The Role of PGE2 Receptor EP4 in Pathologic Ocular Angiogenesis

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PURPOSE. PGE2 binds to PGE2 receptors (EP1–4). The purpose of the present study was to investigate the role of the EP4 receptor in angiogenic cell behaviors of retinal Müller cells and retinal microvascular endothelial cells (RMECs) and to assess the efficacy of an EP4 antagonist in rat models of oxygen-induced retinopathy (OIR) and laser-induced choroidal neovascularization (LCNV).

METHODS. Müller cells derived from COX-2-null mice were treated with increasing concentrations of the EP4 agonist PGE2-OH, and wild-type Müller cells were treated with increasing concentrations of the EP4 antagonist L-161982; VEGF production was assessed. Human RMECs (HRMECs) were treated with increasing concentrations of L-161982, and cell proliferation and tube formation were assessed. Rats subjected to OIR or LCNV were administered L-161982, and the neovascular area was measured.

RESULTS. COX-2-null mouse Müller cells treated with increasing concentrations of PGE2-OH demonstrated a significant increase in VEGF production (P ≤ 0.0165). Wild-type mouse Müller cells treated with increasing concentrations of L-161982 demonstrated a significant decrease in VEGF production (P ≤ 0.0291). HRMECs treated with increasing concentrations of L-161982 demonstrated a significant reduction in VEGF-induced cell proliferation (P ≤ 0.0016) and tube formation (P < 0.0344). L-161982 treatment significantly reduced pathologic neovascularization in OIR (P < 0.0069) and LCNV (P ≤ 0.0329).

CONCLUSIONS. Preliminary investigation has demonstrated that EP4 activation or inhibition influences the behaviors of two retinal cell types known to play roles in pathologic ocular angiogenesis. These findings suggest that the EP4 receptor may be a valuable therapeutic target in neovascular eye disease. (Invest Ophthalmol Vis Sci. 2009;50:5479–5486) DOI:10.1167/iovs.09-3652

Angiogenesis, the formation of new capillaries from an existing vasculature, is a tightly regulated physiological process essential for reproduction, embryonic growth and development, and tissue repair and regeneration.1 In these circumstances, angiogenesis is strictly regulated and briefly activated. Conversely, pathologic processes, such as arthritis and tumorigenesis, are characterized by persistent, poorly regulated angiogenesis. In the eye, pathologic angiogenesis, or ocular neovascularization (NV), is the leading cause of irreversible blindness in developed countries.2–4 Ocular NV is a defining feature of retinopathy of prematurity (ROP), proliferative diabetic retinopathy (PDR), and neovascular age-related macular degeneration (AMD or ARMD). To more effectively prevent and treat ocular NV, a thorough understanding of the cellular and molecular mechanisms involved is necessary.

Retinal NV is often the result of ischemia-induced hypoxia.5,6 In response to retinal hypoxia, several cell types increase their production of proangiogenic growth factors. Of the growth factors involved in retinal NV, vascular endothelial growth factor (VEGF) is recognized as the principal mediator of ocular NV.7–9 Hypoxia-induced VEGF production has been demonstrated most consistently and dramatically in Müller cells, the predominant glial cells within the retina.10–11 Once VEGF is produced and secreted, it binds and activates two cell-surface receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flik-1), with high affinity.12 These receptors are expressed on the surfaces of endothelial cells. VEGFR-2 is the principal receptor involved in VEGF signal transduction leading to angiogenesis.13 VEGFR-2 activation initiates a number of signal transduction cascades leading to angiogenic endothelial cell behaviors such as survival, permeability, proliferation, and migration.12

The cyclooxygenase (COX) enzymes catalyze the biosynthesis of five biologically active prostanoids (prostaglandins and thromboxanes) from membrane-derived arachidonic acid. The prostanoids are PGD2, PGE2, PGF2, PGI2, and TXA2. There is ample evidence of a role for COX-2, the inducible COX isozyme, and its prostanoid metabolites, principally PGE2, in tumor angiogenesis.14–18

