Prolyl Hydroxylase Inhibition During Hyperoxia Prevents Oxygen-Induced Retinopathy in the Rat 50/10 Model

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Purpose. To study the effect of systemic hypoxia-inducible factor prolyl hydroxylase inhibition (HIF PHDi) in the rat 50/10 oxygen-induced retinopathy (OIR) model.

Methods. Oxygen-induced retinopathy was created with the rat 50/10 OIR model. OIR animals received intraperitoneal injections of dimethyloxalylglycine (DMOG, 200 μg/g), an antagonist of α-ketoglutarate cofactor and inhibitor for HIF PHD, on postnatal day (P)3, P5, and P7. Control animals received intraperitoneal injections of PBS. On P14 and P21, animals were humanely killed and the effect on vascular obliteration, tortuosity, and neovascularization quantified. To analyze HIF and erythropoietin, rats at P5 were injected with DMOG (200 μg/g). Western blot or ELISA measured the levels of HIF-1 and Epo protein. Epo mRNA was measured by quantitative PCR.

Results. Alternating hyperoxia and hypoxia in untreated rats led to peripheral vascular obliteration on day P14 and P21. Rats that were treated with systemic DMOG by intraperitoneal injections had 3 times less ischemia and greater peripheral vascularity ($P = 0.001$) than control animals treated with PBS injections. Neovascularization similarly decreased by a factor of 3 ($P = 0.0002$). Intraperitoneal DMOG administration increased the levels of HIF and Epo in the liver and brain. Serum Epo also increased 6-fold ($P = 0.0016$). Systemic DMOG had no adverse effect on growth of rats treated with oxygen.

Conclusions. One of the many controversies in the study of retinopathy of prematurity is whether hyperoxia or alternating hyperoxia and hypoxia creates the disease phenotype in humans. We have previously demonstrated that PHDi prevents OIR in mice exposed to 5 days of sustained 75% oxygen followed by 5 days of 21% oxygen. The 50/10 rat experiments demonstrate that PHDi is also effective in a 24-hour alternating hyperoxia–hypoxia model. The rat OIR model further validates the therapeutic value of HIF PHDi to prevent retinopathy of prematurity because it reduces oxygen-induced vascular obliteration and retinovascular growth attenuation in prolonged and/or alternating hyperoxia.

Keywords: retina, ischemia, angiogenesis, retinopathy of prematurity, hypoxia-inducible factor, prolyl hydroxylase inhibition, erythropoietin

The study of retinopathy of prematurity (ROP) has been at the nexus of a progressive understanding of angiogenesis, vasculogenesis, and neovascularization because of excellent laboratory correlates to human disease.[1] Both the rat and mouse models of oxygen-induced retinopathy have proven the concept of a two-step pathogenesis of pathological neovascularization that begins with retinovascular growth attenuation and vascular obliteration, termed phase 1, and ends in reactive vasoproliferation, known as phase 2.[2] The rat 50/10 model uses fluctuating hyperoxia over a 24-hour cycle of 50% oxygen then 10% oxygen for 14 days, after which rats are returned to room air,[3,4] which stands in contrast to the mouse model which relies on 5 days of sustained hyperoxia. Although there is debate as to which model is more relevant to human ROP (e.g., whether absolute hyperoxia or fluctuating hyperoxia is causative to ROP[5]), the fact remains that oxygen plays a paradoxical role in the care of severely premature infants: it is necessary to sustain life but is simultaneously toxic to developing tissues such as the preterm retina.

The concept of a two-step hypothesis of pathologic neovascularization was first proposed by Ashton et al.,[6] and later proved by Pierce et al.[2] and Alon et al.,[7] who clearly showed that hyperoxia down-regulates critical growth factors, such as vascular endothelial growth factor (VEGF), required for normal blood vessel development. If VEGF was given exogenously during hyperoxia in the mouse, vascular obliteration and retinovascular growth attenuation associated with phase 1 was prevented and therefore phase 2 proliferations never occurred. This was the first observation that replacing gene products down-regulated by oxygen might prevent the disease phenotype.

This observation stimulated the idea that one possible strategy to circumvent the paradoxical role of oxygen was to up-regulate genes that oxygen down-regulates by using a pharmaceutical approach to override oxygen-induced catabolism of hypoxia-inducible factor (HIF).[8,9] HIF-1 and -2 are homologous heterodimers comprised of inducible α and constitutive β subunits.[9–11] The stability of HIF is regulated by prolyl hydroxylases, which induce hydroxylation on two

proline residues (Pro-402 and Pro-564) within the oxygen-dependent degradation domain (ODD) of the α subunit of HIF.[12,13] Hydroxylation makes HIFα a substrate of von Hippel-Lindau protein if proline is hydroxylated within the ODD.[14] The von Hippel-Lindau protein poly-ubiquitinates HIFα to create degradation signal to the 26S proteasome (Fig. 1). Because prolyl hydroxylase (PHD) uses cofactors and cosubstrates such as ascorbate and oxoglutarate, its inhibition can be induced by inhibitory oxoglutarate analogues, such as dimethyloxaloylglycine (DMOG), which competitively inhibits the hydroxylation of HIF-1/2α by displacing the endogenous oxoglutarate cofactor (Fig. 1).[8]

