Sildenafil Attenuates Vaso-Obliteration and Neovascularization in a Mouse Model of Retinopathy of Prematurity

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PURPOSE. We sought to determine the effect of sildenafil on retinal vascular changes in a mouse model of oxygen-induced retinopathy (OIR).

METHODS. Vascular defects in OIR mice were quantified by measuring vaso-oblation at postnatal days 12 and 17 (P12 and P17) and neovascularization at P17 to compare sildenafil-treated to dextrose-treated OIR mice. Retinal HIF1 α protein expression was quantified by Western blotting and normalized to that of β -actin. Right ventricular hypertrophy was measured by Fulton's index as a surrogate for hyperoxia-induced pulmonary hypertension.

RESULTS. At P12, OIR mice treated with sildenafil demonstrated a 24% reduction in vaso-oblation ($P < 0.05$), whereas at P17, treated animals showed a 50% reduction in neovascularization ($P < 0.05$) compared to dextrose-treated controls. Sildenafil-treated OIR mice had stabilization of retinal HIF1 α at P12, immediately after hyperoxia. At P17, sildenafil-treated OIR mice had decreased HIF1 α relative to untreated mice. OIR mice developed right ventricle hypertrophy that was significant compared to that in room air controls, which was abrogated by sildenafil.

CONCLUSIONS. Sildenafil treatment significantly decreased retinal vaso-oblation and neovascularization in a mouse OIR model. These effects are likely due to sildenafil-induced HIF1 α stabilization during hyperoxia exposure. Furthermore, we confirm disease overlap by showing that OIR mice also develop hyperoxia-induced right ventricular hypertrophy, which is prevented by sildenafil. This study is a first step toward delineating a potential therapeutic role for sildenafil in OIR and further suggests that there may be common pathophysiologic mechanisms underlying hyperoxia-induced retinal and pulmonary vascular disease.

Keywords: sildenafil, retinopathy of prematurity, mouse, neovascularization, vaso-oblation

Retinopathy of prematurity (ROP) is a leading cause of blindness in infants.¹ Despite improvements in therapeutic interventions, ROP continues to be a major cause of childhood visual impairment, with approximately 500 cases of ROP-related blindness reported annually.^{2,3} Two major predictors of ROP progression in infants are exposure to hyperoxia and gestational age at birth.^{4,5} Although oxygen therapy is essential for promoting tissue oxygenation in preterm infants, it has the unfortunate side effect of disrupting the normal transition from in utero hypoxia to normoxia, which is essential for healthy vascular and neuroretinal development in the newborn. The hyperoxic environment associated with mechanical ventilation results in phase 1 ROP. The immature retinal vasculature exposed to hyperoxia fails to develop, with the final result being decreased angiogenic activity and subsequent avascular peripheral retina.⁶ The return to normoxia as the infant matures and their lung disease resolves, coupled with metabolic maturation of the retina results in the proangiogenic phase 2 of ROP. Starved for oxygen, the avascular retina undergoes a rapid increase in angiogenesis in an attempt to restore adequate oxygen supply. However, instead of restoring

oxygenation, this rapid angiogenic push results in the formation of disorganized preretinal neovascularization.⁷ As a result of these neovascular tufts, plasma leakage, bleeding, tractional retinal detachments, and sight-threatening complications of ROP can ensue.^{8,9} The extent and severity of the observed neovascularization are believed to correlate with severity of avascular retina during phase 1.¹⁰

Oxygen therapy in premature infants is also associated with pulmonary complications, including bronchopulmonary dysplasia (BPD) and BPD-associated pulmonary hypertension, which are frequent comorbidities in infants who develop ROP.¹¹ Many studies have addressed BPD prevention, but little has been shown to be effective treatment. Inhaled nitric oxide has been shown in animal studies and some human studies to be beneficial, but results have been mixed.¹² Even less is known about the prevention of BPD-associated pulmonary hypertension, but those infants are known to be at a higher risk for ROP, longer hospital stays, and increased mortality.^{11,13} Sildenafil, a phosphodiesterase 5 (PDE5) inhibitor, has been shown to be safe and effective therapy in term infants with pulmonary hypertension, where the pharmacokinetics have been studied.¹⁴ Furthermore, sildenafil was safe and effective in

improving pulmonary hypertension in a small study of BPD-associated pulmonary hypertension.^{12,15}

Given the significant overlap between ROP and BPD-associated pulmonary hypertension in patients, especially realizing the potentially vicious circle associated with lung disease further exacerbating hypoxia and subsequent retinopathy, we wanted to explore the potential effects of sildenafil in ROP. If this approach proved successful, it would provide a noninvasive intervention that could simultaneously address two vascular diseases associated with high morbidity in neonates. To examine this question, we used an established mouse model of ROP, known as oxygen-induced retinopathy (OIR), as originally described by Smith et al.¹⁶ in 1994. This model recapitulates the two phases of ROP as well as the retinal ischemia and neovascularization that are hallmark lesions of ROP in human disease.

