

Protective effect of erythropoietin in neonatal hypoxic ischemia in mice

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The effect of systemic erythropoietin pretreatment on hypoxic-ischemic injury was examined in neonatal mice. Injury was significantly less in cortex, hippocampus, striatum and thalamus of erythropoietin-treated animals (5 U/g vs vehicle) 24 h after hypoxic ischemia and in all of these regions except hippocampus at 7 days. Activated caspase-3- and activated NFκB-immunoreactive

neurons were observed in the injured areas; these areas were smaller in the erythropoietin group. To our knowledge, this is the first report demonstrating persistent neuroprotective effects of erythropoietin in neonatal mice. *NeuroReport* 14:1757–1761 © 2003 Lippincott Williams & Wilkins.

Key words: Caspase-3; Neuroprotection; NFκB

INTRODUCTION

Erythropoietin (EPO) and its receptor are expressed within the central nervous system, where EPO has neurotrophic and neuroprotective effects [1,2]. Systemic administration of this cytokine before, or 6–24 h after, middle-cerebral artery occlusion (MCO) in adult rats and mice dramatically reduces the volume of infarction [3–5]. The mechanism of protection is unclear, but may involve activation of NFκB and transcription of neuroprotective genes [6]. Hypoxic preconditioning increases expression of hypoxia inducible factor-1, which regulates EPO and its receptor; this may underlie induction of ischemic tolerance [7,8]. The present study evaluates the effect of EPO in a mouse model of neonatal hypoxic-ischemia (HI). We quantified brain injury in Nissl-stained sections 0 h to 7 days after HI and examined the expression of activated caspase-3 (aCas3) and activated NFκB (aNfκB) using immunohistochemistry.

MATERIALS AND METHODS

Low vs high dose: CD-1 mice (Charles River) were injected i.p. on postnatal day 7 (P7) with vehicle (0.1% BSA in saline, $n = 11$), or with EPO, 1 U/g ($n = 11$) or 5 U/g ($n = 11$). HI was induced 1 h later: the right common carotid artery was ligated under isoflurane anesthesia, the incision was infiltrated with local anesthetic and sutured, and animals recovered for 90 min before hypoxic exposure (10% oxygen in nitrogen, 50 min at 36°C). After 7 days, anesthetized animals (chloral hydrate, 500 mg/kg, i.p.) were perfused with 4% paraformaldehyde (PAF). Brains were cryopro-

tected, frozen and sectioned at 50 μm. Neuropathological injury was evaluated in sections stained with cresyl violet.

Time course: Mice were treated on P7 with vehicle ($n = 57$) or EPO, 5 U/g ($n = 54$) 1 h before HI. Littermates received vehicle ($n = 10$) or EPO ($n = 11$) before sham surgery. Animals were perfused 0 h, 6 h, 24 h, 48 h or 7 days after HI. Brains were sectioned as described above. Brain injury was evaluated in cresyl violet-stained sections. Immunohistochemistry for aCas3 (1:500, PharMingen 67341A, conformational epitope exposed by activation) and aNFκB (1:800, Chemicon MAB3026, epitope overlaps the nuclear localization signal of p65) was carried out as described [9]. This protocol was approved by the Johns Hopkins University Animal Care and Use Committee.

Neuropathological evaluation of brain injury in sections stained with cresyl violet was conducted by two investigators, blind, as described previously [9], with minor modifications. Injury was scored as 0–4 for cortex (0: no injury, 1: 1–3 small groups of injured cells, 2: one to several larger groups of injured cells, 3: moderate confluent infarction, 4: extensive confluent infarction) and 0–6 for hippocampus, striatum, and thalamus (0–3 for no, mild, moderate or extensive infarction and 0–3 for no, mild, moderate or extensive atrophy); total score: 0–22. The average of the two investigator's scores was used for statistical analysis. Spearman's rank correlation analysis was used for dose-related effects, and Mann-Whitney U and ANOVA were used for the time-course studies, with statistical significance at $p < 0.05$.

RESULTS

Protective effect of EPO: In vehicle-treated controls, atrophy and neuronal injury were observed in cortex, hippocampus, striatum and thalamus. Administration of 1 U/g or 5 U/g EPO, 1 h before HI, produced a dose-related reduction in brain injury, compared to vehicle, 7 days after HI (Fig. 1).

