Published in final edited form as:

Invest Ophthalmol Vis Sci. 2006 March ; 47(3): 1149–1160. doi:10.1167/iovs.05-0531.

Single Periocular Injection of Celecoxib-PLGA Microparticles Inhibits Diabetes-Induced Elevations in Retinal PGE₂, VEGF, and Vascular Leakage

Aniruddha C. Amrite¹, Surya P. Ayalasomayajula², Narayan P. S. Cheruvu¹, and Uday B. Kompella^{1,3}

¹Department of Pharmaceutical Sciences, College of Pharmacy, University of Nebraska Medical Center, Omaha, Nebraska

³Department of Ophthalmology, University of Nebraska Medical Center, Omaha, Nebraska

²Novartis Pharmaceuticals, East Hanover, New Jersey

Abstract

Purpose—To determine whether celecoxib inhibits VEGF secretion from ARPE-19 cells and to investigate further the safety and effectiveness of periocular celecoxib-poly (lactide-co-glycolide; PLGA) microparticles in inhibiting elevations in retinal PGE₂, VEGF, and blood–tissue barrier leakage at the end of 60 days in a streptozotocin diabetic rat model.

Methods—VEGF mRNA and protein expression in ARPE-19 cells was evaluated in the presence of 0 to 10 μ M celecoxib, and cytotoxicity of celecoxib on ARPE-19 and RF6A cells was evaluated over a 0- to 100- μ M concentration range. Celecoxib-PLGA microparticles were prepared by a modified solvent evaporation technique, sterilized by 25 kGy of γ -irradiation, and characterized for size, ζ potential, drug loading, and in vitro release. Normal and streptozotocin-diabetic male Sprague-Dawley rats were divided into five groups: normal, diabetic, diabetic +placebo, normal+celecoxib, and diabetic+celecoxib. Phosphate-buffered saline (PBS) containing celecoxib-PLGA microparticles, placebo PLGA microparticles, or plain PBS in one eye was injected into the posterior subconjunctival (periocular) space in rats under anesthesia. Sixty days after administration, the animals were killed, and retinal PGE₂ secretion, VEGF protein, and blood–retinal barrier leakage were estimated. Blood cell counts, blood chemistry and histology were used to assess the safety of the microparticulate system.

Results—Celecoxib (up to 25 μ M) did not cause significant cytotoxicity in ARPE-19 or RF6A cells. Nanomolar concentrations of celecoxib reduced VEGF mRNA and VEGF protein secretion. Celecoxib-PLGA microparticles (diameter: 1140 $\pm$ 15 nm), containing 14.93% $\pm$ 0.21% of celecoxib sustained in vitro drug release and in vivo drug levels in the retina for 60 days. Diabetes elevated PGE₂ secretion, VEGF protein, the vitreous-plasma protein ratio, and blood–retinal barrier leakage by 3-, 1.7-, 3.1-, and 2.7-fold, and celecoxib-PLGA microparticles significantly reduced these elevations by 40%, 50%, 40%, and 50%, respectively. Neither the placebo-treated eyes nor the contralateral eyes in celecoxib-PLGA microparticle-treated rats showed significant effects. Celecoxib-PLGA or placebo-PLGA particles had no effect on the body weight or blood sugar level of rats. The celecoxib-PLGA microparticles did not cause any changes in blood cell counts or chemistry and caused no histopathological damage to the retina or periocular tissues.

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Corresponding author: Uday B. Kompella, 985840 Nebraska Medical Center, Omaha, NE 68198-5840; ukompell@unmc.edu.

Disclosure: A.C. Amrite, None; S.P. Ayalasomayajula, Novartis Pharmaceuticals (E); N.P.S. Cheruvu, None; U.B. Kompella, None

Conclusions—Nanomolar concentrations of celecoxib can inhibit VEGF mRNA and protein expression from ARPE-19 cells. Periocular celecoxib microparticles are useful sustained drug delivery systems for inhibiting diabetes-induced elevations in PGE₂, VEGF, and blood–retinal barrier leakage. The periocular celecoxib-PLGA microparticles are safe and do not cause any damage to the retina.

Celecoxib, a potent cyclooxygenase (COX)-2 inhibitor is currently being used as an anti-inflammatory agent for the treatment of rheumatoid arthritis and osteoarthritis. It also is under investigation for the treatment of various malignant and premalignant tumors, including colorectal,^{1,2} breast,³ lung,⁴ and prostate cancer.⁵ Through inhibition of Cox-2, celecoxib can significantly reduce the levels of prostaglandins in various tissues.

