Neuroprotection with Erythropoietin Administration Following Controlled Cortical Impact Injury in Rats

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ABSTRACT

This study was designed to determine the effect of erythropoietin (Epo) on cerebral blood flow (CBF), nitric oxide (NO) concentration, and neurological outcome after traumatic brain injury. In one experiment, the hemodynamic effects of Epo were determined after controlled cortical impact injury (CCI) by measuring mean arterial pressure, intracranial pressure, CBF using laser Doppler flowmetry, and brain tissue NO concentrations using an NO electrode. In total, 41 rats were given either Epo (5000 U/kg) or saline s.c. on days 3 before injury. In animals pretreated with saline, L-arginine but not D-arginine administration resulted in a significant increase in tissue NO concentrations and an improvement in CBF at the impact site. Likewise, in animals pretreated with Epo, L-arginine but not D-arginine given postinjury increased brain tissue NO concentrations and increased CBF. In another experiment, 74 rats underwent CCI (3-mm deformation, velocity 5 m/s), and they were given saline or Epo 5000 U/kg s.c. at 5 min, 1 h, 3 h, 6 h, 9 h, or 12 h postinjury. The contusion volume and cell counts of viable neurons in the CA1 and CA3 regions of the hippocampus were assessed at 2 weeks postinjury. The contusion volume was significantly reduced at 5 min, 1 h, 3 h, and 6 h postinjury Epo administration. The neuron density in the CA1 and CA3 region of the hippocampus was increased at 1, 3, and 6 h after injury. These data demonstrate the neuroprotective effects of Epo in traumatic brain injury, and the effects are optimal when Epo is given within 6 h of injury.

Erythropoietin (Epo) has been shown to have neuroprotective effects against a variety of types of experimental brain injury, including experimental cerebral ischemia (hypoxic/ischemic injury) (Kumral et al., 2003), global retinal ischemia caused by increasing intraocular pressure (Junk et al., 2002), bilateral carotid occlusion (Catania et al., 2002), middle cerebral artery occlusion (Sire˘n et al., 2001), focal cerebral ischemia (Bernaudin et al., 1999; Brines et al., 2000), experimental traumatic brain injury (cortical impact injury) (Brines et al., 2000), glutamate neurotoxicity (Morishita et al., 1997), toxin-induced Parkinsonism (1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine-induced Parkinsonism) (Gene et al., 2001), spinal cord ischemia (Celik et al., 2002), spinal cord trauma (Gorio et al., 2002), subarachnoid hemorrhage (Alafaci et al., 2000; Grasso, 2001; Springborg et al., 2002), and axotomy (Iwasaki et al., 2002).

Epo is an attractive candidate for neuroprotection following traumatic brain injury not only because of its effects on the injured brain but also because administration of Epo may improve hemoglobin concentration and therefore cerebral oxygen delivery, possibly reducing the need for blood transfusion. Epo is a 165-amino acid (~30-kDa) glycoprotein responsible for erythropoiesis. Pharmacokinetic studies show that Epo has a half-life ($t_{1/2}$) of 4 to 9 h after intravenous administration and >24 h after subcutaneous injection (Egrie et al., 1988). Brines et al. (2000) reported that intravenous administration of Epo (5000 IU/kg) to normal rats increased the concentration of Epo in cerebrospinal fluid with in 30 min.

For the systemic effects of Epo, there is probably not a compelling need for early administration. However, neuroprotective agents, in general, have the greatest effect when administered either before or very soon following injury. To design a clinical trial of Epo administration for patients with traumatic brain injury, experimental data regarding the optimal time window for Epo neuroprotection would be useful. One previous experimental study in mice has demonstrated reduced contusion volume when Epo was administered within 6 h after injury, but not with administration at 9 h after injury (Brines et al., 2000). Additional clarification of the optimal time window for Epo neuroprotection in traumatic brain injury is needed.

