The Role of Cytochrome P450 Epoxygenases in Retinal Angiogenesis

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Purpose. The purpose of this study was to investigate the role(s) of cytochrome P450 epoxygenases (CYPs) and their products, the epoxyeicosatrienoic acids (EETs), in hypoxia-induced VEGF production and pathologic retinal angiogenesis.

Methods. Human retinal astrocytes, Müller cells, and retinal microvascular endothelial cells (HRMEC) were exposed to hypoxia, and relative *CYP2C* expression was measured by RT-PCR. Astrocyte and Müller cell VEGF production was measured by ELISA after exposure to hypoxia and treatment with the general CYP inhibitor, SKF-525a. Human retinal microvascular endothelial cells were treated with the CYP product, 11,12-epoxyeicosatrienoic acid [EET], or SKF-525a in the presence or absence of VEGF. Proliferation of HRMEC and tube formation were assayed. Oxygen-induced retinopathy (OIR) was induced in newborn rats. Retinal *CYP2C11* and *CYP2C23* expression were measured by RT-PCR. The OIR rats received SKF-525a by intravitreal injection and preretinal neovascularization (NV) was quantified. Retinal VEGF protein levels were measured by ELISA.

Results. Human retinal astrocytes were the only cells to exhibit significant induction of *CYP2C8* and *CYP2C9* mRNA expression by hypoxia. Astrocytes, but not Müller cells, exhibited reduced hypoxia-induced VEGF production when treated with SKF-525a. 11,12-EET induced HRMEC proliferation and tube formation, and SKF-525a inhibited VEGF-induced proliferation. Oxygen-induced retinopathy induced expression of *CYP2C23*, but had no effect on *CYP2C11*. SKF-525a inhibited retinal NV and reduced retinal VEGF levels in OIR rats.

Conclusions. The CYP-derived 11,12-EET may exhibit a proangiogenic biological function in the retina following stimulation by hypoxia in astrocytes. Inhibition of CYP may provide a rational therapy against retinal NV, because it can reduce VEGF production and VEGF-induced angiogenic responses in endothelial cells.

Keywords: retinopathy of prematurity, epoxyeicosatrienoic acid, cytochrome P450, angiogenesis

Angiogenesis is the sprouting of new blood vessels from pre-existing vessels and it occurs in physiologic and pathologic settings. Persistent pathologic angiogenesis contributes to a number of diseases, including cancer and retinopathies. Abnormal retinal angiogenesis, also called neovascularization (NV), results from a vasoproliferative response to retinal hypoxia and occurs in retinopathy of prematurity (ROP), proliferative diabetic retinopathy (PDR), and age-related macular degeneration (AMD).[1–3] These eye diseases account for the majority of vision loss in developed countries, creating an impetus to investigate the cellular and molecular mechanisms underlying retinal NV and develop effective therapies.

Vascular endothelial growth factor (VEGF), a 40 kDa dimeric glycoprotein, is the primary mediator of angiogenesis in ocular diseases.[4] Hypoxia is a potent stimulator of retinal VEGF, and ischemia-induced hypoxia is the major source of VEGF in ROP. The retinal Müller cells and astrocytes have been shown to produce the highest levels of VEGF of all retinal cell types.[5] Anti-VEGF antibodies have been approved by the FDA and are used in the clinic to treat PDR and AMD. However, problems exist with chronic use of anti-VEGF in the clinical setting. For example, anti-VEGF therapy requires multiple intravitreal injections to achieve efficacy, increasing the risk for endophthalmitis. Furthermore, VEGF has demonstrated a neurotrophic role in retinal neurons.[4,6] Additionally, in the case of ROP, anti-VEGF therapies inhibit the normal vascular development in the premature infant. Thus, further development of additional antiangiogenic therapies is warranted.

Cytochrome P450s (CYPs), in addition to cyclooxygenases (COX) and lipoxygenases (LOX), are enzymes that metabolize arachidonic acid (AA) to biologically active eicosanoids. Arachidonic acid is released primarily from the phospholipid bilayer by cytosolic phospholipase A_2– ($cPLA_2$–)–induced cleavage. Cytosolic phospholipase A_2 activity is significantly increased in oxygen-induced retinopathy (OIR), resulting in increased AA intracellular pools available to the CYP, COX, and LOX pathways.[7] Cyclooxygenases, LOX, and their products (prostaglandins, thromboxanes, and leukotrienes) have been well characterized in OIR.[8–11] However, the contribution of AA-derived CYP products to retinal angiogenesis remains largely unknown.