METHODS

Mouse Model of OIR

This study was approved by the Institutional Animal Care and Use Committee at Northwestern University and compliant with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. C57/BL6N mouse pups (Charles River, Wilmington, MA) were placed in a poly(methyl methacrylate) (Plexiglas) chamber with an oxygen controller (Biospherix, Lacona, NY) and exposed to 75% O₂ from postnatal day 7 to 12 (P7–P12), to induce phase 1 OIR, where retinal vaso-obliteration and capillary dropout occurs. Exposure to hyperoxia was continuous, with brief interruptions (less than 15 minutes) for animal care and treatment of neonatal pups. The oxygen controller (Pro-Ox 110; Biospherix) continuously samples the gas within the chamber and regulates gas flow to maintain the target oxygen concentration at 75% ± 1% O₂. Dams were rotated from hyperoxia to room air every 48 hours at the time pups received sildenafil treatment to prevent excessive oxygen toxicity to the adults. After completion of hyperoxia, the pups were returned to room air and euthanized at either P12 or P17 for retinal and heart tissue harvest. Age-matched control pups were maintained in room air during the duration of the experiment.

Eight different litters of C57/BL6 mice were used for quantification of vaso-obliteration and neovascularization and protein analysis at P17, and four matched litters of mice were used for protein analysis and retinal flat mounts at P12.

Sildenafil Treatment

OIR model and room air pups were randomly assigned to treatment with low-dose sildenafil^{17,18} (3 mg/kg, subcutaneously, every other day; Revatio; Pfizer, New York, NY) or dextrose vehicle, for a total of three injections from P7 to P12.

Retina Dissection, Immunostaining, and Flat-Mounting

Eyes for flat mounting were enucleated and fixed in 10% neutral buffered formalin overnight at room temperature. The retinal cups were isolated as previously described,¹⁹ and placed overnight in a 0.5% Triton X-100 solution to improve vascular visibility. The retinas were washed in PBS and then stained overnight with fluorophore-labeled G isolectin B4 (Alexa Fluor 594-conjugated [product no. I21413; Invitrogen, Carlsbad, CA]; 1:100 dilution in 1 mM CaCl₂ in PBS). Following

a 3-hour wash in PBS, the retinal cups were cut into quadrants and flat mounted.

Image Acquisition

Immunostained retinal flat mounts were imaged using an Eclipse 80i upright microscope (Nikon, Tokyo, Japan) with an ES CoolSnap fluorescence intensity camera (Photometrics, Tucson, AZ). Four individual images of retinal quadrants were taken at 5× magnification and merged using Photoshop software (Adobe, San Jose, CA). The resulting composite image of the entire retinal flat mount was used for subsequent image analysis.

Image Analysis and Quantification

Vaso-obliteration was quantified using ImageJ software (National Institutes of Health, Bethesda, MD). Using the “polygon tool,” the entire retinal area was traced and quantified as a number of pixels. The avascular area of the retina was highlighted in the same manner. The avascular area-to-total retinal area ratio was used to determine the percentage of vaso-obliteration.

Neovascularization was quantified using the SWIFT_NV macro set (courtesy of Lois Smith, MD, and Andreas Stahl, PhD) and ImageJ software as described previously.²⁰ Briefly, the composite flat-mount image is divided into quadrants and assigned a threshold value based on fluorescence intensity such that only the areas of greatest intensity (corresponding to neovascularization) are preserved as a “neovascularization” map. The individual maps for each quadrant are then combined to give a neovascularization overlay for the entire retinal flat mount. The area of the overlay can be compared to the overall area of the retina, less any avascular areas subject to vaso-obliteration, to obtain percent neovascularization for each individual flat mount. For each flat mount, quantification was performed by two independent graders (AAF and JCC) in a masked fashion, and the average of their measurements was used for subsequent analysis.

