THE ROLE OF COX-2 IN PATHOLOGICAL OCULAR ANGIOGENESIS

By

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To my parents, John and Nancy, who have given me roots and wings

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AMD	age-related macular degeneration
ANOVA	analysis of variance
BID	2 times a day
COX	cyclooxygenase
ELISA	enzyme-linked immunosorbant assay
Flk-1	fetal liver kinase-1
Flt-1	fms-like tyrosine kinase-1
GC-MS	gas chromatography-mass spectrometry
HRMEC	human retinal microvascular endothelial cell
KDR	kinase-insert-domain-containing receptor
LCNV	laser-induced choroidal neovascularization
mRNA	messenger ribonucleic acid
NSAID	non-steroidal anti-inflammatory drug
NV	neovascularization
OIR	oxygen-induced retinopathy
PDR	proliferative diabetic retinopathy
PKA	protein kinase A
QID	4 times a day
RNA	ribonucleic acid
ROP	retinopathy of prematurity
SD	standard deviation

SEM	standard error of the mean
VEGF	vascular endothelial cell growth factor

CHAPTER I

BACKGROUND AND SIGNIFICANCE

1.1 Angiogenesis

Angiogenesis is the formation of new capillaries from the existing vasculature. Angiogenesis is an essential part of female reproductive functions, embryonic growth and development, and tissue repair and regeneration. However, even in these circumstances, angiogenesis is strictly regulated and briefly activated (1). Many pathological processes, such as arthritis and tumorigenesis, are characterized by persistent, abnormal angiogenesis.

The microvasculature consists of endothelial cells and their basement membranes, and pericytes. The vascular endothelium is relatively quiescent, with one of the most infrequent rates of mitotic division in the body, dividing approximately once every three years (2). Certain pathological conditions, however, change the resting phenotype to an angiogenic phenotype, resulting in the establishment of a new capillary network. Studies have shown that angiogenesis is a well-defined process that occurs in the following stages (3-8):

- Angiogenic growth factors are released in response to tissue injury and hypoxia.
- Endothelial cells produce proteinases that degrade the microvascular basement membrane and the extracellular matrix.

- Endothelial cells migrate through the basement membrane and extracellular matrix.
- Endothelial cells proliferate, and form tubes and lumena.
- Nascent endothelial cell tubes anastomose, forming a vascular network.
- Smooth muscle cells and pericytes are recruited to the vasculature, leading to microvascular stabilization.

1.2 Ocular angiogenesis

Pathological ocular angiogenesis is commonly referred to as ocular neovascularization (NV). NV is a central feature of retinopathy of prematurity (ROP), proliferative diabetic retinopathy (PDR), and age-related macular degeneration (AMD); the leading causes of blindness in infants, working age individuals, and the elderly, respectively (9-11). Although NV tends to occur at a relatively late stage in the course of many ocular disorders, it is nonetheless a highly attractive target for therapeutic intervention, because it represents a final common process in diseases that are multi-factorial in etiology, and it is the event that leads directly to vision loss. Vision loss is due to the pathologic nature of the blood vessels that develop in these disease conditions; they are fragile, non-patent, and leaky, and fibrovascular proliferation and migration into the vitreous ultimately leads to tractional retinal detachment.

A recent review article employed multiple epidemiologic studies and U.S. census data to estimate the prevalence of ROP, PDR, and AMD (12). Based on this work, individuals affected by neovascular forms of ROP, DR, and AMD collectively number well into the millions of Americans. Other retinal conditions with neovascular sequelae, such as sickle cell retinopathy and vascular occlusive disorders, are less prevalent, but remain clinically significant.

Prior to 2005, laser photocoagulation was the prevailing treatment for conditions characterized by ocular NV. Laser photocoagulation involves cauterizing blood vessels with the heat of a fine-point laser beam. This ablative approach offers short-term benefits to certain subgroups of patients (13-15), but is associated with significant adverse effects (16,17). Laser photocoagulation burns and destroys part of the retina, and therefore results in some permanent vision loss. Laser photocoagulation may cause some loss of central and peripheral vision, worsened night vision, and a decreased ability to focus. Moreover, because this approach fails to address the underlying stimuli that initiate pathologic blood vessel growth, it is associated with high rates of persistent and recurrent disease (14,18), accompanied by an increased frequency of severe vision loss (19).

Because of the clear limitations of this treatment for ocular NV, there is a compelling need to develop new, rational therapeutic approaches. One attractive approach is to target the underlying pro-angiogenic stimuli, so as to achieve a sustained therapeutic effect. The development of such treatments depends on a clear understanding of the cellular and molecular processes involved in

angiogenesis, and of the specific characteristics of these processes within the tissues of the eye.

1.3 Molecular mechanisms of ocular angiogenesis

Ischemia-induced hypoxia is a central feature of ROP and PDR, and may worsen AMD, and other neovascular disorders of the eye. It has long been accepted that hypoxia stimulates retinal angiogenesis (20,21). In 1948, Michaelson first proposed that retinal hypoxia stimulates the production of a diffusible angiogenic factor. Since that time, various pro-angiogenic growth factors have been identified, each mediating a number of diverse angiogenic cell behaviors. Of the growth factors involved in retinal angiogenesis, VEGF-A is thought to be a principal mediator (22).

VEGF-A is the prototypical member of the VEGF family of growth factors. This family includes placental growth factor (PlGF), VEGF-A, VEGF-B, VEGF-C, VEGF-D, and the virus encoded VEGF-E. VEGF-A is hereafter referred to as VEGF. VEGF is a 46 kD homodimeric glycoprotein that serves as a cell survival factor, vasopermeability factor, and angiogenic growth factor (23). VEGF protein is produced via alternative splicing of a single VEGF gene, yielding five distinct variants comprised of 121, 145, 165, 189, and 206 amino acids (24). The 121, 145, and 165 variants are diffusible, whereas the larger VEGF variants (189 and 206) remain bound to the cell surface and the extracellular matrix through their interaction with, and binding affinity for, heparin (25). Major splice variants, and the exons that

comprise them, are depicted in Figure 1. Exons 1–5 contain the VEGF receptor-binding domain, while exons 6 and 7 contain heparin-binding domains.

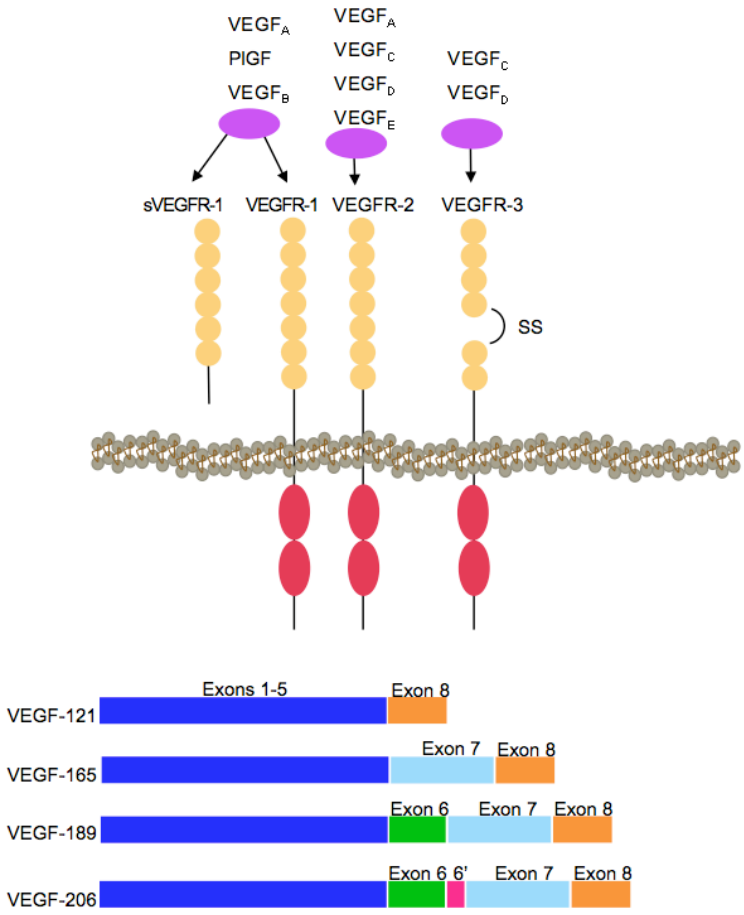

Figure 1. The associations between VEGF family members and the VEGF receptors. Also shown are the major VEGF variants formed by alternative splicing of the VEGF gene. The splice variants differ in their expression of exons 6 and 7, which mediate heparin binding, and therefore, the solubility of the splice variants.

Hypoxia has been shown to regulate VEGF activity at the level of: gene transcription, mRNA stability, translation, and protein secretion (26). Chronic tissue hypoxia induces VEGF production through the transcriptional activator hypoxia-inducible factor 1 (HIF-1). HIF-1 accumulates as a result of low oxygen,

binds to hypoxia response elements (HREs) in the promoter regions of various genes, and stimulates the expression of hypoxia-inducible genes and angiogenic growth factors like VEGF (27-31). In the retina, hypoxia-induced VEGF production has been demonstrated most consistently and dramatically in the Müller cells, the predominant glial cells within the retina (32-34).

VEGF production is also regulated by stimuli other than hypoxia. In fact, a variety of stimuli, including other growth factors, cytokines, hormones, and cellular stressors activate VEGF, particularly at the level of transcription. These stimuli ultimately activate various transcription regulatory factors, which bind to promoter regions on the VEGF gene. Some of the transcription factors that bind to VEGF promoter regions and activate VEGF transcription are: AP-1, AP-2, CREB, Egr-1, HIF, Sp1, Sp3, and STAT3 (for a comprehensive review, see 35).

After VEGF is produced and secreted by Müller cells, and to a lesser extent other cell types, it binds with high affinity to its cognate tyrosine kinase receptors expressed on the surface of endothelial cells, VEGFR-1 (Flt-1) and VEGFR-2 (KDR or Flk-1) (23). VEGF-VEGFR signaling plays a critical role in the physiological angiogenesis that takes place as a normal part of embryonic growth and development. Inactivation of even a single allele of the VEGF gene results in embryonic lethality (36). Formation of blood vessels is severely impaired in mice lacking a single allele of the VEGF gene. VEGF knockout embryos also demonstrate defective vasculogenesis, large vessel formation, capillary sprouting, and remodeling of the yolk sac vasculature. Furthermore, inactivation of either Flt-1 or Flk-1 results in embryonic lethality (37,38). Flt-1 null mice exhibit

vascular disorganization. Flk-1 null mice exhibit severely disrupted hematopoiesis, vasculogenesis, and endothelial cell differentiation. These findings point to the importance of VEGF and its receptors in physiological, developmental angiogenesis. By extension, retinal vascular development is dependent upon VEGF signaling.

Findings from several studies demonstrate the importance of, and interaction between, VEGF and the VEGFRs, to the development of the retinal vasculature (39-45). In both humans and animals, VEGF expression is first seen in astrocytes, which are located adjacent to the inner limiting membrane of the retina. The astrocytes, and VEGF expression, advance from the optic nerve-head towards the retinal periphery. This wave of VEGF expression precedes the development of the retina's superficial vascular net (40,42,46,47). Once the vessels reach the peripheral extent of the retina, VEGF expression disappears from the superficial vascular net, and is instead expressed by Müller cells in the inner nuclear layer of the retina (40). This second wave of VEGF expression is responsible for driving the development of the retina's deep vascular net. In total, these studies demonstrate that VEGF expression in the retina is both temporally and spatially related to the development of the retinal vasculature.

Mice expressing only VEGF-164 demonstrate normal retinal vascular development. Alternatively, mice expressing only VEGF-120 demonstrate retarded venous and arterial development; and mice expressing only VEGF-188 demonstrated normal venous development, but little retinal arterial growth (48).

While both VEGFR-1 and VEGFR-2 play critical regulatory roles in developmental angiogenesis, the neovascular effects of VEGF are primarily mediated through VEGFR-2 signaling (49). Upon VEGF binding, VEGFR-2 dimerization and tyrosine autophosphorylation initiate complex signal transduction cascades that result in diverse endothelial cell behaviors. These VEGF-induced behaviors include survival, proliferation, migration, and nitric oxide production leading to increased vascular permeability (23,50-54). Thus, VEGF signaling through

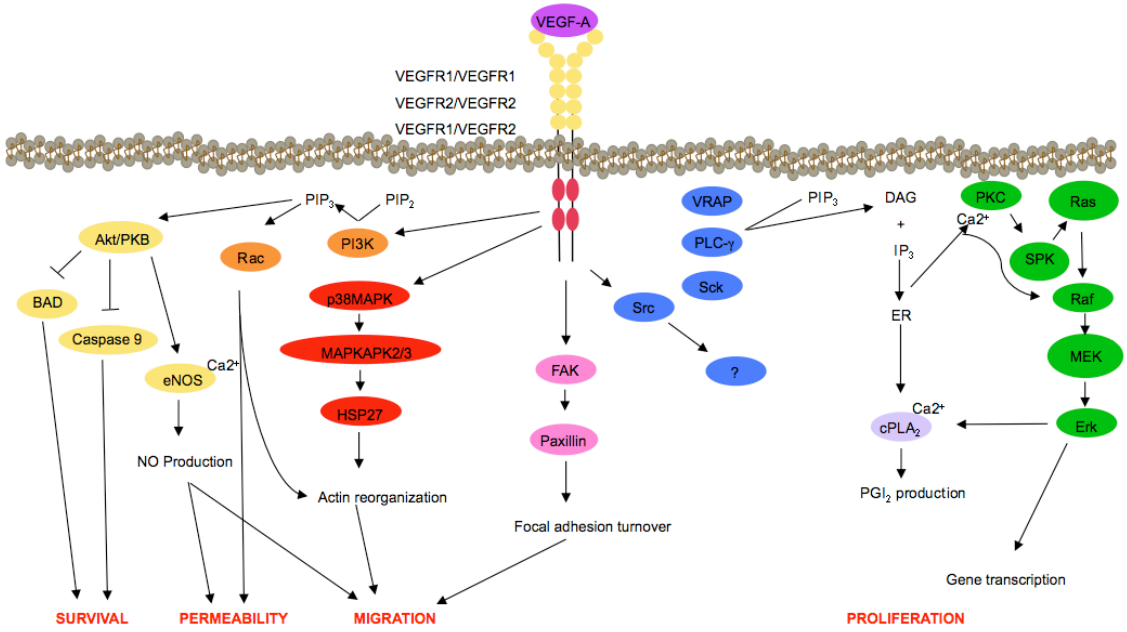

Figure 2. VEGFR-2 signaling pathways leading to angiogenic endothelial cell activities.

VEGFR-2 activates the angiogenic phenotype in endothelial cells, and these behaviors result in retinal NV **(Figure 2)**.

Inhibition of VEGF using anti-sense oligonucleotides, chimeric proteins, monoclonal antibodies, and VEGF peptides has been shown to inhibit oxygen-induced retinal angiogenesis in animals and humans (55-58). Inhibition of KDR, and the use of soluble Flt-1 have both been proven efficacious at reducing the severity of retinal NV in animal models of ocular angiogenesis (59, 60). The results obtained lend support to the strategy of targeting VEGF induction and/or activity as an appropriate ocular therapeutic modality.

1.4 Current treatments

VEGF is widely regarded as a principle growth factor mediating the development of ocular NV (22). This belief has led to the development of a class of therapeutics for the treatment of neovascular AMD based on VEGF antagonism (61,62). These treatments are the only FDA-approved alternatives to ablative laser photocoagulation, previously discussed. VEGF antagonists currently employed in clinical practice include: pegaptanib (Macugen®, Pfizer), an RNA-like aptamer that binds and inhibits VEGF-165, the predominant pathologic splice variant of VEGF; bevacizumab (Avastin®, Genentech), a humanized mouse monoclonal antibody to human VEGF that was approved for systemic administration in the treatment of cancer, but is used off-label in the treatment of AMD; and ranibizumab (Lucentis®, Genentech), the F(ab) fragment

of an anti-VEGF similar to Avastin®, which is approved for use in AMD. The therapeutic efficacy of these VEGF antagonists has been remarkable, although there are important limitations that stem, in part, from the requirement to administer the antagonists by frequently repeated intravitreal injections. In addition to the discomfort and costs associated with the procedures, this approach leads to high peaks levels and prolonged trough levels of antagonist, during which time it is sub-therapeutic. This unfavorable pharmacokinetic profile may contribute to reduced efficacy, as exemplified by the incomplete arrest of disease progression observed in many patients, and the frank resistance to therapy that characterizes others. Periods of very high levels of the antagonist may contribute to both local and systemic toxicity. Morbidity related to the intravitreal injections is another important limitation of the current treatment regimen (63). The most frequent and problematic injection-related toxicity is the development of endophthalmitis, a potentially blinding condition (64), observed at a rate as high as 1.3% per patient per year in clinical studies. This toxicity is expected to rise as the treatment becomes part of standard practice and is performed outside of the rigorous settings of clinical trials in academic centers. Finally, it remains unclear if chronic administration of anti-VEGF drugs will exert a negative influence on the retinal neurons that are known to express VEGF receptors or on the sensitivity and responsivity of the retinal or choroidal vasculature to VEGF stimulation. In fact, it is likely that chronic inhibition of VEGF may lead to increased VEGF receptor expression (our unpublished findings), which may worsen the angiogenic response if the therapy is not maintained.

Thus, although VEGF-centric therapies clearly reduce NV, they do not completely eliminate it. Combination therapies, or therapies that simultaneously target more than one point along the angiogenic cascade (e.g. up- and down-stream of VEGF receptor activation), are an unmet need in ophthalmology. Therapeutics designed to target the angiogenic cascade at more than one point have the potential to provide a more powerful and effective therapeutic target for angiogenic diseases of the eye. In order to design such therapeutics, a solid understanding of the molecules with more than one role in the process is necessary.

1.5 COX-2

The cyclooxygenase (COX) enzymes are responsible for catalyzing the production of prostaglandin H_2 (PGH_2) from membrane-derived arachidonic acid. PGH_2 is an unstable intermediate that is rapidly converted, by tissue- and cell-specific synthases, to biologically active prostaglandins (PGD_2, PGE_2, PGF_2, PGI_2) and thromboxanes (TXA_2) **(Figure 3)**. There exist at least two isozymes of COX, COX-1 and COX-2, the products of two different genes located on two different chromosomes. The two enzymes catalyze identical reactions and retain 60% amino acid sequence identity. COX-1, however, is typically regarded as a housekeeping enzyme whose constitutive expression and prostanoid [prostaglandin (PG) and thromboxane] products are responsible for maintenance of the gastric mucosa, platelet aggregation, and regulation of the renal

vasculature (65). COX-2, on the other hand, is the product of an immediate early gene, and it can be induced by mitogens, cytokines, and tumor promoters (66).

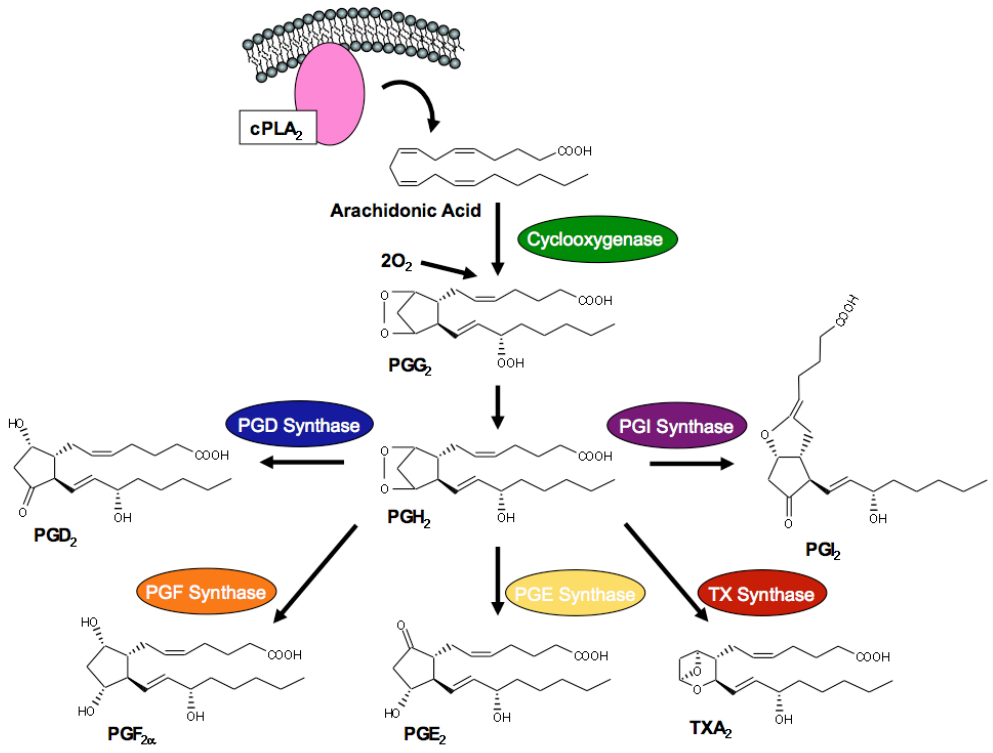

Figure 3. The cyclooxygenase cascade. In response to some stimulus, $cPLA_2$ liberates arachidonic acid from membrane phospholipids. The COX and prostanoid synthase enzymes then convert arachidonic acid to the five biologically active prostanoids.

Once the COX enzymes catalyze their production, prostanoids bind to their cognate G-protein-coupled receptors (GPCRs) on the surface of target cells. The receptors determine the extent and biological activity of the prostanoids. PGF_2, PGI_2, and TXA_2 signal through the FP, IP, and TP receptors, respectively. PGD_2 signals through the DP and CRTH2 receptors. PGE_2 signals through the EP_1, EP_2, EP_3, and EP_4 receptors. Signaling through DP, IP, EP_2, and EP_4

stimulates the activation of adenylyl cyclase (AC), resulting in increased cyclic AMP (cAMP) production. Signaling through EP_3 and CRTH2 results in reduced cAMP production. Signaling through FP, TP, and EP_1 results in calcium mobilization (67). The precise tissue-specific and cell-specific signaling pathways and the biological roles mediated by each of the prostanoid receptors have yet to be determined.

