Erythropoietin activates the phosphorylated cAMP [adenosine 3'/5' cyclic monophosphate] response element-binding protein pathway and attenuates delayed paraplegia after ischemia-reperfusion injury

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Objective: Paraplegia remains a devastating complication of complex aortic surgery. Erythropoietin (EPO) has been shown to prevent paraplegia after ischemia reperfusion, but the protective mechanism remains poorly described in the spinal cord. We hypothesized that EPO induces the CREB (cAMP [adenosine 3'/5' cyclic monophosphate] response element-binding protein) pathway and neurotrophin production in the murine spinal cord, attenuating functional and cellular injury.

Methods: Adult male mice were subjected to 4 minutes of spinal cord ischemia via an aortic and left subclavian cross-clamp. Experimental groups included EPO treatment 4 hours before incision (n = 7), ischemic control (n = 7), and shams (n = 4). Hind-limb function was assessed using the Basso motor score for 48 hours after reperfusion. Spinal cords were harvested and analyzed for neuronal viability using histology and staining with a fluorescein derivative. Expression of phosphorylated (p)AKT (a serine/threonine-specific kinase), pCREB, B-cell lymphoma 2, and brain-derived neurotrophic factor were determined using immunoblotting.

Results: By 36 hours of reperfusion, EPO significantly preserved hind-limb function after ischemia-reperfusion injury ($P < .01$). Histology demonstrated preserved cytoarchitecture in the EPO treatment group. Cords treated with EPO expressed significant increases in pAKT ($P = .021$) and pCREB ($P = .038$). Treatment with EPO induced expression of both of the neurotrophins, B-cell lymphoma 2, and brain-derived neurotrophic factor, beginning at 12 hours.

Conclusions: Erythropoietin-mediated induction of the CREB pathway and production of neurotrophins is associated with improved neurologic function and increased neuronal viability following spinal cord ischemia reperfusion. Further elucidation of EPO-derived neuroprotection will allow for expansion of adjunct mechanisms for spinal cord protection in high-risk thoracoabdominal aortic intervention. (J Thorac Cardiovasc Surg 2015;149:920-4)

See related commentary on pages 925-6.

Delayed paraplegia remains a terrible complication following complex aortic repairs. Despite the evolution of surgical adjuncts to prevent ischemic injuries, the spinal cord remains least tolerant of this insult, resulting in delayed paraplegia in up to 20% of complex cases.¹ Furthermore, a widely accepted preventative pharmacotherapy does not currently exist.

Erythropoietin (EPO) is widely utilized for its hematopoietic effects. In addition, however, it has been widely demonstrated to protect various organs from ischemic injury, including the kidney, the heart, and even the brain.²⁻⁴ Ischemic stroke studies, in particular, prompted exploration in spinal cord ischemia-reperfusion injury. EPO demonstrated significant preservation of spinal cord function in a murine model of complex aortic intervention, but the mechanism of protection was unknown.⁵ EPO can induce expression of multiple neurotrophins with proven roles in tissue protection. B-cell lymphoma 2 (BCL-2) and brain-derived neurotrophic factor (BDNF) were considered to be potential mechanisms because of their known function as apoptotic regulators.^{6,7}

The aim of this study was to elucidate the mechanism of protection of EPO treatment in the ischemic spinal cord. We hypothesized that EPO attenuation of injury would depend on the pCREB (cAMP [adenosine 3'/5' cyclic monophosphate] response element-binding protein)-mediated induction of neurotrophins, including BDNF and BCL-2.

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Abbreviations and Acronyms

AKT	= serine/threonine-specific kinase
BCL-2	= B-cell lymphoma 2
BDNF	= brain-derived neurotrophic factor
CREB	= cAMP [adenosine 3'5' cyclic monophosphate] response element-binding protein
EPO	= erythropoietin
pAKT	= phosphorylated AKT
pCREB	= phosphorylated CREB

MATERIALS AND METHODS

Animal Care

All experiments were approved and monitored by the Animal Care and Use Committee at the University of Colorado at Anschutz Medical Campus, and this investigation adhered to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Male c57/bl-6 mice (12-20 weeks old) were obtained from Harlan Sprague Dawley, Inc Laboratories (Indianapolis, Ind).

Surgical Procedure

Mice were anesthetized with 2% isoflurane. An intraperitoneal dose of 400 IU/kg of heparin was administered to all mice before the procedure. EPO (10 µg/kg) or normal saline was administered via intraperitoneal injections, 4 hours before the operation. The aortic arch was surgically exposed and clamped between the left common carotid artery and the left subclavian artery. A minimum of 90% aortic occlusion was verified using a laser Doppler blood flow monitor (Moor Instruments Ltd, Axminster, United Kingdom). Ischemia was continued for 4 minutes in all ischemic groups. The sham group underwent the same procedure without cross-clamp placement. Core body temperature was maintained at 36.5°C ± 0.5°C using a rectal temperature probe and a heated table (Vestavia Scientific LLC, Birmingham, Ala). All mice were humanely sacrificed after 48 hours of reperfusion, and the spinal cords were harvested for analysis.

