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Suppression of Retinal Neovascularization by Erythropoietin siRNA in a Mouse Model of Proliferative Retinopathy

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Abstract

Purpose—Erythropoietin (EPO), an oxygen-regulated hormone stimulating erythrocyte production, was recently found to be critical for retinal angiogenesis. *EPO* mRNA expression levels in retina are highly elevated during the hypoxia-induced proliferation phase of retinopathy. The authors investigated the inhibition of retinal *EPO* mRNA expression with RNA interference as a potential strategy to suppress retinal neovascularization and to prevent proliferative retinopathy.

Methods—The authors used a mouse model of oxygen-induced retinopathy. Retinal *EPO* and *Epo receptor* (*EpoR*) expression during retinopathy development were quantified with real-time RT-PCR in whole retina and on laser-captured retinal vessels and neuronal layers. Retinal hypoxia was assessed with an oxygen-sensitive hypoxyprobe. A small interference RNA (siRNA) targeting *EPO* or control negative siRNA was injected intravitreally at postnatal (P) day 12, P14, and P15 during the hypoxic phase, and the effect on neovascularization was evaluated in retinal flatmounts at P17.

Results—Retinal *EPO* mRNA expression in total retina was suppressed during the initial phase of vessel loss in retinopathy and was significantly elevated during the hypoxia-induced proliferative phase in all three neuronal layers in the retina, corresponding to an increased level of retinal hypoxia. *EpoR* mRNA expression levels also increased during the second neovascular phase, specifically in hypoxia-induced neovascular vessels. Intravitreal injection of *EPO* siRNA effectively inhibited approximately 60% of retinal *EPO* mRNA expression and significantly suppressed retinal neovascularization by approximately 40%.

Conclusions—Inhibiting *EPO* mRNA expression with siRNA is effective in suppressing retinal neovascularization, suggesting *EPO* siRNA is a potentially useful pharmaceutical intervention for treating proliferative retinopathy.

Proliferative vascular growth characterizes retinopathy of prematurity, diabetic retinopathy, and age-related macular degeneration, the leading causes of blindness in the United States among children, working age adults, and the elderly, respectively. Proliferative retinopathy is modeled in mice with an initial phase of oxygen-induced retinal vessel loss. This loss leads to retinal ischemia and increased expression of hypoxia-induced angiogenic growth factors, triggering retinal neovascularization, the second phase of retinopathy.¹ Retinopathy can be suppressed by preventing vessel loss and thereby reducing tissue hypoxia, which stimulates neovascularization, or by direct inhibition of neovascularization.

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Given that oxygen-regulated growth factors play critical roles in stimulating retinal angiogenesis,² inhibition of these growth factors is a key approach to inhibit retinal neovascularization. One of these hypoxia-regulated factors is vascular endothelial growth factor (VEGF), which is essential for retinal neovascularization. Oxygen through hypoxia-induced factor (HIF) regulates retinal expression of VEGF,³ and inhibition of VEGF inhibits retinal neovascularization in a mouse model of oxygen-induced retinopathy.^{4,5} VEGF inhibitors are now used successfully in the clinic to suppress neovascularization in age-related macular degeneration, though not all patients respond to this treatment. Another (additive) effective pharmaceutical intervention to suppress late-stage vasoproliferation would be of great benefit to prevent vision loss.

