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ORIGINAL PAPER

Up-regulated Endogenous Erythropoietin/Erythropoietin Receptor System and Exogenous Erythropoietin Rescue Retinal Ganglion Cells after Chronic Ocular Hypertension

Qing-Ling Fu · Wutian Wu · Hua Wang · Xin Li · Vincent W. H. Lee · Kwok-Fai So

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Abstract
Aims Recent studies have showed that erythropoietin (EPO) is a neuroprotectant for central nerve system neurons in addition to being a hematopoietic cytokine in response to hypoxia. In this study, we investigate the role of the EPO/EPO receptor (EPOR) system in the rat retina after ocular hypertension injury that mimics glaucoma.
Methods Elevated intraocular pressure was induced by laser coagulation of the episcleral and limbal veins. Expression of EPO and EPOR in the normal and glaucomous retinas was investigated by immunohistochemistry and Western blot. To examine the effects of endogenous EPO on the survival of retinal ganglion cells (RGCs) subjected to hypertensive injury, soluble EPOR was directly injected into the vitreous body. Recombinant human EPO was both intravitreally and systemically administrated to study the effect of exogenous EPO on the survival of RGCs after ocular hypertension injury.
Results Immunohistochemistry studies identified Müller cells as the main source of EPO in the normal retina. Expression of EPO and EPOR proteins was increased significantly 2 weeks after ocular hypertension. RGCs, amacrine and bipolar cells all demonstrated an increased expression of EPOR after ocular hypertension. Neutralization of endogenous EPO with soluble EPOR exacerbated ocular hypertensive injury, suggesting a role of the EPO/EPOR system in the survival of RGCs after injury. Similarly, topical and systemic administration of recombinant human EPO rescues RGCs after chronic ocular hypertension.
Conclusions These results indicate that an endogenous EPO/EPOR system participates in intrinsic recovery mechanisms after retina injury and RGCs might be rescued by exogenous administration of EPO.

Keywords Erythropoietin · Erythropoietin receptor · Retinal ganglion cell · Glaucoma · Intraocular pressure · Endogenous · Neuroprotectant

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Introduction

Erythropoietin (EPO) has been viewed traditionally as a hematopoietic cytokine to stimulate differentiation and proliferation of erythroid progenitor cells

(Jelkmann and Metzen 1996). More recently, EPO and EPO receptor (EPOR) immunoreactivities were detected in the normal cortex, hippocampus (Juul et al. 1998), and retina (Junk et al. 2002). EPO was involved in neurophysiology of the central nervous system (CNS) (Kocsis et al. 2005; Siren and Ehrenreich 2001; Dawson 2002) including neurodevelopment and brain homoestasis (Siren and Ehrenreich 2001; Dawson 2002; Buemi et al. 2002; Siren et al. 2001). Recombinant human EPO (rhEPO) administered peripherally readily penetrates the blood–brain barrier (BBB) and exerts protective effects in injury models of the brain (Siren et al. 2001; Brines et al. 2000), the spinal cord (Gorio et al. 2002), and model of multiple sclerosis (Sattler et al. 2004; Diem et al. 2005). EPO can protect RGC from degeneration induced by acute ischemia-reperfusion injury (Junk et al. 2002), axotomy injury (Weishaupt et al. 2004; Kilic et al. 2005), and light- and genetic-induced degeneration (Grimm et al. 2002; Rex et al. 2004). The recent study has showed that EPO promotes survival of RGCs in DBA/2J glaucoma mice (Zhong et al. 2007). Combination treatment with EPO and vascular endothelial growth factor (VEGF) has a significant and specific biological effect on neurite regrowth of axotomized RGCs (Bocker-Meffert et al. 2002). The neuroprotective properties of EPO may be mediated by the RAS/RAF/ERK or PI-3K/Akt kinase pathways (Siren et al. 2001; Kretz et al. 2005). These signaling cascades are similar to those observed previously for other neurotrophic factors such as brain-derived neurotrophic factor (BDNF) or insulin-like growth factor (IGF)-1.

Prior studies of EPO in different models of brain and retina injury raise the possibility that this cytokine may be an endogenous neuroprotectant for RGCs in glaucoma. Glaucoma is recognized as belonging to a group of neurodegenerative diseases characterized by the slow, progressive degeneration of RGCs and their axons, and loss of visual field (Osborne et al. 1999). Since, elevated intraocular pressure (IOP) is a major risk factor for progression of this optic neuropathy (Sommer, 1989), current pharmacological and surgical treatments of glaucoma have aimed at lowering IOP that may not provide the best clinical efficacy. Active degeneration of the optic nerve and RGCs that is directly responsible for the visual loss remains (Quigley et al. 1995). Therefore, to provide better therapies for treating glaucoma, strategies targeting for glaucomatous optic neuropathy, with the aim of preserving, protecting, and rescuing RGCs and their axons should be investigated.

Junk et al. (2002) showed that systemic administration of rhEPO before or immediately after experimental acute ischemia-reperfusion injury not only reduced histopathological damage but also promoted functional recovery. However, there is no report on the role of endogenous EPO in a chronic glaucoma model. Here we used Argon laser to coagulate episcleral and limbal veins to induce chronic ocular hypertension in rats. We hypothesized that there was an endogenous neuroprotective EPO/EPOR system in the retina after injury. To test this hypothesis, soluble EPOR was injected into the vitreous after chronic ocular hypertension. Furthermore, rhEPO was intravitreally and intraperitoneally injected to test whether exogenous EPO promoted RGCs survival after chronic ocular hypertension and optic nerve transection.

Materials and Methods

Animals

Experiments were carried out according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the University of Hong Kong Animal Ethics Committee. Adult female Sprague–Dawley (SD) rats (250–280 g) were used in the animal models. Three rats were housed per standard laboratory cage and kept in a 12-h dark/light cycle (7:00 a.m./7:00 p.m.) with standard chow and water ad libitum. All operations were carried out in animals anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (8 mg/kg). In addition, 0.5% alcaine (Alcon-Couvreur, Belgium) was applied to the eyes before all operations and antiseptic eye drops (Tobres [Tobramycin 0.3%], Alcon-Couvreur, Belgium) were used to prevent infection after the treatment. Each experiment group contains eight animals.