A total of 57 CYP genes has been identified in humans,[12] and the products of their expression are enzymes that act on a

wide variety of substrates, including xenobiotics, pharmaceuticals, and several endogenous compounds.[12,13] Of these enzymes, 12 metabolize AA, and they belong to the CYP4 family of ω-hydroxylases that yield 20-hydroxyeicosatetraenoic acid (20-HETE) or the CYP2C8/9 and CYP2J2 families that yield four epoxyeicosatrienoic acid region-isomers (5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acid [EET]). In human endothelium, the primary CYP epoxygenases are CYP2C8/CYP2C9 and CYP2J2, whereas the rat homologs are CYP2C11/CYP2C23 and CYP2J6, respectively.[14] Each of the CYP epoxygenases produces EET isomers that vary in relative abundance.[14]

The EETs have multiple vascular functions. They originally were defined as endothelium-derived hyperpolarizing factors, and have since been studied for their anti-inflammatory, vasodilatory, and proangiogenic activities.[15] Cytochrome P450 epoxygenase–derived EET production recently has been shown to have a significant role in promoting endothelial cell proliferation and mitogenesis.[16,17] Particularly relevant to retinal vaosproliferative disease, some CYPs are regulated by hypoxia. Although *CYP2J2* is downregulated in hypoxia, *CYP2C8/9* enzymes are hypoxia-inducible in human umbilical vein endothelial cells (HUVEC) and bovine retinal microvascular endothelial cells (BRMEC).[18,19] The hypoxia-induced EET products are downstream signaling intermediates in the VEGF signaling pathway[20,21]; accordingly, CYP inhibitors reduce EC proliferation and overexpression of *CYP2C8* promotes EC proliferation in vitro.[22] A number of in vivo studies also support a role for EETs in angiogenesis. For example, administration of exogenous EETs induced vascularization in the Matrigel plug assay, and overexpression of rat *CYP2C11* increased muscle capillary density in an ischemic rat hind limb model.[23,24] Furthermore, increased EET production promoted tumor growth and metastases in a number of in vivo cancer models.[25]

The purpose of this study is to investigate the role of CYP2C epoxygenases and their EET products in retinal angiogenesis. Experimental evidence suggests that CYP2C enzymes are expressed in endothelium, are hypoxia-inducible, and their regio-selectivities favor increased 11,12-EET over the other regio-isomers. Therefore, we performed in vitro experiments to establish the effects of 11,12-EET to stimulate human retinal microvascular endothelial cell (HRMEC) proliferation and tubulogenesis. Furthermore, we assessed the effect of a general CYP inhibitor on induction of VEGF in human retinal glial cells and VEGF-induced angiogenic behaviors in HRMEC. We measured the expression of the human *CYP2C* homologs in primary human retinal cells exposed to hypoxia, and determined the expression profile of *CYP2C11* and *CYP2C23* in OIR rats. Additionally, we tested the efficacy of the CYP inhibitor against preretinal NV in vivo, and whether its efficacy correlated with retinal VEGF levels in OIR rats.

Methods

Human Retinal Astrocyte Cell Culture

Primary cultures of human retinal astrocytes (Sciencell, Carlsbad, CA, USA) were plated in tissue culture flasks coated with poly-L-lysine (Sciencell). Astrocytes were cultured in Astrocyte Growth Medium (Sciencell), containing 2% fetal bovine serum (FBS), astrocyte growth supplement, and a penicillin/streptomycin solution. Cultures were incubated at 37°C, 5% CO_2, 20.9% O_2, and 95% relative humidity. In experimental conditions using hypoxia, oxygen levels were maintained at 0.1% using the ProOx 110 (BioSpherix, Lacona, NY, USA). Passages 4 to 6 were used.

Human Müller Cell Culture

Human Müller cells were isolated from human donor tissue within 24 hours post mortem (NDRI, Philadelphia, PA, USA). The retina was dissected from the eye cup and dissociated in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY, USA) containing trypsin and collagenase. Following incubation in dissociation medium, cells are grown in DMEM containing 10% FBS and 1× antibiotic/antimycotic solution, and incubated at 37°C, 5% CO_2, 20.9% O_2, and 95% relative humidity. Experimental conditions using hypoxia were the same as described above. Passages 4 to 6 were used.

Human Retinal Microvascular Endothelial Cell Culture

Primary HRMEC (Cell Systems, Kirkland, WA, USA) were plated in tissue culture flasks coated with attachment factor (Cell Signaling, Danvers, MA, USA). The HRMEC were cultured in phenol red–free endothelial basal medium (EBM; Lonza, Walkersville, MD, USA) containing 10% FBS and endothelial cell growth medium (EGM) with SingleQuots (Lonza). In serum-reduced experimental conditions, 0.5% FBS, and 1× antibiotic/antimycotic solution in EBM was used to maintain cell viability without stimulating additional proliferation. Cultures were incubated at 37°C, 5% CO_2, 20.9% O_2, and 95% relative humidity. Experimental conditions using hypoxia were the same as described above. Passages 8 to 10 were used for the following experiments.

Quantitative Real-Time RT-PCR of *CYP2C8* and *CYP2C9*

Human retinal astrocytes, human Müller cells, and HRMEC were seeded in 6-well plates at 2×10^5 cells/well and maintained under standard tissue culture conditions. At 90% confluency, cells were exposed to either normoxia (20.9% O_2) or hypoxia (0.1% O_2) for 24 hours. After 24 hours, cells were washed twice with cold PBS and total RNA was collected using an RNeasy kit (Qiagen, Valencia, CA, USA). Total RNA isolated from the culture wells was reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Quantitative RT-PCR was performed in duplicate by coamplification of human *CYP2C8* and *CYP2C9* cDNAs versus *B-Actin* (normalization control), using gene-specific TaqMan Gene Expression Assays (Applied Biosystems). The delta Ct method was used to determine relative expression of the *CYP* mRNA normalized to *β-Actin*. All commercial assays were performed according to the manufacturer's protocol.

