Enhanced Oligodendrogenesis and Recovery of Neurological Function by Erythropoietin After Neonatal Hypoxic/Ischemic Brain Injury

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Background and Purpose—Neuronal replacement has recently gained attention as a potential therapeutic target under ischemic conditions. However, the oligodendrogenic infrastructure is equally critical for restoration of brain function and is also sensitive to ischemic injury. Erythropoietin (EPO) is a neuroprotective molecule that stimulates neuronal replacement after neonatal hypoxia/ischemia (H/I) when delivered soon after the onset of reperfusion. Because EPO can improve recovery of neurological function in the absence of tissue protection, we hypothesize that EPO may improve neurological function via enhancement of white matter recovery after H/I. Thus, we sought to determine the effects of delayed administration of EPO on white matter injury and recovery of neurological function after neonatal H/I.

Methods—EPO (1000 U/kg) was injected intraperitoneally at multiple time points beginning 48 hours after H/I in postnatal day 7 rats. The effects of EPO on oligodendrogenesis, white matter injury, and neurogenesis were evaluated using bromodeoxyuridine incorporation and cell-specific immunohistochemistry. Neurological function was assessed by sensorimotor behavioral tests.

Results—Delayed administration of EPO was incapable of reducing brain volume loss but significantly increased oligodendrogenesis and maturation of oligodendrocytes and attenuated white matter injury after H/I. These effects occurred concurrently with enhanced neurogenesis. Delayed EPO treatment improved behavioral neurological outcomes 14 days after H/I injury.

Conclusions—Our study demonstrates that delayed administration of EPO promotes oligodendrogenesis and attenuates white matter injury concurrently with increased neurogenesis. These effects likely contribute to the observed improvement in neurological functional outcomes. (Stroke. 2010;41:1032-1037.)

Key Words: erythropoietin ■ neonatal hypoxia/ischemia ■ neurogenesis ■ oligodendrogenesis ■ white matter injury

Cerebral white matter is highly vulnerable to ischemic injury in adults1 and in neonates,2 with the latter particularly leading to permanent impairment of the brain. During development, oligodendrocyte progenitor cells (OPC) undergo rapid differentiation into mature oligodendrocytes, which are more susceptible to ischemic injury.2 After brain ischemia in adults, there is a delayed increase in the number of mature oligodendrocytes in peri-infarct areas, whereas immature oligodendrocytes proliferate in the regions surrounding the lateral ventricles,3 indicating that ischemic damage may be compensated for, at least in part, by increasing replacement of oligodendrocytes. Thus, it is plausible to propose that postischemic interventions geared toward improving OPC survival and differentiation may greatly improve outcomes in the neonatal ischemic brain.

Erythropoietin (EPO) has emerged as a promising candidate for neuroprotection in animal models of ischemia4,5 and in stroke patients.6 When administered acutely after neonatal ischemia, EPO is neuroprotective and also stimulates angiogenesis and neurogenesis.4 The more clinically relevant delayed (>24 hours after ischemia) administration of EPO does not decrease infarct but has recently been reported to result in a reorganization of white matter in adult rats as detected by MRI.7 The effects of delayed EPO administration on oligodendrogenic replacement and functional recovery in the ischemic neonate are currently unknown. The objectives of this study were to investigate whether delayed administration of EPO stimulates oligodendrogenesis and attenuates white matter injury after neonatal hypoxia/ischemia (H/I), and whether these effects result in improved neurological outcomes.