Immunohistochemistry of Cross-Sections

Paraffin-embedded retinal sections (5 μm) were deparaffinized in xylene and rehydrated through a series of ethanol concentrations. Retinal sections underwent high-temperature antigen retrieval by incubation in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0) at 100°C for 20 minutes. Next, sections were placed in a blocking solution (10% serum with 1% BSA in Tris-buffered saline [TBS]) for 2 hours and then incubated with α-glial fibrillary acidic protein (α-GFAP; 1:200 dilution; Abcam, Cambridge, MA) and α-rod-arrestin (1:10,000 dilution; courtesy of Cheryl Craft, University of Southern California) overnight at 4°C. The following day, sections were washed and incubated in an anti-rabbit immunoglobulin G (IgG) secondary antibody (1:200 dilution; DyLight 594; Abcam) and anti-mouse IgG secondary antibody (1:200 dilution; DyLight 488; Abcam) for 2 hours at room temperature. Sections were washed in 0.1% Sudan black in 70% ethanol for 25 minutes to remove retinal autofluorescence. Sections were washed, mounted, and then sealed. Cross-sections from three different mice in each group were used for this experiment, with the caveat that they centered on the optic nerve.

Western Blot Analysis

Retinas were lysed in 1× Mg lysis buffer (Millipore, Billerica, MA) supplemented with a protease inhibitor cocktail (Sig-

TABLE. Summary of Body Weights per Group

Group	Body Weight, g
P12 Normoxic control, <i>n</i> = 6	6.64 ± 0.2
P12 Normoxic sildenafil, <i>n</i> = 8	6.24 ± 0.27
P12 Hyperoxic control, <i>n</i> = 7	5.85 ± 0.12*
P12 Hyperoxic sildenafil, <i>n</i> = 8	5.83 ± 0.1*
P17 Normoxic control, <i>n</i> = 13	7.62 ± 0.1
P17 Normoxic sildenafil, <i>n</i> = 12	7.28 ± 0.15
P17 Hyperoxic control, <i>n</i> = 18	6.47 ± 0.12*
P17 Hyperoxic sildenafil, <i>n</i> = 17	6.5 ± 0.12*

At both P12 and P17, both OIR groups (vehicle and sildenafil) had statistically lower weights than their normoxic counterparts. There were no statistical differences between normoxic control and normoxic sildenafil mice at either P12 or P17 based on ANOVA with Bonferroni post hoc analysis.

**P* < 0.05 versus age-matched control

ma-Aldrich, St. Louis, MO) and a phosphatase inhibitor cocktail (Millipore) and sonicated. Retina HIF1 α protein expression was assessed via Western blotting as previously described.²¹ Briefly, membranes were blocked at room temperature with 1% BSA (Sigma-Aldrich) in Tris-buffered saline with Tween-20 (TBST) for blocking and incubated overnight at 4°C with HIF1 α primary antibody (Cayman Chemical, Ann Arbor, MI) in blocking buffer at 1:500 dilution or with β -actin primary antibody (Sigma-Aldrich) at 1:5000 dilution, followed by secondary antibody (anti-rabbit and anti-mouse, respectively, 1:1000 dilution; Cell Signaling, Danvers, MA). Membranes were then washed and exposed via chemiluminescence (Supersignal West Femto; Thermo Scientific, Hanover Park, IL). Bands were analyzed using ChemiDoc XRS (Bio-Rad, Hercules, CA) and normalized to those of β -actin. Data are fold change $\pm$ SEM relative to that of control mice.

Measurement of Right Ventricular Hypertrophy

Mouse hearts were dissected, and right ventricles and left ventricles plus septums were weighed. Fulton's index (right ventricle weight divided by left ventricle plus septum weights) was used to assess right ventricular hypertrophy (RVH) as a marker for pulmonary hypertension.^{18,22}

Statistical Analysis

Results are means $\pm$ SEM with “*n*” representing number of animals. Vaso-oblation and neovascularization were compared using a 2-tailed Student's *t*-test between groups. Body

weight and Western blot data were analyzed by ANOVA with post hoc Bonferroni analysis using Prism software (Graphpad Software, Inc., San Diego, CA). Differences were considered statistically significant at a *P* value of <0.05.

