Neonatal Hypoxia/Ischemia Is Associated With Decreased Inflammatory Mediators After Erythropoietin Administration

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Background and Purpose—Erythropoietin (EPO), a hematopoietic growth factor, has been shown to be neuroprotective when administered as either a pretreatment or posttreatment. This study tested the hypothesis that one of the mechanisms of protection afforded by posttreatment with recombinant human EPO (rh-EPO) is an anti-inflammatory effect via inhibition of interleukin (IL)-1β.

Methods—Seven-day-old rat pups were subjected to unilateral carotid artery ligation followed by 90 minutes of hypoxia (8% O₂ at 37°C). Pups were divided into the following groups: control, hypoxia/ischemia, and hypoxia/ischemia plus rh-EPO. In the rh-EPO–treated pups, rh-EPO (5 U/g body weight IP) was administered starting 24 hours after the insult and then for 2 additional days. Samples were collected at 3, 7, 14, and 21 days after the insult. IL-1β mRNA and protein levels were determined by quantitative real-time reverse transcription–polymerase chain reaction and ELISA. Tumor necrosis factor (TNF)-α mRNA levels were determined by colorimetric microplate assay.

Results—rhEPO attenuated brain injury, as assessed by brain weight, and attenuated both the hypoxia/ischemia–induced increases in IL-1β mRNA and protein levels. TNF-α mRNA levels did not increase at 3 to 14 days after the hypoxic/ischemic insult.

Conclusions—Administration of exogenous rh-EPO starting 24 hours after a hypoxic/ischemic insult is neuroprotective in the neonatal rat. This neuroprotective activity prevented the secondary, delayed rise in IL-1β and attenuated the infiltration of leukocytes into the ipsilateral hemisphere. (Stroke. 2005;36:1672-1678.)

Key Words: cytokines ■ growth factors ■ hypoxia ■ inflammation ■ ischemia

The brain exhibits adaptive immunity in response to injury. Inflammatory mediators, ie, cytokines, released by microglia during an innate immune response strongly influence neurons and their ability to process information, making inflammation a leading factor in the pathogenesis of hypoxic/ischemic brain injury. The expression of interleukin (IL)-1β has been shown to increase after a hypoxic/ischemic insult in the neonatal rat, and intracerebroventricular administration of IL receptor antagonists significantly reduces brain injury. Administration of exogenous tumor necrosis factor (TNF)-α markedly exacerbates ischemic injury, and inhibition of TNF-α activity also reduces excitotoxic brain injury in neonatal rats.

It has been reported that recombinant human erythropoietin (rh-EPO) reduces the amount of inflammatory infiltrate in blunt trauma experiments and has an anti-inflammatory effect in the encephalomyelitis model. This data suggests that EPO might act as a protective cytokine in inflammatory pathologies of the central nervous system. We have previously reported that pretreatment with EPO protected the brain from hypoxic/ischemic injury in the neonatal rat and that heatshock protein-27 was involved in this preconditioned protection. In our recent work, rh-EPO administered 24 hours after the insult showed significant brain-protective activity. Therefore, we tested the hypothesis that one of the mechanisms of protection afforded by posttreatment with rh-EPO is an anti-inflammatory effect via inhibition of both IL-1β and TNF-α.

Methods

Neonatal Hypoxia/Ischemia Rat Model

The Animal and Ethics Review Committee at the Louisiana State University Health Sciences Center in Shreveport evaluated and approved the protocol used in this study. Unsexed 7-day-old (day 0=day of birth) Sprague-Dawley (Harlan, Indianapolis, Ind) rats were anesthetized by inhalation with isoflurane (0.1%) in O₂ and subjected to hypoxia/ischemia as previously described. Control littermates were not operated on or subjected to hypoxia.

Experimental Groups

The pups (N=246) for this study were randomly divided into 3 groups: a control group (without surgery and hypoxia/ischemia), hypoxia/ischemia (HI), and hypoxia/ischemia plus rh-EPO treatment.