COX-2 is elevated in various cancers, including colorectal cancer. Patients who regularly take non-steroidal anti-inflammatory drugs (NSAIDs) for the treatment of chronic pain and heart disease demonstrate a 40-50% reduction in the incidence and severity of colorectal cancer (68). NSAIDs are compounds that inhibit the activity of the COX enzymes. These findings led to the suggestion that COX-2 and the prostanoids may act as tumor promoters (69,70). At least one tumor-promoting function of the prostanoids is the stimulation of angiogenesis (71-73).

The prostanoids exhibit angiogenic effects both up- and downstream of growth factor production. Upstream, the prostanoids have been shown to mediate the expression of pro-angiogenic molecules. They have been shown to induce VEGF and bFGF in various cell types (70,74-76). Researchers studying angiogenesis related to various cancers and other neovascularizing conditions have demonstrated, using pharmacological and genetic manipulation of COX-2, that COX-2 inhibition resulted in reduced VEGF production, *in vitro* and *in vivo* (77-80). Downstream, pro-angiogenic factors such as hypoxia, VEGF, bFGF, TNF-α, and IL-1 have been shown to induce endothelial cell expression of COX-2

(81-84), and COX-2-derived prostanoids stimulated proliferation, migration, and tube formation in human umbilical vein endothelial cells (HUVEC) (85). COX-2-selective inhibitors have been shown to block the proliferation and migration of vascular endothelial cells (86-89). Several prostanoids have been shown to induce angiogenesis in various angiogenesis assays and cancer models (72,73,90-92). Furthermore, specific inhibition of COX-2 has been shown to inhibit angiogenesis in the cornea or within experimental tumors (93-96), and this anti-angiogenic effect can be reversed by prostaglandin treatment (81,91).

1.6 COX-2 and the eye

COX-2 has been localized to various ocular tissues, and its expression has been found, or can be induced, in the following structures: cornea, iris, cilliary body, various cell types within the neuroretina, and the retinal pigment epithelium (RPE) (95,97-100). The expression of the COX-2 enzyme in these ocular tissues suggests a functional role for its prostanoid products. Indeed, ocular prostanoids have been shown to mediate the cornea's inflammatory response (101,102), intraocular pressure (103,104), retinal blood flow (105), and maintenance of the blood-retinal-barrier (106). Additionally, inhibition of COX has been effective at reducing the production of VEGF and corneal, retinal, and choroidal NV in relevant models of ocular disease.

In experimental models of corneal angiogenesis, various groups have demonstrated that the non-selective NSAIDs indomethacin and ketoprofen, as

well as the COX-2-selective agent NS-398 significantly reduced the severity of NV (95,107,108). In experimental models of retinal angiogenesis, investigators have demonstrated that inhibiting various points along the COX cascade reduced pathological NV. Cytosolic phospholipase A_2 ($cPLA_2$) is the enzyme responsible for liberating arachidonic acid, the substrate for the COX enzymes, from membrane phospholipids. Rodents treated with a $cPLA_2$ inhibitor demonstrated reduced oxygen-induced retinal NV (109). Non-specific inhibitors of COX such as nepafenac, as well as COX-2-selective inhibitors such as APHS, etodolac, and rofecoxib, have all been shown to have a similar anti-angiogenic effect (77,110,111). In models of laser-induced choroidal NV (LCNV), nepafenac, etodolac, and lumiracoxib (another COX-2-selective agent), all effectively inhibited the development of CNV (77,112,113). These data suggest that COX-2 mediates various aspects of corneal, retinal, and choroidal NV.

In summary, although various groups have demonstrated the efficacy of COX inhibition at reducing ocular NV, little work has been done to determine which of the COX-2 derived prostanoid(s) is (are) involved in mediating VEGF production, downstream angiogenic endothelial cell behaviors, and ocular angiogenesis. Because of this gap in ocular angiogenesis research, we examined the specific involvement of COX-2 and COX-2-derived prostanoids in ocular angiogenesis. Figure 4 very generally depicts our working model of the role of COX-2 in angiogenic cell behaviors, with implications for conditions characterized by retinal NV. The goal of our research was two-fold. We wanted to 1) better understand the role of COX-2-derived prostanoids in ocular angiogenic

disease, and 2) use the knowledge gained through our research to identify more specific therapeutic targets.

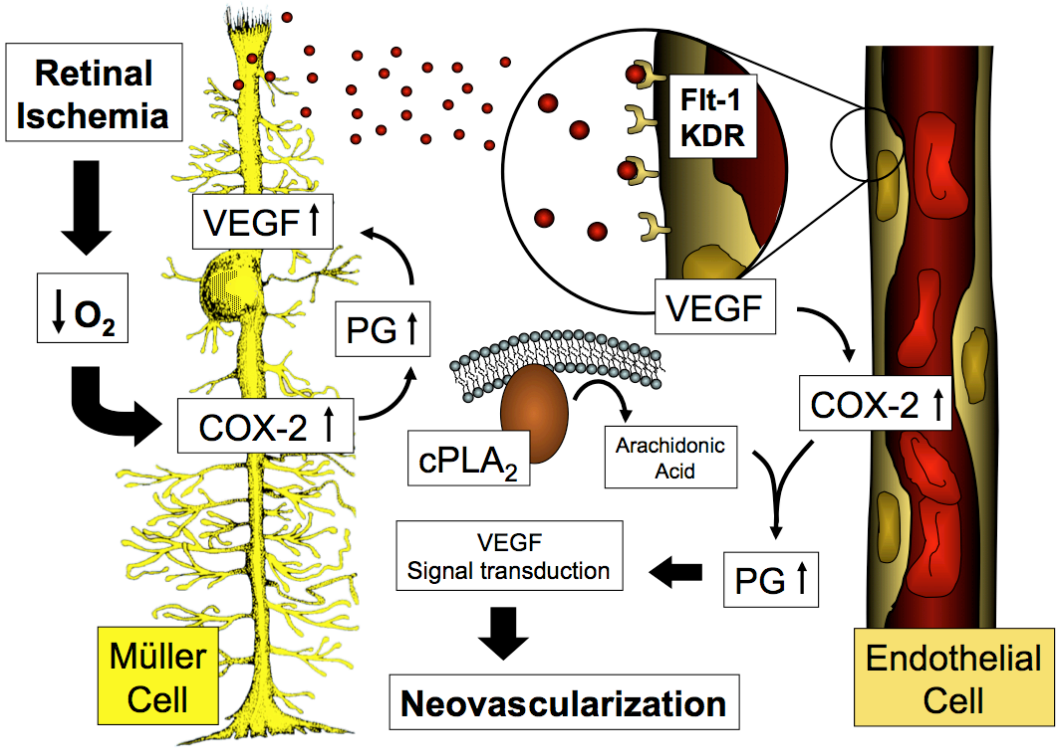

Figure 4. Schematic distillation of retinal angiogenesis. We typically distill retinal angiogenesis into its two most basic components, growth factor production and growth factor consumption. In these studies, we examined the role of COX-2, COX-2-derived prostanoids, and the prostanoid receptors on hypoxia-induced VEGF production by Müller cells and VEGF-induced angiogenic cell behaviors by endothelial cells.

CHAPTER II

EFFECTS OF NEPAFENAC AND AMFENAC ON RETINAL ANGIOGENESIS

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2.1 Abstract

Nepafenac is a potent NSAID that rapidly penetrates the eye following topical ocular administration. In the eye, nepafenac is converted to amfenac, which has unique time-dependent inhibitory properties for COX-1 and COX-2. The purpose of the present study was to investigate the capacity of amfenac to inhibit discrete aspects of the angiogenic cascade *in vitro*, and to test the efficacy of amfenac and nepafenac *in vivo*, using the rat OIR model.

Müller cells were treated with amfenac, celecoxib (COX-2), or SC-560 (COX-1), and hypoxia-induced VEGF and PGE_2 were assessed. Endothelial cells were treated with amfenac, celecoxib, or SC-560, and VEGF-induced proliferation and tube formation were assessed. Rat pups were subjected to OIR, received intravitreal injections of amfenac, celecoxib, or SC-560, and neovascularization (NV), prostanoid production, and VEGF were assessed. Other OIR-exposed pups were treated with topical nepafenac, ketorolac, or diclofenac, and inhibition of NV was assessed.

Amfenac treatment failed to inhibit hypoxia-induced VEGF production. Amfenac treatment significantly inhibited VEGF-induced tube formation and proliferation by endothelial cells. Amfenac treatment significantly reduced retinal prostanoid production and NV in OIR. Nepafenac treatment significantly reduced retinal NV in OIR; ketorolac and diclofenac had no effect.

Nepafenac and amfenac inhibit OIR more effectively than the commercially available topical and injectable NSAIDs used in this study. Our data

suggests that there are COX-dependent and COX-independent mechanisms by which amfenac inhibits OIR. Because it is bioavailable to the posterior segment following topical delivery, nepafenac appears to be a promising advancement in the development of therapies for neovascular eye diseases.

2.2 Introduction

Pathological ocular angiogenesis, or ocular neovascularization (NV), is a pivotal pathologic feature of several prevalent, sight-threatening eye diseases. In developed countries, retinopathy of prematurity (ROP), proliferative diabetic retinopathy (PDR), and age-related macular degeneration (AMD) are the leading causes of irreversible blindness in infants, working-age adults, and the elderly, respectively (9,10,12). Clinical and experimental evidence suggests that ischemia-induced hypoxia is a central etiological factor in retinal NV (114,115). For example, several retinal cell types respond to hypoxia by up-regulating production of vascular endothelial growth factor (VEGF), the principal growth factor promoting retinal NV (32,33,116). Among these retinal cells, Müller cells exhibit the most consistent and dramatic increase in VEGF synthesis and secretion when subjected to experimental hypoxia (32-34). VEGF binds with high affinity to VEGF receptors (VEGFR-1 and VEGFR-2) expressed on the surface of endothelial cells, initiating signal transduction cascades that lead to angiogenic endothelial cell behaviors (51-53,55).

Cyclooxygenase (COX) enzymes are responsible for the biosynthesis of prostanoids [prostaglandins (PG) and thromboxanes] from arachidonic acid. Studies suggest that COX-1, the constitutively active isoform of COX, plays a role in angiogenic cell behaviors and carcinogenesis (117-122). Additionally, evidence suggests that the inducible isoform of COX, COX-2, plays a key role in regulating angiogenesis through the induction of prostanoid synthesis. Prostanoids subsequently induce the expression of pro-angiogenic factors such as VEGF and bFGF in many cell types (75,76), and several prostanoids have been shown to induce angiogenesis in *in vitro* and *in vivo* assays of human angiogenesis and cancer (72,73,90-92). A subset of prostanoids, under some conditions, have been shown to be deleterious to the retinal vasculature in ways other than promoting growth factor production. Prostanoid levels are higher in the retinas of infants than in the retinas of adults (123,124). Prostanoids are involved in maintaining retinal and choroidal blood flow (125,126). Specifically, the infant's retinal prostanoid complement, coupled with their age-dependent responses to the prostanoids, leads to increased retinal vascular relaxation and dilation (124,127,128). This effect is particularly harmful to premature infants on oxygen therapy who do not yet have the ability to auto-regulate retinal and choroidal blood flow; prostanoids serve to enhance oxygen delivery to already-saturated retinal tissue, which is known to worsen the pathology of ROP (125,129). COX-2-dependent production of TXA_2 can lead to endothelial cell cytotoxicity, worsening the retinal microvascular degeneration in ischemic retinopathies (130,131). Prostanoid signaling through the EP_3, EP_4, DP, TP, and IP receptor have all been

implicated in mediating discrete cell behaviors that are invovled in the development or pathology of ischemic retinopathies (110,132-135). Selective inhibition of COX-2 also prevents pathological angiogenesis in the cornea, retina, and experimentally-induced tumors (93-96,111). Therefore, non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit the activity of the COX enzymes may be viable pharmacologic agents for the treatment of retinal neovascularization (NV).

In 2005, the U.S. Food and Drug Administration (FDA) approved the topical NSAID, NEVANAC® (nepafenac; 0.1% ophthalmic suspension) for the treatment of pain and inflammation associated with cataract surgery (136-139). The active ingredient in NEVANAC® is nepafenac, a potent, reversible COX-1 and COX-2 inhibitor (Kulmacz RJ, et al. 2007: EVER E-Abstract e473). Nepafenac is a pro-drug with superior penetration of cornea and scleral tissues (140). It is quickly metabolized *in vivo* by amidases in the iris/cilliary body and retina/choroid to form amfenac (136). Amfenac is an NSAID with antipyretic and analgesic properties, and it inhibits both COX-1 and COX-2 activity (137). Amfenac, like nepafenac, is a reversible inhibitor of both COX-1 and COX-2, but unlike nepafenac, amfenac has unique time-dependent inhibitory properties for both COX-1 and COX-2, implying that with time, amfenac irreversibly binds the enzymes, accounting for amfenac's prolonged activity (Kulmacz RJ, et al. 2007: EVER E-Abstract e473).

Topical ocular administration of nepafenac inhibits posterior segment NV in mouse models of oxygen-induced retinopathy (OIR) and laser-induced choroidal NV (LCNV), and it inhibits the functional abnormalities and retinal

vasculopathy observed in rats with streptozotocin-induced diabetes (77,141). Topical ocular administration of nepafenac reduced retinal VEGF expression in the mouse model of OIR (77). This observation is similar to the reported findings demonstrating the anti-VEGF effects of COX-2 inhibitors in tumor angiogenesis models (142).

In order to better understand its bioactivity, we used *in vitro* assays of angiogenic cell behaviors to determine the capacity of amfenac to inhibit discrete aspects of the angiogenic cascade in the retina. We evaluated the effect of amfenac on hypoxia-induced VEGF production by Müller cells. Then, we looked at the effect of amfenac on VEGF-induced angiogenic cell behaviors in retinal endothelial cells. To further investigate the therapeutic potential of nepafenac for human use, we tested the efficacy of amfenac and nepafenac *in vivo*, using the rat model of OIR developed in our laboratory. This model produces a pattern of pathological pre-retinal NV mimicking that of premature infants with ROP (143). The results of these studies more fully define the mechanism(s) by which nepafenac mediates its anti-angiogenic effect, as well as demonstrate where COX enzymes appear to exert their influence during pathologic retinal angiogenesis.

2.3 Materials and methods

Materials

Nepafenac (NEVANAC®, 0.1% ophthalmic solution), amfenac, and vehicle were synthesized and provided by Alcon Laboratories, Inc. Ketorolac tromethamine (Acular®, 0.5% ophthalmic solution; Allergan, Inc.), diclofenac sodium (Voltaren®, 0.1% ophthalmic solution; Novartis), and celecoxib (Celebrex®; Pfizer) were obtained from commercial sources. SC-560 was purchased from Cayman Chemical (Ann Arbor, MI).

Isolation and culture of primary rat retinal Müller cells

Primary rat retinal Müller cell cultures were established from postnatal day (P)7 Long Evans rat pups according to well-established methods (144). Briefly, enucleated eyes were placed in soaking medium, Dulbecco's Modified Eagle Medium Low Glucose (DMEM; HyClone; Logan, UT) supplemented with 1X Antibiotic/Antimycotic Solution (Sigma; St. Louis, MO), overnight. The following day, eyes were incubated in digestion buffer, comprised of the soaking medium plus 0.1% trypsin and 70 U/ml collagenase, for 60 minutes at 37ºC. Retinas were then dissected, triturated, plated, and grown in DMEM supplemented with 10% fetal bovine serum and 1X Antibiotic/Antimycotic Solution. Cultures were maintained at 37ºC in a 5% CO_2/95% air (20.9% oxygen) atmosphere (normoxia) in a humidified incubator (NuAire; Plymouth, MN). Müller cells were identified by

immunocytochemical staining for cellular retinaldehyde binding protein (CRALBP; Abcam; Cambridge, MA). Passages three to six were used for experiments. For treatment of Müller cells with hypoxia, a CO_2-enriched environment was generated with a BBL^{TM} GasPak Pouch system (Becton-Dickinson; Sparks, MD).

Quantitative real time RT-PCR of VEGF in rat Müller cells

Primary rat Müller cells were seeded in 10-cm Petri dishes at equal density and maintained in normoxia. At 80% confluency, the cells were treated with vehicle (0.1% DMSO) or increasing concentrations of amfenac (0.1 to 10 μM) and placed in hypoxia for 24 hours. Total RNA was isolated from the cells using Trizol reagent (Invitrogen Corporation; Carlsbad, CA). Each RNA sample was quality-controlled for DNA and protein contamination. For VEGF amplification, cDNAs were reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems; Foster City, CA) according to manufacturer's instructions. Quantitative real-time RT-PCR was performed in duplicate by co-amplification of rat VEGF vs. β-actin (endogenous normalization control) in separate tubes, using gene-specific TaqMan Gene Expression Assays according to the manufacturer's instructions (Applied Biosystems; primer and probe sequences used in this assay are proprietary).

Quantification of rat Müller cell-derived VEGF and PGE_2 levels

Primary rat Müller cells were seeded in 12-well plates at equal density and maintained in normoxia. At 80% confluency, cells were treated with vehicle (0.1%

DMSO) or 10 μM amfenac, celecoxib, or SC-560, and then maintained in normoxia or hypoxia for 24 hours. Culture medium from cells was collected and assayed for VEGF and PGE_2 concentration with colorimetric sandwich ELISA kits (R&D Systems; Minneapolis, MN) according to the manufacturer's instructions. Cells were washed with CMF-PBS (Invitrogen), lysed with cold lysis buffer (Promega; Madison, WI), and protein concentration was determined with a bicinchoninic acid assay (BCA; Pierce; Rockford, IL). The amount of VEGF and PGE_2 (pg/ml) in the culture medium was normalized to total protein concentration (mg/ml) of cell lysates.

Culture of human retinal microvascular endothelial cells (HRMEC)

Primary human retinal microvascular endothelial cells (HRMEC; Cell Systems; Kirkland, WA) were seeded in tissue culture flasks coated with attachment factor (Cell Signaling; Danvers, MA) and cultured with endothelial basal medium (EBM; Cambrex; East Rutherford, NJ) supplemented with 10% FBS and EGM single quots (Cambrex). When experimental conditions required serum free (SF) medium, MCDB 131 medium (Sigma) containing 1X Antibiotic/Antimycotic Solution was used. Cultures were maintained at 37ºC in a 5% CO_2/95% air (20.9% oxygen) atmosphere (normoxia) in a humidified incubator.

HRMEC tube formation assay

In vitro tube formation by HRMEC was carried out in 12-well plates coated with growth factor-reduced Matrigel® matrix (Becton-Dickinson). HRMEC were seeded at 3×10^4 cells per Matrigel-coated well in complete culture medium. After 4.5 hours, the culture medium was removed and the cells were treated with SF medium alone or SF medium containing 25 ng/ml VEGF (R&D Systems) in the presence or absence of amfenac, celecoxib, or SC-560 (0.01 to 1 μM). Twenty-four hours later, three images of tubes per well were captured using a DMC digitizing camera (Polaroid; Cambridge, MA) mounted on an IMT-2 inverted microscope (Olympus; Melville, NY). Capillary-like structures were measured using Image J software (NIH; Bethesda, MD), and the mean tube length per area of the field was calculated for each well.

HRMEC cell proliferation assay

VEGF-induced HRMEC proliferation was measured using a modified MTT assay. Each well of a 96-well plate was coated with a fibronectin/hyaluronic acid (HA) matrix and seeded with 3×10^4 cells. Complete medium was added and the cells were incubated for two days. The medium was then aspirated, and the cells were incubated with SF medium overnight. The following day, culture medium was removed and the cells were treated with SF medium alone or SF medium containing 25 ng/ml VEGF (R&D Systems) in the presence or absence of amfenac, celecoxib, or SC-560 (0.01 to 10 μM). Twenty-four hours later, 25 μL of a 5 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

(MTT; Molecular Probes; Eugene, OR) was added to each well and incubated for 4 hours under normal growth conditions. One hundred microliters of lysis buffer [20% SDS in 50:50 dimethylformamide (DMF) and H_2O with 2.0% acetic acid and 0.05% HCl] was then added to each well, and the plates were incubated overnight at 37°C and read (Spectramax 190; Molecular Devices; Sunnyvale, CA) at 570 nm. Absorbance values were translated to cell number using standard curves consisting of six cell densities assayed in quadruplicate. The data obtained from the MTT assay and cell counts using a hemocytometer in the presence of trypan blue (Sigma) were found to be highly correlated (r^2=0.933, data not shown). A standard curve of absorbance at 570 nm vs. HRMEC number was then produced.

Oxygen-induced retinopathy (OIR) in the rat

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 four hours after birth to oxygen exposure chambers where they were subjected to alternating 24 hour periods of 50% oxygen and 10% oxygen for 14 days. Control rats were raised simultaneously in room air. On postnatal day (P)14, the oxygen-exposed rats were returned to room air.

Quantification of retinal prostanoids

On P14, following removal from the oxygen chambers, rats were administered amfenac (0.05 μg; 40 μM) or vehicle by single intravitreal injection, according to a well-established procedure (145). One day later, on P15, retinas were harvested and homogenized. The lipid soluble prostaglandin compounds were extracted with a Sep-Pak C18 column (Waters; Milford, MA) and were nitrogen-evaporated. O-methoxyamine derivatives were formed by treatment with 2% methoxyamine-HCl in water at room temperature for 30 minutes. Compounds were extracted with ethyl acetate and subsequently converted to pentaflurobenzyl esters. The compounds were chromatographed on TLC plates with ethyl acetate/methanol. The compounds were then converted to trimethylsilyl ether derivatives and analyzed by negative ion chemical ionization mass spectrometry coupled with a gas chromatography system (Agilent Technologies; Palo Alto, CA).