RESULTS

Sildenafil Does Not Impact Body Weight in OIR Mice

Average weight for the OIR vehicle-treated group at P12 (*n* = 7) was 5.85 g compared to 6.64 g (*n* = 6) for the normoxic group (*P* < 0.05; Table). Average weight for the OIR vehicle-treated group at P17 (*n* = 18) was 6.47 g compared to 7.62 g (*n* = 13) for the normoxic group (*P* < 0.05; Table). At both P12 and P17, sildenafil had no effect on body weight in either normoxic or OIR mice.

Sildenafil Reduces Retinal Vaso-Obliteration and Neovascularization in OIR Mice

To evaluate the potential therapeutic effect of sildenafil in ROP, we compared the percentages of vaso-oblation and neovascularization in retina of P12 and P17 OIR mice dosed with sildenafil during hyperoxia compared to those in OIR mice receiving vehicle. At P12, OIR mice treated with sildenafil showed significantly reduced avascular area (26.02% $\pm$ 2.0% of total retinal area; *n* = 5; Fig. 1) compared with controls (34.13 $\pm$ 2.0% of total retinal area; *n* = 6, *P* < 0.05; Fig. 1). Of the total mice eyes in each group (Table), some eyes yielded suboptimal quality retinal flat mounts and were excluded from flat mount quantification. No neovascularization was seen in either group at P12. At P17, sildenafil-treated OIR mice showed significantly reduced neovascularization (1.6% $\pm$ 0.3% of total retinal area; *n* = 13) compared with that in vehicle-treated OIR mice (3.2% $\pm$ 0.5% of total retinal area; *n* = 13, *P* < 0.05; Fig. 2). Both graders were masked to the treatment group and were trained extensively in the use of this technique and showed good inter-rater reliability. For quantification of vaso-oblation intra-class-correlation, coefficient was 0.85, whereas for neovascularization it was 0.70.

Increased Expression of Müller Cell GFAP in OIR Mice

Upregulation of GFAP in Müller cells has been shown to occur in response to retinal stresses including mouse OIR.^{16,23} Our data confirm the finding of increased GFAP expression in Müller cells of OIR compared to that in normoxic mice. We

FIGURE 1. Sildenafil reduces vaso-oblation at P12 in the OIR mouse model. P12 OIR mice were treated with dextrose (*n* = 6; [A]) and sildenafil (*n* = 5; [B]). Sildenafil significantly reduced the amount of vaso-oblation seen at P12 versus that in control (26.02% vs. 34.12%, respectively; **P* < 0.05; [C]). Red # indicates area of overexposure; these areas did not affect our assessment of vaso-oblation because they were located in peripheral vascularized retina.

FIGURE 2. Sildenafil reduced neovascularization at P17 in the OIR mouse model. P17 OIR mice were treated with dextrose ($n = 13$; [A]) and sildenafil ($n = 13$; [B]). No differences were seen in the area of vaso-obliteration between sildenafil-treated and control OIR at P17 ([C]; 14.0% vs. 13.9%, respectively). Sildenafil significantly reduced the amount of neovascularization seen at P17 versus that in control ([D]; 1.6% vs. 3.2%, respectively; * $P < 0.05$).

wanted to explore whether the improved vasculature associated with sildenafil treatment had a beneficial effect on Müller cell stress as measured by GFAP expression. Sildenafil treatment did not appear to have any effect on Müller cell GFAP expression in OIR mice at P17 (Fig. 3). Although sildenafil may have had a modest impact on GFAP staining in normoxic glial cells on the retinal surface, we did not see effects on the retinal Müller cells in contrast to the effects seen in the OIR mice (Fig. 3).

Sildenafil Stabilizes HIF1 α During Phase 1 of OIR

Given the fact that sildenafil has been shown to stabilize HIF1 α in the setting of hind-limb ischemia as well as in a different murine model of hyperoxia-induced lung disease,^{24,25} we sought to determine whether sildenafil would stabilize HIF1 α in the retina. At P12, within less than an hour of removal of mice from the hyperoxia chamber, sildenafil significantly increased HIF1 α protein expression in OIR mice compared to that in vehicle mice (Figs. 4A, 4B; 5.3 ± 2.9 -fold vs. 0.9 ± 0.3 -fold [$P < 0.05$; $n = 7$ and 5], respectively). Furthermore, as previously described,²⁶ HIF1 α expression increased with retinal hypoxia during phase 2 OIR. However,

at P17, sildenafil-treated OIR mice had significantly less HIF1 α protein expression than vehicle-treated OIR mice (Figs. 4C, 4D; 1.2 ± 0.2 -fold vs. 2.8 ± 1.1 -fold [$P < 0.05$; $n = 8$ and 7], respectively).

