

Erythropoietin Enhances Neurogenesis and Restores Spatial Memory in Rats after Traumatic Brain Injury

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ABSTRACT

Erythropoietin (EPO) is neuroprotective in models of stroke and traumatic brain injury (TBI) when administered prior to or within the first few hours after injury. We seek to demonstrate that EPO also has neurorestorative effects when administered late (i.e., 1 day) after TBI in the rat. Twelve rats were subjected to TBI. Six rats were treated with EPO daily for 14 days starting 1 day after injury, and an additional six rats were treated with saline. Bromodeoxyuridine (BrdU) was administered daily for 14 days. Memory tests using a Morris Water Maze were performed prior to and after injury and treatment. Animals were sacrificed at 15 days after TBI, and their brains were prepared for histological analysis of damage to the dentate gyrus (DG) and for evaluation of newly formed neurons using double labeling of BrdU and MAP-2. The data revealed a significant improvement in spatial memory and significant increase in the number of newly formed neurons with EPO treatment compared with control animals. These data suggest that EPO treatment initiated 1 day after TBI is neurorestorative by enhancing neurogenesis, as well as neuroprotective.

Key words: erythropoietin; neurogenesis; neuroprotection; rat; traumatic brain injury

INTRODUCTION

ALTHOUGH A NUMBER of therapeutic trials for TBI have been undertaken, no broadly applicable, safe, and efficacious treatment has been identified (Narayan et al., 2002). Recently, attention has focused on potential therapeutic agents that enhance endogenous neuroplasticity including neurogenesis, angiogenesis, and synaptogenesis after brain injury (Yoshimura et al., 2003; Hallbergson et al., 2003; Lu et al., 1999). Erythropoietin (EPO) and its receptors are present in the human cerebral cortex and are up-regulated following hypoxic stimuli

(Jumbe 2002; Sasaki et al., 2000, 2001; Brines et al., 2000; Tong and Nissenson, 2001; Mulcahy, 2000). In animal models, administration of recombinant human EPO prior to or within the first 3 h after onset of experimental global and focal cerebral ischemia as well as traumatic brain injury (TBI) is neuroprotective (Brines et al., 2000; Dame et al., 2001; Cerami et al., 2002; Wang et al., 2004). These findings suggest that exogenous administration of erythropoietic agents may be a potential therapeutic tool for central nervous system injury (Jumbe, 2002). Systemic administration of epoetin alfa, a derivative of erythropoietin, reduced concussive brain injury (Cerami et al.,

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2002). However, previous studies focused on the neuroprotective effect of EPO administered before and immediately after brain injury. We have demonstrated that EPO is effective in improving neurological recovery in the rat after middle cerebral arterial occlusion and also enhances neuroplasticity (Wang et al., 2004a,b). In addition, a novel nonhematopoietic EPO, CEPO, has been shown to significantly improve neurological recovery in animal models of stroke, spinal cord injury and multiple sclerosis (Leist et al., 2004). Whether EPO reduces cognitive deficits and enhances neuroplasticity in the rat after TBI has not been investigated.

In this study, Wistar rats subjected to controlled cortical impact were treated with EPO starting one day after TBI. Our data show that EPO significantly improves spatial memory and concomitantly increases neurogenesis in the dentate gyrus (DG) in rats subjected to controlled cortical impact TBI.

MATERIALS AND METHODS

Animal Models

A controlled cortical impact model of TBI in rat was utilized in the present study (Dixon et al., 1991; Mahmood et al., 2001). Male Wistar rats (300–400 g) were anesthetized with 350 mg/kg/body weight chloral hydrate, intraperitoneally. Rectal temperature was controlled at 37°C with a feedback-regulated water-heating pad. A controlled cortical impact device was used to induce the injury. Briefly, rats were placed in a stereotactic frame. Two 10-mm-diameter craniotomies were performed adjacent to the central suture, midway between lambda and bregma. The second craniotomy allowed for movement of cortical tissue laterally. The dura was kept intact over the cortex. Injury was induced by impacting the left cortex (ipsilateral cortex) with a pneumatic piston containing a 6-mm-diameter tip at a rate of 4 m/sec and 2.5 mm of compression. Velocity was measured with a linear velocity displacement transducer.