We have proven that this approach of HIF PHD inhibition (PHDi) works in the mouse prolonged hyperoxia OIR model.[15] In this investigation, we test whether this same strategy of stabilizing HIF prevents retinopathy in the fluctuating hyperoxia rat 50/10 model. We find that, like in the mouse, HIF PHDi prevents retinovascular growth attenuation and vascular obliteration. Furthermore, systemic administration of HIF PHDi is associated with stabilization of hepatic HIF-1 and increased levels of serum erythropoietin. The fact that this strategy is effective in two disparate models provides further stimulus to pursue this intervention as a possible therapy to prevent neovascularization from ischemic retinopathy by preventing ischemia in the first place.

Materials and Methods

Animal Model

All animal experiments were performed in strict adherence to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and in accordance with the National Institutes of Health and Cleveland Clinic Animal Research Committee guidelines. OIR was induced in Sprague-Dawley rat pups according to a protocol previously established.[4] In brief, within 6 hours after birth, normalized litters of 16 Sprague-Dawley rat pups with their nursing mothers were placed in Plexiglas incubators with an adjustable oxygen sensor and feedback system (ProOx; Biospherix, Lacona, NY) and were subjected to a variable oxygen exposure protocol. According to the protocol we were cycling newborn rats between 50% oxygen for 24 hours and 10% oxygen for 24 hours for seven cycles. At postnatal day (P)3, P5 and P7 the animals were injected with DMOG 200 μg/g body weight or control PBS. At P14 all litters were moved to room air. The weight of each animal was measured at P14 and P21.

Quantification of Vascular Obliteration and Angiogenesis

Animals were anesthetized on day P14 and P21 by intraperitoneal injection of ketamine hydrochloride (40 mg/kg) and xylazine hydrochloride (4 mg/kg). The heart was exposed through the diaphragm and the left ventricle injected with 0.5 mL 0.1% Evans Blue dye through a 30-gauge needle. The systemic spread of dye was confirmed by the presence of blue color throughout the skin and ear of the rat. Animals were killed with a lethal dose of intracardiac anesthetic and the eyes enucleated and placed in fresh 4% paraformaldehyde for 2 hours. Eyecups were dissected and retinal flatmounts created and examined under fluorescent microscopy. Images of retinas were acquired using a Nikon Microphot-2 fluorescent microscope (Nikon, Sendai, Japan). For quantitative analysis of the drop-out area (area of avascular regions in the periphery of the retina), vascular tortuosity, and tufting, retinal images were batch processed using ImageJ (National Institutes of Health [NIH], Bethesda, MD) or Image-Pro Plus 6.1 (Media Cybernetics, Rockville, MD) as previously described.[15]

Briefly, each image was converted to grayscale and thresholded (automated histogram search) to determine total retinal tissue area. For analysis of drop-out area, areas without capillaries were converted to white (gray value of 255) and were measured. Percentage drop-out was calculated as total drop-out area divided by total retinal area for each image. To determine tortuosity of large vessels emanating from the optic nerve, the grayscale images were median filtered to remove noise and contributions of small vasculature. Large vessels within these smoothed images were then segmented using a combination of intensity, length, and aspect ratio filter ranges (in this case the smaller area around the optic nerve was filled with black pixels). Extracted vessels were then skeletonized to produce 1-pixel-width medial lines and pruned to remove branches. Finally, for each vessel the tortuosity index was calculated as the path-length distance divided by the straight line (end-point to end-point) distance. Last, for determination of retinal tufting, the original grayscale image was again used and the areas with tufting were measured. Percentage of tufting was calculated as total tufting area divided by total retinal area for each image. Each DMOG treated group had an $n = 16$ (32 eyes) at P14 and $n = 12$ (24 eyes) for P21; control animals were equal in number.