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group (EPO). In EPO-treated animals, pups received rh-EPO (5 U/kg body weight IP) starting 24 hours after the insult. rh-EPO was then administered at the same dose per day for an additional 2 days. In all groups, pups were humanely killed under deep anesthesia by inhalation with isoflurane at the following designated time intervals: 3, 7, 14, and 21 days.

**Brain Weight**

Brain weight was determined as previously described.7 Brain damage was expressed as the reduction of the ipsilateral (right) hemisphere compared with the contralateral (left) hemisphere.

**Protein Sample Preparation**

Ipsilateral hemispheres were homogenized in extract buffer (0.32 mol/L sucrose, 1 mmol/L EDTA, 5 mmol/L Tris [pH 7.4], 0.1 mmol/L phenylmethylsulfonyl fluoride, 10 μmol/L leupeptin, and 1 mmol/L β-mercaptoethanol) and 0.1% (vol/vol) proteinase inhibitor. Each homogenate was centrifuged at 1000 g for 10 minutes, and the pellet was discarded. The supernatant was centrifuged at 160 000 rpm for 30 minutes and the supernatant collected. All procedures were performed at 4°C. Protein concentrations were determined by bicinchoninic acid assay (Fierce). The samples were kept frozen at −80°C until assay.

**IL-1β Assay**

Protein sample was prepared as described before. IL-1β protein was measured with a rat IL-1β immunoassay ELISA kit, Quantikine M (R&D Systems). Each protein sample (50 μL) was assayed in duplicate. The assay was performed as recommended by the manufacturer.

**RNA Isolation**

Total RNA was isolated from the ipsilateral hemisphere with START-60 (Tel-Test). Brain tissues were treated with RNase-free DNase Promega at 1 U/μg of RNA at 37°C for 30 minutes, followed by phenol-chloroform extraction and ethanol precipitation. RNA quantity was determined by optical density measurement or RNA 6000 Nano-Assay (Agilent Technologies), as recommended by the supplier.

**Quantitative Real-Time RT-PCR**

Total RNA of brain samples from control, HI, and EPO groups at 3, 7, and 14 days after the insult was extracted as described earlier and stored at −80°C. To detect IL-1β gene expression, a TaqMan quantitative 2-step reverse transcription–polymerase chain reaction (RT-PCR) was used to quantify mRNA levels. First-strand cDNA was synthesized with use of a TaqMan RT reagent kit, as recommended by the manufacturer. PCR analyses were conducted with gene-specific primers and fluorescently labeled TaqMan probes (designed by Perkin-Elmer Life Sciences Primer Express software) in a 7700 sequence detector (PE Applied Biosystems). Endogenous control (18S rRNA) was detected by TaqMan ribosomal RNA control reagents. All reagents were purchased from Applied Biosystems. The 18S RNA probe was labeled with a report dye (VIC), and the IL-1β probe was labeled with FAM at the 5′ end and a quencher dye at the 3′ end. The real-time data were analyzed with Sequence Detection Systems software version 1.7. All reactions were performed in duplicate. Each run contained both negative (no template) and positive controls. The following sequence-specific primers were used for IL-1β: forward, 5′-TGAGATCCGTTGAGAACAAC3′; reverse, 5′-CTCACTGCATAGCTCTCTCTCTT-3′; and probe, 5′TGCCAGGCCTGCTCCTGAGATCACCCATG-3′.

**Quantification of TNF-α mRNA**

Total RNA of brain samples from control, HI, and EPO groups at 3, 7, and 14 days after the insult was prepared as described earlier. TNF-α mRNA was determined by a colorimetric microplate assay (Quantikine mRNA kit, R&D Systems) as directed by the manufacturer’s protocol.

