Role of Cytosolic Phospholipase A_2 in Retinal Neovascularization

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Purpose. To identify and characterize the role of cytosolic phospholipase A_2 ($cPLA_2$) in retinal angiogenesis using relevant cell-based assays and a rodent model of retinopathy of prematurity.

Methods. The phosphorylation states of $cPLA_2$ and p38 MAP kinase and the expression of COX-2 were assessed by Western blot analysis in rat Müller cells. The activities of PLA_2 enzymes in rat retinal lysates were assessed using a commercially available assay. Prostaglandin E_2 (PGE_2) and VEGF levels in Müller cell-conditioned medium and in retinal tissue samples were measured by ELISA. Rat retinal microvascular endothelial cell proliferation was measured using a BrdU assay. Efficacy of the $cPLA_2$ inhibitor CAY10502 was tested using the rat model of oxygen-induced retinopathy (OIR) in which neovascularization (NV) was assessed by computer-assisted image analysis.

Results. In Müller cells, hypoxia increased the phosphorylation of $cPLA_2$ and p38 MAP kinase by 4-fold and 3-fold respectively. The $cPLA_2$ inhibitor CAY10502 decreased hypoxia-induced PGE_2 and VEGF levels in Müller cell-conditioned medium by 68.6% ($P < 0.001$) and 46.6% ($P < 0.001$), respectively. Retinal $cPLA_2$ activity peaked 1 day after oxygen exposure in OIR rats. CAY10502 (250 nM) decreased OIR-induced retinal PGE_2 and VEGF levels by 69% ($P < 0.001$) and 40.2% ($P < 0.01$), respectively. Intravitreal injection of 100 nM CAY10502 decreased retinal NV by 53.1% ($P < 0.0001$).

Conclusions. $cPLA_2$ liberates arachidonic acid, the substrate for prostaglandin (PG) production by the cyclooxygenase enzymes. PGs can exert a proangiogenic influence by inducing VEGF production and by stimulating angiogenic behaviors in vascular endothelial cells. Inhibition of $cPLA_2$ inhibits the production of proangiogenic PGs. Thus, $cPLA_2$ inhibition has a significant influence on pathologic retinal angiogenesis. (*Invest Ophthalmol Vis Sci.* 2010;51:1136–1142) DOI:10.1167/iovs.09-3691

Angiogenesis, the formation of new capillaries from existing blood vessels, occurs during physiological processes such as reproduction, growth and development, and wound healing.[1–6] Conversely, diseases such as arthritis, tumor growth, and retinopathies are characterized by pathologic, persistent angiogenesis.[6–8] In the context of the retina, pathologic, persistent angiogenesis is often referred to as retinal neovascularization (NV). Age-related macular degeneration, diabetic retinopathy, and retinopathy of prematurity are potentially blinding conditions characterized by choroidal or retinal NV.

Retinal NV is often caused by tissue hypoxia.[9–11] Hypoxia stimulates the activation of various intracellular signaling pathways, which lead to the production of growth factors and cytokines that stimulate quiescent endothelial cells to develop a neovascular phenotype.[12–17] Of the vasoactive factors identified to date, there is considerable evidence that vascular endothelial growth factor (VEGF) is most consistently and dramatically upregulated by retinal hypoxia.[18] Hypoxia induces VEGF synthesis in a number of retinal cell types, including endothelial cells, astrocytes, retinal pigment epithelial cells, Müller cells, and ganglion cells.[19–23] Müller cells have been shown to be the principal source of VEGF in animal models of retinal NV.[21–23]

Previous studies suggest that cyclooxygenase (COX)/prostaglandin (PG)-dependent signaling mechanisms contribute to retinal VEGF production and neovascular disease.[24–27] The initial step in PG biosynthesis is the liberation of arachidonic acid (AA) from membrane phospholipids by phospholipase A_2 (PLA_2) enzymes. There are at least 19 groups of PLA_2s that are generally classified as cytosolic ($cPLA_2$), secretory ($sPLA_2$), or calcium-independent ($iPLA_2$). PLA_2 is activated in response to a number of stimuli including ischemia, oxidative stress, and cell signaling molecules.[28] $cPLA_2$ is activated when serines 505 and 727 are phosphorylated by p38 and p42/44 MAP kinases.[29] Active $cPLA_2$ then catalyzes the hydrolysis of membrane phospholipids at the sn-2 position, releasing AA directly into the cytoplasm.[30] Free AA either diffuses out of the cell, is reincorporated into phospholipids, or is metabolized by the COX, lipoxygenase, or cytochrome P450 enzymes.[30–32] There are two well-characterized COX enzymes. COX-1, a constitutive isoform, and COX-2, which is responsive to growth factors, cytokines, and environmental stimuli, catalyze the reaction between two molecules of oxygen (O_2) and AA to produce prostaglandin H_2 (PGH_2). Cell-specific synthases catalyze isomerization, oxidation, and reduction of PGH_2 to yield the prostaglandins E (PGE), F (PGF), and D (PGD).[33–35]

