Peroxisome Proliferator-Activated Receptor- β/δ Regulates Angiogenic Cell Behaviors and Oxygen-Induced Retinopathy

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PURPOSE. To develop new therapies against ocular neovascularization (NV), we tested the effect of peroxisome proliferator-activated receptor- β/δ (PPAR- β/δ) agonism and antagonism on angiogenic behaviors and in human retinal microvascular endothelial cells (HRMEC) and on preretinal NV in rat oxygen-induced retinopathy (OIR).

METHODS. HRMECs were treated with the PPAR- β/δ agonist GW0742 and the antagonist GSK0660. Messenger RNA levels of a PPAR- β/δ target gene, angiopoietin-like-4 (*angptl4*) were assayed by qRT-PCR. HRMEC proliferation and tube formation were assayed according to standard protocols. OIR was induced in newborn rats by exposing them to alternating 24-hour episodes of 50% and 10% oxygen for 14 days. OIR rats were treated with GW0742 or GSK0660. Angptl4 protein levels were assessed by ELISA and preretinal NV was quantified by adenosine diphosphatase staining.

RESULTS. GW0742 significantly increased *angptl4* mRNA, and GSK0660 significantly decreased *angptl4* mRNA. GW0742 had no effect on HRMEC proliferation, but caused a significant and dose-responsive increase in tube formation. GSK0660 significantly reduced serum-induced HRMEC proliferation and tube formation in a dose-dependent manner. Intravitreal injection of GW0742 significantly increased total retinal Angptl4 protein, but intravitreal injection of GSK0660 had no effect. Intravitreal injection of GW0742 significantly increased retinal NV, as did GW0742 administered by oral gavage. Conversely, both intravitreal injection and intraperitoneal injection of GSK0660 significantly reduced retinal NV.

CONCLUSIONS. PPAR- β/δ activation exacerbates, and its inhibition reduces, preretinal NV. PPAR- β/δ may regulate preretinal NV through a prodifferentiation/maturation mechanism that depends on Angptl4. Pharmacologic inhibition of PPAR- β/δ may provide a rational basis for therapeutic targeting of ocular NV.

Keywords: retinopathy of prematurity, nuclear transcription factor, angiogenesis, vascular endothelial growth factor, peroxisome proliferator-activated receptor

Angiogenesis is the formation of new capillaries from preexisting vessels. Physiologic angiogenesis occurs during embryonic development, as a part of the wound healing response, and during the menstrual cycle.^{1–6} However, angiogenesis can become dysregulated and contribute to morbidity in diseased tissues. This pathologic angiogenesis, also known as neovascularization (NV), occurs in tumor progression, rheumatoid arthritis, psoriasis, and many other conditions. Ocular NV is a defining feature of retinopathy of prematurity (ROP), diabetic retinopathy (DR), and age-related macular degeneration (ARMD), some of the leading causes of irreversible blindness in the developed world.^{6–11} To more effectively prevent and treat ocular NV, it is necessary to gain a thorough understanding of the cellular and molecular mechanisms regulating angiogenesis in the eye.

Peroxisome proliferator-activated receptors (PPARs) are transcription factors and members of the steroid nuclear hormone receptor family.¹² Three PPAR isoforms have been characterized: PPAR- α (NR1C1), PPAR- β/δ (NR1C2), and PPAR- γ (NR1C3).^{13,14} PPARs bind to genomic DNA at loci called peroxisome proliferator response elements (PPREs). A PPRE is

a consensus sequence that is functionally linked to, and usually located within, the promoter sequence of the PPAR's target gene.^{13,15} PPARs regulate transcription through a classical mechanism that involves: ligand binding to the PPAR, followed by dissociation of PPAR corepressors; heterodimerization between the PPAR and retinoid acid receptor (RXR); association of the heterodimer with coactivators; binding of the ligand-bound PPAR/RXR-containing protein complex to a PPRE; and, finally, transcriptional activation of the target gene.¹³ Other biologically relevant mechanistic variants have been described, suggesting that PPAR- β/δ is a ligand-dependent or -independent transcriptional suppressor.^{13,15}

PPARs are widely known for their roles in lipid metabolism, insulin sensitivity, inflammation, and cell proliferation and/or differentiation.^{15–19} PPAR- α regulates fatty acid uptake and oxidation in a wide variety of tissues.²⁰ PPAR- γ regulates adipocyte differentiation and glucose transport.^{21,22} PPAR- β/δ is expressed ubiquitously and has metabolic regulatory activities that overlap with the other two isoforms.²³ Substantial evidence supports its role in the regulation of cell proliferation and differentiation.^{18,24}

