Erythropoietin Treatment Exacerbates Moderate Injury after Hypoxia-Ischemia in Neonatal Superoxide Dismutase Transgenic Mice

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Keywords

Brain injury · Erythropoietin · Hypoxia-ischemia · Mouse · Superoxide-dismutase

Abstract

The neonatal brain is highly susceptible to oxidative stress as developing endogenous antioxidant mechanisms are overwhelmed. In the neonate, superoxide dismutase (SOD) overexpression worsens hypoxic-ischemic injury due to H₂O₂ accumulation in the brain. Erythropoietin (EPO) is up-regulated in 2 phases after HI, early (4 h) and late (7 days), and exogenous EPO has been effective in reducing the injury, possibly through reducing oxidative stress. We hypothesized that exogenous EPO would limit injury from excess H₂O₂ seen in SOD1-overexpressing mice, and thus enhance recovery after HI. We first wanted to confirm our previous findings in postnatal day 7 (P7) SOD-tg (CD1) mice using a P9 model of the Vannucci procedure of HI with SOD-tg mice from a different background strain (C57Bl/6), and then determine the efficacy of EPO treatment in this strain and their wild-type (WT) littermates. Thus, mice overexpressing copper/zinc SOD1 were subjected to HI, modified

for the P9 mouse, and recombinant EPO (5 U/g) or vehicle (saline) was administered intraperitoneally 3 times: at 0 h, 24 h, and 5 days. Injury was assessed 7 days after HI. In addition, protein expression for EPO and EPO receptor was assessed in the cortex and hippocampus 24 h after HI. With the moderate insult, the SOD-tg mice had greater injury than the WT overall, confirming our previous results, as did the hippocampus and striatum when analyzed separately, but not the cortex or thalamus. EPO treatment worsened injury in SOD-tg overall and in the WT and SOD-tg hippocampus and striatum. With the more severe insult, all groups had greater injury than with the moderate insult, but differences between SOD-tg and WT were no longer observed and EPO treatment had no effect. Increased protein expression of EPO was observed in the cortex of SOD-tg mice given recombinant human EPO compared to SOD-tg given vehicle. This study confirms our previous results showing greater injury with SOD overexpression in the neonatal brain after HI at P7 in a different strain. These results also suggest that EPO treatment cannot ameliorate the damage seen in situations where there is excess H₂O₂ accumulation, and it may exacerbate injury in settings of extreme oxidative stress.

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Introduction

The enzyme Cu/Zn-superoxide dismutase (SOD1) catalyzes the removal of superoxide free radicals generated from mitochondrial respiration, and overexpression of SOD1 has been shown to be beneficial in models of adult ischemia [1]. However, we found overexpression of SOD1 to be detrimental in the setting of neonatal hypoxia-ischemia (HI) [2]. This lack of benefit from increased SOD1 is likely due to the accumulation of H_2O_2 seen in the neonatal brain [3] but not in the adult brain [4]. However, in addition to the detrimental effects of H_2O_2 on cell integrity and survival, the production of H_2O_2 modulates the stabilization and transcriptional activation of HIF-1 α [5], which regulates a number of protective genes that are responsible for homeostasis and also protection and repair after severe oxidative stress in neonatal ischemia. Low levels of H_2O_2 , in particular, stabilize and/or upregulate HIF-1 α , and thereby mediate HI preconditioning protection [6]. One of the genes regulated by HIF-1 α is erythropoietin (EPO). EPO has been shown to reduce cellular injury in vitro [7–10] and to reduce brain injury in in vivo models of neonatal stroke [11–14] and HI [15–19]. EPO may prevent cell death by inhibiting apoptosis. EPO binds to 2 cell surface receptors that form a homodimer that activates Jak2 kinase to phosphorylate both Jak2 and EPO receptor (EPOR). This activates multiple signaling cascades, including NF- κ B and Stat 5, which move into the nucleus and act as transcription factors for the antiapoptotic genes *Bcl-2* and *Bcl-xL*. EPO also reduces necrotic cell death induced by inflammation and it may promote neuroregeneration and angiogenesis [20].

