Beneficial Effect of Erythropoietin on Sensorimotor Function and White Matter After Hypoxia-Ischemia in Neonatal Mice

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ABSTRACT: There are mixed reports on the neuroprotective properties of erythropoietin (EPO) in animal models of birth asphyxia. We investigated the effect of EPO on short- and long-term outcome after neonatal hypoxic–ischemic (HI) brain injury in mice and compared the effect of two different dose regimens of EPO. Nine-day-old mice were subjected to HI, and EPO was injected i.p. at 0, 24, and 48 h after HI in a dose of either 5 or 20 kU/kg. Paw preference in the cylinder rearing test (CRT) was used as a measure of sensorimotor function. Only in female mice, administration of EPO at 5 kU/kg but not 20 kU/kg improved sensorimotor function, reduced striatum atrophy and hippocampal lesion volume, and enhanced myelin basic protein (MBP) staining as determined at 4 and 9 wk after HI. In addition, at 72 h after HI, more Ki67 cells were found in the subventricular zone and dentate gyrus after EPO 5 kU/kg treatment, indicating an increase in progenitor cell proliferation. In conclusion, EPO improves sensorimotor function after neonatal HI and protects against striatum atrophy, hippocampal injury, and white matter loss. The protective effect of EPO is dose-dependent and only present in females. (Pediatr Res 69: 56–61, 2011)

In this study, a mild HI brain injury was induced by unilateral carotid artery ligation followed by 10% O₂ for 45 min, leading to reproducible brain injury (23). The mice were treated with 5 or 20 kU/kg EPO or vehicle at 0, 24, and 48 h after the insult. We investigated the contribution of EPO on short- and long-term sensorimotor function and histological outcome in neonatal mice with a special focus on gender.

METHODS

Animals. Experiments were performed in accordance with international guidelines and approved by the Experimental Animal Committee of the University Medical Center Utrecht. C57BL/6 J mice were bred at the animal facility of Utrecht University, and surgery was performed at the age of 9 d. Mice were housed at 21–23°C in an automatic 12-h light-dark cycle and weaned at the age of weeks. Food and water were available ad libitum. All analyses were performed in a blinded setup.

Experimental model. On postnatal d 9 (P9), 115 mouse pups (58 males and 57 females) underwent HI. The right common carotid artery was isolated and electrocauterized under anesthesia [isoflurane (IsoFlo; Abbott Laboratories Ltd., Berkshire, England): 5% induction, 1.5% maintenance in O₂:N₂O; 1:1]. Pups were allowed to recover for 1.5 h, followed by 45-min hypoxia (humidified 10% O₂, 90% N₂; 35°C). Sham controls underwent anesthesia and skin incision only. Seven mice (four males and three females, 6.1%) died from hypoxia before randomization and treatment, and no mortality occurred after treatment.

EPO treatment. To examine the efficacy of EPO (EPREX, Janssen-Cilag B.V., The Netherlands) treatment in P9 mice after HI, mice were randomly assigned to three groups and treated i.p. with EPO (5 or 20 kU/kg) or vehicle at 0, 24, and 48 h after hypoxia.

Abbreviations: CA, cornu ammonis; CRT, cylinder rearing test; DG, dentate gyrus; EPO, erythropoietin; EPOR, erythropoietin receptor; HE, hematol- lin-eosin; HI, hypoxia-ischemia; MAP-2, microtubule-associated protein 2; MBP, myelin basic protein; P9, postnatal d 9; SVZ, subventricular zone

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Animal groups. Short-term study: sham $n = 6$ (three males and three females); vehicle $n = 16$ (eight males and eight females); EPO5 (5 kU/kg) $n = 12$ (eight males and eight females); and EPO 20 (20 kU/kg) $n = 16$ (eight males and eight females). Long-term study: sham $n = 10$ (five males and five females); vehicle $n = 20$ (10 males and 10 females); EPO5 (5 kU/kg) $n = 20$ (10 males and 10 females); and EPO 20 (20 kU/kg) $n = 20$ (10 males and 10 females).