PPAR- β/δ activation induces differentiation of keratinocytes, colonocytes, and trophoblast giant cells in a ligand-dependent manner.^{24–26} Studies from various laboratories have indicated a direct role for PPAR- β/δ in angiogenic cell behaviors and in the angiogenic component of tumor growth.^{27,28} For example, activation of PPAR- β/δ by a chemically synthesized, high-affinity ligand induces cultured endothelial cells (ECs) to proliferate and form tubes.²⁷ Müller-Brüsselbach et al.²⁸ demonstrated defective tumor angiogenesis in PPAR- $\beta/\delta^{-/-}$ mice transplanted with wild-type tumor cells. Whether PPAR- β/δ activation induces or inhibits cell proliferation appears to be cell type- and tissue-specific.¹⁸ Therefore, it remains to be determined whether PPAR- β/δ plays a similar role in ocular NV.

In this study, in vitro experiments were performed to investigate the influence of PPAR- β/δ on discrete aspects of retinal angiogenesis. First, the effects of PPAR- β/δ on endothelial cell proliferation and tube formation were investigated using human retinal microvascular endothelial cells (HRMECs). Second, to further investigate the therapeutic potential of PPAR- β/δ manipulation, the rat model of oxygen-induced retinopathy (OIR) was used to assess the effects of pharmacologic agonists and antagonists of PPAR- β/δ on the retinal neovascular response. These studies will help to define the role of PPAR- β/δ in mediating pathologic ocular angiogenesis.

METHODS

HRMEC Culture

Primary cultures of HRMECs (Cell Systems, Kirkland, WA) were seeded into tissue culture flasks coated with attachment factor (Cell Signaling, Danvers, MA). HRMECs were grown and cultured in phenol red-free endothelial basal medium (EBM; Lonza, Walkersville, MD) supplemented with 10% FBS, 1× antibiotic/antimycotic solution, and endothelial cell growth supplements (EGM SingleQuots; Lonza), hereafter referred to as growth medium. When experimental conditions required serum-free (SF) medium, EBM with no FBS or growth supplements was used. All cultures were incubated at 37°C, 5% CO₂, and 95% relative humidity (20.9% oxygen). Passages 6 to 8 were used for these experiments.

Quantitative Real-Time RT-PCR (qRT-PCR) of Angiopoietin-Like Protein-4 (*angptl4*) mRNA in HRMEC

HRMECs were seeded in six-well plates at 2×10^5 cells/well and maintained under standard tissue culture conditions. At 80% confluency, the cells were serum starved for 12 hours, then treated on a background of 0.5% serum-containing vehicle (0.1% DMSO) or PPAR- β/δ agonist GW0742 (0.01, 0.1, or 1.0 μ M; Tocris Bioscience, Ellisville, MO), or on a background of 2% serum-containing vehicle or PPAR- β/δ antagonist GSK0660 (0.01, 0.1, or 1.0 μ M; Tocris Bioscience) for 6 hours. Cells were washed twice with cold PBS and total RNA was collected using a commercial kit (RNeasy Kit; Qiagen, Valencia, CA), according to the manufacturer's instructions. Total RNA isolated from the culture wells was reverse transcribed using a high-capacity commercial kit (cDNA Archive Kit; Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Quantitative RT-PCR was performed in duplicate by coamplification of human *angptl4* cDNAs versus β -actin (normalization control) in separate wells, using gene-specific gene expression assays (TaqMan; Applied Biosystems), according to the manufacturer's instructions.

HRMEC Proliferation

HRMECs were seeded at 3×10^3 cells/well in a 96-well plate in growth medium for 8 hours to allow them to settle and attach. Cells were serum-starved for 12 hours and then treated with SF medium containing vehicle (0.1% DMSO) or increasing concentrations of GW0742 (0.01–1.0 μ M), or 2% serum medium or 25 ng/mL vascular endothelial growth factor (VEGF) medium containing vehicle (0.1% DMSO) or increasing concentrations of GSK0660 (0.01–1.0 μ M) for 24 hours. Cells were then labeled with bromodeoxyuridine (BrdU) for 12 hours, and BrdU incorporation was quantified using a colorimetric BrdU ELISA (Roche, Indianapolis, IN), according to the manufacturer's instructions. The experiment was replicated four times.

