Erythropoietin Promotes Neuronal Replacement Through Revascularization and Neurogenesis After Neonatal Hypoxia/Ischemia in Rats

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Background and Purpose—Erythropoietin (EPO) has been well characterized and shown to improve functional outcomes after ischemic injury, but EPO may also have unexplored effects on neurovascular remodeling and neuronal replacement in the neonatal ischemic brain. The current study investigates the effects of exogenous administration of EPO on revascularization and neurogenesis, 2 major events thought to contribute to neuronal replacement, in the neonatal brain after hypoxia/ischemia (H/I).

Methods—Seven-day-old rat pups were treated with recombinant human EPO or vehicle 20 minutes after H/I and again on postischemic days 2, 4, and 6. Rats were euthanized 7 or 28 days after H/I for evaluation of infarct volume, revascularization, neurogenesis, and neuronal replacement using bromodeoxyuridine incorporation, immunohistochemistry, and lectin labeling. Neurological function was assessed progressively for 28 days after H/I by gait testing, righting reflex and foot fault testing.

Results—We demonstrate that exogenous EPO-enhanced revascularization in the ischemic hemisphere correlated with decreased infarct volume and improved neurological outcomes after H/I. In addition to vascular effects, EPO increased both neurogenesis in the subventricular zone and migration of neuronal progenitors into the ischemic cortex and striatum. A significant number of newly synthesized cells in the ischemic boundary expressed neuronal nuclei after EPO treatment, indicating that exogenous EPO led to neuronal replacement.

Conclusions—Our data suggest that treatment with EPO contributes to neurovascular remodeling after H/I by promoting tissue protection, revascularization, and neurogenesis in neonatal H/I-injured brain, leading to improved neurobehavioral outcomes. (Stroke. 2007;38:2795-2803.)

Key Words: erythropoietin ▪ neonatal hypoxia/ischemia ▪ neurogenesis ▪ neuronal replacement ▪ revascularization

After hypoxic/ischemic (H/I) injury, the neonatal brain has only a limited capacity for neuronal replacement.1,2 In both adult stroke models and neonatal H/I, neuronal progenitors proliferate in the subventricular zone (SVZ) and migrate to the lesioned cortex (CTX) and striatum (ST).2–6 Despite the similarity in proliferation and migration patterns of neuronal progenitors in the adult versus neonatal ischemia models, there exists a wide disparity in new neuronal survival. In the adult brain, up to 20% of the new neurons in the ischemic ST remain viable 6 weeks after ischemia,4 whereas less than 1% of new neurons in the neonatal brain survive 2 weeks after H/I.1,2,7 The mechanism responsible for this poor viable neuronal replacement in injured neonatal brains is unclear, but an unfavorable microenvironment and lack of appropriate trophic support to new cells may be a potential underlying cause.4 Therefore, a strategy currently under active investigation in the field is the development of postischemic interventions aiming not only to enhance neurogenesis and neuroprotection, but also to improve the microenvironment for neuronal survival in injured brains.

Enhanced angiogenesis leading to functional vessels plays an important role in postischemic neurovascular unit remodeling.8,9 In addition to the improvement of microcirculation in the ischemic brain, angiogenesis produces endothelial cells that release soluble factors, stimulating neural stem cell proliferation.10 Furthermore, a recent study indicated that formation of a neurovascular niche supports neuronal progenitor migration and differentiation in the adult ischemic brain.11 Thus, angiogenesis may be critical for successful neurogenesis and viable replace-
ment. Whether angiogenesis is altered after neonatal H/I brain injury is currently unknown.

Erythropoietin (EPO) may be an excellent candidate for use in neonatal H/I brain injury to promote posts ischemic neuronal replacement. EPO, through EPO receptor-mediated intracellular signaling, has been shown to stimulate angiogenesis, neurogenesis, and functional recovery in the adult brain. Exogenous EPO enhanced endothelial progenitor cell mobilization from the bone marrow, amplified the production of neural progenitor cell, led to neurovascular remodeling in the adult stroke model, and provided potent neuroprotection and functional recovery in models of neonatal ischemic brain injury. However, EPO-mediated actions on neonatal neuronal replacement and revascularization have not been reported. Therefore, the present study was conducted to investigate the effect of administration of EPO on revascularization and neurogenesis, 2 major events thought to contribute to neuronal replacement, in the neonatal H/I-injured brain.