PGs may exert a proangiogenic influence by inducing the upregulation of VEGF.[36–39] The following lines of evidence suggest a COX/PG-dependent component to retinal VEGF induction and subsequent NV: (1) hypoxia stimulates the upregulation of COX-2 (as well as VEGF) in Müller cells[40]; (2) hypoxia stimulates an approximate 3-fold increase in Müller cell PGE_2 synthase (McCollum GW, et al. *IOVS* 2005;46:ARVO E-Abstract 2974); (3) PGE_2 induces the upregulation of VEGF and basic fibroblast growth factor (bFGF; a potent angiogenesis inducer) in Müller cells[39]; (4) in vitro data show that amfenac, a nonsteroidal anti-inflammatory drug (NSAID), dose dependently inhibits hypoxia-induced VEGF production in Müller cells[41]; (5) cPLA2, COX, and VEGF are coordinately upregulated during the post-oxygen treatment phase (retinal hypoxia) in the

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rat model of oxygen-induced retinopathy (OIR) (Lukiw JW, et al. *IOVS* 2002;46:ARVO E-Abstract 2974) and in retinal endothelial cells exposed to hypoxia[42]; and (6) NSAIDs that inhibit COX and, consequently, PG synthesis, reduce the NV response in rodent models of OIR.[24–27]

In these studies, $cPLA_2$-dependent mechanisms of retinal angiogenesis were investigated. In vitro experiments used Müller and endothelial cells as models of the primary VEGF-producing cell type and the proliferating cell type of neovascular lesions, respectively. Consequently, $cPLA_2$ activity, VEGF levels, and PGE_2 levels were measured in the Müller cells, and proliferation was measured in endothelial cells in response to inhibiting $cPLA_2$. In vivo experiments using the rat OIR model were structured to complement and build on the in vitro studies; to that end, we measured the relative contribution of PLA_2 isoforms, $cPLA_2$ activity, VEGF levels, PGE_2 levels, and neovascular areas with $cPLA_2$ inhibition.

Materials and Methods

Rat Oxygen Treatment

All animal experiments were approved by the Vanderbilt University School of Medicine Animal Care and Use Committee, and they were conducted according to the principles expressed in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Within 4 hours of birth, litters of Sprague-Dawley rat pups and their mothers were exposed to alternating 24-hour periods at 50% and10% oxygen for 14 days. This variable oxygen treatment protocol predisposed the rat pups to OIR. Hereafter, these rats are referred to as OIR rats. Age-matched control rats were maintained in ambient (20.9% oxygen) normoxia. These rats are referred to as room air (RA) rats. After variable oxygen treatment, the OIR rats were returned to room air for up to 6 days, allowing time for retinal NV to develop. We refer to the timing of kill and assessment with two numbers, one representing the time in variable oxygen and one representing the postexposure period. Hence, rats killed immediately on removal from exposure are termed 14(0), whereas rats killed at the end of the 6-day postexposure period are referred to as 14(6).

$cPLA_2$ Activity Assay

Rat pups were killed on 14(0), 14,(1) 14(3), and 14(6). Retinas were collected, and protein was extracted with lysis buffer solution (80 mM Hepes [pH 7.4], 150 mM NaCl, 10 mM $CaCl_2$, 4 mM Triton X-100, 30% glycerol, and 1 mg/mL bovine serum albumin). The protein extracts were then assayed for $cPLA_2$ activity ($cPLA_2$ Assay Kit; Cayman Chemical, Ann Arbor, MI). Samples were either treated with 5 μM bromoenol lactone and thioether amide-PC (Cayman Chemical, Ann Arbor, MI) to block $iPLA_2$ and $sPLA_2$ activity, respectively, or were centrifuged using a membrane filter with a 30,000 MWt cutoff (Amicon Microcon Filter; Millipore Corporation, Bedford, MA) to separate the smaller $iPLA_2$ and $sPLA_2$ enzymes from $cPLA_2$. Both methods yielded similar results. Cytosolic PLA_2 was also pharmacologically inhibited in some samples with either CAY10502, a $cPLA_2$-specific inhibitor, or methylarachidonyl fluorophosphate (MAFP), a $cPLA_2$ and $iPLA_2$-selective inhibitor (Cayman Chemical, Ann Arbor, MI). This experiment was conducted three times. Samples sizes of $n = 4$ were used for experiments examining $cPLA_2$ activity over time and $n = 5$ for experiments testing the relative activity of the PLA_2 isoforms.