Time-course of injury: EPO-treated animals exhibited significantly less injury than vehicle-treated animals in all regions examined, 24 h after HI (Figs. 2 and 3). Cell loss and atrophy increased at later time points in all areas of VEH animals. At 7 days, EPO animals exhibited significantly less injury and/or atrophy than VEH animals in cortex, striatum, and thalamus. In the hippocampus, severe injury was observed in both groups. Sham animals showed no injury.

Histological features of injured neurons 0–48 h after HI are shown in Fig. 4. At 0 h, in some cases, nearly all neurons appeared normal. In other cases in both groups, small round condensed cells and unevenly stained round cells were observed. These cells were found in isolated patches or diffusely in cortex, striatum, hippocampus, thalamus, medial habenula, deep mesencephalic nucleus, cuneiform nucleus, superior colliculus and inferior colliculus. Small round condensed cells were also detected in white matter. The unevenly stained round cells present at 0 h had an abnormal appearance but did not have apoptotic bodies; they could not be categorized as apoptotic, necrotic nor hybrid cells and were not considered injured for neuropathological scoring.

At 6 h cells containing round apoptotic bodies and some necrotic cells with condensed, unevenly stained, irregular shapes were found in vehicle-treated and EPO-treated animals. In both groups, there were some slightly abnormal cells like those observed at 0 h.

At 24 h, numerous apoptotic cells with round, evenly stained apoptotic bodies, necrotic cells that were pyknotic, ruffled and unevenly stained or karyorrhexic, and hybrid cells with morphologic features of both types of cell death were found in a number of areas in vehicle-treated animals and in more limited areas in EPO-treated animals. The slightly abnormal cells detected 0 h and 6 h after HI were no longer present.

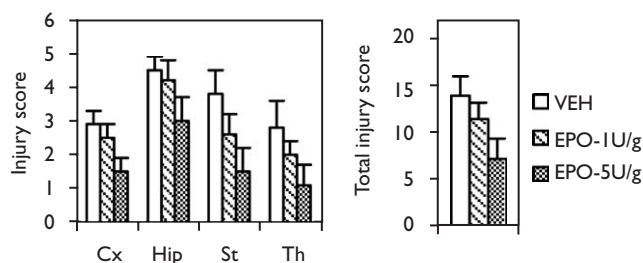


Fig. 1. EPO produced a dose-related reduction in injury in various regions 7 days after HI. Injury score (mean \pm s.e.m.) determined as described in the methods. Linear regression was significant in cortex (Cx, $p < 0.01$), striatum (St, $p < 0.01$), thalamus (Th, $p = 0.05$) and total score ($p < 0.01$); trend observed in hippocampus (Hip, $p = 0.096$).

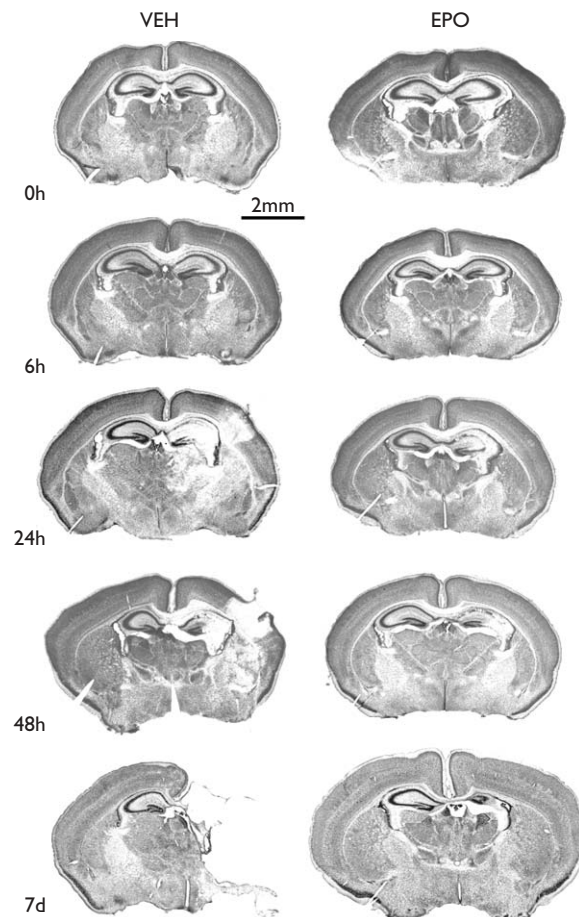


Fig. 2. Time course of injury in representative Nissl-stained sections. Injury produced by HI was remarkably milder in EPO-treated animals than in vehicle-treated animals, beginning at 24 h.