Previous studies demonstrated an elevation in the PGE₂ levels in the diabetic retina.^{6,7} Increased PGE₂ levels are associated with increased breakdown of the blood–retinal barrier.^{8,9} We have shown that the elevation in PGE₂ secretion from diabetic rat retinas is a Cox-2- and not a Cox-1-mediated phenomenon.⁷ Thus, administration of Cox-2 selective inhibitors may be an effective strategy in reducing prostaglandin-induced retinal inflammation and blood–retinal barrier breakdown during diabetes.

VEGF is thought to be a major factor responsible for increased vascular leakage in the initial stages and neovascularization in the later proliferative stages of diabetic retinopathy. It has been demonstrated that the VEGF mRNA upregulation occurs within 1 to 2 weeks after induction of diabetes in rats,^{10,11} and the elevation is observed even at 6 months after diabetes in rat models.¹² In addition, vitreal VEGF is elevated in proliferative retinopathy models and in humans with proliferative diabetic retinopathy.^{13,14} It is also elevated in the aqueous of human subjects with diabetic retinopathy in both the proliferative and nonproliferative forms, and the levels in the vitreous are higher than in the aqueous.¹⁵ Thus, VEGF is an attractive pharmacological target in the treatment of diabetic retinopathy. The increased VEGF levels could be at least in part due to increased secretion of inflammatory mediators such as prostaglandins. Diabetes induces the production of inflammatory mediators via the cyclooxygenase pathway in the rat retina.¹⁰ The products of cyclooxygenase metabolism can stimulate VEGF secretion in tumor cells, cultured retinal cells, and cultured tissues.^{16–22} Many cell types in the eye including the retinal pigment epithelial cells, retinal capillary pericytes, retinal microvascular endothelial cells, Müller cells, and ganglion cells produce VEGF.²³ The RPE cells show elevation in VEGF mRNA early in the course of diabetic retinopathy.²³ In addition, Cox-2 expression is observed in the RPE cells in human diabetic subjects as well as primary cultures.²⁴ Thus, inhibition of the Cox-2 enzyme system is likely to reduce prostaglandin generation, VEGF expression and vascular leakage. In this study, we sought to assess whether celecoxib, a selective Cox-2 inhibitor can inhibit VEGF expression in cultured human RPE cells (ARPE-19).

Oral administration of celecoxib reduces VEGF mRNA and vascular leakage in rats.¹⁰ However, long-term oral administration of high doses of Cox-2 inhibitors such as celecoxib may lead to systemic toxicity.^{25–27} Alternative routes, such as the intravitreal and periocular routes, can provide significantly higher local levels of celecoxib to the retina.²⁸ The periocular routes are safer than the intravitreal route, which may lead to complications such as retinal detachment, endophthalmitis, and cataracts.²⁸ We have previously shown that the retinal availability of celecoxib is 54-fold higher with the periocular route of administration than with the systemic route.²⁹ Diabetic retinopathy is a chronic disorder, and prolonged therapy with multiple doses is required for its treatment. Multiple administrations via the periocular routes are uncomfortable for the patient and can increase the chances of complications. Therefore, a sustained drug delivery system, which could maintain the drug levels for a prolonged period, would be beneficial.

We have demonstrated that biodegradable particulate systems sustain retinal drug delivery of a corticosteroid as well as celecoxib during a 2-week study.^{30,31} In another study, we observed that microparticles and nanoparticles 200 nm, are almost completely retained in the periocular space for at least 60 days.³² Therefore, another objective of this study was to ascertain whether periocularly administered biodegradable celecoxib microparticles reduce diabetes-induced PGE₂ secretion, VEGF expression, and breakdown of the blood–retinal barrier in a rat model of early background diabetic retinopathy at the end of 60 days of diabetes.

Materials and Methods

The ARPE-19 (CRL-2302; a spontaneously arising human retinal pigment epithelial cell line; American Type Culture Collection [ATCC], Rockville, MD) and RF6A (ATCC CRL-1780; spontaneously transformed monkey choroid–retinal endothelium) cells were obtained from the ATCC and were handled in accordance with the privacy guidelines of the Declaration of Helsinki. The cell culture medium (Dulbecco's modified Eagle's medium Ham's-F-12 [DMEM]-F12 for ARPE-19 and Hanks' F12 for RF6A cells), fetal bovine serum (FBS), penicillin-streptomycin, and L-glutamine were purchased from Invitrogen-Gibco (Grand Island, NY). The cells were cultured in either cell culture flasks (T-75 and T-25 cm²) or 96-well plates obtained from BD Bioscience Labware (Franklin Lakes, NJ). An RT-PCR kit was obtained from Promega Corp. (Madison, WI), and the primers for VEGF, VEGF₁₆₅, VEGF receptor 1 (VEGF R1, Flt-1), VEGF receptor 2 (VEGF R2, KDR), and 18S rRNA were custom synthesized (DNA Core Facility, University of Nebraska Medical Center, Omaha, NE). A reverse transcriptase kit (*Taq-Man*) and universal PCR kit (SYBR Green) were purchased from Applied Biosystems, Inc. (ABI; Foster City, CA). Celecoxib was purchased from ChemPacific (Baltimore, MD). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; triazolyl blue), 4.4-kDa FITC-dextran, and polyvinyl alcohol (PVA) were obtained from Sigma-Aldrich (St. Louis, MO). PLGA(85/15) of intrinsic viscosity 0.67 dL/g was obtained from Birmingham Polymers, Inc. (Birmingham, AL). The HPLC grade methylene chloride and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA). An ELISA kit for human VEGF was obtained from Research Diagnostics, Inc. (Flanders, NJ), for rat VEGF from R&D Systems (Minneapolis, MN), and for PGE₂ from Cayman Chemicals (Ann Arbor, MI).

