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Neuroprotective Role of Erythropoietin by Antiapoptosis in the Retina

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Abstract

Erythropoietin (EPO) stimulates red blood cell production, in part by inhibiting apoptosis of the red blood cell precursors. The erythropoietic effects of EPO are circadian stage dependent. Retinal injury due to light occurs through oxidative mechanisms and is manifest by retinal and retinal pigment epithelium (RPE) cells apoptosis. The visual cycle might be circadian coordinated as a means of effectively protecting the retina from the detrimental effects of light-induced, oxygen-dependent, free radical-mediated damage, especially at the times of day when light is more intense. We show that the retinal expression of EPO and its receptor (EPOR), as well as subsequent Janus kinase 2 (Jak2) phosphorylations, are each tightly linked to a specific time after oxidative stress and in anticipation of daily light onset. This is consistent with physiological protection against daily light-induced, oxidatively mediated retinal apoptosis. In vitro, we verify that EPO protects RPE cells from light, hyperoxia, and hydrogen peroxide-induced retinal cell apoptosis, and that these stimuli increase EPO and EPOR expression in cultured RPE cells. Together, these data support the premise that EPO and its EPOR interactions represent an important retinal shield from physiologic and pathologic light-induced oxidative injury.

Keywords

erythropoietin; retina; neuroprotection; retinal pigment epithelium; oxidative stress

Apoptosis is the primary mechanism of abnormal cell death of photoreceptors, retinal ganglion cells (RGC), or retinal pigment epithelium (RPE) cells in degenerative retinal diseases such as age-related macular degeneration, retinitis pigmentosa, or glaucoma, and can be triggered by even moderate oxidation (Grimm et al., 2000; Zarbin, 2004; Wenzel et al., 2005; Zhong et al., 2007). Biochemical reactions of light–electrical energy conversion in

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the retina and the RPE require increased metabolism to satisfy the energy demands. As such, the eye needs increased levels of oxygen, especially when the eye is continuously exposed to light. Light-induced degeneration of photoreceptors depends on rhodopsin bleaching and phototransduction (Wenzel et al., 2005; Grimm et al., 2000). Light insults result in increased production of H₂O₂ in the outer retina, and the production of reactive oxygen species, including free radicals, has been shown to be involved as an early event in light-induced retinal degeneration (Yamashita et al., 1992; Ranchon et al., 2003).

The RPE is also exposed to an oxidative environment as a result of its high oxygen tensions of 70–90 mm Hg, a high metabolic rate, and accumulation of lipofuscin. Moreover, continuous exposure to light causes RPE cells to consume a large amount of oxygen in order to complete the complex processes in the visual cycle, nutrient transport, and phagocytosis. These processes put the RPE into a relatively hypoxic condition with high oxygen demand. In response to light triggers, neuroprotective proteins provide an antiapoptotic effect within the eye. The mechanisms to regulate the expression of these retinal proteins that protect against light-induced oxidative stress remain elusive.

Erythropoietin (EPO) is an oxygen-regulated glycoprotein and a hematopoietic cytokine that stimulates the proliferation, survival, and differentiation of erythroid progenitor cells. It is known to prevent apoptosis and protect neuronal cells from neuronal damage, including experimental central nervous system models of hypoxic and ischemic insults (Jelkmann and Metzen, 1996; Jelkmann, 2005). EPO in the brain and the kidney is regulated by oxygen-dependent mechanisms. These data are derived from models such as traumatic brain injury (Brines et al., 2000), spinal cord injury (Celik et al., 2002), Parkinson's disease (Kanaan et al., 2006), excitotoxicity (Kawakami et al., 2001; Morishita et al., 1997), oxidative stress (Calapai et al., 2000), and chemical neurotoxicity (Genc et al., 2001). EPO can protect RGC from degeneration induced by acute ischemia–reperfusion injury (Liu et al., 2006) and axotomy injury (Weishaupt et al., 2004), and promote survival of RGCs in glaucoma mouse models (Zhong et al., 2007). EPO is also neuroregenerative and stimulates neurogenesis and poststroke recovery (Tsai et al., 2006). In vitro studies have reported that EPO stimulates neuritic outgrowth by postnatal (Böcker-Meffert et al., 2002) and adult RGCs (Kretz et al., 2005). EPO is known to be produced in the retina in response to acute hypoxia via hypoxia inducible factor-1 α (HIF-1 α) stabilization, which confers protection from light-induced retinal degeneration (Grimm et al., 2002). The specific role of EPO was highlighted because only EPO gene expression was significantly affected among the various angiogenic factors in a HIF-1 α -like factor knockdown model (Morita et al., 2003).