At 48 h, apoptotic cells, necrotic cells and hybrid cells were found in many areas of both groups of animals. At 7 days, many necrotic cells, some apoptotic cells, and hybrid cells were found in vehicle- and EPO-treated animals (not shown).

Activated-caspase 3 and activated-NF κ B immunohistochemistry:

A few aCas3-immunoreactive (aCas3(+)) neurons were observed in animals subjected to sham surgery, but more were found after HI. In vehicle and EPO groups at 0 h, some aCas3(+) neurons were seen in cortex, striatum, hippocampus and thalamus on the injured side. At 6 hours after HI, many intensely stained aCas3(+) neurons were found in the striatum; aCas3(+) neurons were also found in cortex, hippocampus and thalamus on the injured side. More aCas3(+) neurons were apparent in vehicle-treated animals than in EPO-treated animals, 6 h after HI (Fig. 5). At 24 h and 48 h, aCas3(+) neurons were apparent in the penumbra and in other areas among non-injured cells. At 7 days after HI, there were almost no intensely aCas3(+) neurons.

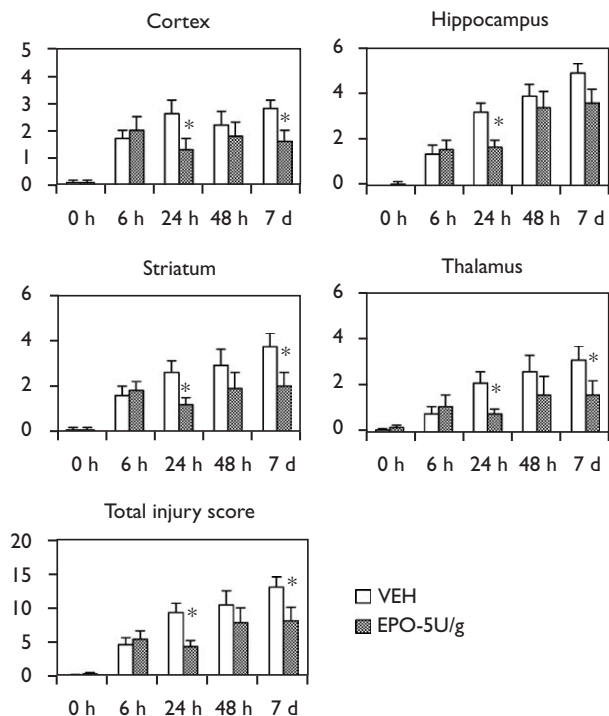


Fig. 3. Semiquantitative analysis of brain injury 0 h to 7 days after HI. Injury scores were significantly smaller in all regions of EPO-treated animals 24 h after HI and in all regions except hippocampus at 7 days. * $p < 0.05$, Mann-Whitney U.

Few activated NF κ B-immunoreactive (aNF κ B(+)) cells were present in either group 0 h after HI. At 6 h, aNF κ B(+) cells were detected in cortex, striatum, hippocampus, and laterodorsal thalamus. Immunoreactivity was found mostly in the cytoplasm, but in some cells in both vehicle- and EPO-treated animals, aNF κ B immunoreactivity was also found in the nucleus (Fig. 6). The cells with aNF κ B(+) nuclei were shrunken and the surface of the nucleus was rough. aNF κ B immunoreactivity reached a peak 24 h after HI; few positive cells were present at 48 h.

