Erythropoietin's Beta Common Receptor Mediates Neuroprotection in Spinal Cord Neurons

Lisa S. Foley, MD, David A. Fullerton, MD, Joshua Mares, BA, Mitchell Sungelo, BA, Michael J. Weyant, MD, Joseph C. Cleveland, Jr, MD, and T. Brett Reece, MD

Department of Surgery, Division of Cardiothoracic Surgery, University of Colorado Denver, Aurora, Colorado

Background. Paraplegia from spinal cord ischemia-reperfusion (SCIR) remains an elusive and devastating complication of complex aortic operations. Erythropoietin (EPO) attenuates this injury in models of SCIR. Upregulation of the EPO beta common receptor (βcR) is associated with reduced damage in models of neural injury. The purpose of this study was to examine whether EPO-mediated neuroprotection was dependent on βcR expression. We hypothesized that spinal cord neurons subjected to oxygen-glucose deprivation would mimic SCIR injury in aortic surgery and EPO treatment attenuates this injury in a βcR-dependent fashion.

Methods. Lentiviral vectors with βcR knockdown sequences were tested on neuron cell cultures. The virus with greatest βcR knockdown was selected. Spinal cord neurons from perinatal wild-type mice were harvested and cultured to maturity. They were treated with knockdown or nonsense virus and transduced cells were selected. Three groups (βcR knockdown virus, nonsense control virus, no virus control; n = 8 each) were subjected to 1 hour of oxygen-glucose deprivation. Viability was assessed. βcR expression was quantified by immunoblot.

Results. EPO preserved neuronal viability after oxygen-glucose deprivation (0.82 ± 0.04 versus 0.61 ± 0.01; $p < 0.01$). Additionally, EPO-mediated neuron preservation was similar in the nonsense virus and control mice (0.82 ± 0.04 versus 0.80 ± 0.05; $p = 0.77$). EPO neuron preservation was lost in βcR knockdown mice compared with nonsense control mice (0.46 ± 0.03 versus 0.80 ± 0.05; $p < 0.01$).

Conclusions. EPO attenuates neuronal loss after oxygen-glucose deprivation in a βcR-dependent fashion. This receptor holds immense clinical promise as a target for pharmacotherapies treating spinal cord ischemic injury.

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Paraplegia after thoracoabdominal aneurysm repairs remains a devastating and elusive problem [1, 2]. Although advances in operative protective techniques have evolved over the past decade, there has yet to be a widespread improvement in the incidence of this complication [3–5]. Furthermore, there are no pharmacologic adjuncts proven to prevent paraplegia from spinal cord ischemia [5, 6].

Ischemia-reperfusion injury, as it occurs in neurologic tissue, has been studied more extensively in the brain than the spinal cord, using ischemic stroke models. These stroke studies have demonstrated that after an ischemic insult there are zones of injury radiating out from the primary lesion. At the center is a core of necrotic tissue, which is irreversibly damaged. This central necrotic core is surrounded by an area of stunned tissue that is severely injured, though has potential for salvage. It is in this region where aberrant blood flow and immunologic processes can advance the lesion to an infarct, ultimately leading to second peak of neurologic decline after the initial insult [7].

Literature and clinical experience has shown that spinal cord injury after aortic surgery mirrors the bimodal injury pattern that characterizes cerebral ischemic injury [8–10]. The first peak in neurologic damage is due to the intraoperative ischemic insult. This injury primarily results in cellular necrosis, and is often subclinical. In response to cytokines released from necrotic cell death, a secondary inflammatory phase develops. Reperfusion delivers immune mediators that combine with proinflammatory cytokines surrounding the ischemic lesion to activate cell death pathways and amplify the damage [7]. Discovering ways to protect this region of vulnerable tissue from secondary insults and progression of injury is of great interest in neurologic research.

Preventing this pathophysiologic response has proven promising in translational brain and spinal cord injury studies. Erythropoietin (EPO) and its receptor are upregulated in the brain and spinal cord after hypoxia and

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Address correspondence to Dr Foley, 12631 E 17th Ave, C302, Aurora, CO 80045; email: lisa.foley@ucdenver.edu.

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inflammatory signaling. It has emerged as a neuroprotective cytokine, with proven efficacy in murine models of spinal cord ischemia-reperfusion injury [11–14]. Two characteristics of EPO highlight its potential role in neuroprotection as well as its potential for therapeutic applications: (1) it is locally produced in the brain in response to ischemic injury and (2) it crosses the blood-brain barrier, likely through a transport mechanism due to its size [15]. These findings have implicated EPO's innate function in neurologic signaling and potential for therapeutic uses.

