

Amelioration of spinal cord compressive injury by pharmacological preconditioning with erythropoietin and a nonerythropoietic erythropoietin derivative

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Object. Spinal cord injury (SCI) is a devastating clinical syndrome for which no truly efficacious therapy has yet been identified. In preclinical studies, erythropoietin (EPO) and its nonerythropoietic derivatives asialoEPO and carbamylated EPO have markedly improved functional outcome when administered after compressive SCI. However, an optimum treatment paradigm is currently unknown. Because the uninjured spinal cord expresses a high density of EPO receptor (EPOR) in the basal state, signaling through these existing receptors in advance of injury (pharmacological preconditioning) might confer neuroprotection and therefore be potentially useful in situations of anticipated damage.

Methods. The authors compared asialoEPO, a molecule that binds to the EPOR with high affinity but with a brief serum half-life ($t_{1/2} < 2$ minutes), to EPO to determine whether a single dose (10 $\mu\text{g}/\text{kg}$ of body weight) administered by intravenous injection 24 hours before 1 minute of spinal cord compression provides benefit as determined by a 6-week assessment of neurological outcome and by histopathological analysis. Rats pretreated with asialoEPO or EPO and then subjected to a compressive injury exhibited improved motor function over 42 days, compared with animals treated with saline solution. However, pretreatment efficacy was substantially poorer than efficacy of treatment initiated at the time of injury. Serum samples drawn immediately before compression confirmed that no detectable asialoEPO remained within the systemic circulation. Western blot and immunohistochemical analyses performed using uninjured spinal cord 24 hours after a dose of asialoEPO exhibited a marked increase in glial fibrillary acidic protein, suggesting a glial response to EPO administration.

Conclusions. These results demonstrate that EPO and its analog do not need to be present at the time of injury to provide tissue protection and that tissue protection is markedly effective when either agent is administered immediately after injury. Furthermore, the findings suggest that asialoEPO is a useful reagent with which to study the dynamics of EPO-mediated neuroprotection. In addition, the findings support the concept of using a nonerythropoietic EPO derivative to provide tissue protection without activating the undesirable effects of EPO.

KEY WORDS • erythropoietin • neuroprotection • spinal cord injury • rat

THE widespread and highly efficacious use of rhEPO for the treatment of EPO-deficient anemia reflects a triumph of biotechnology. However, as a typical member of the cytokine superfamily, EPO also performs other nonhormonal functions. Recently, EPO has been

identified as an important endogenous mediator of the adaptive responses of tissues to metabolic stress, primarily by limiting the extent of injury. Peripherally administered rhEPO penetrates the blood-brain barrier and potentially reduces brain injury after a variety of insults.^{9,17} Additional evidence has shown widespread efficacy of rhEPO in injury models of the spinal cord,^{15,28} subarachnoid hemorrhage,³¹ and retina and heart damage.^{11,34} The mechanisms by which EPO exerts its beneficial effects are incompletely understood. Previous research has suggested that EPO acts in a coordinated fashion at multiple levels to limit the production of tissue-injuring molecules such as glutamate³⁶ and to reverse vasospasm,³⁰ attenuate apopto-

Abbreviations used in this paper: BBB = Basso-Beattie-Bresnahan; EPO = erythropoietin; EPOR = EPO receptor; GFAP = glial fibrillary acidic protein; GSK = glycogen synthase kinase; IgG = immunoglobulin G; JAK = janus kinase; NeuN = neuronal nerve; NF- κ B = nuclear factor- κ B; PBS = phosphate-buffered saline; rhEPO = recombinant human EPO; SCI = spinal cord injury.

Erythropoietin-mediated preconditioning in spinal cord injury

sis,^{15,20} modulate inflammation,⁹ and recruit stem cells.⁵³ A Phase II clinical trial has demonstrated significant improvement in outcome of ischemic stroke patients administered rhEPO intravenously within 8 hours of the onset of symptoms.²¹

Multiple studies have shown that EPO plays a major role in ischemic preconditioning, a well-known phenomenon in which mild ischemia occurring in advance of severe ischemia is markedly protective against injury.^{18,51} Ischemic preconditioning in the nervous system is a powerful protective mechanism that requires protein synthesis and depends on endogenous EPO production. In this regard, *in vitro* studies using cultured neurons and glia have shown that both rapid preconditioning and delayed preconditioning occur after exposure to EPO.^{18,51} Furthermore, pretreatment with EPO has been demonstrated to induce tolerance to transient focal cerebral ischemia in the mouse, as evidenced by a reduction of infarct volume, whereas infusion of soluble EPOR has been shown to significantly reduce the protective effect of hypoxic pretreatment.⁴⁹ These data strongly support the role of EPO as an essential mediator of protection in hypoxic preconditioning. Because many clinical scenarios are characterized by anticipated potential injury (for example, nervous system tumor surgery or vascular malformation surgery), the relevance of pharmacological preconditioning is clear.

