Effect of Erythropoietin Axotomy-Induced Apoptosis in Rat Retinal Ganglion Cells

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Purpose. Erythropoietin (EPO) modulates erythropoiesis by inhibiting apoptosis in erythrocyte progenitors. Recently, EPO has been shown to be protective in experimental models of mechanical trauma, neuroinflammation, cerebral and retinal ischemia, and even in a human stroke trial. However, little is known about EPO signal transduction in vivo and the usefulness of EPO in the prevention of the chronic, purely apoptotic neuronal cell death that contributes to vision loss in glaucoma and the progression of neurodegenerative diseases.

Methods. EPO's effects and signaling in the retinal ganglion cell axotomy paradigm were studied by Western blot analysis and immunohistochemistry, receptor expression was characterized in the retina before and after lesion. EPO was injected into the vitreous body to investigate neuroprotection of axotomized rat RGCs. Moreover, EPO's effects were studied in cultures of immunopurified retinal ganglion cells. Signal-transduction pathways transmitting neuroprotective EPO effects in vivo were characterized by the use of specific kinase inhibitors, immunohistochemistry, and Western blot analysis.

Results. EPO receptors (EPORs) were expressed on RGC somata and dendrites in vivo. EPOR expression did not significantly change after axotomy. Application of EPO prevented death of neurotrophic-factor–deprived immunopurified rat RGCs in vitro, rescued axotomized RGCs in vivo, and prevented caspase-3 activation. EPO-induced Akt phosphorylation and survival-promoting EPO effects were completely abolished by inhibition of PI-3-kinase. EPO neuroprotection followed a bell-shaped dose–response curve in vitro and in vivo, whereas toxic EPO effects were never observed, even at high concentrations.

Conclusions. These data support a potential role for EPO as a therapeutic molecule against predominantly apoptotic neuronal cell death in the context of glaucoma or neurodegenerative diseases and delineate the PI-3-K/Akt pathway as the predominant mediator of EPO neuroprotection in this in vivo paradigm of neuronal cell death. (*Invest Ophthalmol Vis Sci.* 2004;45:1514–1522) DOI:10.1167/iovs.03-1039

Erythropoietin (EPO) was first characterized as a hematopoietic factor.[1] It has been shown to stimulate differentiation and proliferation of erythroid progenitor cells. More recently, EPO and EPO receptor (EPOR) were found to be expressed by brain and retinal tissue,[2–4] cultured neurons,[5–7] and astrocytes.[6–8] Findings that EPO, in addition to its antiapoptotic activity in erythrocyte progenitors,[9] exerts protective effects against light-induced retinal degeneration[10] in retinal ischemia,[11] in various models for neuronal cell death, and even in a human stroke trial,[5,12–14] expanded the role of this cytokine beyond its function in the hematopoietic system.

The EPOR belongs to the cytokine superfamily. On EPO binding, EPORs form homodimers, with subsequent activation of various signal transduction kinases, such as the RAS/RAF/ERK or PI-3K/Akt kinase pathway.[12,15] These signaling cascades have been shown to mediate EPO effects, and are similar to those observed previously for other neurotrophic factors such brain-derived neurotrophic factor (BDNF) or insulin-like growth factor (IGF)-I.[12] The net effect in erythroblasts is an enhanced differentiation and proliferation, resulting from inhibition of apoptosis.[16–18] Some of the downstream events after EPOR activation involve the regulation of bcl-2 family members—for example, upregulation of protective proteins such as bcl-2 and Bcl-XL, or downregulation of the death-promoting gene Bak, as well as upregulation of endogenous inhibitors of apoptosis, such as XIAP.[16,18–21] It was found that EPO activates the RAS/RAF/ERK and PI-3K/Akt pathways also in cultured neurons, and inhibition of these signaling pathways abolished the survival promoting effect of EPO.[12,22] Recently, EPO-induced protection against excitotoxin- and NO-induced apoptosis has been shown to involve crosstalk between the Jak2 and NF-κB signaling pathways.[23] Thus, in vitro results indicate that there is significant overlap between EPO signaling in erythropoid progenitors and neuronal cells, although knowledge about neuroprotective EPO signal transduction in vivo is still scarce.

EPO-mediated neuroprotection was observed in the context of excitotoxic and hypoxic death of cultured motoneurons, C19 teratoma cells, and hippocampal neurons. These findings were further substantiated by the demonstration that EPO protects neurons after experimental mechanical trauma; retinal, cerebral, or spinal cord ischemia; or neuroinflammation in vivo.[10,12,23,24] Moreover, sublethal hypoxia induces upregulation and nuclear translocation of hypoxia-inducible factor (HIF)-1. One of the target genes of this transcription factor is EPO, which is also upregulated by hypoxia and is likely to mediate some of the protective effects of ischemic preconditioning against subsequent severe ischemia, supporting a possible role for the EPO/EPOR system as an endogenous neuronal protectant.[10,23,25–27]

Thus, EPO neuroprotection has mostly been investigated in settings describing ischemic/hypoxic and excitotoxic paradigms that can involve both apoptosis- and necrosis-like types of cell death. EPO has been shown also to suppress caspase activation or the appearance of TUNEL-positive cells in these models, but few data exist regarding the value of EPO in prevention of the RGC death that is thought to contribute to vision loss in glaucoma[28] or chronic neurodegeneration observed in neurodegenerative diseases— conditions that involve predominantly or exclusively apoptosis-like death cascades.

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Submitted for publication September 21, 2003; revised November 19 and December 23, 2003; accepted January 5, 2004.