The mechanism for Epo neuroprotection is also not entirely clear, and multiple pathways may be involved, including...
possibly an effect on the cerebral vasculature through alteration in NO production. Both an increase and a decrease in NO and NOS activity have been described after Epo administration. In vitro studies with prolonged (several days) exposure to Epo increase NOS activity in endothelial cells (Banerjee et al., 2000). In vitro studies using renal arteries suggest that acute administration of Epo can stimulate NO release (Wu et al., 1999). Chronic administration of Epo to rats causes significant hypertension, and it also increased production of NO (Tsukahara et al., 1997). Short-term effects of Epo administration in humans suggest that Epo impairs endothelial function (Wada et al., 1999). In vitro studies using human coronary artery endothelial cells suggest that Epo down-regulates basal and acetylcholine-stimulated NO production and depresses endothelial NOS expression, effects that are inhibited by calcium channel blocking agents (Wang and Vaziri, 1999).

The purpose of this study was to examine the effect of Epo administration at varying time intervals postinjury on neurological outcome after TBI and to clarify the role of the cerebral vasculature in the neuroprotective effects of Epo.

Materials and Methods

Treatment Groups. To achieve these goals, two experiments were performed. The first experiment examined the effect of Epo administration at various times postinjury on neurological outcome assessed by histological indices. The second experiment examined the effect of Epo on L-arginine-induced increases in cerebral blood flow (CBF) after TBI. Both experiments were approved by the Institutional Animal Protocol Review Committee, using guidelines for humane care and use of animals developed by the National Institutes of Health.

Experiment 1: Time Window for Epo Neuroprotection. Seventy-four Long Evans rats were used in this study. The animals were given recombinant human (rh)Epo (5000 U/kg s.c.) or an equal volume of saline at 5 min or 1, 3, 6, 9, or 12 h postinjury. Treatment group was randomly assigned, and the investigator performing the histological outcome assessment was blinded to the treatment group.

Experiment 2: Cerebrovascular Effects of Epo. In total, 41 Long-Evans rats were randomly assigned to one of the following four treatment groups. Each treatment group received either rhEpo (5000 U/kg/ml) or saline, subcutaneously once a day for 3 days before injury and a single dose of either L-arginine (300 mg/kg) or D-arginine (300 mg/kg) 5 min after injury. Forty-one of these animals were used to study the hemodynamic effects (Table 1 lists the numbers of animals per group).

Anesthesia and Surgical Preparation. Rats, weighing 300 to 400 g and fasted overnight, were anesthetized with 3.5% isoflurane in 100% oxygen in a vented anesthesia chamber. Following endotracheal intubation with a 16-gauge Teflon catheter, the rats were mechanically ventilated with 2% isoflurane in 100% oxygen during the surgical preparation and for the impact. Rectal temperature was maintained at 36.5–37.5°C by a heating pad, which was controlled by rectal thermistor. Brain temperature was monitored with a thermocouple microprobe placed in the brain parenchyma and controlled by a heating lamp directed at the head and kept at 37°C.

Production of Brain Injury. The details of the methods to produce the impact injury have been described previously (Cherian et al., 1996). In brief, the head of the rat was fixed in a stereotaxic frame by ear bars and incisor bar. A 10-mm-diameter craniotomy was performed on the right side of the skull over the parietal cortex. The impactor tip, which had a diameter of 8 mm, was centered in the craniotomy site perpendicular to the exposed surface of the brain at an angle of approximately 45° to the vertical. The tip was lowered until it just touched the dural surface. The impactor rod was then retracted, and the tip advanced an additional 3 mm to produce a brain deformation of 3 mm during the impact. The gas pressure applied to the impactor was adjusted to 150 psi, giving an impact velocity of approximately 5 m/s and a duration of approximately 130 ms.

Measurement of Cerebral Blood Flow. Cerebral blood flow was measured by Laser Doppler probes (Perimed, Piscataway, NJ) at the impact site 30 min before injury and continuously for 2 h after injury. Values for the Laser Doppler flow (LDF) were expressed as percentage of change from the preinjury baseline.