Quantification of retinal VEGF levels

On P14, following removal from the oxygen chambers, rats were administered amfenac (0.05 μg; 40 μM) or vehicle by a single intravitreal injection, according to a well-established procedure (145). Because there is a peak in retinal VEGF two days post-oxygen exposure in this model (146), rats were sacrificed on P16 and retinas were harvested and subjected to lysis by homogenization. The total protein concentration of samples was measured by BCA. Retinal VEGF levels were measured with a VEGF colorimetric sandwich

ELISA kit (R&D Systems) according to the manufacturer's instructions. The final mass of retinal VEGF was standardized to total retinal protein.

Quantification of retinal neovascularization (NV)

Using commercially available formulations and drop-tainers, nepafenac (0.03%, 0.1%), ketorolac (0.5%), diclofenac (0.1%) or vehicle was dropped directly onto the cornea two or four times a day, depending upon experiment. Topical dosing was performed between P14 and P19. A separate group of oxygen-exposed rat pups received a single intravitreal injection of amfenac (0.05 µg; 40 µM), celecoxib (0.075 µg; 40 µM), SC-560 (0.07 µg; 40 µM), or vehicle (0.1% DMSO) at P14, after return to room air. Our estimations of vitreous volume indicate that these concentrations of injected NSAIDs lead to vitreous concentrations that fall within the middle range of the concentrations used for *in vitro* assays. Regardless of pharmacologic treatment, all oxygen-exposed rats were sacrificed on P20, 6 days following return to room air. The eyes were enucleated, and retinas were dissected and placed in 10% neutral buffered formalin [CMF-PBS (Invitrogen) with 37% formaldehyde solution (Fisher Scientific; Fair Lawn, NJ)] overnight at 4°C. The retinal vasculature was stained for adenosine diphosphatase (ADPase) activity, according to well-established procedures (147). Images of ADPase-stained retinas were digitized, captured, and displayed at 20X magnification. The total retinal area and the retinal area containing vasculature were independently measured. For each retinal image, pre-retinal vessel tufts were outlined, the pixels within an encircled area were

counted, and the total number of pixels from all areas were summed and converted to square millimeters.

Statistical analysis

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

2.4 Results

Intravitreally-injected NSAID efficacy in rat OIR

The effect of 0.05 μg amfenac on OIR-induced retinal NV was compared to two other NSAIDs, 0.075 μg celecoxib (COX-2 inhibitor) and 0.07 μg SC-560 (relatively COX-1-specific inhibitor). This concentration of amfenac (40 μM) was empirically chosen using the rat model of OIR; the concentrations of celecoxib and SC-560 were matched to this concentration, to standardize treatment. Because amfenac does not possess the tissue-penetration characteristics of its pro-drug, nepafenac, and because celecoxib and SC-560 are not topically formulated, they were delivered directly to the target tissue with a single intravitreal injection. Oxygen-exposed rats received a single intravitreal injection of amfenac, celecoxib, or SC-560 on P14 and were sacrificed on P20. Amfenac significantly ($p \leq 0.005$) reduced the mean area of pre-retinal NV, compared to

vehicle-treated eyes **(Figure 5)**. Celecoxib and SC-560 failed to inhibit OIR-induced retinal NV at the doses tested.

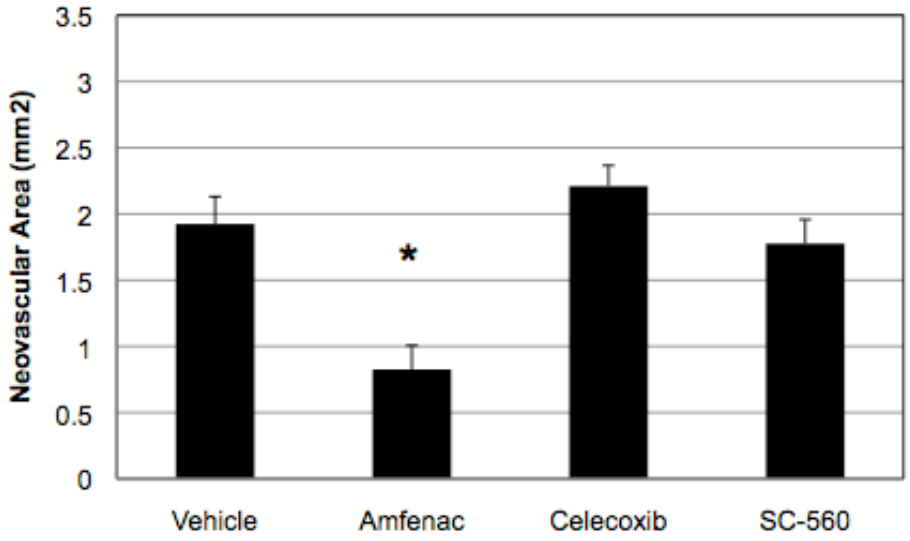

Figure 5. The effect of intravitreally-injected amfenac (40 μM), celecoxib, and SC-560 on the severity of OIR in the rat. Amfenac significantly reduced (* $p \leq 0.005$) OIR-induced retinal NV, but neither celecoxib nor SC-560 demonstrated an effect at the concentrations tested. Each bar represents the mean ± SEM.

Effect of OIR on retinal prostanoids with and without amfenac

Amfenac effectively inhibited NV in the rat OIR model, in contrast to celecoxib and SC-560. Thus, we sought to determine, more specifically, the way(s) in which the bioactive metabolite of nepafenac, amfenac, inhibited pathological angiogenesis. The effects of the OIR model and amfenac treatment on retinal prostanoid levels were surveyed. On P14, oxygen-exposed rats received a single intravitreal injection of vehicle or amfenac (0.05 μg; 40 μM).

One day later, on P15, the retinas were harvested and retinal prostanoid levels were measured. Compared to room air control retinas, the retinas of oxygen-exposed rats demonstrated increased levels of each of the five prostanoids. Intravitreal amfenac treatment significantly reduced levels of PGE_2, PGF_2, TxB_2, and 6-keto-PGF ($p \leq 0.001$) **(Figure 6)**. This data demonstrates that amfenac inhibits COX and prostanoid production as expected, suggesting a possible explanation for the observed anti-angiogenic effect of amfenac in Figure 5.

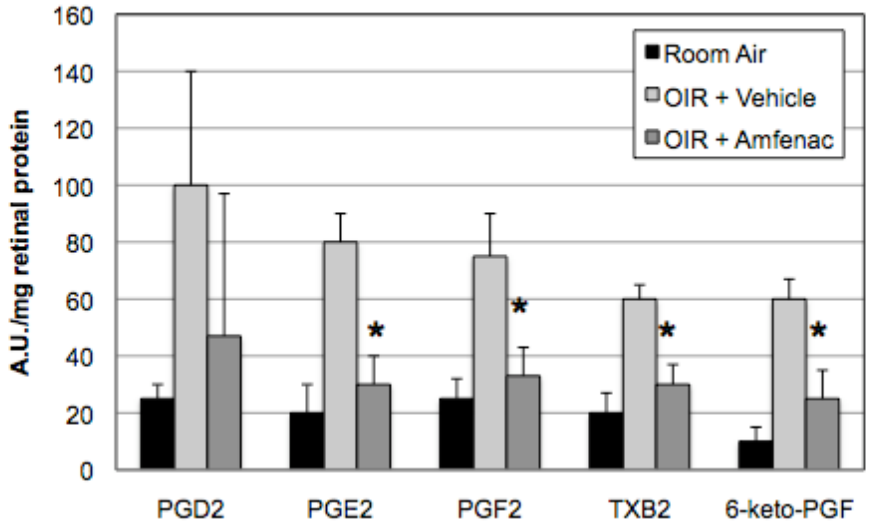

Figure 6. The effect of OIR and amfenac on retinal prostanoid levels. Compared to room air control retinas, the retinas of OIR rats demonstrated increased levels of each of the five prostanoids. Intravitreal amfenac treatment (40 μM) significantly reduced levels of PGE_2, PGF_2, TxB_2, and 6-keto-PGF (* $p \leq 0.001$) in OIR rat retinas. Each bar represents the mean ± SD.

Effect of amfenac on rat Müller cell VEGF expression

Since amfenac decreased retinal prostanoid levels and reduced NV in oxygen-exposed rats, the effect of amfenac on specific angiogenic cell behaviors was

studied using *in vitro* methods. In order to determine whether or not amfenac inhibited hypoxia-induced VEGF production, rat Müller cells were treated with increasing doses of amfenac (0.1 to 10 μM), and placed in hypoxia for 24 hours. Quantitative RT-PCR analysis of VEGF revealed that amfenac exhibited no effect on hypoxia-induced VEGF mRNA expression in rat Müller cells **(Figure 7)**.

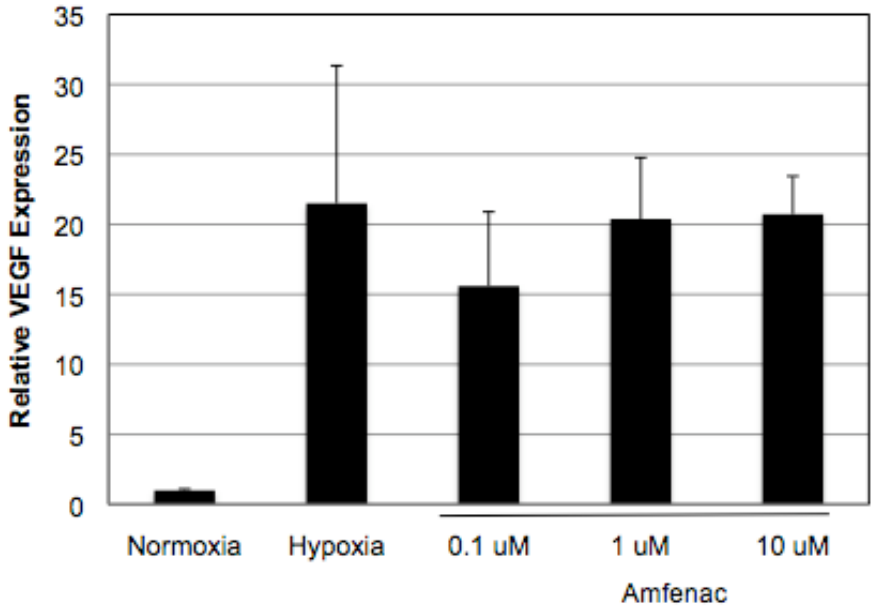

Figure 7. The effect of amfenac on hypoxia-induced VEGF expression in Müller cells. Quantitative RT-PCR analysis of VEGF revealed that amfenac exhibited no effect on hypoxia-induced VEGF mRNA expression in rat retinal Müller cells. Each bar represents the mean ± SD.

Effect of NSAIDs on VEGF and PGE_2 production in rat Müller cells

It is important to note that a change in VEGF mRNA does not always correlate with a change in VEGF protein (Wang FE, et al. IOVS 2004;45:ARVO E-Abstract 3711). Because the production, secretion, and turnover of VEGF protein directly contributes to the pathology observed in the rat OIR model, we

determined the effect of amfenac treatment on VEGF protein in hypoxic rat Müller cells. Rat Müller cells were treated with 1 μM amfenac, celecoxib, or SC-560 and placed in hypoxia for 24 hours. Amfenac, celecoxib, and SC-560 had no

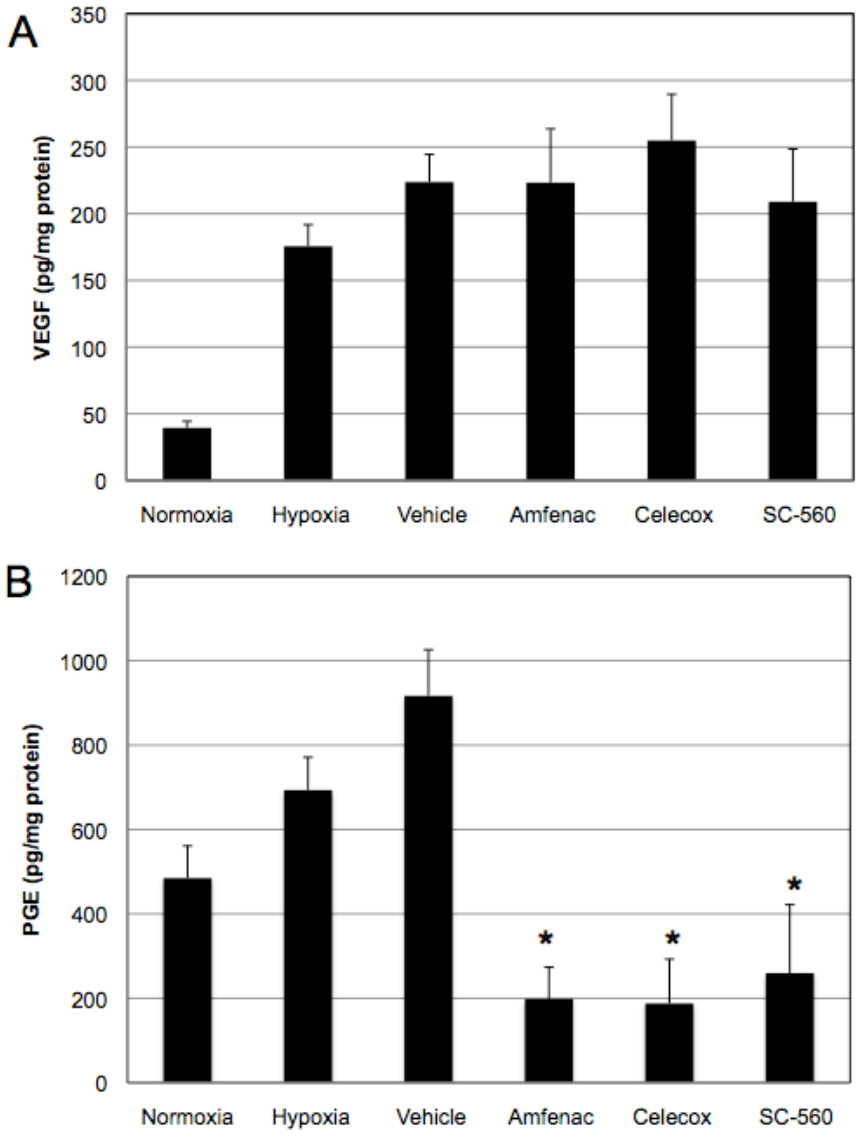

Figure 8. The effect of amfenac, celecoxib, and SC-560 on Müller cell production of VEGF and PGE_2. (A) 1 μM amfenac, celecoxib, and SC-560 had no effect on hypoxia-induced VEGF production in rat retinal Müller cells. (B) However, amfenac, celecoxib, and SC-560 treatment significantly reduced PGE_2 levels in these cells (* $p \leq 0.001$). This finding suggests that SC-560, at this concentration, is non-selective, inhibiting COX-2 as well as COX-1. Each bar represents the mean ± SD.

significant effect on hypoxia-induced VEGF production in rat Müller cells **(Figure 8A)**. However, amfenac, celecoxib, and SC-560 treatment profoundly and significantly reduced PGE_2 levels in these cells ($p \leq 0.001$), implying that hypoxia-induced VEGF expression in rat Müller cells is not affected by pharmacologic manipulation of the COX-2 enzyme **(Figure 8B)**. Since inhibition of COX did not reduce pro-angiogenic VEGF production in rat Müller cells, it cannot explain the inhibition of retinal NV by amfenac.

Effect of amfenac on retinal VEGF production

To complement our *in vitro* data **(Figure 8A)**, we returned to the OIR model in order to determine whether amfenac inhibited retina-wide, as opposed to Müller cell-derived, VEGF production. Amfenac (0.05 µg; 40 µM) was administered by a single intravitreal injection to oxygen-exposed rats upon return to room air. Two days later, retinas were harvested and retinal VEGF levels were measured. As expected, oxygen-exposed rats experienced a 4-fold increase in retinal VEGF compared to room air controls **(Figure 9)**. However, and in agreement with our *in vitro* findings, amfenac treatment demonstrated no significant effect on retinal VEGF in oxygen-exposed rats. Although a modest (20%) reduction in mean VEGF level was observed following amfenac treatment, this result was not statistically significant. Retinal VEGF levels following topical nepafenac treatment were also assayed to determine whether the natural *in vivo* metabolism of amfenac was required in order to achieve a VEGF response, and again no effect was observed (data not shown).

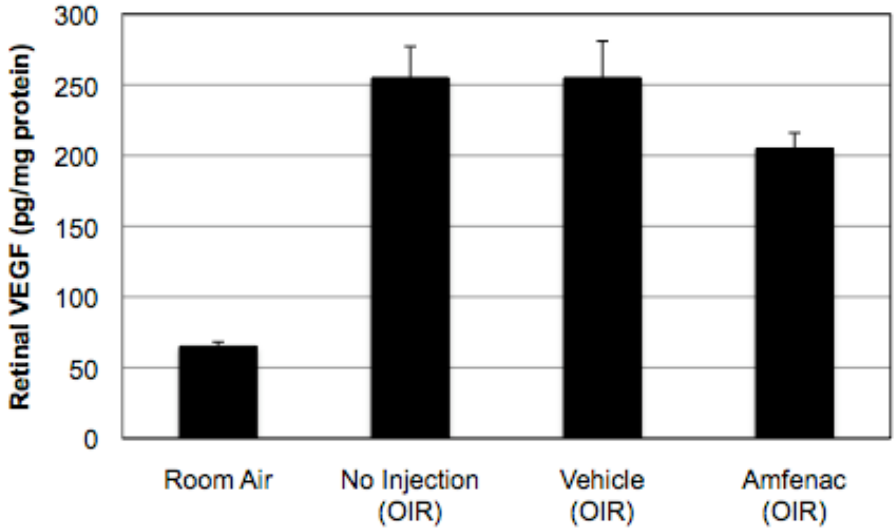

Figure 9. The effect of intravitreally-injected amfenac on retinal VEGF production. In agreement with the *in vitro* findings, 40 μM amfenac treatment demonstrated no significant effect on retinal VEGF levels in OIR rats. Each bar represents the mean ± SEM.

Effect of amfenac on VEGF-induced HRMEC behaviors

Because nepafenac proved ineffective in preliminary *in vitro* assays, we used its bioactive metabolite, amfenac, to determine the effect of the drug on angiogenic endothelial cell behaviors. The effects of amfenac, celecoxib and SC-560 on VEGF-induced tube formation and proliferation were examined. VEGF-induced (25 ng/ml) HRMEC tube formation (as determined by mean tube length) was significantly inhibited by amfenac ($p \leq 0.001$), the COX-2-selective celecoxib (* $p \leq 0.001$; † $p \leq 0.006$), and, at higher concentrations, the relatively COX-1 selective SC-560 (* $p \leq 0.001$; ‡ $p \leq 0.01$) **(Figure 10)**. Amfenac also lead to a significant reduction (32.5%; 10.00 ± 2.12 in VEGF-treated HRMEC vs. 6.75 ± 2.45 in 0.01 μM amfenac-treated HRMEC) in the number of HRMEC branch points in this assay ($p \leq 0.0233$; data not shown). VEGF-induced HRMEC proliferation was significantly inhibited by amfenac and celecoxib ($p \leq 0.001$,

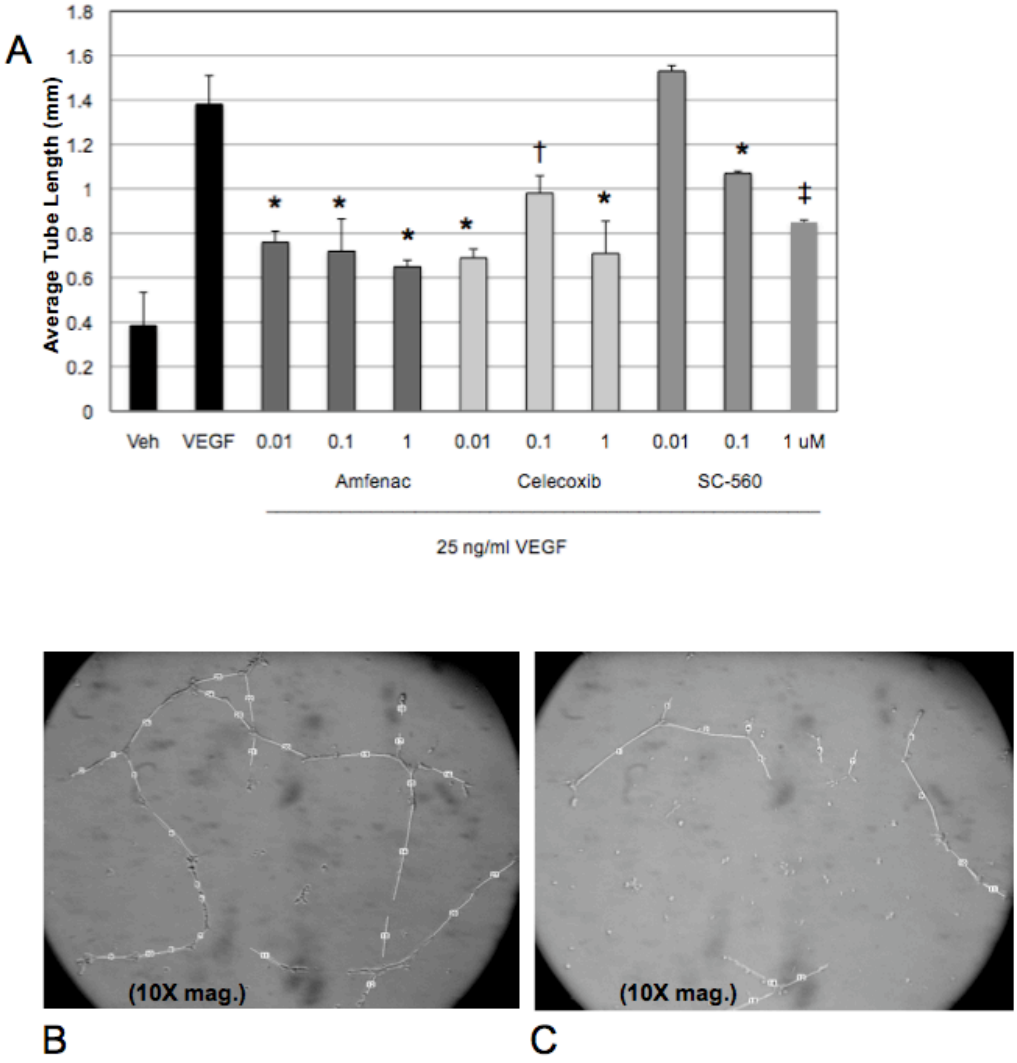

Figure 10. The effect of amfenac, celecoxib, and SC-560 on VEGF-induced HRMEC tube formation. (A) HRMEC tube formation was induced by 25 ng/ml VEGF, and this induction was significantly inhibited by amfenac (* $p \leq 0.001$), the COX-2-selective NSAID celecoxib (* $p \leq 0.001$; † $p \leq 0.006$), and, at higher concentrations, the COX-1 selective NSAID SC-560 (* $p \leq 0.001$; ‡ $p \leq 0.01$). Each bar represents the mean ± SD. (B) A representative image of tube formation in VEGF-stimulated HRMEC. (C) A representative image of tube formation in VEGF-stimulated HRMEC treated with 1 μM amfenac. Amfenac-treated HRMEC demonstrate reduced tube formation.

respectively) in a dose-dependent manner, whereas SC-560 ($p \leq 0.001$) was only inhibitory at the highest concentration tested **(Figure 11)**. Although 10 μM SC-560 significantly inhibited VEGF-induced HRMEC proliferation, this concentration is known to inhibit both COX-1 and COX-2, and to exert COX-independent

effects. These experiments suggest that amfenac, likely through COX inhibition, affects discrete aspects of the angiogenic cascade downstream of VEGFR-2 activation.