Müller Cell Isolation and Culture

Müller cells were isolated from the retinas of 1-week-old Long-Evans rat pups, according the procedure described by Hicks and Courtois.[43] Cells from passages four to six were used in the following experiments. The cells were grown in 10% serum DMEM low glucose (Mediatech, Inc., Manassas, VA) to 70% confluence and were maintained in normoxia for 24 hours. After 24 hours, cells were exposed to hypoxia for 12 hours (BBL GasPak system; Becton, Dickinson and Company, Sparks, MD) in the absence and presence of a $cPLA_2$ inhibitor, CAY10502, at final concentrations of 5, 20, and 50 nM. Some cultures were lysed for Western blot analysis, and others were prepared for PGE_2 and VEGF ELISA (Quantikine Colorimetric Sandwich ELISA; R&D Systems, Minneapolis, MN). When assaying for PGE_2, each experiment included the following controls: no treatment, vehicle treatment (0.1% DMSO), and lipopolysaccharide (LPS; 1 μg/mL) treatment (positive control). These experiments were conducted four times, with $n = 4$ for each treatment group.

Endothelial Cell Isolation and Culture

Rat retinal microvascular endothelial cells (RRMECs) were isolated by the method developed by Matsubara et al.[44] Passages four to seven were used in cell proliferation assays. RRMECs were seeded in 10% serum EBM at 3×10^3 cells/well in a 96-well plate. RRMECs were serum starved for 12 hours and then treated with 1% serum medium in the absence or presence of 25 ng/mL VEGF. Cells treated with VEGF received one of several concentrations (0.1–100 nM) of CAY10502 for 24 hours Cells were then labeled with BrdU for 12 hours, and BrdU incorporation was quantified with a colorimetric ELISA (Roche, Indianapolis, IN). For all treatment groups, $n = 5$.

Assessment of COX-2, p38, and $cPLA_2$ Levels in Müller Cells

For Western blot analysis, 3×10^6 Müller cells were pooled in 300 μL cold lysis buffer (150 mM NaCl, 1.0% TritonX-100, 0.1% SDS, 50 mM Tris-HCl, 100 μg/mL phenylmethylsulfonyl fluoride, 1 mM orthovanadate, 0.3 μg/mL EDTA, 0.5% deoxycholate acid, 50 μM NaF, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin A, and 1.0 mg/mL aprotinin) and were homogenized by sonication at 4°C. The samples were then centrifuged at 5000 rpm for 15 minutes at 4°C. Protein concentrations were determined using a BCA kit (Pierce Biotechnology, Rockford, IL). The volume of each sample was adjusted to a protein concentration of 2.5 μg/μL with cold lysis buffer containing protease inhibitors. Samples were resolved by SDS-PAGE and transferred to 0.2 μm nitrocellulose membranes (Bio-Rad). Nitrocellulose membranes were blocked with TBST-1% bovine serum albumin (Sigma) and probed with primary antibodies. Either goat anti-mouse IgG HRP (Chemicon, Temecula, CA) or goat anti-rabbit IgG-HRP (Chemicon) secondary antibodies were applied to the membranes, which were then developed with enhanced chemiluminescence (Amersham, Piscataway, NJ). The following primary antibodies were used in this experiment: anti-$cPLA_2$, phosphoSer505-$cPLA_2$, -p38, and -phosphoThr180/Tyr182-p38 (Cell Signaling Technology), and anti-COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA). Each Western blot was repeated three times.

Intravitreal Injections

Rats were anesthetized by isoflurane (Terrell, Meridian, ID) inhalation, and a single drop of 0.5% proparacaine (Allergan; Hormigueros, PR) was topically applied to the cornea before intravitreal injection. For all intravitreal injections, the globe was penetrated posterior to the ora ciliaris retinal using a 30-gauge needle with a 19° bevel and a 10-μL syringe (Hamilton Co., Reno, NV). The needle was advanced to the posterior vitreous while a steep angle was maintained to avoid contact with the lens. The injection bolus (5 μL) was delivered near the trunk of the hyaloid artery, proximal to the posterior pole of the retina. After injection, a topical antibiotic suspension (neomycin and polymyxin B sulfates and gramicidin; Monarch Pharmaceuticals, Bristol, TN) was applied. Noninjected eyes were also treated with topical proparacaine and antibiotic to control for the potential of these agents to influence retinal vessel growth.

Drug Treatment

At 14(0), a time of high retinal VEGF expression in this model, eyes from OIR and RA rats remained uninjected, or were injected with 5 μL

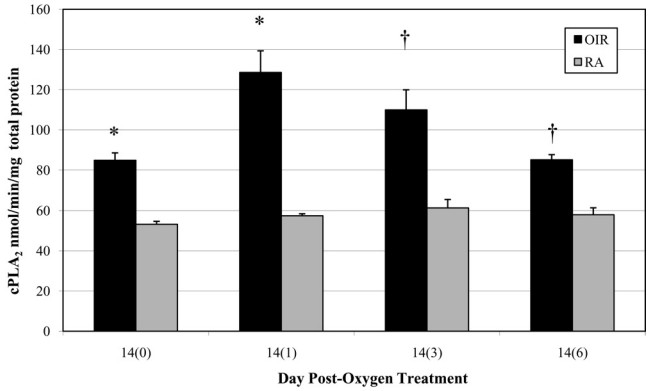

FIGURE 1. Retinal $cPLA_2$ activity ± SEM in OIR and RA rats between 14(0) and 14(6). *$P < 0.01$, and †$P < 0.02$; OIR versus RA.

vehicle (0.1% DMSO), MAFP, or CAY10502 at doses ranging from 0.5 to 100 μM or 2.5 to 100 nM, respectively. These doses were initially chosen based on published IC_{50} data[45–47] and were confirmed empirically.