Astrocyte and Müller Cell VEGF Production

Human retinal astrocytes and human Müller cells were seeded in 6-well plates. At 70% confluency, cells were treated with vehicle (0.1% ethanol) or 5.0 μM SKF-525a. Plates were either maintained in standard culture conditions (37°C, 5% CO_2, 20.9% O_2) or transferred to hypoxic conditions (37°C, 5% CO_2, 0.1% O_2). Cells were treated for 24 hours, media were collected, and cells were lysed. Vascular endothelial growth factor was measured with a VEGF colorimetric sandwich ELISA kit (R&D Systems, Minneapolis, MN, USA) and the normalized to total protein using a BCA assay (Pierce, Rockford, IL, USA).

HRMEC Proliferation

Human retinal microvascular endothelial cells were seeded at 3×10^3 cells/well in a 96-well plate and cultured for 8

hours in 10% FBS medium. Cells then were cultured in serum-reduced (0.5% FBS) medium for 12 hours. To determine if 11,12-EET stimulated proliferation, cells were treated with 0.5% FBS medium containing vehicle (0.1% ethanol) or increasing concentrations of 11,12-EET (0.05–0.5 μM). To determine if CYP inhibition by SKF-525a (ENZO Life Science, Farmingdale, NY, USA) reduced VEGF-induced HRMEC proliferation, cells were treated with 0.5% medium plus 25 ng/mL VEGF containing vehicle (0.1% ethanol) or increasing concentrations of SKF-525a (0.5–5.0 μM). After treatment for 24 hours, cells were labeled with bromodeoxyuridine (BrdU) for 12 hours. The BrdU incorporation was quantified using a colormetric BrdU ELISA (Roche, Indianapolis, IN, USA), according to the manufacturer's protocol.

HRMEC Tube Formation

We coated 24-well tissue culture plates with 300 μL of growth factor reduced Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA). The HRMEC were seeded at 2×10^4 cells/well on polymerized Matrigel. To determine if 11,12-EET stimulated tubulogenesis, cells were treated with 0.5% medium containing vehicle (0.1% ethanol) or increasing concentrations of 11,12-EET (0.05–0.5 μM). To determine if SKF-525a inhibited VEGF-induced tubulogenesis, cells were treated with 0.5% FBS medium plus 50 ng/mL VEGF containing vehicle (0.1% ethanol) or increasing concentrations of SKF-525a (0.5–5.0 μM). After 8 hours of treatment, tubes were observed with an inverted wide field fluorescence microscope (Eclipse Ti-E; Nikon, Tokyo, Japan) and captured at ×2 magnification. Capillary-like structures were measured to determine the mean tube length per field using ImageJ software (National Institutes of Health [NIH], Bethesda, MD, USA), and these values were normalized. The relative tube length per field of each treatment group is reported.

Rat Model of Oxygen-Induced Retinopathy

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 Rivers Laboratories, Wilmington, MA, USA) were transferred to oxygen exposure chambers (BioSpherix), 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 14(0), the oxygen-exposed rats were returned to room air. They remained in room air for an additional six days, hereafter described as 14(1) through 14(6). Age-matched rat litters also were maintained in room air (RA) to serve as controls.

Quantitative Real-Time RT-PCR of *CYP2C11* and *CYP2C23* in Rat Retina

Retinas from 14(2) and room air day-matched controls (P16) were harvested and stored in RNA*later* Solution (Ambion, Grand Island, NY, USA) and frozen until use. Total RNA was isolated from tissue using RNA lysis buffer supplemented with β-mercaptoethanol using an RNeasy kit (Qiagen), according to the manufacturer's instructions. The RT-PCR quantification of rat *CYP2C11* and *CYP2C23* cDNAs versus *β-actin* (normalization control) was performed as described above.

Intravitreal Injections

Rats were anesthetized by isoflurane (Butler Animal Health Supply, Dublin, OH, USA) inhalation. Before intravitreal injection, 0.5% proparacaine (Allergan, Hormigueros, PR) was topically applied to the cornea. The globe was penetrated approximately 0.5 mm posterior to the ora serrata, using a 30-gauge needle with a 19° bevel and a 10 μL syringe (Hamilton Co., Reno, NV, USA). The needle was advanced to the posterior vitreous at a steep angle to avoid contact with the lens. The injection bolus (5 μL) 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, USA) was applied. Noninjected eyes also were treated with topical proparacaine and antibiotic to control for the potential of these agents to influence retinal vessel growth. At 14(0) and 14(3) rats received intravitreal injections of vehicle (0.1% ethanol in PBS) or increasing concentrations of SKF-525a (0.5–5.0 μM).

Quantification of Retinal Vascular Area and Neovascular Area

On 14(6), rats were killed, their eyes enucleated, and their retinas dissected in 4% paraformaldehyde. The retinal vasculature was stained for adenosine diphosphatase (ADPase; Sigma-Aldrich Corp., St. Louis, MO, USA) activity, according to well-established procedures.[26] Images of ADPase-stained retinas were digitized, captured, and displayed at ×20 magnification. For each retinal image, total retinal area, total vascular area, and preretinal vessel tuft area 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.