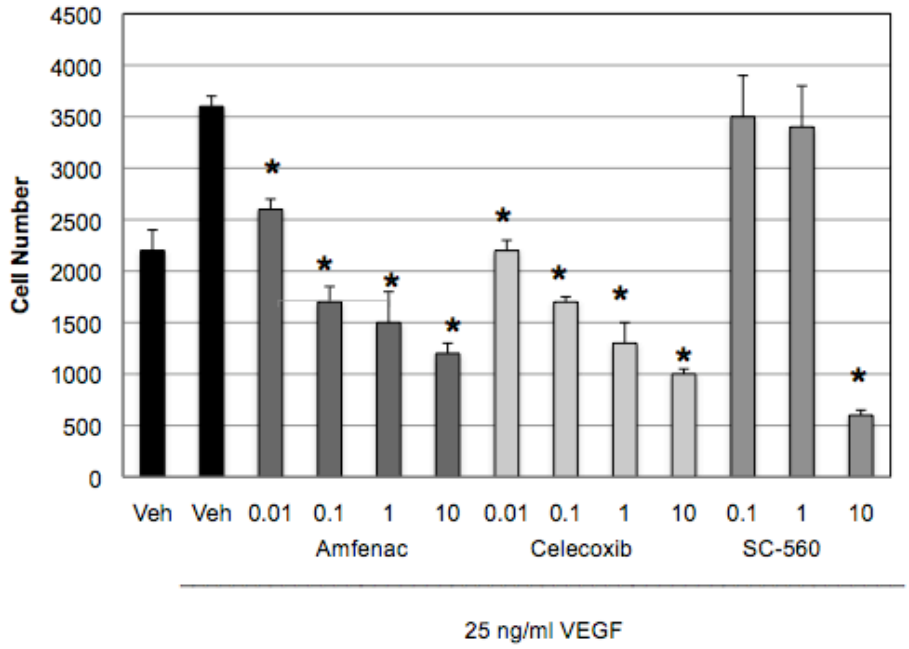

Figure 11. The effect of amfenac, celecoxib, and SC-560 on VEGF-induced HRMEC proliferation. VEGF-induced HRMEC proliferation was significantly inhibited by amfenac and celecoxib (* $p \leq 0.001$) in a dose-dependent manner, and by SC-560 (* $p \leq 0.001$) at the highest concentration tested. The effect of SC-560 at the highest concentration may be attributed to lethality. Each bar represents the mean ± SD.

Topical nepafenac efficacy in rat OIR

Topical administration of a drug that has the capacity to substantially reduce retinal NV would be a promising advancement in the development of therapies for neovascular eye diseases. Thus, we tested the capacity of topical

nepafenac to inhibit retinal NV in the rat model of OIR. Oxygen-exposed rats were treated with topical nepafenac four times daily (QID) or twice daily (BID) from P14 through P19 and were sacrificed on P20. Nepafenac (0.1%) delivered QID or BID significantly reduced ($p \leq 0.001$) the amount of pre-retinal NV in an apparent dose-dependent manner **(Figure 12)**. Like nepafenac, ketorolac and diclofenac are labeled for the treatment of pain and inflammation following cataract surgery. We compared the effect of topical nepafenac (0.1%, QID) to these commercially available, topically formulated NSAIDs, ketorolac (0.5%, QID) and diclofenac (0.1%, QID), on OIR-induced retinal NV. Nepafenac significantly reduced the mean area of pre-retinal NV by 59.3% ($p \leq 0.007$), but neither ketorolac nor diclofenac demonstrated an effect at the tested doses **(Figure 13)**.

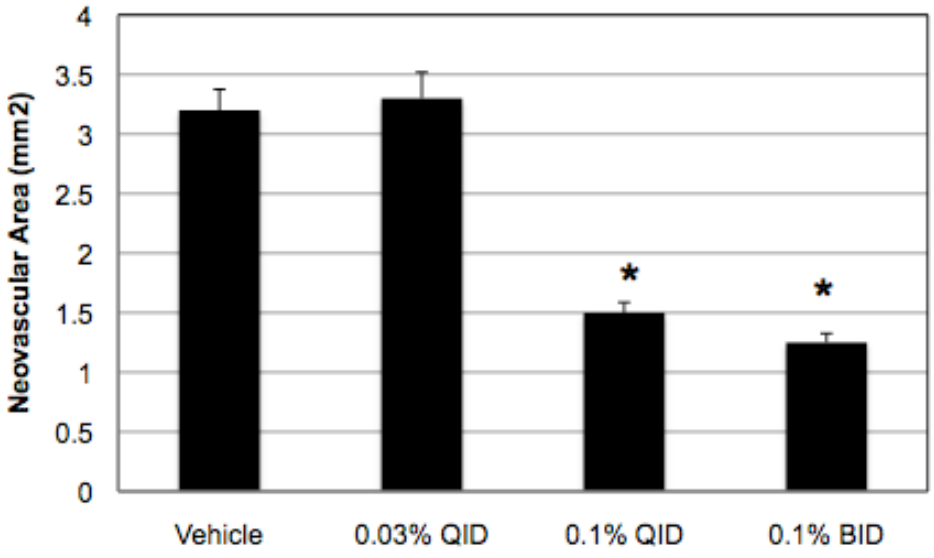

Figure 12. The effect of topical nepafenac on the severity of OIR in the rat. 0.1% nepafenac, given QID or BID from P14-P19, significantly reduced (* $p < 0.001$) OIR-induced retinal NV. Each bar represents the mean ± SEM.

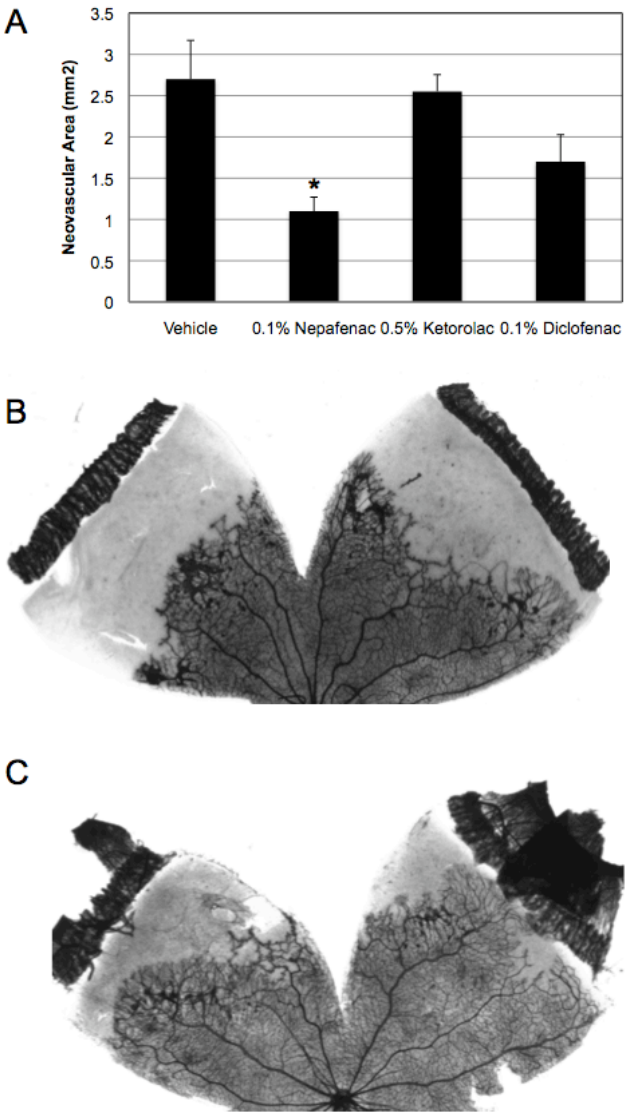

Figure 13. The effect of topical nepafenac, ketorolac, and diclofenac on the severity of OIR in the rat. (A) Drugs were administered topically, QID, from P14-P19. Nepafenac significantly reduced (* $p \leq 0.007$) OIR-induced retinal NV. Ketorolac and diclofenac failed to demonstrate an effect. Each bar represents the mean ± SEM. (B) A representative image of NV in vehicle-treated eyes. (C) A representative image of NV in nepafenac-treated eyes. As demonstrated by representative ADPase-stained retinal flat mounts, nepafenac significantly reduced retinal NV.

2.5 Discussion

The goal of this study was two-fold. First, we used three *in vitro* assays to determine the capacity of amfenac to inhibit discrete aspects of retinal angiogenesis. Using these model systems, we were better able to determine where in the angiogenic cascade COX isoforms exert their influence. Second, in order to further investigate the therapeutic potential of nepafenac as an angiostatic agent for human ocular use, we tested the efficacy of nepafenac *in vivo*, using the rat model of OIR developed in our laboratory.

In 2005, the FDA approved nepafenac for the treatment of pain and inflammation associated with cataract surgery (136-139). In the eye, nepafenac is converted to an active metabolite, amfenac, which like nepafenac is a reversible inhibitor of both COX-1 and COX-2, but unlike nepafenac has unique time-dependent inhibitory properties for both COX-1 and COX-2 (Kulmacz RJ, et al. 2007: EVER E-Abstract e473). Thus, we wanted to determine if, and more specifically how, amfenac inhibited pathological angiogenesis. Amfenac inhibits COX activity and COX-dependent prostanoid production. The cancer literature has shown that COX-2 and the prostanoids are involved in the angiogenesis that occurs during tumor growth (69-73). Moreover, recent studies have shown that COX inhibitors, including topical nepafenac, ameliorate various experimental pathologies in the posterior segment of the eye (77,111,112,138,141,148-151). We tested the hypothesis that amfenac, by virtue of its capacity to inhibit COX

activity, would inhibit pathological angiogenesis. This was done using three NSAIDs with varying selectivities for COX-1 and COX-2. Amfenac is a relatively non-selective NSAID, inhibiting both COX-1 (IC_{50} = 0.25 μM) and COX-2 (IC_{50} = 0.15 μM) (137). Celecoxib is highly COX-2-selective (COX-2 IC_{50} = 0.06 μM, COX-1 IC_{50} = 19 μM) (152). SC-560 is relatively COX-1-selective (COX-1 IC_{50} = 0.009 μM, COX-2 IC_{50} = 6.3 μM) (153). Only amfenac inhibited NV in the rat OIR model **(Figure 5)**.

It is possible that amfenac demonstrates superior ocular pharmacokinetics and bioavailability, and/or pharmacodynamic mechanisms than do celecoxib and SC-560. Or, amfenac may exert distinct COX-independent effects that mediate its angiostatic activity. The non-selective nature of amfenac's COX inhibition may be one possible pharmacodynamic explanation for its superior performance. The importance of inhibiting both COX isoforms during ischemia-induced retinal NV has been suggested by results from studies using COX-1 null and COX-2 null mice (154). Due to its superior performance, we wanted to determine, more specifically, the way(s) in which amfenac inhibited pathological angiogenesis. We surveyed the effects of the oxygen exposure model and amfenac treatment on retinal prostanoid levels **(Figure 6)**. All five of the prostanoids exhibited at least a two-fold increase upon exposure to the OIR protocol, suggesting a potential role in the development of retinal NV. The observed increase in retinal prostanoid production in oxygen-exposed rats could be due to: (1) increased $cPLA_2$ level or activity, which serves to liberate arachidonic acid, the substrate that is converted by COX into prostanoids; (2) increased level or activity of COX-2; (3) increased

prostanoid synthase activity; (4) decreased levels of 15-PGDH, which is the key enzyme responsible for the biological inactivation of the prostanoids; or (5) some combination of these. Regardless of the mechanism by which prostanoids were increased, intravitreal injection of amfenac significantly inhibited the response.

Retinal NV can be studied *in vitro* by distilling it into two basic components: hypoxia-induced VEGF production by retinal, e.g. Müller, cells and VEGF-induced angiogenic behaviors (proliferation and tube formation) in endothelial cells. We tested the capacity of amfenac to inhibit each of these processes *in vitro* so that we could more clearly define its mechanism of action *in vivo*. Amfenac had no effect on hypoxia-induced VEGF expression or production by rat Müller cells **(Figure 7 and Figure 8A)**. This was confirmed *in vivo*: amfenac did not significantly decrease retinal VEGF levels in OIR rats **(Figure 9)**. These data suggest that amfenac likely does not inhibit retinal NV in the rat model of OIR by reducing hypoxia-induced VEGF production. In accordance with our findings, Kern et al. reported that topically applied nepafenac did not reduce the increased retinal VEGF production found in diabetic rats (141). These results suggest that VEGF inhibition is unlikely to be a major contribution to amfenac's anti-angiogenic activity. Our results contradict those of Takahashi et al. who showed that topical nepafenac reduced retinal VEGF mRNA in mice exposed to the OIR model (77). The discrepancy between our findings and Takahashi et al.'s findings may be due to: (1) inherent differences between the rat and mouse models of OIR; (2) inherent differences between the two species; or (3) the fact that Takahashi et al. looked at VEGF mRNA, whereas we looked at protein. It is

important to note that a change in VEGF mRNA does not always correlate with a change in VEGF protein (Wang FE, et al. IOVS 2004;45:ARVO E-Abstract 3711). Because the production, secretion, and turnover of VEGF protein directly contributes to the pathology observed in the rat OIR model, we chose this endpoint. Point number two brings up an important distinction, because it calls into question the universal capacity of NSAIDs to affect VEGF production.

Since amfenac, a potent COX inhibitor, had no effect on VEGF production *in vitro* or *in vivo*, it is unlikely that pharmacologic manipulation of COX-2 affects this process. We tested this hypothesis using three different NSAIDs with varying selectivities for COX-1 and COX-2. The NSAID concentrations used *in vitro* were chosen because they fall within the range that allows us to distinguish between COX-1 and COX-2 effects. Amfenac, celecoxib, and SC-560 significantly inhibited Müller cell PGE_2 production **(Figure 8B)**, indicating that they did, in fact, inhibit COX activity in our cultures. However, the drugs had no effect on hypoxia-induced VEGF production. This demonstrates that hypoxia-induced VEGF production by Müller cells is not diminished by pharmacologic inhibition of the COX-2 enzyme, and that the inhibition of pro-angiogenic VEGF production by Müller cells does not appear to be the mechanism by which amfenac inhibits retinal NV. It is possible that COX-dependent prostanoid production may influence VEGF production by other retinal cell types [as Amrite et al. have shown in retinal pigment epithelial (RPE) cells] (155), although our *in vivo* studies suggest that this is not the case in the rat model of OIR **(Figure 9)**. Therefore,

COX inhibition by NSAIDs likely influences hypoxia-induced angiogenic cell behavior and OIR by a bioactivity unrelated to VEGF induction.

Although we chose to focus on VEGF, it is possible that COX-dependent prostanoid production influences the production of angiogenic factors other than VEGF. Cheng et al. demonstrated that PGE_2 induces bFGF expression in cultured rat Müller cells (75). Others have demonstrated that a different prostanoid, PGF_2, induces bFGF expression in rat osteoblasts and endometrial adenocarcinoma explants (156,157). These and other studies demonstrate that there are other angiogenic factors whose production may be prostanoid-dependent, and thus inhibited by amfenac treatment. We did not assess amfenac's effect on these proteins [namely bFGF, the VEGF receptors, erythropoietin (EPO), adenosine, or insulin-like growth factor (IGF)]. Instead, we chose to look at the effect of amfenac on VEGF-stimulated angiogenic endothelial events because: (1) despite the presence and potential involvement of other, prostanoid-dependent angiogenic factors in the retina, none have been demonstrated to be both necessary and sufficient for the development of retinal NV, as VEGF has; (2) we see increases in VEGF in our model of OIR, but do not see increases in bFGF (158); and (3) HRMEC are exposed and respond to VEGF in human ROP, making it an appropriate means by which to stimulate and manipulate (with amfenac) angiogenic endothelial cell behaviors *in vitro*.

Next, we sought to determine whether the effect of amfenac on retinal NV was being mediated through the inhibition of VEGF-induced angiogenic behaviors in endothelial cells. VEGF binds and activates high affinity VEGF

receptors on retinal endothelial cells (159). Binding of VEGF to VEGFR-2 induces receptor dimerization and tyrosine autophosphorylation, activating complex and incompletely-defined signaling cascades (159). These signal transduction pathways ultimately lead to the induction of various endothelial behaviors necessary for angiogenesis, including proliferation, migration, survival, and the production of nitric oxide that leads to increased permeability. We tested the effect of amfenac, celecoxib, and SC-560 on two of these VEGF-induced behaviors: tube formation and proliferation. Amfenac and celecoxib dose-dependently inhibited both VEGF-induced behaviors. These findings confirm those of Wu et al. who reported that HUVEC demonstrated reduced VEGF-induced proliferation and tube formation when they were treated with NS-398 (a COX-2-selective inhibitor) or with siRNA directed against COX-2 (89). In our studies, the relatively-specific COX-1 inhibitor SC-560 was only mildly effective against tube formation alone **(Figure 10 and Figure 11)**. Notably, 10 μM SC-560 significantly inhibited VEGF-induced HRMEC proliferation. This concentration of SC-560 inhibits COX-2 (in addition to inhibiting the COX-1 target enzyme) and exerts COX-independent effects on HRMEC proliferation, suggesting an explanation for its dramatic effect. Notably, amfenac inhibited two measures of tube formation, mean tube length and the number of HRMEC branch points **(Figure 10)**. The effect of COX-2 inhibition on endothelial cell branching has been documented in the literature, and was confirmed by our study (89). Amfenac, likely through inhibition of COX-2, affects discrete aspects of the angiogenic cascade downstream of VEGFR-2 activation. It is known that VEGF-

stimulated endothelial cells produce PGs (160). It is also known that PGs stimulate proliferation and tube formation, therefore demonstrating angiogenic effects (86,161). Our data suggests that the capacity of nepafenac to inhibit proliferation and tube formation is dependent on its capacity to inhibit pro-angiogenic PG production by COX-2. This *in vitro* data suggests that nepafenac's mechanism of action in ROP is dependent on its capacity to inhibit endothelial cell bioactivities like proliferation and tube formation, two behaviors that are central to the development of pathological ocular NV in ROP.

Safe and effective anti-angiogenic therapies that can be delivered noninvasively remain an unmet need in ophthalmology. Lucentis®, an anti-VEGF antibody fragment (Fab) delivered via intravitreal injection, is the current standard-of-care for neovascular age-related macular degeneration (AMD). During multiple registration studies, intravitreal injections of Lucentis® stabilized vision in over 90% of patients, and improved vision in up to 40% of patients. However, repeated intravitreal injections were necessary for the majority of patients to maintain this level of benefit (162-166). Intravitreal injections require an office visit, are often expensive, can be physically uncomfortable, and they expose the patient to a number of potential vision-threatening complications such as intraocular infection. Topical administration of a drug that has the capacity to substantially reduce retinal NV would be a promising advancement in the development of therapies for neovascular eye diseases. Nepafenac, topically applied to the cornea two or four times daily, significantly inhibited the development of retinal NV in the rat model of OIR **(Figure 12)**. This finding is

consistent with those of Takahashi et al. who reported that topical nepafenac inhibited ischemia-induced retinal NV in mice (77). We hypothesized that the anti-angiogenic effect of nepafenac was due to its capacity to inhibit COX and pro-angiogenic prostanoid production. However, it was unexpected that nepafenac proved to be unique in its capacity to significantly inhibit oxygen-induced retinal NV; ketorolac and diclofenac demonstrated no significant effect **(Figure 13)**. This observation cannot be explained by the COX-2 selectivities of the three compounds, because their respective COX-2 $IC_{50's}$ are within the same range: amfenac = 0.15 μM, ketorolac = 0.086 μM, and diclofenac = 0.038 μM (137,167). A more plausible explanation is that topical nepafenac likely has superior bioavailability to the posterior segment. In early pre-clinical trials, nepafenac exhibited superior corneal penetration and suppressed prostanoid production by the iris/cilliary body and retina/choroid more efficiently and for a longer duration than did diclofenac (136,137). In rabbits, topical administration of 0.1% nepafenac lead to nanomolar concentrations of amfenac in both anterior and posterior segment tissues, above the COX-2 IC_{50}, indicating sufficient penetration for inhibitory activity (Hariprasad SM, et al. IOVS 2009:50:ARVO E-Abstract 5999). Topical administration of nepafenac provided highest concentrations in the sclera > choroid > retina > vitreous. Pharmacologically relevant concentrations in the posterior segment were achieved through a scleral/choroidal distribution. Together, these data suggest that prostanoid synthesis is an important aspect of oxygen-induced retinal NV and that nepafenac's inhibitory effect on retinal NV is due, at least in part, to its capacity to

efficiently penetrate the cornea/sclera and inhibit COX-dependent prostanoid synthesis in the retina.