While therapeutic hypothermia has become the standard of care for newborns with hypoxic-ischemic encephalopathy, it provides incomplete protection to many of the infants treated and may be ineffective in those with exacerbating factors such as intrauterine-acquired infection [21]. EPO shows promise as another therapy for neonatal hypoxic-ischemic injury [22]. A nonhuman primate study [23] and small clinical trials using a multidose regimen based on animal studies have established safety and pharmacokinetics when combined with therapeutic hypothermia [24], and this suggests that EPO may result in less MRI brain injury and improve 1-year motor function [25].

In more severe brain injury after neonatal HI, there is increased oxidative stress. SOD1-overexpressing mice had increased oxidative stress due to the accumulation of H_2O_2 [3]. We hypothesized that injury after neonatal HI in situations of excess H_2O_2 accumulation could be ameliorated with exogenous EPO. To test this hypothesis, we

Fig. 1. Histological injury scores for the whole hemisphere after HI with 50-min hypoxia (scale 0–33). The horizontal line represents the median score. SOD-tg + vehicle was more injured than WT + vehicle ($p < 0.03$), as was WT + EPO ($p < 0.05$) and SOD-tg + EPO ($p = 0.01$).

chose to examine SOD1-overexpressing mice that accumulate excess H_2O_2 , and we tested whether EPO could indeed reverse the severe damage seen in these brains.

Materials and Methods

Mice

Transgenic mice overexpressing human SOD1 (hSOD1-tg) and their wild-type (WT) littermates (C57/Bl6) were used for this study [26]. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at UCSF, in accordance with NIH guidelines for the Care and Use of Laboratory Animals. Genotyping was performed by gel electrophoresis and protein staining for SOD1 as previously described [27].

Hypoxia-Ischemia

For histological analysis, mice underwent HI as previously described [28], with modifications for the postnatal day 9 (P9) mouse model [29]. Specifically, while under isoflurane anesthesia, the left carotid artery was exposed and permanently ligated by electrocoagulation. After a 1-h recovery period with the dam, the mice were placed in chambers floating in a water bath at 37°C and exposed to 10% oxygen and 90% nitrogen for either 50 min ($n = 109$: 77 WT and 32 SOD-tg) or 60 min ($n = 48$: 22 WT and 26 SOD-tg). Recombinant EPO (5 IU/g, R&D Systems) or vehicle (saline) was admin-

istered 3 times, based on previous experiments in rats: immediately, at 24 h, and at 5 days after hypoxia [30]. Mice were anesthetized with Euthasol (Virbac AH, Fort Worth, TX, USA) 7 days after HI and perfused transcardially with cold 4% paraformaldehyde in 0.1 M PB; brains were removed, postfixed overnight, and stored at 4°C in 0.1 M PB until sectioning by Vibratome. Alternate 50- μ m sections through the forebrain were collected for cresyl violet (Nissl) and Perl's (iron) histological stains to assess the degree of injury.

For Western blots, mice underwent HI as above, except all underwent 60 min of hypoxia and received only the immediate administration of EPO ($n = 8$ WT; $n = 6$ SOD-tg) or saline ($n = 5$ WT; $n = 4$ SOD-tg). Sham mice ($n = 3$) were anesthetized and the left carotid artery was exposed but not ligated. Twenty-four hours after HI, mice were anesthetized with Euthasol, their brains rapidly removed, and the cortex and hippocampus from the injured side were dissected on a cold surface, frozen immediately, and then stored at -80°C .

