Dual Switch Mechanism of Erythropoietin as an Antiapoptotic and Pro-Angiogenic Determinant in the Retina

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ABSTRACT: Constant or intense light degenerates the retina and retinal pigment epithelial cells. Light generates reactive oxygen species and nitric oxide leading to initial reactions of retinal degeneration. Apoptosis is the primary mechanism of abnormal death of photoreceptors, retinal ganglion cells, or retinal pigment epithelium (RPE) in degenerative retinal diseases, including diabetic retinopathy and age-related macular degeneration. The current study evaluated the function of erythropoietin (EPO) on angiogenesis and apoptosis in the retina and RPE under oxidative stress. We determined the pro-angiogenic and antiapoptotic mechanism of EPO under stress conditions using a conditional EPO knockdown model using siRNA, EPO addition, proteomics, immunocytochemistry, and bioinformatic analysis. Our studies verified that EPO protected retinal cells from light-, hypoxia-, hyperoxia-, and hydrogen peroxide-induced apoptosis through caspase inhibition, whereas up-regulated angiogenic reactions through vascular endothelial growth factor (VEGF) and angiotensin pathway. We demonstrated that the EPO expression in the retina and subsequent serine/threonine/tyrosine kinase phosphorylations might be linked to oxidative stress response tightly to determining angiogenesis and apoptosis. Neurprotective roles of EPO may involve the balance between antiapoptotic and pro-angiogenic signaling molecules, including BCL-xL, c-FOS, caspase-3, nitric oxide, angiotensin, and VEGF receptor. Our data indicate a new therapeutic application of EPO toward retinal degeneration based on the dual roles in apoptosis and angiogenesis at the molecular level under oxidative stress.

INTRODUCTION

The retinal pigment epithelium (RPE) and retinal cells undergo uncontrolled cell death under oxidative stress leading to neurodegenerative diseases, including diabetic retinopathy (DR) and age-related macular degeneration (AMD).1−5 In response to the stress environment including intense or constant light, endogenous neuroprotective factors provide antiapoptotic effects to shield the retinal and RPE cells.6−10 However, the detailed mechanisms of molecular determinants in apoptosis and angiogenesis remain elusive. Thus, there is a critical need for understanding the intrinsic neuroprotective mechanisms that prevent cell death in the retina.11−14

We have investigated the therapeutic applications of retinal degeneration by focusing on antiapoptotic and neuroprotective mechanisms using a functional proteomics approach.15−31 Previously, we demonstrated that the retinal expression of erythropoietin (EPO), the visual cycle, and the subsequent kinase phosphorylations are interconnected to the circadian clock in anticipation of daily light onset.15 Our data demonstrated that the EPO is a neuroprotective molecule involved in the inhibition of the apoptotic pathway, including BCL-xL and caspase-9.
positive impact on new therapeutic approaches that time-dependent control of EPO expressions may have a clinical application of inhibition of EPO at later pathogenic progression and the addition of EPO at the early stage of retinal diseases. The current data will contribute to our understanding of the neuroprotective and pathogenic roles of EPO, thus guiding the design of pharmacologic modulators of retinal degeneration.

**RESULTS**

Parallel Regulation of EPO and VEGF. First, the working hypothesis of the role of EPO in angiogenesis was tested by the conditional knockdown experiment using EPO-specific siRNA. We verified the knockdown efficiency of EPO in RPE by western blotting analysis. Our data showed that EPO expression was decreased by 80–90% in cells transfected with EPO siRNA as compared to cells transfected with random siRNA sequence and untransfected cells as negative controls. Immunocytochemistry analysis validated the results of western blotting analysis demonstrating that vascular endothelial growth factor (VEGF) was down-regulated upon EPO knockdown in RPE cells, whereas random sequence siRNA and negative control RPE cells showed EPO and VEGF expressions (Figure 1). The knockdown experiment showed the parallel regulation of EPO and VEGF expression that they have a positive functional correlation. The western blotting analysis confirmed the coexpression of VEGF with EPO under knockdown condition.

Next, we tested the hypothesis of whether EPO could regulate downstream of VEGF in angiogenesis. The EPO downstream signaling was examined for major angiogenic molecules including VEGFR1 and angiotensin I/II in the retina (HRP) and RPE (ARPE-19). VEGFR1 and angiotensin I/II increased 6–12 h after EPO treatment (100 U) in the retina and ARPE-19 cells (Figure 2). When EPO is added to RPE cells (200 U) and retinal cells (100 U), VEGF mRNA increased in the RPE (CTL = 176, 3 h = 232, 6 h = 282 as relative amount) and in retinal cells (CTL = 4.45, 3 h = 6.76, 6 h = 8.14, 12 h = 7.56 as relative amount), respectively. Our experiment suggests that the EPO may function as a pro-angiogenic molecule via VEGF/VEGFR/angiotensin signaling. However, EPO treatment decreased VEGFR1 in ARPE-19 cells in 6–12 h, suggesting potential downregulation of angiogenic signaling that is initiated before neovascularization because the initial vessel loss may regulate the severity of neovascularization.