Pharmacological preconditioning clearly depends on expression of the cognate receptor at the time of exposure. In the case of EPO, many tissues/regions exhibit only low levels of expression of EPOR as well as EPO without undergoing metabolic stress. Although positive regulation of cytokine receptors has been reported for many ligands, it is not known to what extent EPO may induce its own receptor in the setting of injury. Study of blood-brain barrier models has shown that EPO induces functional EPOR in capillary endothelial cells with a delay of several hours.⁴³ One tissue with high levels of basal EPOR expression is the spinal cord, where neurons, glial cells, capillaries, and ependymal cells express immunoreactive EPOR protein in the basal, uninjured state.^{32,35,40}

The aim of the present study was to assess how effectively pretreatment with EPO produces pharmacological preconditioning of the spinal cord *in vivo*. The answer to this question has clear relevance for treatment in clinical situations. However, due to its extensive carbohydrate composition, EPO is a long-lived circulating molecule, with a serum half-life of 5 to 6 hours after intravenous injection. Thus, brief signaling by EPO is not possible *in vivo*. With removal of the terminating sialic acid moieties of EPO, the resulting asialoEPO has a very brief half-life (< 2 minutes after intravenous injection) and can be used transiently to initiate signaling.²²

Glial activation is one of the many signals and mechanisms involved in EPO-mediated beneficial effects in the nervous system.^{38,59} In the present study, we also investigated glial response after asialoEPO pretreatment by using GFAP marker detection.

Materials and Methods

Animal Preparation

Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with

international laws and policies. An SCI model in rats was used. Sprague-Dawley rats of similar body weight (275–300 g) were anesthetized by a mixture of oxygen and isoflurane. Body temperature was maintained between 37 and 38°C with a heating pad during all procedures. Surgery was performed using sterile technique and with the aid of a surgical microscope. Animals were subjected to a traumatic injury by using a clip with a 58-g closing force applied extradurally at T-3 for 1 minute.⁵⁰ Immediate paraplegia was observed in every case. After the operation, animals' urinary bladders were compressed manually three times daily until a reflex bladder was established. Highly absorbent bedding was used in cages. Rats were housed in pairs to reduce isolation-induced stress. Animals were maintained in a 12-hour light/dark cycle with water and food freely available at an ambient temperature of 25 to 27°C. No prophylactic antibiotic agents were given.

Treatment Groups and Drug Administration

Fifty-four Sprague-Dawley rats were assigned to one of eight groups. The control group of six rats (Group 1) received saline solution as placebo. In a blinded fashion, a single dose (10 µg/kg of body weight) of asialoEPO (made as described previously²²) was administered by intravenous injection to six rats (Group 2) 24 hours before and to six rats (Group 3) immediately after the injury. In another group of six rats (Group 4) multiple doses of asialoEPO (three daily doses and then twice a week) were administered. Comparison was made to similar administration of EPO (single doses 24 hours before and immediately after injury and multiple doses [Dragon Pharmaceuticals, Inc., Vancouver, BC, Canada]) in three additional groups of six rats each (Groups 5, 6, and 7). Glial response after asialoEPO pretreatment was examined in 12 uninjured rats (Group 8).

Assay of Serum Levels of AsialoEPO and EPO

Serum levels of asialoEPO and EPO were assessed immediately before the spinal cord compression in animals that received drug pretreatment (Groups 2 and 5). Blood samples were drawn from a catheter implanted in the caudal artery. AsialoEPO and EPO concentrations were determined by enzyme-linked immunosorbent assay kits (R & D Systems and Immuno-Biological Laboratories, Hamburg, Germany). Extensive evaluation confirmed that the antibodies used in these kits identified asialoEPO and rhEPO with equal sensitivity. The lower limit of quantification was 1.0 pM. Control animals had undetectable serum EPO levels (rat EPO is not recognized by the antibody in the assay kit).

Detection of GFAP

The extent of glial response as a result of pretreatment with asialoEPO or saline solution was estimated in 12 uninjured animals (Group 8) by GFAP detection using Western blot analysis and immunohistochemical analysis. Twenty-four hours after drug administration, the animals were killed. In six of the 12 rats, the spinal cord was isolated and then homogenized at 4°C in 1 ml of homogenization buffer. The homogenate was separated using a sodium dodecyl sulfate-polyacrylamide gel electrophoresis system (Bio-Rad Laboratories, Hercules, CA), and the proteins were transferred to a nitrocellulose membrane. After being washed with Tris buffered saline solution containing 0.05% Tween-20, the membrane was incubated with a blocking buffer for a period of 2 hours. Then the membrane was incubated with anti-GFAP mouse IgG in blocking buffer at 4°C overnight. After a thorough washing, the membrane was incubated with anti-mouse IgG peroxidase conjugate in blocking buffer for 60 minutes. Blots were developed according to the enhanced chemiluminescence method by using chemiluminescent substrate (LumiGLO; KPL, Gaithersburg, MD) and peroxide reagents (Cell Signaling Technology, Beverly, MA). The relative density of the protein bands was quantified by densitometry using an electrophoresis documentation and analysis system (EDAS 120; Eastman Kodak, Rochester, NY).

