



Recombinant Human Erythropoietin Protects Myocardial Cells from Apoptosis via the Janus-Activated Kinase 2/Signal Transducer and Activator of Transcription 5 Pathway in Rats with Epilepsy



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ABSTRACT

Objective: To investigate the potential mechanisms underlying the protective effects of recombinant human erythropoietin (rhEPO) and carbamylated EPO (CEPO) against myocardial cell apoptosis in epilepsy.

Methods: Rats were given an intra-amygdala injection of kainic acid to induce epilepsy. Groups of rats were treated with rhEPO or CEPO before induction of epilepsy, whereas additional rats were given a caudal vein injection of AG490, a selective inhibitor of Janus kinase 2 (JAK2). At different time points after seizure onset, electroencephalogram changes were recorded, and myocardium samples were taken for the detection of myocardial cell apoptosis and expression of JAK2, signal transducer and activator of transcription 5 (STAT5), caspase-3, and bcl-xl mRNAs and proteins.

Results: Induction of epilepsy significantly enhanced myocardial cell apoptosis and upregulated the expression of caspase-3 and bcl-xl proteins and JAK2 and STAT5a at both the mRNA and protein levels. Pretreatment with either rhEPO or CEPO reduced the number of apoptotic cells, upregulated bcl-xl expression, and downregulated caspase-3 expression in the myocardium of epileptic rats. Both myocardial JAK2 and STAT5a mRNAs, as well as phosphorylated species of JAK2 and STAT5a, were upregulated in epileptic rats in response to rhEPO—but not to CEPO—pretreatment. AG490 treatment increased apoptosis, upregulated caspase-3 protein expression, and downregulated bcl-xl protein expression in the myocardium of epileptic rats.

Conclusions: These results indicate that myocardial cell apoptosis may contribute to myocardial injury in epilepsy. EPO protects myocardial cells from apoptosis via the JAK2/STAT5 pathway in rats with experimental epilepsy, whereas CEPO exerts antiapoptotic activity perhaps via a pathway independent of JAK2/STAT5 signaling.

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Introduction

Epilepsy is a common chronic neurologic disorder that affects approximately 50 million people worldwide.¹ Although the majority of cases can be controlled with medication, some 20% to 30% of patients do not respond to medical treatment and develop intractable epilepsy.² Repeated epileptic seizures often disturb neurohumoral regulation and thereby induce dysfunction of organs other than the nervous tissue.^{3,4} Patients with intractable epilepsy may show ECG abnormalities such as ST-segment depression and T-wave inversion, transient left ventricular dysfunction, and the release of cardiac enzymes into plasma.^{5,6} These manifestations are similar to the symptoms of stress-induced cardiomyopathy.⁵

Additionally, epilepsy is associated with the risk of cardiac ischemia and myocardial infarction.^{6,7} However, the mechanism(s) underlying epilepsy-induced myocardial injury remains unclear.

Erythropoietin (EPO) is a glycoprotein produced by kidneys that promotes the formation of red blood cells in bone marrow. Besides acting as an erythropoiesis-stimulating hormone, EPO has a much broader range of action. Of relevance to the present study, several lines of evidence have indicated that EPO can exert antiepileptic effects and prevent epilepsy-associated impairments.^{8,9} Furthermore, numerous studies demonstrate that EPO is cardioprotective.^{10–14} Interestingly, both the neuroprotective and cardioprotective actions of EPO are dependent on its antiapoptotic activity (ie, antiapoptotic gene upregulation and apoptotic gene downregulation) and the activation of the Janus-activated kinase (JAK)/signal transducer and activator of transcription (STAT) pathway.^{12,15,16} We therefore hypothesized that EPO may protect myocardial cells from apoptosis via the JAK2/STAT5 pathway in epilepsy.

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The primary aim of the present study was to investigate whether apoptosis plays a role in the pathogenesis of myocardial injury in a rat model of epilepsy. Moreover, we examined whether EPO exerts protective effects against myocardial injury in epilepsy and whether the JAK2/STAT5 pathway is involved in this process. Our results indicate that recombinant human EPO protects myocardial cells from apoptosis via the JAK2/STAT5 pathway in kainic acid (KA)-evoked epilepsy.

Materials and Methods

Main reagents and equipment

Reagents and equipment used were stereotaxic apparatus (Setagaya-ku, Tokyo, Japan); dental drills (Saeshin, Daegu, South Korea); micropipettes (Ningbo, Jiangsu, China); EPO (lot No. 20081047; ClonBiotech, Shanghai, China); KA (Sigma, Shanghai, China); TUNEL Assay Kit (Roche, Basel, Switzerland); anti-bcl-xl antibody (sc-8392; Santa Cruz Biotechnology, Texas, USA); anticaspase-3 antibody (sc-7148; Santa Cruz Biotechnology); in situ hybridization kits for detection of JAK2, STAT5a, and STAT5b mRNAs (Haoyang Biological, Tianjin, China); and Western blot assay kits for detection of p-JAK2 and p-STAT5 proteins (Cell Signaling Technology, Boston, USA).

Carbamylation of EPO

EPO was carbamylated by incubation at 37°C with 2000 mM cyanate for 24 hours. These carbamylated EPOs (CEPOs) were followed by extensive dialysis against total 12 L phosphate-buffered saline at pH 7.4 and a temperature of 4°C to remove excess cyanate. After dialysis, EPO was used for the reaction with trinitrobenzenesulfonic acid. The extent of carbamylation was monitored by following the loss of free amino groups using trinitrobenzenesulfonic acid. Fifty microliters of 0.1% trinitrobenzenesulfonic acid was added to 1 mL EPO (500 U/mL in normal saline) and 1 mL 4% sodium hydrogen carbonate (pH 8.4) and this was incubated for 1 hour at 37°C. Absorbance was then measured at 340 nm against a sample blank, and the trinitrobenzenesulfonic acid reactivity was expressed as a percentage of the absorbance obtained for the non-CEPO.