The findings that HRMEC treated with amfenac and celecoxib demonstrate reduced VEGF-induced tube formation and proliferation suggest that there are COX-2-dependent mechanisms through which amfenac inhibits oxygen-induced retinal NV. Amfenac and celecoxib may also inhibit VEGF-induced angiogenic cell behaviors through COX-2-independent mechanisms. For example, Amrite et al. reported that choroidal endothelial cells treated with celecoxib demonstrated reduced proliferation, but that the anti-proliferative effect of celecoxib was independent of its COX-2-inhibitory action (168). Nepafenac appears to be a rational therapeutic strategy for the non-invasive treatment of oxygen-induced retinopathies and other neovascular diseases of the eye, and it appears that nepafenac's mechanism of action is dependent on its capacity to inhibit endothelial cell bioactivities like proliferation and tube formation, two behaviors that are central to the development of pathological ocular NV.

Evidence suggests that oxidative compounds play a role in ROP and other angiogenic diseases of the retina (141,169-171). The retina is particularly susceptible to oxidative damage because it has a high rate of oxygen consumption (172). Furthermore, premature infants have an incompletely developed antioxidant system, leading to a reduced ability to scavenge reactive oxidative species (ROS) (173). This may increase their vulnerability to the effects of damaging oxidative species. These findings have led to a large body of basic and clinical research focused on understanding the effect of antioxidant

supplementation in ROP. A meta-analysis of clinical studies that tested the effect of vitamin E supplementation on the incidence and severity of ROP development demonstrated that vitamin E supplementation led to a 52% reduction in the development of stage 3 ROP (characterized by NV) (174). Vitamin E, superoxide dismutase, and apocynin (an NADPH oxidase inhibitor) have all been shown to prevent the development of pathological features that present in the rat model of ROP (175-178). More recently, Kern et al. have shown that nepafenac demonstrates anti-oxidant activity (141). Nepafenac inhibited diabetes-induced production of superoxide anion (a ROS) in rat retinas. It is known that ROS activates cytosolic phospholipase A_2 ($cPLA_2$) and COX-2, which can lead to the production of potentially pro-angiogenic PGs (179,180). $cPLA_2$ is the enzyme responsible for liberating arachidonic acid, a COX substrate, from membrane-derived phospholipids. cPLA2 has been shown to have a pro-angiogenic effect on retinal cell behaviors (109). These may be additional mechanisms of nepafenac's action: it may prevent the ROS-dependent activation of $cPLA_2$ and/or COX-2 and the resultant PG-induced angiogenic cell behaviors. Although we did not assess nepafenac's anti-oxidant capacity in our model of ROP, it is feasible that a portion of nepafenac's mechanism of action may have been related to its capacity to scavenge damaging ROS.

Various stimuli, including COX-derived PGs, stimulate endothelial nitric oxide synthase (eNOS) (181). Stimulation of eNOS leads to the production of nitric oxide (NO), a potent signaling molecule. NO plays a role in maintaining blood flow and vascular tone. Increased NO production leads to vasodilation,

which can be particularly harmful to the retinas of premature infants on oxygen therapy. In adults, retinal blood flow and choroidal blood flow are tightly regulated; when an adult retina is exposed to hyperoxia, retinal and choroidal blood vessels constrict, limiting excessive oxygen delivery to the retina. This vascular regulation is lacking in infants. Failure of the vasculature to constrict in response to high oxygen, coupled with the vaso-dilatory effect of high NO, means that the infant is particularly sensitive to the deleterious consequences of hyper-oxygenation. This inability to limit oxygen delivery may contribute to the infant's susceptibility to hyperoxia-induced ROP (182). These findings suggest that inhibiting COX-derived PG production will inhibit deleterious NO production, providing protection against hyperoxia-induced retinopathy. We have shown that amfenac inhibits PG production *in vitro* and *in vivo* **(Figure 6 and Figure 8)**. Although we did not assess nepafenac's capacity to inhibit NO production in our model of ROP, it is possible that a portion of nepafenac's mechanism of action may have been related to its capacity to prevent PG-mediated NO production and vasodilation. Notably, NO inhibition may also be detrimental to premature infants on supplemental oxygen therapy. eNOS and NO are required for the development of normal lung vasculature. MacRitchie et al. have shown that there is reduced eNOS in the pulmonary circulation and respiratory tract of preterm lambs on oxygen therapy and suggest that reduced eNOS may play a role in the development of chronic lung disease in the lambs (183). Furthermore, eNOS deficient mice exhibit defective lung vascular development and respiratory distress (184). Therefore, if it is found that nepafenac inhibits NO production and

has a beneficial effect on the ocular vasculature in models of ROP, it's effect on the pulmonary vasculature will need to be carefully assessed in clinical trials.

Pharmacologic inhibition of COX-2 did not have a major effect on hypoxia-induced VEGF production in our models. Despite pharmacologic inhibition, residual COX activity may have remained, which could continue to produce pro-angiogenic prostanoids with the capacity to affect VEGF production (75,76). Alternatively, Lukiw et al. reported that hypoxia-induced VEGF production was directly regulated by HIF-1, and only indirectly regulated through NF-κB-mediated COX-2 in choroidal endothelial cells (185). This suggests that HIF-1-dependent VEGF production may have the capacity to overpower the effect of COX-2-inhibition, and could explain the results of our Müller cell studies. In order to more clearly define the role of COX-2 in this process, it is necessary to assess VEGF production in COX-2 knock-out animals and the cells derived from these animals. These studies are on going.

CHAPTER III

GENETIC DELETION OF COX-2 DIMINISHES VEGF PRODUCTION IN MOUSE RETINAL MÜLLER CELLS

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3.1 Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit COX activity, reduce the production of retinal VEGF and neovascularization in relevant models of ocular disease. We hypothesized that COX-2 mediates VEGF production in retinal Müller cells, one of its primary sources in retinal neovascular disease. The purpose of this study was to determine the role of COX-2 and its products in VEGF expression and secretion. These studies have more clearly defined the role of COX-2 and COX-2-derived prostanoids in retinal angiogenesis.

Müller cells derived from wild-type and COX-2 null mice were exposed to hypoxia for 0-24 hours. COX-2 protein and activity were assessed by western blot analysis and GC-MS, respectively. VEGF production was assessed by ELISA. Wild-type mouse Müller cells were treated with vehicle (0.1% DMSO), 10 μM PGE_2, or PGE_2 + 5 μM H-89 (a PKA inhibitor), for 12 hours. VEGF production was assessed by ELISA.

Hypoxia significantly increased COX-2 protein ($p \leq 0.05$) and activity ($p \leq 0.05$), and VEGF production ($p \leq 0.0003$). COX-2 null Müller cells produced significantly less VEGF in response to hypoxia ($p \leq 0.05$). Of the prostanoids, PGE_2 was significantly increased by hypoxia ($p \leq 0.02$). Exogenous PGE_2 significantly increased VEGF production by Müller cells ($p \leq 0.0039$), and this effect was inhibited by H-89 ($p \leq 0.055$).

These data demonstrate that hypoxia induces COX-2, prostanoid production, and VEGF synthesis in Müller cells, and that VEGF production is at least partially COX-2-dependent. Our study suggests that PGE_2, signaling through the EP_2 and/or EP_4 receptor and PKA, mediates the VEGF response of Müller cells.

3.2 Introduction

Angiogenesis, the formation of new capillaries from the existing vasculature, is a process subject to exquisite regulation. Numerous pathological conditions are characterized by persistent, abnormal angiogenesis. In the eye, pathological angiogenesis, or ocular neovascularization (NV), is the leading cause of blindness in developed countries (9,10,12). Prevalent diseases in which ocular NV is a central feature include retinopathy of prematurity (ROP), proliferative diabetic retinopathy (PDR), and age-related macular degeneration (AMD or ARMD). To effectively prevent and treat ocular NV, a more thorough understanding of the cellular and molecular mechanisms involved in the angiogenic process is necessary.

Neovascularization within the eye is often the result of ischemia, which induces tissue hypoxia (114,115). In response to retinal hypoxia, various pro-angiogenic growth factors are produced, each mediating a number of angiogenic cell behaviors. Of the growth factors involved in retinal angiogenesis, vascular endothelial cell growth factor (VEGF) is thought to be a principal mediator (22). In

response to retinal hypoxia, several cell types exhibit increased VEGF production (32,33,116). This has been demonstrated most consistently and dramatically in Müller cells, the predominant glial cells within the retina (32-34). Notably, Müller cell-specific deletion of VEGF-A significantly inhibits NV in mice exposed to oxygen-induced retinopathy (186).

The cyclooxygenase (COX) enzymes are responsible for catalyzing the production of biologically active prostanoids (prostaglandins and thromboxanes) from phospholipid-derived arachidonic acid. The cancer literature has demonstrated a role for COX-2, the inducible isoform, and its prostanoid metabolites in the angiogenesis that occurs during tumor growth (69-73). Prostanoids can induce the expression of pro-angiogenic factors such as VEGF and bFGF in a wide variety of cell types (75,76).

COX-2 has been localized to various ocular tissues, and its expression has been found, or can be induced, in the following structures: cornea, iris, cilliary body, various cell types within the neuroretina, and the retinal pigment epithelium (RPE) (95,97-100). The expression of the COX-2 enzyme in these ocular tissues suggests a functional role for its prostanoid products. In fact, inhibition of COX has been effective at reducing the production of VEGF and corneal, retinal, and choroidal NV in relevant models of ocular disease (84,107,110-113,187-189). However, although various groups have demonstrated the efficacy of COX inhibition at reducing the production of VEGF and NV, little work has been done to determine which of the COX-2 derived

prostanoid(s) is (are) involved in mediating VEGF production, which stimulates angiogenic endothelial cell behaviors, and results in ocular angiogenesis.

In this study, we used an *in vitro* assay of hypoxia-induced VEGF production combined with cells isolated from COX-2 null mice to more precisely determine the role of COX-2 and its products in stimulating mouse Müller cell VEGF production. These studies are important because VEGF is the growth factor that stimulates the pathological angiogenesis characteristic of ROP, PDR, AMD, and a number of other blinding conditions. These studies have further defined the roles of COX-2 and COX-2-derived prostanoids in this discrete aspect of retinal angiogenesis.

3.3 Materials and Methods

Isolation and culture of primary mouse Müller cells

Primary Müller cell cultures were established from P7 wild-type C57/129 mouse pups and COX-2 null mouse pups on the same background (breeders were a generous gift from Dr. Sudhansu Dey, Cincinnati Children's Hospital Medical Center), according to well-established methods (144). Briefly, enucleated eyes were placed in soaking medium, Dulbecco's Modified Eagle Medium (DMEM; HyClone) supplemented with 1X Antibiotic/Antimycotic Solution (Sigma), overnight. The following day, eyes were incubated in digestion buffer, which is comprised of soaking medium plus 0.1% trypsin and 70 U/ml collagenase, for 20 minutes at 37ºC. Retinas were then dissected, triturated, plated, and grown in

DMEM supplemented with 10% fetal bovine serum and 1X Antibiotic/Antimycotic Solution. Cultures were maintained at 37ºC in a 5% CO_2/95% air (20.9% oxygen) atmosphere (normoxia) in a humidified incubator (NuAire). The identification of Müller cells was confirmed by immunocytochemical staining with a monoclonal antibody against vimentin (Affinity Bioreagents; Golden, CO), an intermediate filament protein normally expressed in Müller cells, and with a monoclonal antibody against cellular retinaldehyde binding protein (CRALBP; Affinity Bioreagents). Passages three to six were used for experiments. Normoxic and hypoxic conditions (< 1.0% oxygen) were generated with an Isotemp 3-gas Laboratory CO_2 Incubator with O_2 control (Kendro Laboratory Products; Asheville, NC) and a Proox Model 110 Gas Oxygen Controller (BioSpherix; Lacona, NY). Appropriate humidity and 5% CO_2 were maintained.

Western blot analysis

Wild-type and COX-2 null mouse Müller cells at 70% subconfluency were exposed to hypoxia for 0, 2, 6, 12, 18, or 24 hours. The lysates were matched for protein concentration, and proteins were resolved by 10% SDS-PAGE minigels (Bio-Rad; Hercules, CA) and transferred to 0.2 µM nitrocellulose membranes (Bio-Rad). Membranes were blocked in TBS containing 0.1% Tween-20 (Sigma) and 1% BSA (Sigma) overnight at 4ºC. The blots were incubated with antibodies recognizing COX-1 and COX-2 (Cayman) for one hour, followed by an anti-rabbit IgG HRP antibody (Promega) for 45 minutes, at room temperature. Following thorough washings, the proteins were visualized with enhanced

chemiluminiscence (ECL; Amersham; Piscataway, NJ). Membranes were stripped and reprobed for β-actin (Sigma). Digitized images of Western blots were quantified using Image J software (NIH). Raw densitometric values were normalized against internal control (β-actin).

Müller cell prostanoid measurement

Wild-type mouse Müller cells at 70% subconfluency were treated with 0.5% serum medium and exposed to either normoxia or hypoxia for 0, 2, 6, 12, 18, or 24 hours. Conditioned medium was collected and the lipid soluble prostanoids were reverse-phase, solid-phase extracted using Sep-Pak C_{18} columns (Waters) and nitrogen-evaporated. O-methoxyamine derivatives were formed by treatment with 2% methoxyamine-HCl in water at room temperature for 30 minutes. Compounds were extracted with ethyl acetate and subsequently converted to pentaflurobenzyl esters. The compounds were chromatographed on TLC plates with ethyl acetate/methanol. The compounds were then converted to trimethylsilyl ether derivatives and analyzed by negative ion chemical ionization mass spectrometry coupled with a gas chromatography system (Agilent Technologies). The amount of each prostanoid (ng/ml) in culture medium was normalized to total protein concentration (mg/ml) of cell lysates using a BCA assay (Pierce). Thus, any variation in prostanoid production due to differences in cell densities was resolved.

Hypoxic induction of VEGF in Müller cells

Wild-type and COX-2 null mouse Müller cells at 70% subconfluency were exposed to hypoxia for 0, 2, 6, 12, 18, or 24 hours. Culture medium from experimental dishes was collected and assayed for VEGF protein concentration with a colorimetric sandwich ELISA kit (R&D Systems) according to the manufacturer's instructions. The assay recognizes the 164 amino acid residue splice variant of mouse VEGF. Cells were washed with cold calcium- and magnesium-free PBS (Invitrogen) and lysed with cold lysis buffer (Promega). 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). Thus, any variation in VEGF production due to differences in cell densities was resolved.

PGE_2 and H-89 treatment of Müller cells

Wild-type mouse Müller cells at 70% subconfluency were serum-starved for 6 hours, and then pre-treated with 5 μM H-89 (Cayman Chemical) for 1 hour. Following pre-treatment, the cells received fresh serum-free medium with H-89, and were treated with 10 μM PGE_2 (dinoprostone; Cayman Chemical) for 12 hours. Culture medium from experimental dishes was collected and assayed for VEGF protein concentration as described above.

Statistical analysis

Data were analyzed with commercial software (JMP; SAS Institute). Analysis of variance (ANOVA) with Dunnet's post-hoc analyses and t tests were used to analyze parametric data.

3.4 Results

Effect of hypoxia on COX-2 protein and activity

Wild-type mouse Müller cells exposed to hypoxia showed increased levels of the COX-2 protein **(Figure 14)**. The densitometry of the COX-2 bands (normalized against the densitometry of the β-actin bands) was quantified, and demonstrated that COX-2 was maximally and significantly ($p \leq 0.05$) induced 6 and 12 hours after hypoxic treatment. Müller cells isolated from COX-2 null mice lack COX-2, as demonstrated by western blot analysis **(Appendix 1)**. COX-1 is the isoform of COX classically recognized as being constitutively active (65). Importantly, hypoxia did not lead to increased COX-1 in wild-type cells **(Figure 14)**. Nor did hypoxia stimulate COX-1 in COX-2 null cells, indicating that COX-1 was not compensating for the absence of the COX-2 enzyme **(Figure 14)**. Due to the fact that COX-2 is an enzyme, an increase in the level of the protein does not necessarily indicate that activity is increased. In order to assess COX-2 activity, we assessed the concentration of PGE_2 in the conditioned medium from hypoxia-treated cells. We chose to look at PGE_2 as a surrogate marker of COX-2 activity

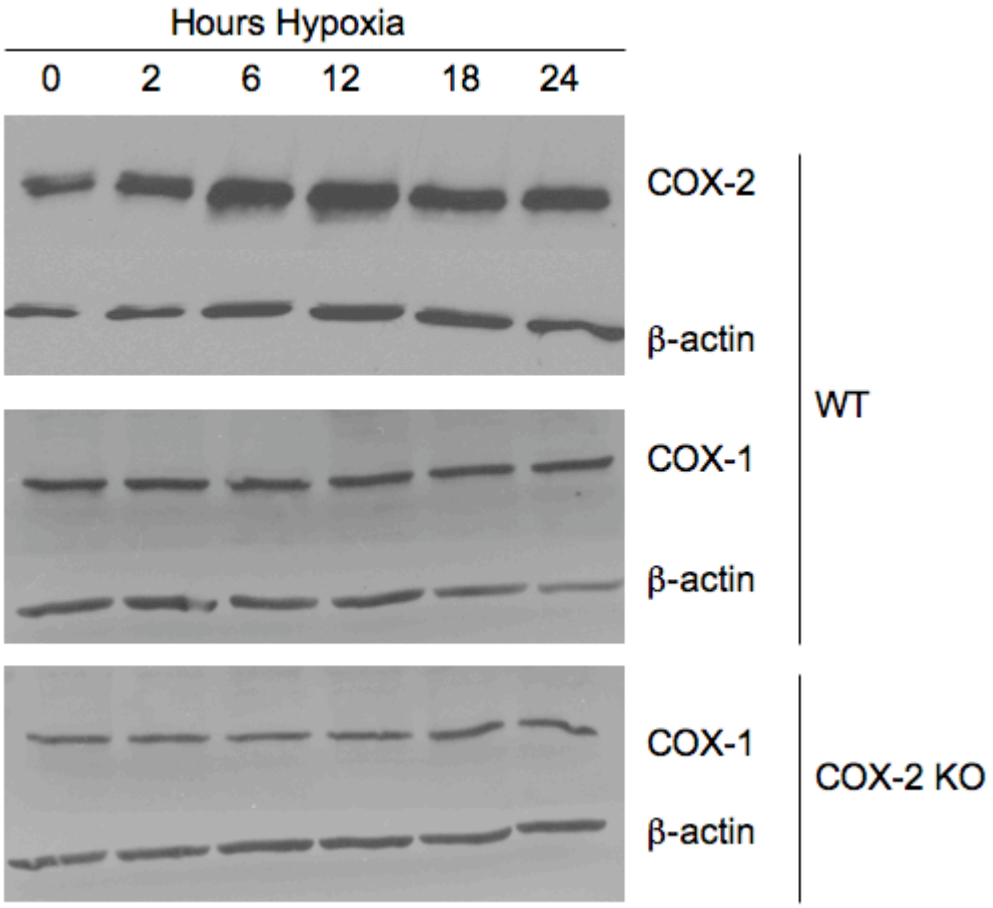

Figure 14. The effect of hypoxia on COX-1 and COX-2 in mouse Müller cells. Wild-type mouse Müller cells demonstrated an increase in COX-2 upon exposure to hypoxia. Neither wild-type nor COX-2 null mouse Müller cells demonstrated an increase in COX-1 upon exposure to hypoxia. Additionally, COX-1 did not appear to be compensating for genetic deletion of the COX-2 gene in null cells.

because COX-2-derived PGE_2 is the prostanoid product most consistently upregulated in angiogenic tumor models (190). Consistent with an increase in COX-2 protein, COX-2 activity, as demonstrated by increased PGE_2 production, is likewise significantly increased (* $p \leq 0.02$; † $p \leq 0.05$; ‡ $p \leq 0.05$) by hypoxia **(Figure 15)**.

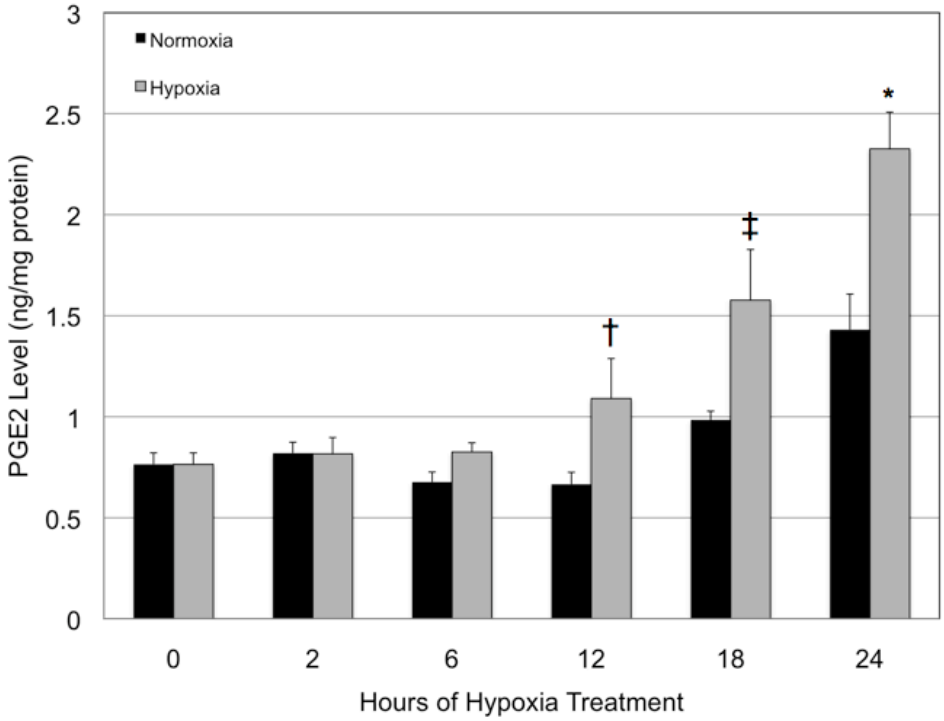

Figure 15. The effect of hypoxia on COX-2 activity in wild-type mouse Müller cells. Consistent with increased COX-2 protein, COX-2 activity was likewise significantly increased (* $p < 0.01$; † $p < 0.025$; ‡ $p < 0.05$) by hypoxia, as determined by the production of COX-2-derived PGE_2. Each bar represents mean + SD.