The prostanoids affect a wide range of physiological and pathologic processes by binding to distinct cell surface G-protein-coupled receptors (GPCRs). PGE2 binds and activates one (or more) of four prostaglandin E (EP) receptors: EP1, EP2, EP3, and EP4.19 The receptors demonstrate distinct, as well as opposing, effects on intracellular signaling events. The EP1 receptor couples to Gq and mediates a rise in intracellular calcium concentration. The EP3 and EP4 receptors couple to Gi and mediate a rise in cyclic adenosine monophosphate (cAMP) concentration. In contrast, the EP3 receptor coultes to Gs, reducing cAMP concentration.

Various groups have determined a direct role for PGE2 and EP4 in angiogenic gene expression,20,21 angiogenic cell behaviors,22–28 and the angiogenic component of tumor growth.22,29–32 However, most of these studies have been conducted using in vitro and in vivo models of colon cancer. It remains to be determined whether the EP4 receptor plays a similar role in ocular NV.

In this study, in vitro experiments were performed to investigate the influence of the EP4 receptor on discrete aspects of retinal angiogenesis. First, prostanoid-mediated VEGF production was assayed to investigate the role of the EP4 receptor in stimulating Müller cell VEGF production. Second, the effect of EP4 receptor antagonism on VEGF-induced endothelial cell proliferation and tube formation was investigated in retinal microvascular endothelial cells (RMECs). Finally, to further
investigate the therapeutic potential of EP4 receptor antagonism for human use, two clinically relevant in vivo models of ocular NV were used. Rat models of retinal and choroidal NV were used to assess the efficacy of EP4 receptor antagonism. These studies will help to define the role of the EP4 receptor in mediating pathologic ocular angiogenesis.

MATERIALS AND METHODS

Isolation and Culture of Primary Mouse Retinal Müller Cells

Primary retinal Müller cell cultures were established from P7 wild-type and COX-2-null mice (a generous gift from Sudhansu Dey, Cincinnati Children’s Hospital Medical Center) according to well-established methods. Briefly, enucleated eyes were placed in soaking medium (Dulbecco’s modified Eagle’s medium low glucose [DMEM]; HyClone, Logan, UT), supplemented with 1X antibiotic/antimycotic solution (Sigma, St. Louis, MO), overnight. The following day, eyes were incubated for 60 minutes at 37°C in digestion buffer composed of the soaking medium plus 0.1% trypsin and 70 U/mL collagenase. Retinas were then dissected, triturated, plated, and grown in DMEM supplemented with 10% fetal bovine serum (FBS) and 1X antibiotic/antimycotic solution. Cultures were maintained at 37°C in a 5% CO2/95% air (20.9% oxygen) atmosphere (normoxia) in a humidified incubator (NuAire, Plymouth, MN). Passages three to six were used for experiments.

Culture of Human Retinal Microvascular Endothelial Cells

Human RMECs (HRMECs; Cell Systems, Kirkland, WA) were cultured in tissue flasks coated with attachment factor (Cell Signaling, Danvers, MA) in endothelial basal medium (EBM; Cambrex, East Rutherford, NJ) supplemented with 10% FBS and endothelial growth supplements (EGM SingleQuotes; Cambrex). When experimental conditions required serum-free medium, EBM with no FBS or growth modifiers was used. Cultures were maintained at 37°C in a 5% CO2/95% air (20.9% oxygen) atmosphere (normoxia) in a humidified incubator.

