Published in final edited form as:
*Invest Ophthalmol Vis Sci*. 2009 November ; 50(11): 5479–5486. doi:10.1167/iovs.09-3652.

# The Role of PGE$_2$ Receptor EP$_4$ in Pathologic Ocular Angiogenesis

**Susan E. Yanni**[1], **Joshua M. Barnett**[2], **Monika L. Clark**[1], and **John S. Penn**[1,2,3]

[1]Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, Tennessee

[2]Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee

[3]Department of Ophthalmology and Visual Sciences, Vanderbilt University School of Medicine, Nashville, Tennessee

## Abstract

**Purpose—**PGE$_2$ binds to PGE$_2$ receptors (EP$_{1\text{-}4}$). The purpose of the present study was to investigate the role of the EP$_4$ receptor in angiogenic cell behaviors of retinal Müller cells and retinal microvascular endothelial cells (RMECs) and to assess the efficacy of an EP$_4$ 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 EP$_4$ agonist PGE$_1$-OH, and wild-type Müller cells were treated with increasing concentrations of the EP$_4$ 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 PGE$_1$-OH demonstrated a significant increase in VEGF production ($P \leq 0.0165$). Wild-type mouse Müller cells treated with increasing concentrations of L-161982 demonstrated a significant decrease in VEGF production ($P \leq 0.0291$). HRMECs treated with increasing concentrations of L-161982 demonstrated a significant reduction in VEGF-induced cell proliferation ($P \leq 0.0033$) and tube formation ($P < 0.0344$). L-161982 treatment significantly reduced pathologic neovascularization in OIR ($P < 0.0069$) and LCNV ($P \leq 0.0329$).

**Conclusions—**Preliminary investigation has demonstrated that EP$_4$ activation or inhibition influences the behaviors of two retinal cell types known to play roles in pathologic ocular angiogenesis. These findings suggest that the EP$_4$ receptor may be a valuable therapeutic target in neovascular eye disease.

---

Corresponding author: John S. Penn, Vanderbilt Eye Institute, Vanderbilt University School of Medicine, 8000 Medical Center East, Nashville, TN 37232-8808; john.penn@vanderbilt.edu..

Disclosure: **S.E. Yanni**, None; **J.M. Barnett**, None; **M.L. Clark**, None; **J.S. Penn**, None

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.[8,10,11] Once VEGF is produced and secreted, it binds and activates two cell-surface receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-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 $PGD_2$, $PGE_2$, $PGF_2$, $PGI_2$, and $TXA_2$. There is ample evidence of a role for COX-2, the inducible COX isoform, and its prostanoid metabolites, principally $PGE_2$, 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). $PGE_2$ binds and activates one (or more) of four prostaglandin E (EP) receptors: $EP_1$, $EP_2$, $EP_3$, and $EP_4$.[19] The receptors demonstrate distinct, as well as opposing, effects on intracellular signaling events. The $EP_1$ receptor couples to $G_q$ and mediates a rise in intracellular calcium concentration. The $EP_2$ and $EP_4$ receptors couple to $G_s$ and mediate a rise in cyclic adenosine monophosphate (cAMP) concentration. In contrast, the $EP_3$ receptor couples to $G_i$, reducing cAMP concentration.

Various groups have determined a direct role for $PGE_2$ and $EP_4$ 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 $EP_4$ receptor plays a similar role in ocular NV.

In this study, in vitro experiments were performed to investigate the influence of the $EP_4$ receptor on discrete aspects of retinal angiogenesis. First, prostanoid-mediated VEGF production was assayed to investigate the role of the $EP_4$ receptor in stimulating Müller cell VEGF production. Second, the effect of $EP_4$ 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 $EP_4$ 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 $EP_4$ receptor antagonism. These studies will help to define the role of the $EP_4$ 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.[33] Briefly, enucleated eyes were placed in soaking medium (Dulbecco's modified Eagle's medium low glucose [DMEM]; HyClone, Logan, UT), supplemented with 1× 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 1× antibiotic/antimycotic solution. Cultures were maintained at 37°C in a 5% $CO_2$/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 SingleQuots; 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% $CO_2$/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 1× antibiotic/antimycotic solution) and then treated with vehicle (0.1% dimethyl sulfoxide [DMSO]) or increasing concentrations (0.1-10 $\mu$M) of the $PGE_2$ $EP_4$ agonist $PGE_1$-OH (Cayman Chemical, Ann Arbor, MI) in 2% serum medium. After 6 hours, culture medium from experimental dishes was collected and assayed for VEGF protein concentration. In a separate experiment, wild-type mouse Müller cells were serum-starved for 12 hours and then pretreated with vehicle (0.1% DMSO) or increasing concentrations (1-5 $\mu$M) of the $EP_4$ antagonist L-161982 in 2% serum medium. Forty-five minutes later, the cells were treated with 10 $\mu$M $PGE_2$ (Cayman Chemical). After 12 hours, culture medium from experimental dishes was collected and assayed for VEGF protein concentration. For both experiments, VEGF protein concentration was measured using the mouse VEGF-164 ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Cells were washed with cold calcium- and magnesium-free PBS (Invitrogen Corporation, Carlsbad, CA) and lysed with cold lysis buffer (Promega, Madison, WI). The amount of VEGF (pg/mL) in culture medium was normalized to total protein concentration (mg/mL) of cell lysates using a BCA assay (Pierce, Rockford, IL). These experiments were 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 $\mu$M) 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 $\mu$L 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 3, 5, or 10 $\mu$M L-161982. The cells were cultured for 24 hours at 37°C in a 5% $CO_2$ 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.[34] On postnatal day (P) 14, the oxygen-exposed rats were returned to room air. Vehicle (0.1% DMSO) or the $EP_4$ antagonist L-161982 (0.01, 0.1, and 0.7 $\mu$M) was administered to oxygen-exposed rats at P14 by intravitreal injection, according to well-established methods.[35] 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.[36] Abnormal retinal neovascularization was measured via computer-assisted image analysis.[35]