Erythropoietin (EPO) is a growth factor regulated by oxygen and HIF. Like VEGF, EPO plays an important role in retinal angiogenesis.⁶ EPO is a hormone produced primarily in the kidney in response to anemia and hypoxia and is then released into circulation to stimulate erythrocyte production in the bone marrow. In addition to its known function of stimulating erythropoiesis, EPO is a multifunctional, proangiogenic, and prosurvival factor. EPO stimulates angiogenesis as potently as VEGF in vitro and in vivo.^{7,8} EPO also promotes endothelial cell and neuronal cell survival.^{9,10} Recently, we found that EPO was produced in the retina and was important for retinal angiogenesis.⁶ Using a mouse model of oxygen-induced retinopathy, we noted that EPO deficiency in the vessel loss phase of retinopathy (phase 1) contributes to initial retinal vessel loss. Early systemic supplementation of EPO prevents retinal vessel loss and thereby prevents subsequent pathologic neovascularization and retinal neuron degeneration.⁶ Conversely, elevated levels of *EPO* mRNA were found in the proliferative stage of retinopathy (phase 2) in this mouse model.^{6,11} Additionally, EPO protein levels were elevated in human vitreous samples from patients with proliferative diabetic retinopathy.^{11,12} These findings suggest that high levels of EPO during the neovascular phase of retinopathy may contribute to pathologic retinal angiogenesis. Suppression of EPO during this phase could be beneficial and might be additive to VEGF inhibition.^{11,12}

We examined whether EPO inhibition in phase 2 of retinopathy with interference RNA suppresses retinal neovascularization in a mouse model of oxygen-induced retinopathy. RNA interference is a novel technology for specifically suppressing gene expression. Small interfering RNAs (siRNAs) are a class of short, double-stranded RNA that interferes with the expression of a specific gene.¹³ Compared with other methods of suppressing gene products, siRNA may be safer and more efficient because of its specificity. This approach has been successfully used to inhibit viral infection and tumor growth.^{14,15} Various siRNAs have been demonstrated to be effective in eyes,^{16,17} but so far no adverse effects were found in human clinical trials with intraocular injection of siRNA.¹⁸

Through the mouse model of oxygen-induced retinopathy (OIR), we found that during the second neovascularization phase of retinopathy, elevated levels of EPO were produced primarily in the neuronal layers in response to increased retina hypoxia. Suppression of EPO with intravitreal injection of EPO siRNA significantly suppressed retinal neovascularization. The suppression of retinal neovascularization likely occurred through decreased signaling of the EPO receptor (EpoR), which is upregulated in retinal vasculature during retinopathy. These data suggest that EPO siRNA might be a specific and effective pharmacologic intervention to prevent the devastating neovascular stage of proliferative retinopathy. The result may also have implications for other diseases, such as cancer, that are dependent on pathologic angiogenesis.

Methods

Animals

These studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by Children's Hospital Animal Care and Use Committee. Unless otherwise indicated, 129S6/SvEv mice (129SVE; Taconic Farms, Germantown, NY) were used for the study.

O₂-Induced Retinopathy (Pathologic Neovascularization)

To induce retinopathy, mice and their nursing mothers were exposed to 75% oxygen from postnatal day (P) 7 to P12.¹ Retinal neovascularization was evaluated at P17, 5 days after return to room air, when the neovascular response was greatest. At P17, mice were given lethal doses of tribromoethanol (Avertin; Sigma, St. Louis, MO), and eyes were fixed in 4% paraformaldehyde for 1 hour. Retinas were isolated and stained overnight at 23°C with fluoresceinated *Griffonia simplicifolia* isolectin B₄ (Alexa Fluor 488-I21411 or Alexa Fluor 594-I21413, 1:100 dilution; Molecular Probes, Eugene, OR) in 1 mM CaCl₂ in PBS. After 2 hours of washes, retinas were wholemounted onto microscope slides (Superfrost/Plus; Fisher Scientific, Pittsburgh, PA) with the photoreceptor side down and embedded in reagent (SlowFade Antifade; Invitrogen, Carlsbad, CA). All experiments were repeated at least three times.

