Reduced Retinal Neovascularization, Vascular Permeability, and Apoptosis in Ischemic Retinopathy in the Absence of Prolyl Hydroxylase-1 Due to the Prevention of Hyperoxia-Induced Vascular Obliteration

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PURPOSE. Prolyl hydroxylases (PHDs) are oxygen sensors that stabilize hypoxia-inducible factors (HIFs) to induce proinflammatory, vasopermeability, and proapoptotic factors. These may be potential targets to reduce the complications of ischemic retinopathies.

METHODS. Oxygen-induced ischemic retinopathy (OIR) was generated as a model for retinopathy of prematurity (ROP) by placing 7-day-old mice in 75% oxygen for 5 days and returning them to the relative hypoxia of room air for 5 days. Neovascularization (NV) and avascular areas were assessed on retinal flat-mounts by image analysis. Blood-retinal barrier breakdown was assessed using [3H]-mannitol as a tracer. Apoptosis was detected with TUNEL staining. HIF-1α and VEGF were quantified using Western blot analysis and ELISA.

RESULTS. PHD1-deficient mice demonstrated reduced hyperoxia-associated vascular obliteration during oxygen-induced ischemic retinopathy. This was associated with subsequent reduced avascular area, vascular leakage, and pathologic NV during the hypoxic phase, which could be accounted for by a reduced expression of HIF-1α and VEGF. Apoptosis in the retina was also reduced in PHD1-depleted mice after 2 days in hyperoxia.

CONCLUSIONS. PHD1 deficiency is associated with a reduction of ischemia-induced retinal NV. The regulatory mechanism in this model appears to be: PHD1 depletion prevents HIF-1α degradation in hyperoxia, which induces VEGF, thus preventing hyperoxia-related vessel loss. Without a vessel deficiency, there would not be relative hypoxia when the mice are returned to room air and there would be no need to initiate angiogenesis signaling. Blocking PHD1 may be beneficial for ischemic retinopathies and inflammatory and neurodegenerative disorders. (Invest Ophthalmol Vis Sci. 2011;52:7565-7573) DOI:10.1167/iovs.11-8002
protect themselves against oxidative damage in hypoxic conditions by switching from aerobic to anaerobic metabolism and by slowing mitochondrial respiration, making inhibition of PHD1 a very promising strategy for preventing ischemic retinopathies. PHD1 inhibition also protects the liver from ischemia and/or reperfusion injury and it promotes survival in mesenchymal stem cells and intestinal epithelial cells. In some systems, inhibition of PHDs promotes angiogenesis, but in other scenarios, it provides neuroprotection without angiogenesis. In the oxygen-induced retinopathy (OIR) model of ROP, nondiscriminative pharmacological inhibition of all PHDs and other oxygen sensors prevents the hyperoxia-induced retinal vessel loss and the subsequent retinal NV that occurs in the hypoxic phase. However, the precise role of the distinct PHD isoforms in the regulation of oxygen homeostasis and the stability of the HIF isoforms is poorly understood.

Several recent studies have highlighted the importance of PHDs in ischemic and inflammatory disorders and suggested that stabilization or activation of HIFs by inhibiting the PHDs may represent a potential therapeutic approach to prevent these disorders. However, this area of research is just emerging and a number of questions remain to be answered. For example, what is the exact role of the three PHD isoforms in ischemic and inflammatory retinopathies and how do the PHD isoforms react with each other and with the different HIF isoforms? In the present study, we show that PHD1 inhibition suppresses retinal NV in the OIR model by stabilizing HIF and VEGF in the hyperoxic phase, thus preventing vascular obliteration, and by reducing the subsequent expression of HIF-1α and VEGF, which would have resulted from the relative hypoxia in normoxic conditions, had vascular obliteration occurred. The results of this study could lead to the development of safer and more efficacious treatments for ROP, diabetic retinopathy (DR), and other ischemic and inflammatory retinopathies.