EPO activates 2 distinct receptors. The classically known receptor is a homodimer of 2 EPO receptor subunits (EPO-R + EPO-R), which mediates hematopoiesis. Activating this receptor on progenitor erythrocytes halts apoptosis, allowing progenitor cells to mature into circulating erythrocytes. A newly identified EPO receptor consists of a heterodimer of an EPO-R subunit paired with the interleukin beta common receptor (βcR) subunit (known as βcR or CD131) that is expressed in various solid organ tissues including the brain, heart, and kidney. This receptor initiates antiapoptotic signaling and mediates tissue protection after injury in these various tissues [16].

βcR subunit expression is low at baseline and induced by hypoxia or metabolic stress. This receptor is primarily expressed in injured or metabolically stressed tissue and is believed to mediate cell preservation pathways. Expression of this receptor on injured cells precedes a local increase in tissue production of EPO temporally. Thus, the expression of this tissue-protective receptor occurs more rapidly than production and diffusion of its ligand, EPO, creating a therapeutic window where exogenous EPO administration could hasten the innate neuroprotective mechanisms.

Our lab has previously demonstrated EPO treatment reduces paraplegia in a murine model of spinal cord ischemia-reperfusion injury [11, 17]. Additionally, we have shown improved outcomes with βcR upregulation and survival after ischemia-reperfusion injury [18]. These mechanisms were both observed but it remained unclear if the βcR was specifically mediating EPO's neuroprotective effects. There is no βcR inhibitor available, limiting the ability to perform blocking studies.

The purpose of this study was to determine if the βcR is essential for EPO-mediated neuroprotection. A murine spinal cord neuron in vitro model that mimics spinal cord ischemia-reperfusion injury was employed to better explore these cellular mechanisms.

We hypothesized that βcR induction by ischemic injury could be blunted using a viral knockdown, and that lack of βcR expression would abrogate EPO's neuroprotective effects.

Material and Methods

Materials

EPO was purchased from Sigma-Aldrich (St. Louis, Missouri). Anti-βcR antibody was purchased from Santa Cruz Biotechnology (Dallas, Texas). Lentiviral vectors containing gene-silencing plasmids were obtained through the Functional Genomics Core at the University of Colorado.

Animals

The University of Colorado Denver Health Sciences Center Animal Care and Use Committee approved all experiments. Experiments adhered to the Guide for the Care and Use of Laboratory Animals. Cells for in vitro experiments were obtained from postnatal day 2 to 3 pups from wild-type (C57/BL6) breeding pairs (Charles Rivers Laboratories, Frederick, Maryland). All cells harvested from a litter of pups were considered n = 1.

Cell Culture

Primary spinal cord neuron cultures were obtained from 2- to 3-day-old wild-type (C57-BL6) mice. The vertebral column was isolated immediately after euthanasia with isoflurane and decapitation. The spinal cord was flushed from the spinal canal en bloc with cold phosphate-buffered saline (pH 7.4). Cord tissue was minced and digested in a solution of Hibernate-A (Invitrogen, Carlsbad, California) with papain (Worthington, Lakewood, New Jersey). Neurons were isolated from the digested cord tissue using an Optiprep (Sigma-Aldrich) density gradient adapted from Brewer [18].

Neurons were plated in 1mL culture media of Neurobasal-A, B27, GlutaMAX (all obtained from Invitrogen), and penicillin or streptomycin (Gibco, New York, New York) on poly-D-Lysine (Sigma-Aldrich)–coated plates at approximately 300,000 cells/well. Cell were cultured in a 37°C, 5% CO_2 humidified atmosphere. Half of the media was replaced every 2 days with fresh media containing AraC (Sigma-Aldrich) to prevent astrocyte growth. The cultures were confirmed to have >90% neurons as seen by morphology on light microscope as well as confirmed with microtubule-associated protein 2–positive neuronal staining. Cells were used at in vitro day 5 for experimentation, which is considered mature for neuronal cultures.

Lentiviral Knockdown of EPO βcR

Lentiviral vectors containing short hairpin RNA gene silencing clones for the βcR subunit of the EPO receptor were obtained through the University of Colorado Denver Functional Genomics Core (University of Colorado Cancer Center, Aurora, CO). Similarly, lentiviral control vectors containing the same plasmid with a nonsense target sequence were obtained. Both the experimental and control plasmids contained a puromycin resistance gene for selection of transduced cells. We obtained 4 variations of a βcR knockdown virus and examined cellular viability after transduction with viability assays (as described subsequently). Additionally, we examined receptor expression levels after treatment to quantify the knockdown success. After identification of a knockdown virus with a high rate of receptor knockdown and minimal effect on viability was identified, this virus was used for all knockdown experiments.

Mature spinal cord neuronal cultures were treated with control or βcR knockdown lentiviral vectors at a total concentration of 10^4 relative infectious units per milliliter for 16 hours with polybrene (2 μL of 1000X). Additionally,