Experimental Groups

Based on earlier studies (Cerami et al., 2002; Wang et al., 2004a), we selected a daily dose of 5000 IU/kg EPO as a treatment protocol. Twelve rats were randomly divided into two groups (6 rats/group): TBI + saline and TBI + EPO. In the TBI + EPO group, rats received 5000 IU/kg EPO intraperitoneally, starting 1 day after TBI and then daily for 14 days. These rats also received 100 mg/kg bromodeoxyuridine (BrdU) intraperitoneally, starting 1 day after TBI, then daily for 14 days. Rats in the TBI +

saline group received the same BrdU and saline injection without EPO.

Tissue Preparation

Rats were anesthetized intraperitoneally with ketamine and xylazine, and perfused transcardially first with saline solution containing heparin, followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4. Their brains were removed, post-fixed in 10% formalin for 1–2 days at room temperature, and then processed for paraffin sectioning.

Floating Section Staining

A series of 50- μ m-thick sections were cut with a microtome through the dorsal hippocampus containing the DG. Three sections with a 100- μ m interval were selected and processed for the floating section staining. Briefly, the sections were deparaffinized in xylene and alcohol, and rinsed in PBS followed by incubation in 0.1% saponin for 3 days. The sections were then treated with 50% formamide in $2 \times$ SSC at 65°C for 60 min, 2 N HCL at 37°C for 20 min and 0.1 M boric acid at room temperature for 3 min. After washing with PBS, 0.3% Triton-1% BSA in PBS was added to block the nonspecific signals at room temperature for 3 h. Mouse anti-BrdU antibody (1:200, DAKO) and rabbit anti-NeuN antibody (1:100, Chemicon) were added and incubated at 4°C for 3 days. After washing, FITC-conjugated anti-mouse antibody (green for BrdU) and CY5-conjugated anti-rabbit (red for NeuN) antibodies were added on the sections (1:200, Jackson Immuno) at 4°C for an additional 3 days. After washing, the sections were washed and mounted with Glycergel. Images were collected with laser confocal microscopy.

Image Acquisition and Analysis

The stained sections were analyzed with a Bio-Rad MRC 1024 (argon and krypton) laser-scanning confocal imaging system mounted onto a Zeiss microscope (Bio-Rad, Cambridge, MA). Sections from each animal were scanned throughout the entire hippocampus in 512×512 -pixel ($1,042.4 \times 1,042.4 \mu\text{m}$) format using a $4 \times$ frame-scan average, and 20 optical sections with a 5- μ m step-size under a $40 \times$ objective and FITC and Cy5-labeled cells were acquired with photomultiplier tubes. FITC and Cy5 fluorochromes on the sections were excited by laser beams at 488 and 530 nm. A series of images was obtained along the DG and subsequently merged using cofocal assistant software. A total of BrdU-only labeled cells (green) and the BrdU and NeuN colabeled cells (yellow) were counted, respectively, in the ipsilateral DG. The average numbers of the three slides were used for statistical analysis.

Spatial Memory Test Procedures

The testing procedure is a modification of the Morris Water Maze tests, described previously (Day et al., 1999; Day and Schallert, 1996; Yamada et al., 1999; Lu et al., 2004). The experimental apparatus consists of a circular water tank (140 cm in diameter and 45 cm high). An invisible platform (15 cm in diameter and 35 cm high) was placed 1.5 cm below the surface of the water. Water temperature was 30°C. The pool was located in a large test room, where there were many clues external to the maze (e.g., pictures, lamps); these were visible from the pool and presumably used by the rats for spatial orientation. The position of the cues remained unchanged throughout the task. Data collection was automated by the HVS Image 2020 Plus Tracking System (US HVS Image, San Diego, CA). For descriptive data collection, the pool was subdivided into four equal quadrants formed by imaging lines.