**METHODS**

**Mice**

Animal use was in accordance with the approved protocols by the Institutional Animal Care and Use Committee of Johns Hopkins University School of Medicine and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The generation of PHD1 knockout mice was described elsewhere, and age- and sex-matched 129 mice (Charles River, Wilmington, MA) were used as controls.

**Mouse Model of Oxygen-Induced Retinal Neovascularization**

The OIR model was produced in PHD1 knockout or control 129 mice according to a method described previously. Briefly, litters of 7-day-old mice (postnatal day P7) were exposed to an atmosphere of 75% oxygen in an airtight incubator for 5 days (P12), after which they were returned to room air for 5 days (P17). For quantification of oxygen-induced retinal NV, mice were killed at P17 and eyes were fixed in PBS-buffered formalin for at least 3 hours. Retinas were dissected, washed, and incubated with griffonia simplicifolia-594 (Invitrogen, Carlsbad, CA) overnight at room temperature. Retinal flat-mounts were prepared and assessed with an epifluorescence microscope (Axiopan2, Zeiss, Germany).

**RESULTS**

**A** Reduced central retinal vascular obliteration of PHD1 knockout mice by hyperoxia in the OIR model. Representative images of P12 retinal vasculatures stained with GSA-lectin. The central avascular area is circumscribed by dashed lines. The quantification results are expressed as the ratio of central avascular area (C) and vascular area (D) to total retinal area. The results are expressed as mean ± SEM of nine individual mouse samples. Scale bar, 200 μm.

**DISCUSSION**

The results of this study could lead to the development of safer and more efficacious treatments for ROP, diabetic retinopathy (DR), and other ischemic and inflammatory retinopathies. The precise role of the distinct PHD isoforms in the regulation of oxygen homeostasis and the stability of the HIF isoforms is poorly understood. Further studies are needed to elucidate the exact role of the three PHD isoforms in ischemic and inflammatory retinopathies and how the PHD isoforms react with each other and with the different HIF isoforms.
Carl Zeiss Meditec, Inc., Thornwood, NY) using commercial software (Image-Pro Plus; Media Cybernetics, Silver Spring, MD).23

Assessment of BRB Integrity

The BRB was assessed using 3H-mannitol as a tracer, as previously described24 and the retina to lung leakage ratio (RLLR) and retina to renal leakage ratio (RRLR) were determined. Briefly, mice were sedated as described above and given an intraperitoneal injection of 1 μCi per gram body weight of [3H]mannitol. One hour after injection, the mice were sedated and retinas from experimental and control eyes were rapidly removed. The posterior portion of the globe was firmly grasped with forceps and a razor blade was used to cut across the cornea and extrude the lens, vitreous, and retina. Retinas were dissected free from lens, vitreous, and any RPE that was extruded, and were placed within pre-weighed scintillation vials within 30 seconds of kill. The thoracic cavity was opened and the left superior lobe of the lung was removed, blotted free of excess blood, and placed in another pre-weighed scintillation vial. A left dorsal incision was made and the retroperitoneal space was entered without entering the peritoneal cavity. The renal vessels were clamped and the left kidney was removed, cleaned of fat, blotted, and placed into a pre-weighed scintillation vial. Superficial liquid was allowed to evaporate for 20 minutes from the open vials. The vials containing the tissue were weighed and tissue weights were calculated. One mL of NCSII solubilizing solution (Amersham, Chicago,