Materials and Methods

Neonatal H/I Rat Model and Drug Administration

All procedures were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. The neonatal H/I
model was performed in postnatal day 7 Sprague-Dawley rats (Charles River Laboratory, Wilmington, Mass) as described.4 In brief, the left common carotid artery was ligated under anesthesia with 3% isoflurane. After a 1.5-hour recovery period, the pups were placed in glass chambers containing a humidified atmosphere of 8% O2/92% N2 and submerged in a 37°C water bath to maintain normothermia. After 2.5 hours of hypoxia, the pups were returned to their dam for the indicated time. Naïve (nonischemic) animals served as controls. Recombinant human EPO was produced and injected in a phosphate-buffered saline intraperitoneally at 1000 U/kg body weight on days 2, 4, 6, 9, and 13 after H/I. Injection of phosphate-buffered saline served as the vehicle control. Bromodeoxyuridine (BrdUrd, 50 mg/kg; Sigma) was injected intraperitoneally according to the indicated regimen. All animals were transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline and their brains were removed. After postfixation and cryoprotection, coronal sections were cut using a microtome. Brain volume loss was determined by calculating the amount of surviving tissue using cresyl violet staining as previously described.5

Evaluation of Oligodendrogenesis, Neurogenesis, and White Matter Injury
Newborn cells were labeled by injection of BrdUrd before euthanization according to the illustrated regimens. For visualization of newborn cells, sections were pretreated with 1 N HCl followed by 0.1 mol/L boric acid (pH 8.5), blocked, and then incubated with anti-BrdUrd (1:2000; BD Biosciences). Oligodendrogenesis and neurogenesis were analyzed 5 and 14 days after H/I. Oligodendrocytes were visualized with anti-NG2 (1:200; Cell Signaling) and antiadenomatous polyposis coli (1:200; Santa Cruz Biotechnology) immunostaining for OPC and mature oligodendrocytes, respectively. Migrating immature neural progenitors were visualized using antidoublecortin (1:1000; Santa Cruz Biotechnology) immunostaining. Secondary IgG antibodies included AlexaFluor 488 (1:500; Molecular Probes) and Cy3 (1:2000; Jackson Immunoresearch). Immunopositive cell densities were calculated as the number of cells in the designated area divided by the area measured by the MCID image analysis system (Image Research, Inc.). To evaluate white matter injury, coronal sections were stained with myelin basic protein (MBP; 1:400; Santa Cruz Biotechnology) and neurofilament 200 (1:200; Sigma) antibodies and images were digitized using confocal microscopy (Olympus Fluoview FV1000; Olympus). The mean intensity value of MBP and neurofilament 200 staining was calculated in the corpus callosum (CC), cortex (CTX), and striatum (ST) as previously reported and expressed as the relative ratio of MBP to neurofilament 200 staining.

Neurological Evaluation
Sensorimotor neurological function was evaluated using foot fault tests and the negative geotaxis test as described.4 Coordination of contralateral limbs was determined by foot fault tests, which analyze the successful rate of the animal using the right foot. Overall motor behavior was evaluated using grid walking to determine total steps taken over the course of 1 minute. Negative geotaxis was determined by testing the time needed for pups to successfully turn and climb up an incline board with their forelimbs.

Statistical Analysis
All values are expressed as mean±SD. Statistical comparisons among groups were determined using analysis of variance followed by post hoc analysis using Fisher probable least-squares difference tests.

Results
Delayed EPO Injection Enhances Proliferation and Maturation of OPC After H/I
Neurotoxic stimuli, such as H/I, and neurotherapeutics may affect the normal course of oligodendrogenesis because of injury and recovery. After H/I in neonates, the number of immature OPC (NG2+) cells was significantly higher than in age-matched controls at 5 days in the CC and CTX in the ipsilateral hemisphere regions of the contralateral hemisphere (Supplemental Figure 1C, F). Similar to total NG2+ cell counts, the number of BrdUrd-labeled NG2+ cells increased significantly at 5 days in the ischemic CTX and CC compared to control animals (Supplemental Figure 1C, F), suggesting that severe stress induces transient proliferation or repair of OPC.