We hypothesize that light-reactive oxygen species, and hypoxic and hyperoxic conditions may regulate the protection mechanisms in retinal and RPE cells through EPO by antiapoptosis. The neuroprotective effect of EPO has been documented in cell cultures and in vivo; however, its effect on primary retinal cell cultures was not reported, including concurrent detailed molecular events. Moreover, to our knowledge, the effect of various oxidative environment conditions such as hypoxia, hyperoxia, bright light, or hydrogen peroxide has never been studied or compared in retinal cell cultures or human RPE cell culture systems. In the present study, we investigate whether EPO and EPO receptor (EPOR) levels might be regulated by time of day and light conditions in vivo. The antiapoptotic

effect of EPO was further compared in the primary rat retinal culture and low-passage human RPE culture under oxidative stress conditions.

MATERIALS AND METHODS

In Vivo Mice Study

Female C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) at 4–5 weeks of age and acclimatized in the animal laboratory. They were housed four per cage and were maintained on food and water ad libitum at a constant temperature with a 12 hr dark/12 hr light regimen (300 lux at cage level). At 15 weeks, the mice were humanely killed, and the left and right eye cups were collected every 4 hr for 24 hr. Eye-cup samples were snap-frozen in liquid nitrogen and stored at -80°C for molecular studies. Red lights were used during the dark periods to facilitate human movements to which mice are insensitive. All animal experiments were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

Protease inhibitor cocktail (Sigma) was used to inhibit nonspecific protein degradation. After removing vitreous and the lens, the dissolved proteins in lysis buffer were centrifuged at $10,500g$ for 1 hr and dialyzed at 4°C against Tris-HCl (10 mM, pH 8.0), EDTA (1 mM), DTT (2 mM), and Triton X-100 (0.1%) after concentrating the sample by Centriprep (Millipore Corp., Bedford, MA). Protein concentration was determined by the Bradford assay. After protein separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to polyvinylidene difluoride (PVDF) membrane for 30 min at 20 V with Tris–glycine buffer (25 mM Tris, 192 mM glycine) containing 20% methanol. The membrane was blocked with 5% nonfat dried milk with Tris-buffered saline Tween-20 (TBST) for 1 hr at room temperature. The membrane was incubated with polyclonal anti-EPO (1:500, R&D Systems) or monoclonal anti-EPOR antibody (1:1,000, R&D Systems) overnight at 4°C and then incubated with secondary antibody linked to horseradish peroxidase. The enhanced chemiluminescence system (GE Healthcare, Piscataway, NJ) was used to visualize the target proteins.

Primary Retinal Cell Culture

Newborn Sprague Dawley rats were handled in compliance with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. Primary cell cultures including neurons, astrocytes, and photoreceptor cells were generated from the retinas of newborn (postnatal day 1 or 2) Sprague Dawley rats as described earlier (Chung et al., 2007). Briefly, retinas were isolated, placed in Hanks' balanced salt solution (Gibco, Rockville, MD) lacking Ca^{2+} and Mg^{2+} , and mechanically dissociated into single cells by triturating with 1,000- μl pipettes. Dissociated cells were placed on poly-L-lysine-coated 24-well or 6-well plates (three retinas per plate). The plating medium was based on Eagle's minimum essential medium (Gibco, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 25 mM KCl. Retinal cultures were maintained at 37°C in a humidified 5% CO_2 incubator and used in experiments after 10 days' culture in vitro.