For immunohistochemical analysis, the spinal cords of the remaining six animals were isolated, fixed in 10% formaldehyde, embedded in paraffin, and processed for histological studies. Five-micrometer-thick sections were obtained from the injured area.

Sections were cut using a microtome, deparaffinized in xylene, and rehydrated in a graded ethanol series. Slides were steamed in 0.01 mol/L of sodium citrate buffer with a pH of 6 for 10 minutes in a microwave oven. Endogenous peroxidase activity was quenched by exposing the slides to a 3% solution of hydrogen peroxide in methanol for 20 minutes, after which the slides were rinsed in PBS for a total of 10 minutes. Slides were incubated with primary antibodies (anti-GFAP used at 1:250 dilution) overnight at 4°C. Slides were rinsed two times in PBS for 10 minutes and incubated for 30 minutes with the specific biotinylated secondary antibody (Vector Laboratories, Inc., Burlingame, CA). The slides were then washed in PBS and incubated for 30 minutes with the avidin–biotin complex (Vector Laboratories, Inc.) followed by a development with 3,3'-diaminobenzidine) for 5 minutes. Slides were counterstained with H & E and examined microscopically to assess GFAP immunoreactivity. All specimens were compared with negative controls performed using as primary antibodies either normal IgG or PBS. In each animal, 10 sections each measuring approximately 6 mm long and each separated by approximately 0.5 mm were randomly selected from the injured zone. Images were photographed through a microscope and captured on a computer, where the images were magnified and evaluated using image analysis software. Cells that contained any brown 3,3'-diaminobenzidine were identified as GFAP positive.¹² An investigator who was unaware of the experimental groups from which each sample was obtained conducted this assessment. The number of GFAP-positive cells was expressed as the mean labeled cell count \pm standard deviation.

Motor Function and Histopathological Evaluation

Postoperatively, locomotor function was evaluated daily by a colleague who was unaware of the treatment groups. Motor function of the injured animals was followed for 6 weeks after SCI by using the BBB locomotor rating scale⁵ and expressed as a BBB score ranging from 0 (paraplegic) to 21 (normal).

Six weeks after injury, the animals subjected to SCI were killed, and the spinal cords were removed and fixed in formalin for histological analysis. Paraffin-embedded tissue was sectioned (5- μ m-thick sections) to determine the extent of injury. For quantitative analysis of tissue damage, a total of 20 sections obtained rostrally and caudally from the injury epicenter were examined with light microscopy by an investigator who was unaware of the experimental treatment. The percentage of posttraumatic cavity area (injured area) in each section was quantitatively determined using a public-domain image program. The relative amount of spared tissue was calculated as a percentage by using the following formula: (total area – injured area)/total area \times 100.⁶⁴

Specimens were also stained by using anti-NeuN (dilution 1:200; Chemicon, Temecula, CA) to identify neurons and then counterstained with H & E. Neurons showing positive immunoreactivity for NeuN were counted in each section. The results of neuronal counting in five randomly selected sections from each animal were averaged with each microscopic field corresponding to 1.6 mm².

Statistical Analysis

All data were expressed as the mean labeled cell count \pm standard deviation. Group comparisons of differences in quantitative measurements were made by analysis of variance, followed by the Dunnett t-test. A probability value less than 0.05 indicated statistical significance.

Results

Serum Levels of AsialoEPO and EPO

Serum levels of asialoEPO administered 24 hours before the spinal cord compression in animals receiving drug pretreatment were undetectable, whereas EPO was detected with a mean serum level of 9 ± 2.1 pg/ml.

Detection of GFAP

Western blot analysis performed using uninjured spinal

cord tissue 24 hours after a dose of asialoEPO or saline solution exhibited a significant marked increase in GFAP in animals pretreated with asialoEPO ($105 \pm 4.7\%$), compared with saline-treated rats ($20 \pm 3.5\%$) ($p < 0.05$), suggesting a glial response to EPO administration (Fig. 1).

Immunohistochemical GFAP detection revealed a higher frequency of GFAP-positive cells in spinal cord sections obtained from animals pretreated with asialoEPO, compared with those from animals treated with saline solution ($p < 0.05$) (Fig. 2).

Further investigation is needed to determine whether such an increase in GFAP immunoreactivity reveals glial activation.

Motor Function

Motor evaluations were performed for 6 weeks after injury. Pretreatment with asialoEPO and EPO significantly improved recovery, compared with pretreatment with saline solution ($p < 0.05$), but was inferior in its effects on recovery to asialoEPO administered at the time of injury ($p < 0.05$). No statistical differences were observed between EPO and asialoEPO pretreatments. Furthermore, EPO administered at the time of injury was as effective as asialoEPO administered at the time of injury. Finally, multiple doses of either agent did not appreciably improve recovery. Figure 3 illustrates these findings.