Several studies have found heterogeneity in NG2+ cell populations, including the capacity to differentiate into oligodendrocytes and astrocytes.10 To determine the fate of OPC maturation into oligodendrocytes, we extended the survival time to 14 days after H/I and immunostained brain sections with the antiadenomatous polyposis coli antibody, which labels only the cell bodies of mature oligodendrocytes, and counterstained with BrdUrd to identify newly generated oligodendrocytes. Interestingly, although H/I increased numbers of NG2+ cells, the number of mature antiadenomatous polyposis coli-positive oligodendrocytes, both total and newly generated, significantly decreased at 14 days after H/I in the CC and CTX of the ipsilateral hemisphere region peripheral to the infarct zone (Figure 1F–H). This decrease was not observed in the contralateral hemisphere, suggesting that the effects of H/I on mature oligodendrocytes are specific to H/I conditions. Taken together, these results indicate that early OPC have a delayed capacity to proliferate but fail to mature or survive after H/I and therefore may afford an extended time window for intervention within 1 week after H/I injury.

We have previously demonstrated that administration of EPO before or at the onset of reperfusion effectively enhances neuronal proliferation, migration, and replacement after neonatal H/I, along with diminished brain volume loss.4 Given that increased BrdUrd-labeled OPC are observed within 1 week after H/I (Supplemental Figure 1F) and acute treatment with EPO promotes cellular proliferation in the neonatal brain,4 we sought to determine whether delayed EPO administration exerted effects on oligodendritic replacement after H/I. Multiple injections of EPO (1000 U/kg BW) and BrdUrd were administered intraperitoneally into pups as diagrammed (Figure 1A). Consistent with other models,7 delayed administration of EPO did not reduce brain volume loss (Figure 1B). However, delayed EPO administration significantly increased the total number of NG2+ cells in the CC, CTX, and ST of the ipsilateral and contralateral hemispheres, much more than that induced by H/I alone or naïve control (Figure 1C–E). The increase in NG2-reactive cells extended to newly proliferating cells, because cells immunoreactive for both NG2 and BrdUrd were significantly increased above control or H/I tissue. In the ipsilateral hemisphere, delayed EPO treatment after H/I restored the number of antiadenomatous polyposis coli-positive cells (both total and newly generated BrdUrd+) to levels similar to those in control brain (Figure 1F–H). Delayed EPO treatment also significantly increased antiadenomatous polyposis coli-immunoreactive cells in all regions of the contralateral hemisphere above control (Figure 1H). Delayed EPO administration thus promotes generation
and survival of oligodendrocytes under lethal and sublethal stress.

**EPO Attenuates White Matter Injury After H/I After an Extended Period**
Whereas delayed EPO administration is ineffective at decreasing total infarct volume, subtle cellular effects may still occur, leading to improved outcomes. Thus, we examined the extent of white matter injury at the cellular level in affected brain areas (Figure 2A) using MBP as a marker of myelina-
tion and neurofilament 200 as a marker of axons. After H/I, a significant and prolonged decrease in MBP immunoreactive density was observed in the CTX and ST (Figure 2B, C) persisted for 14 days after H/I, suggesting severe and long-
term axonal demyelination. Reduced MBP staining in the CC seen at 5 days after H/I was transient, recovering to control levels by 14 days after H/I (Figure 2B–D). Prolonged white matter injury was found only in ischemic tissue and the CTX and ST of the contralateral hemisphere were unaffected (Figure 2E). These results demonstrate that H/I damages white matter in a prolonged fashion.

Delayed EPO treatment after H/I significantly increased the ratio of MBP to neurofilament 200 staining 14 days after H/I in the CC and CTX (Figure 2D). Because there was no effect of EPO on white matter at the earlier 5-day time point, this result indicates that restoration of white matter by EPO is a significantly delayed event. These data illustrate that the time course of white matter restoration after H/I parallels the time frame for EPO-mediated oligodendrogenesis.