Western Blot Analysis

Rats at P5 were injected with DMOG (200 μg/g) prepared as an aqueous solution (10 mg/mL) and the animals were euthanized 3 hours after the injection. Eyecups were prepared by removing the cornea, iris, and lens. The anterior lobe of the liver, brain, and kidneys were obtained from animals humanely killed and then embedded in ice. Organs were snap frozen immediately in dry ice and stored at –80°C. All tissues were placed in RIPA buffer (200 μL per 50 mg of tissue, pH 7.0) containing protease inhibitors, disrupted using a tightly fitted pestle, and centrifuged to remove particulate matter. A bicinchoninic acid protein assay (Pierce, Rockford, IL) was used to measure protein concentration. Lysates were subjected to 4% to 20% SDS-PAGE and electro-transferred to polyvinylidene difluoride membrane for immunoblotting. Membranes were blocked with 5% nonfat dried milk in Tris-buffered saline (TBS) and 0.1% Tween-20 and probed with anti-HIF-1α (Cayman Chemical, Ann Arbor, MI), and after being washed three times for 10 minutes with TBS and 0.1% Tween-20 and secondary antibody hybridization, revealed by chemiluminescence (Western Lightning; PerkinElmer, Waltham, MA); $n = 6$ for each Western blot.

Reverse Transcription and Quantitative PCR

Tissue from liver, kidney, brain, and retina were placed into 1 mL of RNAlater reagent (Qiagen, Germantown, MD) and stored at –80°C. Total RNA was extracted using RNeasy kit (Qiagen) and measured using NanoDrop (NanoDrop, Wilmington, DE) and standard spectrophotometric parameters. One microgram of total RNA from each sample was retrotranscripted to cDNA using QuantiTect Reverse Transcription Kit (Qiagen). One microliter of each cDNA sample was used as template for amplification reactions carried out with the QuantiTect SYBR Green PCR kit (Qiagen) following the manufacturer's instructions. PCR amplifications were performed in a 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA) with primers specific for rat Epo and rat HPRT (supplied by Qiagen). Quantitative PCR data analysis was performed with

