Mutant Erythropoietin Without Erythropoietic Activity Is Neuroprotective Against Ischemic Brain Injury

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Background and Purpose—Erythropoietin (EPO) confers potent neuroprotection against ischemic injury. However, treatment for stroke requires high doses and multiple administrations of EPO, which may cause deleterious side effects due to its erythropoietic activity. This study identifies a novel nonerythropoietic mutant EPO and investigates its potential neuroprotective effects and underlying mechanism in an animal model of cerebral ischemia.

Methods—We constructed a series of mutant EPOs, each containing a single amino acid mutation within the erythropoietic motif, and tested their erythropoietic activity. Using cortical neuronal cultures exposed to N-methyl-D-aspartate neurotoxicity and a murine model of transient middle cerebral artery occlusion, neuroprotection and neurofunctional outcomes were assessed as well as activation of intracellular signaling pathways.

Results—The serine to isoleucine mutation at position 104 (S104I-EPO) completely abolished the erythropoietic and platelet-stimulating activity of EPO. Administration of S104I-EPO significantly inhibited N-methyl-D-aspartate-induced neuronal death in primary cultures and protected against cerebral infarction and neurological deficits with an efficacy similar to that of wild-type EPO. Both S104I-EPO and wild-type EPO activated similar prosurvival signaling pathways such as phosphatidylinositol 3-kinase/AKT, mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2, and STAT5. Inhibition of phosphatidylinositol 3-kinase/AKT or mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 signaling pathways significantly attenuated the neuroprotective effects of S104I-EPO, indicating that activation of these pathways underlies the neuroprotective mechanism of mutant EPO against cerebral ischemia.

Conclusions—S104I-EPO confers neuroprotective effects comparable to those of wild-type EPO against ischemic brain injury with the added benefit of lacking erythropoietic and platelet-stimulating side effects. Our novel findings suggest that the nonerythropoietic mutant EPO is a legitimate candidate for ischemic stroke intervention. (Stroke. 2012;43:00-00.)

Key Words: AKT • cerebral ischemia • ERK1/2 • erythropoietin • erythropoietin mutant • neuroprotection • neurotoxicity

Erythropoietin (EPO) is a potent neuroprotectant against ischemic brain injury both in animal experiments and in the clinic. Thus, using EPO as a neuroprotective approach in stroke, especially in treatment of patients who are not suitable for tissue-type plasminogen activator treatment, represents a potentially exciting new clinical application. However, large doses and multiple administrations of EPO are required for the treatment of stroke. Such a regimen of EPO administration may result in multiple high-risk factors for patients with stroke due to the erythropoietic effects of EPO such as increases in hematocrit, quantity of platelets, and vascular muscle contraction, raising the likelihood of microcoagulation and secondary infarction. EPO may also potentially interact with or be contraindicated with thrombolytic drugs and cause unexpected side effects, as evidenced by a recently failed EPO/tissue-type plasminogen activator combinatorial clinical trial. Accordingly, alternate strategies to reduce erythropoietic activity and other potential side effects of EPO will greatly improve its clinical applicability for the treatment of stroke.

A recent study indicated that a mutant EPO (MEPO) lacking erythropoietic activity was neuroprotective against N-methyl-D-aspartate (NMDA) toxicity in cultured neurons. However, the in vitro model bypasses all the vascular events critical in stroke pathology. Thus, whether MEPO has neuroprotective effects against brain ischemia still remains unknown. In this study, we constructed and subsequently tested a series of MEPOs, each containing a single amino acid mutation within
the erythropoietic motif, and characterized a MEPO that completely lacked erythropoietic activity but retained neuroprotective effects against in vitro neural excitotoxicity. We then further investigated this MEPO in the context of in vivo cerebral ischemia and explored the potential signaling mechanisms underlying the observed neuroprotection.

Materials and Methods

Generation of MEPO Protein
His6-tagged (3’end) EPO cDNAs, each containing a single amino acid mutation, were generated by polymerase chain reaction-based site mutagenesis and transfected into 293 cells. Recombinant protein was purified from the culture medium of 293 stable cell lines overexpressing MEPO using superflow Ni-NTA agarose columns (Quagen).