Animal groups

One hundred eighty-six healthy male Wistar rats, weighing 240 to 260 g, were obtained from the Laboratory Animal Center of Jilin University. Six rats receiving no treatment were used as normal controls. The remaining rats were equally divided into 6 groups: phosphate buffered saline (PBS) group, epilepsy group, EPO group, CEPO group, AG490 (a selective inhibitor of JAK2) group, and ethanol group. These 6 groups were further divided into 5 sub-groups for testing at 0, 2, 6, 12, and 24 hours after seizure onset. This gave a total of 6 rats per time point studied. The PBS group was given an intra-amygdala injection of 1 μ L PBS, whereas all other groups received an equal volume of KA. EPO (250 IU) and CEPO (250 IU) were administered to rats in the EPO group and CEPO group via the caudal vein 24 hours before the induction of epilepsy, respectively, whereas equal volumes of normal saline were given to rats in the PBS and epilepsy groups. The AG490 and ethanol groups were injected with AG490 (0.25 mg, dissolved in ethanol) and ethanol, respectively, via the caudal vein at the time of induction of epilepsy.

Induction of epilepsy

All surgical procedures were performed according to the Guide for the Care and Use of Laboratory Animals. Rats were anesthetized

with 10% chlorpromazine (3.3 mg/kg IP) and fixed in a stereotaxic apparatus with ear bars. A rhomboid skin incision was made along the midline of the skull to expose the skull, bregma, and lambda. A dental drill was then used to make a hole in the skull to expose the dura mater. One microliter of KA (1 mg/mL in PBS) was injected at a speed of 1 μ L/min into the right amygdala (2.5 mm posterior to the bregma, 5.0 mm lateral to midline, 8.0 mm below the skull surface). Before KA injection, a bipolar electrode was positioned into the hippocampal CA3 region (3.0 mm posterior to the bregma, 3.0 mm lateral to midline, and 4.0 mm below the skull surface) in some rats. The needle was slowly withdrawn 5 minutes after injection to ensure complete absorption of KA. At the completion of injection, the skin incision was sutured. The behavior of rats was evaluated according to the Racine scale: stage 0, normal behavior; stage 1, wet dog shakes and facial clonus, including eye blinking, whisker twitching, and rhythmic chewing; stage 2, rhythmic nodding; stage 3, forelimb clonus; stage 4, bilateral forelimb clonus with rearing; and stage 5, rearing, falling, loss of balance, and twitching.¹⁷ Epilepsy was considered successfully induced when rats developed manifestations of stage 4 or above, and spike waves and sharp waves were monitored on a hippocampal EEG.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

At 0, 2, 6, 12, and 24 hours after seizure onset, rats were anesthetized with 10% chlorpromazine and perfused intracardially with normal saline, followed by 10% buffered formalin. Myocardial tissue samples were taken, fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned. After routine deparaffinization and hydration, sections were used to detect the apoptosis of myocardial cells with a TUNEL assay kit according to the manufacturer's instructions. The nuclei of positive cells were stained brown. Quantitative analysis was performed on 5 randomly selected fields using Image-Pro Plus 6.0 software (Media Cybernetics, USA).

Immunohistochemistry

Myocardial tissue sections were prepared as described above and stained by immunohistochemistry using the streptavidin-peroxidase method to detect the expression of bcl-xl and caspase-3 proteins. Positive staining was assessed by the degree of brown color developed. The integrated optical density of positive staining was measured using Image-Pro Plus 6.0 software.

In situ hybridization

In situ hybridization was performed to detect JAK2, STAT5a, and STAT5b mRNAs using commercially available kits according to the manufacturer's instructions. The sequences of primers used for probe synthesis were as follows: JAK2: (i) 5'-GCCGC CACTG AGCAA AAAGG TAAGA CAT-3', (ii) 5'-CACTT TCCTT ATGTT TCCCT CTGCA CCAC-3', (iii) 5'-TCGCT CAACG GCAA GGTCA GGAAG TAT-3'; STAT5a: (i) 5'-GATGG TCTGC TGCTT CCTCA ACAAC TG-3', (ii) 5'-ATGAA CAATG ACGAC CACAG GGAGG GACA-3', (iii) 5'-GGCTT CAGAT TCCAC AGGTT TCGGT C-3'; and STAT5b: (i) 5'-CACTG GGATA AATAA TGTCG CACCT CG-3', (ii) 5'-CCCCA TCGTT CCACT GAGGC TTGAG A-3'. The integrated optical density of positive staining was measured using Image-Pro Plus 6.0 software.

Western blot

Myocardial cells were isolated from the rat myocardium and lysed in radioimmunoprecipitation assay buffer. After centrifugation of lysates (12,000 rpm for 15 minutes at 4°C) the supernatants were quantified by the bicinchoninic acid method.

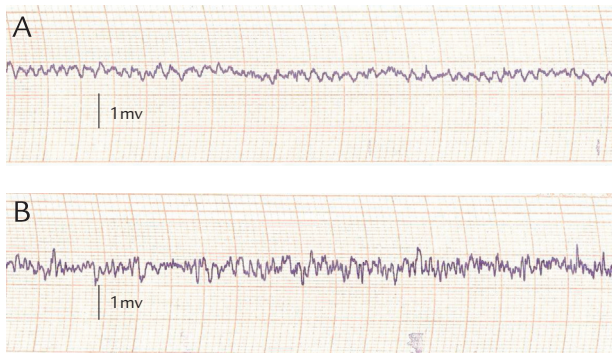


Figure 1. Spectral characteristics of EEG in (A) normal Wistar rats and (B) rats with kainic acid-induced epilepsy. Normal brain waves have a frequency of 5 to 10 Hz and an amplitude below 700 μ V, whereas multiple forms of abnormal waveforms are observed following induction of epilepsy.