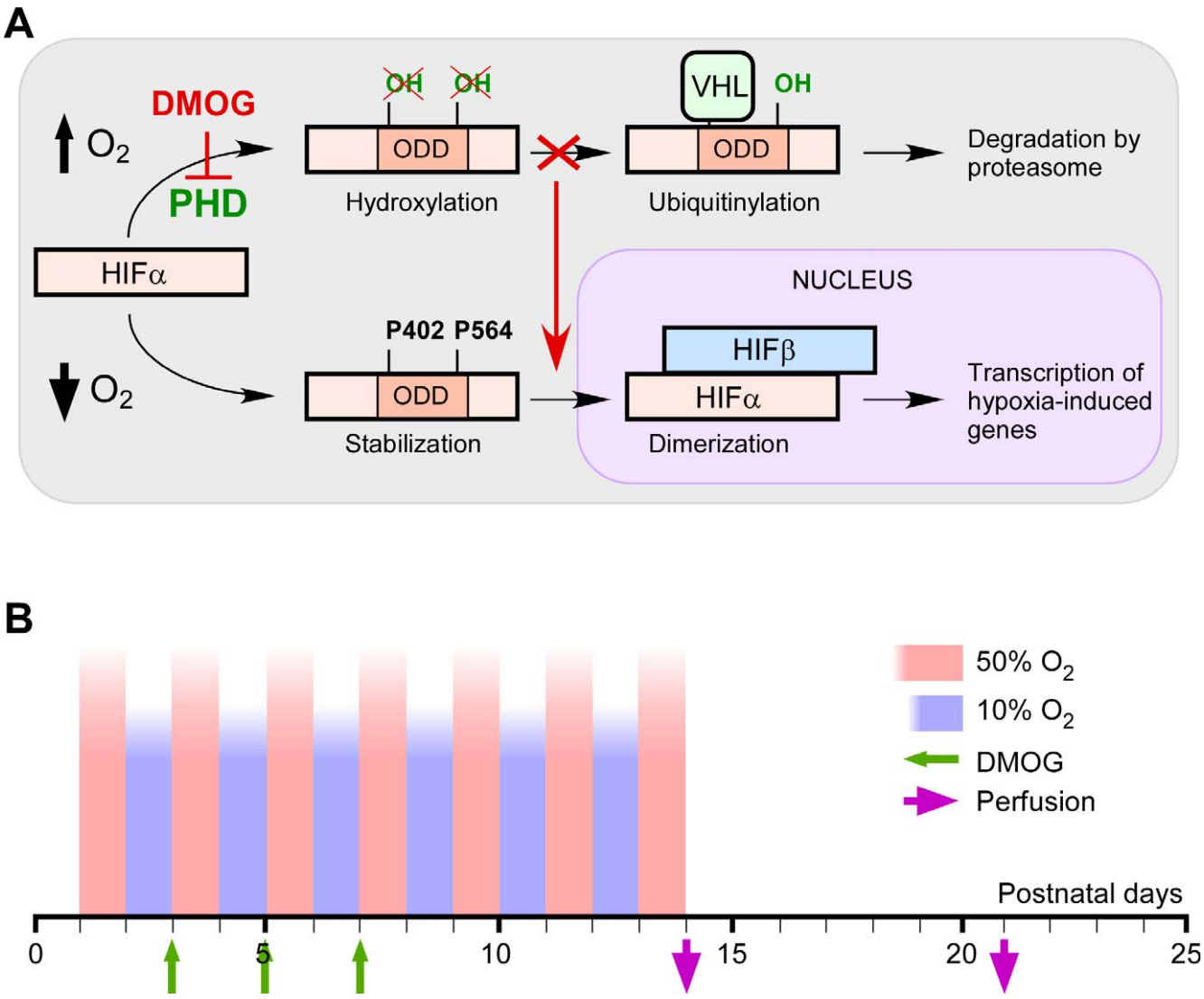

Figure 1. (**A**) Angiogenesis is controlled by oxygen through strict regulation of the HIF-1 pathway. The iron- and ascorbate-dependent HIF PHD enzyme hydroxylates a proline residue in the ODD of HIF-1α which allows the von Hippel Lindau (VHL) protein to target it to the proteasome. PHD uses cofactors of oxygen, iron, ascorbate, and oxoglutarate to achieve hydroxylation. DMOG replaces the oxoglutarate cofactor to inhibit PHD, blocking proline hydroxylation within the ODD, and thereby stabilize HIF-1α, activating the HIF-1 dimer. (**B**) Schema for experimental design. The rat 50/10 model was used to test intraperitoneal (IP) HIF PHD inhibitor DMOG. DMOG injections were given every other day for three injections beginning at P3 (*green arrows*, at P3, P5, P7). P0 rats where placed in alternating hyperoxia/hypoxia using 50% oxygen/10% oxygen with a computer-controlled ProOx chamber that was ventilated to remove trapped CO_2 until P14. At P14, rat pups were moved to room air until P21. The animals were perfused at either P14 or P21.

RQ Managed software (Applied Biosystems) using comparative ΔΔCt method and normalized to HPRT as endogenous control. DMOG-induced effect on Epo mRNA levels was expressed as fold-change differences from control (PBS injected); $n = 6$ for each RT-qPCR.

Epo ELISA

Six hours after intraperitoneal DMOG injection, P5 rat pups were anesthetized by ketamine and xylazine as described above and blood was drawn using a heparinized 27-G needle and syringe. Serum was diluted 1:10 with sample diluent provided by the manufacturer of mouse/rat Epo colorimetric ELISA (R&D Systems, Minneapolis, MN). Epo measurements were performed according to the manufacturer's instructions; $n = 6$ for each measurement.

Statistical Analysis

The size of the difference between the means of values obtained by a masked observer using Image-Pro analysis (Image-Pro Plus 6.1; Media Cybernetics), integrated optical density using ImageJ software (NIH), and absorbance used in the ELISA were compared with the standard error of that difference using the Student's *t*-test. The probability of error associated with rejecting the hypothesis of no difference between treated and untreated groups as a two-tailed test was calculated and is supplied in the figures.

Results

The strategy we utilized to test PHDi in the rat 50/10 model is shown in Figure 1B. We had two goals in developing this

strategy. First, we chose to use systemic administration of the HIF PHD inhibitor because we believe that intraocular injection at P3 changes the phenotype of the animal model even in the control groups and would be analogously impractical in severely premature human infants. Second, we desired to administer treatment early in postnatal life because our hypothesis was that HIF PHDi in the rat 50/10 model would override hyperoxia-induced retinovascular growth attenuation and thereby deprive the model of its substrate for disease by decreasing the peripheral avascular retina.

Critical to the rat 50/10 model is the weight of rat pups. For example, a central component of the success of the rat 50/10 model is litter size, in fact, authors expert in this model recommend that small litters be supplemented with additional pups in order to create reproducible peripheral ischemia, presumably because less rapid growth promotes worse retinopathy. Figure 2 demonstrates near identical growth patterns as measured by weight in PBS sham-treated and DMOG-treated pups injected at P3, P5, and P7.

Using the schema shown in Figure 1B, we administered 200 μg/g bodyweight DMOG using intraperitoneal injection via a 30-gauge needle and 1-mL syringe. Animals were humanely killed at P14, at the end of oxygen cycling and placement into room air, and at P21, at the end of the 50/10 model overall. The effect of DMOG injections at P3, P5, and P7, compared to both control PBS injections in half the littermates and compared to room air–raised pups was assessed at P14 or the end of fluctuating hyperoxia and beginning of relative hypoxia (Figs. 3A–C) and at P21 or the end of the hypoxic phase (Figs. 3D–F). Peripheral avascular zone was calculated by measuring the total avascular area from the ciliary body and pars plana to the avascular/vascular boundary, marked in dashed lines (Fig. 3G). Typical neovascularization is shown in Figure 3H, and arrows mark tortuosity in Figure 3I. Using a computerized automated imaging program we were able to show statically significant reduction of peripheral avascularity and tufting or neovascularization (Figs. 3J, 3K). Although tortuosity was reduced, it was not a statistically significant effect at either P14 or 21 (Fig. 3L). This experiment confirmed that PHDi can override hyperoxia-induced retinovascular growth attenuation because clearly at P14, there is less peripheral avascular tissue in DMOG-treated animals. This presumably reduces the concentration of intravitreal VEGF thereby reducing tufting or intravitreal neovascularization, described by Budd et al.[16]

