Erythropoietin preconditioning suppresses neuronal death following status epilepticus in rats

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Abstract. Status epilepticus (SE) is a grave condition in which the brain undergoes lasting seizures which can lead to neuronal loss. Our previous study suggested that preconditioning with erythropoietin (Epo) suppressed neuronal apoptosis in hippocampus of rats following SE in vivo by inhibiting caspase-3. In this study, we investigated the mechanisms by which Epo preconditioning may exert its anti-apoptotic effects using a lithium-pilocarpine induced SE model in rats. The effects of Epo on neuronal cell death were evaluated using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), and the role of the Bcl-2 protein family, which have been shown to be anti- (Bcl-2, Bcl-w) or pro- (Bid, Bim) apoptotic, was examined with immunofluorescence. We found Epo preconditioning decreased the total number of TUNEL, Bim and Bid positive cells, but increased the total number of Bcl-w and Bcl-2 positive cells. These results suggest that systemic Epo pretreatment protects neurons in an acute phase of SE and may result in further suppression of neuronal apoptosis in hippocampus by regulating the balance between pro- and anti-apoptotic Bcl-2 family proteins.

Key words: epilepsy, seizure, apoptosis, hippocampus, Bcl-2

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INTRODUCTION

Status epilepticus (SE) is an episodic brain disorder arising from excessive, synchronous and sustained discharge of a group of neurons. Experimental modeling and clinical neuroimaging of patients has shown that certain seizures are capable of causing neuronal death. Such brain injury may contribute to epileptogenesis, impaired cognitive function, or an epileptic phenotype.

Pilocarpine-induced seizure models have provided information on the behavioral and neurochemical characteristics associated with seizure activity and can be used to investigate the development of neuropathology in SE (Dal-Pizzol et al. 2000, Gluck et al. 2000). The acute pathology following SE was shown to be ‘ischemic cell change’ and was assumed to arise through hypoxia/ischemia (Meldrum et al. 2002).

Epo is neuroprotective in the central nervous system (Campana and Myers 2003), so it is reasonable to hypothesize that administering exogenous Epo would be of value in treating hypoxic/ischemic injury by reducing neuronal injury following SE. In support of this, our previous results showed that Epo preconditioning suppressed the expression of caspase-3 in hippocampus of rats following SE induced by lithium-pilocarpine (Wen et al. 2006). However, the mechanisms of the neuroprotective effects of Epo are still unclear.

Experimental modeling of brain injury suggests that seizures elicit a mixture of necrotic and apoptotic cell death within vulnerable fields (Araki et al. 2002). Features suggestive of apoptosis following seizures include altered expression of Bcl-2 family proteins, activation of caspases, and neuroprotective effects of caspase inhibitors (Araki et al. 2002). Bcl-2 family proteins, like caspases, are involved in regulating neuronal death and can exert anti- or pro-apoptotic effects. Expression of anti-apoptotic proteins in this family, including Bcl-2, Bcl-xI, and Bcl-w, is altered after seizures in some but not all cases (Ananth et al. 2001, Graham et al. 1996, Henshall et al. 2001, 2002, Schindler et al. 2004). Furthermore, increased expression of the pro-apoptotic Bcl-2 proteins Bim, but not Bad, accompanies seizures (Henshall et al. 2002, Schindler et al. 2004, Shinoda et al. 2004).

In the present study, we examined the effect of Epo preconditioning on neuronal death in hippocampus and seizure activity during lithium-pilocarpine induced SE. The extent of nuclear DNA fragmentation in injured cells in hippocampus was examined using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (Gavrieli et al. 1992). In parallel the expression of Bcl-2 family proteins was examined with immunofluorescence. We provide evidence that Epo reduced an early post-seizure cell death observed at one hour after SE with concomitant pro-survival changes in the expression of the Bcl-2 protein family members.

METHODS

Animals

Adult male Sprague-Dawley rats (n=35) weighing 180–200 g were provided by the Animal Center of the Fourth Military Medical University, P. R. China and their care and handling was conducted in compliance with the Chinese Animal Welfare Act and was approved by the responsible governmental agency at the Fourth Military Medical University. The study animals were housed in individual cages in a controlled environment (constant temperature, 22–25°C; humidity, 50–60%; 12/12h light/dark cycle with lights on at 7 A.M.) for at least one week before being used in the experiment. Animals had free access to standard laboratory food and water. In addition, all efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. Experiments were done in the morning to avoid circadian variations.