Müller Cell VEGF Induction

Müller cells were isolated from wild-type and COX-2-null mice and grown to 70% subconfluence. In one experiment, COX-2-null cells were serum-starved for 12 hours (DMEM supplemented with 1X antibiotic/antimycotic solution) and then treated with vehicle (0.1% dimethyl sulfoxide [DMSO]) or increasing concentrations (0.1–10 μM) of the PGE2 EP4 agonist PGE2-OH (Cayman Chemical, Ann Arbor, MI). In a different experiment, COX-2-null cells were serum-starved for 12 hours and then treated with 1% serum medium in the absence or presence of 25 ng/mL VEGF. Some of the cells treated with VEGF received increasing concentrations (1–5 μM) of L-161982 for 24 hours. Cells were then labeled with BrdU for 12 hours, and BrdU incorporation was independently repeated two times.

HRMEC Proliferation

HRMECs were seeded in 10% serum EBM at 3000 cells/well in a 96-well plate and were allowed to attach and settle. HRMECs were serum-starved for 12 hours and then treated with 1% serum medium in the absence or presence of 25 ng/mL VEGF. Some of the cells treated with VEGF received increasing concentrations (1–5 μM) of L-161982 for 24 hours. Cells were then labeled with BrdU for 12 hours, and BrdU incorporation was quantified with a colorimetric ELISA (Roche, Indianapolis, IN) according to the manufacturer’s instructions. The experiment was independently repeated four times.

HRMEC Tube Formation

Six-well tissue culture plates were coated with 500 μL growth factor-reduced basement membrane matrix (Matrigel; Becton Dickinson, Franklin Lakes, NJ). HRMECs were seeded at 40,000 cells/well and treated with serum-free EBM containing vehicle (0.1% DMSO) or 5, 10, or 50 μM L-161982. The cells were cultured for 24 hours at 37°C in a 5% CO2 atmosphere. Tubes were observed with an IMT-2 inverted microscope (Olympus, Melville, NY), and images were captured with a DMC digitizing camera (Polaroid Corporation, Waltham, MA). Six fields per well were captured for quantitative analysis. The digitized images were imported into ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Capillary-like structures of more than two cell lengths were assessed, and the mean tube length per field of each well was calculated. The average tube length of each treatment group was reported. The experiment was independently repeated three times.

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. Litters of Sprague-Dawley rat pups and their mothers (Charles River Laboratories, Wilmington, MA) were transferred within 4 hours of birth to oxygen exposure chambers, where they received alternating 24-hour periods of 50% oxygen and 10% oxygen for 14 days.46 On postnatal day (P) 14, the oxygen-exposed rats were returned to room air. Vehicle (0.1% DMSO) or the EP4 antagonist L-161982 (0.01, 0.1, and 0.7 μM) was administered to oxygen-exposed rats at P14 by intravitreal injection, according to well-established methods.56 Six days after removal to room air, on P20, the rats were killed, and their retinas were dissected. After dissection, the retinas were stained with ADPase using well-established methods.60 Abnormal retinal neovascularization was measured via computer-assisted image analysis.39

Laser-Induced Choroidal Neovascularization

Laser-induced rupture of Bruch’s membrane was performed to produce CNV in 6-week-old male Brown Norway rats, as previously described.37 Using a hand-held coverslip as a contact lens, an argon laser photocoagulator (532 nm) mounted on a slit-lamp (Coherent Novus Omni; Laser Labs Inc., Tampa, FL) was used to create four lesions in both the left and right eyes of each animal (50-μm spot size, 0.1-second duration, 360 mW). The animals’ eyes were then divided into four treatment groups (vehicle [0.1% DMSO], 0.01 μM L-161982, 0.1 μM L-161982, 1 μM L-161982) and received intravitreal injections at the temporal ora on days 1, 3, and 7 after laser treatment. Fourteen days after laser application, rats were killed and the extent of CNV at the Bruch’s membrane rupture sites was measured. Endothelial cells in CNV lesions were identified by staining choroid-sclera-RPE flatmounts using FITC-conjugated isoelectin B4 (Sigma), and the elastin of the extracellular matrix was identified using an elastin antibody conjugated to Cy3 (Sigma). Areas of abnormal vascular growth were measured via computer-assisted image analysis using high-resolution digital images of the stained choroid-sclera-RPE flatmounts. This experiment was independently repeated two times.

