Erythropoietin upregulates growth associated protein-43 expression and promotes retinal ganglion cell axonal regeneration in vivo after optic nerve crush

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Abstract
In this study, we established a rat model of optic nerve crush to explore the effects of erythropoietin on retinal ganglion cell axonal regeneration. At 15 days after injury in erythropoietin treated rats, retinal ganglion cell densities in regions corresponding to the 1/6, 3/6 and 5/6 ratios of the retinal radius were significantly increased. In addition, the number of growth associated protein-43 positive axons was significantly increased at different distances (50, 250 and 500 µm) from the crush site after erythropoietin treatment. Erythropoietin significantly increased growth associated protein-43 protein levels in the retina after crush injury, as determined by western blot and immunofluorescence analysis. These results demonstrate that erythropoietin protects injured retinal ganglion cells and promotes axonal regeneration.

Key Words: erythropoietin; retinal ganglion cells; axonal regeneration; optic nerve crush; neural regeneration

INTRODUCTION

Injury to the optic nerve can lead to axonal degeneration and retinal ganglion cell (RGC) death, resulting in irreversible vision loss[1-4]. Therefore, therapies should focus on the protection of injured RGCs and the promotion of axonal regeneration[5-7]. Erythropoietin (EPO), a renal cytokine regulating hematopoiesis, is produced by different cell types within the central nervous system. EPO has been shown to be neuroprotective, and it promotes the regeneration of neurons in the central nervous system[8]. EPO may function via an anti-apoptotic mechanism, as well as through anti-inflammatory effects at the site of injury[9]. In vitro experiments show that EPO protects cultured neurons against glutamate toxicity[10-11], and it reduces ischemic neuronal damage and neurological dysfunction in rodent models of stroke[12-15]. EPO also prolongs the lifespan of cultured cortical neurons and promotes neuronal growth[16-17]. Moreover, the administration of EPO has a significant effect on axonal regrowth of fibers in the central nervous system[18]. In vivo studies show that EPO is neuroprotective in animal models of stroke, mechanical trauma and excitotoxic injury[19-25]. Recent evidence indicates that EPO can stimulate postnatal neovascularization by increasing endothelial progenitor cell mobilization from the bone marrow[26]. EPO in the retina has been demonstrated to have a neuroprotective effect on RGCs[27]. Following retinal ischemia, EPO prevents apoptotic cell death and improves functional recovery[28]. In addition, EPO promotes neuronal survival and axonal regrowth in the central nervous system. EPO has a neuroprotective effect on RGCs and significantly reduces the death of primary cultures of RGCs induced by glutamate and nitric oxide[29] in vitro. Furthermore, it stimulates the regeneration of injured RGC axons following optic nerve injury[30-32]. However, these studies only focused on the effects of EPO on axonal regeneration in vitro, and very little data is available on EPO’s actions on axonal regeneration in vivo. To investigate the axonal regeneration-promoting effects of EPO after optic nerve crush, we examined the effects of EPO on RGCs using retrograde 1, 1-dioctadecyl-3, 3′, 3′-tetramethyl-indocarbocyanine perchlorate (DII) labeling of the superior colliculus, as well as by assessing the expression of growth associated protein-43 (GAP-43) in the retina and in the region distal to optic...
nerve crush, using immunofluorescence and western blot analysis.

RESULTS

Quantitative analysis of experimental animals

A total of 72 rats were equally and randomly assigned to three groups: sham-surgery (free of crush), phosphate buffered saline (PBS; optic nerve crush-PBS intravitreal injection) and EPO (optic nerve crush-EPO intravitreal injection) groups. All 72 rats were included in the final analysis.