Disclosure: **J.H. Weishaupt**, None; **G. Rohde**, None; **E. Pölking**, None; **A.-L. Siren**, None; **H. Ehrenreich**, None; **M. Bähr**, None

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After transection of the optic nerve (ON), axotomized RGCs undergo retrograde degeneration, fulfilling all criteria for apoptosis. Besides classic morphologic signs of apoptotic cell death,[29] these criteria include activation of caspase-3 and -9,[30,31] antagonistic regulation of Bax and Bcl-2 expression,[32] downregulation of apoptosis-inhibiting activities of RAS/RAF/ERK or PI-3K/Akt kinases,[33–35] and protective effects of caspase inhibitors[30,31] and neurotrophins.[34–36] Therefore, ON transection in the rat is an elegant and clear-cut in vivo technique for the study of neurodegenerative processes and respective signal-transduction cascades in the central nervous system (CNS). Moreover, it replicates important pathophysiological steps relevant in RGC death and vision loss due to glaucoma.

In this study, we investigated the neuroprotective effects of EPO and the signal transduction and regulation of downstream proteins involved in EPO-mediated neuroprotection after axotomy of RGCs in vivo and after neurotrophin deprivation of cultured immunopurified RGCs.

METHODS

Immunohistochemistry

For immunohistochemical experiments rat retinas were used as described before.[33,37] Briefly, rats were killed with an overdose of chloral hydrate. Eyes were dissected and immediately fixed as eye cups without cornea and lens for 1 hour in 4% paraformaldehyde in PBS at 4°C. Eye cups were immersed in 30% sucrose in PBS overnight at 4°C and frozen in optimal cutting temperature compound (Tissue-tek; Sakura Finetek, Torrance, CA) with liquid nitrogen. Cryostat-cut sections (16 μm) were collected on slides (Superfrost; Fisher Scientific, Pittsburgh, PA), dried at 37°C, and stored at −20°C.

After they were dried for 1 hour at 37°C, cryosections were incubated in 10% normal goat serum (NGS) in PBS containing 0.3% Triton X-100, to block nonspecific binding. Sections were then washed three times in PBS and incubated with primary antibody at 4°C overnight. Immunoreactivity was visualized by incubating the sections with a secondary fluorescent antibody (1:200) after they were washed three times with PBS. Finally, sections were coverslipped (Mowiol; Hoechst, Frankfurt, Germany).

Primary antibodies were directed against EPOR (M-20; Santa Cruz Biotechnology, Santa Cruz, CA) and cleaved caspase-3 (Asp175; Cell Signaling Technology, Beverly, MA). Goat anti-rabbit Cy3-conjugated IgG served as the as secondary antibody (Dianova, Hamburg, Germany). Immunofluorescence was imaged with a microscope (Axioplan; Carl Zeiss Meditec). Immunopositive RGCs were quantified using cryostat sections, cut a maximum distance of 1.5 mm from the ON in at least three different animals per experimental group.

Western Blot Analysis

After SDS-PAGE using 10% or 12% polyacrylamide gels, proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Billerica, MA). Nonspecific binding sites were blocked by incubation in TBST (20 mM Tris [pH 7.6], 140 mM NaCl, and 0.1% Tween 20 [vol/vol]) containing 5% skim milk powder for 1 hour at room temperature. After incubation with primary antibody overnight, blots were washed three times for 10 minutes in TBST and probed with the respective horseradish-peroxidase–conjugated secondary antibody at room temperature for 1 hour. Blots were washed and incubated with enhanced chemiluminescence substrate solution and exposed to autoradiograph film (Hyperfilm ECL; Amersham Pharmacia Biotech, Freiburg, Germany), to visualize immunoreactive bands.

Primary antibodies were directed against phospho-Akt (pAkt, Ser 473; Cell Signaling Technology), Akt (New England Biolabs, Beverly, MA), phosho-p44/42-mitogen activated protein (MAP) kinase (T202/Y204; Cell Signaling Technology), total p44/42-MAP kinase (C-16; Santa Cruz Biotechnology), and EPOR (M-20; Santa Cruz Biotechnology). Secondary antibodies were purchased from Santa Cruz Biotechnology.

Optic Nerve Transection in the Rat

Transection of the ON of adult Sprague-Dawley rats (200–250 g; Charles River, Wiga, Germany), with Fluorogold (Fluorochrome, Englewood, CO) retrograde labeling, tissue processing, and cell counting were performed as described previously.[33,37]

For intravitreal drug administration, erythropoietin (r-Hu-EPO; Roche Molecular Biochemicals, Mannheim, Germany) was diluted in PBS. Wortmannin (WM; Sigma-Aldrich, Diesenhofen, Germany) was dissolved in dimethylsulfoxide (DMSO; Sigma) and diluted in ddH_2O to a final concentration of 0.1 mM. Intravitreal injections (2 μL) of EPO and/or WM on days 0, 4, 7, and 10 after lesioning were performed as described.[33,34] Animals were anesthetized by diethylether. By means of a glass microelectrode with a tip diameter of 30 μm, 2 μL EPO solution, WM, or PBS vehicle was injected into the vitreous space, puncturing the eye behind the cornea–sclera junction, carefully avoiding the lens. Injection of vehicle after axotomy did not lead to an increased number of surviving RGCs compared with axotomy alone.[38]

For neuroprotection studies, rats were killed on day 14 after axotomy, and axotomized eyes were dissected. Animals received an overdose of chloral hydrate and the eyes were removed. Retinas were dissected, flatmounted on gelatin-coated glass slides, and fixed for 20 minutes in 4% paraformaldehyde (PFA) in PBS. RGCs were examined under a fluorescence microscope (Axiovert 35; Carl Zeiss Meditec) using a UV filter (365/420 nm) to detect Fluorogold fluorescence. The number of FG-positive RGCs was determined by counting them in 12 distinct areas of 62,500 μm^2 each (three areas per retinal quadrant at three different eccentricities of one sixth, one half, and five sixths of the retinal radius). Cell counts were performed according to a blind protocol by two different investigators. Overestimation of RGC density by retinal shrinkage due to intraocular injection was excluded.[39] For Western blot analysis and activity assays, retinas were removed and processed as described later.