Glaucoma Model

Argon laser photocoagulation of three episcleral veins and limbal vein in the right eye of SD rats to induce chronic hypertension injury was performed as

previously described (Ji et al. 2004; WoldeMussie et al. 2001). A secondary laser surgery was performed to block the reconnected vascular flow 7 days after injury. The left eyes were not operated and used as controls. Animals were sacrificed 2 weeks post first laser injury. The IOP were measured using a Tonopen XL Tonometer at 0, 3, 7 (before the second laser), 10, and 14 days after the injury. About 10 readings were recorded for each eye in a single measurment. RGCs were labeled by placing a piece of Gelfoam (Pharmacia & Upjohn) soaked with FluoroGold (FG) (6% v/v, Fluorochrome, Denver, CO) on the surface of the superior colliculus (SC) 4 days before the animals were sacrificed. Rimadyl (0.025 mg/ml) in drinking water was given to the animals as an analgesic after the surgery.

Optic Nerve Transection

Transection of the optic nerve leads to an acute optic neuropathy and massive RGC death. The effect of rhEPO treated systemically on promoting RGC survival after acute injury was also tested using an optic nerve transection model. Optic nerve transection was performed by incising the superior conjunctiva. After opening the dural sheath, the optic nerve was completely transected at 1.5 mm behind the globe. The transection of the optic nerve of the right eye was performed at day 0, and the left eye at 2 days before sacrifice as the control. Surviving RGCs were retrogradely labeled by placing a piece of Gelfoam soaked with 6% FG at the ocular stump at 2 days before sacrifice. Animals were sacrificed at 1 week after surgery. Care was taken to maintain the blood supply throughout the operations. The retinas were examined ophthalmoscopically, to assure good blood supply.

Drug Administration

To investigate the effect of endogenous EPO on injured RGCs, soluble EPOR (51.4 kDa) from R&D at 20 ng in 2 μl PBS was given intravitreally for the right eyes at 0, 4, 7, 11 days after the injury (Junk et al. 2002). To examine the effect of exogenous EPO, 2U Epoetin alpha (CILAG AG Internationl, 6300 Zug, Switzerland) was intravitreally injected at 0, 4, 7, 11 days after the first laser coagulation. Control animals received an intravitreal injection of PBS in the right eyes. Epoetin alpha at 5,000 units (42 μg, 1 ml)/kg or normal saline control was also injected i.p. 24 h before and 30 min before the injury to study the effect of rhEPO on the injured RGCs after the systemic treatment.

Quantification of RGCs

Two weeks after laser operation and 1 week after optic nerve transection, rats were perfused with normal saline after the anesthesia. Both eyes of each animal were enucleated and fixed in 4% paraformaldehyde for more than 60 min. Retinas were prepared as flat-mounts and the FG labeled RGCs were counted under fluorescence microscopy using an ultraviolet filter as preciously described (Ji et al. 2004; Cheung et al. 2004). Briefly, the RGCs were quantified under an eyepiece grid of 200×200 μm^2 along the median line of each quadrant, starting from the optic disc to the border at 500 μm intervals. RGCs in eight microscopic fields for each quadrant and a total of 32 fields per retina for all four quadrants were counted. The percentage loss of RGCs was used to examine the survival effects of different treatments. The data was expressed in terms of relative percentage of RGC loss in the injured right eye to the contralateral intact eye, loss of FG-labeled RGCs (% contralateral, mean + SEM).

Immunohistochemistry

RGCs were retrogradely labeled with FG at 4 days before sacrifice. The eyes were enucleated at 2 weeks after injury and were fixed in 4% paraformaldehyde for 1 h. After removing the corneas and lens, the eyecups were fixed further in paraformaldehyde for 4–6 h and then transferred to 30% sucrose solution at 4°C for 16 h. The eyecups were embedded in OCT compound and cryosections (10 μm thick) were prepared at −20°C.

EPOR Immunohistochemistry

The retinal sections were washed with PBS, incubated in 0.5% Triton/PBS for 10 min, and blocked with 10% normal goat/donkey serum and 0.1% triton/PBS for 1 h. Incubation with rabbit polyclonal antibody for EPOR (1:1,000, M-20, sc-697, Santa Cruz Biotechnology) was performed at 4°C for 16 h.

Negative controls were performed by preabsorbing the primary antibodies with a 10-fold (by mass) excess of a specific blocking peptide (sc-697 P, Santa Cruz). Sections were then washed with PBS three times and incubated with Alexa Fluor® 488 goat anti-rabbit IgG (Molecular probes) at room temperature for 2 h. To investigate cell types expressing EPOR, double labeling with bipolar cell-specific marker (mouse monoclonal antibody against PKCα, 1:1,000, Santa Cruz Biotechnology) was performed overnight at 4°C. Sections were incubated with Alexa Fluor® 568 goat anti-mouse IgG for PKCα and Fluor® 488 goat anti-rabbit IgG for EPOR for 2 h after washing with PBS. Goat polyclonal antibody against Calretinin (1:2,000, sc-11644, N-18, Santa Cruz Biotechnology) was used to detect amacrine cells. Alexa Fluor® 488 donkey anti-rabbit IgG for EPOR and Alexa Fluor® 568 donkey anti-goat IgG for calretinin from molecular probes were performed for 2 h. The sections for EPOR expression were also processed for immunoperoxidase staining (DAB). The differences with the above immunofluorencent staining were that the sections were incubated with 1% H_2O_2 instead of 0.5% Triton/PBS for 15 min at the beginning of operation. The sections were incubated with biotin-conjugated secondary antibody (goat anti-rabbit, DakoCytomation) and visualized with diaminobenzidine (DAB) (0.03% DAB, 0.003% H_2O_2, pH 7.2). The nuclei were counterstained by hematoxylin. Sections were mounted with fluorescent mounting medium (DakoCytomation) and analyzed under Carl Zeiss LSM 510 META Confocal microscopy or fluorescent microscopy.