Retinal VEGF Production

The OIR-exposed rats received intravitreal injections of vehicle (0.1% ethanol in PBS) or SKF-525a (5.0 μM) on 14(1). Room air age-matched animals (P15) were injected with vehicle as a control. Retinas were collected on day 14(2), the peak day of retinal VEGF following oxygen exposure,[27] sonicated in lysis buffer, and assayed for VEGF protein concentration with a colorimetric sandwich ELISA kit (R&D Systems). The amount of VEGF (pg/mL) in retinas was normalized to total protein concentration (mg/mL) of retinal lysates using a BCA assay (Pierce).

Statistical Analysis

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

Results

CYP2C23, but Not *CYP2C11*, Is Increased in OIR Retinas

In rat OIR, retinal VEGF peaks at approximately 14(2).[27] Therefore, we chose to investigate retinal CYP expression at this time because VEGF has been previously shown to crosstalk with the CYP pathway.[20,21] Retinas collected on 14(2) from OIR showed a 2.4-fold ($P < 0.0001$) increase in *CYP2C23* mRNA relative to room air controls. Conversely, OIR had no effect on *CYP2C11* expression (Fig. 1).

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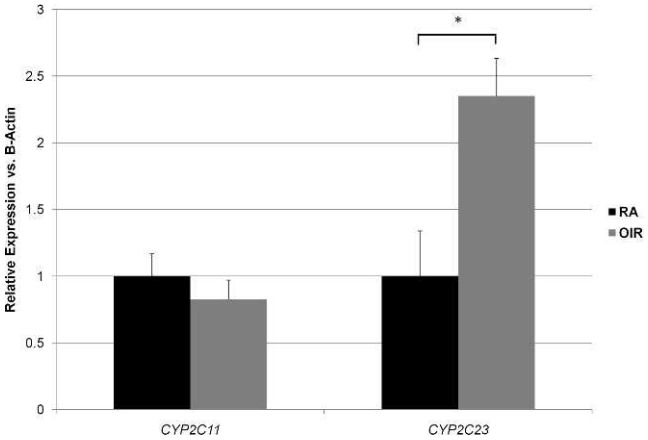

FIGURE 1. *CYP2C11* and *CYP2C23* mRNA expression in rat OIR. Two days after removal from oxygen exposure (14[2]), The *CYP2C23* expression is significantly increased, while *CYP2C11* is unaffected in OIR compared to RA control. Data are presented as mean ± SEM. $^{*}P < 0.0001$, $n = 6$.

Hypoxia Induces *CYP2C* Expression in Human Retinal Astrocytes, but Not Human Müller Cells or HRMEC

Cytochrome P450s are expressed in retinal endothelial cells, Müller cells, and brain astrocytes.[28–31] Therefore, we measured CYP expression in primary HRMEC, human Müller cells, and human retinal astrocytes. Exposure of human retinal astrocytes to hypoxia for 24 hours caused a significant induction of human *CYP2C8* (2.5-fold, $P = 0.0004$) and *CYP2C9* (3.1-fold, $P = 0.0154$, Fig. 2A). However, the same hypoxic conditions did not induce *CYP2C8* or *CYP2C9* in either human Müller cells or HRMEC (Figs. 2B, 2C, respectively).

SKF-525a Reduces VEGF Production by Astrocytes, but Not Müller Cells

In response to hypoxia, Müller cells and astrocytes have been shown to have the greatest capacity to produce VEGF of all retinal cells.[5] Therefore, VEGF production in hypoxic human retinal astrocytes and Müller cells was measured after treatment with SKF-525a for 24 hours (Fig. 3). Müller cells produced 3.6-fold more VEGF than astrocytes when exposed to hypoxia. Hypoxia-induced VEGF production was 11.5-fold in astrocytes and 7.1-fold in Müller cells. SKF-525a had no effect of Müller cell production of VEGF; however, it reduced astrocyte VEGF production by 26.8% ($P = 0.0097$).

11,12-EET and SKF-525a Modulate HRMEC Proliferation

As shown in Figure 4, treatment of HRMEC with increasing concentrations of 11,12-EET stimulated proliferation in a dose-dependent manner. Proliferation was significantly increased by 1.5-fold ($P = 0.0051$) and 1.6-fold ($P = 0.0004$) at the 0.1 and 0.5 μM doses, respectively. General CYP inhibition using SKF-525a significantly inhibited VEGF-induced proliferation by 46% ($P < 0.0001$) at the highest concentration (5.0 μM).

Administration of Exogenous 11,12-EET, but Not SKF-525a, Affects HRMEC Tubulogenesis

Human retinal microvascular endothelial cells were grown on Matrigel in the presence of 11,12-EET or VEGF and SKF-525a.