Human RPE Cell Culture

According to a method described earlier (Hyun et al., 2001), eyes were opened 360° posterior to the ora serrata, and the vitreous and the retinal tissues were removed. They were used in accordance with applicable laws and with the tenets of the Declaration of Helsinki. The remaining eye cups were rinsed with phosphate-buffered saline (PBS) and incubated in 0.25% trypsin in Dulbecco's minimum essential medium (DMEM; Gibco, Grand Island, NY) for 30 min at 37°C. After trypsinization, human RPE cells were placed in DMEM supplemented with 10% FBS and triturated into single cells with gentle pipetting. Cells were transferred to tissue culture flasks (Nunc, Roskilde, Denmark) containing DMEM supplemented with 20% FBS and placed in a 5% CO₂ incubator at 37°C. After proliferation, cells were retransfused with a 0.1% trypsin-EDTA solution (Sigma, St. Louis, MO) for 5 min at 37°C. After triple washes with DMEM, cells were plated onto 24- or 6-well plates (Nunc) at 2×10^4 or 8×10^4 cells/well and allowed to grow to confluence for 2–4 days. Third- or fourth-passage cells were used for experiments. To test protein expression in hypoxic and hyperoxic conditions, the cells were incubated at 37°C in an incubator containing either humidified 5% carbon dioxide and 99% nitrogen (1% O₂) or 5% carbon dioxide and 50% nitrogen (50% O₂).

Assessment of Cell Survival and Death

Survival of retinal cells was quantitatively assessed by using a cell proliferation assay kit (CyQuant NF Cell Proliferation Assay Kit, Molecular Probes, Eugene, OR) according to the manufacturer's protocol. This assay is based on the measurement of cellular DNA contents via fluorescent dye binding. The extent of proliferation was determined by comparing cell counts for samples exposed to various conditions such as hypoxia, hyperoxia, or light with untreated sham-washed controls. Measurement of the fluorescent intensity was done with a fluorescence microplate reader (SpectraMax, Molecular Devices, Union City, CA) with excitation at ~485 nm and emission detection at ~530 nm. Lactate dehydrogenase (LDH) activity in the medium was estimated with an automated microplate reader (UVmax; Molecular Devices, San Francisco, CA) by measuring the rate of decrease in absorbance at 340 nm. All LDH values, after subtraction of background value in sham wash control cultures, were normalized to the mean maximal value (100) in sister cultures exposed for 24 hr to 5 mM H₂O₂, which causes complete cell death. Hoechst 33258 (Molecular Probes, Leiden, Netherlands) staining was performed to determine the effects of EPO on retinal cell death induced by H₂O₂. After rinsing with PBS, the cells were stained for 2 min with 2 µg/ml Hoechst 33258 in PBS and examined with a fluorescence microscope (IX71; Olympus, Tokyo, Japan). Cells with condensed nuclei were considered dead; the others were classified as being alive.

Immunoblots for EPO, EPOR, Hif-1α, Thioredoxin, Hemoxygenase-1, p-Jak2, Cleaved Caspase-3, c-fos, BCL-xL, Rhodopsin, or RPE65

After washing with serum-free medium, cells were suspended in lysis buffer (120 µl per well) containing 20 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 µM Na₃VO₄, 1 µg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride, and centrifuged. Equal amounts of proteins (30–50 µg,

50 μ l per each well) were loaded and electrophoresed on a 10% SDS-PAGE gel and then transferred to PVDF membrane (Millipore Corp., Bedford, MA). The membrane was blocked with 5% nonfat dried milk for 1 hr and incubated overnight at 4°C with the following antibodies: anti-EPO and EPOR, (EPOR, Santa Cruz, CA), anti-Hif-1 α , anti-thioredoxin (TRX), anti-hemoxygenase-1 (HO-1), anti-rhodopsin, anti-RPE65 (Abcam, Cambridge, UK), anti-p-Jak2 anti-cleaved casapse-3 (Cell Signaling, MA), anti-c-Fos, and anti-BCL-xl (Santa Cruz). The secondary antibody was a goat anti-rabbit or anti-mouse IgG (GE Healthcare, NJ), conjugated to horseradish peroxidase. The chemiluminescence substrate (Pierce Biotechnology, IL) was used to visualize immunoreactive proteins.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 1 hr at room temperature and permeabilized with 0.2% Triton X-100 for 10 min. After blocking with 2% bovine serum albumin, fixed cells were incubated overnight at 4°C with the following primary antibodies: anti-mitogen-activated protein kinase 2 (MAP-2; 1/500, Sigma, St. Louis, MO), or anti-glial fibrillary acid protein (GFAP; 1/1,000, Santa Cruz, CA), and anti-EPO or -EPOR (Santa Cruz, CA). The cultures were then treated with a fluorescence-conjugated secondary antibody (Alexa Fluor 488 donkey anti-mouse IgG (1:1,000), or 555 donkey anti-rabbit IgG, Molecular Probes, Leiden, Netherlands) for 2 hr at room temperature. For negative controls, cultures were treated only with a secondary antibody.