Methods

Materials

Recombinant human EPO was produced in-house by standard molecular biology methods. Primary antibodies included the following: mouse anti-bromodeoxyuridine (BrdU; 1:2000; BD Biosciences), goat anti-doublecortin (DCX; 1:1000; Santa Cruz Biotechnology), and mouse anti-neuronal nuclei (NeuN; 1:2000; Chemicon). Secondary IgG antibodies used were: biotin-mouse or -goat (1:1000; Vector Laboratories), Alexa Fluor 488-mouse (1:2000; Molecular Probes), and Cy3-mouse or -goat (1:2000; Jackson ImmunoResearch). Bodies used were: biotin-mouse or -goat (1:1000; Vector Laboratories), and Cy3-mouse or -goat (1:2000; Jackson ImmunoResearch).

Neonatal Hypoxia/Ischemia Rat Model and Drug Administration

Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Postnatal day 7 Sprague-Dawley rats (Charles River Laboratory, Wilmington, Mass) were subjected to 130 minutes H/I as previously described. Briefly, the left common carotid artery was ligated under anesthesia with 3% isoflurane. After a 1.5-hour recovery period, pups were exposed to hypoxia (8% O2) and then returned to their dam for the indicated time. Naive (nonischemic) animals served as controls. Recombinant human EPO was prepared in 0.1% bovine serum albumin in phosphate-buffered saline and injected intraperitoneally at the indicated doses 20 minutes after H/I and again on days 2, 4, and 6. Injection of 0.5% bovine serum albumin in phosphate-buffered saline served as the vehicle control. For identification of newborn cells in the brain, pups were injected intraperitoneally with BrdU (50 mg/kg; Sigma) according to the indicated regimen. All animals were transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline and their brains removed. After postfixation and cryoprotection, coronal sections were cut using a microtome. For assessment of tissue damage, sections from the level of the lateral ventricle were stained with cresyl violet as previously described. Immunochemistry

After blocking with 5% bovine serum albumin in phosphate-buffered saline for 1 hour, sections were incubated with primary antibody at 4°C overnight followed with the appropriate secondary antibody for 1 hour at room temperature. Sections for BrdU staining were pretreated with 1 N HCl for 1 hour at 37°C followed with 0.1 mol/L boric acid (pH 8.5) for 10 minutes at room temperature. To avoid cross-reaction for BrdU and NeuN double immunofluorescence, the M.O.M. kit (Vector Laboratories) was used according to the manufacturer’s instructions.

Evaluation of Functional Revascularization

Functional revascularization after H/I was assessed by quantifying the numbers of functional vessels that showed BrdU labeling in H/I (n=20) and nonischemic rats (n=15). Functional vessels were identified by transcardial administration (5 minutes before euthanasia) of biotinylated-lycopersicin esculentum lectin (tomato lectin, 1.25 mg/kg; Vector Laboratories), which labels endothelial cells only in perfused vessels. Lectin and BrdU stainings were visualized with fluorescence-streptavidin (1:2000; Vector Laboratories) and Cy3-conjugated secondary antibody using a Nikon microscope or Zeiss laser-scanning confocal microscope, respectively. Vessels that showed double labels of lectin and BrdU were counted in a square area (460×460 mm) from the perilesion area (including the ischemic boundary), the distant area (>500 mm apart from the ischemic boundary), and contralateral area.