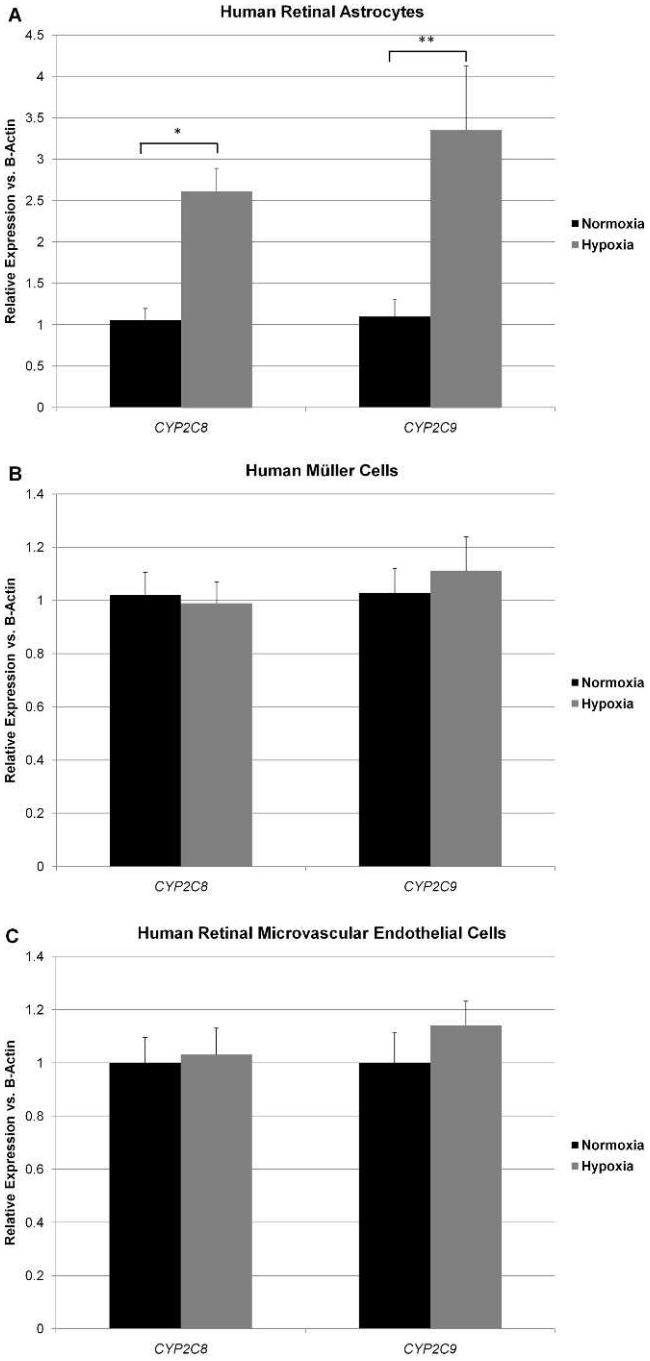

FIGURE 2. The effect of 24-hour hypoxic exposure of CYP-expressing retinal cell types on *CYP2C8* and *CYP2C9* expression. (**A**) Hypoxia significantly induces *CYP2C8* and *CYP2C9* expression in human retinal astrocytes. However, hypoxia had no effect on (**B**) human Müller cells or (**C**) HRMEC. Data are presented as mean ± SEM. $^{*}P < 0.001$, $^{**}P < 0.05$, $n = 7$ to 9.

Tube formation was significantly induced by all doses of 11,12-EET ($P = 0.0135$ at 0.05 μM, $P = 0.0009$ at 0.1 μM, and $P = 0.0078$ at 0.5 μM). Cytochrome P450 epoxygenase inhibition by SKF-525a did not significantly inhibit VEGF-induced tube formation (Fig. 5).

SKF-525a Reduces Retinal NV in OIR

Retinal NV was assessed in the rat model of OIR following intravitreal injection of vehicle or increasing concentrations of