Animal experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Immunopanning of RGCs

For immunopurified rat RGC culture, rat pups were killed on postnatal day 8. RGCs were purified according to a two-step panning protocol described previously.[40] As the culture medium, we used serum-free medium (Neurobasal; Invitrogen-Gibco, Eggenstein, Germany) supplemented with glutamine, cysteine, pyruvate, triiodothyronine, B-27 supplement, and Sato (BSA, transferrine, progesterone, putrescine, sodium selenite). During the first 24 hours, RGCs were additionally incubated with saturating concentrations of forskolin, human BDNF, ciliary neurotrophic factor (CNTF), and insulin. RGCs were withdrawn from neurotrophins by change of medium on culture day 1 and incubated with the respective compounds.

The survival rate of RGCs was determined by an MTT cell survival assay. MTT (5 mg/mL) was added to culture wells (1:10) at culture day 3 and incubated at 37°C for 1 hour. Viability of RGCs was assessed by counting six fields within a culture well at a magnification of ×200. RGCs showing dense blue staining of cell bodies were considered to be MTT-positive.

Survival of RGCs in the different culture conditions was calculated as the mean number of MTT-positive RGCs/mm^2 in three wells per experiment and experimental condition on day 3. Results are expressed as the percentage of additional survival compared with neurotrophin-deprived control RGCs on day 3.

EPO was diluted in cell culture medium without neurotrophins to the respective final concentration in the cell culture well. WM (stock-solution of 10 mM) was first diluted in DMSO and subsequently in cell culture medium. Final concentration in the well was 100 μM. LY294002 and PD98059 stock solutions (20 mM) were diluted in

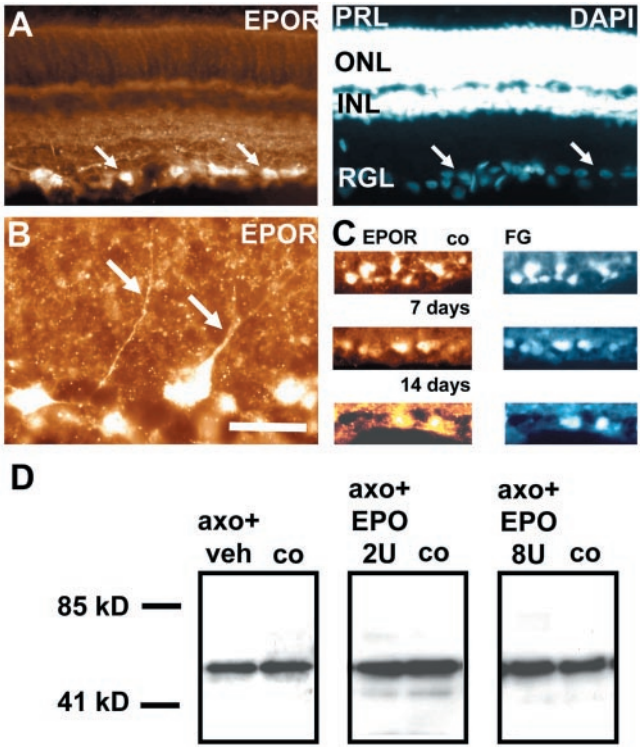

FIGURE 1. Expression of EPOR protein in the rat retina after ON transection. (**A**) EPOR expression (*arrows*) in unlesioned control retina shown with a Cy3-labeled secondary antibody (*left*) and counterstaining with DAPI (*right*). Intense immunoreactivity was found in the RGC layer (RGL). (**B**) High-power magnification showing EPOR expression on neuronal somata and dendrites (*arrows*). (**C**, *top*) EPOR staining of retinal sections with retrograde, gold-fluorescence–labeled RGCs demonstrates EPOR expression predominantly by RGCs, but little if any expression by other, fluorescent tracer–negative, cells in the RGC layer. EPO receptor expression on RGCs persisted until day 7 after axotomy, when the first RGCs had already morphologically disappeared (*middle*), and even RGCs surviving until day 14 were still found to express EPO receptors (*bottom*). (**D**) EPOR expression in retinal protein lysates of axotomized (axo) and unlesioned control eyes (co) was confirmed by Western blot analysis. EPOR expression was not changed significantly compared with control eyes 4 days after axotomy and vehicle treatment, or treatment with EPO (2 or 8 U/eye injected on days 0 and 4 after axotomy). For densitometric analysis of Western blots, see the Methods section. RGL, RGC layer; INL, inner nuclear layer; ONL, outer nuclear layer; PRL, photoreceptor layer; veh, vehicle-treated; co, unlesioned control; axo, axotomy.

cell-culturing medium without neurotrophins to a final concentration of 10 μM.

Statistics

Data are expressed as the mean ± SD, unless otherwise stated. Statistical significance was assessed applying one-way ANOVA followed by the Duncan post hoc test (comparing RGC survival), unpaired *t*-test, or paired *t*-test where appropriate (e.g., comparison of densitometric immunoblot data from axotomized and corresponding contralateral control eyes of the same animal).

RESULTS

Expression of EPORs

With immunohistochemical staining using an antibody raised against the EPOR protein, we found an intense immunoreactivity in the RGC layer of Sprague-Dawley rats (Fig. 1A). Weak EPOR expression, though hardly exceeding background signal, was also found between the RGC layer, the inner and the outer nuclear layers as well as in the photoreceptor layer. EPOR immunoreactivity was detected on the cell bodies and dendrites of RGCs (Fig. 1B). Prelabeling of RGCs by injection of a retrograde tracer (Fluorogold; Fluorochrome) into the superior colliculus, resulting in retrograde, specific staining of RGCs, demonstrated that almost all RGCs were immunoreactive for EPOR protein (Fig. 1C, top).