Cell Culture

ARPE-19 and RF6A cells were cultured in DMEM-F12 medium containing 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine. The medium was changed every 2 days, and all studies were conducted with day-8 cells of passages 20 to 30 for ARPE-19 and passages 35 to 45 for RF6A.

VEGF Secretion Studies

VEGF secretion studies in ARPE-19 cells were performed as previously described.³³ Briefly on day 8, after 12 hours of quiescence, the cell monolayers were incubated with celecoxib (0–10 μ M) for 12 hours. The culture medium was collected at 12 hours and secreted VEGF was quantified by an ELISA method using a kit purchased from Research Diagnostics Inc. The kit is capable of detecting the secreted human VEGF isoforms VEGF₁₆₅ and VEGF₁₂₁. The secreted VEGF levels were normalized to the total protein content in the cell lysates. The kit has no cross-reactivity with recombinant human VEGF-B₁₉₆.

RT-PCR and Real-Time RT-PCR

To determine whether celecoxib reduces VEGF mRNA levels in ARPE-19 cells, semiquantitative RT-PCR was used as previously described.³³ Briefly, after an initial

quiescence period of 12 hours in serum-free medium, confluent ARPE-19 cells, in T-25 flasks, were treated with celecoxib (0–10 μ M). After the 12-hour treatment, total RNA was isolated with a commercial kit (RNA Stat-60; Tel-Test, Friends-wood, TX). Total RNA (3 μ g) was used to amplify VEGF mRNA. The primers used can detect all the splice variants of VEGF. The products were separated by electrophoresis on a 2% agarose gel, and VEGF₁₆₅ (584 bp) and VEGF₁₂₁ (452 bp) mRNAs were identified and quantified with densitometric analysis (Nucleovision Imaging System; Nucleotech, San Mateo, CA). The VEGF mRNA expression was normalized to the expression of 18S rRNA, an internal control.

For real-time relative quantitative RT-PCR, isoform-specific primers for VEGF₁₆₅ were used.³⁴ RNA (1 μ g), isolated as just described, was used in the RT reaction and converted to cDNA using with the reverse transcriptase kit (*TaqMan*; ABI). cDNA equivalent to 200 μ g RNA was then used in the real-time PCR reaction (SYBR Green Universal PCR Master Mix and Prism 7700 Sequence Detection System; ABI). The VEGF₁₆₅ signal was normalized to that of 18s rRNA. The $2^{-\Delta\Delta C_t}$ method was used to calculate the change (*x*-fold) in gene expression with no treatment (0 μ M celecoxib) taken as the control.³⁵ In addition to VEGF mRNA expression, mRNA expressions of VEGF-receptors 1 and 2 were assessed in ARPE-19 cells using real-time PCR with the primer sequences described previously.³⁶

MTT Assay

The cytotoxicity of celecoxib in ARPE-19 and RF6A cells was assessed by incubating the day-8 cell monolayers with 0 to 100 μ M celecoxib for 12 hours after quiescence. The cell viability was assessed with a previously described colorimetric MTT assay.³³

Preparation of Celecoxib-PLGA and Placebo PLGA Microparticles

Celecoxib-PLGA microparticles were prepared using a previously described modified emulsion solvent evaporation method.³¹

Drug Loading

The loading efficiency of celecoxib in the polymeric particles was determined by extracting and quantifying the encapsulated celecoxib. Briefly, celecoxib-polymeric particles (2 mg) were placed in a glass vial, and 4 mL methylene chloride was added and mixed thoroughly at room temperature for 24 hours. The resultant extract was evaporated to dryness under nitrogen, and the dried residue was reconstituted with 1000 μ L acetonitrile-water mixture (70:30). This reconstituted solution was vortexed for 1 minute and centrifuged at 12,000*g* for 5 minutes, and 100 μ L of the supernatant was injected into the HPLC column. Celecoxib was quantified with an HPLC method described previously.^{7,31}

Sterilization of Particles

The celecoxib-PLGA microparticles and the placebo PLGA microparticles were sterilized by γ -irradiation. The particles were kept in polypropylene tubes and irradiated in air at a rate of 230 Gy/min using a Picker V 90 ⁶⁰Co source (Picker International, Inc., Cleveland, OH) for a total dose of 25 kGy.³⁷ Dry ice was used to prevent any increase in temperature during the procedure.