Intense light up-regulates thioredoxin, BCL-xL, and c-FOS in human RPE cells.15 Rat retinal cells under bright light up-regulated phospho-Janus kinase 2 (pJAK2), EPO, and EPOR within an hour. pJAK2 and phospho-Signal Transducer and Activator of Transcription 3 (pSTAT3) increased in human RPE primary cells in hypoxic conditions (1% O2). Increased phosphorylations of JAK2 (0–3 h) and STAT3 (0–12 h) were confirmed as shown by phospho-western blot (Figure 3A). As an upstream regulator of EPO, HIF-1α was examined. Oxidative stress including constant light, hyperoxic, and hypoxic conditions increased the expression of EPO (Figure 3B). EPO treatment (50–200 U) on human RPE cells and rat retinal cells in vitro showed its protective effects under the oxidative environment. EPO inhibited caspase-3 activation in retinal cells under stress, indicating that EPO is antiapoptotic (Figure 3C).

Next, we determined whether EPO and EPOR had a similar pattern of distribution in both neurons and astrocytes. Within 3 h after exposure to hypoxia, the expressions of EPO and EPOR increased in various retinal cells. The number of...
Figure 3. Downstream regulators of EPO. (A) Primary cells from humans and rats were treated with hypoxia (1% O₂) and analyzed using phospho-immunoblotting. pJAK2 increased in 1–3 h under hypoxia, whereas pSTAT3 was up-regulated in 3–12 h in the RPE. (B) HIF-1α was examined using western blot analysis. As an upstream regulator of EPO, HIF-1α increased in hypoxic, hyperoxic, and light conditions. (C) Neuroprotective EPO. Treatment of EPO (50–200 U) on human RPE cells and rat retinal cells under oxidative stress (20 μM H₂O₂) decreased caspase-3 activation in 1–6 h.
colocalized cells with EPO/EPOR with other retinal cell markers also increased by 1% O₂ hypoxic condition (Figure 4A). A quantitative analysis of EPO mRNA using real-time-PCR showed that an elevated EPO mRNA level was detected that the neurite outgrowth increased in 1% O₂ hypoxic condition with more branched neurite of neuronal cells, compared to control. An increase of glial fibrillary acidic protein (GFAP, green; astrocyte marker) expression suggests that the retinal cell differentiation might be stimulated in hypoxia (Figure 4B). Thymocyte differentiation antigen 1 (THY-1, green; neuroglial cell and axon marker) is expressed in the cell surface of the neuronal cell, ganglion cell body, dendrites, and axon after neuronal maturation. In control, THY-1 is observed on the cell body; however, the expression level increased and localization was stretched to the axon in hypoxia (Figure 4B). It seems that hypoxia may promote neuronal maturation and this reaction corresponds to neurite outgrowth. EPO significantly increased in all types of neuronal cells, including neuron, ganglion, and glial cells, in hypoxia. The EPO level increased, and neuronal outgrowth was up-regulated, as shown by increased MAP-2 and THY-1 axonal staining under oxidative stress (40 μM H₂O₂). Besides, glial cells gathered to generate small cell groups representing cell aggregation as a stress response against H₂O₂ treatment.

**On–Off Switch Mechanism of EPO and Nitric Oxide Measurement In Situ.** Next, the environmental effectors of EPO expressions were determined. We postulated that EPO concentration could be controlled by the light condition or circadian clock. Retinas under 12 h light (300 lux)—12 h dark cycles were analyzed for EPO and EPOR expressions at six different time points over a 24 h period (2, 4, 6, 10, 14, and 18 h). During the first 8 h of the dark period as an activity phase (LD 12–LD 20), mice retinal EPO was not detectable (Figure 5). However, 2 h before the light returned (22 h clock time, LD 22), retinal EPO was detected. EPO levels were increased for 2 h after the light turned on and gradually declined and became undetectable in the late light phase (LD 22–LD 6). As a negative control, EPO/EPOR expressions at 22 h light/dark cycles were compared to constant dark cycle at 22 h (DD 22) which shows no expressions in the dark, indicating that light might be indispensable for the EPO expression. Our data demonstrate that the environmental factor including the

![Figure 4](image-url)  
**Figure 4.** EPO and EPOR under oxidative stress. (A) Immunocytochemical analysis of EPO demonstrates that EPO and EPOR expressions and colocalization with neuronal cell markers are increased in 1% O₂ hypoxic condition (red, EPO) and H₂O₂ (red, EPOR), respectively (3 h). Arrows represent the co-localization of EPO (red) and MAP-2 (green, neuronal marker). Scale bar = 30 μm. (B) Immunocytochemical analysis of GFAP and THY-1 under oxidative stress. In hypoxic conditions (1% O₂), an increase of GFAP expression (green, astrocyte marker) was observed. THY-1 (green, neuroglial cell and axon marker) is observed on the cell body in control. The expression level increased, and localization was stretched to the axon in hypoxia. EPO significantly increased in all types of neuronal cells, including neuron, ganglion, and glial cells, in hypoxia. The EPO level was elevated under oxidative stress (40 μM H₂O₂), and neuronal outgrowth increased as shown by THY-1 axonal staining. Arrows represent the colocalization of EPO (red) and GFAP/THY-1 (green). Scale bar = 15 μm.