OIR Mice Develop Significant Right Ventricular Hypertrophy, Which Is Abrogated by Sildenafil

In this study, we sought to determine whether OIR mice developed pulmonary hypertension and RVH as evidence of a common vascular pathophysiology. We used RVH as a proxy for pulmonary hypertension in these very small mice.¹⁸ OIR mice developed RVH as measured by Fulton's index at P12 compared to room air controls (Fig. 5A; 0.39 ± 0.03 -fold vs. 0.27 ± 0.03 -fold [$P < 0.05$, $n = 6$ and 6], respectively). This trend continued at P17 (Fig. 5B; 0.29 ± 0.02 -fold vs. 0.23 ± 0.01 -fold [$P < 0.05$, $n = 17$ and 13], respectively). Sildenafil-treated OIR animals demonstrated significant reduction in RVH versus vehicle-treated OIR animals at both P12 and P17, returning to levels equivalent to RA controls (Figs. 5A, 5B; 0.32 ± 0.02 -fold at P12 vs. 0.39 ± 0.03 -fold and 0.23 ± 0.01 -fold vs. 0.29 ± 0.02 -fold at P17 [$P < 0.05$, $n = 7$, 6 , 13 , and 17], respectively).

FIGURE 3. Vehicle- and sildenafil-treated OIR mice have increased expression of GFAP in Müller glial cells compared to that in room-air control. Immunohistochemistry of paraffin-embedded cross-sections of room air control (*left panel*) mice and OIR (*right panel*) mice at P17 is shown. Vehicle-treated (*top left*) and sildenafil-treated (*bottom left*) normoxic mice at P17, immunostained for rod arrestin (*green*) and GFAP (*red*) do not show activated GFAP-stained Müller cells. In contrast, cross-sections of vehicle-treated (*top right*) and sildenafil-treated (*bottom right*) OIR mice show GFAP staining of Müller cells that spans the entire retina, from internal to external limiting membranes. These cross-sections show intact rod outer segments in both treated and untreated OIR, with overall decreased outer segment length in OIR compared to room air controls.

DISCUSSION

We used an established murine model of OIR to study the retinal effects of PDE5 inhibition during hyperoxic exposure. Retinal vascular flat mounts demonstrated a significant reduction in both retinal vaso-obliteration (at P12) and subsequent neovascularization (at P17), suggesting a potential vaso-protective role for sildenafil in ROP. Our molecular findings suggest that sildenafil is counteracting the detrimental effects of hyperoxic exposure by stabilizing HIF1 α during hyperoxia, thereby promoting physiologic angiogenesis and allowing normal retinal vascularization to occur. This study is the first to explore the effect of PDE5 inhibition in a mouse model of OIR. Similarly, for the first time, we demonstrate that OIR mice have RVH, which is suggestive of pulmonary hypertension. This evidence of growth failure (Table) and RVH (Fig. 5) seen in the OIR mice is consistent with disease seen in humans neonates, in whom those with pulmonary hypertension are at higher risk for growth restriction and ROP.¹¹ Furthermore, we show for the first time, that both retinopathy and pulmonary hypertension are ameliorated by low-dose sildenafil (Figs. 1, 2, 5). Although the response to sildenafil suggests that pathophysiological mechanisms may be similar, further in-depth studies will be required to determine the precise mechanism by which sildenafil stabilizes HIF1 α in the mouse retina.

ROP Pathophysiology: Role of Oxygen and HIF1 α

Since the first identification of the disease in the 1940s, the critical factor in ROP development in the neonate has been exposure to a hyperoxic environment.²⁷ The hyperoxic environment results in phase 1 ROP through ubiquitination and degradation of HIF1 α , resulting in decreased transcription of its downstream targets, including VEGF, and an overall decrease in angiogenic activity.^{6,26,28,29} Decreased angiogenic activity in premature babies occurs at a point when the retina has not been fully vascularized and results in areas of peripheral avascular retina as a result of delayed peripheral