EPO Immunohistochemistry

The primary antibody for EPO was from Santa Cruz Biotechnology (goat polyclonal, N-19, sc-1310). The sections for EPO expression were processed for immunoperoxidase staining (DAB), same as the protocol as EPOR staining with DAB. After incubation with primary antibody for EPO (1:1,000) for 16 h, the sections were washed and incubated with biotin-conjugated secondary antibody (rabbit anti-goat, DakoCytomation) and developed with diaminobenzidine (DAB) (100 mM $NiSO_4$, 125 mM acetate, 10 mM imidozole, 0.03% DAB, 0.003% H_2O_2, pH 7.2). The EPO specific staining was confirmed by co-incubation of the anti-EPO antibody with the EPO-specific blocking peptide (sc-1310 p, Santa Cruz). Images of slides were captured digitally with standardized microscope and camera settings (Axioskop and Axiocam with AxioVision ver. 3.1 Software; Carl Zeiss Meditec, Inc., Dublin, CA).

Western Blotting

To confirm the increase of EPO and EPOR after ocular hypertension, the levels of EPO and EPOR in the retina were measured using Western blotting. Animals were euthanized at 2 weeks post laser coagulation. The retinas were dissected and homogenized in lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA) supplemented with 10% protease inhibitor cocktail and 1% phosphatase inhibitor cocktails from Sigma. Cell debris was removed by centrifugation; the protein concentration of the supernatant was measured using a Bio-Rad DC protein Assay Kit (Bio-Rad Laboratories, CA, USA). A 40-μg aliquot of proteins from each individual animal was subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membrane. The membranes were blocked with 5% non-fat dry milk and 2% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h. Incubations with anti-EPO (1:1,000) or anti-EPOR antibody (1:1,000) in blocking buffer was performed overnight at 4°C. After washing, the membranes were incubated with Horseradish Peroxidase-conjugated secondary antibody (1:2,000, Dako) in blocking buffer for 1 h at room temperature. Immunoreactive proteins were detected using the enhanced chemiluminescence method (ECL, Amersham). Protein loading was controlled using a monoclonal goat antibody against β-actin (1:2,000, C-11, Santa Cruz Biotechnology). All experiments for Western blotting were performed with 4–5 animals in each group and the samples were run on the gels as individual animals.

Data Statistics

Data are mean + SEM. Statistical analysis was evaluated using Student's *t*-test for comparisons between two groups, or by one-way analysis of variance (ANOVA) followed by post-hoc tests (Turkey HSD) for comparisons among three groups.

Results

The Expression of EPO and EPOR in the Normal Retina and Up-regulated Expression after Ocular Hypertension

EPO and EPOR expression in normal and injured eyes was investigated on rat retina sections. The EPO specific staining was confirmed by co-incubation of the anti-EPO antibody with the EPO-specific blocking peptide in the normal retina (Fig. 1A). Immunohistochemistry localized EPO primarily within the nerve fiber layer (NFL) and inner plexiform layer (IPL) in the normal retinas (Fig. 1B). Otherwise, from the morphology of the positive cells, it was easy to see that Müller cells had strong immunohistochemical staining, especially in IPL. Two weeks after ocular hypertension, the immunohistochemical staining for EPO extends to the inner nuclear layer (INL), outer plexiform layer (OPL) and outer nuclear layer (ONL) (Fig. 1C). There is strong staining for Müller cells, which extend through the depths of the retina from the inner limiting membrane to their outer limit. The cell bodies of Müller cells were strongly stained in the INL (Fig. 1C). In order to further examine the change in EPO levels after injury, we investigated the levels of EPO using Western blotting in the retina at normal and 2 weeks after laser coagulation (Fig. 1D). EPO protein levels at 2 weeks after ocular hypertension increased to 1.5 times compared to normal retina ($P < 0.05$).

As for EPOR staining, two strong immunopositive bands were observed in the IPL in the normal retina (Fig. 2A). Minimal staining for EPOR was confirmed in the cells in ganglion cell layer (GCL) (Fig. 2A). However, strong cytoplasmic and membrane immunoreactivity for EPOR was found in the GCL at 2 weeks after the injury (Fig. 2B). There was EPOR positive staining for most of the cells in the INL, but not in outer nuclear layer (Fig. 2B). Similar results were observed with Western blotting. Little EPOR level was noted in the normal retinas. However, laser coagulation induced 3-fold increase for EPOR expression to 2 weeks ($P < 0.001$) (Fig. 2C). These results further confirmed that the EPOR expression significantly increased after chronic ocular hypertension.

In order to examine the retinal cell types of EPOR positive staining, several antibodies for specific cell markers were performed using the retinal sections. Retrograde labeling of RGCs with FG showed that most of the RGCs expressed EPOR in the GCL (Fig. 3A, B, C). Double staining with specific cell markers showed that bipolar cells might mediate the increased expression of EPOR after laser surgeries in the INL (Fig. 3D, E, F), and amacrine cells for the increase in the INL and GCL (Fig. 3G, H, I). Double staining with EPOR and calretinin antibodies also showed that the two strong immunostaining bands in INL for EPOR was in precisely the same position as choline acetyltransferase-immunoreactive (ChATir) terminals of amacrine cells (Fig. 3G, H, I).

Soluble EPOR-induced Aggravating Ocular Hypertension Injury

Prior studies of EPO in different animal models have showed that rhEPO significantly reduced neuronal injury in CNS after damage. Our results showed that the expression of EPO and EPOR increased significantly after chronic ocular hypertension. We hypothesize that an endogenous EPO/EPOR system could be activated and participated in the recovery after retinal injury. In this experiment, we tried to find whether the injection of soluble EPOR exacerbates RGCs loss after ocular hypertension. There were 13.9 ± 1.8% total RGCs lost 2 weeks following ocular hypertension in the PBS group (Fig. 4A). However, 20.7 ± 3.7% RGCs loss was observed after the treatment of soluble EPOR ($P < 0.05$, Fig. 4A). Soluble EPOR may neutralize the endogenous EPO to exacerbate the ocular hypertension injury. These data suggest that the endogenous EPO/EPOR system may participate in the intrinsic recovery mechanisms after ocular hypertension.