Measurement of Nitric Oxide. Nitric oxide was measured using NO electrodes (tip diameter 200 μm, ISO-NOP200; WPI, Sarasota, FL) inserted into the brain at a depth of 1.5 mm at the center of the impact site. The measurement principle of this type of electrode is the oxidation of NO at a working electrode, which is kept at a constant potential of 0.85 V against a silver/silver chloride reference electrode. Selectivity to NO was maintained by a gas-permeable membrane covering the electrode. The redox current proportional to tissue NO concentration was measured with an ISO-NO meter (WPI). NO electrodes were calibrated before and after each experiment by a standard method of chemical regeneration of NO using S-nitroso-N-acetylt-dl-pencillamine and copper sulfate at 37°C. S-Nitroso-N-acetyl-dl-pencillamine (RSNO) decomposes to NO and a disulfide by-product according to the following equation: 2 RSNO + 2NO + RS-SR.

Brain NO concentration was calculated from the current measured with the probe positioned in the brain by means of the in vitro calibration curve. Changes in brain NO was expressed by the change in concentration (nanomolar) from baseline values.

Postoperative Care. Following the impact injury, the surgical wound was sutured closed; the rats were allowed to awaken from anesthesia, and they were extubated. For the first 3 days postinjury, the rats were treated with butyrophenol tartrate, 0.05 mg i.m. every 12 h, for analgesia and enrofloxacin 2.27%, 0.1 ml i.m. q.i.d., to reduce the risk of postoperative infections.

Histopathological Assessment of Outcome. The histological assessment of outcome was performed by personnel who were blinded to the treatment group. At 2 weeks after the impact, the animals were deeply anesthetized, and they were perfused transcardially with 0.9% saline, followed by 10% phosphate-buffered formaldehyde. The entire brain was removed and fixed in 4% Formalin. The fixed brains were examined grossly for the presence of contusion, hematoma, and herniation. The brains were photographed, sectioned at 2-mm intervals, and then embedded in paraffin. Hematoxylin and eosin-stained 9-μm-thick sections were prepared for histological examination. Particular care was made to include the largest cross-sectional area of cortical injury on the cut surface of the embedded sections. The coronal sections were digitized using a Polariod Sprint Scanner (Polaroid Corporation, Waltham, MA) equipped with a PathScan Enabler (Meyer Instruments, Houston, TX).

The injury volume was measured by determining the cross-sectional area of injury in each coronal image and multiplying by the thickness of the tissue between the slices. This slab volume technique was implemented on the image-processing program Optimas.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Physiological parameters at baseline were not significantly different (experiment 2: hemodynamic effects of Epo)</th>
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<tbody>
<tr>
<td>Treatment Group</td>
<td>Epo + L-Arg</td>
</tr>
<tr>
<td>No. of animals</td>
<td>13</td>
</tr>
<tr>
<td>Wt (g)</td>
<td>358 ± 5.8</td>
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<tr>
<td>Baseline parameters</td>
<td></td>
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<tr>
<td>pO2 (mm Hg)</td>
<td>251 ± 38</td>
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<tr>
<td>pCO2 (mm Hg)</td>
<td>40 ± 1.3</td>
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<td>pH</td>
<td>7.38 ± 0.01</td>
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5.2 (Optimas Corporation, Seattle, WA). Neurons in the middle 1-mm segments of the CA1 and CA3 regions of the hippocampus were counted at a magnification of 200×. Neurons were identified by nuclear and cytoplasmic morphology, and individual cells were counted as either normal or damaged. Neurons with cytoplasmic shrinkage, basophilia, or eosinophilia, or with loss of nuclear detail were regarded as damaged. The regions measured were 1 mm long and 1 mm wide (0.5 mm on either side of the long axis of the segment). The total number of neurons and the number of neurons that seemed normal were expressed as neurons per square millimeter.