Effect of hypoxia on VEGF production

We have previously demonstrated the effect of hypoxia on Müller cell production of VEGF (191). We chose to look at a time course of VEGF production by hypoxic Müller cells because we hypothesized that COX-2-derived prostanoids mediate VEGF production in these cells, and we wanted to empirically determine the time course, and therefore likelihood, of these events being mechanistically linked. VEGF levels were significantly ($p \leq 0.0003$) increased over time in hypoxia, peaking at 24 hours **(Figure 16)**. Hypoxic

treatment lasting longer than 24 hours led to cell death, and hence, later time points were not examined.

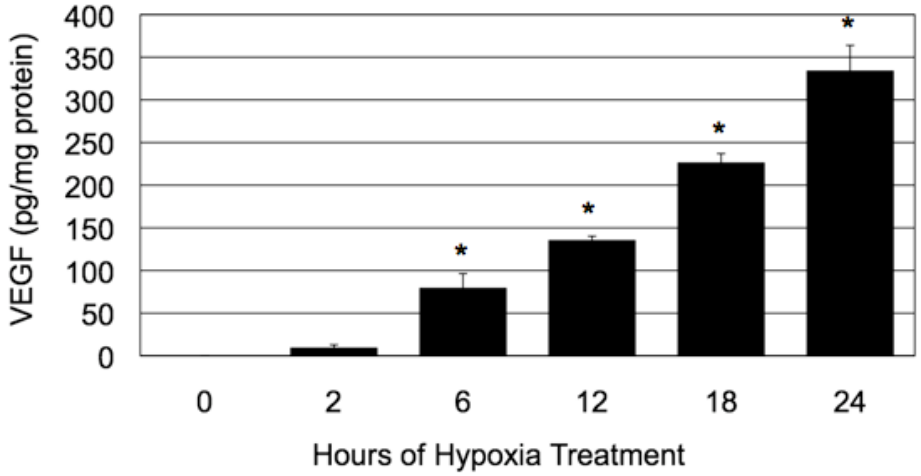

Figure 16. The effect of hypoxia on wild-type mouse Müller cell production of VEGF. Hypoxia significantly increased VEGF production in wild-type mouse Müller cells (* $p < 0.0003$), with a time course that lagged behind that of hypoxia-induced COX-2 protein and activity, suggesting that some degree of VEGF production may be stimulated by COX-2-derived prostanoids. Each bar represents mean + SD.

Effect of COX-2 deletion on VEGF protein

In order to determine the COX-2-dependent effect on hypoxia-induced VEGF production by mouse Müller cells, we cultured wild-type and COX-2 null mouse Müller cells, exposed them to hypoxia for increasing periods of time, and assessed VEGF level. VEGF production was reduced in hypoxic COX-2 null cells, compared to wild-type cells, at every time point. This effect was statistically significant ($p \leq 0.05$) after 12, 18, and 24 hours of hypoxic treatment **(Figure 17)**.

These data demonstrate that VEGF production by mouse Müller cells is at least partially dependent on COX-2 and COX-2-derived bioactive prostanoids.

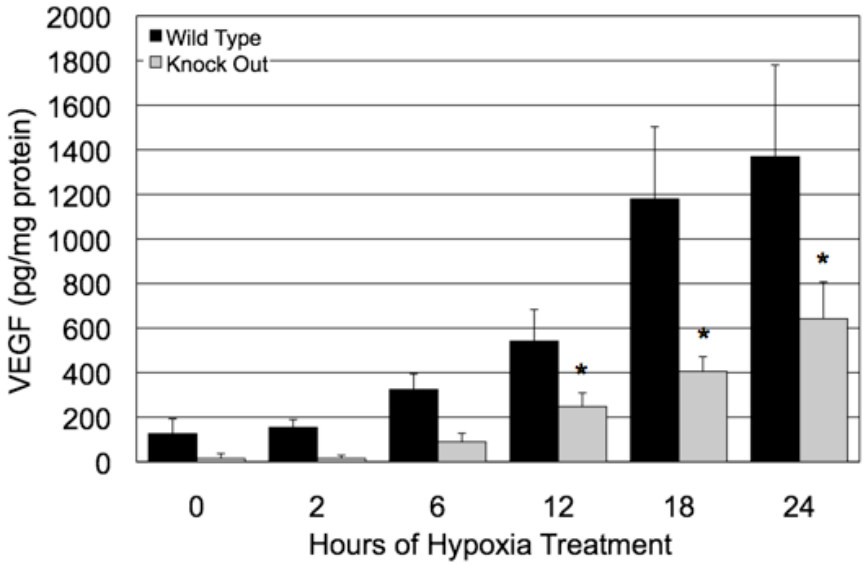

Figure 17. The effect of COX-2 deletion on VEGF protein. VEGF production was reduced in hypoxia-treated COX-2 null cells, compared to wild-type cells, at every time point. This effect was statistically significant (* $p \leq 0.05$) after 12, 18, and 24 hours of hypoxic treatment, indicating that VEGF production is partially COX-2-dependent. Each bar represents mean $\pm$ SD.

Effect of hypoxia on prostanoid production

In order to more clearly define which of the prostanoids might have been influencing hypoxic VEGF production, we analyzed prostanoid production in cells that had been maintained in normoxia and hypoxia for 24 hours. We chose to examine prostanoid production at this time point because the cells remained viable and demonstrated maximal VEGF production. Hypoxic treatment significantly increased ($p < 0.02$) levels of PGE_2. Although levels of PGF_2, PGI_2,

and TXA_2 also were increased by hypoxia, the results were not statistically significant **(Figure 18)**. We performed the same survey in COX-2 null Müller cells, and as expected, baseline (normoxia) prostanoid production was dramatically reduced and the cells failed to demonstrate any hypoxia-reactivity (data not shown). These data demonstrate that several of the prostanoids are increased in the wild-type response to hypoxia, and suggest that PGE_2, particularly, may play an important role in VEGF production by hypoxic mouse Müller cells.

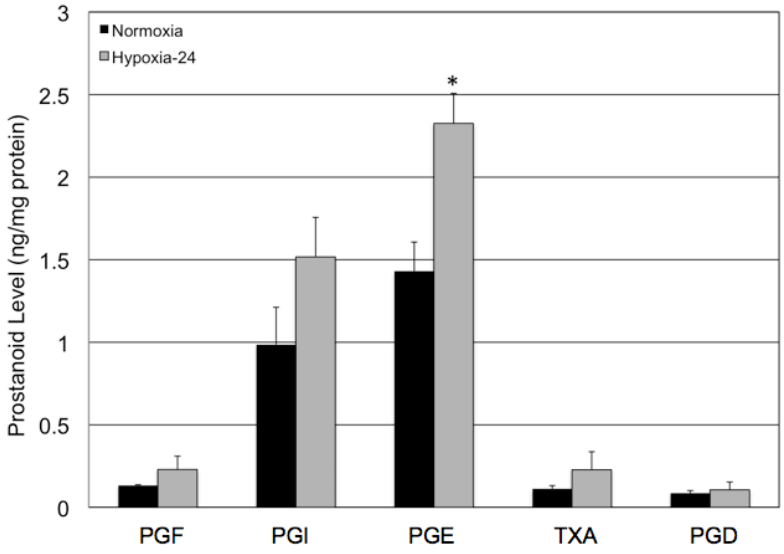

Figure 18. The effect of hypoxia on prostanoid production by wild-type mouse Müller cells. Twenty four hours of hypoxia led to significantly increased (* $p < 0.02$) levels of PGE_2. Although levels of PGF_2, PGI_2, and TXA_2 were increased by hypoxia, the results were not significant. Each bar represents mean $\pm$ SD.

Effect of PGE_2 and PKA inhibitor H-89 on prostanoid production

In order to more clearly define the role of COX-2-derived PGE_2 on VEGF production, Müller cells were treated with PGE_2 and their ability to produce VEGF was assessed **(Figure 19)**. We chose to use wild-type cells because the COX-2

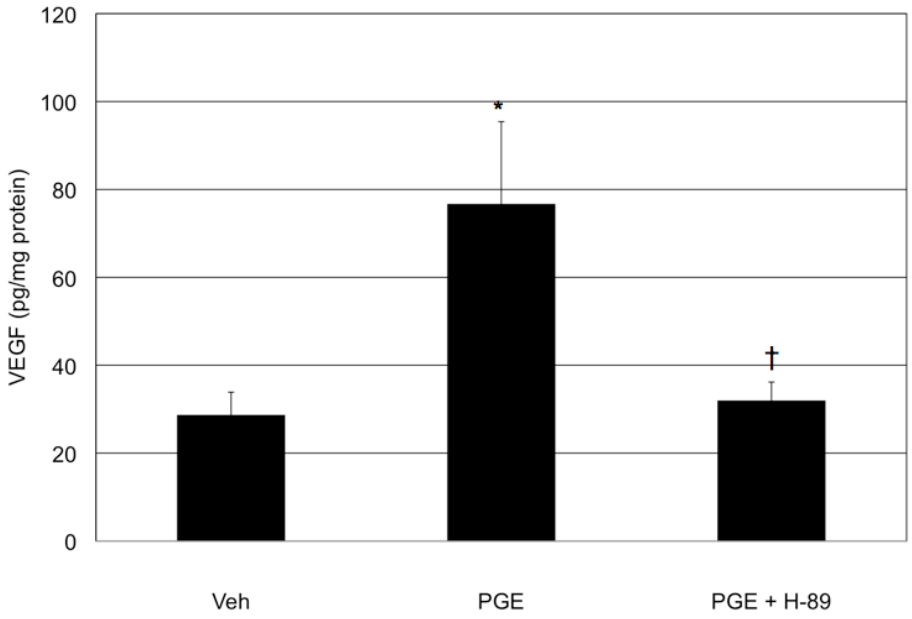

Figure 19. The effect of PGE_2 and PKA inhibition on VEGF production by wild-type mouse Müller cells. Twelve hours of PGE_2 (10 μM) treatment significantly (* $p \leq 0.0039$) increased VEGF production by Müller cells. The PGE_2-induced increase in VEGF was completely inhibited († $p \leq 0.0055$) by treatment with 5 μM H-89, a PKA inhibitor. These data suggest that the effect of PGE_2 on VEGF induction is mediated by the EP_2 and/or EP_4 receptors. Each bar represents mean $\pm$ SD.

null cells do not produce measurable quantities of VEGF following overnight serum-starvation and subsequent treatment with a low serum concentration, as required in this assay. We chose to use PGE_2 (10 μM dinoprostone) because our GC-MS data **(Figure 18)** indicated that this prostanoid was significantly increased by hypoxia. We chose to treat the cells for 12 hours because there

was a 6-12 hour lag time between maximal COX-2 induction and maximal VEGF production, and we believe that these two events are mechanistically linked and that 12 hours may be needed for COX-2 and the prostanoids to affect the VEGF transcription and translation machinery. Dinoprostone significantly (* $p \leq 0.0039$) increased VEGF production. The PGE_2-induced increase in VEGF was completely inhibited († $p \leq 0.0055$) by treatment with the PKA inhibitor H-89. These data suggest that the effect of PGE_2 on VEGF induction is mediated by the EP_2 and/or EP_4 receptors, receptors known to signal through the PKA pathway (192).

3.5 Discussion

Recent studies indicate that inhibiting the COX enzymes is an effective means by which to inhibit VEGF production and NV in relevant models of ocular disease (107,110-13,187-189). COX inhibitors have demonstrated efficacy in corneal, retinal, and choroidal models of angiogenesis. Although these studies have demonstrated the efficacy of COX inhibition at reducing the production of VEGF and NV, little work has been done to determine the mechanism by which COX-2 and its prostanoid products mediate VEGF production and the resultant ocular angiogenesis. In this study, we used an *in vitro* assay of hypoxia-induced VEGF production to examine the role of COX-2 and the prostanoids in mediating VEGF production by mouse retinal Müller cells, the cells that most consistently and dramatically increase production of VEGF in response to angiogenic

stimulation, as a necessary first step in the process of defining a more specific therapeutic target in the treatment of retinal NV (32-34,186).

It is well known that retinal ischemia-induced hypoxia is one driving force behind retinal NV (114,115). It is also known that hypoxic challenge induces COX-2 in various cell types, including vascular endothelial cells, corneal epithelial cells, and various tumor cell lines (83,193-197). We looked at the COX-2 response of mouse Müller cells exposed to hypoxia for increasing periods of time. In response to hypoxia, wild-type mouse Müller cells demonstrated significantly increased levels of COX-2 and prostanoid production **(Figures 14, 15 and 18)**. These data agree with the findings of others (referenced above) and were expected, COX-2 is an immediate early gene, rapidly and transiently induced by a variety of stimuli (198). Herein, we have also shown that wild-type mouse Müller cells demonstrated significantly increased levels of VEGF in response to hypoxia **(Figure 16)**, and VEGF levels were increased with a temporal sequence that lagged behind COX-2 induction and activity. The time sequence of VEGF production by hypoxic mouse Müller cells is consistent with the hypothesis that there are COX-2-dependent aspects of VEGF production.

Previous studies have demonstrated that COX-2 is involved in mediating growth factor production in various cell types, and in response to various stimuli (75,76). The present study has shown that COX-2 was upregulated and activated by hypoxia, and VEGF levels were increased, in an *in vitro* assay of hypoxia-induced VEGF production. We have also shown that, compared to wild-type cells, COX-2 null mouse Müller cells treated with hypoxia produce significantly

less VEGF **(Figure 17).** This finding suggests that COX-2 and its prostanoid products play a role in the VEGF response of Müller cells. These data agree with the findings of others; researchers studying angiogenesis related to various cancers and other neovascularizing conditions have demonstrated, using pharmacologic and genetic manipulation of COX-2, that COX-2 inhibition resulted in reduced VEGF production, *in vitro* and *in vivo* (77-80). Of more relevance to the eye, NSAID use has been effective at reducing the production of VEGF and NV in relevant models of ocular disease (107,110-113,154,187-189). Our findings have important implications for conditions characterized by retinal NV. Retinal hypoxia leads to increased production of VEGF, and VEGF is thought to be a principal mediator of the angiogenesis that occurs in retinal NV (22). The Müller cells most consistently and dramatically increase production of VEGF in response to retinal hypoxia (32-34,186). We have shown that genetic deletion of COX-2 and the resultant reduction in prostanoid synthesis led to a significant reduction in hypoxia-induced VEGF production by Müller cells. However, COX-2 activity leads to the production of five bioactive prostanoid products. Thus, our results led us to investigate which of the prostanoids were involved in VEGF production by mouse Müller cells, in order to define a more selective chemotherapeutic target for the treatment and management of retinal NV.

COX-2 activity results in the formation of five biologically active prostanoids (PGD_2, PGE_2, PGF_2, PGI_2, TXA_2). In response to hypoxia, wild-type mouse Müller cells demonstrated significantly increased levels of PGE_2 **(Figure 18)**, suggesting that this prostanoid might play a role in mediating VEGF

production. In fact, treating Müller cells with PGE_2 significantly increased VEGF production **(Figure 19)**. Of relevance to our focus, Cheng et al., using pure, primary cultures of rat Müller cells, demonstrated that PGE_2 significantly induced VEGF mRNA, with maximal VEGF mRNA noted 2 hours post-prostaglandin treatment (75). Our PGE_2 data confirms what has been shown in the literature – that PGE_2 stimulates VEGF production in widely diverse cell types (132,199-203). PGE_2 affects a wide range of physiological and pathological processes by binding to distinct cell surface G-protein-coupled receptors (GPCRs). PGE_2 binds and activates one (or more) prostaglandin E (EP) receptors: EP_1, EP_2, EP_3, and EP_4 (67). The receptors demonstrate distinct, as well as opposing, effects on intracellular signaling events. Of these receptors, EP_2 and EP_4 couple to G_s and mediate a rise in cAMP concentration and subsequent PKA activity. We chose to focus our attention on these receptors because Müller cells derived from EP_1 and EP_3 null mice failed to inhibit hypoxia-induced VEGF production by Müller cells **(Appendix 2)**. There have been numerous reports in the literature demonstrating a role of these two receptors in mediating PGE_2-induced angiogenic cell behaviors (132,199-201). We sought to determine whether this was the case in our model system. Of particular interest, in our model system, hypoxia led to a 2.5-fold increase in PKA activity (data not shown). As shown in Figure 19, the PKA inhibitor virtually abolished PGE_2-induced VEGF production in mouse Müller cells. These findings agree with a large body of published findings (75,204-1206). Our data suggests that PGE_2 signals through the EP_2 and/or EP_4 receptor(s), activating PKA, which ultimately leads to VEGF production by Müller cells.

Although we have focused our attention on PGE_2 and its cognate receptors, it is possible that one or more of the other prostanoids, specifically PGI_2 or PGF_2, both of which were stimulated by hypoxia **(Figure 18),** play a role in mediating retinal NV. These prostanoids signal through the IP and FP receptors, respectively. A review of these receptors implicates both of them in angiogenic cell behaviors and tumor progression (207). Additionally, the IP receptor is predominantly coupled to G_s, meaning that its activation leads to increased cAMP production and PKA activity. In the future, it will be important to determine whether all prostanoids that demonstrate pro-angiogenic activity in Müller cells signal through PKA, and affect VEGF production similarly. To more clearly define the specific role(s) of the EP_2 and EP_4 receptors, as well as the IP and FP receptors in retinal NV, we have planned studies using pharmacologic agents as well as genetically modified mice and cells derived from their retinas. Preliminary, non-optimized studies have shown that latanoprost, a PGF_2 analog, significantly increased VEGF production by COX-2 null Müller cells **(Appendix 3)**.

We have, for the first time, used COX-2 null mouse Müller cells to show that COX-2 and at least one of its prostanoid products, PGE_2, is involved in VEGF production. Our study implicates PGE_2, signaling through the EP_2 and/or EP_4 receptor(s), in mediating the VEGF response of the cells. Figure 20 depicts our working model, illustrating how COX-2 activation leads to VEGF production in these cells. This information may provide a more selective chemotherapeutic

target for the prevention and/or treatment of conditions in which retinal angiogenesis is a pathologic feature.

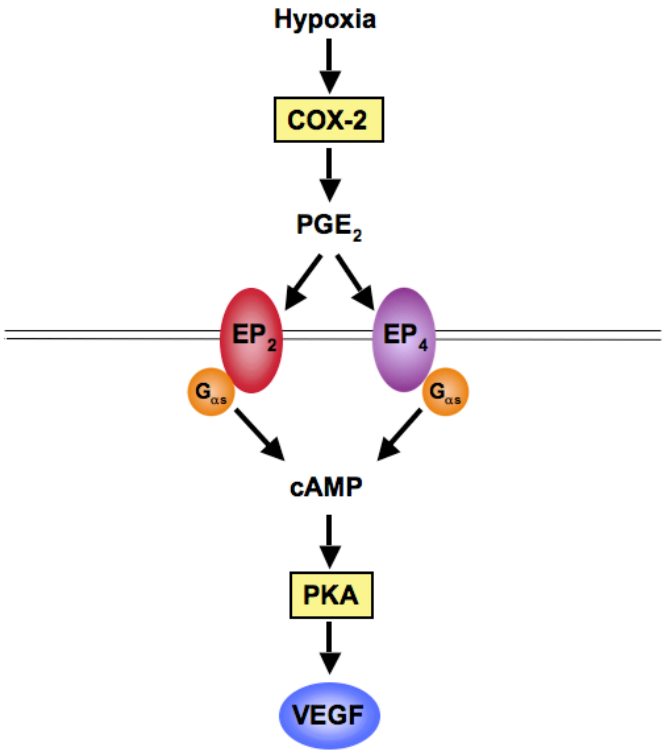

Figure 20. A flowchart demonstrating the way that we hypothesize COX-2 mediates hypoxia-induced VEGF production by Müller cells. In response to hypoxia, COX-2 is up-regulated, leading to increased production of pro-angiogenic PGE_2. PGE_2 binds to the EP_2 and/or EP_4 receptors, activating G proteins that couple to increased cAMP production. cAMP activates PKA, which is involved in mediating VEGF production.

3.6 Acknowledgements

The authors would like to thank Dr. Ginger Milne and Stephanie Sanchez of the Vanderbilt University Eicosanoid Core Laboratory and Dr. Rong Yang for experimental assistance.

CHAPTER IV

PGE_2 RECEPTOR EP_4 IS A POTENTIAL THERAPEUTIC TARGET FOR THE TREATMENT OF PATHOLOGICAL OCULAR ANGIOGENESIS

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4.1 Abstract

PGE_2 binds to PGE_2 receptors (EP_{1-4}). 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 (RMEC), and to assess the efficacy of an EP_4 antagonist in rat models of oxygen-induced retinopathy (OIR) and laser-induced choroidal neovascularization (LCNV).

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 RMEC (HRMEC) 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 neovascular area was measured.

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 ($p \leq 0.0291$). HRMEC 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 pathological neovascularization in OIR ($p < 0.0069$) and LCNV ($p \leq 0.0329$).

Preliminary investigation has demonstrated that EP_4 activation or inhibition influences the behaviors of two retinal cell types known to play roles in pathological ocular angiogenesis. These findings suggest that the EP_4 receptor may be a valuable therapeutic target in neovascular eye disease.