HRMEC Tube Formation

Twenty-four-well tissue culture plates were coated with 400 μ L of growth factor-reduced basement membrane matrix (Matrigel; Becton Dickinson, Franklin Lakes, NJ). HRMECs were seeded at 2.5×10^4 cells/well and treated with SF medium containing vehicle (0.1% DMSO) or increasing concentrations of GW0742 (0.01–1.0 μ M) or 2% serum medium containing vehicle (0.1% DMSO) or GSK0660 (0.01–1.0 μ M) for 24 hours. In another experiment, HRMECs were treated with 0.5% medium containing 25 ng/mL VEGF and vehicle (0.1% DMSO) or GSK0660 (0.01–1.0 μ M) for 12 hours. Tubes were observed with an inverted microscope (IMT-2; Olympus, Melville, NY) and six images/well were captured in a systematic pattern with a digitizing camera (DMC Camera; Polaroid, Cambridge, MA) at $\times 10$ magnification. Capillary-like structures were measured to determine the mean tube length per field using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsbweb.nih.gov/ij/index.html>), and these values were normalized. The relative tube length per field of each treatment group is reported. The experiments were replicated three times.

Rat OIR

All animal procedures used in this study were approved by the Vanderbilt University Institutional Animal Care and Use Committee and were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Within 8 hours after birth, litters of Sprague-Dawley rat pups and their mothers (Charles River Laboratories; Wilmington, MA) were transferred to oxygen exposure chambers in which they were subjected to alternating 24-hour periods of 50% and 10% oxygen for 14 days. On postnatal day 14, referred to as day 14(0), the oxygen-exposed rats were removed to room air. They remained at room air for an additional 6 days, hereafter described as day 14(1) through day 14(6).

Intravitreal Injections

Rats were anesthetized by isoflurane (Butler Animal Health Supply, Dublin, OH) inhalation and a drop of 0.5% proparacaine (Allergan, Hormigueros, PR) was topically applied to the cornea before intravitreal injection. The globe was penetrated approximately 0.5 mm posterior to the ora ciliaris, using a 30-gauge needle with a 19° bevel and 10- μ L syringe (Hamilton Co., Reno, NV). The needle was advanced to the posterior vitreous at a steep angle to avoid contact with the lens. The injection bolus (5 μ L) was delivered near the trunk of the hyaloid artery proximal to the posterior pole of the retina. After injection, a topical antibiotic suspension (Vigamox; Alcon Laboratories, Fort Worth, TX) was applied. Noninjected eyes were also

treated with topical proparacaine and antibiotic to control for the potential of these agents to influence retinal vessel growth.

Treatment Groups

A subset of oxygen-exposed rats was administered vehicle (0.1% DMSO in PBS), GW0742 or GSK0660 (20, 100, or 500 nM) by intravitreal injection on days 14(0) and 14(3). Noninjected animals were used as controls. The dose regime administered by intravitreal injection was determined by preliminary dose/response experiments in OIR rats and yielded an optimal local dose/response profile (see Fig. 4 later in text). We found that GW072 and GSK0660 regulated preretinal NV at lower concentrations than those required to produce responses in our EC proliferation and tubulogenesis assays. These differences may be attributed to the action of these drugs on other retinal cell types and their potential regulation of the angiogenic cascade at multiple points. Another subset of oxygen-exposed rats was administered vehicle (10% ethanol in corn oil) or GW0742 (1.0 or 10 mg/kg) by oral gavage once daily for 6 days [14(0)–14(5)]. The last subset was administered vehicle (1% DMSO in PBS) or GSK0660 (0.2 or 1.0 mg/kg) by intraperitoneal injection on days 14(0), 14(2), and 14(4). Administration routes were chosen based on formulation differences and anticipated systemic bioavailability for the two drugs.

Quantification of Retinal NV

On day 14(6), all rats were euthanized and their retinas dissected. After dissection, the retinal vasculature was stained for adenosine diphosphatase (ADPase) activity, according to well-established procedures.²⁹ Images of ADPase-stained retinas were digitized, captured, and displayed at $\times 20$ magnification. For each retinal image, preretinal vessel tufts were outlined with an irregular polygon, the pixels within the polygon were counted, and the total number of pixels from each polygon in a retina were pooled and converted to square millimeters. The data shown are normalized to NV values from vehicle-treated eyes.

Angptl4 Production in the OIR Rat Retina

Following OIR, rats were intravitreally injected on day 14(1) with vehicle (0.1% DMSO in PBS), 500 nM GW0742, or 500 nM GSK0660. Retinas were collected on day 14(2), sonicated in lysis buffer, and assayed for Angptl4 protein concentration with a colorimetric sandwich ELISA kit (US Biologicals, Salem, MA). The amount of Angptl4 (pg/mL) in retinas was normalized to total protein concentration (mg/mL) of retinal lysates using a bicinchoninic acid assay (Pierce, Rockford, IL).