All sections were examined for injury scoring to obtain a comprehensive view of brain injury (10–15 sections each, for both the cresyl violet and iron-stained sections). We modified our injury scoring system, previously used for the P7 mouse model, for the P9 model so as to reflect the different pattern of injury seen in the older animals. Specifically, we expanded the scoring of the striatum and added the thalamus; these are regions that demonstrate greater injury in the P9 mouse. Thus, 11 regions were assigned a score of 0–3, with 0 = no injury, 1 = small focal areas of cell loss and iron deposition, 2 = patchy areas of cell loss in multiple areas of the region, and 3 = cystic infarction, with a total score of 0–33 for an injured hemisphere. The regions scored were the anterior, middle, and posterior cortex; CA1, CA2, CA3, and the dentate gyrus of the hippocampus; the anterior, middle, and posterior striatum; and the thalamus.

Fig. 3. Histological injury scores for the striatum (**a**, scale 0–9) and thalamus (**b**, scale 0–3) after HI with 50-min hypoxia. The horizontal line represents the median score. **a** In the striatum, SOD-tg + vehicle was more injured than WT + vehicle ($p < 0.05$), as was WT + EPO ($p < 0.04$) and SOD-tg + EPO ($p < 0.02$). **b** In the thal-

amus, there were no differences between groups. Representative photomicrographs of injured striatum and thalamus stained with cresyl violet (top row) or Perl's iron stain (bottom row) are shown below each group. Scale bar, 1 mm.

Western Blots

Brain tissue was homogenized with Dounce homogenizers using NE-PER cytoplasmic extraction reagents, with the addition of HALT protease and phosphatase inhibitors, according to the manufacturer's protocol (Thermo Scientific, Rockford, IL USA). After electrophoresis, proteins were transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). After blocking in 5% non-fat dry milk in TBST for 1 h, the membranes were incubated in blocking buffer containing antibodies recognizing EPO (1:500), EPOR (1:500), and β -actin (1:2,000) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Appropriate secondary HRP-conjugated antibodies (1:2,000) were used, and the signal was visualized with enhanced chemiluminescence. ImageJ was used to measure the optical density (OD) of the blots on radiographic film after scanning.

Statistical Analysis

Injury scores were analyzed by the Mann-Whitney U test. The OD of Western blots normalized to β -actin was analyzed by one-

way ANOVA with the Tukey test for multiple comparisons or the t test, and are expressed as mean $\pm$ SEM normalized to WT sham. Statistical analyses were performed with GraphPad Prism v7.0 (San Diego, CA, USA). $p < 0.05$ was considered statistically significant.

Results

In the group subjected to moderate insult (50 min of hypoxia), the SOD-tg mice had greater injury than their WT littermates, confirming our previous study. EPO treatment did not decrease injury in SOD-tg or WT mice. Indeed, compared to WT + vehicle, injury was worse in both the WT mice and the overexpressors after EPO

Fig. 4. Histological injury scores for the whole hemisphere after HI with 60-min hypoxia (scale 0–33). The horizontal line represents the median score. There were no differences between treatment groups.

(Fig. 1). The hippocampus showed similar results when analyzed separately. The SOD-tg mice had greater injury than the WT mice, and the injury was even worse in the SOD-tg + EPO and WT + EPO groups than in the WT + vehicle group (Fig. 2a). The cortex, however, showed milder injury than the other regions when analyzed separately, and injury here was similar in SOD-tg and WT mice; EPO treatment did not affect cortex injury (Fig. 2b). In the striatum, results were also similar as in the whole hemisphere, that is the SOD-tg mice had greater injury than the WT mice and the injury was worse in the SOD-tg + EPO and WT + EPO groups than in the WT + vehicle group (Fig. 3a). The thalamus displayed more consistent injury across groups, as they all had a median score of 1 (Fig. 3b).

Injury increased substantially in the groups subjected to the more severe insult (60 min of hypoxia), but there were no significant differences between treatment groups (Fig. 4). There were also no differences in the hippocampus (Fig. 5a), cortex (Fig. 5b), striatum (Fig. 6a), or thalamus (Fig. 6b) when analyzed separately.