Samples containing equal amounts of protein (50 μ g) were resolved on 15% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. The blots were blocked for 60 minutes with 5% skimmed milk in PBS + 0.1% Tween-20 at 37°C and then incubated with rabbit anti-p-JAK2 polyclonal antibody (1:1000) or rabbit anti-p-STAT5 polyclonal antibody (1:1000) overnight at 4°C. Sections were washed 3 times with PBS + 0.1% Tween-20, incubated for 1 hour at room temperature with horseradish peroxidase-labeled goat antirabbit immunoglobulin G (1:5000), and then washed again as above. Immunoblotted proteins were subsequently detected using enhanced chemiluminescence. Blots were quantified using Image-Pro Plus 6.0. Glyceraldehyde 3-phosphate dehydrogenase was used to normalize equal protein loading.

Statistical analysis

Statistical analysis was performed using SPSS 11.5 software (IBM-SPSS Inc, Armonk, New York). All data are expressed as mean (SD). The difference between the means of 2 groups was compared using the *t* test. The apoptosis rates, caspase-3 level, bcl-xl, JAK2, and STAT5 were compared among groups by 1-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons. Two-sided values of *P* < 0.05 were considered to be statistically significant.

Results

Hippocampal EEG spectral changes

In normal rats, brainwaves have a frequency of 5 to 10 Hz and an amplitude below 700 μ V (**Figure 1A**). After induction of

epilepsy with KA, multiple forms of epileptic discharges were noted. Typical waveforms observed in epileptic rats included monophasic, biphasic, and polyphasic spike waves, spike-slow waves, paroxysmal rhythmic waves, and postseizure inhibition (**Figure 1B**).

Behavior changes

With respect to strength, the motor seizures were rated on a 5-point scale, where 1=mouth and facial movements, 2=head nodding, 3=forelimb clonus, 4=rearing, and 5=rearing and falling. There is some variation in this progression, but most epileptic rats develop motor seizures (**Figure 2**)

EPO and CEPO protect myocardial cells

TUNEL assays were performed to examine whether KA injection induced myocardial cell apoptosis and if EPO and CEPO exert an antiapoptotic effect. As shown in **Figure 3** and **Table 1**, apoptotic myocardial cells were only occasionally observed in normal control rats. No significant difference was noted in the extent of apoptosis between the normal control and PBS groups (*P* > 0.05). In the epilepsy group, the number of apoptotic myocardial cells began to increase rapidly 2 hours after the onset of seizures. Apoptosis increased with time, peaking at 24 hours; at 24 hours apoptosis was significantly greater in the epilepsy group compared with the PBS group (*P* < 0.01). Pretreatment with either EPO or CEPO reduced the number of apoptotic myocardial cells in epileptic rats. At 24 hours the extent of apoptosis was significantly lower (*P* < 0.01) in both the EPO (15.127% [3.427%]) and CEPO (14.580% [3.104%]) groups compared with the untreated epilepsy group (19.648% [2.191%]). However, there was no significant difference between the EPO- and CEPO-treated groups (*P* > 0.05). AG490 treatment resulted in a time-dependent increase in the number of apoptotic myocardial cells in epileptic rats. The extent of apoptosis at 24 hours was significantly higher in the AG490 group compared with the ethanol control group (*P* < 0.01). Additionally, the apoptosis rates at 2, 6, 12, and 24 hours were significantly higher in the AG490 group than in either the EPO- or CEPO-treated group (all *P* values < 0.01).

EPO and CEPO inhibit the expression of caspase-3

Immunohistochemistry was performed to examine whether myocardial caspase-3 protein expression is altered following induction of epilepsy and whether EPO or CEPO treatment reversed any effect. As shown in **Figure 4** and **Table II**, caspase-3 protein was expressed at low levels in the myocardium of normal rats. No significant difference was noted in the expression intensity of caspase-3 protein between the normal control group and

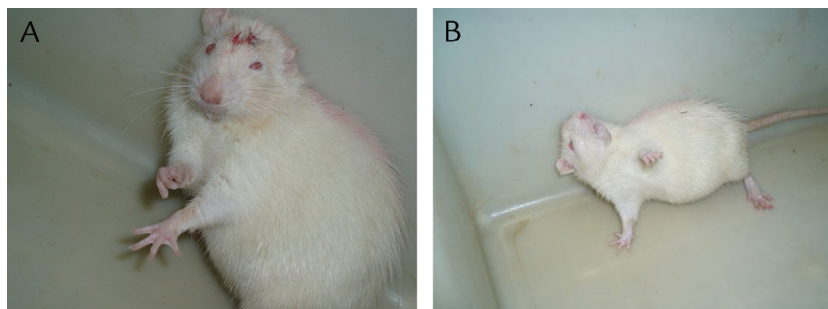


Figure 2. Epileptic rats showing symptoms of (A) Racine scale stage 4, bilateral forelimb clonus with rearing and (B) Racine scale stage 5, rearing, falling, loss of balance, and twitching.