**Statistical Analysis.** The data for CBF and NO are collected every 30 s using the LabVIEW program (National Instruments Corporation, Austin, TX). Values for CBF were expressed as percentage of changes from the preimpact baseline values. Because the laser Doppler method is qualitative, all preinjury values were normalized to 100%. Changes in brain NO were expressed by the change in concentration (nanomolar) from baseline values. A repeated measures analysis of variance (ANOVA) was used for both nitric oxide and cerebral blood flow measurement, with Tukey’s test for multiple comparisons.

Histological outcome measures were compared using one-way (experiment 1) or two-way (experiment 2) analysis of variance followed by Dunnett’s test when multiple comparisons were performed.

**Results**

**Experiment 1: Time Window for Epo Neuroprotection.** The effects of Epo on neurological outcome assessed by histological indices at 2 weeks postinjury are illustrated in Figs. 1 and 2, and examples of the histological appearance are shown in Fig. 3. Seven (9.4%) of the 74 animals died following the impact injury: one animal from the 1-h Epo-treated group, and two animals from the saline-treated, 3-h Epo-treated, and the 9-h Epo-treated groups, respectively. The brains from these seven animals were not available for analysis. One animal in the 1-h Epo-treated group developed a large intraparenchymal hematoma at the injury site, and one animal in the 12-h Epo-treated group had damage to the dura from the impact injury, resulting in a severe penetrating injury of the brain. The results of these two animals were also excluded from the final analysis of outcome. There were no significant differences in the injury severity measured by impact velocity or impact duration among the treatment groups.

The mean contusion volume of the remaining 67 animals at 2 weeks postinjury (Fig. 1) was significantly reduced (p < 0.05 adjusted for multiple comparisons by using Dunnett’s test). Values for neuron density in uninjured animals are 230 ± 15 cells/mm². Erythropoietin in Traumatic Brain Injury 791

![Fig. 1. Contusion volume was significantly reduced by Epo administration when given 5 min and 1, 3, and 6 h after controlled cortical impact injury in rats (p value is for the overall treatment effect by one-way ANOVA test; asterisks indicate those values that are significantly different from the saline-treated value (p < 0.05 by Dunnett’s test)).](image)

The mean neuron density in the CA1 area of the hippocampus (Fig. 2a) was significantly increased when rhEpo was administered at 1, 3, and 6 h after controlled cortical impact injury in rats. Unlike the contusion volumes, for the CA1 neuron preservation administration of rhEpo at 6 h postinjury (Fig. 2a) was significantly increased when rhEpo was administered at 1, 3, and 6 h after controlled cortical impact injury in rats. Unlike the contusion volumes, for the CA1 neuron preservation administration of rhEpo at 6 h post-
Jury had a beneficial effect equal to administration of rhEpo at 3 h postinjury. The neuron density in the CA3 area of the hippocampus (Fig. 2b) followed the same pattern as the CA1 data, but the differences were not as large. Although the overall treatment effect for CA3 neurons was significant \((p = 0.011)\), there were no groups that were significantly different from the saline-treated group when the \(p\) values were adjusted for multiple comparisons.

**Experiment 2: Cerebrovascular Effects of Epo.** Previous studies have shown that L-arginine administration significantly improves blood flow in contused brain after injury and that endothelial NOS is required for this effect of L-arginine (Cherian et al., 2000; Hlatky et al., 2003). This experiment examined the effect that Epo might have on the L-arginine-induced change in pericontusional blood flow.

The pertinent results for the cerebral hemodynamic changes are shown in Figs. 4 and 5. As the previous studies had shown, postinjury administration of L-arginine significantly increased LDF. D-Arginine had no effect on LDF. In the animals pretreated with Epo, the increase in LDF induced by L-arginine administration was even greater than in the animals pretreated with saline.