Histological Evaluation

The histological findings differed markedly among the groups. In particular, animals pretreated with asialoEPO and EPO exhibited a restricted injury with nearly normal architecture of the spinal cord (Fig. 4A and B). In contrast, the group treated with saline solution exhibited extensive cytoarchitectural disruption and edema (Fig. 4C) throughout the cord. The histological appearance of cord from the animals treated with asialoEPO and EPO at the time of injury showed better preservation of the cord cytoarchitecture, consistent with the superior motor scores of those animals (Fig. 4D and E). The appearance of spinal cord from animals treated with asialoEPO or EPO over multiple doses did not present considerable differences, compared to cord from animals treated with a single dose (Fig. 4F and G).

In the quantitative analysis, the percentage of damaged tissue was significantly higher in the spinal cords from the animals treated with saline solution than in the cords from the asialoEPO- and EPO-pretreated animals ($p < 0.05$). Significant reduction in the percentage of cavity area and significant increase in the percentage of spared tissue was mainly associated with asialoEPO and EPO administration at the time of injury ($p < 0.05$). Administration of asialoEPO or EPO over multiple doses did not significantly decrease the percentage of cavity area and did not lead to significant tissue sparing (Fig. 5).

The number of surviving neurons demonstrating positive immunoreactivity for NeuN was significantly higher in the rats pretreated with asialoEPO and EPO than in the rats pretreated with saline solution ($p < 0.05$). No statistically significant difference was observed in the count of NeuN-positive neurons between the animals pretreated with asialoEPO and those pretreated with EPO. Animals in which asialoEPO or EPO was administered at the time

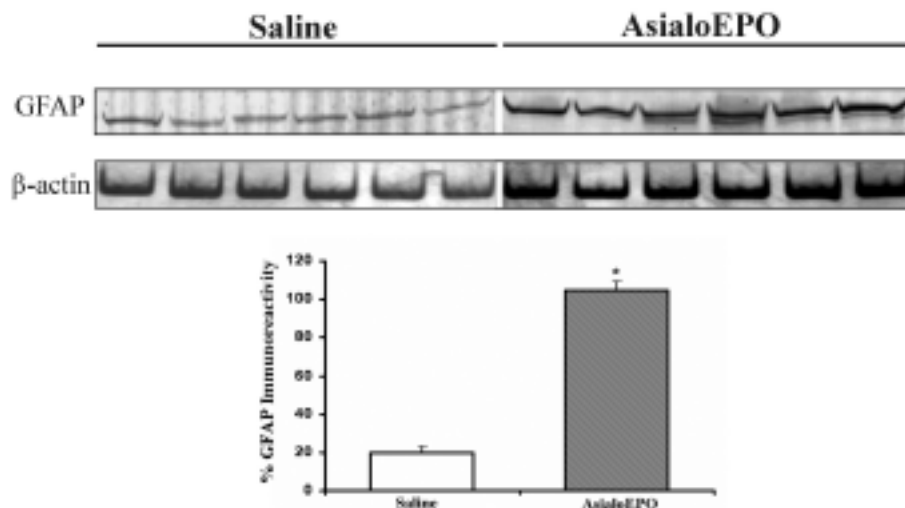


FIG. 1. *Upper:* Western blot analysis demonstrating that asialoEPO administered to uninjured rats increased GFAP expression in spinal cord tissue, compared with the administration of saline solution. The β -actin controls displayed equal protein loading. *Lower:* Bar graph depicting GFAP immunoreactivity estimated by using the densitometry analysis method normalized to percentages. * $p < 0.05$, compared with rats treated with saline solution.

of injury demonstrated a significant increase in the number of NeuN-positive neurons, compared with animals pretreated with asialoEPO or EPO ($p < 0.05$). No statistically significant differences were observed between animals treated with asialoEPO at the time of injury and those treated with EPO at the time of injury, and between animals that were treated with multiple doses of asialoEPO and those treated with multiple doses of EPO. Figure 6 summarizes the total counts of NeuN-positive neurons in each group.

Discussion

Preconditioning-induced tolerance against brain and spinal cord ischemia has been the subject of intensive investigation. Preconditioning may be achieved by preexposing the brain or spinal cord to repetitive short instances of ischemia or hypoxia (ischemic or hypoxic preconditioning),^{4,33} electrical stress,²⁶ or chemical agents (pharmacological preconditioning).^{8,13,19} In the present study, we tested the hypothesis that pretreatment with EPO and its desialated derivative increases tolerance of the spinal cord against compressive injury. Our results demonstrate that pretreatment with asialoEPO and EPO provides significant protection but that single doses of both agents administered at the time of injury provide maximal effect, indicating a better result. Furthermore, we found that additional doses given after an initial dose do not materially improve recovery.