Given the canonical pathway of HIF-1α catabolism induced by HIF PHD (Fig. 1A), the natural expectation of our lab was that systemic PHDi using the well-known inhibitor DMOG would predict that the protective phenotype induced by DMOG would be accompanied by an increased protein concentration of retinal HIF-1α. However, just as in the mouse, we found that retinal HIF concentrations were not increased by systemic PHDi, but rather only hepatic and brain HIF was responsive to intraperitoneal DMOG (Fig. 4A). We did not test intravitreal DMOG injection in the newborn rat because we believe the utility of intravitreal injection is limited in comparison to systemic administration should PHDi become a possible strategy to prevent ROP in humans, and repeated intravitreal injection corrupts the oxygen-induced retinopathy model because these pups are too small at P3 to accomplish this experiment. Even PBS injections may change the outcome of the model through inflammation and associated cytokine release, and certainly repeated injections might damage the lens or retina in this age animal.

We confirmed the up-regulation of HIF-1α in liver and brain by quantitative PCR targeting Epo mRNA, which is regulated by HIF. In synergy with our Western blots, Epo mRNA was increased in liver and brain, but not within statistical significance in retina (Fig. 4B). Serum Epo increased by 6-fold in DMOG-treated animals (Fig. 4C), likely reflecting the hepatic expression of Epo after intraperitoneal DMOG.

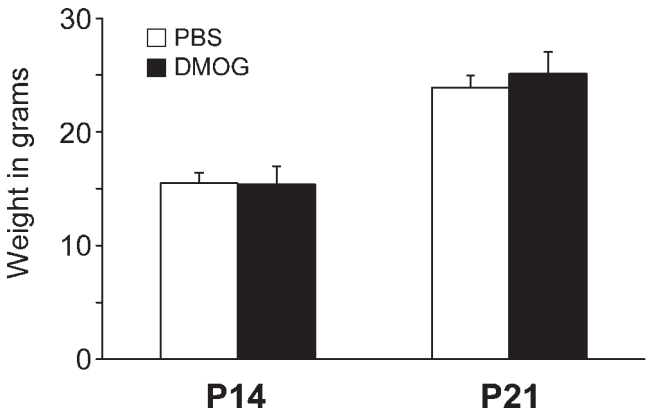

FIGURE 2. Rat pup weight. DMOG injection at P3, P5, and P7 did not result in statistically significant changes in the weight of OIR-treated animals at P14 ($n = 14$) or P21 ($n = 12$). The weight of pups can determine the severity of oxygen-induced retinopathy, therefore all pups were weighed individually to standardize the dose of HIF PHD inhibitor and to make sure there was no weight loss or gain from these treatments.

CONCLUSIONS

This investigation demonstrates that stabilization of HIF-1α protein through inhibition of HIF PHD early in postnatal life of rats prevents oxygen-induced retinovascular growth attenuation, thereby preventing the disease phenotype by decreasing the peripheral avascular retina. In the mouse, we found that HIF PHDi prevents posterior capillary dropout and ischemia during hyperoxia.[15] Analogously, in the rat, which closer approximates the human disease correlate, we demonstrate here that PHDi overrides growth retardation induced by oxygen enabling the neonatal rat retina to grow further peripherally by P14 in comparison to littermate controls.

Our search to identify a systemic approach to protect the retina against hyperoxia damage stems from the paradoxical finding that severely preterm infants require oxygen to stay alive, yet the very same oxygen is toxic to developing tissues. Our approach regarding the use of PHDi comes directly from two sources. First, we and others have made the observation that in the Neonatal Intensive Care Unit, decreasing hyperoxia early in gestational age reduces not only the severity of ROP but even the incidence of ROP overall, implying that gentle stimulation of retinovascular growth deprives the disease of its substrate, which is the peripheral avascular retina.[17–19] This hypothesis is nicely tested in the rat OIR model described here. Second, the landmark studies by Pierce et al.[2] and Alon et al.[7] were the first to show that giving back VEGF during Phase 1 was able to prevent Phase 2 vasoproliferation. Therefore, our strategy was designed to build on this finding but utilized the unique approach of stimulating a transcription factor that regulates the expression of angiogenic growth factors, such as VEGF and Epo, to direct the sequential and coordinated growth of the retina and its vasculature. We believe that the power of this approach is simply that HIF regulates the expression of many genes essential to retinovascular homeostasis and therefore it is sensible to suppose that the approach of stabilizing a transcription factor recapitulates normal development better than one of its gene products.