**Histology**

At 2 weeks after the insult, pups were perfusion-fixed with 10% neutral buffered formalin (0.1 mol/L phosphate-buffered saline). After perfusion, brains were removed and postfixed in the same solution at 4°C. Ten-micron sections were cut on a cryostat (Leica LM 3050S). Adjacent sections were used for Nissl staining and immunohistochemistry. Nissl staining was achieved by placing the slides in cresyl violet. Immunohistochemistry was performed as previously described1 with an ABC staining system (Santa Cruz Biotechnology). Sections were incubated with primary antibodies for IL-1β, CD4, and CD8 (1:200; Santa Cruz Biotechnology) overnight at 4°C. After being rinsed with phosphate-buffered saline, brain sections were then treated with secondary antibodies at room temperature. The brain sections were then mounted, air-dried, dehydrated, and coverslipped.

**Data Analysis**

Data were expressed as mean±SEM. Statistical differences between the control and other groups were compared by 1-way ANOVA and then, when a significant difference was found, the Tukey-Kramer multiple-comparisons procedure. A value of P<0.05 was considered statistically significant.

**Results**

**rh-EPO Preserved Brain Weight**

The top panel of Figure 1 shows representative photographs of brains taken from control, HI, and EPO treatment groups collected 2 weeks after the hypoxic/ischemic insult. Severe brain atrophy occurred in the ipsilateral hemisphere after hypoxia/ischemia, but rh-EPO attenuated brain injury and preserved brain shape.

The lower panel of Figure 1 demonstrates the brain weight of the 3 groups. Severe brain weight loss developed at 1 week and persisted up to 3 weeks after the hypoxic/ischemic insult (P<0.001 vs control). EPO treatment reduced brain weight loss (P<0.001 vs HI) at all 3 time points examined.

Figure 2 shows coronal sections taken from the level of the striatum 2 weeks after the insult. Extensive cerebral cortical atrophy and damage are shown in the ipsilateral hemisphere of the hypoxic/ischemic animals (Figure 2D through 2F). Less damage is shown in the ipsilateral hemisphere of those animals treated with rh-EPO (Figure 2G through 2I).

**rh-EPO Attenuated the Hypoxia/Ischemia–Induced Increase in IL-1β mRNA Expression**

To evaluate the possibility that rh-EPO might have an anti-inflammatory effect via the inhibition of IL-1β, we examined the gene expression of IL-1β by real-time RT-PCR (Figure 3A). After a hypoxic/ischemic insult, expression of IL-1β mRNA increased at 7 (P<0.05 vs control) and 14 (P<0.05 vs control) days. Treatment with rh-EPO decreased the mRNA levels of IL-1β at 7 (P<0.05 vs HI) and 14 (P<0.05 vs HI) days after the insult.

**rh-EPO Attenuated the Hypoxia/Ischemia–Induced Increase in IL-1β Protein**

Changes in IL-1β protein expression (Figure 3B) were analyzed with an IL-1β immunoassay ELISA kit. After a hypoxic/ischemic insult, the level of IL-1β increased in the ipsilateral hemisphere at 3 (P<0.05 vs control and EPO), 7 (P<0.05 vs control and EPO, ANOVA), and 14 (P<0.001 vs control and EPO) days. Treatment with rh-EPO abolished the increase in
IL-1β at 3, 7, and 14 days after the insult. Immunohistochemistry (Figure 4) revealed strong positive staining for IL-1β in the parieto-occipital cortex of the ipsilateral hemisphere 2 weeks after the insult. Limited staining was observed in the sections taken from rh-EPO–treated animals.

rh-EPO Did Not Affect TNF-α mRNA

Changes in the level of TNF-α mRNA (Figure 5) were analyzed at 3, 7, and 14 days after the insult. No differences in TNF-α mRNA were found among the experimental groups at any time point.

Leukocyte Infiltration

To evaluate leukocyte infiltration, immunohistochemistry for CD4 and CD68 was performed (Figure 6). Leukocyte infiltration was visible in the parieto-occipital cortex of the ipsilateral hemisphere in the HI animals; however, extremely

Figure 1. Brain weight. A, The top panel shows brain photographs of pups from control, hypoxia/ischemia, and hypoxia/ischemia plus rh-EPO treatment groups 2 weeks after the hypoxic/ischemic insult. Severe brain atrophy occurred in the hypoxia/ischemia group (arrow) but not in the rh-EPO–treated group (arrowhead). B, The lower panel shows the summary of brain weight represented by ipsilateral/contralateral hemisphere.