Quantification of Retinal Neovascularization and Vaso-obliteration

Quantification of retinal neovascularization was carried out as described previously.^{19,20} Mosaic images covering the entire retina at 5× magnification were taken on a fluorescence microscope (Axio Observer Z1; Zeiss). Neovascular tuft formation was quantified by comparing the number of pixels in the affected areas with the total number of pixels in the retina (Photoshop; Adobe Systems, Mountain View, CA). Vaso-obliteration was quantified by comparing the number of pixels in the avascular areas with the total number of pixels in the retina (Photoshop; Adobe). Percentages of neovascularization or vaso-obliteration in siRNA-treated eyes were compared with values for control retinas. Evaluation was made with the identity of the sample masked (*n* represents the number of eyes quantified). All experiments were repeated at least three times.

EPO siRNA Injection

It has been reported that siRNA can inhibit neovascularization nonspecifically through TLR3-mediated innate immune response in a length-specific manner.²¹ All siRNAs used in this study were of same length, 21mer, to control for any length-related nonspecificity. EPO siRNA (Sigma; sense, 5' GACCCUUCAGCUUCAUUAUATT; antisense, 5' UAUAUGAAGCUGAAGGGUUCTT) were designed with an siRNA design algorithm licensed from Rosetta Inpharmatics (Seattle, WA). Control negative siRNA (Allstars control negative siRNA, catalog no. 1027281; Qiagen, Valencia, CA), which contains no homology to any known mammalian gene, was validated using gene chips and a variety of cell-based assays (sequence not provided by vendor). EPO siRNA and control negative siRNA were reconstituted in siRNA suspension buffer (catalog no. 1024183; Qiagen) according to the manufacturer's directions and were diluted in phosphate-buffered saline for injections. For intravitreal injection, mice were anesthetized with tribromoethanol (Avertin; Sigma), and eyes were dilated. Injections were delivered 1 mm posterior to the limbus with a 33-gauge needle. Varying doses (0.5–2 µg) were tested to identify the optimal dosage. For evaluation of *EPO* mRNA expression, 2 µg EPO siRNA (in 0.5 µL) or control negative siRNA was injected intravitreally to fellow eyes of P12 mice after 5 days of oxygen exposure, and retinas were isolated at P14 for RT-PCR (*n* = 5). For analysis of vessel loss and neovascularization, 0.5 µg

EPO siRNA (in 0.5 μ L) or control siRNA was injected intravitreally to P12 ($n = 11$), P14 ($n = 5$), and P15 ($n = 9$) mice, and retinas were dissected at P17 and wholemounted. Mice without intravitreal injection ($n = 8$) were also subjected to oxygen treatment as no-injection control.

RNA Isolation and cDNA Preparation

Total RNA was extracted (RNeasy kit; Qiagen) from the retinas of one mouse from each of six litters and was pooled to reduce biological variability. Retinas from each time point were lysed in guanidinium isothiocyanate lysis buffer according to the manufacturer's instructions, and RNA was suspended in diethyl pyrocarbonate-treated H₂O. To generate cDNA, 1 μ g total RNA was treated with DNase I (Ambion, Austin, TX) to remove any contaminating genomic DNA. The DNase-treated RNA (100 ng) was then converted to cDNA using murine leukemia virus reverse transcriptase (Invitrogen). All cDNA samples were portioned as aliquots and stored at -80°C .

Quantitative Real-Time PCR Analysis of Gene Expression

PCR primers targeting *EPO* (forward, AGGAATTGATGTCGCCTCCA-20; reverse, AGCTTGACAGAAAGTATCCACTGTG-24), *EpoR* (forward, CAACAGCGGACACATCGAGTT-21; reverse, TGCGGTGATAGCGAGGAGA-19), and an unchanging control gene, *cyclophilin A* (forward, AGGTGGAGAGACCAAGACAGA-22; reverse, TGCCGGAGTCGACAATGAT-19) were designed using PerlPrimer.²² We used the following method to analyze primer sequences for specificity of gene detection. First, only primer sequences that specifically detected the sequence of choice, as determined by means of the NCBI Blast module, were used. Second, amplicons generated during the PCR reaction were analyzed with the first derivative primer melting curve software supplied by Applied Biosystems (Foster City, CA). This analysis determines the presence of amplicons on the basis of their specific melting point temperatures. Quantitative analysis of gene expression was generated using a sequence detection system (ABI Prism 7300; Applied Biosystems, Foster City, CA) and a master mix kit (SYBR Green; Qiagen). Each target gene mRNA copy number was normalized to one million copies of *cyclophilin A* control, which was quantified using a standard curve of known copy number dilutions.