Quantification of Retinopathy

OIR rats were euthanized by decapitation on 14(6). Rat eyes were enucleated, and the neural retinas were dissected and placed in cmf-PBS with 10% formaldehyde solution (37% formaldehyde solution; Fisher Scientific, Fair Lawn, NJ) overnight at 4°C. The retinal vasculature was stained for ADPase activity, according to a previously described method[48] adapted for use herein.[49,50] Images of ADPase-stained retinas were digitized, captured, and displayed at 20× magnification. The total retinal area and the retinal area containing blood vessels were traced on the monitor face with an interactive stylus pen (FTG Data Systems, Stanton, CA).[51] The number of pixels within these areas was converted to square millimeters. Measurements of this parameter were recorded.

To determine the effect of the various treatments on pathologic angiogenesis, the extent of retinal NV was assessed in flat-mounted retinas stained for ADPase activity. Representative retinal flatmounts of vehicle- and CAY10502-treated rats are shown (see Fig. 7). Abnormal preretinal NV was assessed by digitally measuring NV area. Digitized images of the retinas were captured and displayed at 65× magnification. Preretinal vessels were then traced on a computer monitor with an interactive stylus pen (FTG Data Systems). The pixels contained in the areas of NV were totaled for each retina and converted to square millimeter (Photoshop CS; Adobe Systems Inc., San Jose, CA). The operator was masked with respect to the treatment. Where there was a question of the preretinal nature of a vessel tuft, the tissue was evaluated with a light microscope at 200× magnification using the plane of focus. This method of estimation correlates well ($r^2 = 0.947$) with the clock hour method of estimation[52] and yields normally distributed data that allow statistically significant differences between treatment groups to be determined by analysis of variance. The retinas of age-matched RA rats showed no abnormalities and were not included in statistical analyses.

Statistical Analysis

Statistically significant differences in average $cPLA_2$ activities, average PGE_2 and VEGF levels, and average NV areas between treatment and control groups were determined by analysis of variance with a Bonferroni/Dunn post hoc procedure. $P \leq 0.05$ was considered significant. The experiment was repeated three times. For each experiment, n = 8 to 12 eyes for each treatment group.

RESULTS

Effect of OIR on Rat Retinal $cPLA_2$ Activity

Retinal tissue lysates from OIR and age-matched RA rats (Fig. 1) were assayed for $cPLA_2$ activity. At every time point, the $cPLA_2$ activity in OIR retinas was significantly higher than the activity in RA retinas, which was unchanged. The greatest difference was at 14(1), when the $cPLA_2$ activities in OIR and RA retinas were 128.58 ± 36.04 and 57.40 ± 2.78 nmol/min/mg total protein ($P = 0.0076$), respectively.

At least three isoforms—$cPLA_2$, $iPLA_2$, and $sPLA_2$—significantly contribute to total retinal PLA_2 activity. To estimate the relative contribution of $cPLA_2$, we treated retinal tissue lysates from 14(1) OIR rats with isoform-selective inhibitors (Fig. 2). The addition of bromoenol lactone ($iPLA_2$-selective inhibitor) or thioetheramide-PC ($sPLA_2$-selective inhibitor) to retinal lysates did not significantly decrease PLA_2 activity compared with control. When these inhibitors were combined, only a 27.1% ± 11.3% decrease was observed ($P = 0.036$). The addition of MAFP ($cPLA_2$ and $iPLA_2$ inhibitor) resulted in a 76.3% ± 3.5% decrease in activity compared with control ($P < 0.001$), and the more specific $cPLA_2$ inhibitor, CAY10502, showed a 66.6% ± 2.6% decrease in activity ($P < 0.001$). These data suggest that as much as two-thirds of the retinal PLA_2 activity is due to $cPLA_2$. Therefore, inhibiting $cPLA_2$ may be the optimal means by which to inhibit the release of AA in the retina. In Figure 2, bee venom refers to the effect of the individual PLA_2 inhibitors on a sample composed primarily of PLA enzymes with a similar contribution from each of the tested isoforms.

Effect of Hypoxia on the Phosphorylation of $cPLA_2$ and the Expression of Associated Enzymes and Signaling Intermediates

In Müller cells, the phosphorylation of p38 MAP kinase demonstrated a 4-fold increase in response to hypoxia. MAP kinase p38 is an upstream activator of $cPLA_2$.[53] Hypoxia also caused a 3-fold increase in the phosphorylation of Ser505 on $cPLA_2$ and concomitantly induced an approximate 2-fold increase in the level of the COX-2 protein (Fig. 3).