**FIGURE 2.** Reduced apoptosis in the retinas of PHD1−/− mice in hyperoxia. The representative TUNEL staining of 9-day-old wild type (WT) mice (A, B) and PHD1−/− mice (C, D), both of which were exposed to 75% O2 for 2 days. The nuclei were stained blue by DAPI and demonstrated the structures of the eye. Higher magnifications of the regions shown within box “B” (A, inset) and “D” in (C, inset) showed the TUNEL-positive cells, which are much more abundant in WT mice and present specifically in the inner nuclear layer (INL). (E) Double staining of TUNEL and lectin showed colocalization of TUNEL(+) and lectin(+) cells in the superficial plexus of the ganglion cell layer (GCL) (arrow). (F) The quantification showed a significant reduction of TUNEL-positive cells in PHD1−/− mice. The results are expressed as mean ± SEM; n = 16 for WT, and n = 13 for PHD1(KO). Scale bar: (A, C) 200 μm; (B, D, F) 50 μm. IPL, inner plexiform layer.
IL) was added to each vial and the vials were incubated overnight in a 50°C water bath. Solubilized tissue was brought to room temperature (RT) and decolorized with 20% benzoyl peroxide in toluene in a 50°C water bath. The vials were brought to RT and 5 mL of scintillation fluid (Cytoscint ES; ICN, Aurora, OH) and 30 μL of glacial acetic acid were added. The vials were stored for several hours in darkness at 4°C to eliminate chemoluminescence. Radioactivity was counted with a scintillation counter (LS 6500 Liquid Scintillation Counter; Beckman, Brea, CA). The counts per minute (CPM) per mg tissue was measured for lung, kidney, and retina. Retina/lung, retina/kidney, and lung/kidney ratios were calculated and compared.

TUNEL Assay for Detection of Apoptotic Nuclei

The terminal dUTP nick end labeling (TUNEL) assay was performed using a kit (ApopTAG Red In Situ Apoptosis Detection Kit; Millipore, Temecula, CA) and the procedures were performed according to the manual. Briefly, eye sections were fixed in 1% paraformaldehyde for 10 minutes at RT and in ethanol/acetic acid (2:1) for 5 minutes at 20°C and then washed twice for 5 minutes in PBS (pH 7.4). After the tailing of digoxigen-dNTP catalyzed by the TdT enzyme, the sections were incubated with the anti-digoxigenin-rhodamine antibody for 30 minutes at room temperature. For negative controls, deionized water was substituted for the TdT enzyme. Processed sections were mounted with mounting medium containing DAPI (Vectashield Mounting Medium with DAPI; Vector, Burlingame, CA) and viewed with a fluorescence microscope (Axioplan2; Carl Zeiss Meditec, Inc.). The number and locations of TUNEL-positive cells were recorded.

Western Blot Analysis

Retinas were homogenized in ice-cold lysis buffer (150 mM NaCl, 20 mM Tris [pH 7.4], 2 mM ethylenediaminetetraacetic acid, 0.1% Triton X-100, 1 mM EDTA, plus “complete mini” EDTA-free protease inhibitor), and centrifuged at 14,000 g for 10 minutes. Supernatant protein concentrations were determined by the bicinchoninic acid (BCA) method. Twenty to thirty micrograms of supernatant proteins from retina were separated on 4%–20% gradient SDS PAGE gels, and trans-bloted to a nitrocellulose membrane. After blocking, membranes were incubated with primary antibodies overnight at 4°C. The primary antibodies used were anti-HIF-1α and anti-HIF-2α. Blots were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour. Immunoreactive bands were visualized using an enhanced chemiluminescence system (Amersham Life Science, Piscataway, NJ). Densitometric analyses were performed using the NIH Image program. The results were calculated from four independent experiments and expressed as mean ± SEM.
ferrared to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% bovine serum albumin (BSA) or 5% nonfat milk, blots were incubated with primary antibodies for 1 hour overnight, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000), and developed with chemiluminescence reagents (Pierce Technology Co., Holmdel, NJ).

Enzyme-Linked Immunosorbent Assay (ELISA) for VEGF and EPO

Mouse VEGF and erythropoietin (EPO) were measured in retinal protein extracts, the same samples as those used for Western blot analysis, using kits (Quantikine Immunosay kits; R&D Systems, Inc., Minneapolis, MN). All measurements were performed in duplicate according to the manufacturer’s protocols and the results were averaged. The intra-assay and interassay coefficients of variation (CVs) for VEGF were 4% and 15%.