Figure 2. Hypoxic/ischemic changes at the level of the striatum 2 weeks after the insult. Severe brain atrophy occurred in the hypoxia/ischemia group (arrow, B) but not in the rh-EPO–treated group. Also, note the changes in the hypoxia/ischemia animal at a higher magnification (arrow, F). Scale bar, 100 μm (B–I) or 1 mm (A–C).
limited infiltration was observed in sections taken from the rh-EPO–treated animals.

**Discussion**

We have demonstrated that administration of 3 doses (5 U/g body weight daily) of exogenous rh-EPO given 24, 48, and 72 hours after a hypoxic/ischemic insult showed significant brain-protective activity in the neonatal rat. Furthermore, our findings demonstrate that the protection afforded by rh-EPO prevents the secondary, delayed rise in IL-1β and attenuates the infiltration of leukocytes after hypoxia/ischemia.

EPO was first characterized as a hematopoietic growth factor and has been in clinical use by millions of patients for the treatment of anemia.9 During the last 10 years, a prominent role for EPO has been defined in the nervous system, and there is growing interest in the potential therapeutic use of EPO for neuroprotection.10 In vitro, EPO has been shown to protect cultured hippocampal and cortical neurons against glutamate toxicity,11 hypoxia and glucose deprivation,12 and serum deprivation–induced and kainic acid–induced apoptosis.9 EPO has also been shown to be equally beneficial in in vivo experiments. Single and/or multiple systemic adminis-
trations of rh-EPO as a pretreatment or posttreatment have been shown to effectively reduce brain injury after focal ischemia, subarachnoid hemorrhage, and hypoxia/ischemia. The systemic administration of a single dose of rh-EPO also significantly decreased the mean infarct volume after neonatal hypoxia/ischemia and improved long-term neurobehavioral achievements when tested during the subsequent phase of brain maturation and even into adulthood. Previously, we have shown the administration of exogenous rh-EPO before a hypoxic/ischemic insult in neonatal rats to preserve brain morphology and brain weight through inhibition of apoptosis via an upregulation of heatshock protein-27. Here we show that administration of rh-EPO starting 24 hours after the insult was able to attenuate brain injury in much the same manner. This observation of a delayed treatment of rh-EPO being neuroprotective is consistent with a previous study that reported that treatment with rh-EPO beginning 24 hours after embolic stroke significantly improved functional recovery. The neuroprotection afforded by the delayed treatment in this study was not as effective, in terms of brain weight, as our previously reported pretreatment, but it still significantly attenuated brain injury. This suggests that rh-EPO may target events that are delayed or secondary to the initial events surrounding a hypoxic/ischemic insult.

In many in vivo models of central nervous system diseases for which EPO shows a protective effect, inflammation is also

Figure 5. TNF-α mRNA. TNF-α mRNA was determined by a colorimetric microplate assay (Quantikine mRNA kit, R&D Systems) 3, 7, and 14 days after a hypoxic/ischemic insult. No differences in TNF-α mRNA were found among the experimental groups at any time point. Abbreviations are as defined in Figure 1.

Figure 6. Representative photomicrograph of the parieto-occipital cortex of the ipsilateral hemisphere, demonstrating immunohistochemistry for (A–C) CD4 and (D–F) CD68 in (A and D) control, (B and E) HI, and (C and F) HI+rh-EPO. There were some positive cells (arrows) in the HI group (B and E) but extremely limited positive cells in the rh-EPO treatment group (C and F). Scale bar, 50 μm.
an important pathogenic component, induced either by the production of cytokines and chemokines or by leukocyte infiltration or glial activation and proliferation.10 A reduction in the amount of inflammation infiltrate observed in blunt trauma experiments suggests that EPO may play an immunomodulatory role in a manner consistent with known anti-inflammatory agents such as glucocorticoids.13 In a rat model of encephalomyelitis, administration of rh-EPO delayed the onset of disease and decreased clinical scores at peak times.6 The neuroprotection of EPO was attributed to a delay in the increase of TNF-α and an outright decrease in IL-6 levels. In a rat model of cerebral focal ischemia, administration of rh-EPO reduced the influx of inflammatory cells into the region of injury. This decreased the production of proinflammatory cytokines TNF and IL-6, which in turn resulted in a much smaller volume of injury.19