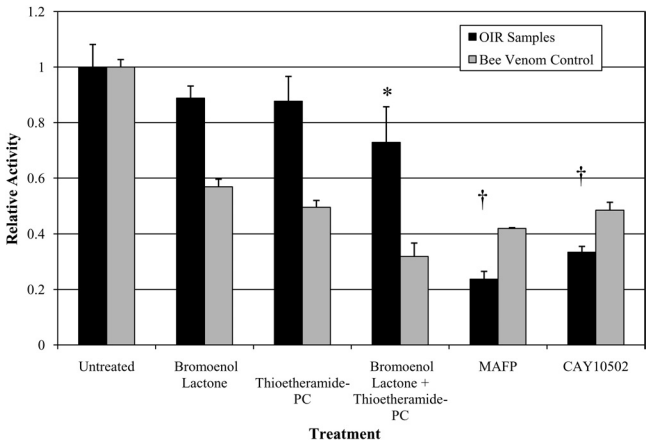

FIGURE 2. Relative PLA_2 activity in OIR retinas at 14(1). This graph demonstrates the contribution of $cPLA_2$ to total PLA_2 activity in OIR retinas. Most of the total PLA_2 activity in the retinal samples is derived from the cytosolic phospholipase family. Bromoenol lactone, $iPLA_2$ inhibitor; thioetheramide-PC, $sPLA_2$ inhibitor; MAFP (methylarachidonyl-fluorophosphonate), $cPLA_2$ inhibitor with some $iPLA_2$ inhibition; CAY10502, $cPLA_{2\alpha}$-specific inhibitor. *$P < 0.05$, and †$P < 0.001$; inhibitor versus untreated.

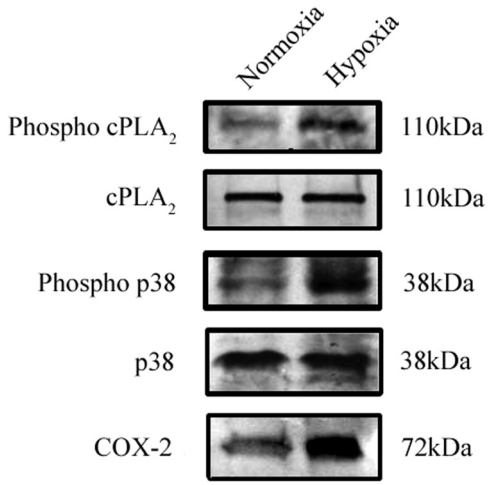

FIGURE 3. The effect of hypoxia on cPLA2 activation and related proteins in rat Müller cells. Hypoxia increases the phosphorylation of cPLA2 and p38 and the total protein levels of COX-2.

Effect of $cPLA_2$ Inhibition on Hypoxia-Induced PGE_2 and VEGF Expression in Rat Müller Cells

Conditioned media from normoxic and hypoxic rat Müller cells treated with LPS (positive control), vehicle, and 5 to 50 nM CAY10502 was assayed for PGE_2 by ELISA (Fig. 4). In both normoxia and hypoxia, LPS treatment increased PGE_2 levels compared with vehicle. A 1.9-fold increase in PGE_2 was observed in hypoxic cells treated with vehicle (492.04 ± 32.5 vs. 260.58 ± 93.15 pg/mg total protein in normoxia; $P = 0.0034$). Notably, CAY10502 inhibited hypoxia-induced PGE_2 production in a dose-dependent manner: 5 nM, 253.24 ± 36.03 ($P < 0.001$); 20 nM, 244.16 ± 40.67 ($P < 0.001$); and 50 nM, 154.50 ± 44.77 pg/mg ($P < 0.001$). There was also a dose-dependent decrease in PGE_2 production by normoxic cultures treated with CAY10502 compared with vehicle; however, only the 50-nM concentration (134.06 ± 25.47 pg/mg [$P = 0.040$]) yielded statistical significance.

Conditioned media from normoxic and hypoxic Müller cells treated with vehicle or 5 to 50 nM CAY10503 was assayed for VEGF by ELISA. Predictably, hypoxia caused a statistically significant increase in VEGF production by untreated and vehicle-treated cultures. VEGF levels were 41.39 ± 4.07 and 77.88 ± 6.62 pg/mg total protein for vehicle-treated normoxic and hypoxic cultures, respectively. CAY10502 treatment caused a dose-dependent decrease in hypoxia-induced VEGF production. The VEGF levels for 5, 20, and 50 nM CAY10502-treated hypoxic cultures were 60.90 ± 7.86 ($P = 0.016$), 57.99 ± 10.63 ($P = 0.019$), and 41.60 ± 5.11 pg/mg ($P = 0.00013$), respectively. However, in normoxic cultures treated with 5 to 50 nM CAY10502, VEGF levels were significantly different from vehicle-treated cultures (41.39 ± 4.07) only at the 50-nM concentration (33.69 ± 2.64 pg/mg; $P = 0.019$). A similar dose-dependent decrease in VEGF production was observed with MAFP treatment (data not shown).