Protective effects of EPO on RGCs after optic nerve crush

To determine RGC densities, retrograde labeling using the fluorescent tracer DiI was used. A large number of DiI-labeled RGCs were present in the sham-surgery and EPO groups 15 days after optic nerve crush. However, many RGCs in the PBS group died, and the microglia were DiI-positive, having phagocytosed RGCs (Figure 1). RGCs situated at the 1/6, 3/6 and 5/6 fractions of the retinal radius were quantified. Statistical analysis showed that compared with the sham-surgery group, the number of RGCs was significantly reduced in the PBS group ($P < 0.01$); and compared with the PBS group, RGC densities were significantly increased in the EPO group ($P < 0.01$; Table 1).

EPO promotes rat RGC axonal regeneration after optic nerve crush

GAP-43 expression was examined to determine the effects of EPO on RGC axonal regeneration. At 15 days after optic nerve crush, animals were sacrificed and longitudinal optic nerve sections were immunostained for GAP-43, an axonal growth-associated protein which is commonly used as a marker of axonal regrowth, to identify regenerating axons (Figure 2). As expected, numerous GAP-43 positive axons passed through the crush site and the distal optic nerve segment in the EPO group. In contrast, the PBS group showed very few GAP-43 positive axons passing through the crush site. The sham-surgery group showed no staining. In addition, the number of GAP-43 positive axons at defined distances from the crush site (50, 250 and 500 µm) was quantified (Table 2). The data show that the number of axons in the EPO group was significantly more than in the PBS group ($P < 0.01$; Table 2), suggesting that EPO promotes RGC axonal regeneration after optic nerve crush.

Figure 1  Morphology of RGCs after optic nerve crush (DiI staining; scale bar: 50 µm in A-C and 100 µm in D-I. Representative photographs of the flat-mounted retinas at the corresponding areas (at 1/6, 3/6 and 5/6 of the retinal radius) showed DiI-labeled RGCs in retinas on day 15 after injury.

Almost all RGCs were labeled and the morphology of the RGCs was round in the sham-surgery (A–C) and EPO (G–I) groups. In the PBS group (D–F), there were a few DiI-labeled RGCs and microglia were obviously labeled at 1/6 and 3/6 radius, and were more numerous at 5/6 retinal radius. RGCs: Retinal ganglion cells; DiI: 1, 1-dioctadecyl-3, 3', 3'-tetramethyl-indocarbocyanine perchlorate; PBS: phosphate buffered saline; EPO: erythropoietin.
EPO promotes GAP-43 expression in rat retina after optic nerve crush

In optic nerve fibers, GAP-43 levels are abundant during development, but expression is absent in adults. GAP-43 expression in adult retina is significantly increased after optic nerve injury. We investigated the effects of EPO on GAP-43 expression in the retina using western blot analysis and immunofluorescence labeling after optic nerve crush. EPO increased GAP-43 expression in the ganglion cell layer of the retina. In comparison, GAP-43 expression was low in the PBS group (Figure 3). Retinal GAP-43 levels in the PBS and EPO groups were higher than in the sham-surgery group, as determined by western blot analysis (P < 0.01; Figure 4). Moreover, the GAP-43 protein level in the EPO group was significantly higher than in the PBS group (P < 0.01; Figure 4), suggesting that EPO upregulates GAP-43 expression in the retina after optic nerve crush.

Table 1  Quantification of surviving retinal ganglion cells (cells/mm²) in different groups

| Group          | Retinal ganglion cell densities |
|----------------|---------------------------------|
|                | 1/6 of the retinal radius       |
| Sham-surgery   | 779.83±96.45                    |
| PBS            | 546.17±86.65                    |
| Erythropoietin | 896.04±47.35                    |

Data are expressed as mean ± SD of six rats in each group. *P < 0.01, vs. sham-surgery group; **P < 0.01, vs. PBS group (one-way analysis of variance). PBS: Phosphate buffered saline.

DISCUSSION

The optic nerve crush model mimics the pathological changes of optic nerve degeneration, and it is suitable for research into optic nerve injury and regeneration. Our results demonstrate that EPO exerts a protective effect after optic nerve injury. Intravitreal injection of EPO enhanced RGC survival and axonal regeneration. The
Erythropoietin (EPO) treatment resulted in significantly increased GAP-43 expression in the retina after optic nerve crush (western blot). Figure 4. Growth associated protein-43 (GAP-43) expression in retina after optic nerve crush (western blot).