RESULTS

Light and Circadian Regulation and EPO/EPOR

The possible connection of EPO/EPOR regulation in relation to visual cycle protein via retinoid metabolism was investigated (Zimmermann et al., 2006). Along with increased levels of EPO after light exposure, the level of rhodopsin in retinal cells increased as early as 15 min after 5,000 lux light exposure in a time-dependent manner (Fig. 1A). Both RPE65 and RPE45, the truncated form of RPE65, increased after 15–30-min exposure to 5,000 lux light in RPE (Fig. 1B). An RPE65 defects is known to trigger a remodeling of the retina that disrupts photoreceptor homeostasis and induces an apoptosis cascade causing retinal degeneration. The in vivo erythropoietic effects of administered EPO is known to depend on the time of its administration (Wood et al., 1998). To investigate EPO regulation in vivo, retinas from mice subjected in vivo to normal 12 hr light (300 lux)–12 hr dark cycles were assessed for EPO and EPOR protein content from mice at six different time points over a single 24-hr period. During first 10 hr of the dark cycle (activity phase), mice retinal EPO was not detectable (Fig. 1C). However, 2 hr before the light returned (e.g., 22 hr clock time), retinal EPO was detectable. Retinal EPO continued to increase for 2 hr after the light turned on and gradually declined and became undetectable in the late light phase (10 hr clock time). The 24-hr pattern of retinal EPOR followed a similar pattern to EPO, with levels beginning to increase at 22 hr in the dark phase, peaking at 2 hr in the light phase, and then declining over the later part of the light cycle. This suggests that the endogenous circadian clock may regulate EPO and EPOR levels such that they increase just before light onset in anticipation of the daily light period.

EPO Neuroprotection in H₂O₂-treated Retina Cells

We performed a cell viability assay by using retina and RPE cell cultures treated with H₂O₂ as an oxidative stress model to study the neuroprotective role of EPO in vitro. At concentration of 20 μ M or higher for 3 hr, H₂O₂ exerted significant toxicity to retinal cell cultures (Fig. 2A,B). H₂O₂-induced retinal cell death was reduced to 40, 46, or 43%, respectively, by 30 min pre-treatment of 50 U EPO (unit/ml) with 40 μ M H₂O₂ for 6, 12, and 24 hr (Fig. 2C,D). In contrast, only a mild protective effect (15%) was observed in the RPE exposed to H₂O₂ by EPO treatment in 24 hr (data not shown). Cell viability was assessed by amount of LDH release measured from the medium of RPE cultures exposed to the indicated concentrations of H₂O₂ for 3, 6, 12, and 24 hr (Fig. 2E).