### 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-$\mu$m spot size, 0.1-second duration, 360 mW). The animals' eyes were then divided into four treatment groups (vehicle [0.1% DMSO], 0.01 $\mu$M L-161982, 0.1 $\mu$M L-161982, 1 $\mu$M 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 isolectin $B_4$ (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 $EP_4$ Agonist, $PGE_1$-OH, on VEGF Production

To investigate the contribution of the $PGE_2$ $EP_4$ receptor to VEGF production, COX-2-null Müller cells were treated with increasing concentrations (0.1-10 $\mu$M) of $PGE_1$-OH, an $EP_4$ receptor agonist. Treatment lasted 6 hours. Agonism of the $EP_4$ receptor significantly ($^*P < 0.0001$; $^\dagger P \le 0.006$; $^\ddagger P \le 0.0165$) increased VEGF production by COX-2-null Müller cells in a dose-dependent manner (Fig. 1).

### Effect of an $EP_4$ Antagonist, L-161982, on $PGE_2$-Induced VEGF Production

To further investigate the contribution of the $PGE_2$ $EP_4$ receptor to VEGF production, wild-type Müller cells were pretreated with increasing concentrations (1-5 $\mu$M) of L-161982, an $EP_4$ receptor antagonist, for 45 minutes, followed by 10 $\mu$M $PGE_2$ stimulation. Treatment lasted 12 hours. Antagonism of the $EP_4$ receptor significantly ($^*P < 0.0066$; $^\dagger P \le 0.0291$) decreased $PGE_2$-induced VEGF production by wild-type Müller cells (Fig. 2).

### Effect of an $EP_4$ Antagonist, L-161982, on VEGF-Induced HRMEC Proliferation

To investigate the contribution of the $EP_4$ receptor to VEGF-induced HRMEC proliferation, HRMECs were treated with VEGF and increasing concentrations (1-5 $\mu$M) of the $EP_4$

receptor antagonist L-161982. L-161982 significantly (*$P < 0.0001$; †$P \leq 0.0033$) inhibited VEGF-induced cell proliferation in HRMECs (Fig. 3).

### Effect of an $EP_4$ Antagonist, L-161982, on HRMEC Tube Formation

To investigate the influence of the $EP_4$ receptor in HRMEC tube formation, HRMECs were treated with increasing concentrations (3-10 $\mu$M) of the $EP_4$ 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 $EP_4$ Antagonist, L-161982, on OIR in the Rat

Figures 1 through 5 demonstrate that $EP_4$ 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 $EP_4$ 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 $\mu$M) 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, $EP_4$ receptor antagonism significantly (0.769 ± 0.141 [0.7 $\mu$M], *$P < 0.0001$; 1.088 ± 0.210 [0.1 $\mu$M], †$P \leq 0.001$; 1.267 ± 0.175 [0.01 $\mu$M], ‡$P \leq 0.0069$ vs. 2.126 ± 0.204 $mm^2$ [vehicle-treated]) inhibited the severity of neovascularization in the OIR model.

### Effect of an $EP_4$ 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 $\mu$M 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 $\mu m^2$ [vehicle-treated], *$P \leq 0.0329$) reduced the severity of the LCNV response at the highest concentration tested (1 $\mu$M), as indicated by a reduced area of choroidal endothelial cell infiltration at the lesion site (Figs. 8, 9).

## Discussion

The COX-2 enzyme leads to the production of five bioactive lipids (prostanoids) that mediate diverse physiological and pathophysiological processes. Of the prostanoids, $PGE_2$ is most consistently increased in angiogenic human tumors.[14-18] We have demonstrated that $PGE_2$ 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 $PGE_2$ on retinal angiogenesis is mediated by the $EP_4$ receptor. To our knowledge, this study is the first to examine and demonstrate a role for the $EP_4$ receptor in retinal angiogenesis.

Müller cells derived from COX-2-null mice exhibit reduced VEGF production (Yanni SE, et al. *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 $EP_4$ agonist $PGE_1$-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 $EP_4$ (data not shown). This suggests that the results in Figure 1 are not attributed to $EP_4$ compensation in COX-2-null cells. We have also demonstrated that $PGE_2$-induced VEGF can be inhibited by the $EP_4$ 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 $EP_4$ receptor.[21,28,38-40] We have also demonstrated that HRMECs treated with the $EP_4$ 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 $EP_4$ receptor differentially regulates angiogenic endothelial cell behaviors, exerting a much stronger influence on proliferation than migration. The fact that only the highest concentration (10 $\mu$M) of L-161982 demonstrated an effect on HRMEC tube formation suggests that the $EP_4$ 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 $EP_4$ 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 $EP_4$ receptor is involved in ERK activation, cell proliferation, and angiogenic cell behavior.[24,26,27] Ideally, the investigators would like to have assessed the effect of $EP_4$ 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 $EP_4$ agonist.