Statistical Analysis

Data were analyzed with commercial software (JMP, SAS Institute, Cary, NC). Analysis of variance (ANOVA) with appropriate post hoc analyses was used to analyze data.
RESULTS

Effect of an EP₄ Agonist, PGE₁-OH, on VEGF Production

To investigate the contribution of the PGE₂ EP₄ receptor to VEGF production, COX-2-null mouse Müller cells were treated with increasing concentrations (0.1–10 μM) of PGE₁-OH, an EP₄ receptor agonist. Treatment lasted 6 hours. Agonism of the EP₄ receptor significantly (*p < 0.0001; †p ≤ 0.006; ‡p ≤ 0.0165) increased VEGF production by COX-2-null cells in a dose-dependent manner (Fig. 1).

Effect of an EP₄ Antagonist, L-161982, on PGE₂-Induced VEGF Production

To further investigate the contribution of the PGE₂ EP₄ receptor to VEGF production, wild-type Müller cells were pretreated with increasing concentrations (1–5 μM) of L-161982, an EP₄ receptor antagonist, for 45 minutes, followed by 10 μM PGE₂ stimulation. Treatment lasted 12 hours. Antagonism of the EP₄ receptor significantly (*p < 0.0066; †p ≤ 0.0291) decreased PGE₂-induced VEGF production by wild-type Müller cells (Fig. 2).

Effect of an EP₄ Antagonist, L-161982, on VEGF-Induced HRMEC Proliferation

To investigate the contribution of the EP₄ receptor to VEGF-induced HRMEC proliferation, HRMECs were treated with VEGF and increasing concentrations (1–5 μM) of the EP₄ receptor antagonist L-161982. L-161982 significantly (*p < 0.0001; †p ≤ 0.0033) inhibited VEGF-induced cell proliferation in HRMECs (Fig. 3).
Effect of an EP4 Antagonist, L-161982, on HRMEC Tube Formation

To investigate the influence of the EP4 receptor in HRMEC tube formation, HRMECs were treated with increasing concentrations (3–10 μM) of the EP4 receptor antagonist L-161982. L-161982 caused a dose-dependent decrease in HRMEC tube formation and significantly (*P < 0.0344) inhibited tube formation at the highest dose tested (Figs. 4, 5).

Effect of an EP4 Antagonist, L-161982, on OIR in the Rat

Figures 1 through 5 demonstrate that EP4 activation or inhibition influences the behaviors of two retinal cell types that are known to play roles in the pathologic ocular angiogenesis characteristic of neovascular retinopathies. Next, the efficacy of the EP4 antagonist L-161982 was tested in the rat model of oxygen-induced retinopathy (OIR). At P14, OIR rats received either vehicle (0.1% DMSO) or L-161982 (0.01, 0.1, or 0.7 μM) by intravitreal injection. Six days after injection, the retinas were dissected, flatmounted, stained, and assessed for extent of neovascularization via computer-assisted image analysis. As shown in Figures 6 and 7, EP4 receptor antagonism significantly (0.769 ± 0.141 [0.7 μM], †P < 0.0001; 1.088 ± 0.210 [0.1 μM], †P = 0.001; 1.267 ± 0.175 [0.01 μM], ‡P ≤ 0.0069 vs. 2.126 ± 0.204 mm² [vehicle-treated]) inhibited the severity of neovascularization in the OIR model.
Effect of an EP4 Antagonist, L-161982, on the Severity of LCNV in the Rat

The efficacy of L-161982 was tested in a second model of ocular neovascularization, the rat model of laser-induced CNV (LCNV). Rats received intravitreal injections of vehicle (0.1% DMSO) or 0.01, 0.1, or 1 μM L-161982 on days 1, 3, and 7 after laser treatment. Rats were killed 14 days after laser treatment. Analysis of stained flatmounts demonstrated that L-161982 significantly (172.666 ± 18.068 [drug-treated] vs. 257.133 ± 12.472 [vehicle-treated], *P = 0.0329) reduced the severity of the LCNV response at the highest concentration tested (1 μM), as indicated by a reduced area of choroidal endothelial cell infiltration at the lesion site (Figs. 8, 9).