We report two principal findings from the present study. First, our results confirm previous observations of a neuroprotective effect after EPO^{15,28} and asialoEPO²² administration in experimental SCI. Second, pharmacological preconditioning through asialoEPO and EPO administration has been shown to trigger a process that provides spinal cord protection against progressive secondary injury after primary spinal cord damage. Our finding of pharmacological preconditioning mediated by EPO and its derivative is in agreement with findings in recent studies showing that

EPO plays a role in ischemic preconditioning.^{18,51} We suggest that a pure triggering of preconditioning can be assumed for asialoEPO, because we found that asialoEPO was undetectable in serum 24 hours after administration. However, it cannot be assumed for EPO, because we detected a mean serum level of 9 pg/ml of EPO before spinal cord trauma was initiated. Pharmacological preconditioning by asialoEPO has been reported in a model of neonatal hypoxia-ischemia.²³ In this study the authors found significant protection when asialoEPO or EPO was administered 4 hours before injury, whereas the same treatment was ineffective when administered at 24 hours and 4 hours before injury. The difference may be related to the low level of EPOR expression in uninjured cerebral cortex, compared with the normal spinal cord, and therefore with a lack of signaling in the brain.

The phenomenon of ischemic preconditioning has been observed in multiple tissues and organs. For example, ischemic preconditioning can provide significant neuroprotection after both focal and global cerebral ischemia^{44,48} and can improve ischemic tolerance in the spinal cord as well.^{1,66,67} Assuming that both traumatic and ischemic central nervous system injuries share several common mechanisms, it is expected that ischemic preconditioning will improve outcome after central nervous system traumatic injuries as well. In fact, ischemic preconditioning has been shown to increase the volume of preserved tissue after traumatic brain injury.⁴⁷ However, the effect of preconditioning on the outcome after traumatic SCI in an animal model has been evaluated in only a few studies,^{26,27,46} and none of these studies has evaluated purely pharmacological preconditioning.

Pharmacological preconditioning depends on expression of a cognate receptor where the drug can be bound at the time of exposure. It has been reported that EPO can confer protection even if given before the insult⁴² and that the effect lasts for at least 3 days.⁵⁵ Erythropoietin exerts its effects through the activation of its receptor (EPOR),

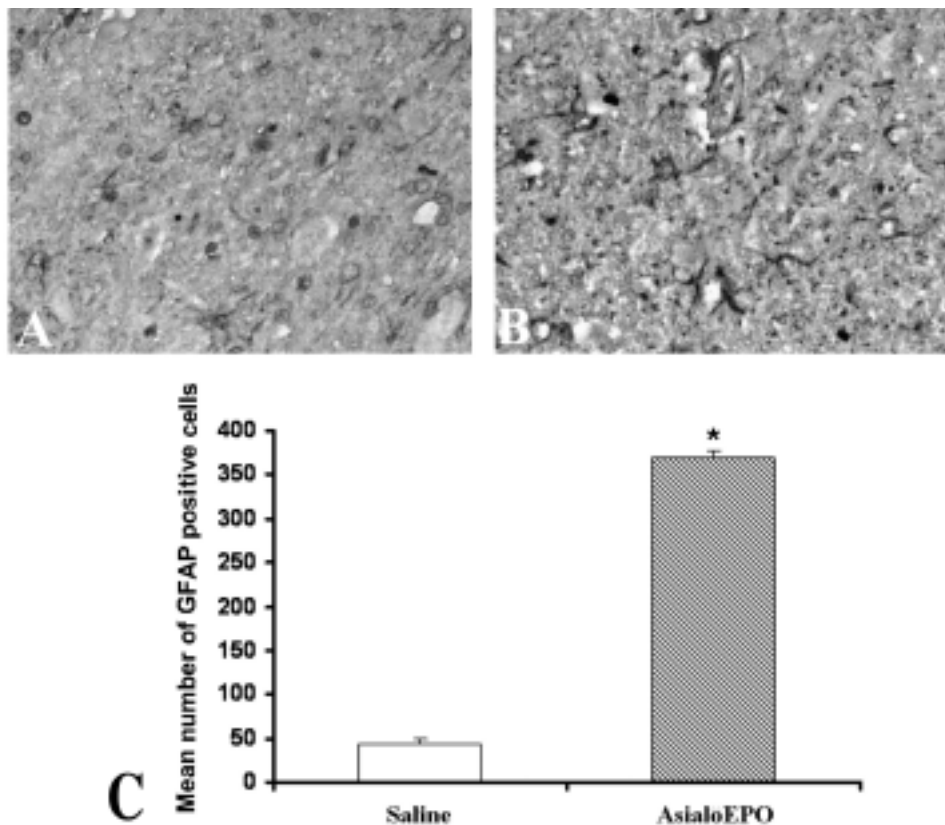


FIG. 2. Photomicrographs of rat spinal cord tissue showing that asialoEPO (A) administered to uninjured animals increased GFAP expression, compared with the administration of saline solution (B). Reactive astrocytes were especially prominent around neurons and vessels. H & E, original magnification $\times 2.5$. Bar graph (C) depicting the mean numbers of GFAP-positive cells in slices of normal rat spinal cord after pretreatment with asialoEPO and saline solution. * $p < 0.05$, compared with rats pretreated with saline solution.