RESULTS

Reduced Central Vascular Obliteration and Apoptosis in the Retinas of PHD1−/− Mice in Hyperoxia

Central vascular obliteration or degradation resulting from hyperoxia leads to retinal NV due to the subsequent relative hypoxia of room air in the model of OIR (Figs. 1 and 2). Sears et al.19 showed that the inhibition of PHDs by dimethyloxalylglycine (DMOG), which inhibits all three isoforms (PHD1, -2 and -3), can protect from the loss of central vasculature and prevent the retinal NV that follows, due to relative hypoxia. We investigated whether a deficiency of PHD1, without inhibiting PHD2 and -3, provides protection to central vessels exposed to hyperoxia. The central avascular area in PHD1−/− mice at P12, after 5 days of hyperoxia, is significantly reduced compared with that of WT mice: 0.87 ± 0.02 mm² per retina for WT mice versus 0.23 ± 0.02 mm² per retina for PHD1−/− mice (P = 3.94 × 10⁻⁶; n = 9). The remnant vascular areas are significantly greater in the PHD1−/− mice than in WT mice: 0.46 ± 0.03 mm² per retina for PHD1−/− mice versus 0.27 ± 0.02 mm² per retina for WT mice (P = 0.0056; n = 9) (Fig. 3).

Consistently, apoptosis is also significantly reduced in PHD1−/− mice at 9 days of age, after a 2-day exposure to 75% O₂: the TUNEL-positive cells for WT mice are 1046 ± 88 cells/mm² (n = 16) and for PHD1−/− mice are 677 ± 80 cells/mm² (n = 13) (P = 0.002; Fig. 2).

Stabilization of HIF-1α in PHD−/− Mice at P12 and Upregulation of HIF-1α and HIF-2α at P14-15 in the OIR Model

To investigate the protective mechanisms due to the absence of PHD1, we first measured the expression of two critical transcription factors at P12 (immediately after the mice were removed from 75% oxygen): HIF-1α and HIF-2α. Western blot analysis results showed that HIF-1α was degraded in WT mice, but stabilized in PHD1−/− mice in hyperoxia (Figs. 3A and 3B). Unfortunately, we were unable to detect HIF-2α, probably because its expression is too low and Western blot analysis is not sensitive enough to detect it.

To ascertain whether the HIF-hypoxia pathway is involved in the effects of PHD1 deficiency on ischemia-associated retinal NV, we further examined the expression of the two HIF transcription factors at P14-15 of OIR. Expression of HIF-1α was barely detected under normoxic conditions and was increased in both PHD1−/− and WT mice, but the degree of elevation was higher in the latter than in the former (P < 0.05; n = 4; Figs. 3C and 3E), which was probably due to the extended effect of the reduced vascular obliteration in PHD1−/− mice, leading to less HIF as a result of reduced hypoxia. Similarly, expression of HIF-2α was barely detected in normoxia and increased in hypoxic retina in both PHD1−/− and WT mice (Figs. 3D and 3F). One of the PHD1−/− mice appeared to show an upregulation of HIF-2α with OIR, which could be accounted for by the fact that each PHD isoform does not uniquely regulate a particular HIF isoform and may influence more than one to different degrees,7,14 but the difference between the two mouse lines was not significant (Figs. 3D and 3F).

Differential Expression Patterns of VEGF in WT and PHD1−/− Mice in the Hypoxic Phase of OIR

Under normoxic conditions, VEGF expression stays at baseline levels in both WT and PHD1−/− mice at the three time points, P12, P14-15, and P17: 60.9 ± 15.3 to 111.5 ± 23.0 pg/mg protein (n = 6) (Fig. 4). Interestingly, the expression of VEGF shows a differential pattern when comparing PHD1−/− and WT mice after the hypoxic phase of OIR. In WT mice, expression of VEGF is lower than baseline levels at P12, when mice are entering the hypoxic phase of OIR (21.0 ± 7.0 pg/mg protein; P < 0.05; n = 6), is up to the peak level at P14-15 (258.0 ± 82.0 pg/mg protein; P < 0.05; n = 6), and is reduced at P17 (still higher than the normal range (P < 0.05; n = 6), but not sensitive enough to detect it.