EPO and EpoR mRNA Expression during Oxygen-Induced Retinopathy

Retinas were isolated at P10, P14, P15, and P17 ($n = 6$ per time point) from hyperoxia and normoxia control C57BL/6 mice. Retinas were combined, and RNA was isolated and converted to cDNA. *EPO* and *EpoR* mRNA expression were compared with *cyclophilin A* expression after quantitative RT-PCR.

Immunohistochemical Staining

Eyes fixed in 4% paraformaldehyde and frozen in OCT were cut into 14- μ m sections, rinsed with PBS, and blocked in PBS with 0.5% Triton X-100 and 5% goat serum. Sections were stained with primary antibodies against EPO (sc-7956; Santa Cruz Biotechnology, Santa Cruz, CA), followed by anti-rabbit secondary antibodies (Alexa 594; Molecular Probes) and thereafter FITC-conjugated *G. simplicifolia* isolectin B₄. For wholemount immunohistochemical staining, retinas fixed in 4% paraformaldehyde for 1 hour were rinsed in PBS, permeabilized overnight at 4°C with 0.5% Triton X-100 in PBS, and stained with isolectin B₄, as described. Retinal wholemounts were prepared as described previously and visualized with a confocal microscope (SP2; Leica, Wetzlar, Germany). For staining of retinal hypoxia, each P15 oxygen-exposed mouse was injected intravitreally (400 μ M, 0.5 μ L) with the oxygen-sensitive drug pimonidazole hydrochloride (Hypoxypore; NPI Inc., Edmonds, WA) 90 minutes before sacrifice. Retinas were dissected, fixed, permeabilized, and stained

with isolectin B₄ as described, followed by primary antibody against hypoxyprobe conjugated with FITC.

Laser Capture Microdissection of Retinal Layers

Eyes from P17 oxygen-treated or control mice were embedded in OCT immediately after enucleation, cut into 8- μ m sections, and collected on RNase-free polyethylene naphthalate membrane slides (Leica). Sections were stained with isolectin B₄, counterstained with hematoxylin and eosin, and dehydrated with both 50% and 75% ethanol, and air dried. Four retinal layers (vessel layer, ganglion cell layer, inner nuclear layer, and outer nuclear layer) were microdissected on a laser capture microdissection (LCM) system (LMD-6000; Leica). RNA was extracted from microdissected tissues (RNeasy Micro Kit; Qiagen), cDNA was created as described, and real-time PCR was performed.

Statistical Analysis

Results are presented as mean $\pm$ SEM for animal studies and mean $\pm$ SD for nonanimal studies. ANOVA with $\alpha = 0.05$ was used for processing the data. Unless otherwise indicated, two-sample *t*-test was used as a posttest.

Results

EPO and *EpoR* mRNA Expression Increases during Development of Retinopathy

EPO and *EpoR* mRNA expression from whole retina during oxygen-induced retinopathy were examined by quantitative real-time PCR. Compared with age-matched normoxia controls, retinal *EPO* mRNA expression was suppressed (0.42-fold at P10) during hyperoxia treatment (P7-P12), when oxygen-induced retinal vessel loss occurs (Fig. 1A). After return to room air, during hypoxia-induced retinal neovascularization (P12-P17), *EPO* mRNA was increased significantly (4.1-fold at P14; 4.5-fold at P15; and 7.5-fold at P17; Fig. 1A) compared with normoxia control. This oxygen-dependent regulation of *EPO* during retinopathy is consistent with our previous observations.⁶ Expression of *EpoR* mRNA, on the other hand, did not change during the first phase of retinopathy (0.94-fold at P10). During the neovascular phase of retinopathy, *EpoR* mRNA expression increased (1.8-fold at P15 and 2.0-fold at P17; Fig. 1B). The increased expression of *EPO* mRNA corresponded to increased levels of retinal hypoxia, as visualized by oxygen-sensitive pimonidazole (hypoxyprobe). In P15 oxygen-exposed mice, the immunofluorescent localization of hypoxyprobe showed the deposition of protein adducts (marker of hypoxia) in the central avascular retina area distal from major blood vessels but not in the peripheral vascularized retina (Fig. 1C).