DISCUSSION

In this study, pretreatment with 1 or 5 U/g EPO reduced brain injury caused by unilateral hypoxic-ischemic insult in 7-day-old mice in a dose-dependent fashion. Quantitative neuropathological rating showed progressive evolution of injury in control animals over 7 days after the insult. EPO did not reduce early signs of neuronal injury in any region at 6 h, but did significantly protect cortex, striatum, thalamus and hippocampus when assessed at 24 h. At 7 days EPO provided significant protection in cortex, striatum and thalamus but not in hippocampus. The observation that EPO did not block the earliest signs of morphological injury up to 6 h following HI but provided protection at later time points suggests that a delay is required for the cytokine to induce its neuroprotective effect or that it acts at a point downstream in the cascade of neuronal damage. Our results are consistent with a recent report showing that EPO protected neonatal rats from HI injury when examined 72 h later [10]. Studies *in vitro* show that there is a delay of 4–

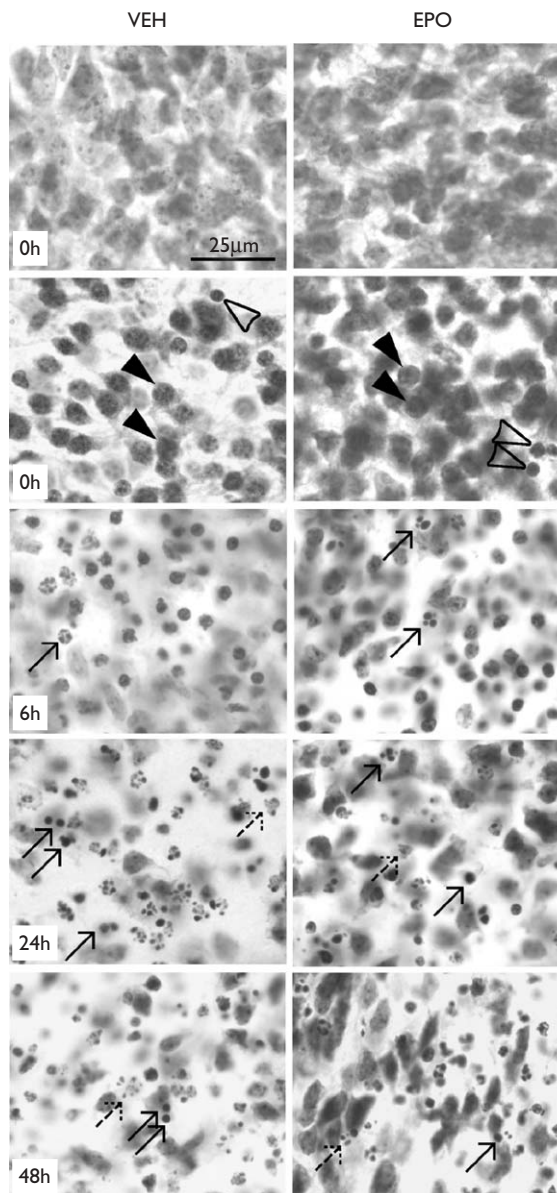


Fig. 4. Cytopathology in striatum 0–48 h after HI in vehicle- and EPO-treated animals, contralateral (top row) or ipsilateral to the ligation (remaining rows). At 0 h many unevenly stained round cells (filled arrowheads) and small round condensed cells (open arrowheads) were detected on the ligated side. At later time points, apoptotic cells with evenly stained round chromatin clumps (solid arrows) and necrotic cells with small, pale chromatin clumps (broken arrows) were observed; these cells were more frequently observed in vehicle-treated than in EPO-treated animals (Cresyl violet).

8 h in the onset of protection against glutamate toxicity after EPO administration that may be related to synthesis of protein [11]. In adult mice, 5 U/g EPO produces peak serum levels ~4 h later that remain elevated for 20–30 h [4]. Thus, a longer delay in onset of neuroprotection would be anticipated *in vivo*.

A time-dependent continuum of apoptotic and necrotic morphological signs have been reported after HI, and

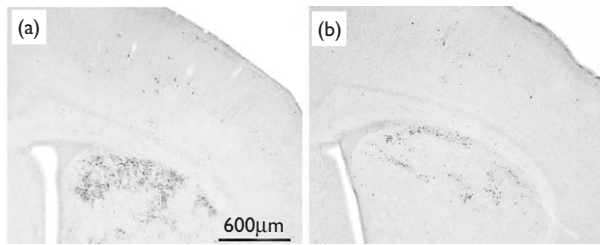


Fig. 5. Activated caspase 3 immunohistochemistry in the striatum, 6 h after HI, in vehicle-treated (a) and EPO-treated (b) animals. Activated caspase 3-immunoreactive cells were distributed across larger areas in vehicle-treated animals than in EPO-treated animals.