![Expression of VEGF in PHD1−/− and WT mice in the hypoxic and hyperoxic phases of OIR](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933248/ on 06/25/2017)
not significantly less than at P14: 204.0 ± 31.0 pg/mg protein), while in PHD1−/− mice, VEGF expression was significantly greater than normal at each time point (P < 0.05; n = 6), but decreased gradually from P12-P17: 414.0 ± 5.0 pg/mg protein at P12, 262.0 ± 82.4 pg/mg protein at P14-15, and 147.0 ± 30.0 pg/mg protein at P17 (Fig. 4). Another interesting observation is that VEGF expression is approximately 20 times higher in PHD1−/− mice than in WT mice after 75% O2 exposure for 5 days (at P12). However, we did not detect any neovascularization in any of the three retinal vascular layers: superficial, intermediate, or deep (data not shown); although VEGF protein is increased dramatically, suggesting a survival rather than proangiogenic activity of VEGF in this condition. These results show that VEGF levels are markedly elevated in PHD1−/− mice immediately after the hyperoxic phase, but thereafter are not different from WT mice with OIR. In most cases, EPO levels were too low to be detected in hyperoxic and hypoxic tissue with the total protein from one whole retina (200–300 mg).

**Suppression of Ischemia-Induced Retinal NV and Retinal Vascular Leakage in PHD1−/− Mice**

OIR was generated in 7-day-old pups of PHD1−/− and PHD1+/+ genotypes, as described in the Method section (Figs. 5 and 6). The mice were euthanatized at P17, when retinal NV is peaking in this model, and the retinas were harvested, fixed, and flat-mounted for griffonia simplcifolia iselectin B-4 (GSA-lectin) staining. The area of retinal NV tufts in the PHD1−/− mice (Fig. 5C) is much less than that of the PHD1+/+ mice (Fig. 5A). Further quantification showed that the retinal NV tufts were reduced by 59%: 0.69 ± 0.05 mm² for
DISCUSSION

A previous report\textsuperscript{19} shows that inhibition of PHD by an inhibitor that is not isoform-specific inhibited the hypoxia-induced central retinal vessel loss and subsequent retinal NV associated with OIR. The present study shows that this activity is largely or entirely dependent on the PHD1 isoform. We have also demonstrated that retinal NV is markedly inhibited in the absence of PHD1 due to the prevention of vascular obliteration in the hyperoxic phase. The inhibition of pathologic angiogenesis in these hypoxia-associated models appears to be due to the destabilization of HIF-1\textalpha and, as a result of this, a reduction of VEGF expression. It is known that PHD isoforms have different effects in different systems and the present results contrast with previous reports that show PHD1 predominantly regulates HIF-2\textalpha and HIF-1\textalpha is affected to a much lesser degree in muscle\textsuperscript{13} and cell cultures\textsuperscript{7} as a means of rendering cells more tolerant to hypoxia by shifting metabolism to an anaerobic state.\textsuperscript{1,13} The present study demonstrates that in the retina, PHD1 predominantly regulates HIF-1\textalpha. We found little or no effect on HIF-2\textalpha in the OIR model, but it is possible that we were not able to detect a slight upregulation of HIF-2\textalpha.

PHD1–3 mediate proteosomal degradation of HIF-1\textalpha by the ubiquitination-dependent Von Hippel-Lindau (VHL) complex, while factor inhibiting HIF-1 (FIH-1)-mediated hydroxylation leads to the inhibition of HIF-1\textalpha transactivation. This PHD-mediated activity is dependent on substrate oxygen and 2-oxoglutarate, a Krebs cycle intermediate, and cofactor Fe\textsuperscript{2+}, thus under hypoxic conditions, the PHDs are less active, leading to HIF stabilization. PHD-independent stabilization of HIF-1\textalpha can be achieved in hypoxic melanoma cells by bcl-2 via the molecular chaperone heat shock protein 90 (HSP90), which protects HIF-1\textalpha from proteosomal degradation.\textsuperscript{20} In an oxygen independent reaction, HSP90 can bind to HIF-1\textalpha, competing with receptor of activated protein kinase C, which mediates PHD and/or Von Hippel-Lindau-independent ubiquitination and proteosomal degradation of HIF-1\textalpha.\textsuperscript{21} Other posttranslational modifications of HIF-1\textalpha have also been reported.\textsuperscript{22-24} It is not known if alternative mechanisms for HIF-1\textalpha stabilization are operative in retina and, to our knowledge, no alternative mechanisms for HIF-2\textalpha stabilization have been reported.