Up-regulation of EPO/EPOR in Hypoxic, Hyperoxic, or Light-exposure Condition

We tested the hypothesis that perturbed oxygen homeostasis, including hypoxia, hyperoxia, and excessive reactive oxygen species, may induce neuroprotective responses in the retina and the RPE. To test whether EPO and EPOR are up-regulated in these conditions, retina and human RPE cells were exposed to 1% O₂, 50% O₂, or 5,000 lux light. In retinal and RPE cells, cells appeared morphologically viable, and no cell death was noticed after exposure to 1 or 50% O₂ until 6 hr (data not shown). Percentage intensity of DNA fluorescence confirmed that no significant cell death occurred in these conditions (Figs. 3A,D and 4A,C). However, cytotoxicity of retinal cells was seen in 15 min in bright light exposure (5,000 lux), whereas no significant cell death occurred with exposure to light for 60 min in RPE cells (Figs. 3F and 4E). Immunoblots for EPO and EPOR of retinal cells showed that expression of EPO and EPOR were increased as early as 1 hr after exposure to hypoxia, and continued to increase until 6 hr (Fig. 3B). Up-regulation of EPOR seemed to be more prominent than the increase of EPO. Similar findings were seen in RPE cells after 3 hr exposure to hypoxia (Fig. 4B). Immunocytochemistry of MAP-2 (neuronal marker) or GFAP (astrocyte marker) and EPO/EPOR antibody in sham-washed retinal cells showed that EPO and EPOR were not limited to either neurons or astrocytes, but variable amounts were present in both cell types. EPO and EPOR had a similar pattern of distribution in both neurons and astrocytes. Three hours after exposure to hypoxia, EPO and EPOR increased in neurons and astrocytes (Fig. 3C). In hyperoxic condition, EPO and EPOR expression was up-regulated in 1–3 hr in retinal and RPE cells (Figs. 3E and 4D). Increase of EPOR was more prominent than that of EPO seen in hypoxic conditions. As early as 15 min after light exposure in retinal and RPE cells, EPO expression increased until 60 min. In the light, EPOR expression was also increased (Figs. 3G and 4F).

Upstream and Downstream Regulators of EPO in the Retina and the RPE Cell Culture

Increased expressions of Hif-1 α in hypoxia/hyperoxia in the retina and the RPE cells (Fig. 5A,B) were observed after 1–3 hr exposure. However, light exposure does not seem to induce up-regulation of Hif-1 α in both retinal and RPE cells (data not shown). Instead, up-regulation of TRX and heme oxygenase-1 (HO-1) in RPE cells exposed to 5,000 lux light was seen after 15 min (Fig. 5C). HO-1 was chosen as a positive marker of oxidative stress in the retina and the RPE cells. HO-1 exerts a protective or adaptive effect in the context of oxidative stress or oxygen toxicity. For example, HO-1 is an inducible mechanism for

protection against hypoxic lung injury in vivo (Zhang et al., 2004). The level of p-Jak2 in retinal and RPE cells increased in hypoxia, hyperoxia, or light exposure, as depicted in Figures 6A,B. Until 30 min of light exposure, the level of cleaved caspase-3 in retinal cells was not changed, indicating that endogenous neuroprotective or antiapoptotic proteins including EPO/EPOR might be affording protection. At 60 min, increased level of cleaved caspase-3 with significant cell death suggested that neuroprotective factors in the cell were insufficient to prevent acute neuronal injury (Fig. 6C). To understand apoptotic pathway in this condition, expression of other antiapoptotic proteins were investigated. Immunoblots for BCL-x1 and c-fos showed that neuroprotective effects of EPO may involve up-regulation of these early markers in stress-induced condition (Fig. 6D,E). As shown here, EPO-mediated neuroprotective effects may be mediated through Jak2, BCL-x1, and c-fos. Other downstream regulators such as Stat3 might be required for antiapoptotic BCL-x1 induction as shown in motor neurons (Schweizer et al., 2002).