Design

A total of 35 rats were randomly assigned to four groups: (i) control (n=7), (ii) administered Epo alone (Epo; n=7), (iii) administered pilocarpine alone (Pilo; n=7), and (iv) administered both Epo and pilocarpine (Epo + P; n=14). In the Pilo and Epo + P groups, all rats also received lithium chloride (3 mEq/kg i.p.; Sigma-Aldrich, St. Louis, MO). The next day, they were injected with methylscopolamine bromide (1 mg/kg s.c.; Sigma-Aldrich) to limit the peripheral effects of the convulsant, and thirty minutes later, SE was induced by injecting pilocarpine hydrochloride (30 mg/kg, s.c.; Sigma-Aldrich). The Epo and Epo + P group received recombinant human (rh) Epo (10 U/g, i.p.) (Wang X et al. 2004) 4 hours before pilocarpine hydrochloride injection.
The Racine grading scale for seizures was adopted: grade 0, arrest; grade 1, facial twitches; grade 2, chewing, nodding; grade 3, forelimb clonus; grade 4, more than one limb clonus; grade 5, tonic clonic seizure (Racine 1972).

Tissue preparation

One hour after SE onset, rats were deeply anesthetized (thionembutal, 50 mg/kg, i.p.) and transcardially perfused with 0.9% saline solution followed by 4% ice-cold phosphate-buffered (PB) paraformaldehyde (PFA). The brains were then removed and post-fixed in 4% PFA for 12 hours and then immersed sequentially in 20% sucrose solutions in 0.1 M PB (pH 7.4) until they sank. Coronal sections were cut on a freezing microtome (Jung Histocut, Model 820-II, Leica, Germany) at a thickness of 30 μm for immunofluorescence, and 10 μm for TUNEL.

TUNEL assay

TUNEL was performed according to the manufacturer’s protocols (In Situ Cell Death Detection Kit, POD, Roche, Penzberg, Germany), with minor modifications. Briefly, fixed brain slices were sectioned at 10 μm. Cryo-sectioned brain slices were placed in PBS (containing 4% PFA, PH 7.4) and incubated at 20°C for 20 min. After the slides were washed for 5 min with PBS, they were incubated for 1 hour at room temperature with 10% normal goat serum containing 0.3% Triton X-100. Then slides were rinsed twice with PBS for 5 min and incubated for 1 hour in a humidified container at 37°C with a TUNEL reaction mixture containing the following: 5 μl enzyme solution (terminal deoxynucleotidyl transferase in storage buffer); 50 μl label solution (fluorescein-nucleotide mixture in reaction buffer). After washing three times in PBS, slides were directly observed under a fluorescence microscope (Olympus BX-51, Olympus Co, Japan).

Fluorescence immunolabeling

For double-immunofluorescence labeling, sections were processed for immunocytochemistry using one of the following combinations of primary antibodies (all from Chemicon, Teme...
Fig. 1. Epo preconditioning suppresses neuronal death in hippocampus. Few, if any, TUNEL-positive cells were detected in the control (A–C) and Epo groups (D–F), but large numbers were seen in the CA1/CA3 pyramidal cell layer in Pilo group (G–I). TUNEL-positive cells were markedly decreased in the Epo + P group (J–K). The granule cell layer (GCL) of the dentate gyrus was unaffected (data not shown). Blue: DAPI-positive cells; green: TUNEL-positive cells. Scale bar: 100 μm.

**Epo preconditioning suppressed neuronal death in hippocampus**

The TUNEL technique was used to evaluate SE-associated cell death in the stratum pyramidale of hippocampal fields CA1/CA3. In the control and Epo groups, few TUNEL-positive cells were visualized (8.3 ± 4.4 and 7.4 ± 3.5/mm², respectively). In contrast, TUNEL-positive cells were numerous in the Pilo group rats (56.8 ± 18.5/mm²) representing about a 588% increase compared to the control group (t₁₋₂=4.86, P<0.01) (Fig. 1). TUNEL-positive cells were also found, albeit sparsely, in the Epo + P group (26.4 ± 12.3/mm²). Thus, Epo preconditioning decreased the number of TUNEL-positive cells by 54% compared to the Pilo group (t₁₋₂=2.14, P<0.05).