Neuroprotective Effect of rhEPO after Ocular Hypertension Injury

We hypothesized that exogenous application of EPO may have neuroprotective benefits. To test this idea, we administered rhEPO intravitreally in this chronic glaucoma model. Injection of 2 U EPO/eye on days 0, 4, 7, and 10 after first laser coagulation enhanced RGC survival by −1.5 ± 3.08% compared with survival in vehicle-injected control eyes on day 14 injury ($P < 0.001$, Fig. 4A).

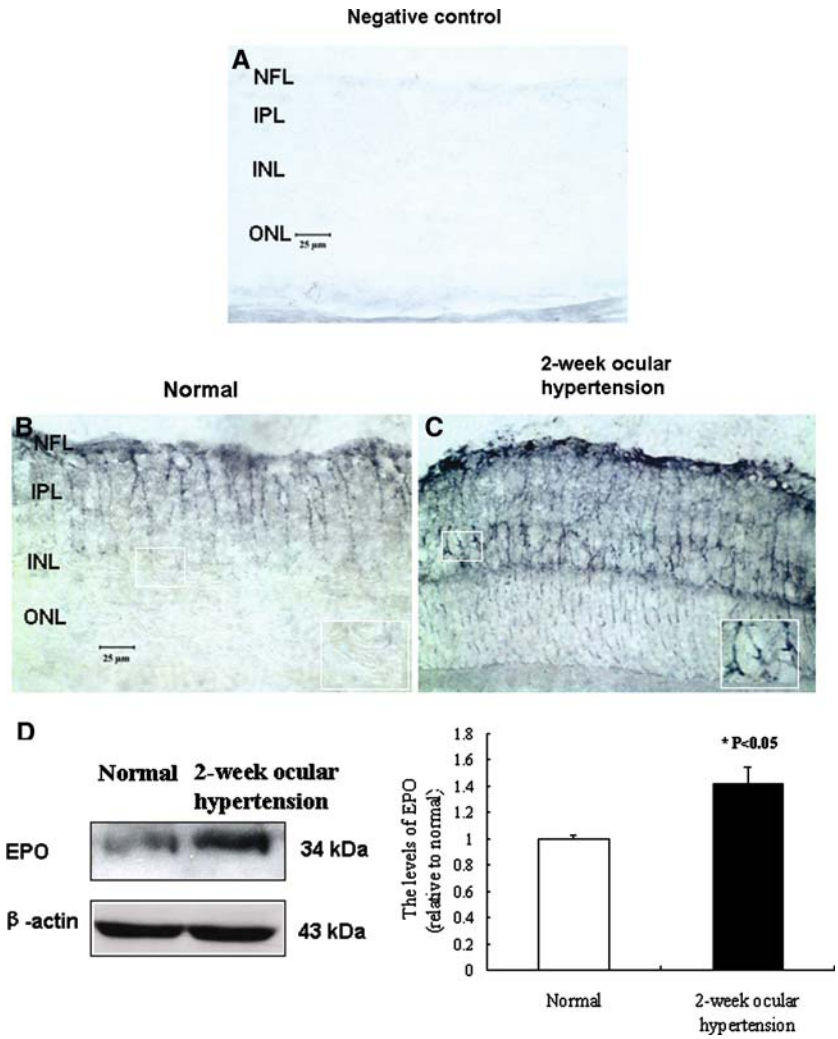

Fig. 1 Expression of EPO protein in the normal and ocular hypertensive retina. (**A**) Negative control for EPO expression in normal retina shown with diaminobenzidine. There was no non-specific staining for EPO. (**B**) EPO expression in normal retina shown with diaminobenzidine. Intense immunoreactivity was found in the NFL and IPL. Müller cells had strong immunohistochemical staining, especially in the IPL. The cell bodies of Müller cells are not stained with EPO (amplified image in box). (**C**) EPO expression extends to the whole thickness of the neural retina from the inner limiting membrane to outer limiting membrane at 2 weeks after ocular hypertension injury. The cell bodies of Müller cells are stained with EPO (amplified image in box). GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, ONL: outer nuclear layer. Scale bar in 25 μm. (**D**) EPO expression in retinal protein lysates of normal and ocular hypertension retinas was confirmed with Western blot analysis. Densitometric analysis demonstrated significant increase for EPO expression at 2 weeks after ocular hypertension ($P < 0.05$). The number of samples for each group is six (Mean + SEM)

Many data have showed that rhEPO administered peripherally readily penetrates the BBB. The systemic administration of a drug has more advantages than the local administration especially for CNS diseases in the clinic. The effect of rhEPO on the survival of RGCs was also investigated by the injection i.p. 24 h or 30 min before the first laser coagulation in our experiment. In the saline control group, the amount of RGCs loss was 12.8 ± 4.2% RGCs 2 weeks after ocular hypertension (Fig. 4B). After injection of rhEPO, a marked neuroprotective effect was observed, with no loss of RGCs (loss = −1.9 ± 1.5%) (Fig. 4B). These results suggest that exogenous application of EPO provides a