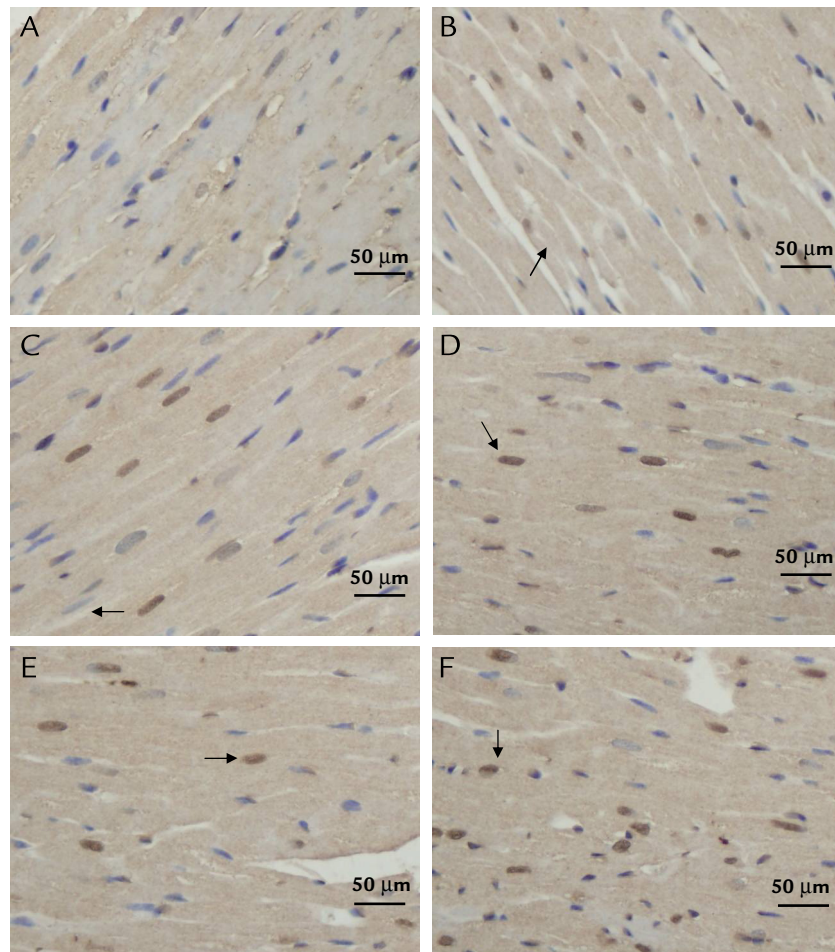


Figure 3. Recombinant human erythropoietin (rhEPO) and carbamylated erythropoietin (CEPO) protects myocardial cells from apoptosis in rats with KA-induced epilepsy. Apoptosis was measured in control (ie, phosphate buffered saline) and epileptic rats as well as epileptic rats treated with rhEPO, CEPO, ethanol, or AG490 by terminal deoxynucleotidyl transferase dUTP nick end labeling assay at 0, 2, 6, 12, and 24 hours after the appearance of seizures. The figures show representative images (400 × ; 24 hours after onset of seizure) of terminal deoxynucleotidyl transferase dUTP nick end labeling staining for apoptotic cells in the myocardium of (A) control and (B) epileptic rats, as well as epileptic rats treated with (C) ethanol, (D) rhEPO, (E) CEPO, or (F) AG490. The nuclei of positive cells were stained brown (shown by arrows).

PBS group ($P > 0.05$). In the epilepsy group, the expression of caspase-3 protein in the myocardium began to be upregulated rapidly 2 hours after the onset of seizures. The expression intensity of caspase-3 protein increased with time, peaking at 24 hours. The expression intensity of caspase-3 protein at 24 hours was significantly higher in the epilepsy group than in the PBS group ($P < 0.01$). Pretreatment with either EPO or CEPO reduced the expression of caspase-3 protein in epileptic rats, and the expression intensity of caspase-3 protein decreased with time, reaching the lowest level at the 24-hour time point. The expression intensity of caspase-3 protein

at 24 hours was significantly lower in the EPO and CEPO groups than in the epilepsy group (32,895.12 [5685.755] and 32,627.12 [5144.080] vs 43,922.68 [6459.093]; both P values < 0.01). However, there was no significant difference in the expression intensity of caspase-3 protein between the EPO and CEPO groups ($P > 0.05$). AG490 treatment enhanced the expression of caspase-3 protein in epileptic rats, and the expression intensity of caspase-3 protein increased with time, peaking at 24 hours. The expression intensity of caspase-3 protein at 24 hours was significantly higher in the AG490 group than in the ethanol-treated group ($P < 0.01$).

Table I

Apoptotic rate (%) in different time points and groups.

Time (h)	Control	Phosphate buffered saline	Epilepsy	Erythropoietin	Carbamylated erythropoietin	Ethanol	AG490
	Mean (SD)						
0	1.84 (0.86)	1.92 (1.05)	1.92 (0.98)	1.96 (0.96)	2.02 (0.93)	1.83 (0.73)	2.00 (1.02)
2	–	2.06 (0.86)	5.04 (1.80)	3.82 (1.84) [§]	4.04 (1.67) [§]	4.98 (1.74) [†]	7.91 (1.73)
6	–	2.15 (0.93)	10.22 (2.27)	8.06 (1.79) [§]	7.96 (1.84) [§]	10.15 (1.83) [†]	13.14 (2.62)
12	–	2.01 (0.95)	16.03 (1.91)	13.08 (2.07) ^{†,§}	12.90 (2.06) ^{†,§}	15.15 (2.02) [†]	19.74 (2.05)
24	–	2.03 (0.87)	19.65 (2.19)	15.13 (3.43) ^{†,§}	14.58 (3.10) ^{†,§}	19.91 (2.14) [†]	25.04 (2.12)

* Compared with phosphate buffered saline group, $P < 0.01$.

† Compared with epilepsy group, $P < 0.01$.

‡ Compared with epilepsy group, $P < 0.05$.

§ Compared with AG490 group, $P < 0.01$.

|| Compared with epilepsy 0 h group, $P < 0.01$.