Effect of $cPLA_2$ Inhibition on VEGF-Induced RRMEC Proliferation

RRMECs stimulated with VEGF in serum-free growth medium demonstrated a 69.2% increase in proliferation compared with cells maintained in serum-free medium alone. RRMECs treated with 35 or 50 nM CAY10502, demonstrated significant reductions in VEGF-induced proliferation (64.3% [$P = 0.010$] and 84.1% [$P = 0.012$]), respectively) compared with cultures treated with VEGF alone. This experiment was also conducted using human retinal microvascular endothelial cells (HRMECs), yielding identical results (data not shown).

Effect of $cPLA_2$ Inhibition on PGE_2 and VEGF Induction in Rat OIR

At 14(0), OIR and age-matched RA rats received intravitreal injections of vehicle or 2.5, 25, 100, or 250 nM CAY10502. Retinal tissues were collected at 14(1), and PGE_2 levels were determined by ELISA. As expected, OIR induced retinal PGE_2. In vehicle-injected eyes, OIR rats exhibited retinal PGE_2 levels of 9.125 ± 1.36 compared with 3.682 ± 0.69 ng/mg total protein in the RA rats (Fig. 5). In the OIR rats, 2.5 or 25 nM CAY10502 did not significantly affect retinal PGE_2 production, whereas 100 or 250 nM decreased the OIR-induced PGE_2 levels to 5.71 ± 1.37 ($P = 0.012$) and 2.83 ± 1.11 ng/mg ($P = 0.00036$), respectively. Notably, the OIR-induced retinal PGE_2 production was completely abolished at the highest concentration of PLA_2 inhibitor. There was no statistically significant effect of CAY10502 on PGE_2 production in RA rats except at the 250-nM (highest) concentration, which reduced PGE_2 to 2.45 ± 0.55 ng/mg ($P = 0.031$ vs. RA vehicle treatment).

In vehicle-treated eyes, OIR rats exhibited retinal VEGF levels of 279.87 ± 52.24 compared with 100.19 ± 4.88 pg/mg total protein for RA rats (Fig. 5). None of the concentrations of CAY10502 significantly affected retinal VEGF levels in RA rats. In contrast, CAY10502 treatment caused a dose-dependent decrease in retinal VEGF in OIR rats. The retinal VEGF levels of the 25, 100, and 250 nM-treated eyes were 259.07 ± 47.04 (P = NS), 207.64 ± 42.62 ($P = 0.043$), and 167.69 ± 34.58 pg/mg ($P = 0.0039$), respectively. MAFP treatment caused a similar dose-dependent decrease in retinal VEGF in OIR rats (data not shown).

Effect of $cPLA_2$ Inhibition on OIR Severity

CAY10502-injected eyes demonstrated a dose-dependent inhibition of retinal NV (Fig. 6). Injection of 100 nM CAY10502 resulted in a 53.1% reduction in NV compared with vehicle treatment ($P < 0.0001$). Figure 7 contains representative flat-mounted retinas from vehicle-treated eyes (Fig. 7A) and 100 nM CAY10502-treated eyes (Fig. 7B). Compared with vehicle, the CAY10502-treated retina has fewer and less severe neovascular lesions.

A similar demonstration of efficacy was conducted using the less specific $cPLA_2$ and $iPLA_2$ inhibitor, MAFP. These experiments were conducted with concentrations ranging from 0.5

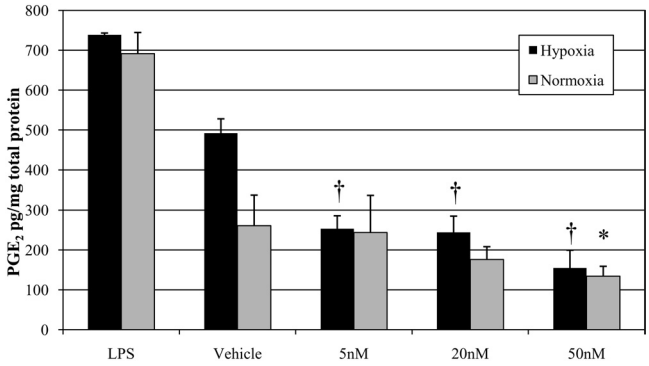

FIGURE 4. The effect of $cPLA_2$ inhibition on PGE_2 in normoxic and hypoxic rat Müller cells. Cells were pretreated in normoxia for 24 hours and then were treated with normoxia or hypoxia plus drug for 12 hours. The $cPLA_2$ inhibitor CAY10502 led to a dose-dependent reduction in PGE_2 production. $^*P < 0.05$, and $\dagger P < 0.001$ relative to vehicle (0.1% DMSO).