part of the cytokine receptor type I superfamily. Many tissues exhibit EPOR at baseline without undergoing metabolic or traumatic stress. High levels of basal EPOR expression have been found in the spinal cord, where neurons, glial cells, capillaries, and ependymal cells express immunoreactive EPOR protein in the basal, uninjured state.^{35,40} In preclinical studies EPO has been shown to be neuroprotective after SCI.^{15,28} Although the mechanisms by which EPO acts as a neuroprotectant are still a matter of controversy, an increasing amount of evidence has suggested that EPOR activation after EPO binding inhibits neuronal apoptosis.^{15,20} Prevention of neuronal apoptosis involves the activation of JAK-2 and NF- κ B signaling pathways.²⁰ In particular, it has been suggested that the binding of EPO to its receptor induces the activation of JAK-2, leading to phosphorylation of the inhibitor of NF- κ B, subsequent translocation of the transcription factor NF- κ B from the cytoplasm to the nucleus, and eventual transcription of neuroprotective genes. In addition, EPO appears to prevent apoptotic injury through an Akt-dependent mechanism.³ One of a variety of enzymes involved in pathways that promote cell survival, Akt has been shown to block cellular apoptotic degradation through inhibition of GSK-3 β activity.⁷ Glycogen synthase kinase-3 β has been shown to be involved in the signaling pathway of

preconditioning in the heart⁶¹ and to play a significant role in the regulation of apoptosis in neurons,² vascular smooth-muscle cells,⁴¹ and cardiomyocytes.⁶⁵ In addition, GSK-3 β has been shown to be suppressed by EPO.⁵⁶

Erythropoietin-mediated neuroprotection after SCI includes other triggering events such as restoration and maintenance of vascular autoregulation. Spinal cord injury is associated with an early loss of vascular autoregulation, leading to the development of vascular hyperpermeability and tissue edema that ultimately cause degeneration of spinal cord white matter tracts.^{14,24} Antagonization of such processes provides rapid motor recovery.^{45,47}

In preclinical injury models of cerebral vasospasm induced by subarachnoid hemorrhage^{31,57} or splanchnic artery constriction in the setting of septic shock,⁵⁸ it has been shown that EPO can reverse vascular spasm, thus providing neuroprotection. This vascular effect of rhEPO appears to depend on modulation of the activity of inducible nitric oxide synthase.⁵⁸ The neuroprotective effect of rhEPO has been shown to depend on inhibition of nitric oxide production;¹⁰ thus, it is reasonable to hypothesize that similar mechanisms may be relevant within the spinal cord. Furthermore, inflammatory cells are involved in the late damage that occurs to the oligodendrocytes that provide the myelin for axons within the spinal cord.⁶⁰ Recombinant human EPO appears to reduce the inflammatory infiltrate and

Erythropoietin-mediated preconditioning in spinal cord injury

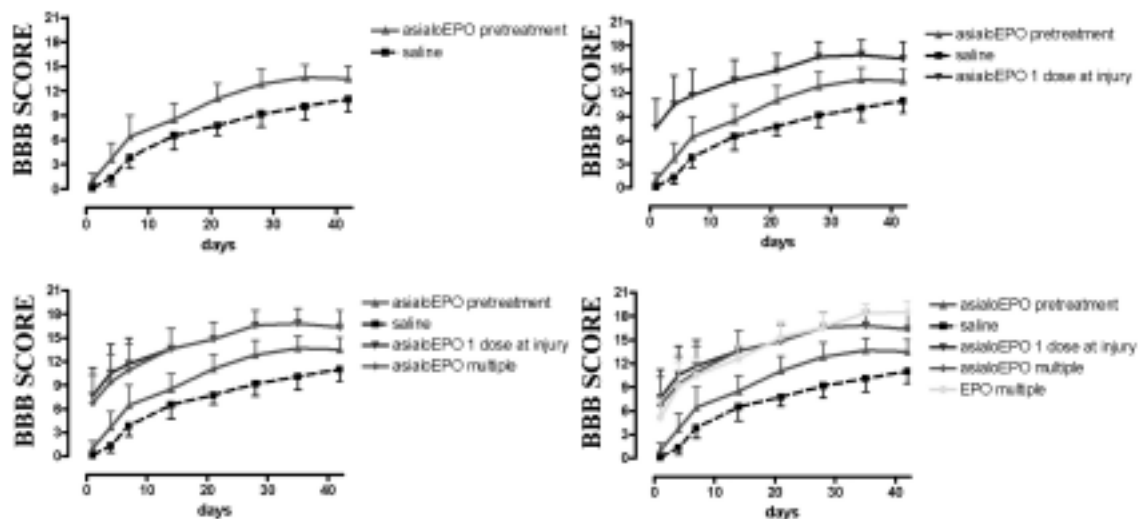


FIG. 3. Graphs depicting locomotor outcome (BBB score) evaluated for 6 weeks after SCI in rats pretreated with asialoEPO, EPO, or saline solution. Pretreatment with asialoEPO significantly improved recovery, compared with saline solution (*upper left*; * $p < 0.05$), but was inferior to asialoEPO administered at the time of injury (*upper right*; * $p < 0.05$). Although EPO administration at the time of injury was as effective as asialoEPO administration at the time of injury (data not shown), multiple doses of either agent did not appreciably improve recovery (*lower left and right*; * $p < 0.05$).