![Figure 5](image-url)  
**Figure 5.** On–off switch mechanism of EPO during the day. Mouse retinas were collected every 4 h (2, 6, 10, 14, 18, and 22 h) in 12 h light—12 h dark cycles over a 24 h period. During the first hours of the dark cycle (LD12–LD20), mice retinal EPO was not detectable. However, 2 h before the light returned (LD22), EPO was increased. Retinal EPO increased for 2 h after the light turned on and gradually declined and became undetectable in the late light phase (LD22–LD6). As a negative control, EPO/EPOR expressions at 22 h in 12 h light/dark cycle were compared to EPO/EPOR in constant dark period at 22 h. EPO is turned on at night during LD22 (22 h clock time, LD20). EPO expression showed a similar pattern to EPO regulation, peaking at 2 h in the light phase and declining over the later part of the light cycle, whereas EPOR was detectable at all times. EPO/EPOR at 22 h light/dark cycle was compared to constant dark cycle at 22 h (DD 22) which shows no expressions in the dark, indicating that light might be indispensable for the EPO expression. Our data demonstrate that the environmental factor including the
light or the endogenous circadian clock may regulate EPO expressions as the on–off switch mechanism that they increase just before light onset.

To test the potential mediators of the light-EPO signaling pathway based on EPO–protein interactome, we determined real-time nitric oxide production quantitatively in light-stressed RPE cells. EPO and hypoxic conditions stimulate EPOR and nitric oxide production through the MAPK pathway. Nitric oxide is produced by RPE cells to help protect from oxidative stress; however, there is no understanding of how much nitric oxide is produced and what duration is required to up-regulate EPO expression. We measured nitric oxide concentration directly in situ from cultured cells exposed to different light stresses, including light/dark cycles, continuous illumination, and various light intensities (200–7000 lux). It is observed that intense light up-regulates nitric oxide concentration in ARPE-19 cells compared to dark conditions (Figure 6).

Nitric oxide was produced in 20 h under light exposure in normoxia. However, the production of nitric oxide was more prominent under hypoxic–normoxic transition suggesting that a change of oxygen concentration is the main determinant of EPO regulation. Light induces the formation of nitric oxide in an oxygen-dependent manner (Figure 7). Like the other potential mediator of EPO downstream signaling, the calcium influx into the cell was analyzed in vitro (Figure 8). When EPO (25 U) was added into the cell, the calcium-binding experiment suggests that calcium influx (500, 2000 s), as well as neurite growth (1440 s), increased in the retina, as shown in Figure 9.

EPO Interacting Network. To understand the EPO network, a comprehensive protein/metabolite interaction map was established using proteomics and bioinformatics tools including two-dimensional (2D)-electrophoresis, mass spectrometry, STRING 11, and OmicsNet software. One hundred one proteins were analyzed and collected from proteomics study after the immunoprecipitation (IP) experiment using ARPE-19 cells and anti-EPO antibody. EPO interacting proteins were identified by mass spectrometry analysis and database search. The interaction network depicted the protein molecules as the nodes of the graph while the interaction as the edges (Figure 10). Most of the interactors were connected by multiple lines suggesting that the interaction was derived from more than one source of information. The EPO interactome showed 101 nodes, 1677 edges, 33.2 average node degree, 0.67 average local clustering coefficient, 543 expected number of edges, and PPI enrichment p-value < 1.0 \times 10^{-16}. All interacting proteins and metabolites are described and summarized (Supporting Information; Tables S1 and S2).

The protein–protein interactome map confirmed that EPO could be involved in JAK/STAT signaling (JAK2, STAT1, STAT5AB), energy metabolism, and insulin pathway (AKT, mTOR, MAPK, and IRS2) as shown in Figure 10. EPO is shown to interact with VEGFA as an upstream regulator of angiogenesis and is also involved in oxygen signaling and
phosphorylations (HIF1A, HIF3A, FOXO3, and PPP2CA) (Supporting Information; Figures S1–S3). EPO down-regulates caspases as an antiapoptotic molecule and controls other apoptotic molecules, including BCL2, PP2A, p53, PTEN, and SIRT1, as shown in the apoptotic network (Supporting Information; Figure S1). However, EPO up-regulates VEGF and angiotensin as a pro-angiogenic protein (Supporting Information; Figure S2).

Protein interaction clustering shows that nitric oxide and calcium could be involved in the EPO network. Nitric oxide regulates AKT, HIF1A, JAK2, EGFR, VEGFA, NFkB, and EGFR positively, whereas calcium influx was responsive to EGFR, JUN, FOS, SOCS3, and PLCG1. The inflammation initiation mechanism of EPO can be explained through indirect interactions with PTPN6/11, LYN, and NFkB (Supporting Information; Figure S3).

