCTION

Epilepsy is a relatively common serious neurological disease that affects the normal life of 65 million people around the world (1, 2). Epilepsy patients are usually discriminated against, misunderstood and even disgraced by others due to their symptoms. The pressure of unpredictable chronic disease deprives the patients of the autonomy of activity of daily living (3). Although epilepsy can be treated in most cases, the current treatment is not effective in all patients, so it is necessary to conduct deeper studies on the pathogenesis of epilepsy.

A large number of studies have confirmed that epilepsy can cause the apoptosis of brain neurons (4-7). Erythropoietin (EPO), a glycoprotein composed of 165 amino acids, is stable in peripheral circulation (8). In some cases, EPO and EPO-R can be expressed in multiple organs such as the brain (9, 11). It is believed that EPO has neuroprotective and therapeutic effects on different neurological diseases, including stroke, ischemia, multiple sclerosis, schizophrenia, brain injury and epilepsy (12-14). Besides, the protective effect of EPO on the nerve is related to its anti-apoptotic activity (15). However, there are few studies on the therapeutic effect of EPO on epilepsy.

The transforming growth factor-β (TGF-β) family plays crucial roles in such aspects as cell proliferation, survival, apoptosis, dormancy, autophagy and senescence (16-18). A related study has indicated that TGF-β is able to induce or inhibit partial programmed cell death (19-20). Smads are the only TGF-β receptor substrates capable of transmitting signals. The transcriptional regulation by the signal transduction of the TGF-β family is a dynamic process, during which the inactive state or possibly active suppression can be transformed into target gene-activated transcription by recruiting Smad complexes (21). Whether EPO could activate the TGF-β/Smad signaling pathway, thus providing a theoretical basis for the research and clinical treatment of epilepsy as well as potential therapeutic methods.

MATERIALS AND METHODS

Main materials

Sprague-Dawley (SD) rats (Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China), phosphate-buffered saline (PBS) (Gibco, Rockville, MD, USA).
USA), pentylenetetrazol (PTZ) and dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA), SB431542 (TGF-β1/Smad inhibitor, Selleck, Houston, TX, USA), recombinant human EPO (rHuEPO), primary antibodies against TGF-β1, phosphorylated smad3 (p-smad3), tumor necrosis factor receptor 1 (TNFR1), caspase-3 and β-Actin and horseradish peroxidase (HRP)-labeled secondary antibodies (Abcam, Cambridge, MA, USA), and one-step terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis assay kit (Beyotime Biotechnology, Shanghai, China).

Grouping of rats and establishment of epilepsy model

This study was approved by the Animal Ethics Committee of The Yancheng Clinical College of Xuzhou Medical University. The epilepsy model induced by PTZ is the most widely used at present, which is simple in modeling and low in cost, and can show the characteristics of aphasia attack and myoclonic attack of epilepsy. The healthy male SD rats aged 8 weeks old were prepared into animal models by reference to the experimental methods of Motte et al. (22) and adjustments were made according to the results of previous experiments: PTZ was intraperitoneally injected at a dose of 20 mg/kg and then at 10 mg/kg every 10 min. The epilepsy symptoms of the rats were assessed according to the Racine's scale (six grades) (23): grade 0 (no response), grade I (facial spasm and rhythmic mastication), grade II (rhythmic nodding), grade III (unilateral forelimb clonus), grade IV (bilateral forelimb clonus accompanied by standing) and grade V (tumble and generalized tonic-clonic seizure). The epileptic seizures at grade IV and V as well as the symptom of generalized convolution for 30 min and longer indicated successful modeling. Then the model rats were screened and randomly divided into 4 groups: PTZ model group (n=6), PTZ + rHuEPO intervention group (n=6), PTZ + SB431542 + rHuEPO intervention group (n=6) and PTZ + SB431542 intervention group (n=6). The rats receiving an equal dose of saline were enrolled in the control group.

Mode of administration

PTZ model group: At 30 min after successful modeling of epilepsy, saline was intraperitoneally injected into the rats. PTZ + rHuEPO intervention group: The rats were intraperitoneally injected with 50 μg/kg rHuEPO at 30 min after successful modeling of epilepsy (24). PTZ + SB431542 + rHuEPO intervention group: The rats were given cerebral ventricle injection of 5 μL of SB431542 (2 mg/mL) before administration with PTZ and intraperitoneal injection of rHuEPO (50 μg/kg) at 30 min after successful modeling. PTZ + SB431542 intervention group: 5 μL of SB431542 (2 mg/mL) was injected into the cerebral ventricle of the rats at 30 min after successful modeling, and saline was injected intraperitoneally at 30 min after successful epilepsy modeling. The rats in the control group were intraperitoneally injected with saline in the same volume and frequency.

Observation of rat behaviors

The behavior changes of the rats were observed after the intraperitoneal injection of relevant drugs, and the degree and time of epileptic seizure in each group were recorded based on Racine's scale (six grades).