Given the central role that HIF plays in metabolism, angiogenesis, and hematopoiesis, it is natural that HIF

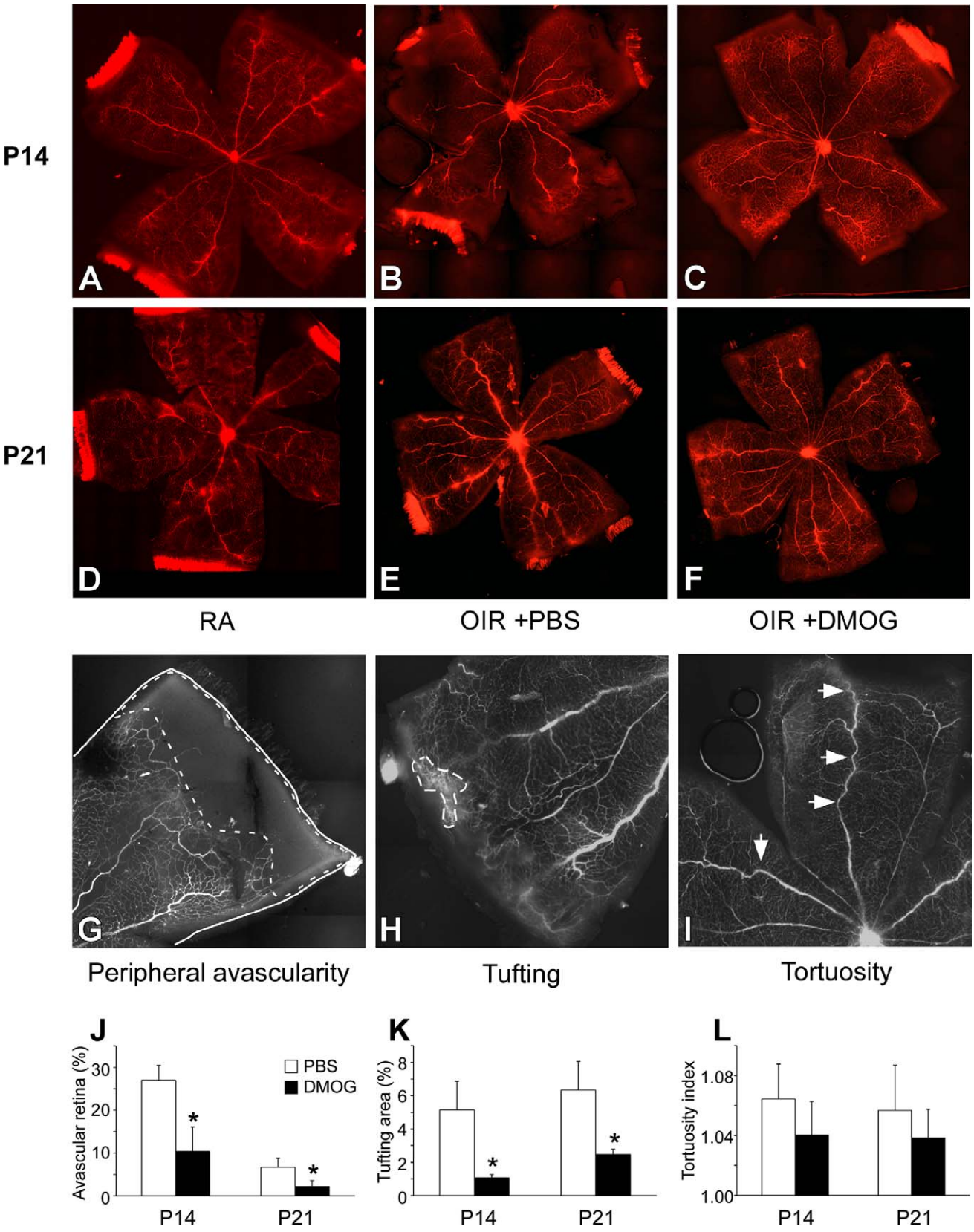

FIGURE 3. Perfused and flat-mounted rat retina at P14 and P21 with statistical evaluation of tortuosity, avascularity, and neovascularization (tufting). Inhibition of retinopathy was induced by intraperitoneal administration of DMOG at P3, P5, and P7. Representative flatmounts at P14 of OIR from (**A**) rat pups raised in room air ($n = 12$), (**B**) pups raised in hyperoxia and sham injected with PBS ($n = 14$), and (**C**) pups raised in hyperoxia and injected with DMOG ($n = 16$). Representative flatmounts are next shown at P21 of (**D**) rats raised in room air ($n = 10$), raised in hyperoxia and injected with PBS ($n = 10$) and (**E**) rat pups raised in hyperoxia and injected with DMOG ($n = 10$). Magnified images showing a quarter of the retina can also be seen in Figures 3G through 3I. Representative examples are marked with *dashed lines* to show the border of avascular/vascular retina, areas of