4.2 Introduction

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, pathological processes, such as arthritis and tumorigenesis, are characterized by persistent, poorly regulated angiogenesis. In the eye, pathological angiogenesis, or ocular neovascularization (NV), is the leading cause of irreversible blindness in developed countries (9,10,12). 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 (114,115). In response to retinal hypoxia, several cell types increase their production of pro-angiogenic growth factors. Of the growth factors involved in retinal NV, vascular endothelial growth factor (VEGF) is recognized as the principal mediator of

ocular NV (32,55,116). Hypoxia-induced VEGF production has been demonstrated most consistently and dramatically in Müller cells, the predominant glial cells within the retina (32-34,186). 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 (23). These receptors are expressed on the surface of endothelial cells. VEGFR-2 is the principle receptor involved in VEGF signal transduction leading to angiogenesis (49). VEGFR-2 activation initiates a number of signal transduction cascades leading to angiogenic endothelial cell behaviors such as survival, permeability, proliferation, and migration (23).

The cyclooxygenase (COX) enzymes catalyze the biosynthesis of five biologically active prostanoids (prostaglandins and thromboxanes) from membrane-derived arachidonic acid. The prostanoids include 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 (69-73).

The prostanoids affect a wide range of physiological and pathological 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 (67). 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 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 (199,208), angiogenic cell behaviors (209-215), and the angiogenic component of tumor growth (209,216-219). However, the majority 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 (RMEC). 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 pathological ocular angiogenesis.

4.3 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 Dr. Sudhansu Dey, Cincinnati Children's Hospital Medical Center) according to well-established methods (144). Briefly, enucleated eyes were placed in soaking medium, Dulbecco's Modified Eagle Medium Low Glucose (DMEM; HyClone) supplemented with 1X Antibiotic/Antimycotic Solution (Sigma), overnight. The following day, eyes were incubated in digestion buffer, comprised of the soaking medium plus 0.1% trypsin and 70 U/ml collagenase, for 20 minutes at 37ºC. Retinas were then dissected, triturated, plated, and grown in DMEM supplemented with 10% fetal bovine serum and 1X Antibiotic/Antimycotic Solution. Cultures were maintained at 37ºC in a 5% CO_2/95% air (20.9% oxygen) atmosphere (normoxia) in a humidified incubator (NuAire). Passages three to six were used for experiments.

Culture of human retinal microvascular endothelial cells (HRMEC)

Human retinal microvascular endothelial cells (HRMEC; Cell Systems) were cultured in tissue flasks coated with attachment factor (Cell Signaling) in endothelial basal medium (EBM; Cambrex) supplemented with 10% FBS and EGM single quots (Cambrex). When experimental conditions required serum free (SF) medium, EBM with no FBS or single quots was used. Cultures were

maintained at 37ºC 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% sub-confluency. 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% DMSO) or increasing concentrations (0.1 to 10 μM) of the PGE_2 EP_4 agonist, PGE_1-OH (Cayman Chemical), 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 pre-treated with vehicle (0.1% DMSO) or increasing concentrations (1 to 5 μM) of the EP_4 antagonist, L-161982, in 2% serum medium. Forty-five minutes later, the cells were treated with 10 μM 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) according to the manufacturer's instructions. Cells were washed with cold calcium- and magnesium-free PBS (Invitrogen Corporation) and lysed with cold lysis buffer (Promega). 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). These experiments were independently repeated two times.

HRMEC proliferation

HRMEC were seeded in 10% serum EBM at $3x10^3$ cells per well in a 96-well plate, and allowed to attach and settle. HRMEC 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 to 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 of growth factor-reduced Matrigel (Becton-Dickinson). HRMEC were seeded at 40,000 cells/well and treated with serum-free EBM containing vehicle (0.1% DMSO) or 3, 5 or 10 μM L-161982. The cells were cultured for 24 hours at 37ºC in a 5% CO_2 atmosphere. Tubes were observed with an IMT-2 inverted microscope (Olympus), and images were captured with a DMC digitizing camera (Polaroid Corporation). Six fields per well were captured for quantitative analysis. The digitized images were imported into Image J software (NIH). 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 (OIR)

All animal procedures used in this study were approved by the Vanderbilt University Institutional Animal Care and Use Committee and were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Litters of Sprague-Dawley rat pups and their mothers (Charles River Laboratories) were transferred within four hours after birth to oxygen exposure chambers where they received alternating 24 hour periods of 50% oxygen and 10% oxygen for 14 days (143). On post-natal day 14 (P14), 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 μM), was administered to oxygen-exposed rats at P14 by intravitreal injection, according to well-established methods (145). Six days after removal to room air, on P20, the rats were sacrificed, and their retinas dissected. Following dissection, the retinas were stained with ADPase, using well-established methods (171). Abnormal retinal neovascularization was measured via computer-assisted image analysis (145).

Laser-induced choroidal neovascularization (LCNV)

Laser-induced rupture of Bruch's membrane was performed to produce CNV in 6-week-old, male Brown Norway rats, as previously described (220). Using a hand-held cover slip as a contact lens, an argon laser photocoagulator

(532 nm) mounted on a slit-lamp (Coherent Novus Omni) was employed 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 the following treatment groups [vehicle (0.1% DMSO); 0.01 μM L-161982; 0.1 μM L-161982; 1 μM L-161982] and received an intravitreal injection at the temporal ora 1, 3, and 7 days following laser treatment. Fourteen days after laser application, rats were sacrificed to measure the extent of CNV at the Bruch's membrane rupture sites. Endothelial cells in CNV lesions were identified by staining RPE-Bruch's membrane-choroid flatmounts using FITC-conjugated isolectin 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 flat-mounts. This experiment was independently repeated two times.

Statistical analysis

Data were analyzed with commercial software (JMP; SAS Institute). Analysis of variance (ANOVA) with appropriate post-hoc analyses were used to analyze data.

4.4 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 to 10 μM) of PGE_1-OH, an EP_4 receptor agonist. Treatment lasted 6 hours. Agonism of the EP_4 receptor significantly (* $p < 0.0001$; † $p \leq 0.006$; ‡ $p \leq 0.0165$) increased VEGF production by COX-2 null Müller cells, in a dose-dependent manner **(Figure 21)**.

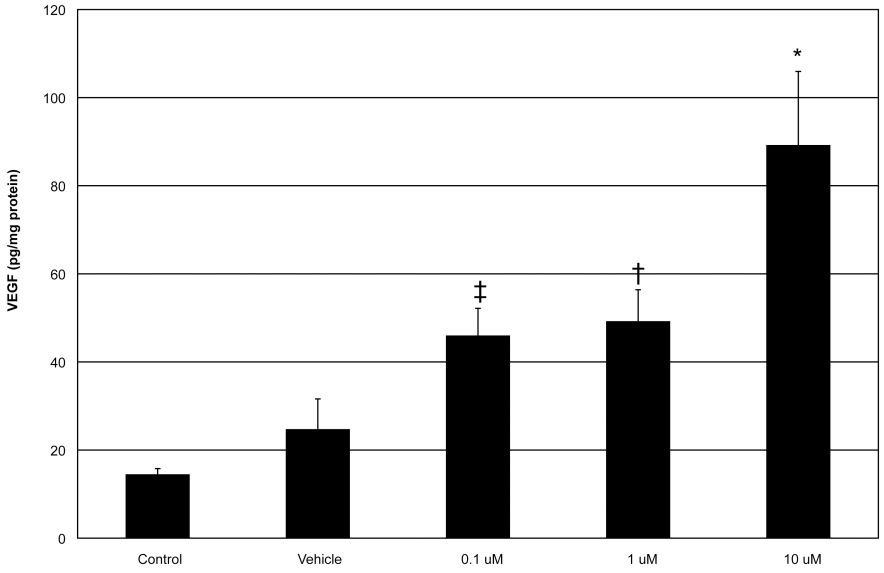


Figure 21. The effect of an EP_4 agonist, PGE_1-OH, on VEGF production in COX-2 null mouse Müller cells. PGE_1-OH significantly increased VEGF production by COX-2 null cells. Each bar represents the mean ± SD. * $p < 0.0001$; † $p \leq 0.006$; ‡ $p \leq 0.0165$ (Dunnet's post-hoc analysis). For each bar, n = 4.

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 pre-treated with increasing concentrations (1 to 5 μM) of L-161982, an EP_4 receptor antagonist, for 45 minutes, followed by 10 μM PGE_2 stimulation. Treatment lasted 12 hours. Antagonism of the EP_4 receptor significantly (* $p < 0.0066$; † $p \leq 0.0291$) decreased PGE_2-induced VEGF production by wild-type Müller cells **(Figure 22)**.

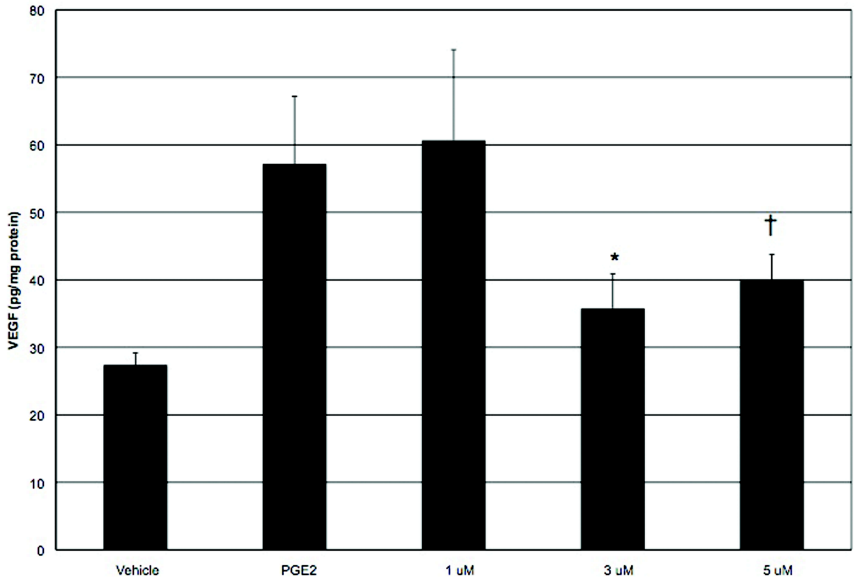


Figure 22. The effect of an EP_4 antagonist, L-161982, on PGE_2-induced VEGF production by wild-type mouse Müller cells. L-161982 pre-treatment significantly decreased PGE_2-induced VEGF production by wild-type mouse Müller cells. Each bar represents the mean ± SD. * $p < 0.0066$; † $p \leq 0.0291$ (Dunnet's post-hoc analysis). For each bar, $n = 4$.

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, HRMEC were treated with VEGF and increasing concentrations (1 to 5 μM) of the EP_4 receptor antagonist, L-161982. L-161982 significantly (* $p < 0.0001$; † $p \leq 0.0033$) inhibited VEGF-induced cell proliferation in HRMEC **(Figure 23)**.

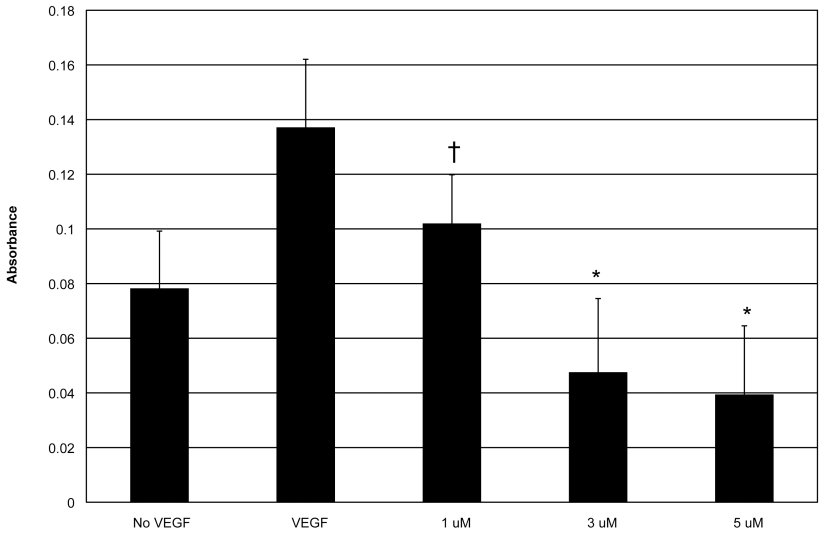


Figure 23. The effect of an EP_4 antagonist, L-161982, on VEGF-induced HRMEC proliferation. HRMEC proliferation was stimulated with 25 ng/ml VEGF. L-161982 significantly decreased VEGF-induced cell proliferation in HRMEC. Each bar represents the mean ± SD. * $p < 0.0001$; † $p \leq 0.0033$ (Dunnet's post-hoc analysis). For each bar, n = 11.

Effect of an EP_4 antagonist, L-161982, on HRMEC tube formation

To investigate the influence of the EP_4 receptor on HRMEC tube formation, HRMEC were treated with increasing concentrations (3 to 10 μM) 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 **(Figure 24 and 25)**.

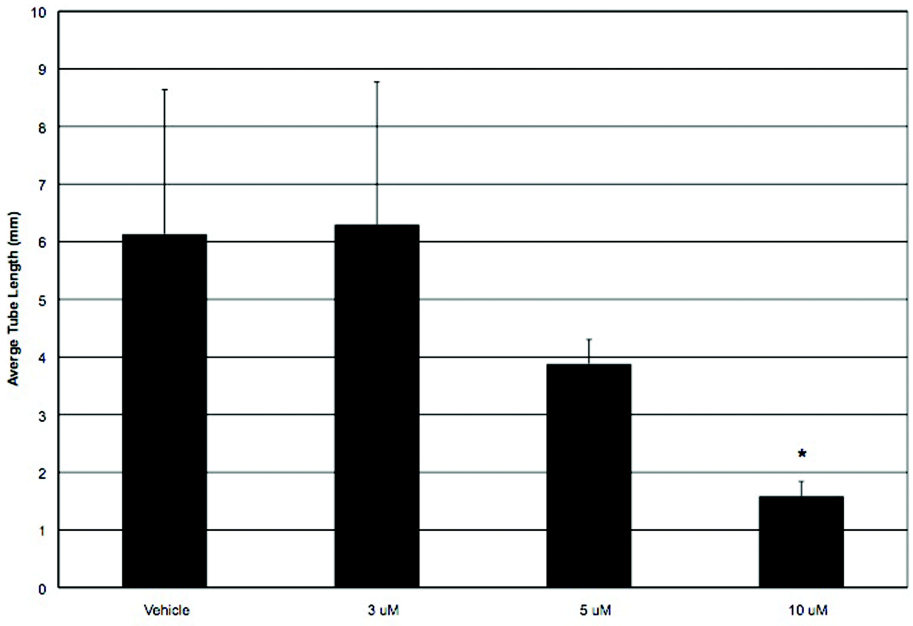


Figure 24. The effect of an EP_4 antagonist, L-161982, on HRMEC tube formation. L-161982 significantly decreased tube formation in a dose-dependent manner. Each bar represents the mean ± SD. * $p < 0.0344$ (Dunnet's post-hoc analysis). For each bar, n = 3.

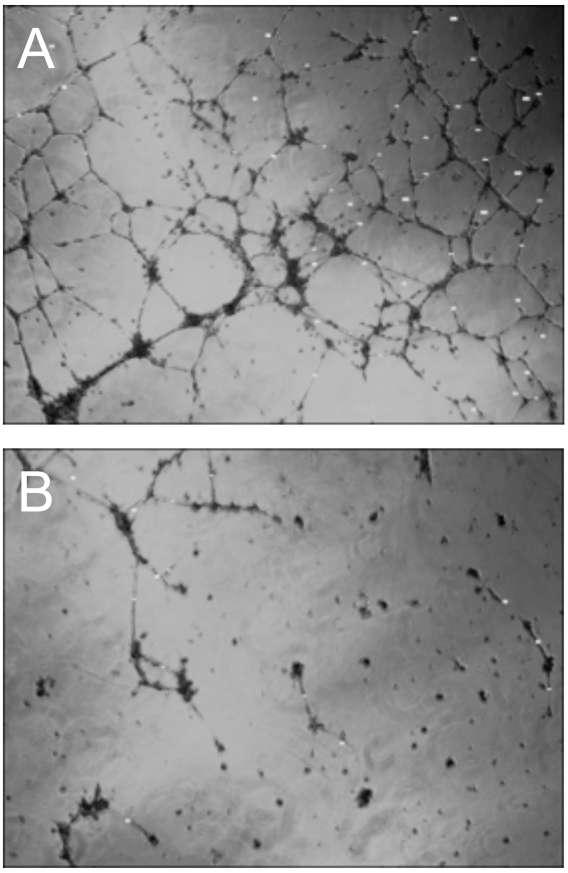


Figure 25. The effect of an EP_4 antagonist, L-161982, on HRMEC tube formation. L-161982 (10 μM) significantly decreased tube formation, as depicted in representative photomicrographs. A. HRMEC treated with vehicle (0.1% DMSO); B. HRMEC treated with 10 μM L-161982.

Effect of an EP_4 antagonist, L-161982, on OIR in the rat

Figures 21 through 25 demonstrate that EP_4 activation or inhibition influences the behaviors of two retinal cell types that are known to play roles in the pathological ocular angiogenesis characteristic of neovascular retinopathies. Next, the efficacy of the EP_4 antagonist L-161982 was tested in the rat model of 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 post-injection, the retinas were dissected, flat-mounted, stained, and assessed for extent of neovascularization via computer-assisted image analysis. As shown in **Figures 26 and 27**, EP_4 receptor antagonism significantly [0.769 ± 0.141 (0.7 μM) * $p < 0.0001$; 1.088 ± 0.210 (0.1 μM) † $p \leq 0.001$; 1.267 ± 0.175 (0.01 μM) ‡ $p \leq 0.0069$ vs. 2.126 ± .204 mm^2 (vehicle-treated)] inhibited the severity of neovascularization in the OIR model.

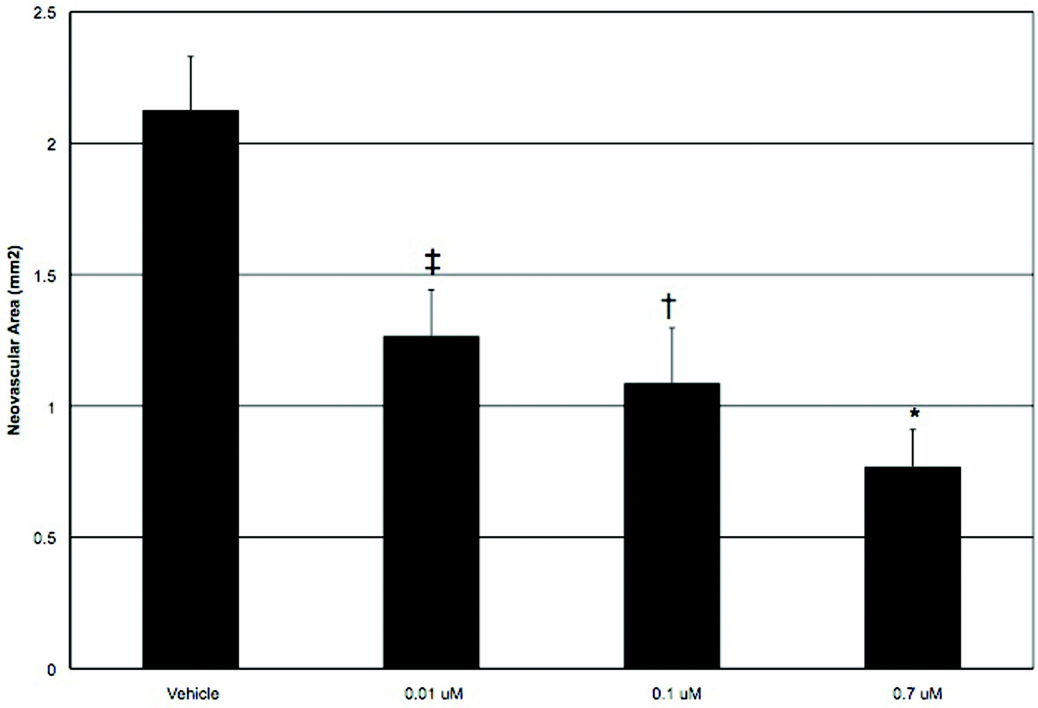


Figure 26. The effect of an EP_4 antagonist, L-161982, on the severity of oxygen-induced retinopathy in the rat. L-161982 significantly decreased the severity of OIR in a dose-dependent manner. Each bar represents the mean ± SEM. * $p < 0.0001$; † $p \leq 0.001$; ‡ $p \leq 0.0069$ (Dunnet's post-hoc analysis). For vehicle, n = 9; 0.01 and 0.1 μM, n= 10; 0.7 μM, n = 11.

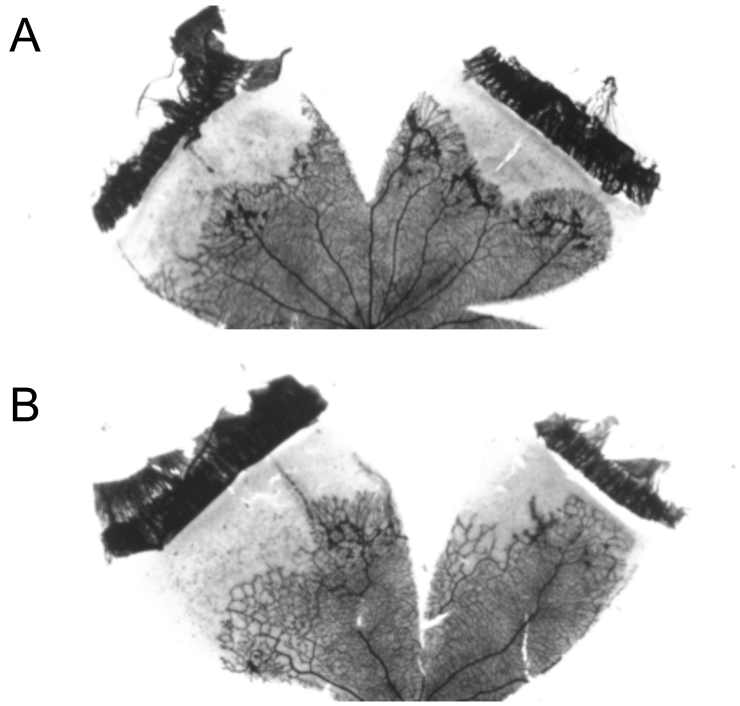


Figure 27. The effect of an EP_4 antagonist, L-161982, on the severity of oxygen-induced retinopathy in the rat, as visualized by representative ADPase-stained retinal flat mounts. L-161982 significantly decreased the severity of OIR. A. Eye treated with vehicle (0.1% DMSO); B. Eye treated with 0.7 μM L-161982.