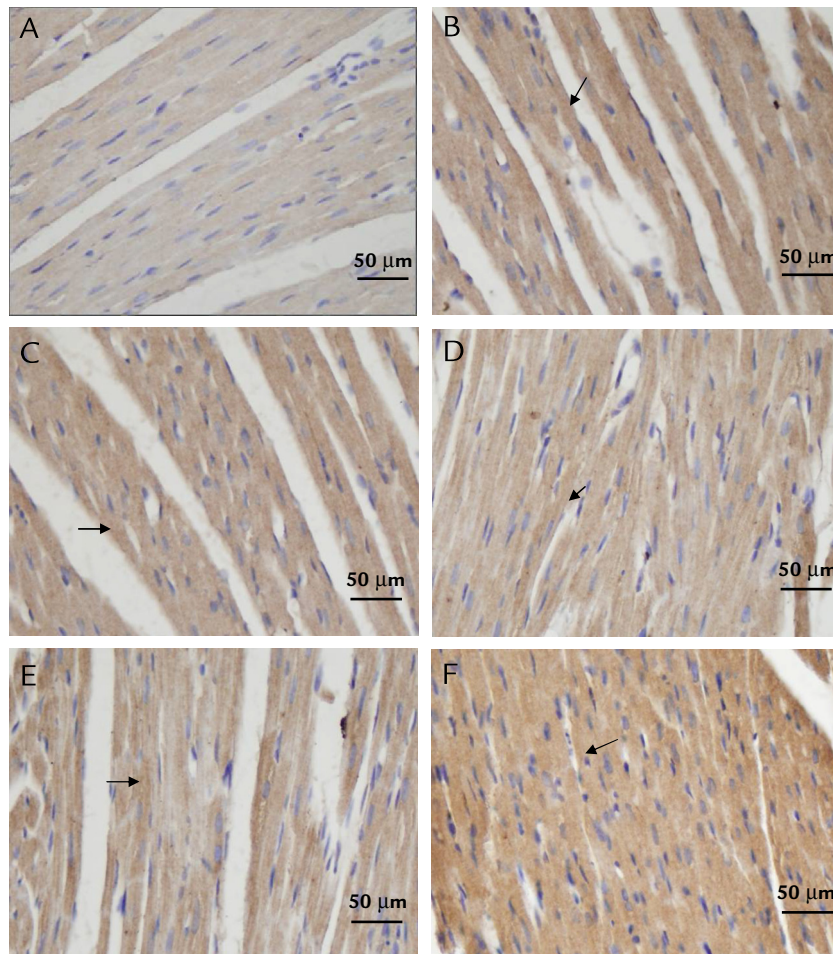


Figure 4. Recombinant human erythropoietin (rhEPO) and carbamylated erythropoietin (CEPO) downregulate the expression of caspase-3 protein in the myocardium of rats with kainic acid-induced epilepsy. Using immunohistochemistry the expression of caspase-3 protein in the myocardium was determined at 0, 2, 6, 12, and 24 hours after the onset of seizures. The figures show representative images ($400\times$; 24 hours after onset of seizure) of caspase-3 expression in the myocardium of (A) control and (B) epileptic rats as well as epileptic rats treated with (C) ethanol, (D) rhEPO, (E) CEPO, or (F) AG490. The cytoplasm of positive cells is stained brown (shown by arrows).

Additionally, the expression intensity of caspase-3 protein at 24 hours was significantly higher in the AG490 group than in the EPO and CEPO groups (both P values < 0.01).

EPO and CEPO enhance the expression of bcl-xl

To examine whether bcl-xl protein expression in the myocardium is altered and whether EPO and CEPO affect such alteration in epileptic rats, immunohistochemistry was performed. As shown in **Figure 5**, bcl-xl protein was expressed at comparatively low levels in the myocardium of normal rats. No significant difference

was noted in the expression intensity of bcl-xl protein between the normal control group and PBS group ($P > 0.05$). In the epilepsy group, the expression of bcl-xl protein in the myocardium began to be upregulated rapidly 2 hours after the onset of seizures. The expression intensity of bcl-xl protein increased with time, peaking at 24 hours. The expression intensity of bcl-xl protein at 24 hours was significantly higher in the epilepsy group than in the PBS group ($P < 0.01$). Pretreatment with either EPO or CEPO further enhanced the expression of bcl-xl protein in epileptic rats, and the expression intensity of bcl-xl protein increased with time, peaking at 24 hours. The expression intensity of bcl-xl protein at 24 hours

Table II
Integrated optical density of caspase3 protein expression in different time points and groups.

Time (h)	Control	Phosphate buffered saline	Epilepsy	Erythropoietin	Carbamylated erythropoietin	Ethanol	AG490
	Mean (SD)						
0	8368 (1470)	8358 (1034)	8197 (1306)	8254 (1828)	8017 (1257)	7724 (1628)	8326 (1833)
2	-	8288 (992)	14,483 (2877) [†]	13,662 (2351)	13,932 (2621)	13,925 (2947) [†]	14,738 (2539)
6	-	8199 (992)	19,247 (3051) [*]	17,909 (2515)	17,571 (2947)	18,864 (3073) [*]	20,029 (4066)
12	-	8380 (871)	29,481 (4705) [*]	25,744 (3953)	25,017 (4243)	28,770 (5434) [*]	32,942 (5442)
24	-	8286 (999)	43,923 (6459) [*]	32,895 (5686) ^{†‡}	32,627 (5144) ^{†‡}	43,923 (6459) [*]	53,640 (6427) [§]

* Compared with PBS group, $P < 0.01$.

† Compared with epilepsy group, $P < 0.01$.

‡ Compared with AG490 group, $P < 0.01$.