Bioactivity Assays of MEPO
In vitro bioactivity was determined using a proliferation assay in the EPO-dependent murine myeloid cell line 32D-EPOR. 32D-EPOR cells (2×10⁵ cells/well) were factor-starved for 4 hours in RPMI 1640 medium (Invitrogen) and then incubated in the presence of MEPOs or wild-type EPO (1 U/mL). Live cells were counted using trypan blue exclusion 72 hours later. For in vivo erythropoietic activity bioassay, C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine) were intraperitoneally injected with S104I-EPO or wild-type EPO twice weekly for 4 weeks at a dose of 5000 U/kg body weight. Hemoglobin and platelet levels were determined before the first EPO administration and then analyzed every 2 weeks.

NMDA Neurotoxicity
Primary cortical neurons at 11 days in vitro from pregnant Sprague-Dawley rats were pretreated with MEPOs or wild-type EPO (1 U/mL) for 8 hours and then challenged with NMDA (200 μmol/L) for 15 minutes. The neurons were then returned to normal culture medium supplied with the same concentration of MEPOs or wild-type EPO. Cell death was analyzed by nuclear staining (Hoechst 33258) and lactate dehydrogenase release 24 hours after NMDA treatment.

Murine Model of Focal Ischemia, Drug Administration, and Determination of Infarct Volume
All procedures were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Temporary focal ischemia was induced by left middle cerebral artery occlusion (MCAO) for 60 minutes as previously described. Animals were randomized and S104I-EPO, wild-type EPO, or vehicle (saline) was administered intraperitoneally at the concentration indicated at the onset of reperfusion and again at 24 and 48 hours of reperfusion.

Statistical Analysis
Results are presented as mean±SEM. The statistical significance of the difference between means was analyzed by the Student t test for single comparisons or by analysis of variance followed by post hoc Bonferroni/Dunn tests for multiple comparisons. Differences among groups were regarded as significant if P<0.05.

Results

Generation of Neuroprotective MEPOs That Lack Erythropoietic Activity
To obtain MEPOs that lack erythropoietic activity, we constructed and subsequently tested 12 EPO mutants, each containing a single amino acid mutation within 3 EPO receptor-binding motif regions that are essential for EPO’s erythropoietic activity: the amino terminal (amino acids 1–17), internal (amino acids 99–109), and carboxyl-terminal motif (amino acids 147–151). The bioactivity of these MEPOs was analyzed using an in vitro proliferation assay in the EPO-dependent myeloid cell line 32D-EPOR. As shown in Figure 1A, the survival and proliferation of 32D-EPOR cells depends entirely on the existence of EPO in the medium. Mutations within the carboxyl-terminal motif (N147A, N147K, and G151A) either increased or only partially reduced EPO bioactivity, whereas mutations within the amino-terminal motif (R14E, R14A, R14Q, and Y15I) significantly...
reduced but did not completely abolish EPO bioactivity. However, mutations within the internal motif (S100E, R103A, R103E, S104I, and L108K) resulted in complete loss of EPO bioactivity (Figure 1A), consistent with previous reports, indicating that the internal motif is essential for EPO bioactivity.7,8

Next, we tested whether the MEPOs retain neuroprotective effects against excitotoxic neural injury. MEPOs significantly reduced NMDA-induced neuronal cell death with an efficacy similar to that of wild-type EPO (Figure 1B). However, there was a nonsignificant trend toward better protection against NMDA toxicity after mutations within the internal motif compared with mutations within the amino terminal. Taken together, these data suggest that, although all amino terminal or internal motif MEPOs assayed retained equivalent neuroprotection against NMDA neurotoxicity compared with wild-type EPO, mutations within the amino-terminal motif resulted in only a partial loss of EPO bioactivity. In contrast, mutations within the internal motif not only retained neuroprotective effects, but also caused a complete loss of EPO bioactivity. We therefore chose mutants with mutations within the internal motif for further assessment.

Characterization of S104I-EPO
Amino acid residues 100, 103, 104, and 108 are exposed on the surface of the EPO molecule; thus, change of charge may potentially result in EPO binding to new targets due to charge–charge interactions. Among the 5 mutants targeting the internal motif, only S104I-EPO is a neutral amino acid substitution, whereas the other mutations either increased or decreased the charge. Interestingly, the 293 cells secreted higher levels of S104I-EPO into the medium compared with other MEPOs. The mechanism for this remains unclear, but it is likely due to impaired EPO secretion mechanism caused by charge change. Based on these observations, we chose S104I-EPO for further investigation.