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 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 following laser treatment. Rats were sacrificed 14 days post-laser treatment. Analysis of stained flatmounts demonstrated that L-161982 significantly [172.666 ± 18.068 (drug-treated) vs. 257.133 ± 12.472 μm^2 (vehicle-treated); * $p \leq 0.0329$] 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 **(Figures 28 and 29)**.

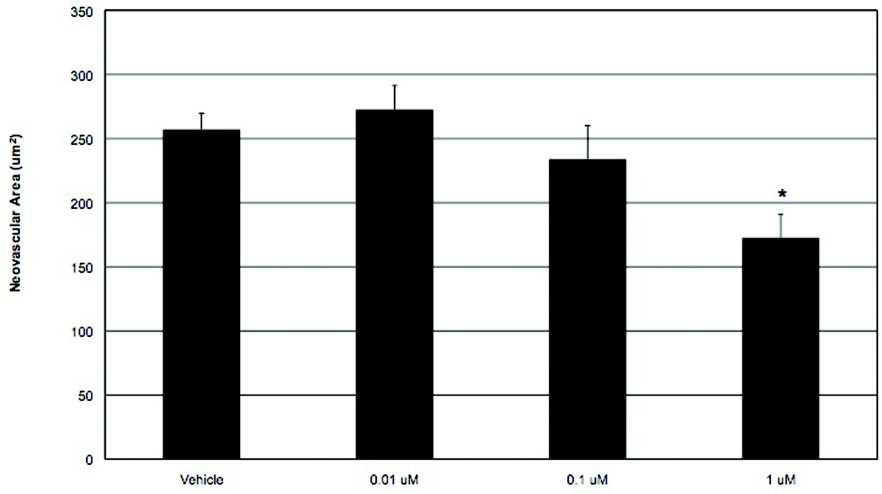


Figure 28. The effect of an EP_4 antagonist, L-161982, on the severity of laser-induced choroidal neovascularization in the rat. The highest concentration of L-161982 significantly decreased the severity of LCNV. Each bar represents the mean ± SEM. * $p \leq 0.0329$ (Fisher's LSD post-hoc analysis).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

4.5 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 (69-73). 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 lab suggest that the effect of PGE_2 on retinal angiogenesis is mediated by the EP_4 receptor. This hypothesis is based on the following preliminary findings: 1) Müller cells derived from EP_1 and EP_3 knockout

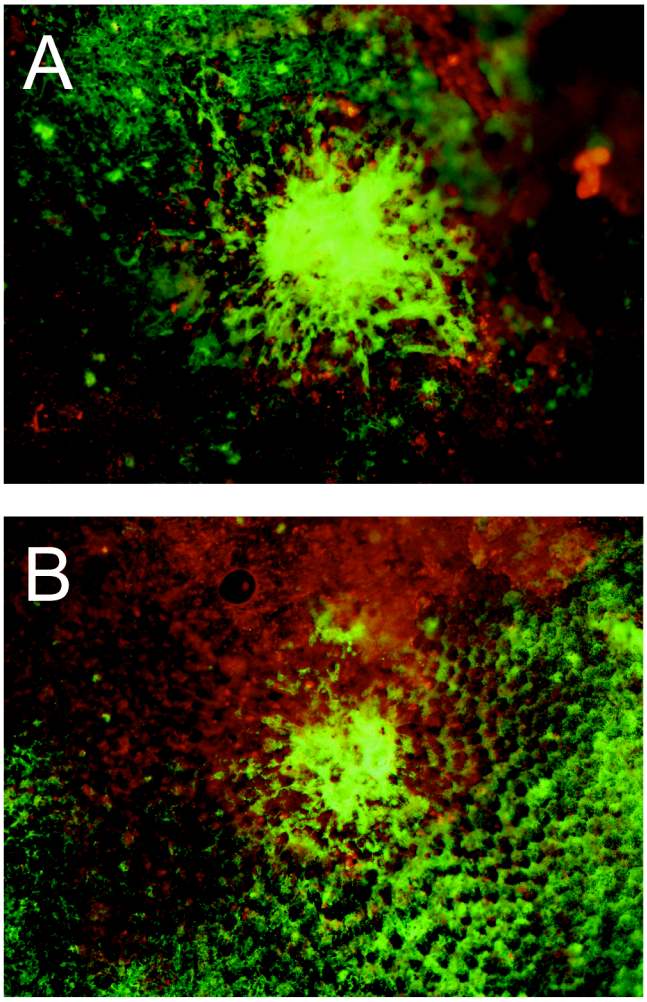


Figure 29. The effect of an EP_4 antagonist, L-161982, on the severity of laser-induced choroidal neovascularization in the rat, as visualized by isolectin B4 (green) and elastin (red) stained RPE-Bruch's membrane-choroid flat mounts. The highest concentration of L-161982 was able to significantly decrease 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 μM L-161982.

mice fail to inhibit angiogenic cell behavior, as measured by hypoxia-induced VEGF production **(Appendix 2)**; and 2) specific agonists of EP_{1-3} fail to elicit a VEGF response in COX-2 knockout Müller cells. 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 due to the absence of COX-2 and pro-angiogenic prostanoid production. We have demonstrated that VEGF can be stimulated in COX-2 null Müller cells by the EP_4 agonist PGE_1-OH **(Figure 21)**. Compared to 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 21 are not due 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 **(Figure 22)**. 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 compliments 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 (199,201,216,221,222). We have also demonstrated that HRMEC treated with the EP_4 antagonist L-161982 exhibit reduced VEGF-induced cell proliferation and tube formation **(Figures 23 – 25)**. 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 μM) 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, in order 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 (211,213,214). Ideally, the investigators would have liked to assess 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 is not possible for the following reasons: in culture, COX-2 null mouse RMEC (MRMEC) 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 HRMEC, in order to use

knockdown cells for agonist studies. However, only 60% knockdown was obtained, despite trying 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. In the future, it will be of great value to attempt to isolate MRMEC from COX-$2^{+/-}$ mice (cells that have less COX-2 protein than wild-type cells), followed by siRNA knockdown of residual COX-2. This experimental approach may yield MRMEC lacking COX-2 activity, which can be used in the assays described above. Alternatively, other means of achieving stable knockdown should be explored.

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 pathological 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 **(Figures 26 – 29)**. In both models, L-161982 was injected into the vitreous cavity. Thus, L-161982 may be more bioavailable at sites of pre-retinal NV than at sites of sub-retinal NV, explaining the drug's superior performance in OIR vs. LCNV. L-161982, at high concentrations, binds and

activates the angiotensin II AT1 receptor, which has angiogenic activity (223). Of particular relevance, the angiogenic activity of the AT1 receptor has been demonstrated in a mouse model of oxygen-induced retinopathy (224,225). Additionally, L-161982 has the following Ki values for other prostanoid receptors (in μM): 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 (207). 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. In order 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 confound of AT1 receptor activation, studies using EP_4 null cells and animals are currently underway. Preliminary data suggests 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 the EP_4 receptor to be pro-angiogenic, and that EP_4 receptor inhibition elicits an anti-angiogenic effect (209,214,216,218,219). 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.

CHAPTER V

CONCLUDING REMARKS

COX-2 and the prostanoids are up-regulated in many types of human cancers (70,226-228). These findings implicate these proteins in the angiogenesis that occurs as a part of the disease process. In fact, COX-2 inhibition has proven to be an effective means by which to inhibit tumor growth in animal models of disease (69,229-231). In large part due to data derived from coxib-treated animals, there have been several hundred clinical trials designed to assess the efficacy of COX-2 inhibitors, alone or as a therapeutic adjuvant, on the regression of human tumors. Unfortunately, many of the human clinical trials using COX-2 inhibitors were suspended because of the occurrence of adverse cardiovascular effects (232,233). Broad inhibition of COX-2 and all five of the downstream prostanoids appears to be a therapeutic strategy of limited clinical utility. This is likely because the prostanoids mediate a wide variety of physiological, as well as pathophysiological, processes. Selectively inhibiting one component of the COX-2 enzymatic cascade (e.g., one of the five prostanoids, or one of the nine receptors) may provide a more rational approach, one that is safer and more tolerable, but remains highly effective.

In the eye, various groups have demonstrated that COX inhibitors reduced the severity of pathological ocular angiogenesis in animal models (77,95,107,108,110-113,189). However, little work has been done to determine

which of the COX-2-derived prostanoid(s) is (are) involved in mediating VEGF production, downstream angiogenic endothelial cell behaviors, and ocular angiogenesis. Because of this gap in ocular angiogenesis research, we examined the specific involvement of COX-2, COX-2-derived prostanoids, and prostanoid receptors in ocular angiogenesis. The goal of our research was two-fold. We wanted to: 1) better understand the role of COX-2-derived prostanoids in ocular angiogenic disease, and 2) use the knowledge gained through our research to identify more specific therapeutic targets.

In order to better understand ocular angiogenesis, we typically distill it into its two most basic components: growth factor production by Müller cells, and growth factor consumption by (and stimulation of angiogenic cell behaviors in) endothelial cells. Surprisingly, NSAID-treated Müller cells did not demonstrate reduced hypoxia-induced VEGF production **(Figure 8A)**. This finding contrasted with a body of scientific data, collected from a variety of cell types, which demonstrated the capacity of NSAIDs to inhibit pro-angiogenic VEGF production (79,155,234-236). However, COX-2 is an enzyme, and therefore despite NSAID treatment, it is likely that residual COX-2 activity remained, which stimulated prostanoid production, and resulted in VEGF induction. In order to address this possibility, we isolated Müller cells derived from COX-2 and prostanoid receptor knockout animals. We showed that genetic deletion of COX-2 significantly reduced hypoxia-induced VEGF production **(Figure 17)**, the principal growth factor responsible for the development of retinal NV. Furthermore, and more specifically, we showed that of the COX-2-derived prostanoids, PGE_2 was

significantly increased in response to hypoxia **(Figure 18)**, and a stable analog of PGE_2 stimulated VEGF production by Müller cells **(Figure 19)**. This finding complements that of Cheng et al., who demonstrated that PGE_2 stimulated an increase in VEGF mRNA in cultured rat Müller cells (75). It is also likely that one of the other COX-2-derived prostanoids, specifically PGF_2 or PGI_2, both of which were increased by hypoxia, participates in VEGF production by Müller cells. In order to address this question unequivocally, future experiments should be designed using COX-2 null cells and stable analogs of each of the prostanoids, alone and in combination. Preliminary, non-optimized studies have shown that latanoprost, a PGF_2 analog, significantly increased VEGF production by COX-2 null Müller cells **(Appendix 3)**. This finding suggests that the FP receptor may constitute a rational therapeutic target in neovascular eye disease, either alone or in combination with other prostanoid receptors. This experiment, and a complementary experiment using a PGI_2 analog, must be optimized and repeated. Studies are underway to determine the effects of FP and IP antagonism on hypoxia-induced VEGF production by Müller cells.

Our Müller cell data motivates a complementary set of experiments to be performed downstream, in endothelial cells. Specifically, it is of importance to understand: which prostanoids are increased by VEGF-treatment of RMEC; do RMEC increase production of the same prostanoids as Müller cells, under relevant treatment conditions (i.e., VEGF treatment); does genetic deletion of COX-2 affect VEGF-induced RMEC angiogenic cell behaviors (survival, proliferation, migration, permeability); does one prostanoid, or several in

combination, rescue the phenotype (if there is one) of COX-2 null RMEC? The most ideal venue for many of the experiments listed above is COX-2 null RMEC. While we have successfully isolated these cells, we have been unsuccessful in our attempts to amplify them, or with achieving adequate knockdown with siRNA. Future efforts should be spent optimizing one of these two methodologies. It will be of great value to attempt to isolate MRMEC from COX-$2^{+/-}$ mice (cells that have less COX-2 protein than wild-type cells), followed by siRNA knockdown of residual COX-2. This experimental approach may yield MRMEC lacking COX-2 activity, which can be used in endothelial cell assays. Alternatively, other means of achieving stable knockdown in RMEC should be explored. Stable analogs of PGF_2 and PGI_2, as well as inhibitors of FP and IP, are currently being assessed in angiogenic endothelial cell assays. Once these experiments have been completed, the vision research community will have a more thorough understanding of the role of COX-2 in endothelial cell angiogenic behaviors. This information, in conjunction with the Müller cell data, will facilitate our understanding of the molecular basis of ocular angiogenic disease, as well as the development of a more specific therapeutic target, or combination of targets.

As a first step towards understanding the mechanisms of, and developing a novel therapeutic target for, ocular angiogenic diseases, we demonstrated that EP_4 receptor antagonism significantly inhibited angiogenic cell behaviors in two retinal cell types that are known to play roles in the pathological ocular angiogenesis characteristic of neovascular retinopathies. Specifically, EP_4 receptor antagonism inhibited Müller cell production of VEGF and endothelial cell

proliferation and tube formation. Additionally, antagonism of the EP_4 receptor *in vivo* significantly inhibited NV associated with both OIR and LCNV **(Chapter IV)**. It is important to mention that although we did achieve significant inhibition of NV with the EP_4 antagonist, its therapeutic effect was much less dramatic than that of other compounds tested in the lab. In fact, intravitreal injection of an EP_4 receptor antagonist was less effective than topical administration of a broad COX inhibitor, in our model of OIR **(Figures 12 and 26)**. This finding suggests that: 1) the COX inhibitor may have exerted COX-independent anti-angiogenic effects; and/or 2) the EP_4 receptor is not the sole prostanoid receptor with a role in this pathology. Thus, additional experiments should be designed to ascertain precisely which prostanoids are increased in VEGF-treated endothelial cells and their specific roles in angiogenic endothelial cell behaviors (as described above), compared to the prostanoids involved in Müller cell angiogenic activity, and then a list of prostanoid receptors with potential involvement both up- and downstream of VEGF production can be generated. The results of those studies might suggest that targeting a different prostanoid or prostanoid receptor, alone or in combination with EP_4, will provide enhanced therapeutic efficacy, which is the ultimate goal of this project, and virtually all studies conducted in the Penn lab.

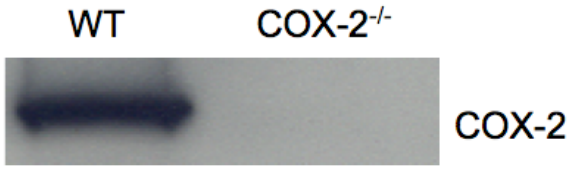
Based upon our findings, we presented a simplified model **(Figure 20)** of the way that we expect PGE_2 to affect VEGF transcription in Müller cells. The EP_4 receptor is typically coupled to a G_s G-protein, which increases the activity of adenylyl cyclase, and levels of intracellular cAMP (71). It has been established that increased cAMP activates PKA, leading to phosphorylation of CREB (and

other) transcription factors. Phosphorylated CREB attracts CREB-binding protein (CBP), and the CREB-CBP protein complex binds to the CREB response element (CRE) on the VEGF gene, stimulating transcription. This over-simplified model may be the primary mechanism by which the EP_4 receptor affects VEGF production in other cell types (215). However, prostanoid receptors activate widely different intracellular signaling pathways in both tissue- and cell-specific manners. In fact, Fujino et al., in two different studies, demonstrated 1) that the EP_4 receptor leads to transcriptional activation in a PI3K/ERK-dependent manner (237); and 2) that the EP_4 receptor can couple to a G_i G-protein, inhibiting cAMP production in certain cell types (238). Future experiments should be designed to assess specifically: 1) which type of G-protein is activated by EP_4 stimulation of Müller cells (G_s, G_i, or G_q); 2) which downstream signaling intermediates the G-protein activates or inhibits (PKA, PKC, PI3K); and 3) specifically how their activation leads to transcription of the VEGF gene (which transcription factors are activated, and the promoter regions of the VEGF gene they bind). These studies will provide the vision research community with a deeper understanding of the role of prostanoids in at least one angiogenic cell behavior. A complementary set of experiments should be conducted in RMEC in order to ascertain the signaling intermediates that mediate angiogenic cell behaviors coupled to EP_4 (and other prostanoid receptor) activation.

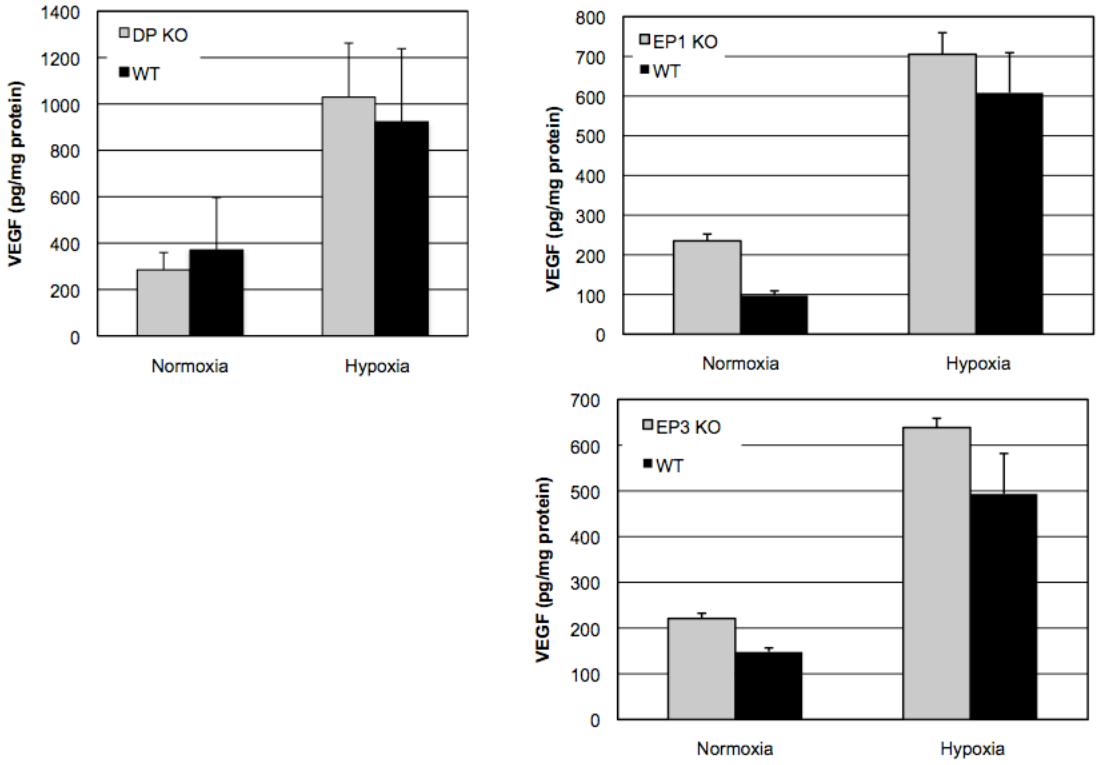
In conclusion, these studies have contributed much of what is currently known about the role of COX-2 and the prostanoids in pathological ocular angiogenesis. Although our findings are significant, additional experiments may

yield discoveries that, in conjunction with the results presented herein, will ultimately benefit the vision community in the form of a precisely targeted, highly effective pharmacotherapeutic for diseases such as ROP, PDR, and AMD.

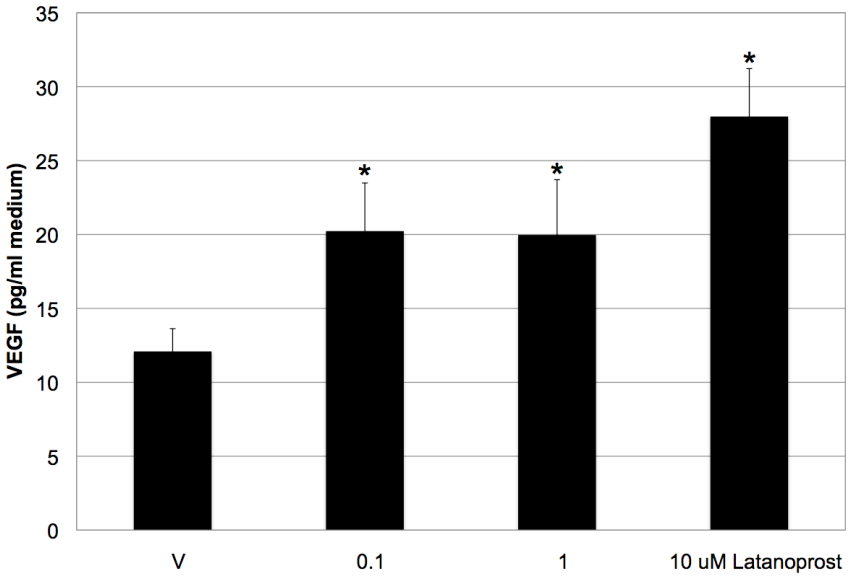
APPENDICES



Appendix 1. Western blot demonstrating the lack of COX-2 protein in Müller cells isolated from COX-2 null mice. Müller cells were isolated from wild-type and COX-2 null mice. The cells were cultured in DMEM with 10% FBS and 1X antibiotic/antimycotic, and exposed to hypoxia for 24 hours. Levels of COX-2 protein were assessed by western blot analysis.



Appendix 2. Müller cells isolated from DP, EP_1, and EP_3 null mice fail to demonstrate reduced hypoxia-induced VEGF production. Müller cells were isolated from wild-type and receptor knockout mice, cultured in DMEM with 10% FBS and 1X antibiotic/antimycotic, and exposed to hypoxia for 24 hours. VEGF was quantified by ELISA.



Appendix 3. Preliminary study demonstrating the effect of latanoprost, a PGF_2 analog, on VEGF production by COX-2 null Müller cells. COX-2 null Müller cells were serum-starved overnight, and then treated with increasing concentrations of latanoprost in 2% serum DMEM. Treatment lasted 6 hours. VEGF was quantified by ELISA. * $p < 0.0044$

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