Histology. For the short-term study, mice were terminated by 300 mg/kg pentobarbital natrium (Alfasan International BV, Woerden, Holland) at 72 h after HI and perfused with 4% paraformaldehyde in PBS. Brains were paraffin embedded, and coronal sections (8 μm) were cut out between −0.10 and −1.18 mm from bregma. Deparaffinized sections were incubated with mouse anti-microtubule-associated protein 2 (MAP-2; 1:1000, Sigma Chemical Co.-Aldrich, Steinheim, Germany), rabbit-anti-cleaved caspase-3 (1:800, Cell Signaling), rabbit-anti-Ki67 (1:400, Abcam) followed by biotin-labeled secondary antibodies and revealed using Vectastain ABC kit (both Vector-Labs, Burlingame, CA) and diaminobenzamidine (Sigma Chemical Co.-Aldrich).

MAP-2 loss was quantified using photoshop CS 4 (Adobe Systems, Mountain View, CA). Both hemispheres (at −0.10 and −1.12 mm from bregma), stratum at (−0.10 mm from bregma), and hippocampus alone (at −1.12 mm from bregma) were outlined on full-section images, and MAP-2 loss was calculated as $100 - [(\text{MAP-2 positive area ipsilateral by contralateral hemisphere}) \times 100]$. For each animal, three consecutive sections on the same levels were calculated and averaged as the final ratio.

Cleaved-caspase-3 staining was scored using a semiquantitative approach in the ipsilateral hippocampal area with the following scale: 0 = 90–100% caspase-3* cells (or no brain tissue), 1 = 50–90% caspase-3* cells, 2 = 10–50% caspase-3* cells, and 3 = 0–10% caspase-3* cells. Scoring was divided into four regions: cornu ammonis (CA), CA1, CA2, CA3, and CA4. For each animal, the cells in five randomized sections between −1.12 and −1.68 mm from bregma were scored and averaged as the final score.

Progenitor cell proliferation was determined by Ki67 staining. The number of immunopositive cells was counted on the level of ipsilateral subventricular zone (SVZ; between −0.10 and −0.56 mm from bregma) and dentate gyrus (DG; between −1.12 and −1.68 mm from bregma). For each animal, five randomized sections were counted and averaged as the final cell number.

For the long-term histological study, mice were terminated at 10 wk after HI as described for the short-term study. Brains were mounted in paraffin from which coronal sections (8 μM) were cut between −0.14 and −0.58 mm from bregma. Brain sections were stained with hematoxylin-eosin (HE). Deparaffinized sections were then incubated with mouse-anti-myelin basic protein (MBP; 1:1600, Sternberger Monoclonals, Lutherville, MD) followed by biotin-labeled secondary antibodies, and staining was revealed using Vectastain ABC kit (Vector-labs, Burlingame, CA) and diaminobenzamidine (Sigma Chemical Co.-Aldrich). For HE staining, both hemispheres were outlined in paraffin from which coronal sections (8 μM) were cut between −0.14 and −1.58 mm from bregma, stratum at (−0.14 mm from bregma), and hippocampus alone (at −1.58 mm from bregma) were outlined on full-section images, and the ratio of ipsi- and contralateral areas was calculated with photoshop CS 4. For MBP staining, the images were made binary, and the white proportion of right (ipsilateral) forepaw contacts was calculated as follows: paw preference = (right − left)/(right + left) × 100 (26).

RESULTS

MAP-2 loss. Loss of ipsilateral MAP-2 staining as determined at 72 h after HI was used as a marker of early gray matter area loss. In our P9 model of neonatal HI brain damage, the loss of MAP-2 chiefly occurred in ipsilateral striatum and hippocampal area. There is no significant neuroprotective effect of EPO treatment (5 or 20 kU/kg) compared with vehicle treatment on the level of MAP-2 staining at this early time point after treatment (Fig. 1A and B).