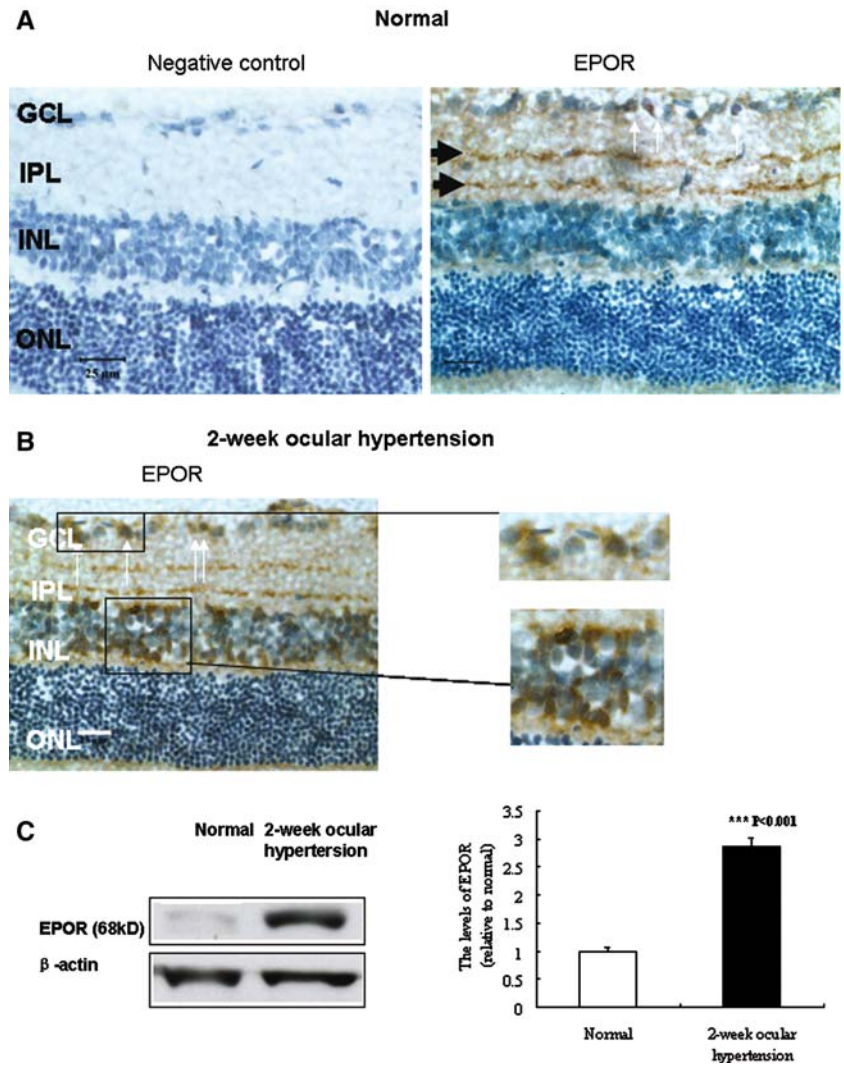

Fig. 2 Expression of EPOR protein in the normal and ocular hypertensive retina. Graphs (**A**–**B**) show the EPOR expression in the normal retina shown with diaminobenzidine. The nucleus were counterstained by hematoxylin. There was no non-specific EPOR expression in the normal retina (**A**). EPOR protein was predominantly expressed in the IPL with two strong immunohistochemical staining bands in the normal retina (black arrows) (**A**). Minimal EPOR staining was found in some cells in GCL (white arrows). Graphs (**B**) show that the expression of EPOR was increased in the INL and GCL at 2 week after ocular hypertension injury. The increase of EPOR protein following ocular hypertension was confirmed with Western blotting (**C**). Densitometric analysis demonstrated a significant increase ($P < 0.01$). The number of samples for each group is six (Mean + SEM). GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, ONL: outer nuclear layer. Scale bar is 25 μm

significant neuroprotective effect after ocular hypertension. In order to further examine the neuroprotective activity of rhEPO after retinal injury, the effect of rhEPO administrated systemically on promoting RGC survival after acute injury was also tested using an optic nerve transection model. Similar results were observed at 7 days after the injury (Fig. 4C). There were 44.0 ± 2.4% RGCs lost 1 week following optic nerve transection in the saline group. However, significantly more surviving RGCs were observed in the rhEPO treated rats (loss = 22.6 ± 4.3%, $P < 0.01$). These data suggest that systemic treatment of rhEPO has significant neuroprotective activity on the survival of RGCs in an ocular hypertension model of glaucoma (chronic injury) and optic nerve transection (acute injury).

IOP profile

Photocoagulation of limbal and episcleral veins using Argon laser is a good approach to induce chronic ocular hypertension in animals which can be monitored by measuring changes in the intraocular pressure or neuronal survival of RGCs (Schori et al. 2001). Elevated IOP is an important risk factor for progression of glaucomatous optic neuropathy. IOP in the contralateral left eye of animals was about 13 mmHg (Fig. 5) and remained at the same level following injury until sacrifice (Ji et al. 2004). The IOP of the laser-treated right eye in the PBS, saline control groups, and soluble EPOR, rhEPO treatment groups increased after the first laser surgery reaching to approximately 22 mmHg and remained at this level