FIGURE 5. The effect of an EP4 antagonist, L-161982, on HRMEC tube formation. L-161982 (10 μM) significantly decreased tube formation, as depicted in representative photomicrographs. (A) HRMECs treated with vehicle (0.1% DMSO). (B) HRMECs treated with 10 μM L-161982.

FIGURE 6. The effect of an EP4 antagonist, L-161982, on the severity of OIR in the rat. L-161982 significantly decreased the severity of OIR in a dose-dependent manner. Each bar represents the mean ± SEM. *P < 0.0001; †P = 0.001; ‡P = 0.0069 (Dunnett’s post-hoc analysis). For vehicle, n = 9; for 0.01 and 0.1 μM, n = 10; for 0.7 μM, n = 11.

DISCUSSION

The COX-2 enzyme leads to the production of five bioactive lipids (prostanoids) that mediate diverse physiological and pathophysiological processes. Of the prostanoids, PGE2 is most...
consistently increased in angiogenic human tumors.\textsuperscript{14–18} We have demonstrated that PGE\textsubscript{2} is increased in in vitro experiments that model retinal angiogenic cell behaviors and in in vivo models of retinal angiogenesis (data not shown). Preliminary studies conducted in our laboratory suggest that the effect of PGE\textsubscript{2} on retinal angiogenesis is mediated by the EP\textsubscript{4} receptor. To our knowledge, this study is the first to examine and demonstrate a role for the EP\textsubscript{4} receptor in retinal angiogenesis.

Müller cells derived from COX-2-null mice exhibit reduced VEGF production (Yanni SE, et al. \textit{IOVS} 2007;48:ARVO E-Abstract 51), presumably because of the absence of COX-2 and proangiogenic prostanoid production. We have demonstrated that VEGF can be stimulated in COX-2-null Müller cells by the EP\textsubscript{4} agonist PGE\textsubscript{2}-OH (Fig. 1). Compared with wild-type cells, COX-2-null cells in culture do not demonstrate any significant difference in the protein level of EP\textsubscript{4} (data not shown). This suggests that the results in Figure 1 are not attributed to EP\textsubscript{4} compensation in COX-2-null cells. We have also demonstrated that PGE\textsubscript{2}-induced VEGF can be inhibited by the EP\textsubscript{4} receptor antagonist L-161982 (Fig. 2). To our knowledge, this study is the first to use primary cultures of Müller cells derived from COX-2-deficient mice. Our Müller cell data complements a growing body of data in the literature; various cell types and model systems have been used to demonstrate that VEGF production is at least partially dependent on the EP\textsubscript{4} receptor.\textsuperscript{21,26,38–40} We have also demonstrated that HRMECs treated with the EP\textsubscript{4} antagonist L-161982 exhibit reduced VEGF-induced cell proliferation and tube formation (Figs. 3–5). Notably, L-161982 significantly inhibits HRMEC proliferation at a concentration lower than that required to inhibit HRMEC tube formation. Under our assay conditions, tube formation requires little, if any, cell proliferation. The finding that L-161982 more effectively inhibits HRMEC proliferation than tube formation suggests that the EP\textsubscript{4} receptor differentially regulates angiogenic endothelial cell behaviors, exerting a much stronger influence on proliferation than migration. The fact that only the highest concentration (10 μM) of L-161982 demonstrated an effect on HRMEC tube formation suggests that the EP\textsubscript{4} receptor might not play an important role in vascular reorganization (as modeled by this assay) but may play a more important role in sprouting angiogenesis. Additional experiments could be used to corroborate the role of EP\textsubscript{4} in sprouting angiogenesis in vitro. Additionally, to more clearly define the activity of L-161982, it will be necessary to explore the signal intermediates affected by drug treatment. Our HRMEC data also complement the literature, which demonstrates that in...
other cell types, the EP4 receptor is involved in ERK activation, cell proliferation, and angiogenic cell behavior.\textsuperscript{24,26,27} Ideally, the investigators would like to have assessed the effect of EP4 agonism in VEGF-induced HRMEC assays (proliferation and tube formation). The appropriate way to perform this experiment is in the absence of endogenous prostaglandin production and influence. Therefore, cells isolated from COX-2-null mice are the optimal experimental venue. Unfortunately, this approach was not possible for the following reasons: in culture, COX-2-null mouse RMECs (MRMECs) lose their EC phenotype and do not survive passaging, rendering them useless in in vitro assays of the type required. After unsuccessfully trying this approach, the authors investigated siRNA knockdown of COX-2 in HRMECs to use knockdown cells for agonist studies. However, only 60% knockdown was obtained, despite having tried several siRNA sequences alone and in combination. In these cases, enough residual COX-2 activity remained to confound the results obtained using knockdown cells treated with the EP4 agonist. These experiments indicate that the EP4 receptor mediates distinct angiogenic cell behaviors in two retinal cell types that are known to play roles in the pathologic ocular angiogenesis characteristic of neovascular retinopathies. This finding is significant because it suggests that EP4 receptor inhibition has the potential to affect the ocular angiogenic cascade at more than one point, providing a more powerful and effective therapeutic target for angiogenic diseases of the eye and other tissues.