We expanded our injury scoring system to reflect this different pattern of injury, increasing the potential score

for the striatum and adding the thalamus (Table 1). With 50 min of hypoxia, the median injury score for all 56 WT + vehicle brains was 14, slightly below the theoretical median of 16.5. The hippocampus median for all WT brains was 6, precisely at the theoretical median, and the cortex median for all WT brains was 3, well below the theoretical median of 4.5. The striatum median for all WT brains was 3, below the theoretical median of 4.5, but above that previously seen in the P7 model [31]. The thalamus median for all WT brains was 1, again below the theoretical median of 1.5, but the majority (62.5%) displayed histological injury, unlike in the P7 model. Mortality was much lower in the P9 mouse model than in the P7 model, where it was typically 50% with the C57bl/6 strain [32].

Expression of EPO and EPOR after HI

In the cortex, EPO expression was higher 24 h after HI in the SOD + EPO group than in the SOD + vehicle group ($p = 0.01$; Fig. 7a), as was EPOR expression ($p < 0.05$; Fig. 7b). In the hippocampus, there were no differences in EPO or EPOR expression (Fig. 7c, d).

Mortality

There was no mortality with the milder insult of 50 min of hypoxia. With the greater insult (60 min of hypoxia), 2 male WT and 1 female SOD-tg mouse died.

Sex Differences

There were no differences in injury scores between male and female mice when analyzed separately.

Discussion

With the more moderate injury induced by the 50-min exposure to hypoxia after carotid ligation in P9 mice, we confirmed our previous result seen in P7 CD1 mice that the SOD-tg mice had greater injury than their WT littermates, despite the different background strain and HI paradigm. This was not seen with a more severe insult of 60-min exposure to hypoxia. We used a historical comparison of the P7 versus P9 models of HI. Specifically, in the P7 model, the cortex and hippocampus sustain the bulk of the injury, with cystic infarction in the cortex being common; in the hippocampus, injury is primarily to the pyramidal cell layer with the dentate gyrus being relatively spared. Injury is often comparatively mild in the striatum and thalamus in the P7 rodent [32, 33].

Since the overall injury was relatively mild with 50 min of hypoxia, we repeated the experiments with a stronger

Fig. 5. Histological injury scores for the hippocampus (**a**, scale 0–12) and cortex (**b**, scale 0–9) after HI with 60-min hypoxia. The horizontal line represents the median score. There were no differences between groups in the hippocampus or cortex. Representa-

tive photomicrographs of injured hippocampus and cortex stained with cresyl violet (top row) or Perl's iron stain (bottom row) are shown below each group. Scale bar, 1 mm.

insult (i.e., 60 min of hypoxia). Injury was indeed increased and the appearance of cystic infarction also increased accordingly; 22 out of 45 brains (46%) had cystic infarction in ≥ 1 regions. The WT + vehicle brains had the greatest increase in injury with the longer hypoxia exposure, sustaining a 50% increase in median score for the whole hemisphere as well as in the hippocampus, cortex, striatum, and thalamus. The increase in injury was more moderate in the SOD-tg + vehicle (15%), WT + EPO (25%), and SOD-tg + EPO (8%) groups. These groups showed greater injury in the 50-min hypoxia experiments in comparison to the WT + vehicle group, suggesting that the increased oxidative stress did not further increase injury in these groups beyond that of the WT + vehicle group. Consequently, differences between treatment

groups were lost with the more severe insult, perhaps suggesting that a greater susceptibility to oxidative stress can be seen not only between P7 (preterm human model) and adult rodents [4], but also between the preterm and term models (P9). The Western blots were all done with 60 min of hypoxia, limiting these results to the more severe injury paradigm.