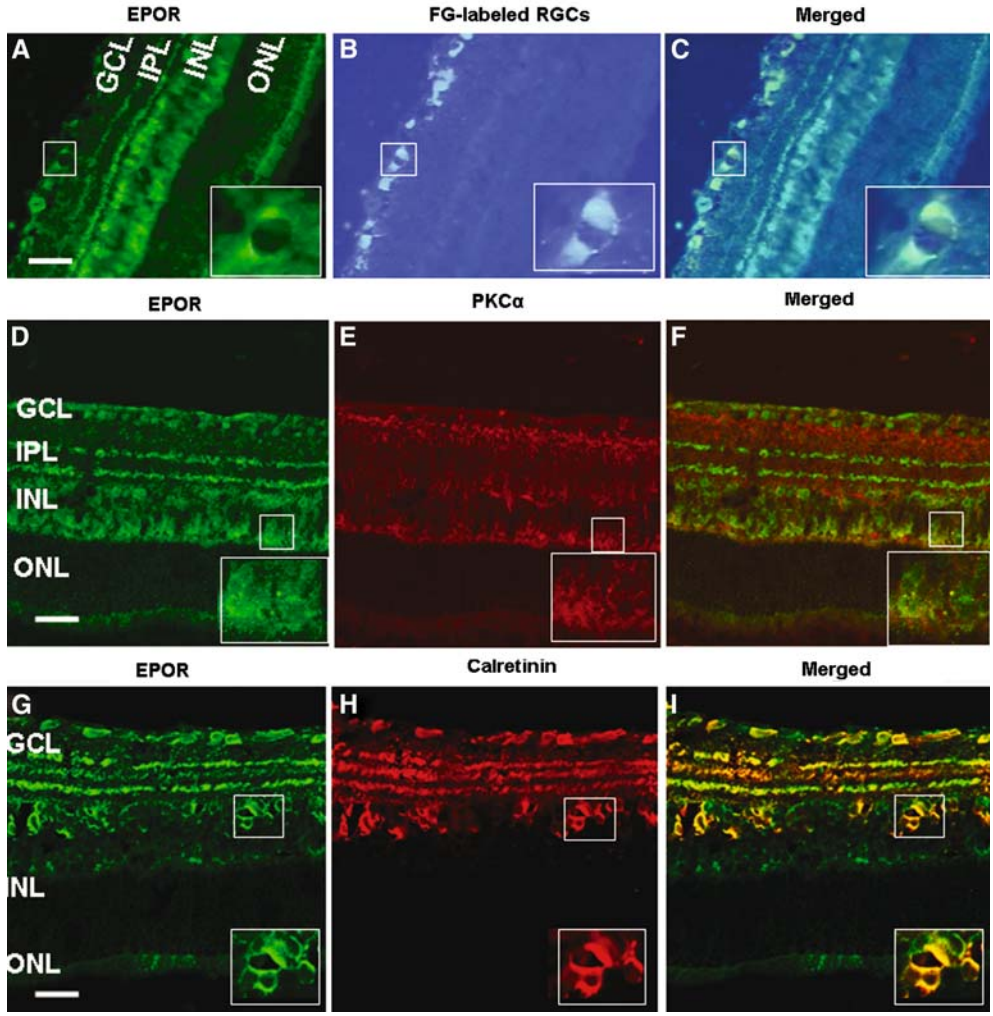

Fig. 3 Expression of EPOR protein stained with specific cell markers in the ocular hypertensive retina. Retrogradely labeling of RGCs with Fluorogold (**B**) demonstrated strong membrane and cytoplasma staining for EPOR (**A**, **C**). Double labeling demonstrated the co-localization EPOR protein (**D**, **G**, green) with bipolar (**E**, red) and amacrine (**H**, red) neurons after the injury (**F**, **I**, orange). The number of samples for each group is six (Mean + SEM). GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, ONL: outer nuclear layer. Scale bar is 50 μm

throughout the experimental period. Treatment of rhEPO and soluble EPOR had no effect on the IOP, in a manner analogous that of ciliary neurotrophic factor (CNTF) (Ji et al. 2004).

Discussion

In this study, we have identified ocular hypertension induced significant up-regulation of EPO and EPOR. Intravitreal injection of soluble EPOR exacerbated ocular hypertensive injury, which provides evidence supportive of an endogenous EPO/EPOR system in the survival of RGCs after injury. Similarly, intravitreal and systemic administration of rhEPO also promotes RGCs survival after ocular hypertension. These results further establish EPO as a neuroprotective agent in chronic glaucoma injury.

EPO and EPO Receptor Expression in the Retina

We found that the normal retina expresses EPO primarily in Müller cells located in the IPL, and the expression of this cytokine extends to the whole

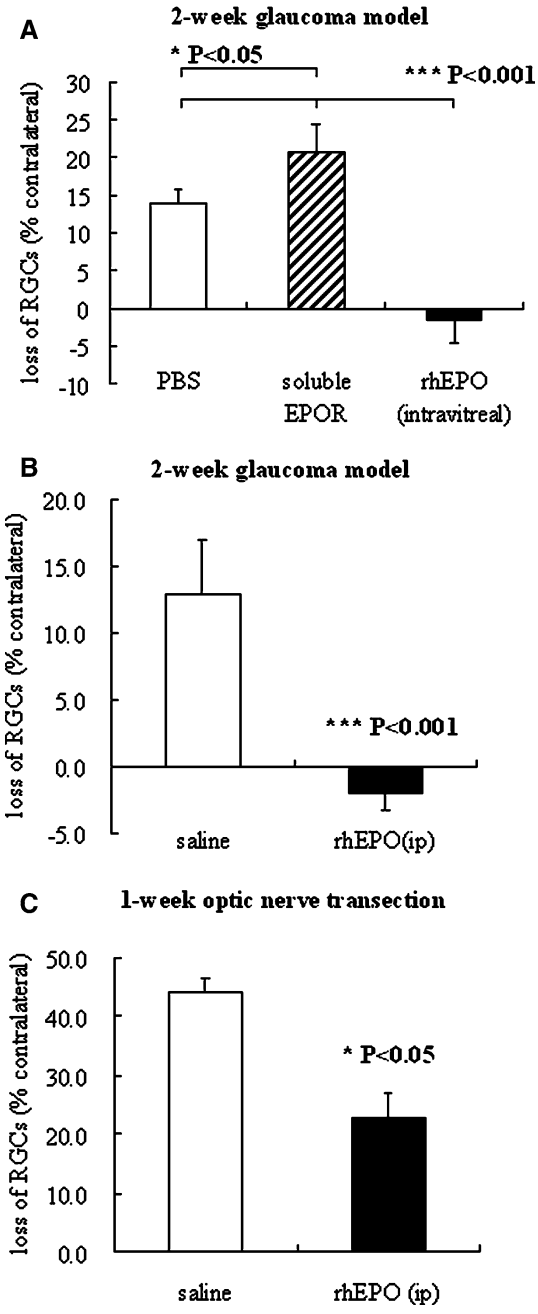

Fig. 4 The effects of soluble EPOR and recombinant human EPO in the ocular hypertensive and optic nerve transection eyes. Figures showing percentage loss of RGCs in the ocular hypertensive eyes after counting the Fluorogold labeled RGCs from the whole flatmount retinas. (**A**) Intravitreal injection of soluble EPOR exacerbates ocular hypertensive injury compared to PBS groups after counting the Fluorogold labeled RGCs from whole flatmounts after 2 weeks ocular hypertension ($P < 0.05$). Application of EPO to the vitreous body protects the injured RGCs at 2 weeks after laser coagulation ($P < 0.001$). (**B**) rhEPO administered peripherally significantly promotes the survival of RGCs at 2 weeks after ocular hypertension ($P < 0.001$). (**C**) rhEPO injected intraperitoneally promotes the survival of injured RGCs at 1 week after optic nerve transection ($P < 0.05$). i.p.: intraperitoneal. The animal number for each group is six