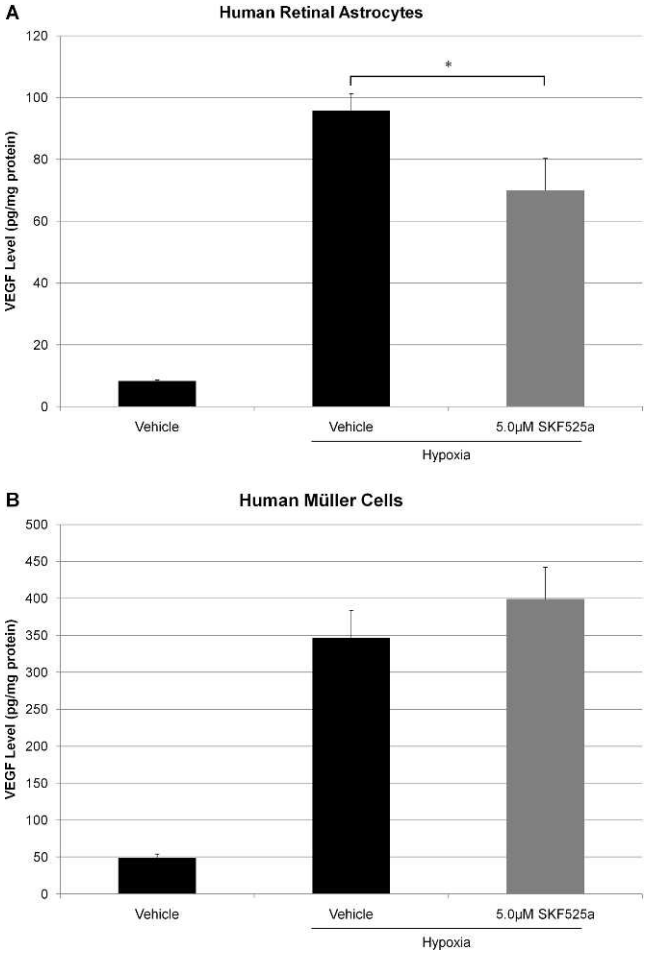

Figure 3. The effect of SKF-525a on hypoxia-induced VEGF production in retinal glial cells. (**A**) Human retinal astrocytes and (**B**) human Müller cells were exposed to either normoxia (20.9% O_2) or hypoxia (0.1% O_2) for 24 hours. The VEGF levels were measured in the culture medium and normalized to total protein. SKF-525a significantly decreased VEGF levels in cultures of hypoxic astrocytes, but not in cultures of hypoxic Müller cells. Representative figures are shown with mean ± SEM. $^*P < 0.01$, $n = 3$ to 9.

SKF-525a. SKF-525a at 5.0 μM significantly inhibited NV formation by 48% ($P = 0.0239$). Representative retinal quadrants and SKF-525a efficacy are shown in Figures 6A and 6B, respectively.

SKF-525a Attenuates Retinal VEGF Production in OIR

Animals were injected on 14(1) with vehicle or SKF-525a, and collected on 14(2), when retinal VEGF is known to peak in this model.[27] Soluble VEGF protein was upregulated 5.6-fold in OIR retinas compared to room air controls. SKF-525a (5.0 μM) significantly reduced OIR VEGF induction by 34% ($P = 0.0229$), as shown in Figure 7.

Discussion

Hypoxia is a known inducer of CYP2C expression in BRMEC, and subsequently causes increased EET levels.[19,28] Additionally, EETs are downstream effectors in the VEGF signaling pathway, promoting endothelial cell proliferation, tube formation, and migration.[20,32] Therefore, EET production is likely to facilitate an enhanced retinal neovascular response. However, to our knowledge, reduction of EETs by CYP epoxygenase inhibition has never been tested as a therapeutic modality against retinal NV.

In BRMEC, 11,12-EET is the only EET product significantly increased when cells are stimulated by hypoxia.[28] In the present study, we showed that *CYP2C23* is the primary OIR-induced CYP epoxygenase in the rat retina (Fig. 1), and CYP2C23 preferentially produces the 11,12-EET regioisomer.[33] Therefore, we chose to use 11,12-EET in our in vitro experiments. In this study, we show for the first time to our knowledge that 11,12-EET induces proangiogenic cell behaviors in HRMEC (Figs. 4, 5).

There are a number of possible cell types that produce cytochrome P450-derived EET products in the retina. Brain-derived astrocytes were the first source identified as hypoxia-stimulated producers of proangiogenic EETs.[30,31] Additionally, BRMEC demonstrated hypoxia-induction of CYP2C mRNA expression over 24 hours.[28] The retina also contains a large population of Müller glial cells that express CYP epoxygenases and the EET-metabolizing enzyme, soluble epoxide hydrolase, in a high abundance, yet their response to hypoxia has never been tested.[29] In the present study, using primary human retinal cell types, we demonstrated that astrocytes are likely the predominant cell type contributing to hypoxia-induced *CYP2C* expression in the retina (Figs. 1, 2).

Currently, no EET receptor has been identified. However, data suggest that EETs likely bind and activate G-coupled protein receptors and/or peroxisome proliferator-activated receptors.[17,34–36] The proangiogenic effects of EETs are mediated at least in part by activation of PI3K/Akt, ERK, and p38 MAPK.[20,24,37] Yang et al.[20] demonstrated that CYP activation in mouse primary lung endothelial cells led to phosphorylation of ERK1/2 and Akt, and knockdown of the primary mouse CYP epoxygenase, Cyp2c44, inhibited VEGF-induced ERK and Akt phosphorylation, and subsequent tube formation.[20] Another study by Potente et al.[38] showed that EET activates PI3K/Akt, inhibiting FOXO1 and FOXO3b, and thereby downregulating p27Kip1, a cyclin-dependent kinase inhibitor. Subsequently Cyclin D1 increases, inducing proliferation by promoting cell cycle progression.[38] Therefore, it is not surprising that increased EET signaling potently induced proangiogenic behaviors in HRMEC.

The general CYP epoxygenase inhibitor, SKF-525a, inhibits neuronal nitric oxide synthase ($IC_{50} = 90$ mM) and blocks the potassium channel Kir6.1 ($IC_{50} = 4.4$ mM).[32,39] Previous studies in models of hypertension have shown that at 50 μM, SKF-525a inhibits AA metabolism by CYPs by 90%.[40] However, at this concentration, we observed loss of cell viability in our experiments (data not shown). We do not expect off-target effects of the drug, because our experiments were performed using substantially lower doses. In our in vitro experiments, SKF-525a significantly inhibited VEGF-induced proliferation; however, we did not observe any effect on VEGF-induced tube formation. Yet, in studies using HUVEC, the EET antagonist 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE) inhibited VEGF-induced tube formation.[21] Additionally, Webler et al.[21] showed that VEGF stimulated CYP2C promoter activity and induced *CYP2C8* expression. In the present study, we did not observe a VEGF-induced increase in *CYP2C8* expression (data not shown). Therefore, VEGF induction of tubulogenesis likely occurs by CYP-independent mechanisms in HRMEC.