The brain tissue NO concentration (Fig. 5) showed the same general pattern as the cerebral blood flow. Tissue levels of NO decreased by an average of 10 nmol in the D-arginine group pretreated with Epo and 7 nmol in the D-arginine group pretreated with saline. L-Arginine in both Epo- and saline-pretreated groups restored the brain tissue concentration.

There were no significant differences in physiological parameters between groups (Table 1). In addition, the injury severity measured by impact velocity and duration was similar in all of the groups.

The interaction of Epo and L-arginine on cerebral blood flow did not result in a significantly improved neurological outcome as measured by histological indices at 2 weeks postinjury. Similar to the findings of experiment 1, there was a significant main effect of Epo on contusion volume \((p = 0.045)\), with Epo decreasing contusion volume from 12.5 ± 8.0 to 7.7 ± 5.8 mm\(^3\), but there was not a significant effect of arginine or interaction with arginine for contusion volume or for neuronal loss in the hippocampus.

**Discussion**

The mechanism of the neuroprotective action of Epo administration is not fully understood, but multiple potentially beneficial effects have been identified. The effect of Epo administration on NO production has been variable from study to study, but a number of studies suggest that NO production is increased after Epo administration. Numerous studies suggest that Epo administration up-regulates NOS, increases NO production (del Castillo et al., 1995; Tsukahara et al., 1997; Wu et al., 1999), or dilates vessels in a manner that suggests NO production by endothelium (Gene et al., 2001). In physiological circumstances where endogenous Epo production is increased, such as in athletes training at high altitudes, production of NO is also increased (Schena et al., 2002). Finally, in some pathological conditions, Epo administration has been found to dilate cerebral vessels. In a subarachnoid hemorrhage model, Epo reversed the vasoconstriction that occurred in intracranial vessels (Grasso, 2001). A
single dose of rhEpo given peripherally has been shown to preserve autoregulation of CBF (Springborg et al., 2002). However, chronic administration (1 week) of Epo caused attenuated depressor responses to endothelium-dependent vasodilators that may have suggested inhibition of NOS activity (Noguchi et al., 2001). Some studies have suggested that Epo inhibits eNOS protein expression (Wang and Vaziri, 1999) and reduces NO concentration in the brain after ischemia (Calapai et al., 2000). Some studies suggest that Epo administration does not alter NO synthesis (López Ongil et al., 1996; Yamamoto et al., 2000).

The findings of experiment 2 from the present study, where pretreatment with Epo augmented the increase in cerebral blood flow induced by L-arginine postinjury, suggest that Epo may up-regulate or stimulate endothelial nitric-oxide synthase. Some limitations of the findings should be noted. Endothelial nitric oxide activity was not actually measured, and this would have to be done to clearly implicate this as a mechanism of Epo. Using the laser Doppler method to assess blood flow, it is not possible to determine whether Epo pretreatment increases the baseline blood flow, since all measurements are normalized to the preinjury level. Although postinjury blood flow was improved, there was not a significantly better outcome with the combined Epo and L-arginine treatment. Epo has other mechanisms of neuroprotection that may be more important than these vascular effects. Epo also has a systemic pressor effect that could preserve better perfusion of the brain in focal and incomplete ischemia models (del Castillo et al., 1995; Vaziri et al., 1995).

A number of studies implicate Epo activities in apoptosis pathways (Yatsiv et al., 2005). In a global ischemia model in gerbils, expression of Bcl-xL was markedly increased in the hippocampus of animals given Epo intraventricularly (Wen et al., 2002). Activation of neuronal Epo receptors prevented apoptosis induced by N-methyl-D-aspartate or NO through activation of nuclear factor-κB by the Janus tyrosine kinase (Digicaylioglu and Lipton, 2001). Studies involving free radical-induced injury in cerebral microvascular endothelial cells showed that constitutive Epo is present in endothelial cells but that it is insufficient to prevent cellular injury. Signaling through the Epo receptor, however, remains biologically responsive enough to exogenous Epo administration to offer significant protection against nitric oxide-induced injury. Exogenous Epo maintains both genomic DNA integrity and cellular membrane asymmetry through parallel pathways that prevent the induction of apoptotic protease-activating factor 1 and preserve mitochondrial membrane potential in conjunction with enhanced Bcl-xL expression. Consistent with the modulation of apoptotic protease-activating factor 1 and the release of cytochrome c, Epo also inhibits the activation of caspase 9- and caspase 3-like activities (Chong et al., 2003a). Through pathways that involve the initial activation of protein kinase B, Epo maintains mitochondrial membrane potential. Subsequently, Epo inhibits caspase 8-, caspase 1-, and caspase 3-like activities linked to cytochrome c release through mechanisms that are independent of the mitogen-activated protein kinase systems of p38 and c-Jun NH₂-terminal kinase (Chong et al., 2003b).