To determine the EPO–metabolite interactions, the EPO metabolome map was generated using bioinformatic tools including OmicsNet software (https://www.omicsnet.ca/) with S2 EPO–interacting metabolites (Supporting Information; Figure S4, Table S2). The metabolome map shows the interactions of EPO—regulating factors including creatine, citrate, ATP/ADP, ATP/AMP, cholic acid, phosphoenolpyruvate, 4-aminobutanoate, pyridoxine phosphate, pyruvate, methyl malonate, icosaapentaenoic acid, taurochenodeoxycholate, O2, nitric oxide, and CO2.

Protein–metabolites interaction in the EPO metabolome confirmed VEGF signaling pathway and its metabolites interaction (nitric oxide and calcium) and HIF1 signaling (pyruvate, lactate, ATP, oxygen, nitric oxide, calcium, and iron) in the EPO network. EPO may control angiogenesis through AKT, phosphatidylinositol-3 kinase (PI3K), HIF1, and VEGF expressions. The metabolome map confirms that EPO could be a dual switch to regulate both apoptotic and angiogenic signaling based on time points (light/dark, circadian clock), energy, and microenvironment (ATP/ADP, O2/CO2) (Figure 11).

■ DISCUSSION

Neurodegenerative diseases of the retina that include AMD and DR under oxidative stress are initiated from uncontrolled apoptosis and angiogenesis, especially in the aged population worldwide. Many risk factors and biomarkers of these retinal diseases have been reported; however, early molecular mechanisms of the disease development through EPO signaling are elusive.32–34 Multiple factors of retinal disorders are involved in the occurrence, including aging, genetic predispositions, DNA methylation, nutrition, oxygen tension, intense/prolonged light, and diabetic conditions. Key characteristics of the diseases include the compromised structural integrity of microvasculatures, overproduction of reactive oxygen/nitrogen species (ROS/RNS), mitochondrial disruption, and uncontrolled retinal cell death. As a result, antiangiogenic, anti-oxidative, anti-inflammatory, and antiapoptotic strategies remain the most promising methods to treat these irreversible progressive neurologic degenerations.23,24,27
previously, we demonstrated that EPO is an antiapoptotic molecule to protect the retina through the HIF1α pathway. A long-term (6 months) study on intravitreal EPO injection proved EPO’s protective effect on microvasculatures and neuronal cells in the diabetic retina. EPO counteracts vascular VEGF-induced angiogenic reactions by reinforcing tight junctions and decreasing blood vessel permeability in the brain barrier; however, the molecular mechanisms of EPO have been controversial.

Angiogenic factors, including angiopoietin-like 4, were prevented by EPO in the ischemic retina. High levels of ROS formed in the retina may lead to cell damage and inflammation that can facilitate the progress of retinal degeneration. We demonstrated that nitric oxide is induced under oxidative stress as a free radical signaling molecule with potential apoptotic and angiogenic targets, as well as physiological effectors. Nitric oxide reacts with superoxide to generate peroxynitrite that can convert tyrosine into nitrotyrosine in the pathogenesis of DR.

EPO up-regulates glutathione S-transferase, glutamine cysteine ligase, and heme oxygenase as antioxidant enzymes, through nuclear factor erythroid 2-related factor 2 (Nrf-2) under oxygen imbalance conditions. EPO/EPOR is the major molecular switch for cell survival, where a very low concentration of EPO (1−10 pM) can activate EPOR and cause dimerization and autophosphorylation of the receptor. ERK, PI3K, JAK2, and Ras pathways are the dominant downstream network of EPO.

The mitochondrial membrane potential is maintained by the inhibition of cytochrome c release from mitochondria that may lead to the antiapoptotic mechanism of EPO.

The activation of specific mechanisms by EPO may differ in various cell types in neuronal cells under specific oxidative conditions. For example, EPO and EPOR mRNAs are significantly higher in the RPE than in the retina, indicating that EPOR-mediated molecular events might be dominant in the RPE.

In mouse mesenchymal-derived cells, the antiapoptotic and anti-inflammatory properties of EPO might be through CD131 (beta-common receptors) for stress response and tissue repair, although higher EPO concentration (2−20 nM) may be required in this circumstance. Expressions of EPOR and endothelial nitric oxide synthase (eNOS) are induced by exogenous EPO in primary human endothelial cells to a much higher degree under hypoxic conditions than in normoxia.

The current study used in vitro models including ARPE-19, HRP, rat retina cells, and human primary RPE cells as well as in vivo murine models to demonstrate the crosstalk between EPO and VEGF/HIF/nitric oxide signaling. Nitric oxide generation also coincided with PP2A tyrosine nitration in mouse retina and disruption of the cytoskeleton structure within RPE, indicating a deleterious effect of nitric oxide in the retina and RPE.

The effect of nitric oxide varied at different time points in various oxidative stress conditions, including constant/ intense light or an oxygen imbalance environment. Nitric oxide inhibited superoxide production by inhibiting NADPH oxidase activity in neutrophil, which might protect tissues from neutrophil-mediated inflammation. As a result, the multifaceted nature of EPO and nitric oxide requires systematic variable controls and proper biological models to unveil the intricate interactions between the two pathways.