Evaluation of Neurogenesis and Neuronal Replacement

Neurogenesis and neuronal replacement were assessed at 3 distinct levels: proliferation in the SVZ, migration of immature progenitors to the CTX and ST, and differentiation of mature neuronal phenotypes in the CTX and ST. BrdU” (proliferating) cells were counted in the drosolateral SVZ. DCX” (migrating immature neurons) cell densities in the CTX and ST were calculated as the number of cells in the designated area divided by the area measured by the MCID image analysis system. BrdU”NeuN” (neuronally differentiated) cells in the CTX and ST were counted in the square area (460×460 μm) of the ischemic boundary.

Neurological Evaluation

To evaluate sensorimotor neurological function, righting reflex and gait testing were performed 2, 4, 6, and 7 days after H/I. Righting reflex was determined by placement of the animal on its back and subsequent measurement of time needed for the animal to right itself. Gait testing was expressed as the first day that the animal successfully moved off a circle within 30 seconds. Coordination of contralateral limbs was determined by footprint tests, which were administered 1, 2, 3, and 4 weeks after H/I. Overall motor behavior was evaluated using grid walking to determine total steps taken over 1 minute. These assessments were performed according to previous reports.

Red Blood Cells and Hematocrit

Measurement of red blood cells and hematocrit was determined by hemocytological counting from blood samples obtained through intracardiac aspiration during euthanasia 28 days after H/I from animals injected with either the vehicle or 1000 U/kg EPO.

Statistical Analysis

All values are expressed as mean±SD. Statistical comparisons among groups were determined using analysis of variance followed by post hoc Fisher’s probable least-squares difference tests. P<0.05 was considered statistically significant.

Results

Functional Neovascularization Is Decreased After Hypoxia/Ischemia

Functional vessels were detected by staining for tomato lectin injected intracardially immediately before euthanasia, which allows for identification of vessels receiving active blood flow and precludes nonspecific labeling of microglia. In the CTX of control animals, the number of functional vessels continuously rose through postnatal day 21 (Figure 1C–D), reflecting ongoing neovascular formation during postnatal development. After H/I, the number of functional vessels compared with age-matched controls was dramatically de-
creased in the perilesion area 1 day after H/I. Although a modest increase in functional vessels was observed 5 days after H/I compared with earlier time points, the number of functional vessels remained significantly below age-matched controls over the entire time course analyzed (Figure 1C–D).

In regions distant to the infarct, functional vessels were decreased 1 to 3 days after H/I but rapidly increased thereafter and returned to control levels 7 days after H/I (Figure 1C–D). Contralateral tissue (hypoxic, but not ischemic) did not significantly differ from H/I tissue distant from the necrotic core in terms of the number of functional vessels (data not shown), indicating that hypoxia alone induces substantial but transient loss of neurovascular formation.

In normal neonates, the active proliferative process of angiogenesis peaks 5 to 9 days after birth and plateaus approximately 3 weeks postnatally as determined by [3H]thymidine uptake. Consistent with this, we found that BrdU uptake in progenitor cells associated with functional vessels was increased in control animals within the first 3 days assayed corresponding with postnatal days 7 to 9 (Figure 1F).
Under confocal microscopy, H/I induced significant loss of BrdU-positive (BrdU\(^+\)) cells on functional vessels in the perilesion region 1 to 5 days after H/I (Figure 1E–F). However, BrdU labeling in the perilesion area returned to the control level 7 days post-H/I (Figure 1F), suggesting that restoration of vascular-associated proliferation occurs 1 week after severe stress. Surprisingly, BrdU\(^+\) cells on stained vessels in the distant regions significantly increased 5 days after H/I compared with control tissue (Figure 1F), indicating that sublethal H/I stress can stimulate proliferation of vessel-related progenitor cells. These data demonstrate that functional revascularization is suppressed within the first 5 days after H/I in regions immediately surrounding the infarct zone. Given this timeframe, we proceeded to hypothesize that therapeutics geared toward enhancement of angiogenesis such as EPO may be best administered within 1 week after H/I.