Preparation of tissue specimens

At 24 h after the corresponding treatments in different groups, the rats underwent anesthesia using overdose anesthetics. Then the thoracic cavity was opened, the aorta of the left ventricle was intubated and connected to the perfusion system, and the right atrial appendage was cut open timely to discharge the perfusate. Next, the skull was opened to obtain the brain after perfusion fixation with 500 mL of normal saline and 500 mL of 4% paraformaldehyde in sequence. The brain tissues at the optic chiasm and mamillary body were vertically transected and the middle brain tissues containing the hippocampus were fixed in 4% paraformaldehyde for 24 h, followed by routine dehydration, transparentization and embedding in paraffin. After that, the hippocampus tissues were sliced to 5 μm-thick sections for later use.

Western blotting (WB)

The middle brain tissues containing the hippocampus were acquired after perfusion with 500 mL of normal saline. The total proteins were extracted and their concentration was determined via bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). Subsequently, the proteins were separated via 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Next, the membrane was sealed in 5% skim milk powder and 0.1% Tris-buffered saline-Tween 20 and incubated with TGF-β1, p-smad3, TNFR1 and caspase-3 primary antibodies at 4°C by gently shaking overnight. After that, HRP-labeled secondary antibodies were added for incubation, and the proteins to be detected were subjected to exposure using an electrochemiluminescence (ECL) reagent. β-actin detected using the same WB was taken as the control.

One-step TUNEL apoptosis assay

The tissue sections prepared as mentioned above were subjected to the one-step TUNEL apoptosis assay according to the kit instructions. Specifically, the sections were deparaffinized in xylene for 5-10 min and deparaffinized again in fresh xylene for 5-10 min, in absolute alcohol for 5 min, in 90% ethanol for 2 min, in 70% ethanol for 2 min and in distilled water for 2 min. Later, DNase-free protease K (20 μg/mL) was added in drops for reaction at 20-37°C for 15-30 min, followed by washing with PBS 3 times. Thereafter, the samples were added with 50 μL of TUNEL assay solution for incubation in the dark at 37°C for 60 min and washed with PBS 3 times, followed by mounting using an anti-fluorescence quenching medium. Finally, the samples were observed and photographed under a fluorescence microscope.

Statistical analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for data recording and processing. The data from different treatment groups were presented as mean ± standard deviation (x±s). Comparison between groups was performed using a One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). P<0.05 suggested that the difference was statistically significant.
Results

Observation of rat behaviors in different treatment groups
After the intraperitoneal injection of 20 mg/kg PTZ, convulsion and myoclonus were observed in the rats. Following the injection of PTZ once every 10 min, the rats soon manifested vigorous contraction and convulsion of extensors of forelimbs and hind limbs as well as generalized tetanic contraction. Furthermore, uncontrolled body posture persisted for several minutes, and generalized tonic-clonic seizures occurred for over 30 min after tetanic contraction. After the intervention with rHuEPO, the duration of clonus was shortened remarkably compared with that in the other three groups \( (P<0.01) \). After the treatment with SB431542, the duration of clonus in the rats undergoing intervention with rHuEPO was markedly shortened. However, it was not significantly different from that in the PTZ group \( (P>0.05) \) but notably longer than that in the PTZ + rHuEPO intervention group \( (P<0.01) \) (Figure 1). These findings suggested that rHuEPO could effectively control the seizure and the effect could be neutralized by SB431542. The results of the one-step TUNEL apoptosis assay indicated that there was an extremely small number of apoptotic cells (approximately 8.76%) in the control group. Besides, PTZ promoted neuronal apoptosis (apoptosis rate: 63.32%), and rHuEPO could reduce the number of apoptotic neurons in the rats induced by PTZ (apoptosis rate: 19.83%). Moreover, there were more apoptotic neurons in the rats treated with SB431542 inhibitor (apoptosis rate: approximately 48.15% and 52.61%). The results indicated that rHuEPO could inhibit apoptosis induced by PTZ and SB431542 could neutralize the effect of rHuEPO.

Impacts of different treatments on TGF-β/Smad signaling in the hippocampus of rats
After rat modeling by means of PTZ, the expressions of TGF-β1 and p-smad3 in the TGF-β/Smad signaling pathway were not significantly different from those in the control group \( (P>0.05) \). However, the intraperitoneal injection of rHuEPO could markedly up-regulate the expressions of TGF-β1 and p-smad3 \( (P<0.01) \), and this effect could be repressed by SB431542 \( (P<0.05) \). Moreover, administration with only SB431542 to the rats could down-regulate the expressions of TGF-β1 and p-smad3 as expected \( (P<0.05) \) (Figure 2). The above results showed that SB431542 could evidently inhibit the expressions of TGF-β1 and p-smad3, thus repressing the up-regulation of TGF-β1 and p-smad3 by rHuEPO.