These experiments indicate that the $EP_4$ 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 $EP_4$ 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 $EP_4$ 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 angiotensin II AT1 receptor, which has angiogenic activity.[41] Of particular relevance, the angiogenic activity of the AT1 receptor has been demonstrated in a mouse model of OIR.[42,43] Additionally, L-161982 has the following $K_i$ values for other prostanoid receptors (in $\mu$M): 0.024 for $EP_4$, 0.71 for TP, 1.90 for $EP_3$, 5.10 for DP, 5.63 for FP, 6.74 for IP, 19 for $EP_1$, and 23 for $EP_2$. Some of these receptors have demonstrated angiogenic activity, as detailed in the literature.[44] Thus, the in vivo concentrations chosen should be selective for $EP_4$. For this reason, we chose to inject low concentrations of L-161982 in the OIR and LCNV models. studies using $EP_4$ null cells and animals are under way To complement the data presented herein and to more clearly define the specific role(s) of the $EP_4$ 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 $EP_4$ receptor is proangiogenic and that $EP_4$ receptor inhibition elicits an antiangiogenic effect.[22,27,29,31,32] The data presented here suggest that the $EP_4$ 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 $EP_4$ receptor antagonism may be a rational therapeutic strategy for the treatment of human neovascular eye disease.

## Acknowledgments

Supported by National Institutes of Health Grants EY07533 and EY01826, an unrestricted grant from Research to Prevent Blindness, Inc., and a Research to Prevent Blindness Senior Scientific Investigator Award (JSP).

## References

1. Li WW, Talcott KE, Zhai AW, et al. The role of therapeutic angiogenesis in tissue repair and regeneration. Adv Skin Wound Care. 2005; 18:491–500. [PubMed: 16365547]
2. Rahmani B, Tielsch JM, Kat J, et al. The cause-specific prevalence of visual impairment in an urban population: the Baltimore Eye Survey. Ophthalmology. 1999; 103:1721–1726. [PubMed: 8942862]
3. Lee P, Wang CC, Adamis AP. Ocular neovascularization: an epidemiologic review. Surv Ophthalmol. 1998; 43:245–269. [PubMed: 9862312]
4. Steinkuller PG, Du L, Gilbert C, et al. Childhood blindness. J AAPOS. 1999; 3:26–32. [PubMed: 10071898]
5. D'Amore PA. Mechanisms of retinal and choroidal angiogenesis. Invest Ophthalmol Vis Sci. 1994; 35:3974–3979. [PubMed: 7525506]
6. Casey R, Li WW. Factors controlling ocular angiogenesis. Am J Ophthalmol. 1997; 124:521–529. [PubMed: 9323943]
7. Aiello LP, Northrup JM, Keyt BA, et al. Hypoxic regulation of vascular endothelial growth factor in retinal cells. Arch Ophthalomol. 1995; 113:1538–1544.

8. Pierce EA, Avery RL, Foley ED, et al. Vascular endothelial growth factor/vascular permeability factor expression in a mouse model of retinal neovascularization. Proc Natl Acad Sci U S A. 1995; 92:905–909. [PubMed: 7846076]
9. Aiello LP, Pierce EA, Foley ED, et al. Suppression of retinal neovascularization in vivo by inhibition of vascular endothelial growth factor (VEGF) using soluble VEGF-receptor chimeric proteins. Proc Natl Acad Sci U S A. 1995; 92:10457–10461. [PubMed: 7479819]
10. Robbins SG, Conaway JR, Ford BL, et al. Detection of VEGF protein in vascular and non-vascular cells of the normal and oxygeninjured retina. Growth Factors. 1997; 14:229–241. [PubMed: 9386988]
11. Robbins SG, Rajaratnam VS, Penn JS. Evidence for upregulation of vascular endothelial cell growth factor by hypoxia. Growth Factors. 1998; 16:1–9. [PubMed: 9777366]
12. Byrne AM, Bouchier-Hayes DJ, Harmey JH. Angiogenic and cell survival functions of vascular endothelial growth factor (VEGF). J Cell Mol Med. 2005; 9:777–794. [PubMed: 16364190]
13. Gille H, Kowalski J, Li B, et al. Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2): a reassessment using novel receptor-specific vascular endothelial growth factor mutants. J Biol Chem. 2001; 276:3222–3230. [PubMed: 11058584]
14. Kawamori T, Rao CV, Seibert K, et al. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. Cancer Res. 58:409–412. 998. [PubMed: 9458081]
15. Williams CS, Mann M, DuBois RN. The role of cyclooxygenases in inflammation, cancer and development. Oncogene. 1999; 18:7908–7916. [PubMed: 10630643]
16. Form DM, Auerbach R. PGE2 and angiogenesis. Proc Soc Exp Biol Med. 1983; 172:214–218. [PubMed: 6572402]
17. Ziche M, Jones J, Gullino PM. Role of prostaglandin E1 and copper in angiogenesis. J Natl Cancer Inst. 1982; 69:475–482. [PubMed: 6180207]
18. Diaz-Flores L, Gutierrez R, Varela H. Angiogenesis: an update. Histol Histopathol. 1994; 9:807–843. [PubMed: 7534514]
19. Hata AN, Breyer RM. Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. Pharmacol Ther. 2004; 103:147–166. [PubMed: 15369681]
20. Hatazawa R, Tanaka A, Tanigami M, et al. Cyclooxygenase-2/prostaglandin E2 accelerates the healing of gastric ulcers via EP4 receptors. Am J Physiol Gastrointest Liver Physiol. 2007; 293:G788–G797. [PubMed: 17673547]
21. Hatazawa R, Tanigami M, Izumi N, et al. Prostaglandin E2 stimulates VEGF expression in primary rat gastric fibroblasts through EP4 receptors. Inflammopharmacology. 2007; 15:214–217. [PubMed: 17943254]
22. Rao R, Redha R, Macias-Perez I, et al. Prostaglandin E2-EP4 receptor promotes endothelial cell migration via ERK activation and angiogenesis in vivo. J Biol Chem. 2007; 282:16959–16968. [PubMed: 17401137]
23. Kabashima K, Sakata D, Nagamachi M, et al. Prostaglandin E2-EP4 signaling initiates skin immune responses by promoting migration and maturation of Langerhans cells. Nat Med. 2003; 9:744–749. [PubMed: 12740571]
24. Cherukuri DP, Chen XB, Goulet AC, et al. The EP4 receptor antagonist, L-161982, blocks prostaglandin E2-induced signal transduction and cell proliferation in HCA-7 colon cancer cells. Exp Cell Res. 2007; 313:2969–2979. [PubMed: 17631291]
25. Hawcroft G, Ko CW, Hull MA. Prostaglandin E2-EP4 receptor signalling promotes tumorigenic behaviour of HT-29 human colorectal cancer cells. Oncogene. 2007; 26:3006–3019. [PubMed: 17130837]
26. Pozzi A, Yan X, Macias-Perez I, et al. Colon carcinoma cell growth is associated with prostaglandin E2/EP4 receptor-evoked ERK activation. J Biol Chem. 2004; 279:29797–29804. [PubMed: 15123663]
27. Ma X, Kundu N, Rifat S, et al. Prostaglandin E receptor EP4 antagonism inhibits breast cancer metastasis. Cancer Res. 2006; 66:2923–2927. [PubMed: 16540639]