(A) Representative western blots for GAP-43 in the retina. Erythropoietin (EPO) treatment resulted in significantly increased GAP-43 expression. (B) Quantification of western blot bands for GAP-43 protein levels from six independent experiments. aP < 0.01, vs. sham-surgery (sham) group; bP < 0.01, vs. phosphate buffered saline (PBS) group (one-way analysis of variance).
injections were used. All surgeries were performed aseptically and on the right eye alone. Animals were anesthetized by intraperitoneal injection of 10% chloral hydrate solution (420 mg/kg). A 1.0–1.5 cm incision was made in the skin above the right orbit. The optic nerve of the right eye was exposed under an operating microscope (Alcon, Fort Worth, TX, USA), and the sheath was opened longitudinally. Using special forceps (force at 40 g; Suzhou Medical Instrument Factory, Jiangsu, China), the optic nerve was crushed within the sheath, 1 mm behind the optic nerve head, for 9 seconds, avoiding injury to the ophthalmic artery. Nerve injury was verified by the appearance of a clearing at the crush site; the vascular integrity of the retina was verified by fundoscopic examination after dilating the pupil with atropine [31]. In the sham-surgery group, the optic nerve of the right eye was exposed, and the sheath was opened longitudinally, but the crush procedure was not performed. The skin was then closed with sutures, and bacitracin ointments were applied to the wound. Animals with postoperative complication (e.g., cataract) were excluded from analysis. Using this optic nerve crush technique, all RGC axons were irreversibly damaged and neuroprotection could be measured by analyzing the delay in apoptosis.

**Drug administration**

All intravitreal injections were performed using a glass microelectrode (WPI, Florida, USA) connected to a Hamilton precision syringe (Hamilton, Reno, NV, USA), puncturing the eye at the cornea-sclera junction. The lens was not punctured. EPO (R&D Systems, Minneapolis, MN, USA) was diluted in PBS. Except for the sham-surgery group which had no intravitreal injection, the PBS and EPO groups received an intravitreal injection of 3.5 μL PBS or EPO (6 U EPO (8.3 μg = 1 000 U EPO)), respectively, for each time point. The intravitreal injections were performed four times: immediately after optic nerve crush (day 0), and at days 3, 6 and 9.

**Quantity of Dil-labeled RGCs in the retina**

At 15 days after optic nerve injury, deeply anesthetized animals were transcardially perfused with 4% paraformaldehyde. The eyes were rapidly enucleated, rinsed, punctured with a needle through the pupil, and then fixed for 1 hour in freshly prepared 4% paraformaldehyde. The retinas were dissected and washed with PBS. After a final wash, four cuts were made with fine iris scissors from the edge to the center of the retinas to flatten them. They were mounted with the RGCs facing up on glass slides in glycerol, and the coverslip was sealed with clear nail polish. The slides were stored in the dark at -20°C until analysis. Labeled RGCs were examined with a confocal microscope (Axiovert 35; Zeiss, Oberkochen, Germany) using a rhodamine filter (560 nm for Dil). RGC densities were determined by quantifying labeled RGCs in 12 distinct areas of 62 500 μm² each (four areas per retinal quadrant at three different eccentricities of 1/6, 3/6 and 5/6 of the retinal radius). Cell number was quantified with a computerized image-analysis system (Image Pro Plus Version 6.0; Media Cybernetics, Silver Spring, MD, USA) in duplicate by two independent investigators in a blind fashion. The average number of RGCs in 12 distinct areas was used to calculate the mean density of RGCs for each retina. Secondary Dil-stained activated microglia, after RGC phagocytosis, were separated using morphologic criteria and excluded from examination [32]. All averaged data are expressed as mean RGC densities (cells/mm²) ± SD.