EPO protects neuronal cells in hypoxic-ischemic conditions of the central nervous system. Oxygen-dependent mechanisms of EPO include spinal cord injury, excitotoxicity, traumatic brain injury, Parkinson’s disease, oxidative stress, and chemical neurotoxicity.

EPO promotes the survival of RGCs in a glaucoma mouse model, stimulates neurogenesis, and facilitates post-stroke recovery. Retinal ganglion cells (RGCs) were protected by EPO from degeneration induced by acute ischemia-reperfusion and anatomy injury.

EPO promotes the survival of RGCs in a glaucoma mouse model, stimulates neurogenesis, and facilitates post-stroke recovery. Retinal ganglion cells (RGCs) were protected by EPO from degeneration induced by acute ischemia-reperfusion and anatomy injury.

Our experiments suggest that EPO could be a pro-inflammatory molecule since it triggers the expression of angiotensin I/II, which may act as an inflammatory agent by enhancing vascular permeability through VEGF. Oxygen imbalance condition (1% O2) increased EPO two-fold, whereas nine-fold for VEGF suggesting that EPO-induced angiogenesis signaling through VEGF is amplified under hypoxia. Our data demonstrated that EPO and VEGF expressions are regulated in parallel and they may communicate directly or indirectly through downstream regulators, including STAT3 and JAK2 phosphorylations. The basal level of VEGF is higher in the RPE than in the retina, whereas VEGFR increased in the retina but not in RPE under oxidative stress. Inducible nitric oxide synthase generates nitric oxide under oxidative stress including light exposure in RPE cells.

Protein phosphatase 2A (PP2A, Y169−NO2) and tubulin B5 (Y159−NO2) were nitrated by light exposure. Neuroprotective or neurotoxic role of nitric oxide is determined by its local concentration and cell type to inhibit ischemic injury in delayed cell death.

The erythropoietic effects of administered EPO are known to depend upon the time of administration. EPO contributes to neovascularization and accelerates pathological angiogenesis in the proliferative stage, showing
little therapeutic effect at later stages of retinal degeneration. The current experiments support the protective effects of EPO on RPE and neuronal cell populations when exposed to light-induced oxidative stress in the early time points.

The protein interactome suggests that the mechanism of EPO signaling could be relayed through specific protein domains, including SH2 (false discovery rate = 2.83 × 10−11), tyrosine kinase domain (false discovery rate = 6.22 × 10−11), S/T kinase domain (false discovery rate = 6.22 × 10−11), PCI domain (false discovery rate = 5.12 × 10−8), PAS domain (false discovery rate = 1.18 × 10−7), and PH domain (false discovery rate = 1.29 × 10−5). Further, the EPO network at the molecular level indicates dual roles in apoptosis and angiogenesis on the ATP-dependent mechanisms (Supporting Information; Figures S1–S3, Table S3). The EPO metabolome map indicates that EPO could be regulated by the balance of ATP/ADP and O2/CO2 as supported by EPO reactions (Supporting Information; Figure S4, Table S3).

The addition of 25 U EPO into the cells in the calcium-binding experiment suggests that calcium influx (500, 2000 s), as well as neurite growth (1440 s), increased in a time-dependent manner. Our result is consistent with calcium influx induced by EPO through PLC-γ1 activation which leads to the activation of TRPV1 and TRPVI-Akt-AMPK-eNOS complex association, eNOS activation, and nitric oxide production.

The clinical limitations exist as the addition of EPO could be pathogenic at the later stage of DR. In the long term goal, we will determine the time point and concentration of EPO addition in the clinical model. In addition, the detailed molecular mechanism will be examined using a metabonomics approach of lipids and small molecules in the retina after EPO (as well as EPO siRNA) additions.

In conclusion, the current study demonstrated that the retinal expressions of EPO, HIF, and subsequent phosphorylations of JAK2/STAT3 are tightly linked to apoptosis and angiogenesis through VEGF, angiostatin, and caspases. Biochemical experiments indicate that potential retinal degeneration mechanisms include the changes of key molecules including HIF1, VEGF, angiostatin, nitric oxide, and calcium in the EPO proteome–metabolome network. The understanding of antiapoptotic and pro-angiogenic EPO mechanism implies an important positive impact on new therapeutic approaches to time-dependent control of EPO. The ant apoptotic EPO is an important effector at the early stage of retinal diseases, whereas inhibition of EPO at a later stage may protect the retina from uncontrolled apoptosis and pathological angiogenesis.