Using immunohistochemistry, we did not observe a change in EPOR expression by RGCs or other retinal cells 7 days (Fig. 1C, middle) or 1, 2, and 5 days (data not shown) after transection of the ON, compared with unlesioned retinas. Even 14 days after ON transection, we found EPO receptor immunoreactivity on surviving RGCs, although most RGCs underwent apoptosis within 14 days after axotomy (Fig. 1C, bottom). Western blot experiments confirmed EPOR expression in protein lysates prepared from control retinas of Sprague–Dawley rats. Again, we did not observe an up- or downregulation of EPOR protein expression after axotomy compared with unlesioned retinas. On day 4 after axotomy, 1 day before the earliest RGC death can be detected after ON transection,[34,41,42] immunoblot analysis failed to reveal a significant difference in retinal EPOR expression between control retinas and retinas after axotomy and intravitreous vehicle injection (Fig. 1D). Densitometric analysis of the Western blot revealed a mean signal ratio between axotomized and control eyes of 0.93 ± 0.80 (n = 18; mean ± SD) 4 days after axotomy. When EPO was injected on days 0 and 4 after axotomy (2 or 8 U/eye), we found a relative EPOR expression compared with control eyes of 1.38 ± 1.42 (n = 14) and 1.41 ± 1.35 (n = 11), respectively. However, this increase in EPOR expression on EPO injection did not reach statistical significance.

Protective Effect of EPO In Vivo

After ON transection, which results in axotomy of all RGCs, approximately 85% of all RGCs die within 14 days by a degenerative process fulfilling all criteria of apoptosis.[29,43] The effect of EPO on the survival of RGCs was investigated by repeated intraocular injections of various EPO concentrations. Injection of 2 U EPO/eye on days 0, 4, 7, and 10 after ON transection enhanced RGC survival by 92% compared with survival in vehicle-injected control eyes on day 14 after axotomy (872 ± 243.5 vs. 455 ± 95.7 surviving RGCs/mm^2; $P < 0{,}05$; mean ± SD; Figs. 2A, 2B).

Testing different EPO doses, we found a bell-shaped dose–response curve with increasing rescue rates at doses of between 0 and 2 U EPO injected on days 0, 4, 7, and 10 (Fig. 2D). Increasing the EPO dose to 4 or 8 U/eye resulted in a decline of RGC rescue rates, with RGC densities at 8 U EPO/eye not significantly different from vehicle control values (Figs. 2C, 2D). However, a decrease in RGC survival below the control (i.e., toxic effects) was never observed within the concentration range tested.

EPO-Promoted RGC Survival after Neurotrophic Factor Deprivation

EPOR has been shown to be expressed by glial cells.[6–8,44] To show that EPO exerted a direct neuroprotective effect on RGCs that was not secondary to glial EPOR activation and subsequent release of neuroprotective factors, we used immunopurified rat RGCs. We used a two-step immunopurification protocol[40] to purify RGCs to near homogeneity and then cultured them in serum-free medium, supplemented with the neurotrophic factors forskolin, human BDNF, CNTF, and insulin. After 1 day in culture, neurotrophins were withdrawn, and the number of surviving RGCs was determined by MTT assay

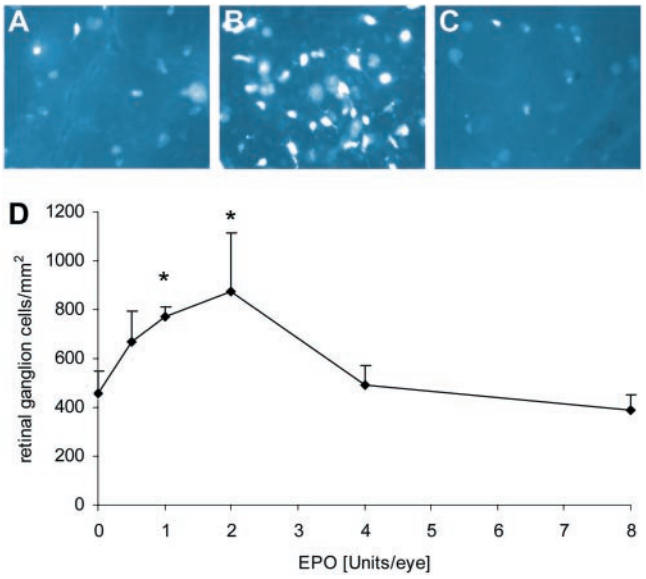

FIGURE 2. EPO protected axotomized rat RGCs in vivo. Retinal wholemounts of retrogradely labeled RGCs 14 days after axotomy under treatment with vehicle (**A**), EPO intravitreally injected at a dose of 2 (**B**) or 8 (**C**) U/eye on days 0, 4, 7, and 10 after ON transection. (**D**) EPO dose–response-curve showing the number of surviving RGCs/mm^2 in retinal wholemounts 14 days after axotomy and treatment with various EPO doses (injection on days 0, 4, 7, and 10 after axotomy; $n = 4$ - 6; mean ± SD). *$P < 0.05$ compared with vehicle injection.

after two further days in culture. When EPO was added concomitantly with the neurotrophin deprivation, survival of immunopurified RGCs was enhanced significantly (Figs. 3A–C). Again, we found a bell-shaped dose–response curve, with the maximum at an EPO concentration of approximately 0.6 U/mL (Fig. 3C).

Retinal PKB/Akt, but not ERK1/2, Activation In Vivo after Intravitreal Injection of Neuroprotective EPO

To investigate the intracellular signaling pathways mediating EPO neuroprotection in vivo, we studied activation of two potential EPO signal-transduction pathways, the PI-3-kinase/Akt pathway and the ERK1/2-MAP kinase pathway, that have been shown to mediate the survival-promoting effects of various neurotrophins in vitro, including EPO.[12,15]

In contrast to other studies that demonstrated upregulation of the phosphorylated, active form of ERK1/2 on administration of EPO in vitro, we failed to show any change in the abundance of both phosphorylated and total amounts of ERK1/2 in retinal protein lysates after axotomy and intraocular injection of EPO, at least at the neuroprotective concentrations used in this study (2 U/eye on days 0 and 4 after axotomy). In accordance with previous findings, axotomy alone did not influence phospho-ERK1/2 levels, while robust ERK1/2 activation was shown by intraocular injection of BDNF (not shown).