**Preparation of retina and optic nerve samples**

After the rats were transcardially perfused with 4% paraformaldehyde, the eyes were enucleated with at least 5 mm of optic nerve attached, and bisected. The eyes were dissected as eye cups, without the cornea or lens, and the optic nerves were dissected free from connective tissue. The eye cups and optic nerves were immediately fixed overnight, and transferred to 30% sucrose solution and stored overnight at 4°C. The tissues were then embedded, frozen and cut. Longitudinal sections (16 μm for optic nerve) were obtained by cutting through the globe or optic nerve along the anterior-posterior axis. The sections were collected on gelatin-coated glass slides and stored at -80°C for subsequent use.

**Immunofluorescence labeling for GAP-43 expression in the retina and optic nerve**

Retinal/optic nerve sections were dehumidified at 37°C for 1 hour, and nonspecific binding was blocked by application of 10% normal goat serum. Primary antibody (mouse anti-rat GAP-43 monoclonal antibody, 1: 100, sc-17790; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were applied at 4°C overnight. Negative controls were treated with PBS or serum. Secondary antibodies (Cy3-labeled anti-mouse IgG or anti-mouse fluorescein isothiocyanate antibody, 1: 100; Invitrogen, Carlsbad, CA, USA) were applied for 45 minutes at room temperature. Cell nuclei were counterstained with 4, 6-diamidino-2-phenylindole (1: 1 000; Sigma-Aldrich, St. Louis, MO, USA). Immunoreactivity was examined with a confocal microscope (Axiovert 35; Zeiss). For evaluation of RGC axon regeneration, GAP-43 positive axons were quantified as previously described [33]. Briefly, the optic nerve sections processed for anti-GAP-43 immunoreactivity were photographed using a confocal microscope. Images of whole sections were assembled from single pictures taken with a 20 × objective. Using a calibrated ocular (Zeiss) to measure distance, the number of GAP-43 positive axons crossing a line at distance 50, 250, 500 μm from the end of the crush site was determined. By measuring the cross-sectional width of the nerve at the point of quantification, axon counts were converted into axon crossings per unit nerve width (axons per mm) and the average of these over the four sections was obtained. For all immunohistochemical staining, three sections per eye were examined and there were six rats in each group.
Western blot analysis for retinal GAP-43 expression

Eighteen rat retinas were used for western blot analysis. Total retinal protein was extracted from pulverized samples using modified radioimmunoprecipitation (modified RIPA) buffer with a Halt™ protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, Illinois, USA). Protein concentrations were determined using the Bradford protein assay (Bio-Rad, Hercules, CA, USA)\(^{(40)}\). Each retina served as an individual sample (\(n=6\) per group). Equal amounts of protein (20 \(\mu\)g/ lane) were separated on polyacrylamide gels and then electrotransferred onto a nitrocellulose membrane. After blocking for 3 hours in Tris-buffered saline with 0.1% Tween-20 (Sigma-Aldrich) for 2 hours and incubated with alkaline phosphatase conjugated secondary antibodies in Tris-buffered saline with 0.1% Tween-20 (1:500; Sigma-Aldrich) for 2 hours and then washed and incubated with alkaline phosphatase conjugated secondary antibodies in Tris-buffered saline with 0.1% Tween-20 (1:500; Sigma-Aldrich) for 2 hours and developed using NBT/BCIP substrate (Promega, Madison, WI, USA). The densities of the bands on the membrane were scanned and analyzed with Image Pro Plus version 6.0 (Media Cybernetics).

Statistical analyses

Data are expressed as mean ± SD, unless otherwise stated. Statistical analyses were performed using SPSS 19.0 software (SPSS, Chicago, IL, USA). One-way analysis of variance was used to compare data among three groups, followed by post-hoc tests. Independent samples \(t\)-test was applied to compare data between two groups. A value of \(P<0.05\) was considered statistically significant.

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