DISCUSSION

Our data demonstrate that the circadian organization of retinal EPO and EPOR elaboration, anticipating daily light onset, is a potentially effective endogenous physiologic strategy for retinal protection from light-induced, oxygen-mediated damage. These findings are consistent with documented circadian organization of the full range of oxidative damage protecting enzyme systems in a number of tissues (Hardeland et al., 2003). The glutathione system is among the most important and ubiquitous oxidation protectors. The reduced to oxidized glutathione ratio (GSH/GSSG) is tightly coordinated each day in all organ systems where oxidative damage must be limited. In the heart, these systems peak during the activity phase each day, when cardiac function is most active and oxygen consumption is greatest (Scheving et al., 1988). A sevenfold circadian difference in the GSH/GSSG ratio characterizes nucleated human bone marrow cells. The physiologic function of the human bone marrow is red and white blood cell precursor proliferation to replace blood cells. DNA synthesis (S) phase of the cell cycle is the most sensitive cell cycle phase to oxidative DNA damage (Hrushesky, 1985). Human bone marrow DNA synthesis is circadian clocked to the end of the daily sleep and beginning of the daily activity phase (Mauer, 1965; Smaaland et al., 1991). The GSH/GSSG ratio is highest at this time of day, when most DNA synthesis is ongoing; it is virtually seven times higher than 12 hr later in the day (Smaaland et al., 1992). Additionally, oxidative damage to human bone marrow by doxorubicin is abrogated when this agent is given in the early morning, when these defenses are most robust (Bellamy et al., 1988). Our circadian data indicate that retinal protection from light is also coordinated each day to anticipate daily light onset and that EPO and EPOR expression anticipate daily light.

In vitro, it is shown that treatment with EPO significantly increased the viability of rat retina and human RPE cells exposed to oxidative stress. When under oxidative stress, up-regulation of EPO confers a neuroprotective function against retinal degeneration. Our aim was to explicate the role of the light and oxidative stress in the control of neuroprotective protein expression in the retina and the RPE. Our questions include whether EPO can protect retina and RPE cells against oxidative- or light-induced apoptotic neurodegeneration. Susceptibility of light-induced retina degeneration was known to be directly associated with regeneration of 11-*cis* retinoid and phototransduction pathway. Light initiates vertebrate

vision to isomerize 11-*cis*-retinyl imine chromophore of rhodopsin to all-trans isomer. In the photoreceptor and the RPE, 11-*cis*-retinal is regenerated through the visual cycle for continued vision. The photoisomerization signal passes through the G protein-coupled receptor (GPCR) pathway to activate photoreceptor cells that are hyperpolarized. Two different apoptotic pathways, GPCR dependent and independent, were reported (Wenzel et al., 2005). Light-induced retinal degeneration in an animal model occurs only when the visual cycle is functional. A study of RPE65 knockout mice showed that light damage only occurs when the retina is supplied with 11-*cis* retinal, the opsin chromophore (Wenzel et al., 2005). Additional evidence, including RPE65 L450M mice showing slow rhodopsin regeneration, halothane anesthesia as inhibition method of 11-*cis*-retinal regeneration, and 13-*cis*-retinoic acid as a putative RPE65 inhibitor, imply that continuous regeneration of 11-*cis*-retinal is one of the key steps to induce retina degeneration (Wenzel et al., 2005). Light-induced cone/rod degeneration proceeds in biochemical reactions, leading to cellular apoptosis of the RPE. During photon absorption, light-induced toxic oxidative radicals are generated and concentrated in the retina. These damaged membrane segments are removed by RPE phagocytosis. To maintain membrane integrity of the outer segment, RPE supplies oxygen, secreted proteins, lipids, and retinoid. Thus, survival of the RPE is a prerequisite to maintain the functional retina. It should be noted that RPE cells seem to have higher expression of EPOR compared with the retina. Our data showed that rhodopsin and RPE65 are also up-regulated in the light in a parallel fashion to EPO induction.

Mouse studies have shown that increased EPO can protect the retina against light-induced damage after the retina is exposed to a hypoxic environment (Grimm et al., 2002). This response to ischemic preconditioning is triggered by Hif-1, which targets EPO. As a downstream pathway of EPO signaling, we observed up-regulation of BCL-xl and c-fos at an early time point in the retina and the RPE cells exposed to light. Phosphorylation of Jak2 was increased as a downstream mediator of EPO.

