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

Joshua M. Mares, BA,[a] Lisa S. Foley, MD,[a] Marshall T. Bell, MD,[a] Daine T. Bennett, MD,[a] Kirsten A. Freeman, MD,[a] Xianzhong Meng, MD, PhD,[a] Michael J. Weyant, MD,[a] Joseph C. Cleveland, Jr, MD,[a] David A. Fullerton, MD,[a] Ferenc Puskas, MD,[b] and Thomas Brett Reece, MD[a]

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.

From the Division of Cardiothoracic Surgery,[a] Department of Surgery, and Department of Anesthesia,[b] University of Colorado, Aurora, Colo.

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Address for reprints: Thomas Brett Reece, MD, 12631 E 17th Ave, Room 6602, MS C310, Aurora, CO 80045 (E-mail: brett.reece@ucdenver.edu).

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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% isofluorane. 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 spinals 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

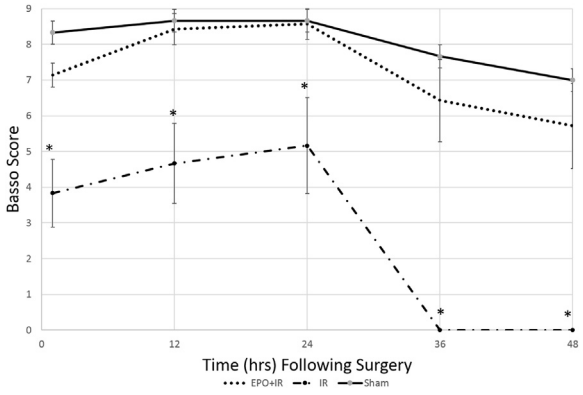

FIGURE 1. Hind-limb function, as measured by Basso score in the first 48 hours after surgery. *EPO*, Erythropoietin; *IR*, ischemia reperfusion.

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