The implication that proinflammatory cytokines play a role in the inflammatory response20 that follows a hypoxic/ischemic insult stems from the existence of a significant association between abnormalities in neurologic outcome and high cytokine concentrations measured from umbilical cord blood taken from infants exposed to chorioamnionitis or perinatal asphyxia.21 IL-1β and TNF-α are early-response cytokines that are synthesized and secreted by microglia, astrocytes, and neurons. The biologic effects of these proinflammatory cytokines include the stimulation and synthesis of other cytokines, induction of leukocyte infiltration, influence of glial gene expression, and stimulation of the synthesis of trophic factors.22 The administration of an rh-IL-1 receptor antagonist (rh-IL-1ra) has been shown to attenuate brain damage caused by a hypoxic/ischemic insult in neonatal rats,3,5 suggesting that IL-1 plays a prominent role in the progression of hypoxia/ischemia–induced brain damage. Studies have shown that mRNA levels of IL-1β peak 4 hours after a hypoxic/ischemic insult in neonatal rats22 and that the bioactivity of IL-1 increases transiently, reaching a peak 6 hours after the insult.5 In addition to this first rise in IL-1β levels, a secondary rise in expression of IL-1β starting at 3 days and extending up to 14 days after the insult has also been observed in the ipsilateral hemisphere.2 In the present study, we also observed increases in the mRNA and protein levels of IL-1β at 3, 7, and 14 days after a hypoxic/ischemic insult. We found that administration of rh-EPO starting 24 hours after the insult attenuated the secondary increase in IL-1β mRNA and protein levels and decreased leukocyte infiltration into the injured area. Whether or not rh-EPO has a direct or indirect effect on IL-1β remains to be determined. However, it has been suggested that in terms of anti-inflammatory effects, rh-EPO may attenuate ischemia-induced inflammation by reducing neuronal death rather than by having direct effects on inflammatory cells that express EPO receptors.19 Certainly, the antiapoptotic effects of rh-EPO23 have been established, and it stands to reason that administration of rh-EPO during the time points, 24 to 72 hours, at which apoptotic changes in this model24 occur would lead to a decrease in cell death. However, additional work in this area is certainly warranted to draw any definitive conclusions concerning the anti-inflammatory actions of rh-EPO. TNF-α levels have been shown to increase 2- to 3-fold in the ipsilateral hemisphere from 3 to 5 hours after injury.22 However, in the present study, we examined TNF-α mRNA levels 3, 7, and 14 days after an insult and found no difference between any of the groups at any time point evaluated. These current data, coupled with data from previous studies, suggests that TNF-α may not play a prominent role in inflammatory responses that occur from 3 to 14 days after a hypoxic/ischemic insult.

A definitive mechanism(s) by which exogenous administration of rh-EPO exerts its neuroprotection has not been fully elucidated. However, its neuroprotective action may involve inhibition of glutamate release, stimulation of angiogenesis, stimulation of neurogenesis, antioxidative actions, antiapoptotic actions, and anti-inflammatory actions.9,18 Some of these actions may be mediated by nuclear factor-κB, Janus kinase 2 (Jak2), Akt, and heatshock protein-27.7 In conclusion, the administration of exogenous rh-EPO starting 24 hours after a hypoxic/ischemic insult showed significant brain-protective activity in the neonatal rat. This neuroprotective activity prevented the secondary, delayed rise in IL-1β and attenuated the infiltration of leukocytes into the ipsilateral hemisphere. Because rh-EPO has been shown to have an excellent safety profile, this agent may be beneficial in treating neonates experiencing from hypoxic/ischemic brain damage in the perinatal period.

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