EPO siRNA Inhibits Pathologic Retinal Angiogenesis

To examine whether specific inhibition of *EPO* with siRNA during phase 2 of retinopathy with elevated expression of *EPO* would prevent pathologic vessel proliferation, *EPO* siRNA or negative control siRNA was injected into fellow eyes of P12 mice immediately after removal from 5-day treatment of 75% oxygen. Retinal expression of *EPO* mRNA was quantified at P14, 2 days after siRNA injection, and retinal flatmounts were examined at P17, a time with maximal neovascular response.¹ Intravitreal injection of *EPO* siRNA suppressed retinal expression of *EPO* mRNA to 44% $\pm$ 11% of the level found in eyes injected with same-length control siRNA at P14 (Fig. 2). By P17, 5 days after injection, the inhibitory effect of *EPO* siRNA diminished (data not shown). Eyes injected with *EPO* siRNA at P12 were significantly protected (approximately 40%) from pathologic neovascularization at P17 (5.9% $\pm$ 1.5%) compared with fellow eye controls (10.2% $\pm$ 1.4%; $P \leq 0.001$) (Figs. 3A, 3B). In addition, *EPO* siRNA injected at P14 and P15 also suppresses retinal neovascularization at P17 by approximately 55% and approximately 40%, respectively (Fig. 3B). These data suggest that

elevated retinal levels of EPO during the proliferative phase of retinopathy contribute to retinal neovascularization and suppression of EPO. EPO siRNA is effective at protecting the retina from pathologic vessel proliferation. Injection of EPO siRNA at P12 and P14 (but not at P15) also resulted in less vaso-obliviation at P17, suggesting increased revascularization with EPO siRNA treatment. In uninjected eyes subjected to hyperoxia, the average vaso-obliviation is approximately 12% and the average neovascularization is approximately 16% higher than in eyes with intravitreal injection, as expected.

Localization of EPO and EpoR in Retina during OIR

We localized EPO in retinal cross-sections from P17 mice through immunohistochemistry. EPO protein was localized mostly in the inner retina, especially in the ganglion cell layer in oxygen-treated retinas (Fig. 4A). Given that EPO protein can diffuse in the retina and EpoR antibody specificity has been controversial,²³ we performed real-time RT-PCR on laser capture microdissected retinal vessels and neuronal layers to quantitatively localize *EPO* and *EpoR* mRNA expression. Compared with normoxia controls, *EPO* mRNA expression was increased in all neuronal layers of retina in oxygen-treated P17 mice, with especially dramatic upregulation in the inner and outer nuclear layers (Figs. 4B, 4D). Expression of *EPO* mRNA by retinal vessels was negligible (data not shown). In contrast, *EpoR* mRNA was expressed in all retinal layers (Fig. 4D), with the most significant upregulation in neovascular vessels isolated from oxygen-treated eyes (Figs. 4C, 4E).