Statistical Analyses

Data were analyzed with commercial software (JMP; SAS Institute, Cary, NC) using ANOVA with Student's post hoc analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of PPAR- β/δ Agonism and Antagonism on *angptl4* mRNA

HRMECs treated with 0.5% serum medium plus 0.01, 0.1, and 1.0 μ M GW0742 for 6 hours exhibited a significant increase in *angptl4* mRNA expression at every dose ($P < 0.0001$; Fig. 1A). Treatment with 2% serum plus GSK0660 significantly decreased *angptl4* mRNA expression in a dose-responsive fashion ($P = 0.0035$, $P = 0.0092$, and $P = 0.0002$, respectively; Fig. 1B).

Effect of PPAR- β/δ Agonism and Antagonism on HRMEC Proliferation

Treatment of HRMECs with SF medium plus 0.01, 0.1, or 1.0 μ M GW0742 for 24 hours had no effect on proliferation. HRMECs treated with 2% serum medium plus 0.01, 0.1, or 1.0 μ M GSK0660 for 24 hours exhibited dose-dependent decreases in HRMEC proliferation. There was a 39% ($P = 0.0034$) reduction in serum-induced proliferation at the highest concentration tested (Fig. 2A). GSK0660 inhibited VEGF-induced proliferation by 33.1% ($P < 0.0001$) at the 1.0 μ M concentration (Fig. 2B).

Effect of PPAR- β/δ Agonism and Antagonism on HRMEC Tube Formation

Increasing concentrations of GW0742 in SF medium increased the average HRMEC tube length in a dose-dependent manner. Increases of 89.5% ($P < 0.0001$) and 118% ($P = 0.0028$) were observed at the 0.1 and 1.0 μ M GW0742 concentrations, respectively. Increasing concentrations of GSK0660 in 2% serum medium reduced the average serum-induced HRMEC tube length in a dose-dependent manner. We observed percentage decreases of 18.7% ($P = 0.04$) and 40% ($P = 0.0067$) at the 0.1 and 1.0 μ M concentrations, respectively. These data and representative images are shown in Figures 3A, 3B. GSK0660 inhibited VEGF-induced tube formation by 50.6% ($P = 0.0016$) at the 1.0 μ M concentration (Fig. 3C).

Effect of Local Administration of PPAR- β/δ Agonists and Antagonists on the Severity of OIR

Intravitreal administration of GW0742 increased the retinal NV response in OIR rats. Injecting GW0742 at 20 nM, 100 nM, and 500 nM increased NV by 123% ($P = 0.0001$), 47.5% ($P = 0.0578$), and 73% ($P = 0.0077$), respectively. Treatment with GSK0660 decreased NV by 58.5% ($P = 0.0084$) at 20 nM, 56.9% ($P = 0.0109$) at 100 nM, and 44.4% ($P = 0.0142$) at 500 nM. These data are shown in Figure 4.

Effect of Systemic Administration of PPAR- β/δ Agonists and Antagonists on the Severity of OIR

A similar trend was observed when GW0742 was administered by oral gavage for 6 consecutive days. As shown in Figure 5, GW0742 increased retinal NV by 109.1% ($P = 0.0067$; 10 mg/kg). Conversely, intraperitoneal administration of GSK0660 on days 14(0), 14(2), and 14(4) significantly reduced retinal NV. Retinal NV was decreased by 50.3% ($P = 0.0062$) at the low concentration and 59.4% ($P = 0.0017$) at the high concentration. These data and representative images are shown in Figure 5.

Effect of PPAR- β/δ Agonists and Antagonists on Angptl4 Production in OIR Retinas

Intravitreal administration of GW0742 on day 14(1) significantly induced retinal Angptl4 production by 89.8% ($P = 0.0246$) when assayed 24 hours later. Intravitreal injection of GSK0660 had no effect on retinal Angptl4 production 1 day following injection. These data are shown in Figure 6.

DISCUSSION

This study is the first to examine the role of PPAR- β/δ in retinal angiogenesis, using in vitro models of angiogenic cell behaviors and an in vivo model of preretinal NV. Our experiments were performed using GW0742, a chemically synthesized, highly selective PPAR- β/δ agonist, and GSK0660, a potent competitive antagonist that also demonstrates inverse agonist activity.^{30,31}

FIGURE 1. The effect of GW0742 and GSK0660 on *angptl4* expression in HRMEC. (A) Quantitative RT-PCR analysis of *angptl4* mRNA revealed significant activation of PPAR- β/δ with GW0742 treatment. (B) GSK0660 treatment led to a significant reduction in *angptl4* expression. Each bar represents the mean $\pm$ SEM.