Studies by Brines et al. (2000) noted that the inflammatory response to traumatic injury and to ischemia was markedly reduced in Epo-treated animals. More recent studies have shown that Epo inhibits activation of microglia, possibly by reducing phosphatidylserine exposure (Chong et al., 2003b; Kang et al., 2003). In a mouse model of closed brain injury, improved neurological recovery was accompanied by reduced activation of glial cells when erythropoietin was administered and 1 and 24 h after injury (Yatsiv et al., 2005). Erythropoietin has also been demonstrated to inhibit the rise in interleukin-1β and infiltration of leukocytes in the involved hemisphere following a hypoxic-ischemic insult (Sun et al., 2005).

Most of the previous experimental studies examining the neuroprotective effects of Epo have administered the drug either before injury or shortly thereafter (Bernaudin et al., 1999; Alafaci et al., 2000; Calapai et al., 2000; Catania et al., 2002; Celik et al., 2002; Gorio et al., 2002; Junk et al., 2002). However, a few studies have directly addressed the issue of time window for neuroprotective effects of Epo after various types of brain injury. Both in vitro studies of injury processes, such as excitotoxicity, and in vivo studies in standardized injury/ischemia models suggest that the earlier that Epo is given, the better the neuroprotective effect. When given beyond 6 h postinjury, there is minimal neuroprotection with Epo in all of these studies. These previous studies are consistent with the findings of experiment 1.

For the mechanisms of Epo that may involve later processes, such as angiogenesis, and neurogenesis, most studies have still administered the Epo immediately after injury. Two recent studies, however, have reported that administration of Epo at 24 h after ischemia or trauma improved neurological function (assessed with behavioral testing) (Wang et al., 2004; Lu et al., 2005). In the stroke model, this recovery of function was not accompanied by a reduction in infarct volume, but it was associated with increased density of microvessels at the infarct boundary zone, suggesting that angiogenesis may be involved in recovery of function (Wang et al., 2004). In the trauma model, increased numbers of newly formed neurons were observed in the dentate gyrus of Epo-treated animals, suggesting that neurogenesis may have been enhanced by administration of Epo (Lu et al., 2005). Administration of erythropoietin and epidermal growth factor in combination intraventricularly promoted regeneration of the damaged cerebral cortex and improved recovery of spontaneous and skilled motor tasks in a rat model of stroke (Kolb et al., 2007). Cortical regeneration and functional recovery occurred even when growth factor administration was delayed for up to 7 days after the stroke.

In summary, the actions of Epo are complex after TBI. Vascular effects of Epo may play some role in preserving cerebral blood flow after trauma, and this could be beneficial, but it is probably not the major neuroprotective mechanism of Epo. The optimal neuroprotective effect occurs if Epo is administered within 3 h of injury, although there still maybe some neuroprotection, especially for hippocampal neuronal preservation, if Epo is administered within 6 h of injury. Similar to the study by Brines et al. (2000), there is no significant neuroprotective effect with Epo administration beyond 6 h postinjury in the cortical impact injury of TBI. There may still be some beneficial effects of Epo administration at later times, but the mechanism is likely to be different (Lu et al., 2005).
Acknowledgments

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References


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