Impacts of different treatments on apoptotic proteins in the hippocampus of rats
The expressions of apoptotic proteins TNFR1 and caspase-3 were up-regulated as expected after the modeling with PTZ \( (P<0.01) \). The rHuEPO injected intraperitoneally could prominently down-regulate the expressions of TNFR1 and caspase-3 \( (P<0.01) \), but this effect was inhibited markedly by SB431542 (Figure 3). These findings indicated that rHuEPO could inhibit apoptotic proteins induced by PTZ and SB431542 could neutralize the effect of rHuEPO.

Discussion

The classification of epilepsy is fairly complicated, but status epilepticus (SE) can be induced by any type of epileptic seizure. SE generally refers to frequent epileptic seizures in a short time, resulting in constantly unclear individual consciousness during the seizures. Large quantities of research results have demonstrated the apoptotic features of neurons after epilepsy through morphological features of neurons after epilepsy through morphological....
observation and biological tests on various SE models induced by chemical, electrical, systemic or local administration of epileptogenic agents (12, 13). As a receptor antagonist commonly used to prepare the animal models of epilepsy, PTZ mainly acts on the γ-aminobutyric acid receptor, significantly affects the activity of the chloride channel and leads to overexcitation of the neurons, thereby triggering epilepsy (25). In this experiment, the rat model of epilepsy was successfully established using PTZ, and the forms and intensity of epileptic seizures were similar to those reported in China and foreign countries (25, 26).

Numerous investigations have testified that epileptic seizures will cause neuronal apoptosis in the brain. Previous evidence (14) showed that massive neuronal apoptosis in hippocampus CA3 and CA4 regions is observed at 24-48 h after epilepsy induced by kainic acid injection in the rats. Thereafter, increasingly more studies have illustrated that the neurons exhibit apoptotic features after epilepsy in individuals. Faherty et al. (7) revealed that at 30-48 h after epilepsy in mice, the expression of caspase-3 is up-regulated in the hippocampal tissues, and cell apoptosis can be detected via TUNEL assay. In this research, the rat model of epilepsy was established using PTZ, and the apoptosis of a large number of neurons in the hippocampus was detected through the TUNEL assay, which is similar to the results of previous studies.

EPO is generated by the fetal liver, and adult kidney as well as developing and mature central nervous system. The EPO produced by the liver and kidney can stimulate erythropoiesis, while the EPO in the central nervous system participates in brain maturation and neuronal protection and repair. Genc et al. (27) found that EPO plays a neuroprotective role in the cultures of the brain and neurons. According to some studies, EPO needs to be applied at 3-8 h in advance to induce the neuroprotective effect, while the positive results of other investigations manifested that the application of EPO before, during and after the reaction can also exert the neuroprotective effect (28, 29). In the present experiment, the application of rHuEPO after epileptic seizures could effectively reduce the symptoms of epilepsy, and it was also indicated in TUNEL assay results that rHuEPO could alleviate the PTZ-induced neuronal apoptosis.

In order to further verify the correlations of rHuEPO with the TGF-β/Smad signaling pathway and cell apoptosis, the expressions of proteins related to the TGF-β/Smad signaling pathway and neuronal apoptosis were detected. The study of Zhu et al. (30) revealed that exogenous TGF-β1 can protect neurons from in vitro and in vivo injuries. TGF-β1 is expressed by neurons in the hippocampus CA1 region under physiological conditions, and its expression is up-regulated within the first hour after ischemia. The endogenous TGF-β1 expressed in neurons after transient forebrain ischemia in rats may play roles in pathological processes such as DNA degradation and delayed neuronal death. Meanwhile, TGF-β1 is able to effectively inhibit caspase-3 activation and reduce neuronal apoptosis in rat hippocampal cultures (31, 32), but the loss of TGF-β1 leads to neuronal death and increases microglial cells in the mouse brain (33). In this experiment, the rHuEPO applied after epileptic seizures could efficiently up-regulate the expressions of TGF-β1 and its downstream receptor substrate p-smad3 in the TGF-β1/Smad signaling pathway. After the application of TGF-β inhibitor SB431542, however, those expressions were repressed prominently. Moreover, the expressions of apoptosis-related proteins (TNFR1 and caspase-3) were measured, and it was manifested that rHuEPO could potently down-regulate the expressions of TNFR1 and caspase-3 after epilepsy for the first time, and it was found that rhEPO had a protective effect on epilepsy, which suggested that rhEPO may be useful even after epileptic seizures.

**Conclusions**

In conclusion, the inhibitory effect of EPO on neuronal apoptosis in epilepsy rats may be realized by activating the TGF-β/Smad signaling pathway, which relieves neuronal apoptosis and ameliorates the symptoms of epilepsy.

**Competing interests**

The authors declare no competing interests.

**References**