Fig. 2. Effects of Epo preconditioning on the expression of Bcl-w and Bim. Brain sections were immunostained for Bcl-w (green) and Bim (red). Bcl-w and Bim are expressed at a low level in hippocampus of control group (A–C). Epo increased the expression of Bcl-w, but did not alter the expression of Bim (D–F). SE induced a robust increase of Bcl-w positive cells, while the Bim positive cells increased slightly (G–I). In Epo + P group, cells immunopositive for Bim decreased, while those for Bcl-w increased (J–K). Scale bar: 100 μm.

**Effect of Epo preconditioning on the expression Bcl-2 family proteins**

In order to elucidate the protective mechanism of Epo preconditioning, we examined the expression of Bcl-w, Bim, Bcl-2, and Bid in CA1/CA3 area of hippocampus by immunofluorescence (Figs 2 and 3). As shown in Fig. 4A, the Epo group had 59% more cells containing Bcl-w than did the control group (394 ± 49.4 vs. 248 ± 56.6/mm², respectively; t₁₋₂=2.14, P<0.05). Compared to these groups, SE significantly increased the number of Bcl-w immunopositive cells (508 ± 63.4/mm²; t₁₋₂=3.42, P<0.05) (Fig. 2), and Epo preconditioning further elevated Bcl-w expression by 57% (912 ± 43.3/mm²; Epo + P vs. Pilo, t₁₋₂=2.65, P<0.01) (Fig. 4A).

Generally, the number of Bim-positive cells changed much less than those expressing Bcl-w (Fig. 4A). Bim
expression slightly increased in the Epo group (264 ± 64.3/mm² vs. 236 ± 64.3/mm² in control; \( P > 0.05 \)), but was significantly elevated in the Pilo group (318 ± 46.7/mm², \( F_{3,16} = 3.69, P < 0.05 \)). Interestingly, compared to the latter group, Bim-positive cells decreased in the Epo + P group by 44% (177 ± 35.6/mm², \( t_{4.5} = 2.05, P < 0.05 \)) (Fig. 4A).

As seen with Bcl-w containing cells, Epo increased also the number of Bcl-2 positive cells by 126% compared to the control group (531 ± 39.7 vs. 235 ± 47.5/mm², respectively; \( t_{4.5} = 2.26, P < 0.05 \)) (Fig. 3). Rats in the Pilo group had even higher numbers of Bcl-2 containing cells (627 ± 58.3/mm²; \( F_{3,16} = 3.31, P < 0.05 \)) and Epo preconditioning further increased Bcl-2 expression by 2.26-fold (838 ± 45.2/mm², \( t_{4.5} = 2.96, P < 0.01 \)) (Fig. 4B).

The control and Epo groups had equivalent numbers of Bim positive cells (297 ± 58.4 and 288 ± 37.4/mm², respectively). Bid expression increased by 22% in the Pilo group (350 ± 68.2/mm²) although insignificantly so. Interestingly, compared to the Pilo group, Bid positive cells decreased by 36% with Epo preconditioning (225 ± 34.7/mm², \( t_{4.5} = 1.95, P < 0.05 \)).

Fig. 3. Effects of Epo preconditioning on the expression of Bcl-2 and Bid. Brain sections were immunostained for Bcl-2 (green) and Bid (red). Bcl-2 and Bid are expressed in hippocampus of the control group (A–C). Epo increased the expression of Bcl-2, but didn’t alter the expression of Bid (D–F). In Pilo group, Bcl-2 positive cells increased significantly, but Bid positive cells increased less dramatically (G–I). In the Epo + P group, Bid positive cells decreased in the CA1/CA3 area of hippocampus, while Bcl-2 containing cells increased (J–K). Scale bar: 100 μm.

Fig. 4. Quantitative analysis of the relative expression of Bcl-2 family proteins. Each column represents the mean value obtained from 21 sections of hippocampus. For each type, the relative number of positive cells is normalized to the sum of the numbers of the control group. Bars indicate SEM. *\( P < 0.05 \) and **\( P < 0.01 \), as compared to controls. \( \dagger P < 0.05 \) and \( \ddagger P < 0.01 \), as compared to Pilo group.

**DISCUSSION**

This study showed that experimentally-induced SE elevates neuronal cell death in CA1/CA3 field of hippocampus and that pre-treating with Epo suppresses this effect. Furthermore, a mechanism by which Epo exerts this effect was suggested involving regulation of the Bcl-2 family of proteins that play a role in apoptotic signaling pathways. We showed that Epo up-regulated expression of the anti-apoptotic Bcl-w and Bcl-2 proteins, while down-regulating the pro-apoptotic Bim and Bid in hippocampal neurons. These changes, apart of being induced in an acute phase of seizure-connected
cell pathology, may inhibit further propagation of pro-apoptotic signalling and suppress delayed (apoptotic) cell death in the affected tissue region.