S104I-EPO completely lost the ability to induce myeloid proliferation, even at a high concentration (100 U/mL, equivalent to 25 nmol/L; Figure 2A). To further confirm whether S104I-EPO loses its erythropoietic activity, hemoglobin levels in mice were measured after injection of a high dose of either S104I-EPO or wild-type EPO (both at 5000 U/kg) twice weekly. As shown in Figure 2B, S104I-EPO did not induce the production of hemoglobin even when continuously present at high concentrations over 4 weeks, whereas wild-type EPO significantly increased hemoglobin level at Weeks 2 and 4 (by 15% and 39%, respectively). EPO has been reported to stimulate the production of platelets, which may increase the likelihood of microcoagulation and secondary stroke; we therefore also measured platelet levels in mice. S104I-EPO administration did not induce the production of platelets at least up to 4 weeks posttreatment, whereas wild-type EPO significantly increased the platelet counts (Figure 2C).

To determine whether S104I-EPO retains neuroprotective effects, we first tested it in an in vitro model of NMDA neurotoxicity in primary neuronal cultures. As illustrated, pretreatment with S104I-EPO significantly decreased the number of neurons containing condensed nuclei in cultures (Figures 2D-g, 2D-h, and 2D-i) and prevented necrotic cell death after NMDA treatment as determined by lactate dehydrogenase release (Figure 2D-j). Importantly, no significant difference in neuroprotective efficacy was observed between S104I-EPO and wild-type EPO, suggesting that S104I-EPO and wild-type EPO are equally effective in protecting neurons against NMDA neurotoxicity with a mixed cell death phenotype.

S104I-EPO Is Neuroprotective Against Focal Cerebral Ischemia
The neuroprotective effects of S104I-EPO were further determined in the murine model of focal ischemia. S104I-EPO treatment significantly decreased infarct volume in a dose- and time-dependent manner (Figure 3A–B) with an efficacy similar to that of the wild-type EPO. S104I-EPO provided optimal protection at a concentration of 5000 U/kg administered intraperitoneally, a dose that has been widely used in the field to test the neuroprotective effect of wild-type EPO in rodent models of cerebral ischemia.5,9 At this dose, S104I-EPO reduced infarct volume by 53% (Figures 3A and 3C). S104I-EPO still exerted significant neuroprotection at 2000 U/kg, but the effect disappeared when administered at doses <1000 U/kg.

To determine the temporal time frame for neuroprotection, we examined whether postischemic administration of S104I-EPO would be capable of decreasing ischemic infarct volume. Injection of S104I-EPO immediately after the ischemic period provided optimal protection. However, S104I-EPO exerted significant neuroprotective effects even when administered up to 4 hours after ischemia, although the effects were diminished and lost when administered 6 hours after ischemia. Physiological parameters (blood pressure, gases, and glucose) and recombinant cerebral blood flow were measured with no significant differences between groups (data not shown).

Administration of S104I-EPO Improves Neurological Outcomes After Ischemia
We next tested whether the neuroprotective effect of S104I-EPO could be translated into functional improvement. S104I-EPO significantly improved neurological deficit scores as assessed at 72 hours after ischemia (Figure 4A). To further determine the impact of S104I-EPO on neurological recovery, 2 behavioral tests, the Rotorod and corner tests, were performed during a 7-day recovery period. As illustrated in Figure 4B, S104I-EPO-treated mice performed significantly better on the Rotorod at all time points tested after ischemia compared with vehicle-treated mice. Similarly, S104I-EPO-treated mice decreased preferential turning behavior toward the nonimpaired (right) side as assessed by the corner test, indicating improved bilateral motor behavior compared with vehicle-treated mice. No significant differences were found for functional outcomes between S104I-EPO-treated and wild-type EPO-treated mice, suggesting that S104I-EPO improves neurological outcomes with an efficacy similar to that of wild-type EPO.
S104I-EPO and Wild-Type EPO Exert Neuroprotective Effects Through Similar Signaling Pathways

We next sought to determine if MEPO and wild-type EPO activate similar cell survival pathways. Previous studies suggested that EPO derivatives do not bind to EPO receptors, but rather to a tissue-protective receptor complex, the common β receptor/EPO receptor heteroreceptors. However, the downstream survival signaling pathways relevant to ischemic neuroprotection may be similar to those activated by...