Assessment of Hind-Limb Motor Function

The Basso Mouse Scale for Locomotion was used to assess and quantify the extent of motor dysfunction in mice after ischemia. The scale scores the hind-limb function of mice on a scale of 0, indicating complete paralysis, to 9, indicating normal function. Function was scored at 12, 24, 36, and 48 hours after reperfusion.

Histopathology of Spinal Cord Cross-Sections

Spinal cord samples were harvested and immediately preserved in a 10% formalin solution. The spinal cords were then embedded in paraffin and sectioned into 5 micrometer-thick sections. Hemotoxylin and eosin stains of the spinal cord sections were obtained to analyze the number of viable neurons.

Western Blot Analysis

Spinal cords were flash frozen at -80°C and later homogenized in ethylenediaminetetraacetic acid-free complete lysis-M buffer (Roche Diagnostics, Indianapolis, Ind). Protein quantification was determined using a NanoDrop (Thermo Scientific, Wilmington, Del). Samples were then loaded onto a 4% to 20% tris(hydroxymethyl)aminomethane (Tris) hydrochloride gradient gel (Bio-Rad Laboratories, Inc, Hercules, Calif) and subsequently run in Tris-glycine buffer. After the gel was run, the protein was transferred to a nitrocellulose membrane. Nonspecific binding was blocked by incubating the membrane in 5% bovine serum albumin. The membrane

was then incubated in 1:500 rabbit anti-mouse BCL-2, BDNF, pCREB, or pAKT (serine/threonine-specific kinase) primary antibody overnight at 4°C. Excess antibody was washed using a solution containing Tris-buffered saline and Tween (BioVision, Inc, Milpitas, Calif). Anti-rabbit antibody was then placed on the membranes and allowed to incubate for 1 hour; the membranes were washed again to remove any leftover antibody. Using enhanced chemiluminescence, the target bands were exposed on the film, and the band density was determined using ImageJ software (National Institutes of Health, Bethesda, Md).

Statistical Analysis

Data were collected using the ImageJ software for Western blot, and blind observer quantification of functional outcomes and microscopic samples. The StatView statistical analysis program was used for all analyses (SAS Institute, Cary, NC). Functional outcomes were compared using analysis of variance. All additional comparisons were evaluated with an unpaired 2-tailed *t* test.

RESULTS

Erythropoietin significantly preserved hind-limb function in the mice subjected to aortic occlusion ($P < .05$) (Figure 1). Throughout the 48-hour assessment of hind-limb function, the treatment group demonstrated the most significant preservation of hind-limb function. In contrast, all mice lacking EPO treatment developed permanent paraplegia by 36 hours.

Histologic analysis, using hemotoxylin and eosin stains of the spinal cord cross-sections, revealed a significant reduction in neuronal viability in control mice subjected to spinal cord ischemia and reperfusion (Figure 2). Although the EPO treatment group showed a minor decrease in neuronal viability compared with the sham group, the reduction was not significant. In addition to decreased neuronal viability, the control group had a higher level of vacuolization, an additional marker of cellular injury, relative to the sham and treatment groups.

Pathway Activation in Nonischemic Mice Subjected to EPO or Normal Saline Injection

Levels of both activated AKT and activated CREB protein expression were significantly higher after EPO treatment (Figure 3). Levels of AKT phosphorylation increased by

FIGURE 1. Hind-limb function, as measured by Basso score in the first 48 hours after surgery. EPO, Erythropoietin; IR, ischemia reperfusion.

FIGURE 2. Histologic analysis, using hemotoxylin and eosin stains, of the spinal cord cross-sections, shows a significant reduction in neuronal viability in control mice subjected to spinal cord ischemia and reperfusion. *IR*, Ischemia reperfusion; *EPO*, erythropoietin.

$183.3\% \pm 9\%$ ($P < .01$); CREB phosphorylation increased by $171.4\% \pm 16\%$ ($P = .03$). Levels of BCL-2 and BDNF were examined at 12 and 24 hours (Figure 4). The BDNF level increased significantly through 24 hours, with the most prominent increase at 12 hours ($P = .011$); the BCL-2 levels were elevated through 24 hours ($P = .042$).

DISCUSSION

These data support the hypothesis that EPO attenuates spinal cord ischemia-reperfusion injury through the pCREB-mediated induction of neurotrophins, including BDNF and BCL-2. EPO is a 30.4-kDa glycoprotein in the type 1 cytokine family and is most known for its role in

hematopoiesis. Various tissues express EPO mRNA (messenger ribonucleic acid), including kidney, brain (cortex, hippocampus, pituitary), myocardium, lung, and spinal cord.^{8,9} The discovery that hypoxia induces a 100-fold increase in human brain EPO production established its intrinsic presence in neural tissue and generated research into its role after neurologic injury.¹⁰

Erythropoietin's most known function is in hematopoietic progenitor cells where it prevents their programmed cell death, allowing survival and maturation, causing an increase in circulating red blood cells. This action is mediated through a homodimer of EPO receptor components.¹¹ In addition, EPO has been shown to be an effective

FIGURE 3. Levels of activated AKT and activated CREB protein expression after EPO treatment. *AKT*, Serine/threonine-specific kinase; *CREB*, cAMP [adenosine 3'5' cyclic monophosphate] response element-binding protein; *pAKT*, phosphorylated AKT; *pCREB*, phosphorylated CREB; *IR*, ischemia reperfusion; *NS*, nonsignificant; *EPO*, erythropoietin.