28. Muller M, Sales KJ, Katz AA, et al. Seminal plasma promotes the expression of tumorigenic and angiogenic genes in cervical adenocarcinoma cells via the E-series prostanoid 4 receptor. Endocrinology. 2006; 147:3356–3365. [PubMed: 16574793]
29. Yang L, Huang Y, Porta R, et al. Host and direct antitumor effects and profound reduction in tumor metastasis with selective EP4 receptor antagonism. Cancer Res. 2006; 66:9665–9672. [PubMed: 17018624]
30. Chell SD, Witherden IR, Dobson RR, et al. Increased EP4 receptor expression in colorectal cancer progression promotes cell growth and anchorage independence. Cancer Res. 2006; 66:3106–3113. [PubMed: 16540660]
31. Kitamura T, Itoh M, Noda T, et al. Combined effects of prostaglandin E receptor subtype EP1 and subtype EP4 antagonists on intestinal tumorigenesis in adenomatous polyposis coli gene knockout mice. Cancer Sci. 2003; 94:618–621. [PubMed: 12841871]
32. Mutoh M, Watanabe K, Kitamura T, et al. Involvement of prostaglandin E receptor subtype EP(4) in colon carcinogenesis. Cancer Res. 2002; 62:32.
33. Hicks D, Curtois Y. The growth and behaviour of rat retinal Müller cells in vitro, 1: an improved method for isolation and culture. Exp Eye Res. 1990; 51:119–129. [PubMed: 2387332]
34. Penn JS, Henry MM, Tolman BL. Exposure to alternating hypoxia and hyperoxia causes severe proliferative retinopathy in the newborn rat. Pediatr Res. 1994; 26:724–731. [PubMed: 7898981]
35. Barnett JM, McCollum GW, Fowler JA, et al. Pharmacologic and genetic manipulation of MMP-2 and -9 affects retinal neovascularization in rodent models of OIR. Invest Ophthalmol Vis Sci. 2007; 28:907–915. [PubMed: 17251494]
36. Penn JS, Tolman BL, Lowery LA. Variable oxygen exposure causes preretinal neovascularization in the newborn rat. Invest Ophthalmol Vis Sci. 1993; 34:576–585. [PubMed: 8449677]
37. Bora PS, Sohn JH, Cruz JM, et al. Role of complement and complement membrane attack complex in laser-induced choroidal neovascularization. J Immunol. 2005; 174:491–497. [PubMed: 15611275]
38. Inoue H, Takamori M, Shimoyama Y, et al. Regulation by PGE2 of the production of interleukin-6, macrophage colony stimulating factor, and vascular endothelial growth factor in human synovial fibroblasts. Br J Pharmacol. 2002; 136:287–295. [PubMed: 12010778]
39. Spinella F, Rosanò L, Di Castro V, et al. Endothelin-1-induced prostaglandin E2-EP2, EP4 signaling regulates vascular endothelial growth factor production and ovarian carcinoma cell invasion. J Biol Chem. 2004; 279:46700–46705. [PubMed: 15347673]
40. Bradbury D, Clarke D, Seedhouse C, et al. Vascular endothelial growth factor induction by prostaglandin E2 in human airway smooth muscle cells is mediated by E prostanoid EP2/EP4 receptors and SP-1 transcription factor binding sites. J Biol Chem. 2005; 280:29993–30000. [PubMed: 15970595]
41. Fujita M, Hayashi I, Yamashina S, et al. Blockade of angiotensin AT1a receptor signaling reduces tumor growth, angiogenesis, and metastasis. Biochem Biophys Res Commun. 2002; 294:441–447. [PubMed: 12051731]
42. Lonchampt M, Pennel L, Duhault J. Hyperoxia/normoxia-driven retinal angiogenesis in mice: a role for angiotensin II. Invest Ophthalmol Vis Sci. 2001; 42:429–432. [PubMed: 11157878]
43. Nagai N, Noda K, Urano T, et al. Selective suppression of pathologic, but not physiologic, retinal neovascularization by blocking the angiotensin II type 1 receptor. Invest Ophthalmol Vis Sci. 2005; 46:1078–1084. [PubMed: 15728568]
44. Wang MT, Honn KV, Nie D. Cyclooxygenases, prostanoids, and tumor progression. Cancer Metastasis Rev. 2007; 26:525–534. [PubMed: 17763971]