**MATERIALS AND METHODS**

**In Vitro Experiments: ARPE-19 Cells.** Human retinal pigment epithelial cells (ARPE-19; American Type Culture Collection, Manassas, VA) were cultured in dishes (56.7 cm², Nunc, Naperville, IL) or flask (25 cm², Corning, St. Louis, MO) containing Dulbecco’s modified Eagle’s medium (DMEM) with fetal bovine serum (10%, Sigma, St. Louis, MO) and streptomycin/penicillin (1%, Hyclone, Logan, UT). Cells were maintained in a humidified CO2 incubator (5%, 37 °C). Cells were trypsinized (5 min, 37 °C, 0.1% trypsin–EDTA; Sigma, St. Louis, MO) and centrifuged (125g for 7 min). Cells were plated (6- or 24-well plates; 8 × 10⁴ or 2 × 10⁴ cells/well) and allowed to grow to confluence (2–4 days).

**Human and Rat Primary Retinal Cell Culture.** Human retinal progenitor cells (HRP) were donated (Dr. Harold J. Sheeldo, University of North Texas Health Science Center). Primary rat cell cultures including neurons, astrocytes, and photoreceptor cells were generated from the retinas of the newborn Sprague-Dawley rats (Postnatal Day 1 or 2). Retinas were isolated, placed in Hanks balanced salt solution (Gibco, Rockville, MD) lacking Ca²⁺ and Mg²⁺, and mechanically dissociated into single cells. The plating medium was based on Eagle’s minimum essential medium (MEM, Gibco, Rockville, MD), supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 25 mM KCl.

**Human RPE Primary Cell Culture.** RPE cells were obtained postmortem from human donor eyes (Utah Lions Eye Bank, Moran Eye Center, UT; Lion’s Eye Bank, Portland, OR; Asan Medical Center). The protocol adhered to tenets of the Declaration of Helsinki for research involving human tissue. Eyes were opened 360° posterior to the ora serrata, and the retinal tissues and the vitreous were removed. The remaining tissues were rinsed with phosphate-buffered saline and incubated for 30 min at 37 °C in 0.25% trypsin in DMEM. RPE cells were plated in DMEM with fetal bovine serum (10% FBS). 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 triple washes with DMEM, cells were plated in 6- or 24-well plates (8 × 10⁴ or 2 × 10⁴ cells/well) and allowed to grow to confluence for 2–4 days (third- or fourth passage).

**Cell Treatment.** siRNA Transfection: ARPE-19 cells were seeded in 24-well plates at a density of 6 × 10⁴ cells/well with DMEM (0.1 mL) containing FBS (10%) and streptomycin/penicillin (1%). ARPE-19 cells were treated with 175 ng (final concentration of 23 nM) of siRNA against EPO (Sigma, sense = 5′ GACCUCUCAGCUCAUAATTT, antisense = 5′ UAUAUGGCUAAGGCUAGCTT) and random sequence control siRNA (Allstars control negative siRNA, Qiagen, Valencia, CA) in 100 μL of culture medium without serum. Transfection was conducted with HiPerFect transfection reagent (Qiagen, Valencia, CA), and cells were harvested 48 h post-transfection. Proteins were assayed by western blot for detection of EPO (ab226956, Abcam; sc5290, Santa Cruz), AIF (EMD Millipore), BCL-XL (sc-634, Santa Cruz Biotechnology), caspase-3 (EMD Millipore), BAK (ab32371, Abcam), and actin (sc-47778, Santa Cruz Biotechnology). Protein localization and expressions were also analyzed by immunocytochemistry.

**Analysis of Antiapoptotic and Pro-Angiogenic EPO on Downstream Regulators.** To examine the antiapoptotic and pro-angiogenic function, EPO (25, 50, 100, 200 U; R & D Systems, Minneapolis, MN) was added to retinal (HRP and rat retina) and RPE (human primary and ARPE-19) cells under stress conditions to analyze downstream effectors of EPO. A dose–response relationship between EPO and oxidative stress was developed using 1, 6, 10, 21, and 50% O2. Hypoxic or hyperoxic conditions were used in an incubator containing 5% CO2 and oxygen (1, 6, 10, 21, and 50%). For oxidative stress conditions, confluent ARPE-19 cells were exposed to bright fluorescent light (7000–10,000 lux), H2O2 (or tert-butyl hydroperoxide, 200 μM), and reoxygenation (2 h hypoxic condition, and then 21% O2) in serum-free medium for different time intervals in a CO2 incubator. Cells grown in 25 cm² flasks were used to measure nitric oxide release in situ. Proteins were extracted using ice-cold lysis buffer (pH 7.4, 0.5 M Tris–HCl, 1.5 M NaCl, 2.5% deoxycholic acid, 10 mM EDTA, 10% NP-40, protease inhibitor cocktail). Cell lysates...
were centrifuged at 10,000g for 30 min at 4 °C to pellet cell debris. Proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting.

**Immunocytochemistry to Determine the EPO Localization.** Cells were fixed with paraformaldehyde (4%) for 1 h at room temperature and permeabilized with Triton X-100 (0.2%, 10 min), followed by blocking with bovine serum albumin (2%). Fixed cells were incubated at 4 °C overnight with the primary antibodies including anti-MAP-2 (1/500, Sigma, St. Louis, MO), anti-GFAP (1/1000, Santa Cruz, CA), anti-EPO and EPOR (Santa Cruz, CA). Cells were incubated with a fluorescence-conjugated secondary antibody at room temperature (2 h, Alexa Fluor 488 donkey anti-mouse IgG, 1:1000; donkey anti-rabbit IgG, Molecular Probes, Leiden, Netherlands). Cells with a secondary antibody only were used as a negative control.