To our knowledge, this is the first demonstration that CYP inhibition can reduce neovascularization in an in vivo model of retinopathy. It recently has been shown that retinal *Cyp2c* expression is increased in mice exposed to hyperox-

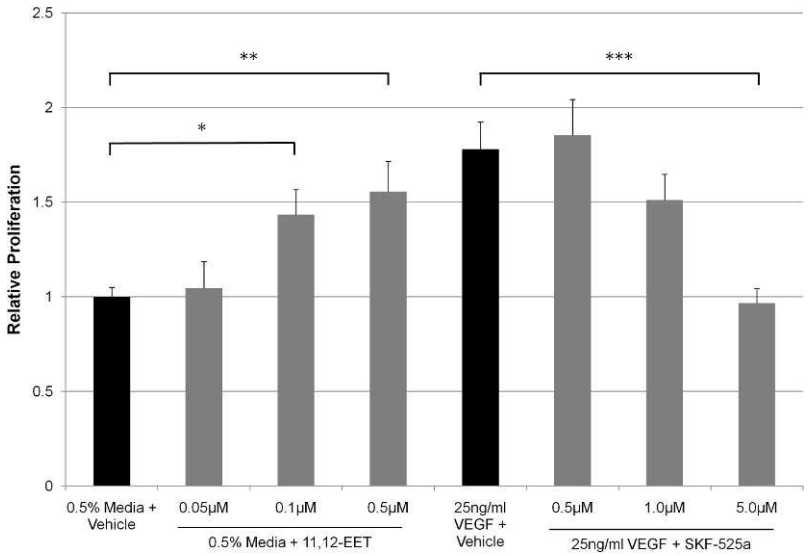

Figure 4. The effect of 11,12-EET or SKF-525a on VEGF-induced proliferation in HRMEC. Treatment of HRMEC with 11,12-EET led to a dose-dependent increase in proliferation. Treatment with SKF-525a inhibited VEGF-induced proliferation at the highest concentration. Data are presented as mean ± SEM. $^{*}P < 0.01$, $^{**}P < 0.001$, $^{***}P < 0.0001$, $n = 9$.

ia.[41] In addition, human *CYP2C8* overexpression in OIR-exposed mice fed ω-3-enriched diet promoted retinal angiogenesis.[41] Similarly, we showed an upregulation of *CYP2C23* expression in rats during the post-oxygen exposure period in OIR rats. Moreover, we showed that inhibition of CYP activity, and presumably the consequent decrease in EET levels, reduces retinal NV. In rat OIR, retinal VEGF normally peaks at 14(2).[27] In this study, OIR rats receiving intravitreal injections of SKF-525a showed reduced retinal VEGF levels at 14(2) (Fig. 7), perhaps contributing to the inhibition of retinal NV we observed at 14(6). Induction or exogenous addition of EETs has been shown to induce

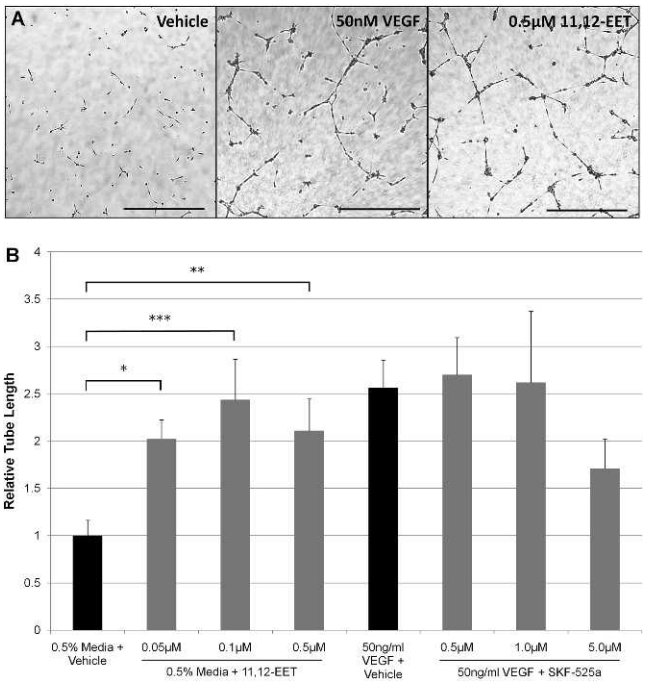

Figure 5. The effect of 11,12-EET or SKF-525a on VEGF-induced tube formation in HRMEC. (**A**) Representative images from vehicle, 50 ng/mL VEGF or 11,12-EET (0.5 μM). *Scale bars*: 500 μm. (**B**) HRMEC treatment with 11,12-EET significantly induced tube formation at all doses used. SKF-525a did not significantly inhibit VEGF-induced tube formation. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 9$.