Discussion

EPO is an oxygen-regulated hormone known to promote erythropoiesis. It is essential for development, as both EPO and EpoR null mice are embryonically lethal.²⁴ We recently found that EPO produced in the retina is critical for retinal angiogenesis.^{6,11} Local and systemic EPO production can contribute to retinal angiogenesis.⁶ Retinal *EPO* mRNA expression is highly elevated during the proliferative phase of retinopathy. This study investigated the strategy of inhibiting pathologic vessel proliferation by inhibiting EPO, when endogenous *EPO* expression is elevated, through RNA interference. We found intravitreal injection of EPO siRNA effectively suppresses endogenous expression of *EPO* mRNA in the retina. Inhibition of EPO with siRNA during the neovascular phase of retinopathy significantly suppressed destructive vessel proliferation directly (since injection at P15 caused inhibition of neovascularization). There was also an indirect effect of EPO siRNA treatment, decreasing neovascularization by increasing vascular regrowth from P12 to P15. This finding is consistent with those of a previous study that used blocking antibody against EPO to suppress retinal neovascularization in mice and suggests that restoring EPO to physiologic levels in retinopathy in phase 2 or phase 1 of retinopathy might be a useful therapeutic strategy.¹¹

Oxygen regulates *EPO* expression through HIF. The increased level of retinal EPO in the second phase of retinopathy, after oxygen-induced vessel loss (P7-P12) and on return to room air (P12-P17), corresponded to an increased level of retinal hypoxia in the now hypovascular retina. *EpoR* expression also increases with the development of retinopathy, suggesting marked increases of EPO signaling in response to hypoxic and ischemic injury in the retina. We found that hypoxia induces increased expression of *EPO* mRNA in all neuronal layers. The upregulation of *EPO* mRNA is especially prominent in the outer and inner nuclear layers. Increased *EPO* mRNA expression during retinopathy development likely reflects a self-regulation mechanism to increase tissue protection because EPO is reported to be a potent neuronal and vascular survival factor. However, given that EPO is also a potent angiogenic factor, the upregulation of *EPO* mRNA during retinopathy, combined with the increased expression of *EpoR* in neovascular vessels, likely helps to precipitate vessel proliferation, which can become pathologic.

Our observation of an increase in *EpoR* in vessels supports and expands on previous findings of strong *EpoR* staining in microvessels in human retinas with diabetic retinopathy.²⁵ Upregulation of *EpoR* has also been implicated in other pathologic conditions, such as prostate cancer²⁶ and ischemia-induced brain injury,²⁷ suggesting the upregulation of the EPO/*EpoR* system plays a prominent role in pathologic angiogenesis and neurovascular protection.

The regulation of EPO by oxygen parallels the regulation of VEGF. Just as VEGF is suppressed by oxygen, so too is EPO suppressed in the vessel loss phase of retinopathy, and correction of EPO deficiency improves vessel stability and therefore diminishes hypoxia and subsequent hypoxia-induced neovascularization.⁶ Conversely, in the second neovascular phase of retinopathy, in response to hypoxia, EPO is highly upregulated in a manner similar to that for VEGF.^{6,11} VEGF inhibition suppresses neovascularization^{4,5}; the suppression of EPO with siRNA also suppresses vessel proliferation. Our data suggest that the two phases of retinopathy require opposite approaches to normalize retinal vessels (Fig. 5). In the first vessel loss phase, supplementing EPO helped protect retina from vessel loss, diminished hypoxia, and ultimately helped prevent hypoxia-driven retinal neovascularization. In contrast, in the second neovascular phase, EPO inhibition was beneficial for preventing neovascularization. Inhibition of EPO alone, however, did not totally resolve neovascularization, indicating other factors such as VEGF are also at work in promoting vessel proliferation. EPO and VEGF are independently regulated in retinopathy,^{6,11} suggesting the suppression of EPO is likely to be as effective and might be additive to VEGF inhibition.

Although EPO siRNA is likely to be useful for inhibiting proliferative retinopathy, oversuppression of EPO could be harmful because EPO is a neurotrophic factor and is required for retinal survival.²⁸ If the research is to be translated to the clinic, dosing must be carefully monitored to inhibit vessel proliferation without impairing neuronal and vascular survival.

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Figure 1.