Figure 2. S104I-EPO completely loses erythropoietic activity but is neuroprotective. A, In vitro bioactivity assay of S104I-EPO as assessed by live cell counting using trypan blue exclusion in the 32D-EPOR cells. Data are mean±SEM from 3 independent experiments. B-C, In vivo erythropoietic and platelet-stimulating activity assay by measuring hemoglobin and platelet levels. Data are presented as mean±SEM, n=8 per group. *P<0.05, **P<0.01 versus wild-type EPO injection group. D, Phase contrast (a–d) and Hoechst staining (e–h) of primary neurons challenged with NMDA. NMDA induces both apoptotic-like (red arrows) and necrotic-like cell death (yellow arrows). Pretreatment with either EPO (c, g) or S104I-EPO (d, h) reduced both types of neuronal death. i, Quantitative counting of cell death induced by NMDA neurotoxicity. j, Relative LDH release compared with NMDA treatment alone. #P<0.05 versus NMDA alone. Data are mean±SEM from 3 independent experiments. EPO indicates erythropoietin; NMDA, N-methyl-D-aspartate; LDH, lactic dehydrogenase.

Figure 3. S104I-EPO reduces infarct volume. A, Dose efficacy of S104I-EPO on reduction of infarct volume. B, Temporal profile of S104I-EPO (5000 U/kg) on reduction of infarct volume. C, Representative photographs of TTC-stained sections of mouse brains. The same dose of wild-type EPO was used as control in all groups. All quantitative data are mean±SEM, n=8 per group. *P<0.05 versus vehicle group, **P<0.01 versus vehicle group. EPO indicates erythropoietin; TTC, 2,3,5-triphenyl tetrazolium chloride.
Therefore, we tested whether MEPO mediates neuroprotection through PI3K/AKT, mitogen-activated protein kinase/ERK1/2, and STAT5 signaling, because these pathways are critical to the neuroprotective effects of EPO.\(^2,11\) Primary neurons were treated with S104I-EPO, and the activation of signaling molecules was analyzed by Western blot (Figure 5A). Similar to wild-type EPO, S104I-EPO increased the phosphorylation of AKT and ERK1/2 as early as 30 minutes after exposure. Increased phospho-AKT and phospho-ERK1/2 levels were observed over the time course assessed, including 24 hours after exposure. S104I-EPO also increased the level of phosphorylated STAT5, beginning 16 hours after S104I-EPO exposure. To determine the role of these signaling pathways in the context of MEPO-mediated neuroprotection, primary neurons were challenged with NMDA in the presence or absence of either the PI3K inhibitor LY294022 (Figure 5B) or the ERK1/2 inhibitor PD98059 (Figure 5C). Both inhibitors significantly decreased the protective effects of EPO and S104I-EPO against NMDA neurotoxicity. Finally, we tested the relevance of these signaling pathways in MEPO-mediated neuroprotection against cerebral ischemia in mice. Intracerebroventricular injection of either LY294002 or PD98059 led to a diminished neuroprotective effect of S104I-EPO, and combined injection of LY294002 and PD98059 completely abolished S104I-EPO-induced neuroprotection. Injection of LY294002 or PD98059 alone did not show a significant difference compared with the vehicle control group. These data suggest that the PI3K/AKT and ERK1/2 signaling pathways may synergistically mediate the neuroprotective effect of MEPO. Furthermore, the data underscore that both S104I-EPO and wild-type EPO may activate similar signaling pathways to induce neuroprotection.

**Discussion**

EPO exerts a potent neuroprotective effect against ischemic brain injury in both animal experiments and clinical trials.\(^3,12\) However, several critical limitations have hampered the potential for extensive use of EPO as a clinically therapeutic tool after stroke. Of primary concern, the delivery of EPO to the brain through systemic administration is technically

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**Figure 4.** S104I-EPO treatment improves neurological outcomes. S104I-EPO treatment improves neurological outcomes as determined by neurological deficit scores (A), Rotorod test (B), and corner test (C). *P<0.05 versus vehicle group. EPO indicates erythropoietin.