Moreover, we did not observe a change in protein expression or phosphorylation of various proteins or other signal-transduction kinases that were demonstrated to be involved in EPO-induced signaling and apoptosis regulation in other paradigms. By Western blot analysis, neither p38 nor JNK displayed any difference in the level of their phosphorylated form or total protein. Moreover, the levels of bcl-2, Bcl-Xl, or XIAP were not affected by EPO treatment at the dose that was neuroprotective in our model (2 U/eye; data not shown).

In contrast, we found the amount of pAkt to be clearly increased by the same treatment protocol, whereas total amounts of Akt protein remained unchanged, as shown by Western blot and densitometric analyses of at least five paired samples (axotomy+EPO/WM injection compared with unlesioned contralateral control eyes) in each experimental group (Figs. 4A, 4B). For each experimental group, we calculated the mean ratio of arbitrary densitometric units of axotomized versus corresponding unlesioned eyes. PAkt levels showed that the ratio of axotomized and EPO-treated eyes compared with unlesioned control eyes increased significantly to 2.58 ± 1.21, whereas total Akt protein levels remained constant (ratio 1.03 ± 0.10). This increase could be blocked by cotreatment with WM, a small molecule kinase inhibitor that blocks activity of PI-3-kinase, which is located in the same pathway directly upstream of and activating Akt (pAkt levels of axotomized and EPO/WM-injected versus control eyes 1.12 ± 0.21; total Akt protein ratio 1.01 ± 0.02). Axotomy and vehicle injection alone did not result in a significant change in pAkt or Akt levels (Figs. 4A, 4B). Because we used total retinal lysates, it is important to note that retinal Akt and pAkt immunoreactivity has been shown to be localized in RGCs before.[45,46]

WM Blocks EPO Neuroprotection

The results outlined demonstrated that EPO induces Akt phosphorylation in a PI-3-kinase–dependent manner. To demonstrate the functional significance of EPO-induced Akt phosphorylation in vivo, we assessed RGC survival under a combined treatment with EPO and the PI-3-kinase inhibitor WM. Injection of 2 μL 0.1 mM WM, together with EPO (2 U/eye) on each injection day (days 0, 4, 7, and 10 after axotomy) completely abolished the neuroprotective effects of EPO. Whereas 455 ± 95.7 (mean ± SD) surviving RGCs/mm^2

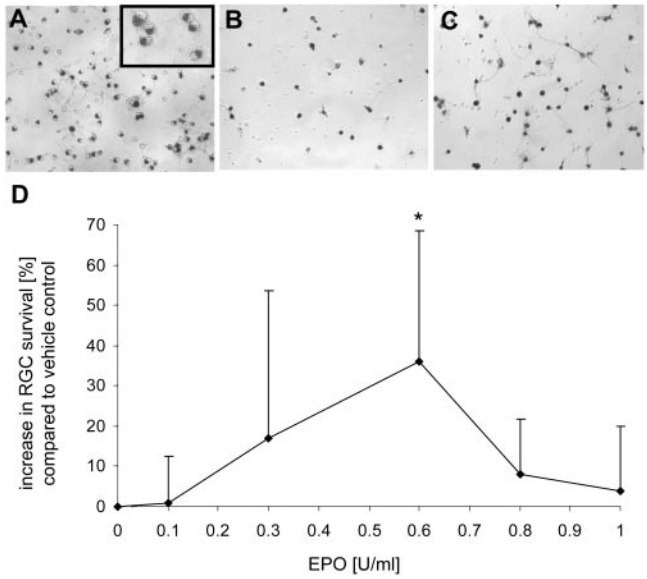

FIGURE 3. EPO promotes survival of immunopurified rat RGCs after neurotrophic factor deprivation. RGCs from 6- to 8-day-old rat pups were immunopurified and cultured under full neurotrophic support for 24 hours before neurotrophins were withdrawn. At the same time, different concentrations of EPO or vehicle were added, and the number of surviving RGCs was determined by MTT staining, after an additional 2 days in vitro. (**A**) Immunopurified RGCs cultured under full neurotrophic support for 24 hours; (*inset*) high-power magnification. (**B**) Vehicle-treated RGCs after two additional days of neurotrophic factor deprivation. (**C**) neurotrophic factor deprivation and treatment with EPO (0.6 U/mL) for 2 days. (**D**) Bell-shaped dose–response curve showing survival of RGCs compared with the vehicle-treated control after EPO treatment. $P < 0.05$ compared with 0, 0.1, and 1.0 U/mL.