**Effect of Exogenous Erythropoietin on Brain Damage**

To detect the optimal dose of EPO necessary for neuroprotection in our neonatal H/I model, EPO (500, 1000, or 2000 U/kg) or vehicle was injected intraperitoneally multiple times after H/I (Figure 2A). Tissue loss (volume) was effectively reduced by 1000 U/kg EPO in both the CTX and ST compared with the vehicle control (Figure 2B–C). At the highest dose (2000 U/kg), EPO did not result in significant tissue protection, potentially as a result of thrombotic effects because of EPO-induced erythropoiesis. Based on these data, we chose 1000 U/kg as the optimal dose for subsequent studies of functional revascularization and neurogenesis.

To ensure that the effective dose regimen (1000 U/kg per Figure 2A) of EPO did not stimulate erythropoiesis, red blood cells and hematocrit levels were measured 28 days after H/I. No significant difference between EPO-treated and control animals was evident (red blood cells: 832±85×10\(^4\) versus 767±75×10\(^4\)/mm\(^3\), \(P>0.05\); hematocrit: 43.4±2.1 versus 42.8±1.0%, \(P>0.05\)). This suggests that the therapeutic dose of EPO is unlikely to stimulate erythropoiesis and lead to subsequent thrombosis.

**Exogenous Erythropoietin Enhances Functional Revascularization After Hypoxia/Ischemia**

To determine whether EPO induces functional vascular remodeling after H/I in the neonatal model, EPO or vehicle and BrdU were administered as diagrammed (Figure 2A), and tomato lectin was injected intracardially 5 minutes before animal euthanasia. Exogenous EPO induced a significant increase in the number of functional vessels after H/I in both perilesion and distant areas compared with vehicle-infused H/I animals (Figure 3A–B).

BrdU-labeled (BrdU\(^+\)) cells present on functional vessels were significantly increased by EPO treatment in both the perilesion and distant areas compared with vehicle-infused H/I animals (Figure 3A, C). In the contralateral (nonischemic, but hypoxic) area, EPO treatment did not significantly increase vessel number compared with vehicle (data not shown). These data indicate that exogenous EPO enhances functional revascularization in the ischemic hemisphere of hypoxic animals.

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**Figure 2.** Post-H/I EPO administration reduces infarct volume. A, EPO injection and BrdU labeling diagram. B, Representative pictures of cresyl violet staining from animals treated with vehicle or 500, 1000, or 2000 U/kg EPO after H/I. Scale bar, 1 mm. C, Infarct volumes in the CTX and ST 7 days after H/I (n=16 for vehicle, n=12 for EPO500, n=12 for EPO1000, and n=8 for EPO2000). *\(P<0.05\), **\(P<0.01\) versus vehicle control.

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**Erythropoietin Enhances Cell Proliferation, Neuronal Migration, and Replacement After Hypoxia/Ischemia**

To examine the potential effects of EPO on progenitor cell proliferation, EPO and BrdU were administered as illustrated (Figure 2A). Exogenous EPO significantly increased BrdU\(^+\) cells in the ipsilateral SVZ compared with vehicle-infused H/I animals (Figure 4A–B) but not in the contralateral (nonischemic) side (Figure 4B). This result is in contrast to
the previous study using adult mouse brain, which showed that EPO delivered directly into the SVZ increased neuronal progenitor proliferation in the absence of ischemic insult. The percentage of the BrdU/H11001 cells colabeled with DCX, a protein that is expressed in migrating immature neurons, was significantly increased in the ischemic SVZ compared with vehicle H/I animals (Figure 4B–C), but not in the nonischemic hemisphere (Figure 4B). These data suggest that EPO can stimulate proliferation of neuronal progenitors in the H/I brain. Because of the fact that both proliferating cells and damaged cells engaged in active DNA repair can incorporate BrdU, we pursued further experiments assessing the actual presence of immature neurons in the ischemic zone.