vascular maturation in human ROP, which is distinct from the vaso-obliteration seen and quantified in the posterior retina of the mouse OIR model.³⁰ The return to normoxia paired with metabolic maturation of the retina results in phase 2 ROP, where the relative hypoxia in the avascular retina leads to HIF1 α stabilization, resulting in a rapid increase in transcription of downstream targets, including VEGF in a proangiogenic fashion, in an attempt to restore oxygen to the retina.⁵ However, this angiogenic drive adversely leads to aberrant angiogenesis on the surface of the retina, with neovascular tuft formation leading to sight-threatening complications of ROP in premature infants. Although this simplified scheme with 2 distinct disease phases may have been true for neonatal ROP in previous decades, present-day ROP is more complex, related to repeated large-scale fluctuations in oxygen saturation, complicated by episodes of apnea, concomitant lung immaturity, and the need for prolonged mechanical ventilation with supra-physiologic oxygen. As shown in our results, the mouse OIR model also develops hyperoxia-induced pulmonary hypertension, as shown by Fulton's index, indicating disease overlap between ROP and lung vasculature.

Sildenafil Treatment as a Systemic Approach to Promote Developing Retinal and Lung Vasculature

In human infants, ROP and BPD-associated pulmonary hypertension are frequent comorbidities.¹¹ To model ROP as described in this study, mice were placed in 75% O₂ from P7 to P12, whereas to model BPD, the mice were in 75% O₂ from P0 to P14 with associated pulmonary hypertension, suggesting a relative similarity in the disease process.¹⁸ Given this disease overlap in which BPD and ROP are frequent comorbidities,¹¹ it seems that a systemic approach that targets both the lung and retinal vascular beds may offer a less invasive and perhaps more effective intervention by addressing vascular signaling abnormalities that underlie both retinopathy and pulmonary vascular disease. This would be a paradigm shift, as current approaches to ROP continue to be invasive (laser or intravitreal injections),³¹ with a continued risk of permanent, severe vision

FIGURE 4. Sildenafil stabilizes HIF1 α protein expression during hyperoxia. Retina protein lysates were subjected to Western blotting for HIF1 α . At P12, sildenafil treatment significantly increased HIF1 α expression relative to that of vehicle-treated OIR mice ([A], $\#P < 0.05$, $n = 7$ and 5); and a representative blot is shown (B). At P17, sildenafil-treated mice demonstrated significantly less HIF1 α expression relative to that of vehicle-treated OIR mice ([C], $\#P < 0.05$, $n = 8$ and 7); and a representative blot is shown (D).

loss threatening premature infants despite advances in ROP management and earlier therapy.³² Our findings of disease overlap support the hypothesis that ROP and BPD-associated pulmonary hypertension, two common diseases associated with prematurity, might share a common vascular pathophysiology. In rat models of BPD with associated pulmonary hypertension, sildenafil has been shown to be effective at normalizing lung architecture and decreasing pulmonary hypertension.^{33,34} More recently, we have shown that very-low-dose sildenafil can effectively prevent pulmonary vascular remodeling and RVH in a mouse model of BPD with associated pulmonary hypertension.¹⁸ The mechanism by which sildenafil normalizes the pulmonary vasculature is an area of active investigation.^{24,25} Consistent with these previously reported findings in the lung, our study shows that low-dose sildenafil is sufficient to stabilize retinal HIF1 α during hyperoxia in OIR. Our observations show that treatment with low-dose sildenafil during hyperoxia results in a reduction in vaso-obliteration in the first phase and neovascularization in the second phase of retinopathy in mouse OIR. Reduction in neovascularization could be explained by an overall reduction in retinal angiogenesis or, alternatively, as a direct result of reduction in the initial severity of vessel loss during hyperoxic exposure. Given that we observed a significant reduction in overall avascular retina at P12 (Fig. 1), it is likely that sildenafil is

FIGURE 5. Sildenafil abrogates hyperoxia-induced RVH in OIR mice. Fulton's index (right ventricle weight divided by left ventricle plus septum weights) showed P12 (A) and P17 (B) OIR mice had a significantly increased RVH versus age-matched room air controls ($*P < 0.05$). Sildenafil significantly decreased RVH at both P12 and P17 versus that in vehicle-treated OIR ($\#P < 0.05$).

altering the initial hyperoxia-induced delayed vascular maturation and vaso-obliteration, rather than simply limiting neovascular progression. The relatively short (1.3-hour) half-life of sildenafil³⁵ coupled with fact that treatment was given only during hyperoxia further suggests that the medication would have little effect on the angiogenic, second phase of OIR.