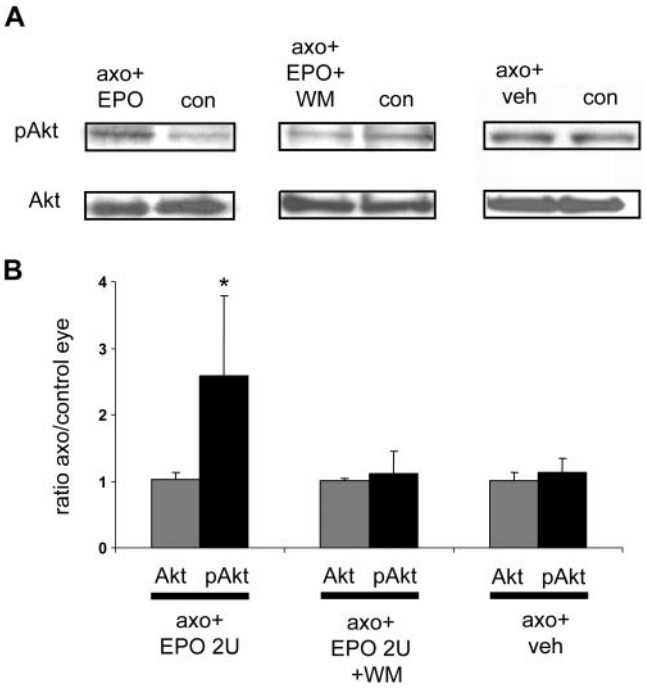

FIGURE 4. Retinal Akt activation in vivo after intravitreal injection of neuroprotective EPO doses. Axotomy and injection of EPO at the neuroprotective dose of 2 U/eye (on days 0 and 4 after axotomy) resulted in a clearly enhanced Akt phosphorylation (pAkt) compared with unlesioned control retinas on day 4 after axotomy (**A**; *top left*), which could be blocked by additional injection of the PI-3-kinase inhibitor WM (*top middle*). Axotomy and vehicle treatment alone did not result in a significant change of pAkt levels in retinal protein lysates when compared with unlesioned control eyes (*top right*). Total Akt levels remained constant under all three experimental conditions (*bottom panels*). (**B**) Densitometric analysis of at least four different experiments. Ratios of arbitrary densitometric units of total Akt and pAkt (pAkt) were calculated between axotomized and vehicle/EPO/WM-treated eyes, and corresponding contralateral control eyes. (▨) Ratios of total Akt levels; (■) ratios of pAkt levels. $^*P < 0.05$ when compared with the total Akt level.

were counted after axotomy and vehicle injection, EPO treatment significantly increased RGC survival to 872 ± 243.5 surviving RGCs/mm^2. Under combined injection with EPO and WM, the number of surviving RGCs was reduced to 423 ± 34.9 surviving RGCs/mm^2 (Fig. 5; Table 1). This experiment suggested that most, if not all, survival promoting EPO effects were conveyed by activation of the PI-3-kinase/Akt signal-transduction pathway.

EPO PKB/Akt–Dependent Suppression of the Activation of Caspase-3

Caspase-3 is one of the main cysteine proteases involved the execution phase of programmed cell death. Its activation, an important hallmark of apoptosis, has been shown in RGCs after transection of the ON.[47] To confirm further the neuroprotective potential of EPO and the predominant role of the PI-3-kinase/Akt pathway for mediating neuroprotective EPO effects in this paradigm, we examined caspase-3 cleavage after intravitreal EPO treatment. Using an antibody that recognizes specifically the active p20 fragment of caspase-3, we performed immunostaining of retinal sections of axotomized or unlesioned eyes. To obtain comparable sections for quantification of immunopositive RGCs, we prepared 16-μm cryostat sections with a maximum distance of 1.5 mm from the optic disc from at least three different animals per experimental group.

We found 2.92 ± 1.03 (SD) intensively stained cells per section in the ganglion cell layer 4 days after ON transection (n = 13; mean ± SD; Fig. 6), but no positive immunostaining in control retinas (data not shown). This confirms previous findings of approximately three RGCs per retinal section that are immunopositive for an antibody raised against the active fragment of caspase-3 4 days after ON transection.[37]

Injection of 2 U EPO on days 0 and 4 after axotomy reduced the number of cells immunoreactive for the p20 fragment of caspase-3 by 47% to 1.54 ± 0.97 per section (n = 19; $P < 0.01$; Fig. 6B). Further supporting the notion that activation of Akt is the main upstream mediator of the antiapoptotic EPO effect in this model, coapplication of WM, together with EPO completely abolished this effect. In the EPO/WM-treated experimental group, 3.91 ± 2.08 (n = 12) cells immunopositive for activated caspase-3 were found per section in the ganglion cell layer (Fig. 6B). Injection of WM alone did not significantly change the number of RGCs immunopositive for activated caspase-3 4 days after ON transection (Fig. 6B). Retrograde labeling of RGCs by a fluorescent tracer (Fluorogold) demonstrated that only RGCs, but not amacrine cells that are also present in the RGC layer, became immunopositive for caspase-3 after axotomy (not shown).

DISCUSSION

The present study described EPO neuroprotection of axotomized rat RGCs and characterized the intracellular signaling pathway responsible for transmitting these effects.

EPO Receptor Expression by Axotomized and Control Rat RGCs In Vivo

First, we studied the expression of EPO receptors within the rat retina of Sprague-Dawley rats. We found constitutive expression of EPORs on both somata and dendrites of almost all RGCs, which are the cell type specifically and quantitatively affected by the axotomy caused by ON transection. EPOR immunoreactivity was also found at the margins of the inner nuclear layer and the photoreceptor layer, albeit to a much lesser extent. The finding that EPORs were expressed in the brain and were even found to be expressed by neurons was the first hint that the role of EPO could be expanded beyond its function as a hematopoietic factor, as was identified a few years ago.[3–7] The fact that we found robust EPOR expression in control rat RGCs, using both immunohistochemistry and immunoblot techniques, contrasts with previously reported immunohistochemical data describing only minimal EPOR ex-

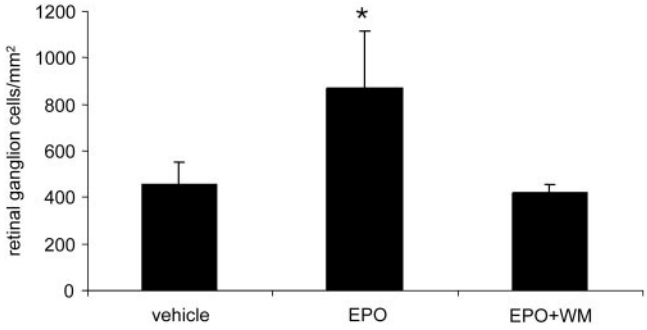

FIGURE 5. WM blocked neuroprotective effects of EPO in vivo after axotomy. Additional injection of 2 μL of 0.1 mM WM on each injection day (day 0, 4, 7, and 10 after axotomy) completely abolished neuroprotection by the most efficient EPO dose in the ON transection paradigm (intravitreal injection of 2 U EPO on each injection day). Mean ± SD $^*P < 0.05$.