FIGURE 4. Levels of BCL-2 and BDNF at 12 and 24 hours. *BCL-2*, B-cell lymphoma 2; *BDNF*, brain-derived neurotrophic factor; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *NS*, nonsignificant; *EPO*, erythropoietin.

neuroprotective agent after ischemia-reperfusion injury. These neuroprotective properties have been demonstrated in other animal models, including cerebral ischemia and spinal cord contusion.¹²⁻¹⁴ An additional heterodimer receptor consisting of EPO-receptor and β common-receptor (β CR), a shared subunit for type 1 cytokines GM-CSF (granulocyte-macrophage colony stimulating factor), interleukin (IL)-3, and interleukin-5, has been identified in neural tissue and mediates these tissue-protective actions of EPO.¹⁵ Activation of this receptor complex upregulates pro-survival signaling pathways, including STAT (signal transducers and activators of transcription) and AKT.¹⁶

The present study expands on previous research in identification of EPO-mediated neuroprotective pathways within the spinal cord after ischemia-reperfusion injury. The thoracic aortic cross-clamp model used is particularly relevant in replicating the functional injury observed clinically. Within this model, a spectrum of injury is reproducible by varying cross-clamp times, ranging from delayed paraplegia within 4 minutes to dense paraplegia at 8 minutes. The 4-minute cross-clamp time was chosen to mimic the delayed functional decline seen clinically.

Discovery of EPO-mediated motor preservation within this model prompted further exploration into mechanistic pathways. Previous studies have implicated the transcription factor CREB as a crucial regulator of cell survival pathways in neurons. The activation of CREB is dependent on the phosphorylation of Ser133 by a diverse set of kinases.¹⁷ One such kinase is the serine/threonine-specific kinase AKT.¹⁸ Once in its active conformation, CREB mediates the transcription of neurotrophins and other neuroprotective proteins important for neuronal survival.¹⁹

Neurotrophins are a group of proteins that regulate distinct, yet specific, neuronal responses.²⁰ Among the neurotrophins activated by CREB is the endogenous neuroprotective agent BDNF.²¹ The suggested mechanism of the neuroprotection exhibited by BDNF is reduction of both inflammation and apoptosis.⁷ The neuroprotective effects of BDNF have been demonstrated in many cerebral models in which BDNF was shown to revive damaged neurons after ischemia and other traumatic injury.^{22,23} Furthermore, inhibition of endogenous BDNF increased the vulnerability of neurons to death after global forebrain ischemia.²⁴ Treatment with EPO increased the expression of BDNF levels at both 12 and 24 hours, with the most prolific increase occurring at 12 hours.

Another neuroprotective protein induced after CREB activation is BCL-2, which plays a pivotal role in the regulation of apoptosis. This protein inhibits apoptosis by affecting the permeability of the mitochondrial membrane.⁶ In doing so, BCL-2 helps mediate the levels of apoptotic factors released from mitochondria. As with BDNF, BCL-2 levels were significantly higher at 12 and 24 hours after pretreatment with EPO. However, BCL-2 expression was more pronounced at 24 hours. Activation of this pathway was also identified in dexmedetomidine-mediated attenuation of delayed paraplegia.²⁵

All studies have limitations. The current model utilizes lower-body ischemia to create an injury that mimics clinical delayed paraplegia. Although the mode of ischemia is different, the delayed neurologic injury progresses in a similar way to the pathology seen in both open and endovascular aortic interventions. In addition, the intraperitoneal delivery of EPO differs from the method that would be used with patients. We have previously seen equal efficacy between

intraperitoneal and intravenous administration with the medication having similar reach into the tissue levels.

Despite these limitations, we believe the data and conclusions derived from this study are valid and potentially beneficial to patients undergoing complex aortic intervention. We hope to pursue further studies of how to more optimally induce the protective subunit of the EPO receptor without induction of the hematopoietic pathways. Further, we would like to use this induction to better prepare the spinal cord in advance of the injury for the metabolic strain of the ischemia and reperfusion injury.

In summary, pretreatment with EPO in a murine model of ischemia and reperfusion afforded significant functional preservation through AKT/CREB induction of neurotrophins. This result provides further evidence for the efficacy of EPO in spinal cord injury, but it also reveals the need for further lines of study of how to expand these protective mechanisms. In conclusion, the role of EPO in clinical protection from delayed paraplegia may be a significant part of complex aortic intervention in the near future.

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