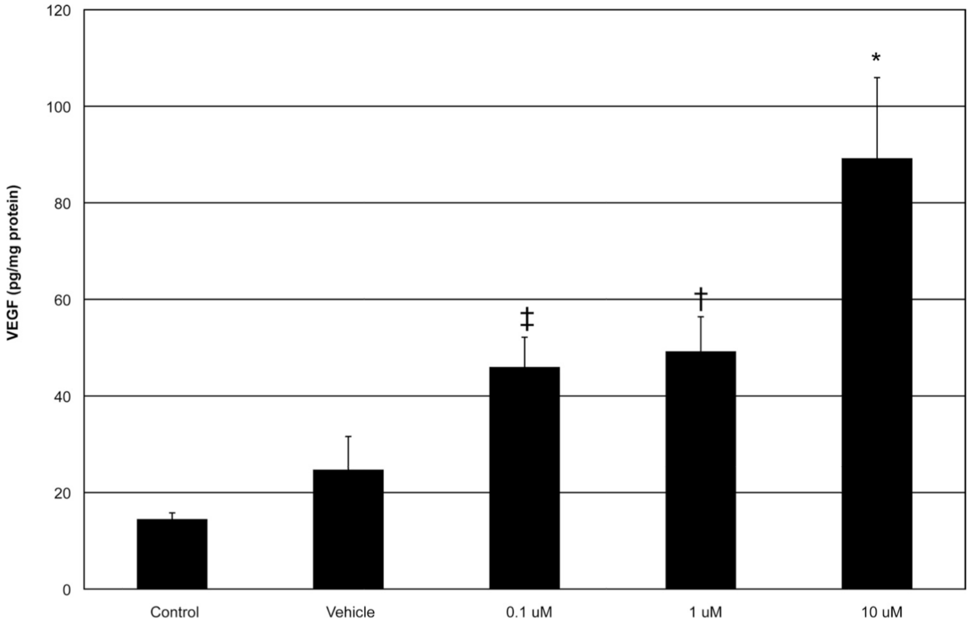

**Figure 1.**

The effect of an $EP_4$ agonist, $PGE_1$-OH, on VEGF production in COX-2-null mouse Müller cells. $PGE_1$-OH significantly increased VEGF production by COX-2-null cells. Each bar represents the mean ± SD. $^*P < 0.0001$; $^\dagger P \leq 0.006$; $^\ddagger P \leq 0.0165$ (Dunnett's post hoc analysis). For each bar, $n = 4$.

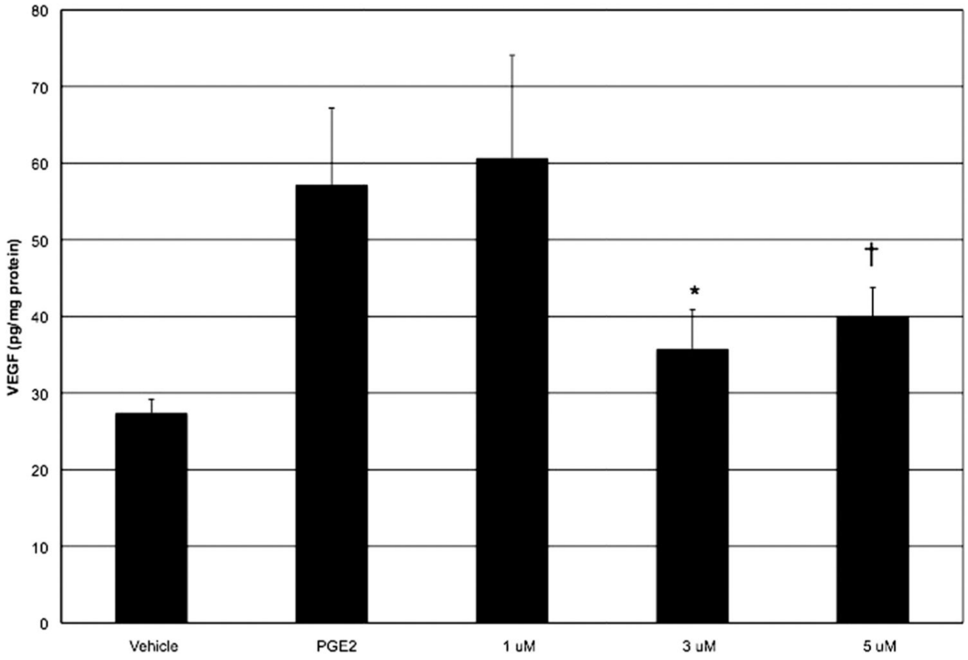

**Figure 2.**

The effect of an $EP_4$ antagonist, L-161982, on $PGE_2$-induced VEGF production by wild-type mouse Müller cells. L-161982 pretreatment significantly decreased $PGE_2$-induced VEGF production by wild-type mouse Müller cells. Each bar represents the mean ± SD. $^*P < 0.0066$; $^\dagger P \leq 0.0291$ (Dunnett's post hoc analysis). For each bar, $n = 4$.