Role of cGMP in Angiogenesis: Sildenafil Promotes HIF1 α Stabilization

Whereas the vasodilator effects of PDE5 inhibition have been well characterized in the retina,³⁶ cGMP elevation has recently been shown to impact vascular repair and angiogenesis through several additional mechanisms.³⁷ One of these mechanisms is through increased cGMP-activated protein kinase G, which has been shown to increase HIF1 α expression.²⁴ Elevated HIF1 α expression exerts proangiogenic effects through several downstream effectors, including VEGF.³⁸ In a rodent hind-limb ischemia model, increased HIF1 α not only improved overall VEGF levels it also promoted increased endothelial progenitor cell numbers and capillary angiogenesis at the site of injury.²⁴ Given the fact that HIF1 α expression is inhibited during phase 1 of ROP,²⁶ we hypothesized that upregulation of HIF1 α expression via PDE5 inhibition could have a beneficial vaso-protective effect on ROP. We demonstrated that sildenafil treatment resulted in increased HIF1 α protein expression relative to that in vehicle-treated OIR mice at P12, immediately after hyperoxia (Fig. 4). We postulate that sildenafil treatment in phase 1 of the OIR model counteracts the antiangiogenic effects of hyperoxia and promotes more normal vessel development. This is reflected in the reduction in vaso-obliteration seen in the sildenafil-treated OIR mice at P12 (Fig. 1). When sildenafil-treated OIR mice develop relative hypoxia on moving from a hyperoxic to normoxic environ-

ment, neovascularization at P17 is reduced as a result of a pre-existing more robust vascular network (Fig. 2), and statistically lower HIF1 α protein levels at P17 (Fig. 4), reflecting an overall reduction in hypoxic angiogenic drive.

Comparison to Other Approaches of Systemic HIF1 Manipulation for OIR Prevention

Sears et al.³⁹ and Trichonas et al.⁴⁰ used another approach to stabilize HIF1 during hyperoxia in OIR by administering dimethylxaloylglycine, an inhibitor of prolyl hydroxylase, the enzyme that hydroxylates HIF α , a first step toward its degradation. That study showed significant reduction in retinal vaso-obliteration and neovascularization in both mouse and rat OIR. Interestingly, unlike our finding of elevated retinal HIF1 α at P12, those authors were not able to identify elevated retinal HIF1 α as a corollary to improved OIR in their model, suggesting instead that HIF stabilization in the liver exerts its effect on the retina in a paracrine and endocrine manner.

Sildenafil Does Not Completely Abrogate Müller Cell Stress Response

Parallel with improved overall vasculature, we were hoping to find downregulation of GFAP expression in Müller cells as an indication of reduced hypoxic stress in the retina. However, our results show no qualitative differences in GFAP expression in a comparison of sildenafil-treated to untreated OIR mice. This result is consistent with incomplete abrogation of the Müller cell gliotic response to hypoxia in these eyes and may suggest the need to continue sildenafil therapy beyond P12 and the hyperoxic exposure in OIR, a question that deserves further exploration (Fig. 3). In addition, it will be important to study the effect of sildenafil on the various downstream targets of HIF1 α , and especially HIF2 α , which in some studies was shown to be expressed at a higher level than HIF1 α in the retina and is suggested to play a prominent role in OIR through regulation of the Müller cell hypoxic response.⁴¹ As shown in our data, although sildenafil was effective at protecting the vasculature during hyperoxia, it did not completely abolish neovascularization or Müller cell stress. Although GFAP expression on the surface glial cells of the retina appears brighter in the sildenafil-treated normoxic retina, we were most interested in the absence of GFAP expression on Müller cells traversing the retina (a nonspecific indicator of retinal stress response) in both normoxia groups, as compared to the OIR groups.