Active-caspase-3 staining. To determine whether EPO treatment had an effect on apoptotic cell death, we scored active caspase-3 staining in the hippocampal area and calculated the scoring ratio of ipsilateral/contralateral areas. There was an obviously lower score after HI (either vehicle or EPO-treated animals) in CA1–CA4 areas of the ipsilateral hippocampus, indicating that apoptotic cell death was ongoing in these regions at 72 h after the insult. The i.p. administration of EPO (5 or 20 kU/kg) at 0, 24, and 48 h after the insult had no significant antia apoptotic effect (Fig. 1C).

Progenitor cell proliferation. Ki67, which is expressed during mitosis, is considered as a reliable marker for proliferating cells (27). In the ipsilateral SVZ and DG, the number of Ki67-positive cells was reduced at 72 h after HI. At this time point, more Ki67-positive cells were observed on both areas in females only after EPO 5 kU/kg treatment compared with the vehicle group (p < 0.001), but no effect was found after EPO 20 kU/kg administration (Fig. 1D and E).

Paw preference (CRT). At 9, 4, 2, and 9 wk after HI, we assessed sensorimotor function using the CRT. Sham-treated animals did not show any paw preference during rearing in the CRT. HI caused a preference (~15% at 9 d, ~30% at 4 wk, and ~25% at 9 wk) to use the unimpaired forepaw. EPO treatment did not improve performance in the CRT of male or female animals when assessed at 9 d after the insult. However, EPO-treated female but not male animals showed a significant improvement of sensorimotor function at 4 wk after HI. Interestingly, the improvement obtained in females of the EPO 5 kU/kg group was ~10% stronger than that obtained in females of the 20 kU/kg treatment group (p < 0.001, p < 0.01, respectively). At 9 wk after HI, the EPO 5 kU/kg-treated female mice still showed improvement of sensorimotor function (p < 0.001). Surprisingly, we no longer detected sensorimotor improvement in the 20 kU/kg EPO-treated female animals at this late time point (Fig. 2). Sensorimotor function in males was not improved by EPO treatment at any of the time points tested.

Analysis of ipsilateral surface area. The effect of EPO on long-term brain damage was determined at 10 wk. The morphological changes in the brain as visualized after HE staining are shown in Figure 3. The lesion volumes of the ipsilateral hemispheres were similar in male and female mice after HI. In addition, we did not detect a significant effect of EPO treatment (5 and 20 kU/kg) in the ratio of ipsilateral/contralateral hemispheres in males or females (Fig. 3A and B). However, when measuring the striatum and hippocampus area alone, a ~30% reduction of striatum tissue atrophy and a ~40% reduction of hippocampal tissue loss were indicated in females only after EPO treatment at 5 kU/kg (p < 0.01).

In contrast to what was observed for total area loss as determined after HE staining, the data in Figure 3C and D show that a significant reduction in the HI-induced loss of MBP staining was obtained only in females after treatment with 5 kU/kg EPO compared with vehicle (p < 0.01). However, there was no effect in either males or females of 20 kU/kg EPO.
DISCUSSION

In this study, we found no neuroprotective effect of EPO in our histological parameters when determined 24 h after the last EPO treatment (72 h after HI). In the long term, we observed a positive effect of EPO treatment on sensorimotor function, striatum atrophy, hippocampal lesions, and white matter injury when using 5 kU/kg of EPO. However, the beneficial effect of EPO was only observed in female animals.