Müller cells in mixed retinal cell cultures also expressed EPOR, as shown by colocalization with GFAP in immunocytochemistry. Müller cell expression of EPOR further supports a protective role of glial cells toward neuronal injury because these cells provide structural and nutritional support for the survival of neurons. In cerebral ischemia, a rapid and marked up-regulation of EPOR occurs, followed by an increase in local EPO production (Li et al., 2007). Our results demonstrate that EPO may act as an innate neuroprotecting molecule in the retina under abnormal oxygen conditions in hypoxia, in hyperoxia, and in the light. These conditions are considered to be the most harmful risk factors to the retina in many neurodegenerative diseases. Furthermore, the EPO/EPOR system might play an important role in the protection of retinal neurons during the daytime or during highly oxidative stress such as continuous light exposure. Induction of EPO expression by light exposure in vitro may represent its functional role in the light. Along with the up-regulation of EPO, the activation of caspase-3 was suppressed, as shown in retinal cell cultures after intense light exposure. However, continuous exposure to light for 60 min was cytotoxic to retinal cells with induction of caspase-3 activity. This suggests that endogenous neuroprotective systems including EPO/EPOR may not be sufficient in such oxidative stress conditions. Originally, EPO was identified almost 30 years ago (Miyake et al., 1977) as the substance in red blood cell differentiation. New roles of EPO as a remarkable tissue protecting protein in the

nervous system are worth revisiting to find a new approach to treat neurodegenerative diseases (Brines and Cerami, 2005; Jelkmann, 2007).

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Fig. 1.

Light and circadian regulation of EPO/EPOR. Retinal cultures derived from rat or early passages of human RPE cells were exposed to light. Proteins were separated by SDS-PAGE and visualized by Western blot test. **A:** Rhodopsin was up-regulated in retinal cells after exposure to 5,000 lux light. **B:** RPE65 was increased after 15 min exposure to 5,000 lux light in the RPE. Truncation of RPE65 to 45 kDa was also seen at 30 min. **C:** Expression of EPO/EPOR in the 12 hr light/12 hr dark in vivo. Two hours' exposure to room light (300 lux) rapidly increased the expression of EPO. Continuous exposure to light subsequently decreased EPO. Expression of EPO was recovered after prolonged incubation in the dark.

Fig. 2.

Exogenous EPO treatment reduced cell death in H₂O₂-treated retina cells. **A:** Phase-contrast photomicrographs of sham-washed control or culture treated with 40 μM H₂O₂ for 12 hr. Marked cell death occurred in H₂O₂-treated culture. Scale bar = 30 μm. **B:** Amount of LDH release was measured from the medium of retinal cultures exposed to the indicated concentrations of H₂O₂ for 3, 6, 12, and 24 hr (mean ± SD, ★*P* < 0.05, *n* = 6). All LDH values, after subtraction of background value in sham-washed control cultures, were normalized to the mean maximal value (100) in sister cultures exposed for 24 hr to 5 mM H₂O₂, which causes complete cell death. **C:** Hoechst 33258 staining of control cells exposed to 40 μM H₂O₂, or cells treated to both H₂O₂ and EPO in retinal cultures (scale bar = 25

µm). **D:** Bars denote the percentage of LDH release in retinal cell cultures after 3, 6, 12, and 24 hr treatments with 40 µM H₂O₂ alone, or with the addition of EPO 50 U. At 6 hr or later, exogenous EPO showed significant cytoprotective effects of retina cells in oxidative stress.

E: Amount of LDH release was measured from the medium of RPE cultures exposed to the indicated concentrations of H₂O₂ for 3, 6, 12, and 24 hr (mean ± SD, ★*P* < 0.05, *n* = 6). All LDH values, after subtraction of background value in sham-washed control cultures, were normalized to the mean maximal value (100) in sister cultures exposed for 24 hr to 5 mM H₂O₂, which causes complete cell death.

Fig. 3. Up-regulation of EPO/EPOR expression in the retina exposed to hypoxia (1% O₂; A–C), hyperoxia (50% O₂; D,E), or light (5,000 lux; F,G). **A:** Data represent percentage intensity of DNA fluorescence that is proportional to the number of surviving cells by the cell proliferation assay kit (mean ± SD, ★*P* < 0.05, *n* = 6) of retinal cultures exposed to 1% O₂ for 1, 3, and 6 hr, respectively. No significant cell death was observed. **B:** Immunoblots for EPO and EPOR. As early as 1 hr after exposure to hypoxia, expressions of EPO and EPOR were both increased until 6 hr. Increase of EPOR appeared to be more prominent than EPO