neovascularization, and with *arrows* to show vascular tortuosity (**G–I**). Quantification of tortuosity, peripheral vascular drop-out, and tufting shows a statistically significant decrease (denoted by *asterisks*) in peripheral vascular drop-out and tufting, but not tortuosity in DMOG-injected animals at P14 (**J–L**). P21 (**J–L**) shows statistical significance (denoted by *asterisks*) in all categories except tortuosity. These experiments demonstrate that HIF PHDi can override retinovascular growth retardation by oxygen, thereby depriving the disease of its substrate, the peripheral avascular retina.

regulation is complex and extends beyond the HIF PHDi paradigm illustrated in Figure 1. In addition to hydroxylation of proline residues close to the N-terminal transactivation domain by PHDs under oxygenated conditions, HIF is also regulated by another member of the iron-dependent dioxygenases family called FIH (factor inhibiting HIF). Under well-oxygenated conditions, FIH hydroxylates a conserved asparaginyl residue in the C-terminal transactivation domain that prevents binding of p300 and CBP.[20,21]

Signaling pathways also regulate HIF activity via S-nitrosylation.[22] Nitric oxide can bind covalently to thiols such as Cys 533 in mice and Cys 520 in humans within the ODD, stabilizing HIF-1α.[23] In contrast, iron and ascorbate are endogenous negative regulators of HIF activity through the role they play as cofactors for PHDs.[24] Krebs cycle intermediates are also known to regulate HIF activity through competition with the 2-oxoglutarate (α-ketoglutarate) cofactor to PHD. The HIF hydroxylases are inhibited by citrate, isocitrate, succinate, fumarate, malate, oxaloacetate, and pyruvate.[25–29] Finally, these direct metabolic effects are also tempered by the effect of metabolism on reactive oxygen species (ROS) formation in the mitochondria. ROS are hypothesized to play a role in ROP in humans and rats.[30,31] Blockade of ROS formation has been associated with increased prolyl hydroxylation and therefore decreased HIF activity.[32–34]

The experiments described within this investigation also correlate to genetic manipulation of PHD in the mouse. PHDs are an evolutionarily conserved subfamily of dioxygenases that all use Fe(II) and ascorbate as cofactors and oxoglutarate and oxygen as cosubstrates.[35] PHDs have been identified in plants, prokaryotes, and mammalian cells. In the latter group, four isoforms have been identified: PHD1/EGLN2/HPH3, PHD2/EGLN1/HPH2, PHD3/EGLN3/HPH1, and a recently characterized protein named as P4HTM. In two studies that examine PHD1 and 2 deficiency, reduction of oxygen-induced retinopathy in mice was achieved by inducible systemic PHD2 deficiency beginning at P1 using oral tamoxifen and systemic PHD1 ablation in a nonconditional PHD1 knock-out mouse.[36,37]