These two compounds permitted a clear demonstration of a regulatory role for PPAR- β/δ in ocular NV.

Preliminary studies conducted in our laboratory have shown that PPAR- β/δ is expressed in HRMECs and in rat retina (Penn JS, et al. *IOVS* 2009;50:ARVO E-Abstract 2958). PPAR- β/δ activity level is tightly correlated with the induced expression of *angptl4* mRNA, providing a convenient surrogate marker of PPAR- β/δ activation.^{31,32} GW0742 treatment significantly increased *angptl4* mRNA in quiescent HRMECs at all concentrations (Fig. 1A). GW0742 is approximately 2000- and 1000-fold more selective for PPAR- β/δ (EC₅₀ = 0.001 μ M) over PPAR- γ (EC₅₀ = 2.0 μ M) and PPAR- α (EC₅₀ = 1.1 μ M), respectively (human forms).³⁰ Conversely, GSK0660 treatment decreased *angptl4* mRNA expression in serum-stimulated HRMEC (Fig. 1B). These findings demonstrate the effect of activation or deactivation of PPAR- β/δ on expression of PPAR- β/δ target genes, and confirm the reliability of our pharmacologic reagents in a relevant experimental setting.

In this study, treatment with PPAR- β/δ agonists GW0742 or GW501516 (data not shown) did not affect HRMEC proliferation at concentrations known to activate PPAR- β/δ (Fig. 2). Ligand activation of PPAR- β/δ has been reported to decrease or increase EC proliferation.^{27,33} Piqueras et al.²⁷ reported increased proliferation of human umbilical endothelial vein cells (HUVECs), human aortic endothelial cells (HAECs), and a fused human endothelial/A549 cell line (EAHy926), but only after 72 hours of treatment with the PPAR- β/δ agonist, GW501516. Since VEGF is a principal growth factor involved in the proliferative response of ECs,⁸ these authors performed experiments to determine whether PPAR- β/δ agonism increased VEGF expression and if blockade of the VEGF receptors, VEGFR1 and VEGFR2, influenced the observed GW501516-induced EC proliferation. GW501516 treatment induced VEGF mRNA and protein in the conditioned medium of EAHy926, and expression of dominant negative PPAR- β/δ abrogated this effect. Furthermore, pretreatment with the

VEGF receptor 1/2 antagonist, cyclo-VEGFI, reduced GW501516-induced proliferation.²⁷ Stephen et al.³⁴ reported similar findings: 14 days of treatment with GW501516 induced HUVEC proliferation, VEGF-A expression, and Flt-1 (VEGFR1) expression. We assessed HRMEC proliferation after 1 day, and up to 14 days, of exposure to GW0742 or GW501516, but did not observe any effect (data not shown). We conclude that HRMEC proliferation may be independent of GW0742 and GW501516 treatment. Comparison of our data with those of other studies supports the notion that pharmacologic activation of PPAR- β/δ has mixed effects on EC proliferation. Similar observations have been reported for cancer cell proliferation.¹⁸

GSK0660 is a specific PPAR- β/δ antagonist/inverse agonist; we found that it decreases serum-induced HRMEC proliferation and, to our knowledge, this is a novel finding. The GSK0660 IC₅₀ values (human PPARs) for PPAR- β/δ are, respectively, 0.155 μ M and >10 μ M for - α and - γ .³¹ Consequently, minimal inhibition, if any, of PPAR- α and - γ occurred in our proliferation experiments. Therefore, GSK0660 inhibition of PPAR- β/δ reduces microvascular EC proliferation, which is an important component of retinal NV.

In addition to EC proliferation, retinal NV also depends on cell migration and vascular remodeling: two EC angiogenic behaviors that are modeled by in vitro VEGF-dependent tubulogenesis assays.³⁵ In normal retinal vascular development, tubulogenesis allows for the eventual maturation of new, patent vessels.⁸ The literature is replete with studies suggesting that PPAR- β/δ plays a role in cellular differentiation, an important component of tubulogenesis.^{25,27,36–40} Accordingly, we tested GW0742 in a tube formation assay and observed a significant, dose-dependent induction of HRMEC tube formation. Although PPAR- β/δ activation is likely to be largely responsible for the tube induction at all the concentrations tested, PPARs- α and - γ also may have been activated at the 1.0 μ M GW0742 concentration in these experiments. That activation of these other PPAR isoforms contributed to