Role of cGMP in Developing Photoreceptors: Potential Risks of Sildenafil

Although our data are promising, it must be acknowledged that sildenafil at higher doses has also been shown to inhibit PDE6, which is critically involved in photoreceptor visual phototransduction.⁴² This possible undesirable effect is one reason we chose to use a low-dose of sildenafil for our studies. Mutations in the gamma subunit of PDE6 that decrease enzyme activity are linked to photoreceptor degeneration in rd/rd null mice.⁴³ The rd/rd null mutants experience more than 10-fold elevation of cGMP before onset of photoreceptor degeneration.⁴⁴ Thus, use of cGMP-elevating drugs must be approached with great caution. Interestingly, null mutants exhibit normal photoreceptor development up until P8 (whereas, rod outer segments are absent) but show completely degenerated photoreceptors, “rodless retina,” by P20, signaling that there is a key developmental window where cGMP levels may be vitally important. Photoreceptor degeneration in this model is

thought to be a result of continuous “dark current,” with unchecked free Ca²⁺ influx and activation of apoptosis in photoreceptor outer segments.⁴³ Heterozygotes, however, have a completely normal photoreceptor phenotype.⁴⁵ Because we used very low doses of sildenafil in order to minimize the PDE6 inhibition, we did not expect or encounter photoreceptor degeneration. Interestingly, a recent study in rats gave sildenafil antenatally to pregnant dams (from embryonic day 11.5–20.5), and found no abnormalities in the pups’ retinal structure or function, by performing electroretinography at P30.⁴⁶ A critical difference between that study and ours is that sildenafil was given during embryogenesis, when photoreceptor development is incomplete and consists of rod and cone precursors, which specifically do not have outer segments. In mice, and in human premature babies,⁴⁷ photoreceptor outer segment maturation occurs postnatally. Thus, in this report, we demonstrate for the first time that the photoreceptor outer segments in OIR mice are generally less developed than those in room air controls, which is consistent with previous mouse and human electrophysiological results.^{48–51} We subsequently saw no qualitative differences between photoreceptor outer segment staining in sildenafil-treated and that in vehicle eyes (Fig. 3). While these qualitative results are encouraging, any attempt to use sildenafil clinically in premature infants will need to be preceded by careful preclinical studies exploring the long-term effects of PDE inhibition on photoreceptor development and retinal function.

Existing Data for Retinal Side Effects of Sildenafil in Human Infants and Adults

Despite data suggesting sildenafil may be useful in preterm infants for BPD-associated pulmonary hypertension, there has been significant concern about targeting PDE5 in premature infants for fear of potential adverse ophthalmological effects, although these remain controversial.^{52,53} Whereas case reports have linked sildenafil treatment with potentially enhanced ROP progression and severity,⁵³ subsequent retrospective cross-sectional studies have found no adverse outcomes in premature infants with ROP who were treated with sildenafil for pulmonary hypertension.^{54,55} Previous studies of the effects of PDE5 inhibitors in the eye have focused largely on retinal and choroidal blood flow, showing increased choroidal congestion and ophthalmic artery velocity and flow, with controversial results in the retinal vasculature.^{56,57} Using ex vivo human retinal tissue to study the distribution of PDE5, researchers have shown that in addition to retinal and choroidal vasculature, PDE5 was expressed in retinal ganglion and bipolar cells.⁵⁸ In humans, studies have evaluated visual function and electroretinographic changes in adults treated with PDE5 inhibitors^{59,60} and found no effects on white or blue/yellow perimetry except in a single subject who received acute high doses, which was thought to be an idiosyncratic reaction.⁶⁰ Given this clinical controversy and lack of data in OIR models, we sought to explore the effect of PDE5 inhibition on retinal vasculature in the mouse OIR model.

CONCLUSIONS

In summary, this study shows that administration of low-dose sildenafil during hyperoxia results in significant decrease of retinopathy and RVH in an OIR mouse model. Our findings suggest a potential preventative role for sildenafil (through HIF1 α stabilization) as a way to reduce the vaso-obliterative response associated with hyperoxia in immature vessels, with exciting implications for neonatal vascular maturation in both retina and lung. While a strictly biphasic vascular response is

true in mouse OIR and high oxygen exposure in human ROP observed in previous decades, the rat OIR model, as well as modern human ROP, have shown that rapid fluctuations in O₂ exposure may be associated with a more complex biological picture.^{31,61} Hence, as in the complex situation in neonatal BPD and pulmonary hypertension,^{1,2} further studies are needed in order to determine the most efficacious dosage and optimum time window of administration of sildenafil in ROP, before this therapy can be translated to the clinical arena. Furthermore, additional studies will need to characterize the long-term functional and structural health of the newborn retina following sildenafil use. Overall, this study underscores the importance of cGMP-mediated signaling in the developing retinal vasculature, which may present a potentially powerful new therapeutic target for the treatment of ROP as well as other hyperoxia-induced diseases of prematurity.

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