up-regulation. **C:** Immunocytochemistry for cell markers of retina (MAP-2 or GFAP, green) and EPO or EPOR antibody (red) at 3 hr after exposure to hypoxia and sham-washed control. Expression of EPO/EPOR increased after exposure to hypoxia. Scale bar = 30 μ m. **D:** Data represent percentage intensity of DNA fluorescence that is proportional to the number of surviving cells (mean $\pm$ SD, $\star P < 0.05$, $n = 6$) of retinal cultures exposed to 50% O₂ for 1, 3, and 6 hr, respectively. No significant cell death was seen. **E:** Immunoblots for EPO and EPOR show that 3 hr after exposure to hyperoxia, EPO and EPOR expression increased. **F:** Data represent percentage intensity of DNA fluorescence that is proportional to the number of surviving cells (mean $\pm$ SD, $\star P < 0.05$, $n = 6$) of retinal cultures exposed to 5,000 lux light for 15, 30, and 60 min. At 15 min or longer exposure, significant cytotoxicity was observed. **G:** Immunoblots for EPO and EPOR show that EPO expression increased as early as 15 min after exposure to the light, until 60 min. Levels of EPOR also increased.

Fig. 4. Up-regulation of EPO/EPOR expression in the RPE exposed to hypoxia (1% O₂; A,B) hyperoxia (50% O₂; C,D), or light (5,000 lux; E,F). **A:** Data represent percentage intensity of DNA fluorescence that is proportional to the number of surviving cells (mean $\pm$ SD, $\star P < 0.05$, $n = 6$) of human RPE cell cultures exposed to 1% O₂ for 1, 3, and 6 hr. No significant cell death was seen. **B:** Immunoblots for EPO and EPOR show that 3 hr after exposure to hypoxia EPO/EPOR expression increased. **C:** Data represent percentage intensity of DNA fluorescence that is proportional to the number of surviving cells (mean $\pm$ SD, $\star P < 0.05$, $n = 6$) of RPE cell cultures exposed to hyperoxia for 1, 3, and 6 hr. No significant cell death was seen. **D:** At 3 hr after exposure to hyperoxia, EPO and EPOR expression increased. **E:**

Data represent percentage intensity of DNA fluorescence that is proportional to the number of surviving cells (mean $\pm$ SD, ★ $P < 0.05$, $n = 6$) of human RPE cell cultures exposed to 5,000 lux light for 15, 30, and 60 min. No significant cell death was seen. **F**: As early as 15 min after exposure to light, EPO/EPOR expression increased until 60 min.

Fig. 5.

Upstream regulators of EPO in the retina and the RPE. Retinal cultures derived from rat or early passages of human RPE cells were exposed to 1% O₂, 50% O₂, or 5,000 lux light. Proteins were separated by SDS-PAGE and visualized by Western blot test. **A:** Increased expression of Hif-1α in the retina under hypoxic and hyperoxic conditions. **B:** Increased expression of Hif-1α in the RPE at hypoxia or hyperoxic conditions **C:** Up-regulation of TRX and HO-1 in the RPE exposed to light.

Fig. 6.

Downstream regulators of EPO in the retina and the RPE. Retinal cultures derived from rat or early passages of human RPE cells were exposed to 1% O₂, 50% O₂, or 5,000 lux light. Proteins were separated by SDS-PAGE and visualized by Western blot test. **A:** Expression of p-Jak2 increased in the retina exposed to 1% O₂, 50% O₂, or 5,000 lux light. **B:** Expression of p-Jak2 increased in the RPE exposed to 1% O₂, 50% O₂, or 5,000 lux light. **C:** Expression of activated caspase-3 after exposure to light in retinal cells. Until 30 min of light exposure, the level of cleaved caspase-3 in retinal cells was unchanged, indicating that

endogenous neuroprotective or antiapoptotic proteins including EPO/EPOR against light stress were up-regulated. At 60 min, increased level of cleaved caspase-3 was compatible with significant cell death. Expression of c-fos and BCL-xl in the retina (**D**) and RPE cells (**E**). These antiapoptotic proteins were up-regulated in the light.

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