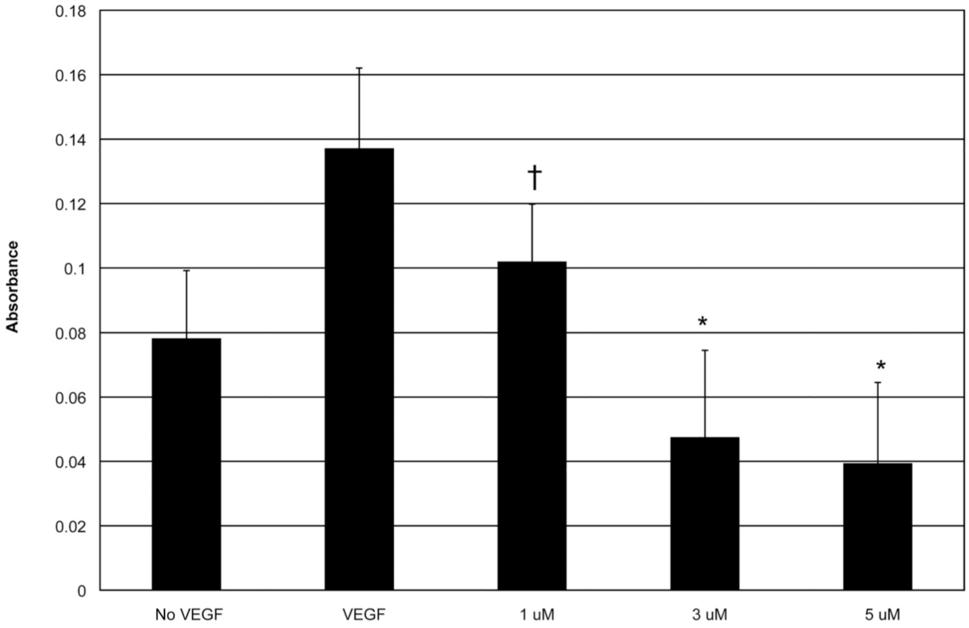

**Figure 3.**

The effect of an $EP_4$ antagonist, L-161982, on VEGF-induced HRMEC proliferation. HRMEC proliferation was stimulated with 25 ng/mL VEGF. L-161982 significantly decreased VEGF-induced cell proliferation in HRMECs. Each bar represents the mean ± SD. $^*P < 0.0001$; $^\dagger P \leq 0.0033$ (Dunnett's post hoc analysis). For each bar, $n = 11$.

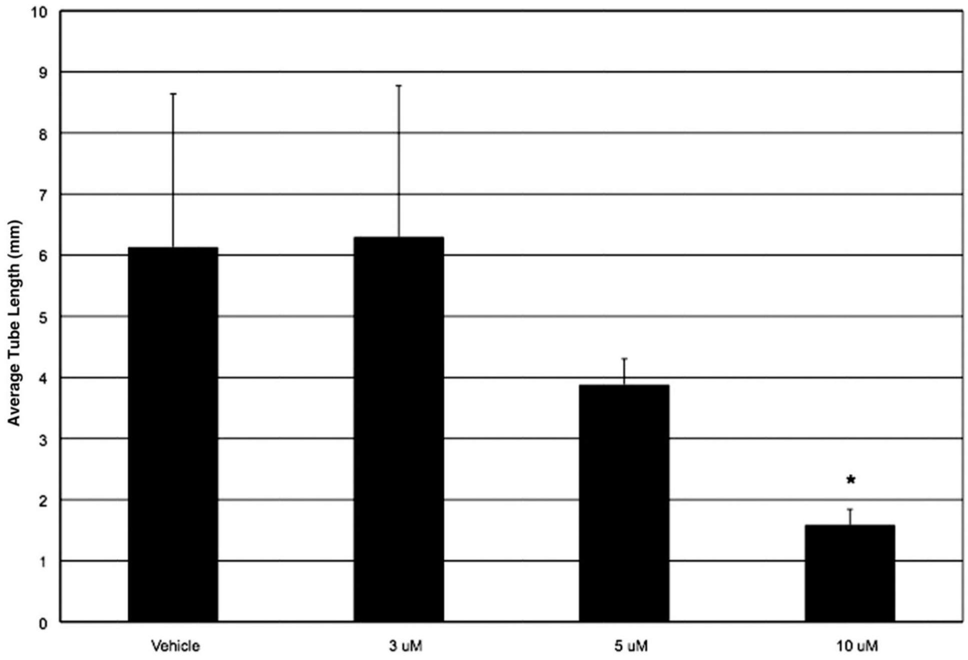

**Figure 4.**

The effect of an $EP_4$ antagonist, L-161982, on HRMEC tube formation. L-161982 significantly decreased tube formation in a dose-dependent manner. Each bar represents the mean ± SD. *$P < 0.0344$ (Dunnett's post-hoc analysis). For each bar, $n = 3$.