FIGURE 2. The effect of GW0742 and GSK0660 on HRMEC proliferation. **(A)** GW0742 exhibited no effect on HRMEC proliferation. GSK0660 treatment led to a dose-dependent reduction of serum-induced proliferation. **(B)** GSK0660 treatment results in a dose-dependent reduction of VEGF-induced proliferation. Each bar represents the mean $\pm$ SEM.

increased tube formation is unlikely, because ligand activation of these receptors has been reported to be antiangiogenic.²⁷ We also found that GSK0660 inhibited tube formation in a dose-dependent manner, and this effect is solely PPAR- β/δ -dependent at the concentrations tested (Fig. 3). Piqueras et al.²⁷ demonstrated that GW501516 induced EAHy926 cells to form microvascular tubes and increased VEGF mRNA in these cells. Additionally, the observed GW501516-induced tube

formation was blocked with the VEGFR1/2 receptor antagonist cyclo-VEGFI, indicating that a VEGF signaling component exists downstream from PPAR- β/δ activation in these cells.²⁷ In preliminary experiments, we found no evidence for GW0742-induced expression of VEGF or VEGFR1 and VEGFR2 in HRMEC; however, we did observe a significant increase of proangiogenic *angptl4* mRNA by qRT-PCR at all concentrations (Fig. 1A). Experimental evidence suggests that Angptl4 has a

FIGURE 3. The effect of GW0742 and GSK0660 on HRMEC tube formation. (A) Representative images of tube formation in HRMECs treated with SF medium with vehicle, 2% serum with vehicle, 1.0 μ M GW0742 in SF media, and 1.0 μ M GSK0660 in 2% serum media. (B) GW0742 treatment induced tube formation in a dose-dependent manner. HRMEC tube formation was induced by 2% serum, and this induction was significantly inhibited with increasing concentrations of GSK0660. (C) GSK0660 inhibits VEGF-induced tube formation. Each bar represents the mean $\pm$ SEM.

FIGURE 4. The effect of local administration of GW0742 and GSK0660 on OIR-induced NV. GW0742 and GSK0660 were intravitreally injected. GW0742 significantly increased OIR-induced NV at the lowest and highest concentrations. At all concentrations, GSK0660 significantly decreased retinal NV. Each bar represents the mean $\pm$ SEM.

prodifferentiation biologic function, an important component of tube formation.^{36,40,41} Although PPAR- β/δ activation is likely responsible for the majority of the induction of *angptl4* mRNA, PPAR- γ also induces it,⁴¹ and at the 1.0 μ M GW0742 concentration, PPAR- γ may contribute to the observed increase. Interestingly, PPAR- β/δ siRNA-mediated knockdown blocked GW501516-induced human keratinocyte differentiation and additional evidence suggested that Angptl4 signaled prodifferentiation events downstream from PPAR- β/δ activation.³⁶ These data suggest, and we hypothesize, that increased PPAR- β/δ -dependent transcription of *angptl4* promotes HRMEC tubulogenesis by mediating HRMEC differentiation/maturation. Conversely, GSK0660 decreased *angptl4* mRNA expression in our qRT-PCR experiments (Fig. 1B). Given the PPAR- α , - β/δ , and - γ IC50 values for GSK0660 cited above, it is highly probable that the GSK0660-dependent decrease in *angptl4* mRNA we observed is solely attributed to inhibition of PPAR- β/δ , perhaps explaining the observed GSK0660-dependent decrease in HRMEC proliferation and tube formation.

To explore the therapeutic potential of targeting PPAR- β/δ , we assessed the efficacy of PPAR- β/δ -directed compounds using the rat model of OIR. This model consistently produces preretinal NV that mimics human ROP.^{42,43} Following oxygen exposure to induce OIR, local (intravitreal) and systemic (oral gavage) administration of GW0742 led to a significant increase in NV response (Figs. 4, 5). In the case of systemic administration, we did not determine GW0742 serum concentrations. However, GW0742 serum concentrations in mice that were fed rodent chow supplemented with GW0742 at 1 and 10 mg/kg were 440.4 and 2270 nmol/L, respectively.^{44,45} The EC50 values for murine PPARs are: - β/δ , 50 nM; - α , 8900 nM; and - γ , >10,000 nM.^{45,46} Therefore, the murine serum concentrations were well below those expected to activate PPARs- α and - γ . Relatively few studies have explored the effect of GW0742 on angiogenesis. Gaudal et al.⁴⁷ reported that GW0742 induced angiogenesis in mouse skeletal muscle. Furthermore, Wagner et al.⁴⁸ reported similar findings in heart muscle. It is well established that the neovascular response in the rat model of OIR has a significant endothelial cell proliferation component, and our cell culture experiments suggest that GW0742 does not initiate EC proliferation. This