retina at 2 week after ocular hypertension. This finding is in accordance with prior studies of brain that have identified astrocytes as the main cellular source of EPO. Müller cells are the principal supporting glial cells of the retina and play important roles in protein synthesis, intracellular transport, and secretion. Müller cell expression of EPO and active response of Müller cells for EPO after ocular hypertension further supports a glial protective response to neuronal injury. These findings contrast a report that described amacrine and bipolar neurons as the main cellular source of EPO, and this cytokine was decreased after acute ischemia (Junk et al. 2002). These differences in EPO expression in retinal injury may be related to the severity of injury.

In our study, EPOR was mainly detected in the ChATir terminals of amacrine cells in IPL of the normal rat retina. Most of the signal compression and information processing is performed by amacrine cells in the IPL. Amacrine cells receive signals from a large number of connecting bipolar and/or horizontal cells and directly modulate the function of ganglion cells through inhibitory and/or stimulatory transient responses. Some types of amacrine cells have been identified. ChATir amacrine cells have a bipolar, triangular or stellate appearance, and give rise to processes coursing in the inner plexiform layer. They release acetylcholine transiently at the onset and offset of a light stimulus. It has been suggested that they may be involved in ON/OFF responses in directionally selective ganglion cell responses. The strong expression of EPOR in the same position of ChATir terminals of amacrine cells suggests the important function of EPOR on the synaptic connections between amacrine cells and ON/OFF RGCs in distinct layers of the IPL.

We also confirmed weak expression of EPOR in the GCL. Same as our results, Junk et al. (2002) also found that minimal EPOR immunoreactivity was noted in the normal retinas stained with the EPOR antibody from Santa Cruz. Furthermore, the observation of up-regulation of EPOR after injury using

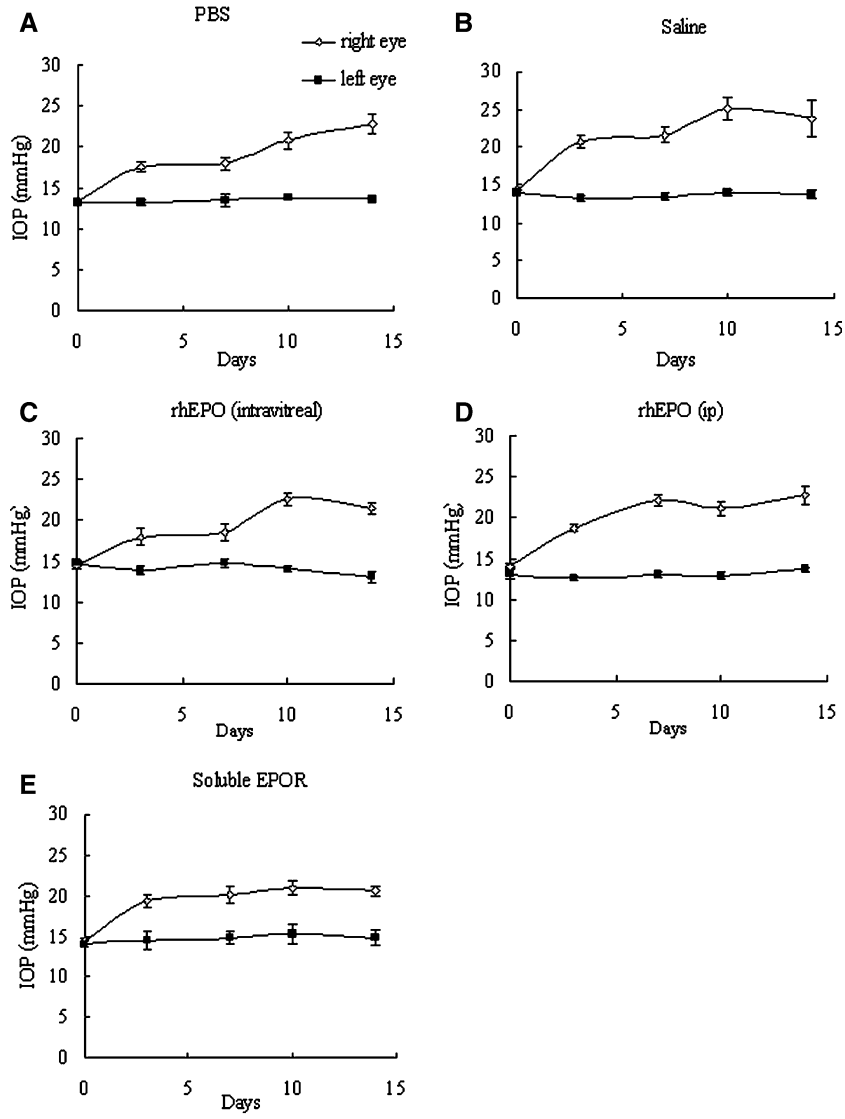

Fig. 5 IOP profile after laser treatment. Graphs showing the IOP profile for the four treatment groups. All animals received two laser photocoagulations. An increase of about 1.7 times in IOP was observed in the laser-treated eyes in all groups at 2 weeks after ocular hypertension. The values of IOP are expressed as the mean ± SEM of the experimental or control eyes for a particular time point. The animal number for each group is six

immunohistochemistry and Western blot is in agreement with previously reported increased EPOR protein expression after acute retinal ischemia injury (Junk et al. 2002) or hypoxia (Grimm et al. 2002) and brain injury (Bernaudin et al. 1999; Sadamoto et al. 1998), but contrasts with the study of Weishaupt et al. describing unchanged EPOR protein after optic nerve transection (Weishaupt et al. 2004). These differences in EPOR expression may be related to the severity of injury and/or species differences. Pronounced up-regulation of EPO and EPOR after chronic ocular hypertension indicates an important role for EPO/EPOR system in the retina after injury. The increased of EPO and EPOR expression may provide endogenous response similar to that of CNTF (Ji et al. 2004) and BDNF (data not shown) promoting neuronal survival.