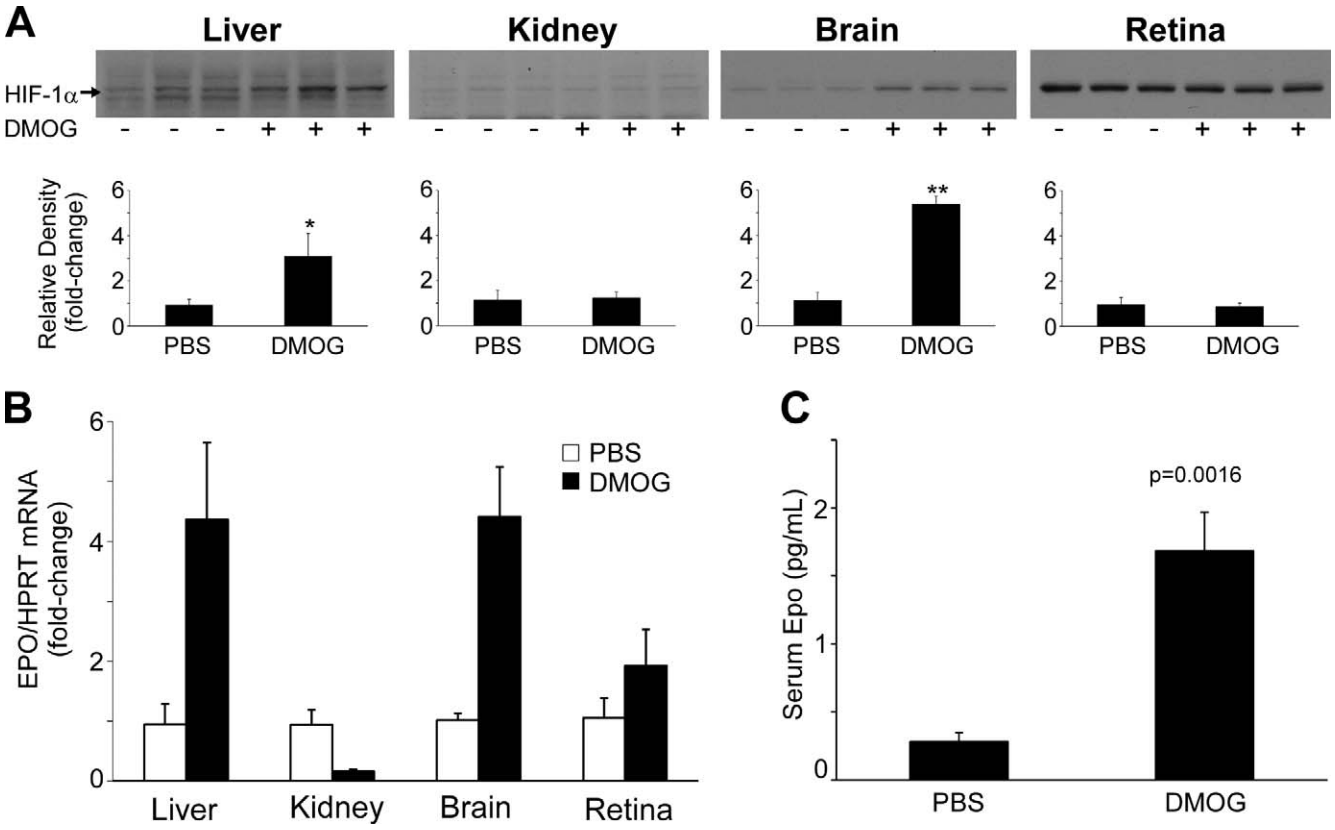

Figure 4. Western Blot of HIF-1, quantitative PCR for erythropoietin, and serum erythropoietin levels after systemic PHD inhibition. Immunoblot ($n = 3$) using anti-HIF-1α antibody demonstrates up-regulation of HIF-1α protein 6 hours after intraperitoneal injection of DMOG 200 μg/g (**A**). DMOG has tropism for liver in rat as well as brain, but does not affect kidney or retinal HIF-1α protein levels with statistical significance. Animals were euthanized 6 hours after injection, buried in ice, and organs snap frozen in ethanol/dry ice mixture. The up-regulation of HIF in brain is a surprising result, and as yet it is unknown whether it follows stimulus from liver induction of HIF or direct inhibition of brain HIF prolyl hydroxylase. All densitometric values are first normalized to control lanes (PBS injection). Epo PCR ($n = 3$) correlates nicely with Western blot indicating that HIF gene product (Epo) follows HIF-1α stabilization (**B**). Again, liver and brain demonstrate greatest stabilization of HIF-1α and up-regulation of Epo mRNA. Increased expression of Epo ($n = 3$) at P5, 6 hours after intraperitoneal DMOG injection, correlates with an increase in serum Epo concentration (**C**). ELISA for serum Epo synthesis is 6-fold elevated compared to PBS-injected control animals. *Represents statistically significant data. **Represents very statistically significant data.

It is the latter two reports from Duan et al.[36] and Huang et al.[37] that challenge the importance of our finding in the mouse, and now here in the rat, which is that systemic inhibition of all three PHD isoforms leads to prevention of oxygen-induced retinopathy by hepatic stabilization of HIF-1α. Clearly oxygen induces down-regulation of retinal HIF.[38] And we know that direct injection of PHDi into the adult rat eye increases HIF-1α protein expression (data not shown). It may well be that the elegant experiments of Duan et al.[36] and Huang et al.,[37] and our experiments in both the rat and mouse, prove that PHD deficiency and inhibition works to protect retinal vasculature from hyperoxia either by locally stabilizing HIF or by globally inducing the liver to act as a large endocrine organ that produces enough relevant cytokines through hepatic HIF stabilization to rescue the retina from growth retardation. Certainly, a systemic approach offers the possibility that other organs systems, such as the lung, intestine and brain, which suffer from hyperoxia-induced suppression of oxygen-related factors of the preterm infant, might also benefit from PHDi. Although early relative hypoxia in human neonates is associated with decreased ROP, worrisome trends in mortality have recently been associated with reduced oxygen levels in two randomized prospective trials.[39,40] Therefore, PHDi might help the severely premature infant gain the benefit of oxygen supplementation (reduced mortality) while avoiding down-regulation of vital growth factors that normally occurs in phase 1. Further studies are in progress with regard to ablation of hepatic HIF and whether systemic PHDi prevents the disease phenotype in these conditional knock out mice as well.

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