§ Compared with ethanol group, $P < 0.01$.

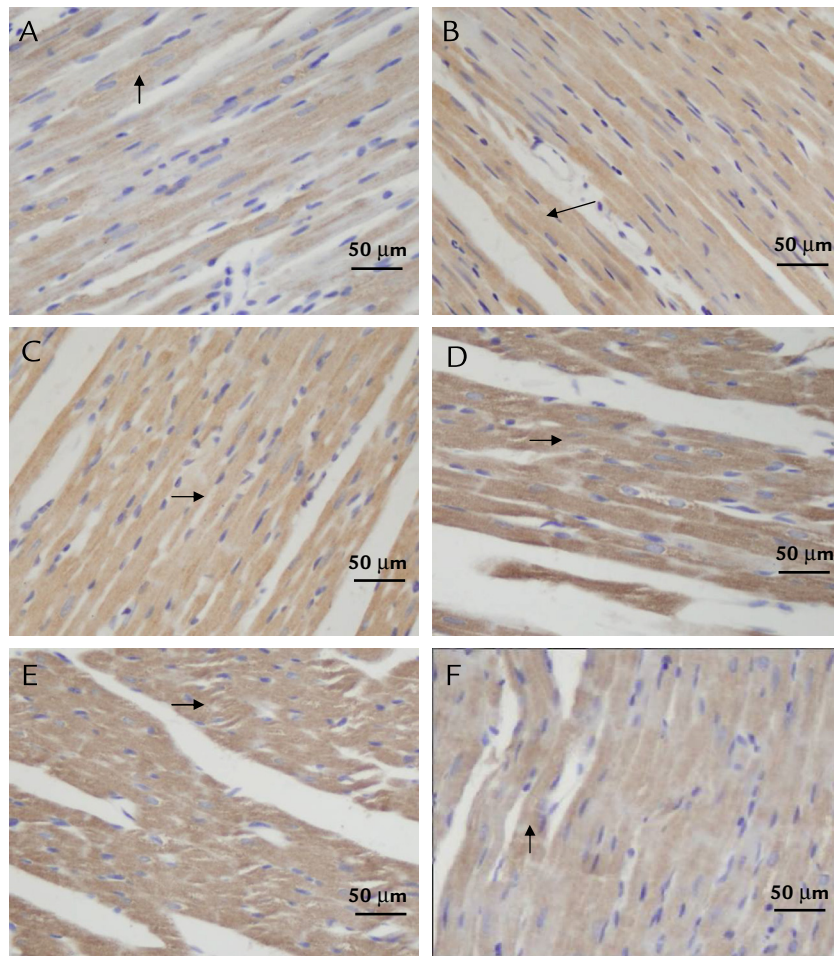


Figure 5. Recombinant human erythropoietin (rhEPO) and carbamylated erythropoietin (CEPO) upregulate expression of bcl-x1 protein in the myocardium of rats with kainic acid-induced epilepsy. Myocardial bcl-x1 protein expression was determined by immunohistochemistry at 0, 2, 6, 12, and 24 hours after the onset of seizures. Representative images (400 ×; 24 hours after onset of seizures) of bcl-x1 expression in the myocardium of (A) control and (B) epileptic rats and epileptic rats treated with (C) ethanol, (D) rhEPO, (E) CEPO, or (F) AG490. The cytoplasm of positive cells is stained brown (shown by arrows).

was significantly higher in the EPO and CEPO groups than in the epilepsy group (both P values < 0.01). AG490 treatment reduced the expression of bcl-x1 protein in epileptic rats, and the expression intensity of bcl-x1 protein decreased with time, reaching lowest levels at 24 hours. The expression intensity of bcl-x1 protein at 24 hours was significantly lower in the AG490 group than in the ethanol group (P < 0.01). Additionally, the expression intensity of bcl-x1 protein at 2, 6, 12, and 24 hours was significantly lower in the AG490 group than in the EPO and CEPO groups (all P values < 0.01).

EPO upregulates the expression of JAK2 mRNA

In situ hybridization was performed to examine whether JAK2 mRNA expression in the myocardium is altered in the experimental model of epilepsy and whether EPO and CEPO treatment could reverse such alterations. As shown in **Figure 6A**, JAK2 mRNA was expressed at low levels in the myocardium of normal rats, and no significant differences were detected in expression levels between the normal control and PBS-treated groups (P > 0.05). In the epilepsy group, expression of JAK2 mRNA in the myocardium increased as early as 2 hours after the onset of seizures and reached an observed maximum at 24 hours (P < 0.01 compared with the PBS group). AG490 treatment reduced the expression of JAK2 mRNA in epileptic rats with expression intensity decreasing with time and reaching lowest levels after 24 hours (P < 0.01

compared with the ethanol-treated control group). Pretreatment with EPO enhanced the expression of JAK2 mRNA in epileptic rats, and the expression intensity of JAK2 mRNA increased with time, reaching an observed maximum at 24 hours. The expression intensity of JAK2 mRNA at 24 hours was significantly higher in the EPO group than in the epilepsy group (P < 0.01). In contrast, pretreatment with CEPO showed no significant influence on the expression of JAK2 mRNA in epileptic rats. No significant difference was noted in the expression intensity of JAK2 mRNA at 24 hours between the CEPO group and epilepsy group (P > 0.05).