in this manner is likely to reduce the contribution of late injury to neurological deficit.²⁸

Additional possible mechanisms through which EPO could achieve neuroprotective effects include neuronal protection from glutamate toxicity by activation of calcium channels,⁵² production of antioxidant enzymes in neurons,³⁷ and neoangiogenesis, which improves blood flow

and tissue oxygenation in the border zone of an ischemic area.⁶

Although a prominent role in ischemic preconditioning has been proposed for EPO,^{20,54} the exact mechanism of protection afforded by EPO and its derivative by preconditioning is not clearly understood. As for the downstream processes, it is noteworthy that the mechanisms of neuro-

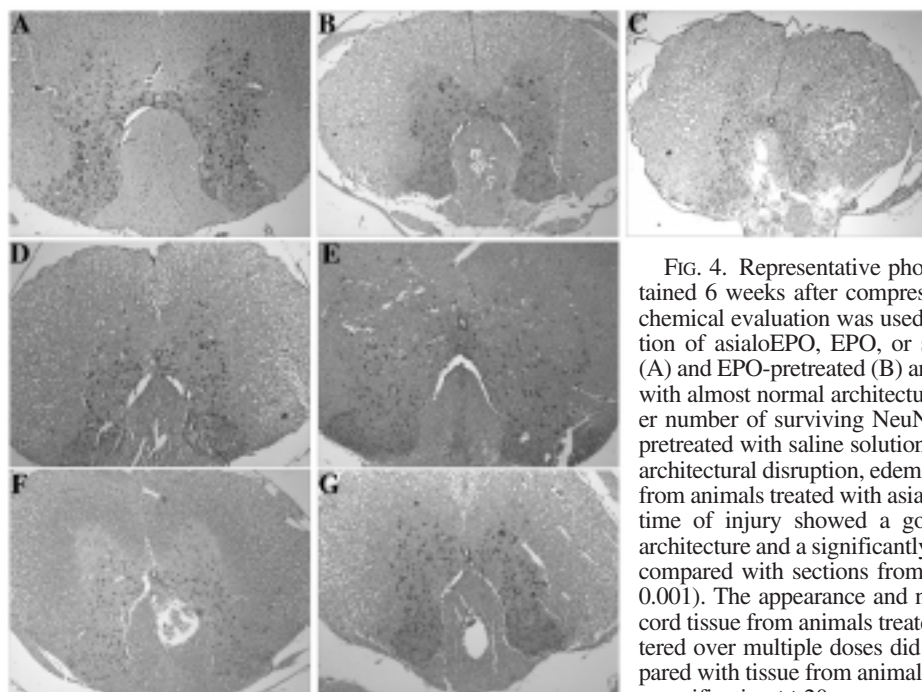


FIG. 4. Representative photomicrographs of rat spinal cord tissue obtained 6 weeks after compressive injury. Neuronal nerve immunohistochemical evaluation was used to identify living neurons after administration of asialoEPO, EPO, or saline solution. Both asialoEPO-pretreated (A) and EPO-pretreated (B) animals presented with a restriction of injury, with almost normal architecture of the spinal cord and significantly higher number of surviving NeuN-positive neurons, compared with animals pretreated with saline solution (C; $p < 0.05$), which exhibited wide cytoarchitectural disruption, edema, and few NeuN-positive neurons. Sections from animals treated with asialoEPO (D) and EPO (E) administered at the time of injury showed a good preservation of the spinal cord cytoarchitecture and a significantly greater number of NeuN-positive neurons, compared with sections from animals pretreated with both agents ($p < 0.001$). The appearance and number of NeuN-positive neurons in spinal cord tissue from animals treated with asialoEPO (F) or EPO (G) administered over multiple doses did not present considerable differences, compared with tissue from animals treated with a single dose. H & E, original magnification $\times 20$.

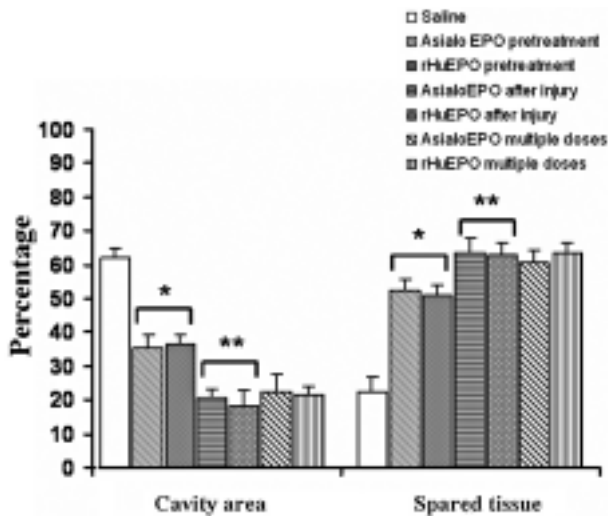


FIG. 5. Bar graph depicting percentages of damaged and spared tissue and cavitation volume assessed at 6 weeks after SCI in rats treated with asialoEPO, EPO, or saline solution. The percentage of cavity area was significantly higher in rats pretreated with saline solution, compared with asialoEPO-pretreated animals and EPO-pretreated animals (* $p < 0.05$). Dramatically less posttraumatic cavity area was mainly observed in animals that received asialoEPO and EPO treatment at the time of injury, compared with animals that received a single pretreatment dose (** $p < 0.05$). Administration of asialoEPO or EPO over multiple doses did not significantly decrease the percentage of damaged tissue or increase the percentage of spared tissue. RHuEPO = rhEPO.