HIF-1\textalpha and HIF-2\textalpha have been reported to have distinct cellular distributions in ischemic retina with HIF-1\textalpha being primarily associated with neuronal cells in the inner retina and HIF-2\textalpha being largely restricted to Müller cells and astrocytes.\textsuperscript{25} Our data shows that HIF-1\textalpha, which is normally upregulated in the hypoxic conditions of the OIR model, but not HIF-2\textalpha, is decreased in the absence of PHD1. These findings suggest that vascular endothelial cells and RPE cells, which secrete VEGF during oxidative stress,\textsuperscript{26,27} may also be associated with HIF-1\textalpha. In fact, HIF-1\textalpha has been reported as the predominant isoform in cultured RPE cells with the other isoforms being negligible.\textsuperscript{28} HIF-1\textalpha levels would be expected to be high in the absence of PHD1, regardless of the oxygen levels, so the apparent protection of the retina in the absence of PHD1 must cause a reduction of HIF-1\textalpha levels by alternative mechanisms.

Inhibition of the hypoxia→HIF-1\textalpha→VEGF pathway due to PHD1 deficiency leads to inhibition of ischemia-induced retinal NV. The regulatory mechanism by PHD1 in this model appears to be: lack of PHD1 prevents the degradation of HIF-1\textalpha in the hyperoxic state, which induces the expression of VEGF, thus preventing hyperoxia-related vessel loss. Without a vessel deficiency, there would not be relative hypoxia when the mice are returned to room air and there would be no need for signaling to initiate angiogenesis. The proposed cascades in which PHD1 is involved in this model are shown in Figure 7. Because PHD1 was already blocked before the hypoxic insults in these models and HIF-1\textalpha and VEGF levels would be expected to be increased in the absence of PHD1, therefore, inhibition of PHD1 may be more effective as a preventative strategy. There is no current evidence that blocking PHD1 would reduce established retinal NV, but its suppression of retinal NV by 59%, and its reduction of BRB breakdown and
Hyperoxia

\[ \downarrow \]

HIF1α

\[ \downarrow \]

VEGF

1st Hyperoxia phase

Vessel degeneration

\[ \downarrow \]

hypoxia

\[ ? \]

HIF1α

\[ \downarrow \]

VEGF

2nd Hypoxia phase

Retinal NV

\[ \rightarrow \]

Wild type

PHD1(KO)

Activation

Inhibition

SDF-1/CXCR4, that are implicated in vascular cell proliferation and survival and could play a role in ocular disease. These genes include erythropoietin (EPO), which like VEGF is up-regulated in hypoxic conditions in the absence of PHD1, plasminogen activator inhibitor-1, endothelin-1, and nitric oxide synthase 2.40,41 HIF-1α and its target gene, EPO, protect the retina from ischemic injury and retinal degeneration,42 but the RPE does not appear to be a source for EPO, as it is for HIF-1α and VEGF,43 so an alternative source within the retina must exist.

Oxidative stress is not only implicated in AMD and ROP, but plays a role in a variety of disease processes. Oxidative stress is involved in inflammation and neuroprotection, as well as angiogenesis and vascular permeability. It has been specifically associated with diabetic retinopathy,44,45 atherosclerosis,46 Alzheimer’s Disease, Parkinson’s Disease, epilepsy, amyotrophic lateral sclerosis, and other inflammatory and neurodegenerative disorders,47–48 suggesting that regulation of the PHDs could have beneficial effects in these types of disorders.

References


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