**Figure 5.** S104I-EPO activates the same survival signaling pathways as wild-type EPO. A, S104I-EPO activates the phosphorylation of AKT, ERK1/2, and STAT5 in primary neuronal cultures. AKT inhibitor LY294002 (LY) and ERK1/2 inhibitor PD98059 (PD) attenuated the neuroprotective effect of S104I-EPO against NMDA neurotoxicity as assessed by Hoechst staining of nuclei. The same dose of wild-type EPO was used as control in all groups. B, Effect of AKT and ERK1/2 inhibitors on infarct volume in S104I-EPO-treated groups. Data are mean±SEM, *P<0.05 versus S104I-EPO-treated groups. EPO indicates erythropoietin; ERK, extracellular signal-regulated kinase; NMDA, N-methyl-D-aspartate.
challenging. EPO does not easily cross the blood–brain barrier and possesses a relatively low affinity for receptors involved in tissue protection compared with receptors for erythropoietic function. The delivery of EPO to the brain can be enhanced by addition of protein transduction peptide, but this still requires doses higher than that used for the treatment of kidney anemia. Second, the activation of signaling pathways diminishes 24 to 48 hours after EPO exposure (unpublished observations), raising the possibility that multiple administrations of EPO may be required for treatment of stroke and rekindling of signaling pathways over the course of the injury. Multiple administrations of high doses of EPO may result in increased risk factors for patients with stroke such as increased hematocrit and platelet quantity, which could give rise to secondary infarction. Accordingly, alternate strategies to develop nonerythropoietic EPO derivatives will greatly improve the potential for clinical application in the treatment of patients with stroke.

Three types of EPO derivatives have been generated that lack erythropoietic activity yet retain neuroprotective effects: asialoerythropoietin, carbamylated EPO, and MEPO. Of all these options, MEPO possesses the optimal translatable potential as a therapy. For example, the plasma half-life of intravenously injected asialoerythropoietin is only 1.4 minutes due to rapid clearance. Thus, continuous administration of asialoerythropoietin is likely needed to compensate for clearance before crossing the blood–brain barrier. Although a single injection of asialoerythropoietin has been shown to be neuroprotective against ischemic brain injury, the timing of this injection may require precise correlation with blood–brain barrier compromise, a situation with limited clinical applicability. Carbamylated EPO, in which all 8 lysine residues are chemically modified to homocitrulline, has also been demonstrated to exert ischemic neuroprotection. However, MEPO has several major advantages over carbamylated EPO in potential clinical applicability. First, the generation and purification of MEPO is fairly straightforward and does not require the complex chemical modifications necessary for carbamylated EPO. The option of MEPO thus significantly reduces the production/cost value compared with carbamylated EPO. Second, MEPO contains a single amino acid substitution. The conversion of all 8 lysine residues to homocitrulline to generate carbamylated EPO. Third, MEPO activates the same signaling pathways in neurons such as PI3K/AKT and mitogen-activated protein kinase/ERK1/2.

The precise mechanism underlying the neuroprotective effect of MEPO remains unknown. A previous study indicated that EPO derivatives incapable of binding to classical EPO homoreceptors may mediate neuroprotection through binding to a tissue protective receptor consisting of both the common β receptor and the EPO receptor, forming a heteroreceptor that activates similar survival signaling pathways activated by wild-type EPO such as PI3K/AKT and mitogen-activated protein kinase/ERK1/2. Consistent with this concept, we found that both S104I-EPO and wild-type EPO activate the same signaling pathways in neurons such as PI3K/AKT, mitogen-activated protein kinase/ERK1/2, and STAT5 (Figure 5A). Inhibition of these signaling pathways using either the PI3K inhibitor LY294002 or the ERK1/2 inhibitor PD98059 not only attenuated the neuroprotective effect of S104I-EPO against NMDA-induced neurotoxicity (Figure 5B–C), but also inhibited the neuroprotective effect of S104I-EPO against ischemic brain injury in the MCAO model (Figure 5D). Interestingly, combined application of LY294002 and PD98059 completely abolished the neuroprotective effect of S104I-EPO in ischemia, suggesting that PI3K/AKT and mitogen-activated protein kinase/ERK1/2 pathways may synergistically mediate the neuroprotective effect of S104I-EPO. The observation that STAT5 activation is delayed until 16 hours after addition of S104I-EPO suggests that STAT5 signaling might occur downstream of either PI3K/AKT or mitogen-activated protein kinase/ERK1/2 signaling. The further characterization of neuroprotective signaling triggered by MEPO warrants additional investigation, including the identification of the receptor required for neuroprotection, a deeper understanding of downstream signaling molecules, and the exploration of parallel signaling pathways.

In summary, S104I-EPO completely lacks erythropoietic activity but retains neuroprotective effects against in vitro NMDA neurotoxicity and ischemic brain injury in a murine model of MCAO with an efficacy similar to that of wild-type
EPO. Furthermore, although S104I-EPO activates survival signaling pathways similar to those of wild-type EPO, the nonerythropoietic feature of S104I-EPO avoids important clinical confounds for the patient with stroke. Thus, the novel S104I-EPO should be explored as a therapeutic agent for the treatment of stroke and other neurological diseases.

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None.

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