Despite other animal studies showing improvements with EPO, especially in stroke models [13], 2 studies on rats treated with hypothermia and EPO showed no added improvement of EPO over hypothermia only [34, 35]. Much of the work showing improvement with EPO in HI has been in the P7 rat model [15, 36, 37]. The difference in age as small as that between P7 and P9 has a significant effect on outcome. One study in P9 mice showed

Fig. 6. Histological injury scores for the striatum (**a**, scale 0–9) and thalamus (**b**, scale 0–3) after HI with 60 min of hypoxia. The horizontal line represents the median score. There were no differences between groups in the striatum or thalamus. Representative

photomicrographs of injured striatum and thalamus stained with cresyl violet (top row) or Perl's iron stain (bottom row) are shown below each group. Scale bar, 1 mm.

Table 1. Median injury score (range) and *p* value for each treatment group

Injured region	50-min hypoxia				60-min hypoxia			
	WT + vehicle	SOD-tg + vehicle	WT + EPO	SOD-tg + EPO	WT + vehicle	SOD-tg + vehicle	WT + EPO	SOD-tg + EPO
Whole hemisphere	14 (3–33)	17 (10–22)	16 (9–25)	18.5 (10–21)	21 (16–28)	19.5 (16–22)	20 (14–28)	20 (14–33)
Hippocampus	6 (0–12)	7 (5–9)	7 (3–11)	8 (5–9)	9 (7–12)	8 (7–9)	9 (7–12)	8 (7–12)
Cortex	3 (1–9)	3.5 (3–6)	3 (1–7)	4.5 (2–6)	6 (3–6)	4.5 (3–6)	5 (2–6)	5 (3–9)
Striatum	3 (0–9)	5 (2–6)	5 (1–6)	5 (2–6)	6 (4–7)	5 (3–6)	6 (2–7)	6 (1–9)
Thalamus	1 (0–3)	1 (1–2)	1 (0–2)	1 (1–2)	2 (2–3)	2 (2–2)	2 (2–3)	2 (1–3)

Fig. 7. Protein expression in the cortex (**a, b**) and hippocampus (**c, d**) 24 h after HI normalized to WT sham. **a** EPO expression is higher in SOD-tg + EPO (* $p < 0.01$) than in SOD + vehicle. **b** EPOR expression is also higher in SOD-tg + EPO (* $p < 0.05$) than in SOD + vehicle. **c** EPO expression in the hippocampus did not differ across treatment groups. **d** EPOR expression was not different across treatment groups. WT Veh, wild-type + vehicle; WT EPO, wild-type + erythropoietin; SOD Veh, superoxide dismutase + vehicle; SOD EPO, superoxide dismutase + erythropoietin.

improvements only in female mice [18]. The injury created in these models is not as severe as that seen in this study at a different age. Indeed, an association of age with the severity of injury has shown mixed results in human studies. In a recent study of early prophylactic high-dose EPO in very preterm infants, there were no statistically significant differences in neurodevelopmental outcomes at 2 years, despite positive changes in MRI at earlier ages between the placebo- and EPO-treated groups [38].

In this study, there was no improvement in injury scores with EPO treatment, and with prolonged hypoxic

insults, there was worsened injury, despite an increase in EPO levels in the cortex. These findings suggest that in situations of extreme hypoxic-ischemic damage, there is little chance of rescue with these neuroprotective therapies. In situations with more moderate insults, therapies such as EPO may be able to impact the trajectory of damage. These important findings suggest that EPO may not be beneficial in situations of extreme oxidative stress when used immediately after the insult. Possibly, exogenous EPO treatment during the early phase interferes with endogenous repair responses and consequently

worsens the injury. Given the variety of results, continued studies with EPO, especially those on hypoxic-ischemic encephalopathy, are warranted. Attention should be paid to differences in developmental age, species, and strain, as well as the dosage and timing of EPO administration. In particular, delayed rather than immediate EPO treatment has greater clinical relevance and may enhance repair mechanisms, especially when multiple doses are given over days or weeks [14].

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Disclosure Statement

The authors have no conflict of interests to disclose.

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