Gender effects of posthypoxic neuroprotective interventions have been described after hypothermia and after treatment with the drug 2-iminobiotin (24,28). Moreover, in those studies, only females benefited from these neuroprotective interventions. The results of this study on the gender-specific effects of EPO treatment after neonatal HI are in line with those of the study by Wen et al. (29). These authors described gender-specific long-term protective effects of EPO treatment in a neonatal stroke model (29). Although it has been shown convincingly that circulating estradiol can lower the sensitivity for HI injury in females (30), it is not likely that female sex hormones play already a major role in P9 neonatal mice.
Indeed, we did not observe any gender difference in the extent of long-term HI brain injury in either sensorimotor function or brain damage in the vehicle group. In a clinical investigation using peripheral blood cells from healthy individuals, the EPOR alleles, EPORA 1 and EPORA 10, were present in a significantly higher frequency in females than in males (31). Although there is no report about the gender difference of EPOR in the human brain, we would like to propose that the higher frequency of EPOR in females may contribute to the gender-dependent neuroprotection of EPO. In any case, our results indicate that gender has to be taken into account even early in life, when designing strategies to protect the neonatal brain against injury.

It has been reported that EPO does not cross the blood-brain barrier in a detectable amounts when given at doses appropriate for erythropoiesis (200–400 U/kg) (32). Although the dose of EPO used in our studies is clearly above the range used for anemia treatment, there is no consensus as to the optimal dose, dose frequency, or dosing interval when using EPO to treat brain damage (33). Pharmacokinetics of EPO have demonstrated that i.p. administration produced a higher plasma concentration than s.c. administration. Meanwhile, i.p. injection also resulted in a more pronounced penetration into the brain than s.c. injection after HI (34). We administered EPO i.p. in our study and used a dose that is well above the dose used for treatment of anemia. Therefore, we expect that in our study EPO could reach the brain in sufficient amounts.

In our study, the beneficial effect of 5 kU/kg EPO on sensorimotor function, striatum atrophy, hippocampal lesions, and white matter damage in females was stronger and lasted longer than the effect of a higher (20 kU/kg) dose. Interestingly, an inverted U-shaped dose-response curve for EPO has also been described in a neonatal rat HI model (11); 5 kU/kg injection resulted in more neuroprotection than 2.5 or 30 kU/kg; three injections had a stronger beneficial effect than one injection, although increasing treatment to seven injections did not further improve the effect of EPO. It is not completely clear which mechanisms underlie the loss of positive effects at high (>20 kU/kg) doses of EPO.

Toxicity of EPO at high dosage has been reported in animal experiments. In a neonatal rat HI model, administration of EPO at 20 kU/kg significantly increased the number of degenerating cerebral neurons (35). However, in our female animals, a mild improvement of sensorimotor function was shown at 4 wk post-HI after EPO treatment with 20 kU/kg, but the improvement did not last into the adult period (9 wk post-HI). Although we could not check the hematocrit values in blood of EPO-treated animals at this age of the animals, the toxicity of the high dose of EPO may partially result from increased hematocrit-associated side effects, such as hypertension and thromboembolism, which may increase the infarction volume of the brain and neuronal cell death (36).

In addition, two types of EPO receptors have been identified in an in vitro study: a homodimeric (EpoR/EpoR) receptor and a heterodimeric (CD131/EpoR) receptor (37). The protective action of EPO occurs when EPO binds to the heterodimeric CD131/EpoR receptor, but protective effects of EPO will be lost if all EPO is bound by homodimeric receptors (37). EPO itself is also involved in the degradation of EPOR (38). It is possible that extremely high doses of EPO will promote the degradation of EPOR leading subsequently to a loss of the protective effects of EPO.
The rodent model of neonatal HI injury has been widely used since it was developed in 1981 (39), with some variation in the hypoxic duration (23,40,41). Previous studies have indicated that treatment with EPO was only effective provided in the hypoxic duration (23,40,41). Previous studies have used since it was developed in 1981 (39), with some variation

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improved sensorimotor function. In conclusion, EPO treat-

duce the loss of MBP staining in females. However, we do not

rule out the possibility that proliferation and differentiation of

progenitor) cell proliferation in the SVZ and DG was in-


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