For the training trials, each animal received one trial per day for a 5-day session, with a 2-day break between the final training session and brain injury. The rats then were tested on days 1, 4, 8, and 15 after TBI or surgery. At the start of a trial, the rat was placed randomly at one of four fixed starting points, randomly facing either toward the wall or inwardly (designated North, South, East, and West) and allowed to swim for 90 sec or until they found the platform. The platform was located in a randomly changing position within the NE quadrant throughout the test period (e.g., sometimes, equidistant from the center and edge of the pool, against the wall, near the center of the pool, and at the edges of the NE quadrant). The goal was to make the daily mean of trials comparable to that of a standard probe trial without removing the platform and without being confounded by a single starting place (as in the standard probe trial). The animal learns to search extensively in the correct quadrant, because the platform is located somewhere within the NE quadrant but its precise location cannot be predicted.

In the standard fixed placement of the platform, the animals, once they learn the location, do not need to spend much search time in the quadrant, and when the platform is removed for the occasional brief probe trial, the animal quickly discovers its absence and may spend only a small amount of time searching for it again in that location. Moreover, the maladaptive preservative performance can increase the percentage of time an animal spends in the vicinity of the location from which a fixed-location platform is removed during a probe trial.

In each training session, the latency to escape onto the hidden platform was recorded. If the animal found the platform, it was allowed to remain there for 15 sec before being returned to its home cage. If the animal was unable to find the platform within 90 sec, the training

was terminated and a maximum score of 90 sec was assigned. The percentage of time traveled within the NE (correct) quadrant was calculated relative to the total amount of time spent swimming before reaching the platform.

Statistical Analysis

Data were analyzed by ANOVA followed by *t*-test for multiple comparisons. Paired *t*-test was used to test the difference of cell counts between the ipsilateral and the contralateral hemispheres or between different groups. All measurements were performed by observers blinded to individual treatment.

RESULTS

Erythropoietin Enhances the Restoration of Spatial Memory

All rats were trained for 5 days before surgery. At the first day of training, the percentage of the mean time spent in the correct quadrant (containing the platform) was approximately 25% (chance level), which then persistently increased during the subsequent training days, reaching about 50% after the five daily training sessions (Fig. 1).

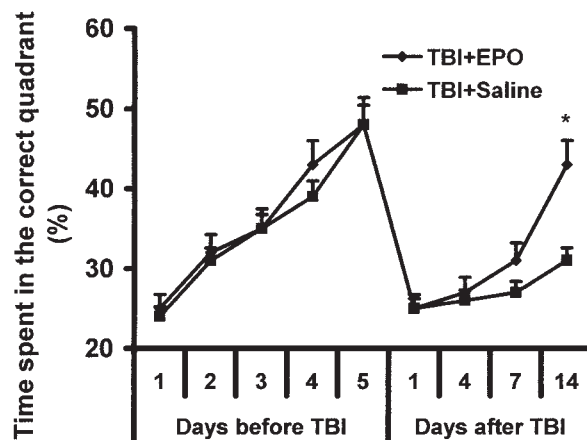


FIG. 1. Plot shows the temporal profile of the time spent in the correct quadrant (mean \pm SD%). Percentage of time spent in the correct quadrant by the rats receiving 5 days of training reached \sim 50% and then significantly declined to \sim 25%, resembling the first-day training level at 1 day after traumatic brain injury (TBI). At 15 days after TBI treatment (14-day treatment), the percentage of time spent by the erythropoietin (EPO)-treated rats in the correct quadrant was significantly elevated versus the saline-treated TBI rats ($*p < 0.05$). These data demonstrate that TBI in the controlled cortical impact model in rat significantly reduces the spatial memory and EPO treatment promotes restoration of spatial memory.