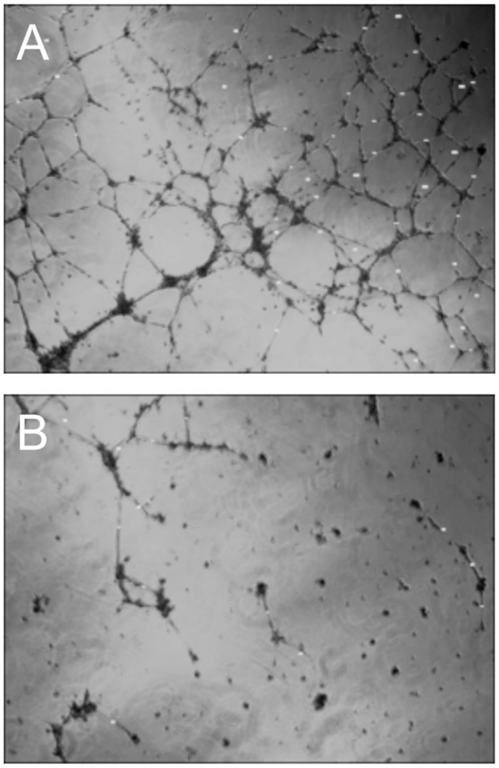

**Figure 5.**

The effect of an $EP_4$ antagonist, L-161982, on HRMEC tube formation. L-161982 (10 $\mu$M) significantly decreased tube formation, as depicted in representative photomicrographs. (**A**) HRMECs treated with vehicle (0.1% DMSO). (**B**) HRMECs treated with 10 $\mu$M L-161982.

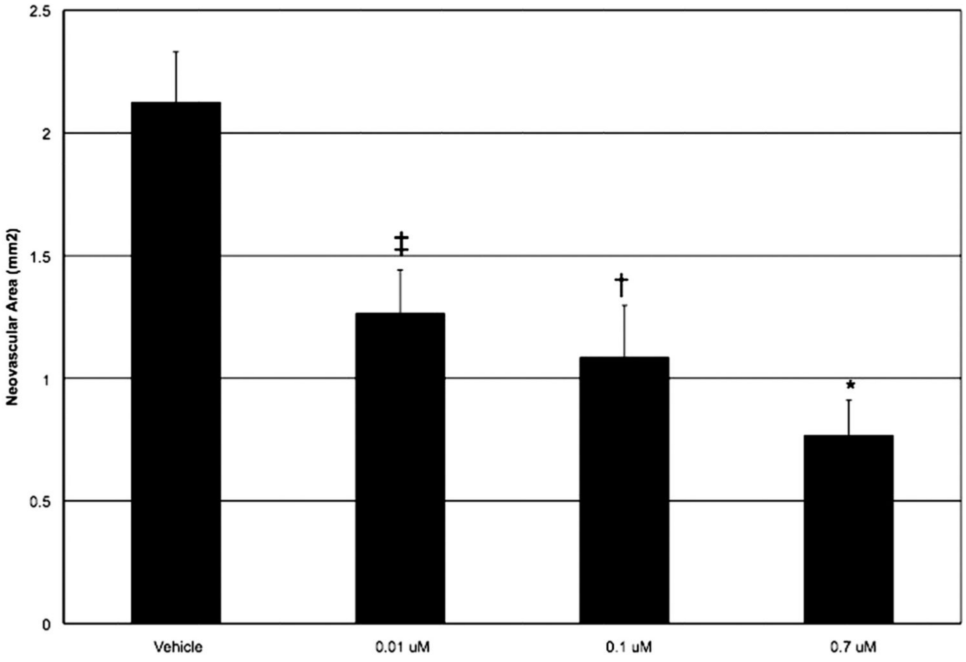

**Figure 6.**

The effect of an $EP_4$ 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. *$P < 0.0001$; †$P \leq 0.001$; ‡$P \leq 0.0069$ (Dunnett's post-hoc analysis). For vehicle, $n = 9$; for 0.01 and 0.1 $\mu$M, $n = 10$; for 0.7 $\mu$M, $n = 11$.

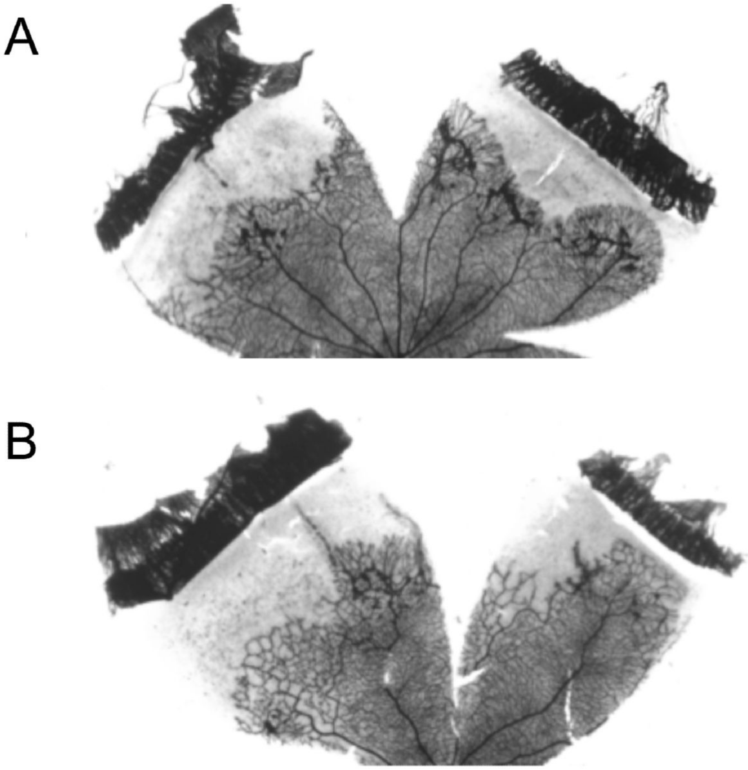

**Figure 7.**

The effect of an $EP_4$ antagonist, L-161982, on the severity of OIR in the rat, as visualized by representative ADPase-stained retinal flatmounts. L-161982 significantly decreased the severity of OIR. (**A**) Eye treated with vehicle (0.1% DMSO). (**B**) Eye treated with 0.7 $\mu$M L-161982.