Endogenous Neuroprotective Effect of EPO after Ocular Hypertension Injury

Up-regulation of EPOR and EPO after ocular hypertension injury supports the hypothesis that the EPO-dependent recovery pathway may be up-regulated in the injured retina. Since, there is still a 13% loss of RGCs, this suggests that the EPO-mediated

neuroprotection is inadequate to offer full protective effect. On the contrary, treatment with soluble EPOR significantly augmented the loss of RGCs at 2 weeks after ocular hypertension. These results indicate that the injected soluble EPOR neutralized endogenous EPO, inhibiting the binding of retinal EPO with neuronal EPOR. These findings are in agreement with prior studies showing that soluble EPOR potentiated neuronal damage in global cerebral ischemia (Sakanaka et al. 1998) and acute retinal ischemia injury (Junk et al. 2002). These data indicates that an endogenous EPO/EPOR system might participate in intrinsic recovery mechanisms after chronic ocular hypertension. Previous studies have showed that EPO is an endogenous retinal survival factor to the photoreceptors against photochemical injury (Becerra and Amaral 2002).

Neuroprotective Effects of rhEPO after Chronic Ocular Hypertension

Glaucoma is a common eye disease that can cause irreversible loss of vision if left undiagnosed and untreated. There is a lack of therapies preventing RGC death and existing pharmacological and surgical treatments, aimed at lowering IOP, are not fully effective in protecting RGCs and their axons. The model used in this report is considered to mimic well the slow, progressive neuropathy produced by elevated IOP in humans, by partially reducing aqueous humor outflow, similar to the elevated episcleral venous pressure in glaucoma (Morrison et al. 2005). So far, EPO has been shown to protect RGCs from acute ischemia injury (Junk et al. 2002), glutamate and nitric oxide toxicity (Yamasaki et al. 2005), axotomy (Kilic et al. 2005), and light induced degeneration (Rex et al. 2004). We demonstrate that the topical administration of rhEPO appears to have a protective effect on RGC viability in an in vivo rat model of ocular hypertension, which is similar to prior study of EPO on the survival of RGCs in a glaucoma model (Tsai et al. 2005). Grimm et al. also described the neuroprotection in mice that constitutively over-express EPO (Grimm et al. 2004). Furthermore, our findings have showed that systemic pretreatment of rhEPO significantly promotes the survival of RGCs in ocular hypertension glaucoma injury. Even in extreme conditions of neurotrauma such as optic nerve transection, exogenous EPO provided a significant level of RGC protection for up to a week. Many drugs to treat eye diseases were performed by local administration partly because of the limitation of penetrating the BBB. Although in the classic view the BBB is considered to be impermeable to large molecules, recent study clearly establishes that some large molecules such as EPO can be specifically transported into the brain across the capillary endothelium to affect brain function (Siren et al. 2001; Brines et al. 2000; Gorio et al. 2002). It suggests that there is an intersection between peripheral EPO system and retinal EPO system. It also provides a potential for EPO to be administrated systemically. The retina is an extremely metabolically active sheet of neural tissue with the highest oxygen consumption (per weight) of any human tissue. The retina has a dual blood supply from the central retina vessels and choroidal circulation. It is possible for rhEPO to arrive anywhere of the retina along with the blood flow. Both the barrier of the inner limiting membrane of vitreous and high frequency of infection limit the application of drugs by intravitreal injection. Of course, the dosage for the systemic administration is much higher than that of the local administration. In our experiment, 5,000 unit rhEPO/kg was intraperitoneally injected for adult SD rats, which is much more than the dosage of rhEPO intravitreally injected (Weishaupt et al. 2004). It is likely that the mode of action of EPO is to bind directly to EPOR present on the RGC cell membrane to activate the neuroprotective pathway. The exogenous EPO can remedy the limitless of endogenous EPO/EPOR system. The up-regulation of EPOR in RGCs after injury further promotes the protective effect of exogenous EPO. These findings have direct clinical relevance to glaucoma disease. Thus, like neurotropins CNTF & BDNF, EPO may represent a novel treatment for glaucoma.

Furthermore, enthusiasm for rhEPO as a potential neuroprotective therapeutic must be tempered, however, by the knowledge that it also leads to potentially harmful increases in the red cell mass and platelet aggregability. In this study, two injections of rhEPO lead to a little increase of circulating red cell mass, the level of hematoglobin, hematocrit, and mean corpuscular volume (data not shown). Actually, many clinical situations will likely require multiple doses of rhEPO, which will strongly stimulate erythropoiesis. So molecules retaining the beneficial neuroprotective

actions of EPO, but not stimulating the bone marrow are desirable. AsialoEPO, generated by total enzymatic desialylation of rhEPO, possesses a very short plasma half-life and is fully neuroprotective (Erbayraktar et al. 2003). This short-lived EPO has the same high affinity for EPOR as rhEPO and is fully protective in animal models of stroke, spinal cord injury, and peripheral neuropathy (Erbayraktar et al. 2003; Wang et al. 2004; Grasso et al. 2006). Identification of the neuroprotective EPO derivative with a brief plasma half-life potentially offers important advantages over rhEPO and would allow for multiple or chronic dosing strategies in chronic glaucoma or other neurodegenerative diseases.

In summary, these data provide evidence that EPO/EPOR system plays an endogenous neuroprotective role in the survival of RGCs after ocular hypertension injury. Systemic administration of rhEPO rescued the RGCs after chronic and acute retinal injury. EPO, especially the polypeptides that retain only the neuroprotective activity of the molecule can be used as therapeutic for treatment intervention of brain injury and neurodegenerative diseases.

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