SE is a neurologic trauma associated with high mortality and long-term disability (Manno 2003, Wasterlain et al. 1993). Depending on the extent and the location of the insult, survivors develop permanent neurological dysfunctions such as cerebral palsy, epilepsy and mental retardation (Berger and Garnier 1999, Mercuri and Cowan 1999). SE in human and animal models causes significant cerebral damage and increases the risk of subsequent seizures, along with a characteristic pattern of neuronal cell loss preferentially in the hippocampus (DeGiorgio et al. 1991, Manno 2003, Mikati et al. 2003). Selective neuronal loss following SE was assumed to arise through hypoxia/ischemia and experimental modeling of such brain injury suggests that seizures elicit a mixture of necrotic and apoptotic cell death within vulnerable fields (Araki et al. 2002).

In recent years, accumulated evidence has shown that the renal cytokine Epo displays a number of biological actions within the central nervous system that are not related to its effect on erythropoiesis. Both Epo and its receptor are expressed in neurons and glial cells of human and rodent brains (Vairano et al. 2002). Epo has also been shown to cross the blood-brain barrier via a receptor-mediated mechanism (Brines et al. 2000) indicating that it has the potential for a direct central effect. Our previous study suggested that Epo preconditioning reduced neuronal apoptosis induced by SE in vivo through a mechanism involving the inhibition of caspase-3 activation (Wen et al. 2006). However, the neuroprotective mechanism of Epo preconditioning in hippocampus of epileptic rats was still unclear.

Apoptosis is cell death process that is regulated by a variety of genes, and is determined by their relative expression levels. The Bcl-2 gene family is comprised of more than 20 different members that either positively or negatively regulate apoptosis (Cory and Adams 2002, Liou et al. 2003) executed by specific downstream proteases named caspases.

In the present study, our results revealed that SE increased acute neuronal death in hippocampus of rats and that Epo preconditioning significantly reduced this increase. Furthermore, Epo’s regulation of the expression of the Bcl-2 family proteins offers a potential mechanism through which it could suppress SE-induced apoptosis. The mechanism is supported by the following lines of evidence. First, Epo preconditioning itself up-regulated the expression of Bcl-2 and Bcl-w, which oligomerize and thereby neutralize pro-apoptotic Bim and Bid protein function, but may also confer protection through more direct actions on mitochondrial membrane integrity, Ca$^{2+}$ mobilization (Cory and Adams 2002, Polster and Fiskum 2004), and perhaps via blocking nuclear AIF translocation (Strosznajder and Gajkowska 2006). Second, pretreatment with Epo down-regulated the expression of Bid and Bim and ultimately suppressed neuronal apoptosis. Bim is normally sequestered to dynein, a microtubule-associated protein, but after seizures its interaction with Bcl-w increases, resulting in a conformation that has been interpreted as cell death-promoting in human epileptic brain (Shinoda et al. 2004). Similarly, increased expression of Bim and Bid has also been associated with promotion of apoptosis. In an extrinsic apoptotic pathway, activation of initiator caspase-8 and -10 can trigger the mitochondrial apoptotic pathway via cleavage of Bid and translocation of the truncated Bid to mitochondria (Wang K et al. 1996). Activation of the mitochondrial apoptotic pathway triggers cytochrome C release, which then interacts with Apaf-1 to activate the initiator caspase-9, and subsequently the effector caspase-3 (Cohen 1997). Our previous study suggested that Epo preconditioning inhibited the activation of caspase-3 (Wen et al. 2006). Because the caspase-3 (executioner caspase) can cleave key structural and functional proteins within the cell such as actin (Meshima et al. 1997) and the inhibitor of caspase-activated DNase (Sakakura et al. 1998), as well as providing feedback loops for further processing of caspases (Thornberry and Lazebnik 1998), the inhibition of caspase-3 activation can directly suppress apoptosis.

CONCLUSION

To summarize, these data provided the first evidence that Epo preconditioning, except of a direct neuroprotection in the acute phase of seizure-induced cell injury, can suppress apoptotic neuronal cell death by regulating expression of the Bcl-2 family. The present study affords a novel insight into the protection of the brain from SE injury.

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