EPO upregulates the expression of STAT5a mRNA

The expression of STAT5 mRNA in the myocardium of epileptic rats was similarly detected by in situ hybridization. STAT5a mRNA was expressed at low levels in the myocardium of normal rats (**Figure 6B**) and no significant difference was detected between the normal control and PBS groups (P > 0.05). In the epilepsy group, the expression of STAT5a mRNA in the myocardium was significantly (P < 0.05) increased 2 hours after the onset of seizures and continued to increase during the 24-hour observation period relative to the PBS group. AG490 treatment reduced the expression of STAT5a mRNA in epileptic rats, with expression intensity decreasing with time and reaching its lowest level at 24 hours (P < 0.01 compared with the ethanol group). Pretreatment

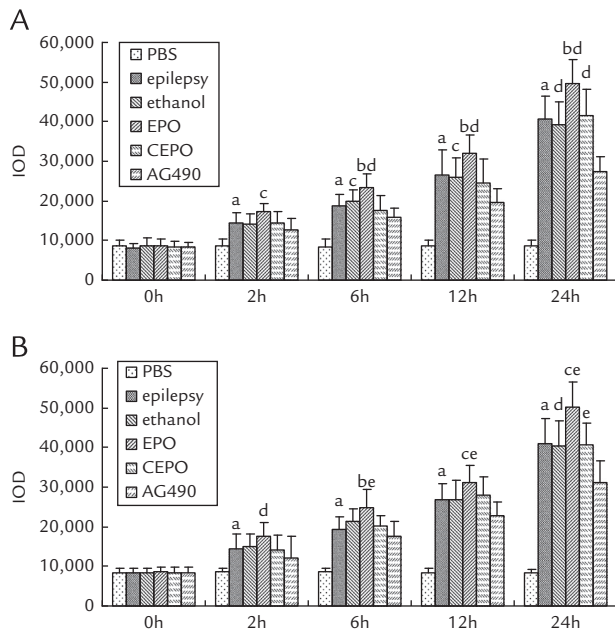


Figure 6. Recombinant human erythropoietin (rhEPO), in contrast to carbamylated erythropoietin (CEPO), significantly upregulates expression of Janus kinase 2 mRNAs in the myocardium of rats with kainic acid-induced epilepsy. Myocardial expression of Janus kinase 2 mRNA for control (ie, phosphate buffered saline) and epileptic rats and epileptic rats treated with rhEPO, CEPO, ethanol, or AG490 was determined by in situ hybridization at 0, 2, 6, 12, and 24 hours after the onset of seizures. The integrated optical density of mRNA staining was measured using Image-Pro Plus 6.0 software (Media Cybernetics, USA). Data shown are expressed as mean (SD) (n = 6). (A) $P < 0.01$ versus the phosphate buffered saline group, (B) $P < 0.01$ versus the epilepsy group, (C) $P < 0.05$ versus the epilepsy group, and (D) $P < 0.01$ versus the AG490 group.

with EPO enhanced expression of STAT5a mRNA in epileptic rats with the expression intensity increasing with time and reaching highest observed levels at 24 hours. The expression intensity of STAT5a mRNA at 24 hours was significantly higher in the EPO group than in the epilepsy group ($P < 0.01$). In contrast, pretreatment with CEPO showed no significant influence on the expression of STAT5a mRNA in epileptic rats. Thus, no significant difference was noted in the expression intensity of STAT5a mRNA at 24 hours between the CEPO-treated and epilepsy groups ($P > 0.05$).

STAT5b mRNA was also lowly expressed in the myocardium of normal rats. No significant difference was noted in the expression intensity of STAT5b mRNA between the normal control group and PBS group ($P > 0.05$). In the epilepsy group, the expression of

STAT5b mRNA in the myocardium was slightly upregulated 2 hours after seizure onset. However, there was no significant difference in the expression intensity of STAT5b mRNA between the epilepsy group and PBS group ($P > 0.05$). Pretreatment with either EPO or CEPO had no significant influence on the expression of STAT5b mRNA in epileptic rats.

EPO promotes the activation of JAK2 and STAT5 proteins

Western blotting was performed to examine whether the levels of p-JAK2 and p-STAT5 proteins in the myocardium were altered in epilepsy, and if EPO and CEPO treatment influences such changes. As shown in **Figure 7**, p-JAK2 levels were upregulated in the epilepsy and ethanol groups. AG490 treatment reduced the level of p-JAK2, whereas pretreatment with EPO enhanced the level of p-JAK2. In contrast, pretreatment with CEPO had no significant influence on the level of p-JAK2 in rats with KA-induced epilepsy. Similar results were also obtained for p-STAT5 levels (**Figure 7**).

Discussion

Apoptosis has been implicated in many heart disorders.^{18,19,20} Although it is clear that myocardial injury can occur in patients with intractable epilepsy,^{5,6,17,21,22} it has not previously been known whether myocardial cell apoptosis is a contributing factor. Importantly, our novel data reveal that myocardial cell apoptosis increases after induction of experimental epilepsy and suggest that this pathway may indeed be involved in epilepsy-related myocardial damage. At a mechanistic level, we demonstrated that the induction of epilepsy upregulates the expression of caspase-3 protein, a critical regulator of apoptosis, providing further evidence to support myocardial cell apoptosis occurring in epilepsy. Although bcl-xl, an antiapoptotic bcl-2 family protein, is also upregulated in parallel with caspase-3, we speculate that this is a self-defense mechanism of the body that occurs in response to apoptosis. Therefore, these findings suggest that myocardial cell apoptosis may contribute to myocardial injury in epilepsy and be of clinical significance.

EPO can protect multiple organs, such as the heart and brain, against various types of injuries.^{11,23,24} Multiple lines of evidence demonstrate that EPO exerts protective effects dependent on its antiapoptotic action via upregulation of antiapoptotic genes and downregulation of apoptotic genes.^{10,25} In the present study, we demonstrated that EPO pretreatment significantly reduced the rate of apoptosis in myocardial cells, upregulated bcl-xl expression, and downregulated caspase-3 expression, suggesting that EPO is able to prevent myocardial injury in epilepsy by antagonizing apoptosis.