TABLE 1. Summary of In Vivo RGC Counts

Experimental Group	Animal	RGCs/mm^2	Mean	SD	SEM
Axotomy+vehicle injection	1	586			
	2	468			
	3	389			
	4	379	455	95.7	47.8
Axotomy+injection of 0.5 U EPO/injection	1	757			
	2	585	671	122.1	86.3
Axotomy+injection of 1 U EPO/injection	1	756			
	2	721			
	3	816			
	4	796	772	42.2	21.8
Axotomy+injection of 2 U EPO/injection	1	1072			
	2	869			
	3	544			
	4	736			
	5	1139	872	243.5	108.7
Axotomy+injection of 4 U EPO/injection	1	571			
	2	412			
	3	488	490	79.5	45.9
Axotomy+injection of 8 U EPO/injection	1	424			
	2	308			
	3	424	386	66.5	38.4
Axotomy+injection of 2 U EPO/injection+WM 0.1 mM	1	411			
	2	469			
	3	385			
	4	427	423	34.9	17.4

pression by normal mouse RGCs,[10] but is in accordance with another study that also reported strong EPOR protein expression by rat RGCs.[2] Therefore, although a quantitative comparison between immunohistochemical data of different studies is hardly possible, levels of EPO expression in unlesioned eyes may vary in specific cell types between species or may even be strain dependent.

An increase in neuronal and glial EPOR expression has been shown to be induced by ischemia or hypoxia, suggesting a possible role for endogenous EPO in the retina's and brain's response to injury.[10,11,27,48] However, in our model representing subacute to chronic neuronal cell death, we did not detect an upregulation of EPOR protein expression in our quantification of immunoblots and evaluation of immunohistochemical stainings. We hypothesize that upregulation of EPOR expression upon a lesion is not an obligate observation, but may depend on the type or acuteness of the insult. In addition, the substantial EPOR expression that was found in RGCs of control animals could have prevented further upregulation of EPOR protein expression.

After axotomy and treatment with EPO, we found a trend toward increased EPOR expression, using doses of both 2 and 8 U per eye. Although these differences did not reach statistical significance, we can at least conclude from this finding that EPO application does not lead to a compensatory downregulation of EPO receptors by RGCs, as it can be found in several other receptor/ligand systems.[49]

Neuroprotective EPO Effects after Axotomy or Trophic Factor Deprivation of Rat RGCs

As we found EPOR to be expressed by unlesioned RGCs and also at least 1 week after axotomy, we investigated possible neuroprotective effects of EPO on the survival of RGCs. To date, EPO has been shown to protect neurons in several settings of neuronal cell death and to reduce apoptosis. However, most of these paradigms, such as in vitro oxygen-glucose deprivation,[27] experimental retinal and cerebral ischemia,[11–13] spinal cord ischemia,[24] or even human stroke,[14] relate to ischemic or hypoxic conditions in which predominantly necrotic cell death is observed. As an exception, one study characterizes EPO as a protecting factor against light-induced apoptosis in photoreceptors,[10] and another report showed EPO-mediated neuroprotection against death of neonatal motoneurons after sciatic nerve transection (i.e., peripheral nervous system lesion).[50] We demonstrate profound EPO neuroprotection against axotomy-induced death of CNS neurons, using a chronic in vivo cell death paradigm with exclusively apoptotic features. Furthermore, our model replicates important steps in glaucoma's pathologic course, as axonal lesions resulting from increased ocular tension are thought to induce retrograde RGC death and subsequent vision loss,[51–53] although care should be taken when applying the results of the ON transection model to human glaucoma, because this model, in contrast to other animal models that have been described for this disease, also lacks important aspects of glaucoma (e.g., elevation of intraocular pressure).[53]

Moreover, in our study EPO protected trophic-factor–deprived, immunopurified RGCs in culture, excluding secondary effects of cell types other than neurons. The relatively mild effect in this in vitro model system may have occurred because EPO was added directly after, not before, neurotrophic factor deprivation, as in other in vitro studies.

These data support the idea that EPO could be beneficial against RGC death in the context of glaucoma, as well as in traumatic brain injury or spinal cord lesion. Moreover, investigating the effectiveness of EPO in settings of apoptotic and chronic degeneration of CNS neurons, addresses the question of whether EPO, besides its potential to ameliorate the se-