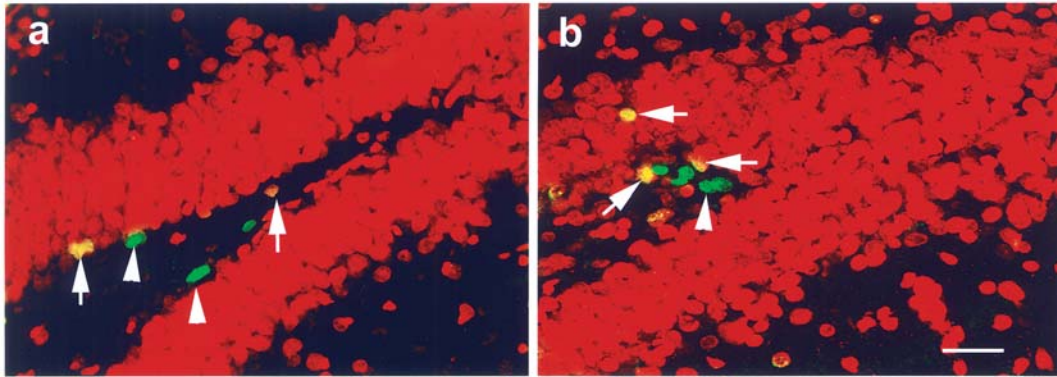


FIG. 2. Photographs show the floating double fluorescent staining for BrdU (green) and NeuN (red) to identify the newly generated cells and neurogenesis in the dentate gyrus (DG) of the ipsilateral hemisphere of the saline-treated (a) and the erythropoietin (EPO)-treated (b) groups. Arrows indicate the BrdU-labeled cells colabeled with NeuN (yellow). Bar = 50 μ m.

The average time spent in each of the other quadrants decreased over the 5-day training trial to about 17% (below chance). At day 7 after the start of the experiment, all rats were subjected to TBI, and at day 1 after TBI, the rats showed a significantly diminished percentage of time spent in the correct quadrant, close to the chance levels of the time spent at the start of the training (Fig. 1). These data demonstrate that damage to brain in this model with the current degree of injury causes significant dysfunction of spatial memory in rats, which can be used as a target for the treatment of the injury. The degree of pre-training was purposely limited so that overtraining did not occur.

Rats were tested at day 1 after TBI just prior to treatment, and at days 4, 8, and 15 after TBI (3, 7, and 14 days after initiation of treatment). The percentage of time spent in the correct quadrant was significantly higher in the EPO-treated group versus the saline-treated group at day 15 ($43.0 \pm 6.6\%$ vs. $31.0 \pm 3.5\%$) after TBI ($p <$

0.05) (Fig. 1). These data demonstrate that EPO administration reduces the dysfunction of the spatial memory caused by the brain damage in this model.

EPO Increases the Number of BrdU-Labeled Cells in the DG

In this study, our focus is on spatial memory recovery after TBI, consequently BrdU-positive cells as an index of neurogenesis were counted in the DG of the hippocampus but not in other areas of brain. BrdU-labeled cells were observed mainly in the subgranular zone and the granular cell layer, and a few were found in the molecular layer of both the EPO-treated and the saline-treated groups (Fig. 2a,b). Some BrdU-labeled cells expressed NeuN, a marker for mature neurons in the granular cell layer (Fig. 2a,b). However, few BrdU-labeled cells were detected in the hilus of the DG in the EPO-treated group (Fig. 2b). EPO administration for 14

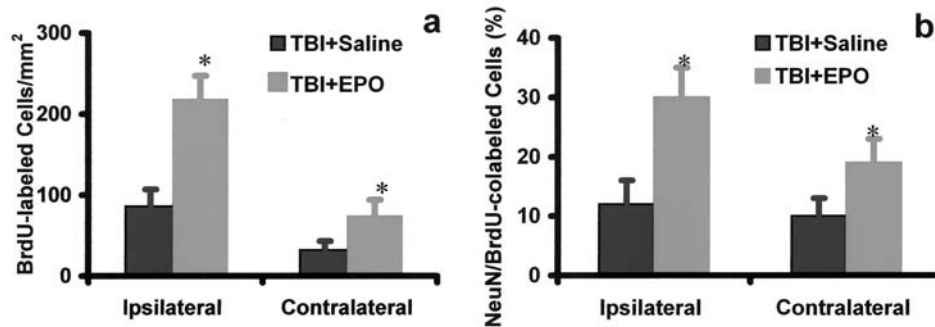


FIG. 3. Bar graphs show the density of BrdU-labeled cells (a) and the percentage of BrdU-labeled cells colabeled with NeuN (b) in the dentate gyrus (DG) after traumatic brain injury (TBI) in rat. $n = 6$ rats/group. * $p < 0.05$, compared to the saline-treated group.

days significantly increased the number of BrdU-labeled cells in both the contralateral and ipsilateral DG (51 ± 10 cells/mm² and 142 ± 17 cells/mm², respectively) compared to the saline-treated group (17 ± 7 cells/mm² and 36 ± 12 cells/mm²; $p < 0.05$) (Fig. 3a). Counting the percentage of BrdU-labeled cells colabeled with NeuN, EPO administration significantly increased the percentage of newly generated cells that differentiated into mature neurons in the granular cell layers of both the contralateral and ipsilateral DG (Fig. 3b). These data demonstrate that EPO administration increases neurogenesis in the DG in rats after TBI.