Previous studies have shown that activation of the JAK/STAT pathway inhibits apoptosis of myocardial cells and contributes to cardioprotection.^{16,26} AG490 is a selective inhibitor of JAK2 that can effectively block tyrosine phosphorylation of JAK2 and STAT activation.²⁷ Our results showed that AG490 treatment increased the percentage of apoptotic myocardial cells, upregulated the expression of caspase-3 protein, and downregulated the expression of bcl-xl in epileptic rats. This suggests that activation of the JAK2-STAT5 pathway can exert cardioprotection in epilepsy by inhibiting myocardial cell apoptosis. Further, this finding is consistent with the previous observation that STAT5 can regulate the transcription of genes that encode bcl-2 family members and caspases.²⁸

EPO-mediated cardioprotection from injury has been attributed to activation of the JAK/STAT pathway and downstream antiapoptotic events.^{12,15,16} Our results demonstrated that EPO treatment could activate the JAK2/STAT5a pathway and thereby inhibit myocardial cell apoptosis by upregulating the expression of

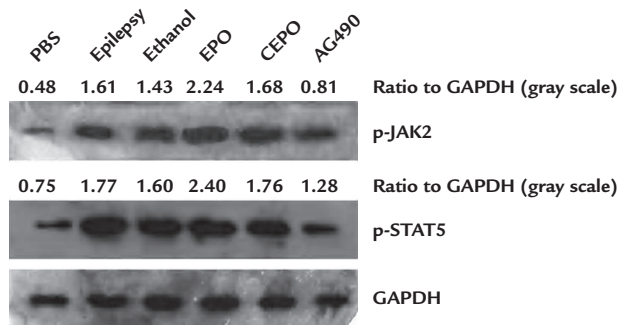


Figure 7. The levels of p-Janus kinase 2 (JAK2), p-STAT5a, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the myocardium of control (ie, phosphate buffered saline [PBS]) and epileptic rats as well as epileptic rats treated with recombinant human erythropoietin (EPO), carbamylated EPO (CEPO), ethanol, or AG490 were determined by Western blot 24 hours after the onset of seizures. Recombinant human EPO, in contrast to CEPO, significantly upregulates the levels of p-JAK2 and p-STAT5a in the myocardium of rats with kainic acid-induced epilepsy.

caspase-3 protein and downregulating the expression of bcl-xl in epileptic rats. In contrast, EPO pretreatment induced no obvious changes in the expression levels of STAT5b mRNA. Interestingly, although the induction of epilepsy also increased the expression of JAK2 and STAT5a mRNAs in the myocardium, the degree of upregulation of JAK2 and STAT5a mRNAs in response to epilepsy alone was weaker than when it was combined with EPO pretreatment. More importantly, the number of apoptotic myocardial cells was elevated in rats with epilepsy but was reduced in those rats pretreated with EPO. Therefore, it appears that epilepsy-induced activation of the JAK2/STAT5a pathway, similar to bcl-xl upregulation, represents a self-defense mechanism in response to myocardial injury. Thus, these data suggest that EPO pretreatment can protect myocardial cells from apoptosis via the JAK2/STAT5 pathway in rats with KA-evoked epilepsy.

Despite potent protective effects in various tissues, EPO may cause unwanted secondary effects. For example, long-term use of high-dose EPO can lead to erythrocytosis and subsequent cardiovascular dysfunction.^{29–31} CEPO may be an ideal alternative to EPO because the former does not stimulate erythropoiesis but can still provide tissue protection.³² It has been demonstrated that CEPO protects the myocardium from ischemia-reperfusion injury but does not raise the hemoglobin concentration.³³ Instead of binding to the classical EPO receptor, CEPO signaling requires formation of a heterodimer consisting of the EPO receptor and the β common receptor. Therefore, CEPO exerts tissue protection perhaps via a mechanism that differs from that of EPO.³³ In the present study, we demonstrated that CEPO showed a similar antiapoptotic effect to EPO in the myocardium of epileptic rats. However, CEPO pretreatment had no significant influence on the expression of JAK2 and STAT5 mRNAs and proteins, suggesting that its antiapoptotic action in the myocardium of rats with KA-induced epilepsy occurs via a pathway independent of JAK2/STAT5 signaling. This result is consistent with a previous observation that CEPO does not activate the JAK2/STAT5 pathway *in vitro*.³² Based on previous data, we speculate that CEPO exerts cardioprotection against myocardial injury in epilepsy, possibly by inhibiting myocardial apoptosis via a phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)-dependent mechanism.²⁵

This epileptic model has been successfully established by KA microinjected into nucleus amygdalae in rats, this model is applicable for temporal lobe epilepsy. However, cost and a complicated operation may be limiting factors for the use of the model. The use of KA and stereotaxic apparatus may be associated with higher treatment costs. On the other hand, pathologic physiology change of this model is not consistent with epilepsy induced by specific factors, including trauma and stroke. In that respect, this model never appears to represent a sensitive and relevant approach.

Conclusions

The data from this study provide the first evidence that myocardial cell apoptosis may contribute to myocardial injury in epilepsy. Furthermore, EPO protects myocardial cells from apoptosis via the JAK2/STAT5 pathway in rats with KA-evoked epilepsy. In contrast, CEPO exerts antiapoptotic action in the myocardium of rats with KA-induced epilepsy perhaps via a pathway independent of JAK2 and STAT5. CEPO may therefore represent a rational and effective therapeutic alternative for myocardial injury in epilepsy.

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Sui-sheng Wu conceived and designed the study. Bao-Xin Ma, Jie Li and Hua Li contributed equally to this work.

Conflicts of Interest

The authors have indicated that they have no conflicts of interest regarding the content of this article.

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