**In Vivo Experiment.** Female C57BL/6j mice of 4–5 weeks old (17.8 ± 1.1 g) were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were maintained on food and water ad libitum at a constant temperature with a 12 h dark/12 h light (300 lux). The mice were euthanized at 15 weeks, and the right and left eyes were collected every 4 h for 24 h. Eyecup samples were snap-frozen in liquid nitrogen and stored at −80 °C.

**Protein Analysis.** After removing vitreous and the lens, retina samples (or ARPE-19 cells or primary RPE cells) were homogenized in lysis buffer (15% glycerol, 120 mM NaCl, 25 mM Tris, 0.3% Triton X-100, 10 mM EDTA, 0.2% sodium orthovannadate, 2 mM PMSF, and protease inhibitor cocktail). Cells were lysed by repeated freeze–thaw cycles and sonication (3 × 5 min), centrifuged (10,500g, 1 h), and dialyzed against Tris–HCl (10 mM, pH 8.0), DTT (2 mM), Triton X-100 (0.1%), and EDTA (1 mM) after Centriprec purification (Millipore Corp., Bedford, MA). Proteins were analyzed by SDS–PAGE and transferred to polyvinylidene difluoride membrane (20 V, 30 min) with Tris-glycine buffer (192 mM glycine, 25 mM Tris, 20% methanol). The membrane was blocked for 1 h (5% nonfat dried milk, Tris-buffered saline, Tween-20) and incubated with anti-EPO antibody (polyclonal, 1:500, R&D Systems) or anti-EPOR antibody (monoclonal, 1:1000; anti-EPO and EPOR (Santa Cruz, CA). Cells were incubated with a fluorescence-conjugated secondary antibody at room temperature (2 h, Alexa Fluor 488 donkey anti-mouse IgG, 1:1000; donkey anti-rabbit IgG, Molecular Probes, Leiden, Netherlands). Cells with a secondary antibody only were used as a negative control.

**Cell Survival Assay.** The survival of retinal cells was analyzed quantitatively using a cell proliferation assay kit (CyQuant NF Cell Proliferation Assay Kit, Molecular Probes, Eugene, OR).

**Hoechst 33258 staining** (Molecular Probes, Leiden, Netherlands) was performed to determine the effects of EPO on retinal cell death induced by H2O2. After rinsing with PBS, the cells were stained using Hoechst 33258 (2 μg/mL in PBS, 2 min) and examined with a fluorescence microscope (IX71; Olympus, Tokyo, Japan).

**Direct Nitric Oxide Measurement.** Nitric oxide was measured by chemiluminescence detection with Sievers 280i nitric oxide analyzer (200 mL/min ambient air, 5% CO2 sweep gas; GE Instruments, Boulder, CO). The nitric oxide analyzer was equilibrated with a 5% CO2 incubator for 30 min before the experiment. ARPE-19 cells in T25 flask under an oxidative environment (200 μM l- butyl hydroperoxide; 7000 lux of fluorescent light; reoxygenation = 1% O2 for 2 h then 21% O2) were directly connected to nitric oxide analyzer using a customized adaptor. Quantitative analysis of intrinsic nitric oxide generation was also measured using 4,5-diaminofluorescein-2-diaceatac (DAF-2DA, 5 μM, 15 min; Sigma) under stress conditions (7,000–10,000 lux light, 16–24 h). Cells were washed three times with PBS and fixed (3 min, 2% glutaraldehyde). Images were acquired using a Zeiss Axiovert 200 M Aps Teme fluorescence microscope with 63× objective lens magnification.

**IP of EPO–Interacting Proteins.** ARPE-19 cells were rinsed (Modified Dulbecco’s PBS), lysed using IP lysis buffer containing Tris (25 mM), NaCl (150 mM), EDTA (1 mM), NP-40 (1%), glycerol (5%), and protease inhibitor cocktail (pH 7.4), and incubated on ice (3 × 5 min periodic sonication), followed by centrifugation (10 min, 13,000g). Protein samples were loaded (200–400 μL, 1 mg/mL), and amino-linked protein-A beads were used to immobilize anti-EPO antibody (sc5290, Santa Cruz) with a coupling buffer (1 mM sodium phosphate, 150 mM NaCl, pH 7.2), followed by incubation (25 °C, 2 h) with sodium cyanoborohydrate (3 μL, 5 M). Columns were washed using a washing buffer (1 M NaCl), and then, protein lysate was incubated in the protein A antibody column with gentle rocking overnight at 4 °C. The unbound proteins were washed three times using a washing buffer to remove nonspecific binding proteins. The interacting proteins were eluted by incubating with elution buffer for 5 min at 25 °C. Eluted proteins were separated using 2D-SDS–PAGE and visualized using Coomassie blue (Pierce, IL) or silver staining kit (Bio-Rad, Hercules, CA).