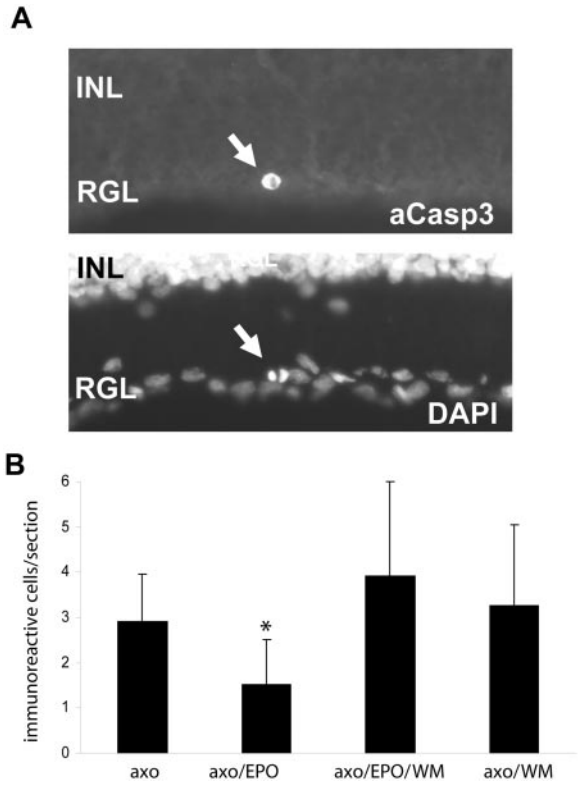

FIGURE 6. EPO PKB/Akt dependently suppressed activation of caspase-3 in axotomized RGCs in vivo. (**A**) Example of an RGC (*arrows*) regarded as immunoreactive for activated (cleaved) caspase-3 (aCasp-3) in the RGC layer 4 days after axotomy (*top*). DAPI staining (*bottom*) shows the condensed and fragmented nucleus of the same cell. (**B**) Quantification of the number of RGCs per retinal section that were immunopositive for activated caspase-3. EPO treatment (axo/EPO) significantly reduced caspase-3 cleavage when compared with axotomy alone. This protection was abolished by coadministration of WM (axo/EPO/WM). WM had no significant influence on the number of immunoreactive RGC when injected alone after ON transection (axo/WM). At least 12 sections obtained from at least three different animals per experimental group. $^*P < 0{,}01$. RGL, RGC layer; INL, inner nuclear layer.

quelae of acute ischemic stroke, might be used for the treatment of chronic neurodegenerative diseases, such Parkinson's or Alzheimer's disease or amyotrophic lateral sclerosis.

As others have found in various cell culture models of neuronal cell death,[12,54] the survival-promoting action of EPO followed a bell-shaped dose–response curve in our in vitro paradigm of immunopurified RGC culture. Increasing EPO concentration in the cell culture medium above an optimal dose resulted in a decline of the neuronal rescue rate, which finally returned to control values. However, we did not observe toxic effects, even at the highest concentrations. We were then able to confirm this particular dose–response behavior of EPO in vivo, which might have relevance for the design of upcoming clinical trials. EPO's effects in clinical studies, as in the recent human stroke study that demonstrated beneficial effects in the context of cerebral ischemia,[14] may be further improved by an optimized dose regimen in the future.

Overall, although EPOR protein was expressed at least by most RGCs, not all RGCs were protected from apoptosis. We hypothesize that this may be because neuroprotective EPO signal transduction relied on only one intracellular kinase pathway in our paradigm: the PI-3-kinase/Akt pathway. In addition, in our model, RGC axons remain transected, and this sustained proapoptotic stimulus could account for the reduced, but still significant RGC death, despite treatment with EPO. Similarly, other neuroprotectants (e.g., BDNF, NT, IGF-I, GDNF, caspase- or CDK5-inhibitors) show only partial rescue rates after ON transection.[31,35–38,42] Although EPO was not as effective as BDNF, NT-4, or caspase-3 inhibitor, it is important to note that this neuroprotective molecule, which is already routinely used in non-neurologic or non-ophthalmologic clinical settings, conferred better neuroprotection for RGCs than NT-3, GDNF, IGF-I or caspase-9 inhibitor 14 days after ON transection.[31,35,36,38,42]

Neuroprotective EPO Signal Transduction in Axotomized Rat RGCs In Vivo

Regarding intracellular transmission of EPO's effects, several signal transduction pathways have been shown to be involved in EPO signaling, depending on the paradigm and biological function of EPO. The best characterized among them are the PI-3-kinase/Akt pathway, which transduces EPO survival signaling in several in vitro systems, and the ERK1/2 kinase pathway, which has also been shown to contribute to EPO survival signaling, both in neurons and in erythrocyte progenitors.[12,54,55] Recently, crosstalk between Jak2 and NF-κB signaling pathways has been shown to be necessary for EPO protection against excitotoxin- and NO-induced apoptosis of cultured cerebrocortical neurons.[23] However, regarding the in vivo situation in the retina and brain, information about signal transduction of antiapoptotic EPO effects is very limited. In our paradigm, we found no evidence for an involvement of the ERK1/2 pathway in EPO signal transduction. Rather, it seemed the PI-3-kinase/Akt pathway was solely responsible for the survival-promoting effect of EPO. This finding is consistent with the fact that, in this in vivo model, the ERK1/2 pathway did not contribute to intracellular antiapoptotic signaling of other neurotrophins. BDNF, for instance, resulted in a robust phosphorylation of ERK1/2 in rat retinas, but pharmacologic inhibition of this kinase pathway did not impair BDNF-induced protection of RGCs after ON transection.[34] In contrast, the PI-3-kinase/Akt mediated neuroprotective action of BDNF and IGF-I in axotomized RGCs in vivo.[34,35]

In general, the relevance of the ERK1/2 kinase pathway in cell survival has remained ambiguous, as several studies have either failed to demonstrate a role for this pathway in cell survival, or have even shown death-promoting effects.[35,56–58] In neurons, the relevance of this kinase cascade is better established in the context of neurite extension and differentiation.[59–64] Both the ERK1/2 and the PI-3-kinase/Akt pathway are also involved in intracellular EPO signaling in erythrocyte progenitors. Also, in this non-neuronal cell type, ERK1/2 activation is more important for proliferation and differentiation of these progenitor cells, whereas PI-3-kinase/Akt seems to transmit predominantly prosurvival signals.[65–67]

Effect of EPO Treatment on Caspase-3 Activation after Axotomy of RGCs

Caspase-3 is activated in the ON transection model, and inhibition of this protease exerts profound protective effects after ON protection.[33] In line with the results obtained with IGF-I and BDNF, which reduce caspase-3 cleavage by Akt activation,[34,35] EPO administration diminished the number of RGCs immunopositive for the active cleavage product of procaspase-3. The PI-3-kinase inhibitor WM completely blocked this effect, further supporting an at least predominant role of the PI-3-kinase/Akt pathway in RGC survival in our model.

CONCLUSION

In summary, in our study, EPO was neuroprotective in an in vivo model replicating important pathophysiological features

of glaucoma and chronic neurodegeneration with solely apoptosis-like cell death, and the results outline the signal-transduction pathway transmitting this effect. Our data provide evidence that EPO could be useful in the treatment of glaucoma or neurodegenerative diseases such as Parkinson's disease or amyotrophic lateral sclerosis.

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