discrepancy may reflect differences in the cytokine/growth factors available in our in vitro and in vivo model systems, differences between the species (human RMEC versus rat OIR), differences in stimulation of PPAR- β/δ target genes in diverse cell types within the eye, or because in vivo GW0742 may exert its influence by cell proliferation-independent mechanisms, all of which have the potential to affect the NV response of the retina. It is well established in the rat OIR model that retinal VEGF increases postoxyg exposure.^{8,49} We administered GW0742 to OIR rats 1 day following removal to room air [day 14(1)] and assayed retinal VEGF 24 hours later, when secreted VEGF is at its highest concentration in the retina. We found no effect of this drug on OIR-induced retinal VEGF (data not shown). Our in vitro results suggest that GW0742 agonism of PPAR- β/δ drives tube formation, an angiogenic cell behavior that is dominated by maturation/differentiation, rather than proliferation.⁴⁰ Additionally, GW0742-induced PPAR- β/δ activation in HRMECs increased proangiogenic *angptl4* mRNA, and the results of other studies suggest that Angptl4 may mediate prodifferentiation signaling events downstream from PPAR- β/δ .³⁶ These data are supported by our findings that retinal Angptl4 protein increased significantly following intravitreal injection of GW0742 (Fig. 6). Therefore, we propose that GW0742 agonism of PPAR- β/δ promotes preretinal NV via an Angptl4-dependent prodifferentiation/maturation mechanism in the rat model of OIR.

Conversely, GSK0660 treatment reduced preretinal NV in rat OIR. The drug was administered by local (intravitreal [IVIT]) injection or by systemic (intraperitoneal [IP]) injection. GSK0660 is rapidly cleared from the blood.³¹ Our doses of GSK0660 administered by IP injection were based on a report describing the efficacy of GSK0660 against copper-induced liver damage in mice. The authors report that doses as high as 10 mg/kg/day had no toxicity.⁵⁰ Our in vivo data are in agreement with our in vitro data because GSK0660 inhibited HRMEC proliferation and differentiation, suggesting that pharmacologic inhibition of PPAR- β/δ may represent a reasonable therapy against retinal NV. We also injected OIR rats with GSK0660, as we did for GW0742, to test for any effects on retinal VEGF and we found none (data not shown). Any blockade of the differentiation/maturation component of

FIGURE 5. The effect of systemic administration of GW0742 and GSK0660 on OIR-induced NV. (A) Representative images of retinal quadrants in rats treated with vehicle by oral gavage, 10 mg/kg GW0742 by oral gavage, and 1.0 mg/kg GSK0660 by intraperitoneal injection. (B) GW0742 was administered by oral gavage (1.0 and 10 mg/kg) and GSK0660 by intraperitoneal injection (0.2 and 1.0 mg/kg). GW0742 significantly increased the NV area at the highest concentration tested, whereas GSK0660 significantly decreased the NV area at both concentrations tested. Each bar represents the mean $\pm$ SEM.

the neovascular response may be related to reduced HRMEC *angptl4* mRNA expression, because we observed a GSK0660-dependent decrease of *angptl4* in HRMEC (Fig. 1), although we did not observe a decrease in Angptl4 protein in GSK0660 OIR retinas (Fig. 6). We hypothesize that local expression and secretion of Angptl4 protein by retinal endothelial cells induces their maturation and differentiation via an autocrine mechanism that is critical to the neovascular response. However, the Angptl4 levels produced by EC may be relatively small compared with cumulative contributions of other retinal cells, such that we were unable to detect any GSK0660-dependent reduction in total retinal Angptl4. Additionally, although we observed a substantial PPAR- β/δ activation-dependent increase in retinal Angptl4 protein, there is no reason to assume that GSK0660 should produce the opposite results since PPAR- β/δ transcriptional mechanisms and their inhibition may vary according to the cell type. Consistent with the notion that Angptl4 is critical, in the mouse model of OIR *angptl4*-null mice demonstrated delayed normal development of the retinal vasculature and decreased retinal NV compared with wild-type controls.⁵¹