**Proteomics Approach to Determine the EPO Interaction.** Crosstalk between EPO, apoptotic, and angiogenic pathways was determined using a proteomics approach. EPO–interacting proteins from IP were analyzed using 2D gel electrophoresis (DIGE), mass spectrometry, and western blot. EPO itself can be cytotoxic in high concentrations (>200 U), so the EPO level was maintained below 200 U.

For 2D SDS–PAGE, proteins (150 μg) were mixed with rehydration buffer (200 μL, 50 mM DTT, 2 M thiourea, 8 M urea, and 2% CHAPS) with destreak buffer (0.5%, pH 3–10, GE Healthcare). Isoelectric focusing was conducted using 11 cm Immobiline dry strips (Bio-Rad) with linear pH 3 to 10 and pH 4 to 7 gradients. Proteins were analyzed using the Ettan IPGphor-3 equipment (1 h at 500 V, 1 h at 1000 V, 2 h at 6000 V, and 40 min at 6000 V; GE Healthcare). The IPG strips were incubated in equilibration buffer I (0.375 M Tris–HCl–pH 8.8, 6 M urea, 20% glycerol, 2% SDS, and 50 mM DTT) for 20 min at 25 °C. The gel pieces were destained, washed, and vacuum-dried. Proteins were digested using trypsin (13 ng/μL; sequencing grade, Promega, Madison, WI) in NH4HCO3 (25 mM) and incubated overnight at 37 °C. Peptides were extracted (50 μL, 50% MeCN/5% formic acid, 15 min, 37 °C), combined, and dried. The peptides were purified using the C18 ZipTip (Millipore) eluting with 5 μL of a 75% MeCN/0.1% TFA mixture. Matrix-assisted laser-desorption-ionization tandem time-of-flight mass analysis (Bruker Ultraflex MALDI-TOF-TOF) was performed using Flex analysis 2.0 and Biotools 2.2 software. One microliter of purified peptides was crystallized with an equal volume of a freshly prepared α-cyano-4-hydroxycinnamic acid matrix solution (saturated, 0.5 mL/sample) in MeCN (50%)/TFA (0.1%). The search criteria include zero missed trypsin cleavage, methionine oxidation, and 50 ppm mass accuracy tolerance.
 Protein–Protein Interactome and Metabolome Mapping. EPO interacting proteins were collected using our proteomics data, PubMed database, National Center for Biotechnology Information. One hundred one EPO–interacting proteins were analyzed and collected based on their accession number, gene name, protein name, and type of model as selection criteria (Supporting Information Table S1). All the proteins were validated using the Uniprot Knowledgebase (UniProtKB); STRING database v11 (http://string-db.org/) was used for the construction of the network. The protein–protein interactions of Homo sapiens were selected in the database, and the interaction network was constructed using the “add more interactors” option in the STRING database. The first shell (50/20/10 interactions) and second shell (50/20/0 interactions) were compared, and various confidence scores (0.9/0.7/0.4, respectively) were examined for the map. A comprehensive network of protein interaction generated from databases of physical interactions and curated biological pathways knowledge. The EPO network was further analyzed by clustering the interactors in five overlapping clusters using the k-means clustering algorithm. The k-means algorithm was optimized as an unsupervised clustering method based on the adjacency matrix, which identifies molecules based on pre-specified criteria.

The EPO metabolome map was established using bioinformatics tools including OmicsNet (https://www.omicsnet.ca/) with 52 EPO–interacting metabolites. OmicsNet is a visual network system that generates interactions among genes, proteins, miRNAs, metabolites, or transcriptome in three-dimensional space. EPO–interacting metabolites were validated using their Kyoto Encyclopedia of Genes and Genomes (KEGG) and PubChem identification number (https://pubchem.ncbi.nlm.nih.gov/rest/pug). These metabolites carry protein interactions on their edges. The metabolome map validated our protein interactome to uncover any hidden interactions and the molecular mechanisms between EPO–metabolites interactions. We confirmed the EPO metabolites with the PubMed database using rat, mouse, and human models.

Statistical Analysis. Values are presented as the mean of three independent experiments. Two-group comparisons were analyzed by the 2-tailed t-test. Multiple comparisons were evaluated by ANOVA and Tukey or Dunnet tests, as appropriate. P < 0.05 is considered statistically significant.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c02763.

Apoptotic mechanism by EPO–interacting proteins; angiogenic mechanism by EPO–interacting proteins; mechanistic dissection of EPO Network; EPO metabolome map; EPO–interacting proteins with their accession number, gene name, protein name, and models; EPO–interacting metabolites; and EPO reactions including kinases and ATP (PDF)

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ABBREVIATIONS

AMD, age-related macular degeneration; DAPI, 4′,6-diamidino-2-phenylindole; DIGE, differential gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium; DR, diabetic retinopathy; IP, immunoprecipitation; ROS, reactive oxygen species; RPE, retinal pigment epithelial; siRNA, small interfering RNA

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