Although proliferation of SVZ indicates that exogenous EPO is capable of stimulating neurogenesis, neuronal replacement necessitates the migration of new neurally destined cells to the lesion site. Thus, tissue sections were visualized with an antibody to DCX by fluorescent microscopy to detect whether the EPO-induced, newborn cells in the SVZ successfully migrated to the CTX and ST 7 days after H/I. Although DCX positive (DCX/H11001) cells were counted from the entire CTX, immunoreactive cells were observed primarily in the perilesion region (Figure 4D). A substantial basal level of immature neuronal migration occurred in naïve neonatal brain as evidenced by the presence of DCX+/cells in both the CTX and ST (Figure 4E). This observation was unique to the naïve neonatal brain as compared with the naïve adult brain, because DCX− cells could no longer be detected at postnatal day 35 and thereafter (data not shown). After H/I, a significant increase in the number of DCX+ cells occurred at 7 days in the CTX and ST in both the ischemic and nonischemic hemispheres (Figure 4D–E).

Exogenous EPO induced a further increase of DCX+ cell numbers in the ipsilateral (ischemic) CTX and ST compared with vehicle controls, whereas no difference between EPO and vehicle was evident in the contralateral (nonischemic) hemisphere (Figure 4D–E). EPO injection after H/I led to a significant increase in DCX+ cells that either incorporated BrdU or expressed the mature neuronal cell marker NeuN in their nuclei (Figure 4F), indicating that these DCX+ cells were newly synthesized and destined to a neuronal phenotype.

To quantify long-term neuronal replacement, cells expressing both NeuN and BrdU were counted 28 days after H/I in the presence of either EPO or vehicle (Figure 5A). Although substantial migration of immature neurons had occurred 7 days after H/I (Figure 4), few of these neurons survived as mature (NeuN/) neurons long-term after H/I (Figure 5B–D). However, injection of EPO led to a significant increase in the number of NeuN+/BrdU− cells compared with the vehicle in both the ST and CTX of the ischemic hemisphere (Figure 5B–D). No NeuN+/BrdU− cells were observed in age-matched control tissue (data not shown). Taken together, these results indicate that exogenous EPO significantly increased progenitor cell migration and neuronal replacement into ischemic areas after H/I.

Erythropoietin Infusion Improves Neurological Outcomes After Hypoxia/Ischemia

After H/I, a significant impairment of sensorimotor behavior was observed (Figure 6). The observed differences were not because of alterations in overall motor activity, because there was no significant difference in total number of steps between groups (Figure 6A). Contralateral limb faults were signifi-
cantly higher in vehicle-treated animals compared with controls, even 28 days after H/I (Figure 6B). Similarly, directed movement (as evidenced by gait testing) was significantly delayed in H/I animals (Figure 6C). Although impairment in righting reflex was evident up to 6 days after H/I, no significant difference was observed between groups at 7 days (Figure 6D), indicating that behavioral compensation is possible in H/I neonatal animals over time. The neurological deficiencies were reversed by post-H/I infusion of EPO (Figure 6B–D). Thus, exogenous EPO delivered post-H/I is effective at improving neurological outcomes after H/I.

Discussion
The current study demonstrates that EPO administered over 1 week after H/I resulted in reduced infarct volume, significant enhancement of functional revascularization and neuronal replacement in the ischemic hemisphere, and improved neurological outcomes in the neonatal rat. These results provide the first evidence that EPO treatment promotes the process of neurovascular unit remodeling after neonatal H/I brain injury in addition to its known tissue-salvage neuroprotective effect.