DISCUSSION

The present study shows, for the first time, that treatment of TBI with EPO promotes restoration of spatial memory and enhances neurogenesis in the DG in rat.

Via a 5-day training regimen, rats learned and remembered spatial cues. Thereafter, they spent more time finding the platform in the correct quadrant since the location of the platform was changed. TBI destroyed this spatial memory, leading the rats to swim purposelessly in the pool after brain injury. Treatment with EPO promotes restoration at 15 days after TBI compared to the saline treatment, thereby demonstrating the benefits of EPO treatment on memory restoration in TBI. EPO-induced neurogenesis of the DG may contribute to spatial memory restoration in this model of TBI, although the therapeutic mechanisms are unclear. EPO gene expression can be induced and regulated by hypoxia, ischemia and brain trauma (Tong and Nissenson 2001; Cerami et al., 2002; Leist et al., 2004; Wen et al., 2004; Martinez-Estrada et al., 2003; Aydin et al., 2003; Yu et al., 2002). However, when comparing pre- and post-TBI functional data, endogenous expression of the EPO gene is apparently not sufficient to restore damaged brain tissue and corresponding functional deficits. Exogenous administration of EPO has been tested in many model systems (e.g., treatment of stroke with EPO stimulates neurogenesis and angiogenesis in the injured brain and improves functional recovery) (Wang et al., 2004a).

Inhibition or reduction of neurogenesis in the DG of the rodent has been shown to reduce spatial memory (Rhodes et al., 2003; van Praag et al., 1999). The negative impact of blocking neurogenesis has also been found in rodents subjected to radiation escaping from a closed container (Rola et al., 2004; Raber et al., 2004). Our previous studies on the amplification of neurogenesis by means of cell-based or pharmacologically based therapies after stroke and TBI (Lu et al., 2003; Mahmood et al., 2004) demonstrate a significant improvement in func-

tional outcome and are consistent with concomitant EPO-mediated neurogenesis and functional improvement after TBI.

An additional finding in this study is that EPO promotes differentiation of the newly generated cells into neuronal phenotype in the DG. Differentiation of the newly generated cells in the DG is dependent on the tissue microenvironment (i.e., the presence of angiogenesis and production of growth factors, such as brain-derived neurotrophic factor (BDNF) (Kempermann et al., 2004). Therefore, BDNF and vascular endothelial growth factor (VEGF) induced by EPO, as previously shown (Wang et al., 2004b), may also play an important role in the survival and differentiation of neural progenitor cells in the DG after TBI. However, in-depth studies are needed to demonstrate whether the newly generated neurons function as mature neurons.

EPO has an anti-apoptotic and neuroprotective effect in many organs including brain (Buemi et al., 2003; Yu et al., 2002). Thus, preventing the death of newly generated cells as a way to increase the number of newly generated neurons cannot be ruled out. Many studies have shown that a large portion of the newly generated cells in the subventricular zone and the DG die by undergoing apoptosis (Kempermann et al., 2003). However, in this study, we focus solely on the number of newly generated cells as the end point after EPO treatment. Additional studies are needed to evaluate the dynamic changes of apoptosis at early time points, and the relative role of neuroprotection and brain plasticity (e.g., neurogenesis on improvement of spatial memory after TBI) in improving functional recovery after TBI.

CONCLUSION

Our data demonstrate that EPO treatment promotes spatial memory restoration and enhances the presence of the newly formed neurons in the DG after TBI in the rat.

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ERYTHROPOIETIN ENHANCES NEUROGENESIS AFTER TBI

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