As an initial step in determining therapeutic potential, we tested the efficacy of the EP4 antagonist L-161982 in rat models of OIR and LCNV and have shown that this compound reduced the severity of neovascularization in both model systems (Figs. 6–9). In both models, L-161982 was injected into the vitreous cavity. Thus, L-161982 may be more bioavailable at sites of preretinal NV than at sites of subretinal NV, explaining the drug’s superior performance in OIR versus LCNV. L-161982, at high concentrations, binds and activates the angiostatin II AT1 receptor, which has angiogenic activity.\textsuperscript{41} Of particular relevance, the angiogenic activity of the AT1 receptor has been demonstrated in a mouse model of OIR.\textsuperscript{42,43} Additionally, L-161982 has the following Kᵢ values for other prostanoid receptors (in nM): 0.024 for EP4, 0.71 for TP, 1.90 for EP3, 5.10 for DP, 5.63 for EP2, 6.74 for IP, 19 for EP3, and 23 for EP2. Some of these receptors have demonstrated angiogenic activity, as detailed in the literature.\textsuperscript{44} Thus, the in vivo concentrations chosen should be selective for EP4. For this reason, we chose to inject low concentrations of L-161982 in the OIR and LCNV models. Studies using EP4 null cells and animals are under way to complement the data presented herein and to more clearly define the specific role(s) of the EP4 receptor in ocular neovascularization, without the confounding factor of AT1 receptor activation. Preliminary data suggest that the pharmacologic data presented here will be validated by studies using genetically modified mice and cells derived from their retinas.

Various models of in vivo angiogenesis and tumor growth have similarly demonstrated that the EP4 receptor is proangiogenic and that EP4 receptor inhibition elicits an antiangiogenic effect.\textsuperscript{22,27,29,31,52} The data presented here suggest that the EP4 receptor exerts its angiogenic influence by promoting VEGF production by Müller cells and that antagonism of the receptor inhibits VEGF production by Müller cells and endothelial cell proliferation and tube formation. These novel findings suggest that EP4 receptor antagonism may be a rational therapeutic strategy for the treatment of human neovascular eye disease.

**References**