**Delayed EPO Injection Enhances Neurogenesis and Improves Neurological Outcomes After H/I**
We have previously shown that EPO administered immediately after H/I injury enhances neuronal migration and replacement and also improves neurological outcomes. Therefore, we next sought to determine if similar effects could also be observed in the more clinically relevant delayed EPO administration and correlate to oligodendrogenesis. Consistent with our previous report, a significant increase in the number of newly generated migrating progenitor cells (BrdUrd/antidoublecortin-positive) occurred at 10 and 14 days after H/I in the CTX (Figure 3A, B) and ST (Figure 3C, D). Delayed administration of EPO after H/I
significantly increased antidoublecortin-positive and BrdUrd+/antidoublecortin-positive cells in ischemic CTX (Figure 3A, B) and ST (Figure 3C, D) compared to controls and vehicle-treated H/I animals.

Finally, to test whether the increased oligodendrogenesis and neurogenesis could be translated into neurofunctional improvement, sensorimotor neurological function was evaluated using foot fault tests and negative geotaxis test. A decrease in the total steps taken in vehicle-treated and EPO-treated H/I groups compared to controls was only evident 10 days after H/I, which recovered by 14 days (Figure 4A). Delayed EPO treatment significantly decreased the fault rate in H/I animals at the later time points of 10 and 14 days after H/I for the contralateral forelimb (Figure 4B) and hindlimb (Figure 4C). EPO-treated H/I animals significantly improved their negative geotaxis response at later time points compared to vehicle-treated H/I animals (Figure 4D). These data suggest that delayed administration of EPO after H/I does not affect short-term motor outcomes, but rather significantly improves outcomes at an extended period 14 days after injury in a time frame concurrent with oligodendrogenesis and neuronal replacement.

**Discussion**

The present study investigated the state of brain oligodendrogenesis and neurological functional outcomes after delayed administration of EPO to H/I-treated neonatal rats. The findings demonstrate that EPO administration delayed by 24 hours was able to: (1) induce a prolonged increase in oligodendrogenesis and maturation in the ischemic hemisphere and in the corpus callosum; (2) attenuate white matter injury concomitant with migration of neuronal precursors into the ischemic region; and (3) improve neurological functional outcomes after neonatal H/I.

Administration of EPO to ameliorate damage to the ischemic brain has proven effective if acutely administered within 6 hours of ischemia. However, its potential clinical value as a postischemic therapeutic has remained questionable because of its inability to reduce infarct size in the adult brain. Interestingly, in the neonatal brain, delayed administration of EPO was also ineffective at decreasing infarct volume but significantly improved neurological function outcomes. These findings demonstrate that gross preservation of neural tissue is not a definitive requirement for the recovery of neurological function. Rather, the restoration of neurological function may be influenced by a variety of factors, such as reorganization of surviving tissue and oligodendrocytic architecture. Our data show a correlation between increased oligodendrogenesis, neuronal migration, and white matter recovery with improved neurological function outcomes. Promotion of oligodendrogenesis may thus function in 2 manners: prevention of further degradation of existing neurons via remyelination and promotion of neuronal migration.

The observed changes in the contralateral hemisphere demonstrate that EPO may also potentially aid brain tissue that is only indirectly stressed by H/I. Increased oligodendrogenesis was observed in the contralateral hemisphere,
whereas EPO treatment further enhanced oligodendrogenesis in all regions of the contralateral hemisphere above control. This result is intriguing, because little damage is observed within the contralateral hemisphere. One possible explanation is that severe stress (ie, ipsilateral hemisphere) damages oligodendrocytes, whereas mild injury (ie, contralateral) acts as a preconditioning signal to induce the production of protective factors, such as endogenous EPO, which in turn stimulates neurogenesis and oligodendrogenesis. In line with this, upregulation of EPO and EPOR was observed after hypoxia in the central nervous system\textsuperscript{11,12} and EPO induces neurogenesis in response to hypoxia-only insult and in the

**Figure 3.** Delayed EPO administration induces neurogenesis. Representative images and quantification of antidoublecortin (DCX) and BrdUrd double-labeling in the CTX (A, B) and ST (C, D) after H/I. Scale bar, 500 µm. E. Confocal (left) and 3-dimensional (middle) image of BrdUrd\textsuperscript{*}/DCX\textsuperscript{*} cells in the CTX and confocal image of BrdUrd\textsuperscript{*}/DCX\textsuperscript{*} cell in the ST (right). Scale bar, 10 µm. *P<0.05, **P<0.01 vs control; ##P<0.01 vs H/I vehicle.