Currently, there is little information regarding the mechanistic details of Angptl4-dependent proangiogenic signaling in the retina. It is possible that GW0742-induced PPAR- β/δ -dependent *angptl4* expression promotes angiogenic EC behaviors via an autocrine loop in OIR rats. As discussed above, GSK0660 could block an Angptl4-dependent autocrine loop operating in retinal endothelial cells. Paracrine mechanisms may also operate. For example, retinal pigment epithelium cells (ARPE-19) cultured in high glucose express and secrete higher Angptl4 levels compared with normal glucose controls. Conditioned medium from the high-glucose cultures induces retinal EC-tubulogenesis, and this effect was blocked by Angptl4-targeted RNAi knockdown.⁵² It follows that PPAR- β/δ drugs could act by either blocking or activating *angptl4* expression in non-EC retinal cell types and regulate any putative Angptl4-dependent paracrine signaling mechanisms in the neovascular retina. Angptl4 is an orphan ligand and, consequently, the angiogenic signaling mechanisms downstream of Angptl4 in EC are poorly understood. Thus, it is not known whether PPAR- β/δ drugs directly target Angptl4, its downstream signaling intermediates, and/or regulate the

FIGURE 6. The effect of intravitreally injected GW0742 and GSK0660 on total retinal Angptl4 production. GW0742 and GSK0660 were injected at 500 nM concentration and retinas were collected 1 day later. Following GW0742 injection, Angptl4 was significantly increased. GSK0660 had no effect on Angptl4 production in the retina. Each bar represents the mean $\pm$ SEM.

expression of these signaling intermediates in EC. These questions raise important considerations for future studies.

Even though our data suggest that PPAR- β/δ inhibition may warrant continued investigation as a therapeutic modality against retinal NV, we also realize that caution must be exercised due to the many potentially beneficial PPAR- β/δ -related biologic functions. PPAR- β/δ has been shown to be antiapoptotic, anti-inflammatory, neuroprotective, and to suppress tumorigenesis.^{53–60} Additionally, pharmacologic activation of PPAR- β/δ protects against EC dysfunction.⁴⁵ Consequently, any studies that are designed to explore pharmacologic inhibition of PPAR- β/δ in the retina should carefully address potential deleterious side effects related to retinal inflammation and retinal cell death. These studies should also carefully monitor the overall health of the experimental subjects since PPAR- β/δ has several known positive effects on metabolic homeostasis and cardiovascular disease, and given its controversial role in carcinogenesis.^{53,61} As a crude indicator of potential toxicity, we administered 20 nM GW0742 or GSK0660 via IVIT injection, to 7-day-old room-air rats, and assessed the retinal avascular area 3 days later. We found no difference between vehicle and the drug-injected groups, indicating that these drugs have no effect on the normal development of the retinal vasculature and no gross retinal vascular toxicity. Additionally, there were no differences between the body weights of drug- versus vehicle-injected OIR rats in this study (data not shown). Although we propose an Angptl4-dependent mechanism, our normal vascular development data after pharmacologic manipulation contrast with data describing vascular development in the Angptl4^{-/-} mouse.⁵¹ There are a number of possible explanations for this difference: (1) species differences between rat and mouse vascular development, (2) the difference caused by deleting a gene as opposed to reducing its activity pharmacologically, (3) differences in the bioavailability of the pharmacologic agents to different layers of the retina and vasculature, and (4) compensatory changes in response to life-long absence of a

gene product as opposed to the temporary (3 days) reduction of its activity in this model.

The method of drug administration is a critical parameter that must be addressed. In our studies, GSK0660 was efficacious against retinal NV when administered by IVIT or IP injection. Intravitreal injection has the advantages of producing high levels of drug at active sites of neovascular disease, but deleterious side effects are associated with this route of drug administration, including endophthalmitis, cataractogenesis, and glaucoma.^{62–72} Systemic administration could avoid these side effects, but it is hampered by the need for repeated dosing to obtain target concentrations of active drug in diseased tissues. It also needlessly exposes disease-free organs and tissues to active drug. These factors must be carefully considered since PPAR- β/δ is ubiquitously expressed and has multiple beneficial physiologic functions. Given these considerations, optimization of drug delivery is of particular importance for future studies.

Notably, PPAR- β/δ regulates the transcription of numerous genes, and it is of critical importance that future studies precisely define the PPAR- β/δ -dependent components of NV in retinal tissue. This is necessary to develop more precisely targeted therapeutic strategies. New therapies for retinal NV remain desirable, because many patients receive limited benefit from the currently available VEGF-directed drugs, which, although widely successful, nonetheless have drawbacks (potential toxicity to neurons, elevated intraocular pressure, refractory responses, and so forth).^{73,74}

This study is the first to demonstrate that PPAR- β/δ may play a key role in mediating retinal NV. Pharmacologic manipulation of PPAR- β/δ affects angiogenic endothelial cell behaviors that contribute to the retinal neovascular response. These findings are significant because they suggest that PPAR- β/δ may be a novel therapeutic target for the treatment of retinal neovascular disorders, such as ROP.

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