In normal neonates, the active process of angiogenesis peaks 5 to 9 days after birth and plateaus approximately 3 weeks postnatally. In our study, during the period of new vessel accumulation, a limited number of newly formed neuronal progenitors migrated away from the SVZ to the ST and CTX in naïve animals (Figure 4). After H/I, functional revascularization in the ischemic boundary was severely suppressed over the first week and never completely restored compared with age-matched naïve animals (Figure 1). Despite the finding that the number of neuronal progenitors increased within the first week in the SVZ, ST, and CTX after H/I compared with controls (Figure 4), long-term maturation of these neurons in H/I animals was rare. Postischemic infusion of EPO led not only to improved functional revascularization 7 days after H/I, but also to increased neuronal proliferation, migration, and long-term neuronal replacement in the ischemic territory. These findings support the concept that early therapeutic vascular protection and angiogenesis is closely correlated with a supportive microenvironment for long-term neuronal replacement.
We and others have observed increased labeling of BrdU in the SVZ after H/I (Figure 4).\textsuperscript{2,7,24,25} BrdU can be incorporated into the genome of both proliferating cells and cells engaged in active DNA repair, and distinguishing between these 2 populations has proved to be difficult. It is possible that the increased BrdU labeling observed after H/I could be because of active repair of damaged cells in the SVZ, populations of which have been found to be susceptible to neonatal H/I injury,\textsuperscript{26} where cell death in the susceptible populations peaked 12 to 48 hours after injury. Because EPO has been demonstrated in vitro to be capable of stimulating the production of neuronal progenitors,\textsuperscript{13,27} and our BrdU injection timeframe was significantly delayed, our results are likely to indicate newly formed progenitors as opposed to damaged preexisting cells. Nonetheless, EPO increased the number of BrdU$^+$ cells in the SVZ (Figure 4), indicating that either increased proliferation or increased DNA repair capacity had been stimulated by EPO.

Interestingly, we found a significant difference in BrdU labeling and neuronal migration responses to EPO in the hypoxic/ischemic versus hypoxic/nonischemic hemisphere (Figures 3 and 4). Moreover, our data from the ischemic cortex included the periinfarct as well as distant regions, and we observed greater levels of neuronal progenitor migration in the periinfarct region (Figure 4D) than in the distant region. A similar effect was observed on revascularization, in which systemic EPO injection led to increased revascularization in the ischemic hemisphere (Figure 3) but had no significant effect in the nonischemic (contralateral) hemisphere of hypoxic animals. Therefore, the degree or nature of the injury itself may elicit differential actions of EPO. One potential mechanism for these differential actions of EPO under different degrees of injury may be the regulation of EPO receptors. A previous study demonstrated that exogenous EPO upregulated the expression of EPO receptor in the ischemic hemisphere after neonatal H/I.\textsuperscript{14} Further studies to delineate the mechanisms unique to H/I compared with hypoxia alone may provide interesting insight into the actions of exogenous EPO under different settings.

A previous study found that, under normal conditions, a single systemic injection of EPO (5000 U/kg) is capable of crossing the blood–brain barrier but that EPO was found primarily in neurons surrounding the capillary network.\textsuperscript{28} Thus, our results may be biased toward EPO exerting effects...
on the vascular and perivascular system, and improved delivery of EPO into periinfarct zones may increase the survival of migrating neurons in ischemic brain regions. Nonetheless, the current study and previous reports correlating EPO-mediated improved neurological outcome without decreased infarct volume in adult stroke models8 underscore the potential importance of the contribution of neurovascular unit remodeling in postischemic outcomes.

Perinatal H/I brain injury often leads to long-term neurodevelopmental disabilities; thus, early intervention is critical for neonatal prognosis. EPO is currently used clinically with few known risks for the treatment of anemia in premature newborns; therefore, this agent may be easily applicable in treating newborns with H/I brain injury.

Acknowledgments
We thank Carol Culver for editorial assistance and Pat Strickler for secretarial support.

Sources of Funding
This work was supported by National Institutes of Health grants NS43802, NS45048, and NS36736 (to J.C.) and NS051576 (to J.L.) from the National Institute of Neurological Disorders and Stroke. J.C. is a recipient of the Established Investigator Award (0240135N) from the American Heart Association. J.C. was also supported in part by the Geriatric Research, Education and Clinical Center, Veterans Affairs Pittsburgh Health Care System (Pittsburgh, Pa). J.L. was also supported by the Department of Veterans Affairs merit review program.

Disclosures
None.

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*Stroke*. 2007;38:2795-2803; originally published online August 16, 2007;
doi: 10.1161/STROKEAHA.107.483008

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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