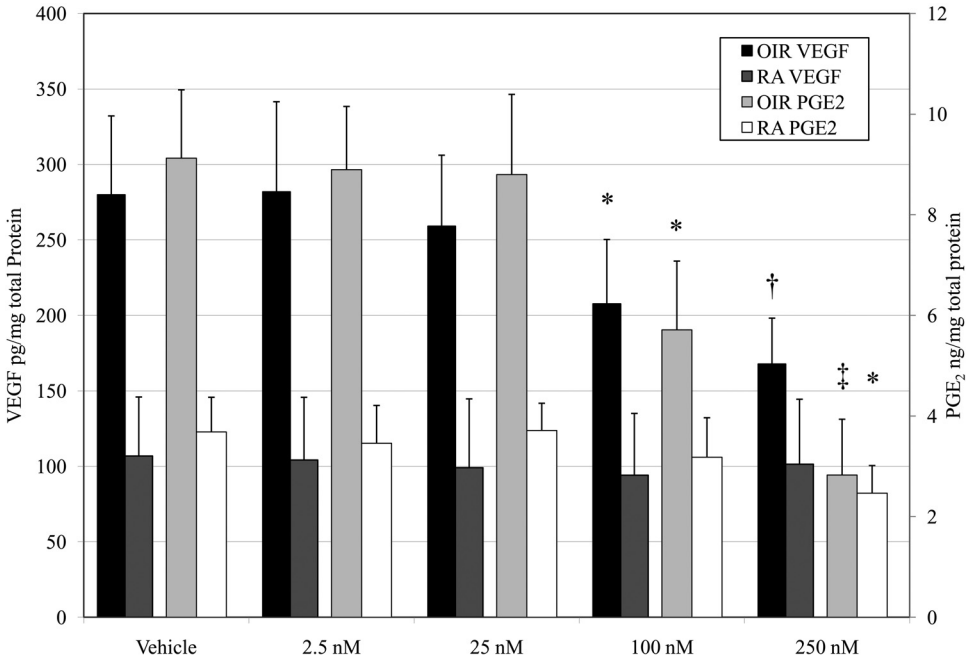

FIGURE 5. The effect of $cPLA_2$ inhibition on retinal VEGF and PGE_2 in OIR rats 24 hours after removal from oxygen treatment. $cPLA_2$ inhibition by CAY10502 demonstrates a dose-responsive reduction on both retinal VEGF (*dark bars with axis on the left*) and PGE_2 (*light bars with axis on the right*). $^*P < 0.05$, $\dagger P < 0.01$, and $\ddagger P < 0.001$ relative to vehicle.

to 100 mM and resulted in a similar pathology-response profile (data not shown).

DISCUSSION

In tumor angiogenesis, studies suggest PGE_2 is a proangiogenic inducer of VEGF.[54,55] Cheng et al.[39] showed that treating rat Müller cells with PGE_2 increased VEGF and bFGF secretion. Because retinal Müller cells are a primary source of VEGF in neovascular retinopathies, this study and others[54,55] suggest that the COX/PG pathway plays a role in the pathologic condition. More knowledge of this pathway will improve our understanding of retinal NV. With a goal to further this knowledge, this study investigated $cPLA_2$, a molecule upstream of prostaglandin synthesis.

Our central premise regarding the pathogenesis of retinal NV is not novel: retinal ischemia causes retinal hypoxia, leading to the induction of vasoactive factors, which activate vascular endothelial cells. To investigate the role of $cPLA_2$ in this, we have used assays of hypoxia-induced VEGF production in Müller cells and VEGF-induced proliferation in endothelial cells. Although we have found $cPLA_2$ to be ubiquitously produced throughout the retinal tissue (data not shown), Müller cells were specifically used in these studies because these cells have been shown to be associated with the largest induction of VEGF in the retina in response to hypoxia.[21,54,55] In Müller cells, $cPLA_2$ is expressed; p38 MAP kinase (an enzyme that activates $cPLA_2$) and $cPLA_2$ are phosphorylated/activated. The molecular weight of $cPLA_2$ is 85 kDa. However, it has been reported to have an molecular weight of 110 kDa by SDS-PAGE, and our findings are consistent with this observation.[56,57] These findings are also consistent with a role for $cPLA_2$ in retinal NV. Moreover, in agreement with our previous findings and those of other studies,[40] we observed increased COX-2 levels in OIR retinas and in hypoxic Müller cells (Barnett JM, et al. *IOVS* 2005;46:ARVO E-Abstract 4188). These coordinated events are expected to lead to increased levels of proangiogenic PGs because activation of $cPLA_2$ liberates AA from membrane phospholipids, and AA is a necessary substrate for PG production by COX enzymes. We also observed that retinal $cPLA_2$ activity is increased in OIR rats relative to RA rats. Accordingly, we found elevated levels of PGE_2 in OIR retinas and hypoxic Müller cells and, as expected, elevated levels of VEGF.