protection require time to develop through the induction and expression of antideath proteins. Our findings suggest that very rapid changes occur in the setting of injury, allowing cells to respond to EPO and its analog, consistent with acute preconditioning. We found that asialoEPO administration 24 hours before injury was followed by an increase in GFAP immunoreactivity, which suggests a glial protective response to neuronal injury. On the other hand, previous reports have included a description of a rapidly recruitable pool of EPORs that become activated after SCI and serve to promote neuronal survival.³² Although asialoEPO and EPO have been shown to bind to EPOR with similar affinities,²² recent evidence has suggested that the hematopoietic and tissue-protective activities could be separated and that the hormonal and neuroprotective actions of EPO can occur through different signaling systems.³⁹ Specifically, the receptor complex mediating the neuroprotective effects of EPO has been reported to be associated with the common β receptor subunit, also known as CD131, which is the signal-transducing component of granulocyte-macrophage colony-stimulating factor, interleukin-3, and interleukin-5 receptors.¹⁶

Thus, it is reasonable to assume that endogenous EPO, by interacting with EPOR or other cytokine receptors, acts in a protective manner that becomes rapidly activated after injury and serves to promote neuronal survival. This process could explain the increased immunoreactivity of EPOR observed in neurons and endothelial cells of the spinal cord in the 1st hours after injury.³² Conversely, the limited recovery and permanent disability that the vast majority of neuronally injured patients demonstrate sug-

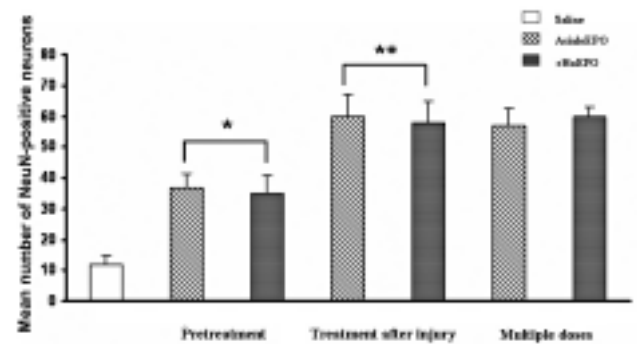


FIG. 6. Bar graph depicting the mean numbers of NeuN-positive neurons after SCI in rats pretreated with saline solution, rats pretreated with asialoEPO or EPO 24 hours before SCI, rats treated with asialoEPO or EPO over multiple doses (* $p < 0.05$, compared with rats pretreated with saline solution; ** $p < 0.05$, compared with rats that received a single pretreatment dose).

gest that the endogenous system does not fully prevent neuronal damage, which is likely due partly to the presence of proinflammatory cytokines. Despite the lack of direct evidence that endogenous EPO mediates preconditioning, we have shown that EPO functions as an exogenous preconditioning agent. It is safe, well tolerated, can be administered systemically, and crosses the blood-brain barrier. In the first clinical trial of rhEPO in patients with acute stroke, improvement in clinical outcome was reported for treated patients.^{21,29} Because the protective action of EPO lasts only approximately 3 days,⁵⁵ it will likely need to be administered chronically in the treatment of neurological diseases. However, untoward side effects could occur with long-term administration. The long-term consequences of lengthy EPO treatment may include polycythemia (production of hyperreactive platelets), which can predispose the patient to thrombosis, especially in the setting of injury.^{25,62,63} A nonerythropoietic tissue-protective EPO derivative with a brief plasma half-life potentially offers important advantages over rhEPO and would allow for multiple or chronic dosing strategies in neurodegenerative diseases and other diseases of the nervous tissue.²² Preoperatively, asialoEPO may be used to precondition the brain and spinal cord before neurosurgical procedures or other surgical procedures that put the brain and spinal cord at risk of injury. Finally, because the fully protective effects of the EPO derivative appear with administration immediately after injury, asialoEPO may be especially useful in the treatment of stroke or trauma in clinical settings.

Conclusions

In the present study conducted in rats, the results indicate that EPO and its analog asialoEPO do not need to be present at the time of injury to provide tissue protection after experimental SCI. This finding, consistent with pharmacological preconditioning, has clear relevance for the "window of opportunity" for treatment in clinical situations where potential injury can be anticipated. Furthermore, the use of a nonerythropoietic EPO derivative with a brief plasma half-life offers significant advantages, com-

pared with use of rhEPO, because potentially safer multiple or chronic dosing strategies may be possible with the derivative in the treatment of pathological conditions of the nervous system.

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