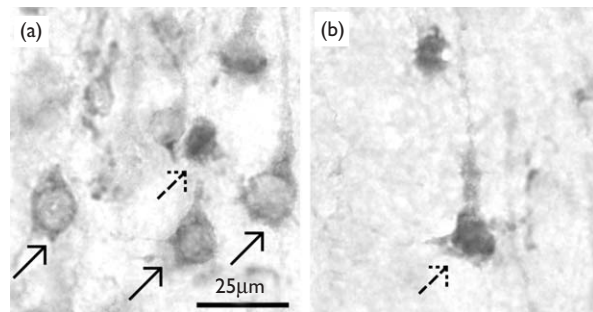


Fig. 6. Activated NFκB immunohistochemistry in the cortex, 6 h after HI. Activated NFκB immunoreactivity was in the cytoplasm (a, solid arrows) of many neurons in the injured regions, but in some cells in both EPO-treated (shown) and vehicle-treated animals it was also found in the nucleus (a,b, broken arrows).

studies of apoptosis in the neonatal rat indicate that cells continue to commit to cell death for several weeks after the injury [9,12,13]. The prolonged evolution of injury *in vivo* suggests that administration of EPO after HI may provide some protection. Delayed EPO administration, up to 6 h after focal brain ischemia, reduced infarct volume in adult rats [4]. Thus, the time window for EPO administration that provides neuroprotection may be long enough to be clinically useful.

Chronic intracerebroventricular administration of EPO prevents ischemia-induced learning disability in adult gerbils and preserves neurons in the hippocampus [14]. In the present study, an initial decrease in injury scores in the hippocampus of EPO animals did not persist 7 days after HI. The hippocampus is especially vulnerable to HI and protection may require a greater dose of EPO than that used in the present study. Apoptosis persists in the hippocampus of neonatal rats up to 7 days after HI [9], and the single dose of EPO used here may not be sufficient to protect against apoptosis at later time points.

The mechanism of this neuroprotective effect remains unclear; however, several possible mechanisms have been suggested. In cultured neurons, EPOR-mediated activation of Jak2 leads to phosphorylation of IκB and nuclear translocation of NFκB [6]. In our study, aNFκB was found in the cytoplasm and in the nucleus of neurons within injured regions, but was not observed in neurons in other areas. The subcellular distribution of aNFκB positive cells

did not show remarkable differences between vehicle- and EPO-treated animals, in contrast to the study *in vitro* [6]. Further studies are required to determine mechanisms of EPO-mediated neuroprotection in developing mice.

EPO may limit apoptosis by maintaining or increasing expression of Bcl-x_L, by reducing caspase activation, or through a signaling cascade that involves increased Akt1 phosphorylation or transient increases in intracellular calcium [11,14,15]. In the present study, aCas3 immunoreactive neurons were observed as early as 0 h after HI and were still abundant 48 h after HI, which indicates a prolonged role of aCas3 in HI, as reported in developing rats [9]. The areas in which aCas3(+) neurons were detected were larger in VEH animals than EPO animals, which suggests that EPO limits the activation of caspase 3 and therefore reduces apoptosis. The expression of Bcl family members that limit apoptosis would be an interesting focus for further studies in this model.

Although EPO pretreatment is protective within the dose range used in the present study, high systemic EPO levels may be detrimental. In adult transgenic mice with 4-fold over-expression of EPO in brain and elevated cerebral blood flow (CBF), cerebral infarct volumes were smaller after permanent MCO [16]. In contrast, infarct volumes were larger in mice that over-express EPO in both brain and plasma; these mice had normal CBF and elevated hematocrit. Thus, while modest increases in EPO improve CBF, possibly by increasing NO synthesis [17], high doses of EPO can exacerbate ischemic brain injury, possibly through increased blood viscosity. The dose of EPO used in the present study is protective, but there is likely to be an upper limit to the protective range.

CONCLUSION

Systemic administration of EPO protects the neonatal mouse brain against hypoxic-ischemic injury in a dose-dependent fashion. The mechanism of neuroprotection remains unclear, although some differences in aCas3 expression were noted between VEH and EPO animals. Further studies using post-HI administration of EPO will be required to evaluate the therapeutic potential of this drug in neonatal hypoxic ischemia.

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