**Figure 4.** Delayed EPO administration improves neurological outcomes after H/I. A. Quantification of total steps among groups. Foot fault testing from contralateral forelimb (B) and hindlimb (C). D. Assessment of negative geotaxis. Control (n=15), vehicle-treated (n=25), and EPO-treated animals (n=21). *P<0.05, **P<0.01 vs control; ##P<0.01 vs H/I vehicle.
normal brain. The contralateral hemisphere undergoes a sublethal hypoxic challenge and, because of transhemispheric diaschisis, may have alterations in electric activity, cerebral blood flow, and metabolites. Further studies examining the effects of EPO in sublethal stress conditions will be interesting because, in addition to providing protection to severe injury, sublethal injury may also be a potential target of EPO.

The current study supports other works demonstrating that neonatal H/I has differential effects on the various stages of maturing oligodendrocytes. In particular, immature OPC are more resistant to neonatal H/I as compared to mature oligodendrocytic populations, and the number of late OPC is increased by EPO treatment. EPO has been demonstrated to possess oligodendro-protective capacity; however, we also demonstrate that delayed EPO treatment induces OPC proliferation (BrDUrd-) as opposed to simple cellular protection. This is consistent with the observed effects of EPO on neuronal populations, in which EPO is capable of exerting neuroprotection and stimulation of neurogenesis. Whereas the mechanism for EPO-stimulated cell replacement is currently unknown, both neurons and OPC express EPO receptors. The signaling mechanisms underlying cell replacement in the brain may further identify potential therapeutic targets and points for intervention.

Interestingly, the effects of delayed EPO administration were not detectable until a later (14 days) time point. The observation that EPO exerts effects on white matter injury is novel, because until now no effects of delayed EPO administration had been observed at earlier time points in neonatal H/I. The longer 14-day time frame extends a recent report that described EPO as ineffective at attenuating white matter injury after neonatal H/I, with an end point of 72 hours. Consistently, we found that shorter survival time points (5 days) exhibited no significant differences between H/I and EPO–H/I groups in terms of cell replacement, whereas longer survival (14 days) resulted in significant differences in white matter cell counts and sensorimotor behavior. These data argue that EPO facilitates postischemic white matter restorations rather than a direct protective effect on preexisting oligodendrocytes. Current and previous studies suggest that oligodendrogenesis and neurogenesis are closely related to neurological outcome improvement after ischemia. Conceptually, oligodendrogenesis and neurogenesis may have a delayed rather than immediate effect on neurological outcomes, based on its long temporal and complex spatial pathophysiological processes. This was supported by our study showing that longer survival (14 days) rather than shorter survival time points (5 days) resulted in improved sensorimotor behavior. It is tempting to speculate that delayed EPO administration also has effects on oligodendrogenesis/neurogenesis and functional profiles at even longer periods, and this warrants further study.

The present study indicates that therapeutics, such as EPO, with positive effects on oligodendrocytic replacement also yield improved functional outcomes in the absence of gross tissue protection. The interplay between white matter restoration, neurogenesis, and neurological function outcomes after H/I in neonates may effectively improve postischemic therapies.

Acknowledgments
The authors thank Carol Culver and Armando P. Signore for editorial assistance, and Pat Strickler for secretarial support.

Sources of Funding
This project was supported by National Institutes of Health/NINDS grants NS43802, NS45048, NS6736, NS6118 (J.C.), and NS053473 (G.C.), VA Merit Review grants (J.C. and G.C.), and AHA Scientist Development grant 0630006N (G.C.).

Disclosures
None.

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Stroke. 2010;41:1032-1037; originally published online April 1, 2010; doi: 10.1161/STROKEAHA.109.570325

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