EPO and *EpoR* expression during oxygen-induced retinopathy. Real-time PCR quantification of (A) *EPO* and (B) *EpoR* mRNA expression in age-matched mouse retinas under normoxia or oxygen treatment. Copy number of mRNA/million copies of cyclophilin A control mRNA at P10, P14, P15, and P17 ($n = 6$ per group). (C) Representative retinal whole mounts from P15 oxygen-treated mouse showing deposits of hypoxyprobe at site of tissue hypoxia during OIR. Original magnification, $\times 5$. A portion of the image was enlarged (right, white frame). Vessels were visualized with isolectin B₄ staining (red). Hypoxyprobe staining (green) is evident in central avascular retina but not in peripheral vascularized retina.

Figure 2.

EPO siRNA effectively inhibited *EPO* expression. Fellow eyes of oxygen-treated mice were injected at P12 with negative control siRNA or EPO siRNA. At P14, retinas were isolated, and expression of *EPO* mRNA was quantified with quantitative real-time PCR ($n = 5$; $**P \leq 0.005$, comparing matched fellow eyes). Retinal *EPO* levels after control or EPO siRNA treatment ranged from 200 to 500 copies per 10^6 *cyclophilin A* control but are compared with control to preserve the fellow eye comparisons.

Figure 3.

EPO siRNA suppresses vaso-obliteration and neovascularization in retinopathy. Fellow eyes of oxygen-treated mice were intravitreally injected with negative control siRNA or EPO siRNA at P12, P14, and P15. **(A)** Representative retina whole mount at P17 showing vaso-obliteration (VO; green) and neovascularization (NV; yellow). Vessels were visualized with isolectin B₄ staining (red). Original magnification, $\times 5$. **(B)** Areas of VO and NV were quantified as percentages of total retina area. P12, $n = 11$; P14, $n = 5$; P15, $n = 9$. *** $P \leq 0.001$; * $P \leq 0.05$.

Figure 4.

Localization of EPO and EpoR in oxygen-induced retinopathy. **(A)** Representative retinal cross-sections from P17 oxygen-treated mouse immunolabeled with EPO antibody (red), isolectin B₄ to visualize vessels (green), and DAPI (blue). **(B)** Quantitative localization of EPO and EpoR expression in response to oxygen-induced retinopathy with LCM. Representative cross-section from P17 normoxia retina stained with hematoxylin and eosin for LCM. Three neuronal layers were laser captured from normoxia and oxygen-treated P17 retinas: ganglion cell layer (GCL), inner nuclear layer (INL), and outer nuclear layer (ONL). **(C)** Cross-sections of normoxia control and OIR retinas stained for vessels with isolectin B₄ (red). Normoxic vessels and angiogenic tufts were captured by LCM. **(D)** Quantification of

EPO and *EpoR* mRNA expression in microdissected retinal neuronal layers with RT-PCR. Samples were normalized to 10^6 copies of cyclophilin. (E) Quantification of *EpoR* mRNA expression in vessels from normoxic mouse and angiogenic tufts from oxygen- treated mouse at P17. *EPO* expression in vessels and tufts was negligible (data not shown). Scale bar, 50 μm .

Figure 5.

Schematic representation of EPO control of blood vessel development during retinopathy. Opposite approaches of EPO intervention are required during the two phases of retinopathy to normalize retinal vessels. During the first phase of retinopathy, vascular growth ceases and normal vessels degenerate. High oxygen exposure (as occurs in animal models and in some premature infants) and compromised kidney function (as in some patients with diabetes) suppress EPO, further contributing to inhibition of vessel growth. Supplementation of EPO during this phase may prevent vessel loss through systemic recruitment of bone marrow–derived proangiogenic stem cells into the retina and through local mechanisms such as prosurvival (NF-κB), antiapoptosis (caspase) pathways. During the second neovascular phase of retinopathy, hypoxia increases EPO expression, which may precipitate vessel proliferation. Inhibition of EPO (e.g., with siRNA) during this phase is likely beneficial.