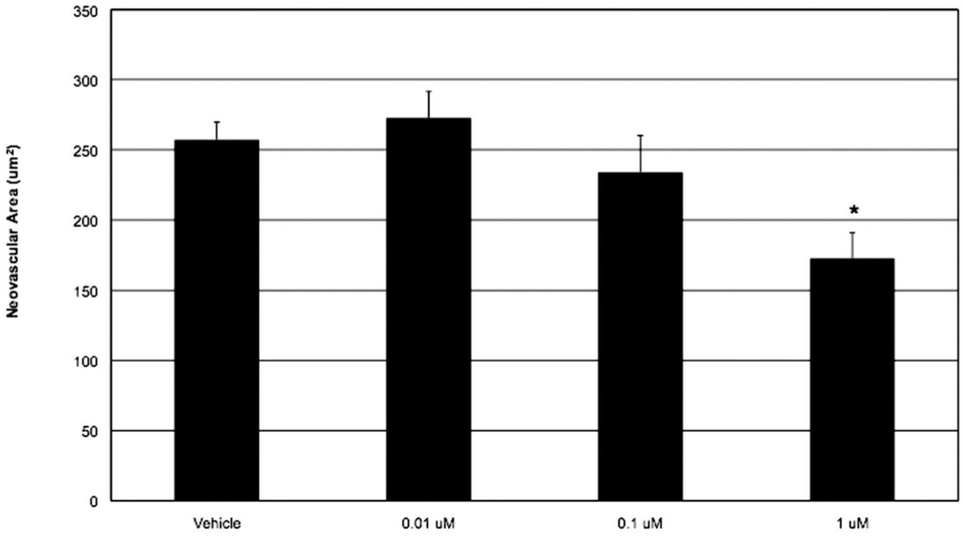

**Figure 8.**

The effect of an $EP_4$ antagonist, L-161982, on the severity of LCNV in the rat. The highest concentration of L-161982 significantly decreased the severity of LCNV. Each bar represents the mean ± SEM. *$P \leq 0.0329$ (Fisher's LSD post hoc analysis). For vehicle and 1 $\mu$M, $n = 16$; for 0.01 $\mu$M, $n = 28$; for 0.1 $\mu$M, $n = 24$.

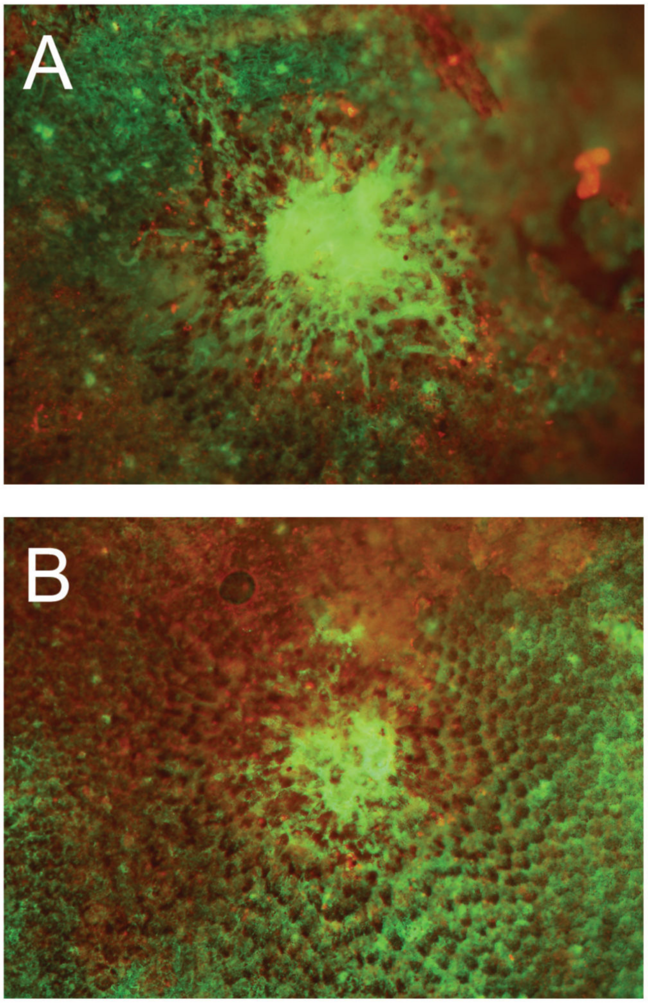

**Figure 9.**

The effect of an $EP_4$ antagonist, L-161982, on the severity of LCNV in the rat, as visualized by isolectin $B_4$ (*green*)-stained and elastin (*red*)-stained choroid-sclera-RPE flatmounts. The highest concentration of L-161982 significantly decreased the severity of LCNV, as indicated by decreased choroidal endothelial cell infiltration around the laser-induced wound site. (**A**) Eye treated with vehicle (0.1% DMSO). (**B**) Eye treated with 1 $\mu$M L-161982.