Inhibition of PLA_2 using either a $cPLA_2$-specific compound or a $cPLA_2$ and $iPLA_2$-selective compound reduced or eliminated the elevation of PGE_2 and VEGF in hypoxic Müller cells. Furthermore, the same inhibitors decreased VEGF-induced

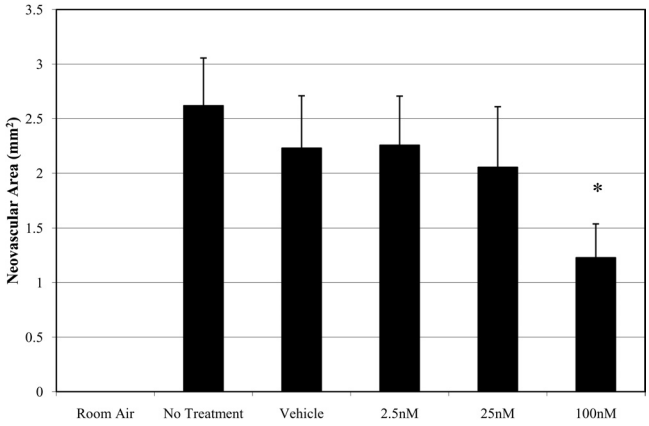

FIGURE 6. The effect of $cPLA_2$ inhibition on NV area in rat OIR. The $cPLA_2$ inhibitor CAY10502 led to a dose-dependent inhibition in NV area. $^*P < 0.0001$ relative to vehicle.

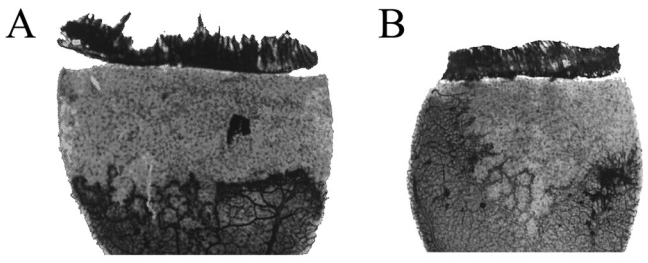

FIGURE 7. Comparison of two representative retinal quadrants from eyes treated with 0.01% DMSO vehicle (**A**) or the $cPLA_2$ inhibitor CAY10502 at 100 nM (**B**).

RRMEC and HRMEC proliferation. Several studies have shown AA release and both the cyclooxygenase and lipoxygenase pathways to be influential in endothelial cell proliferation induced by basic fibroblast growth factor, platelet-derived growth factor, and serum-containing VEGF.[55,58,59] Similarly, this study's decreased endothelial proliferation, shown by $cPLA_2$ inhibition, was likely the result of consequentially blocked VEGF-induced AA release. The effects of inhibiting PLA_2 in vitro predicate our working hypothesis, modeling events of central importance to the onset of retinal angiogenesis in the in vivo setting, and our results suggest the importance of $cPLA_2$ as a positive regulator of that process.

We further investigated the potential role of $cPLA_2$ in vivo using rat OIR. In this biphasic animal model, phase 1 is OIR induced and leads to the attenuation of normal retinal vascular development, producing a substantial peripheral avascular zone. Phase 2 occurs when OIR rats are removed from exposure to RA. In phase 1, retinal avascularity leads to OIR-induced production of vasoactive factors and activation of vascular endothelium. In this model, the most important of these factors is the VEGF that is largely produced by the hypoxic Müller cells in the retinal avascular zone.[21,54,55]

Collectively, the in vivo data suggest a mechanistic link between OIR-induced increases in retinal $cPLA_2$ activity, PGE_2 and VEGF and imply that $cPLA_2$ activity is important in the pathogenesis of rat OIR. CAY10502 reduced the retinal PLA_2 activity at its maximal activity on 14(1) by approximately 66%, indicating that a majority of this activity can be attributed to the cytosolic PLA_2 isoform. Retinal PGE_2 and VEGF were increased in OIR rats, and intravitreal administration of CAY10502 significantly inhibited retinal levels of both. The peak in OIR-induced retinal $cPLA_2$ activity, and increased retinal PGE_2 and VEGF levels, all preceded the appearance of preretinal NV, which typically occurred at approximately 14(3) in this model.

The angiostatic capacity of the $cPLA_2$-specific CAY10502 was tested in the rat OIR model, and the compound proved significantly efficacious at 100 nM. A similar efficacy trial was performed using the less specific inhibitor MAFP, which targets both $cPLA_2$ and $iPLA_2$. The two inhibitors showed similar potency in vivo, suggesting that the proangiogenic effect of retinal $cPLA_2$ outweighs the contribution of $iPLA_2$.

In light of our findings, $cPLA_2$ is implicated in the pathogenesis of OIR and, by extension, human neovascular retinopathies. Notably, it appears to exert its influence both upstream and downstream of VEGF receptor activation. Although this is an important and potentially attractive feature of $cPLA_2$, its value as a chemotherapeutic target for angiogenic conditions is limited. PLA_2 enzymatic activity yields free AA, which in turn serves as a substrate for COX enzymes and PG production. PGs confer a wide range of bioactivities, many of which are completely unrelated to angiogenesis. Thus, inhibition of either PLA_2 or COX represents a nonselective therapeutic strategy with the potential for a variety unintended side effects, a complication that has been clearly demonstrated.[60] Our current efforts are focused on identifying the specific PGs and PG receptors responsible for the induction of VEGF and the promotion of angiogenic endothelial cell activities. We believe that inhibition of these targets holds significant therapeutic promise.

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