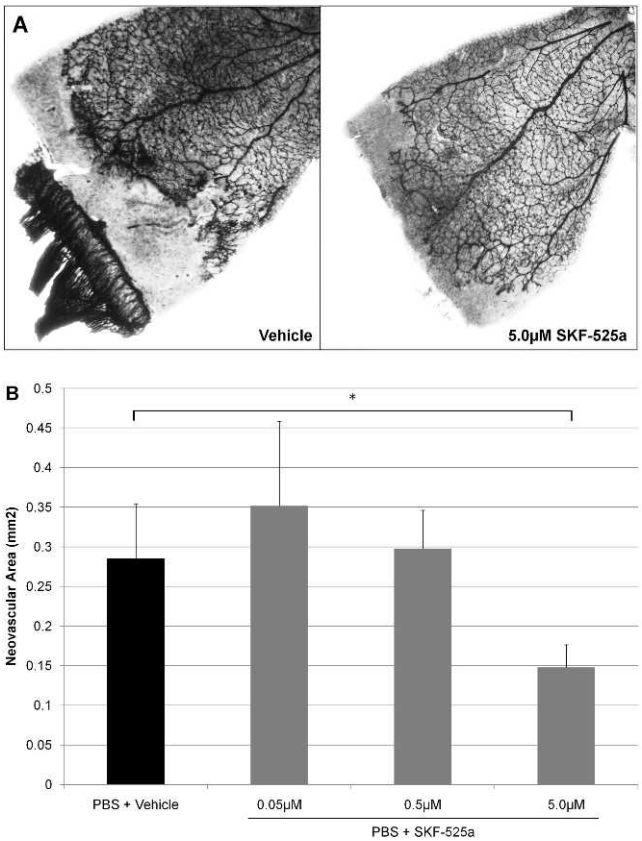

Figure 6. The effect of intravitreal injection of SKF-525a on OIR-induced pre-retinal NV. (**A**) Representative images from vehicle-treated and 5.0 μM SKF-525a-treated OIR retinas. (**B**) At the highest injected concentration (5.0 μM), NV was significantly inhibited. Data are presented as mean ± SEM; $^{*}P < 0.05$, $n = 12$.

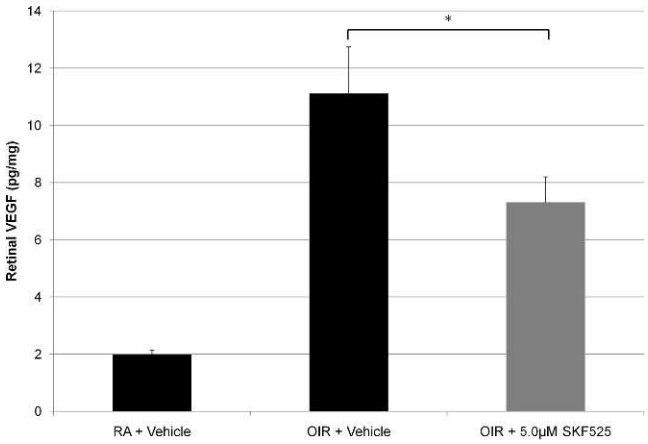

Figure 7. The effect of intravitreal injection of SKF-525a on VEGF production in rat OIR. Injection of 5.0 μM SKF-525a significantly reduced retinal VEGF protein level at 14(2) compared to vehicle injection in rat OIR. Data are presented as mean ± SEM. $^*P < 0.05$, $n = 18$.

VEGF.[42] Inhibition of CYP2C9 by sulfaphenazole suppressed the hypoxia-induced transcriptional activity of the VEGF hypoxia response element in human umbilical arterial endothelial cells.[43] In human dermal microvascular endothelial cells, 14,15-EET induced VEGF via a Src-STAT-3-dependent mechanism.[42] However, in the retina, endothelial cells are not the major source of hypoxia-induced VEGF; Müller cells and astrocytes are the primary producers of VEGF of the retinal cell types.[5] We found that inhibition of CYP epoxygenases in hypoxia-induced primary human Müller cells had no effect on VEGF production; however, it significantly reduced production of VEGF by astrocytes (Fig. 3). This suggests that astrocytes are the primary cell type involved in SKF-525a inhibition of retinal VEGF.

A previous study from Scicli et al.[44] demonstrated the proangiogenic activity of the 20-HETE product derived from the CYP4A family. In this study, inhibitors of CYP4A demonstrated potent antiangiogenic capacity in VEGF-induced HUVEC proliferation and VEGF-induced corneal vasculature growth.[44] However, we believe that CYP epoxygenase inhibitors may provide better efficacy in retinal angiogenesis, because they can inhibit hypoxia-induced VEGF production by astrocytes and VEGF-induced angiogenic behaviors in retinal microvascular endothelial cells.

In this study, we have identified a novel contribution of CYP-derived epoxyeicosatrienoic acids to retinal angiogenesis. The CYP inhibitor, SKF-525a, reduced VEGF production by retinal astrocytes, VEGF-induced proangiogenic behavior in HRMEC and NV area in the rat model of oxygen-induced retinopathy. Our findings suggest that CYP-directed therapeutics may provide a promising target for inhibition of retinal NV, because it can partially inhibit VEGF induction and downstream signaling of VEGF.

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