In Vitro Drug Release from Celecoxib-PLGA Particles

The in vitro release of celecoxib from the PLGA particles was performed at 37°C with dialysis membrane bags (molecular weight cutoff: 10,000; Spectrum Laboratory, Gardena, CA), as described previously.³⁰ Briefly, a 0.5-mL suspension of either plain celecoxib (20 μ g) or celecoxib-PLGA particles containing 20 μ g celecoxib was taken into the dialysis bag,

and the unit was allowed to float in 50 mL of release medium (PBS [pH 7.4] containing 0.025% sodium azide as a preservative). At discrete time intervals, 1 mL of the release medium was removed and replaced with fresh release medium. The released celecoxib was analyzed by HPLC assay. The release between sterilized and nonsterilized celecoxib-PLGA particles was also compared.

Particle Size and ζ -Potential Measurement

The particle size and the ζ potential were measured with a ζ -potential analyzer (Zeta Plus; Brookhaven Instruments Ltd., New York, NY) that employs the dynamic light-scattering technique for particle size measurement. The software calculates the effective diameter based on the Stokes-Einstein equation as a diameter of an equivalent hydrodynamic sphere from *z*-average diffusion coefficients. The particle size measurements were performed after dilution in PBS.

Subconjunctival Administration

All animal studies were performed in male Sprague-Dawley rats weighing 150 to 180 g. The animals were divided into five groups: (1) normal rats that did not have diabetes and did not receive any microparticles; (2) diabetic rats that had diabetes but received no microparticles; (3) diabetic+placebo-treated rats that had diabetes and were injected with placebo PLGA microparticles; (4) normal+celecoxib-treated rats that did not have diabetes and received celecoxib-PLGA microparticles; and (5) diabetic+celecoxib-treated rats that were diabetic and received celecoxib-PLGA microparticles. Diabetes was induced by intraperitoneal injection of streptozotocin (60 mg/kg). The blood sugar level was assessed 24 hours after administration of streptozotocin, and the animals with blood sugar level above 250 mg/dL were deemed diabetic. One day after streptozotocin injection, the animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg). After this, 50 μ L of sterile suspension of celecoxib-PLGA microparticles (equivalent to 750 μ g celecoxib), placebo-PLGA microparticles (amount equivalent to celecoxib-PLGA microparticles), or plain PBS (pH 7.4) was injected into the posterior subconjunctival space of one eye (ipsilateral) using a 27-gauge needle. The normal+celecoxib microparticle group animals received microparticles equivalent to 750 μ g of celecoxib on the same day as the diabetic animals received the microparticles. The animals were killed on day 60 after subconjunctival administration. Just before death, the blood sugar levels were tested again to confirm diabetic status. All the animal experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

PGE₂ Secretion from Isolated Rat Retinas

On the 60th day after subconjunctival injection, the rats were euthanatized with pentobarbital sodium (250 mg/kg). The eyes were enucleated, and the retina was isolated immediately. The retinas were weighed and placed in 2-mL centrifuge tubes. PBS (200 μ L) was added, and the retinas were incubated at 37°C in a metabolic incubator for 2 hours. After 2 hours, the tubes were centrifuged at 7000g, and the supernatant was used for PGE₂ estimation with an ELISA kit according to the manufacturer's recommendations (Cayman Chemicals). The secreted PGE₂ levels were normalized to the wet retina weight.

Retinal VEGF Protein

The retinas were isolated and homogenized in 200 μ L of PBS. The tubes were centrifuged at 7000g for 10 minutes (Marathon MicroA centrifuge; Fisher Scientific), and the supernatant was used for the estimation of VEGF (VEGF ELISA; R&D Systems; Minneapolis, MN). This kit detects the 164 and 120 isoforms of VEGF in the rats. The VEGF levels were normalized to the protein content measured using a protein estimation kit (Pierce; Rockford,

IL). The kit has no cross-reactivity with recombinant mouse VEGF-B₁₉₆ and 73.4% and 90.8% cross-reactivity with recombinant mouse VEGF₁₆₄ and VEGF₁₂₀, respectively.

Blood–Retinal Barrier Leakage

Vitreous-Plasma Protein Ratio—For the vitreous-to-plasma protein ratio, just before death, 1 mL of blood was withdrawn and placed into centrifuge tubes containing EDTA. The blood was centrifuged at 13,000g to separate the plasma. The supernatant was collected, diluted appropriately, and used for the plasma protein estimation. For the vitreal protein estimation, the eyes were enucleated after death, and the vitreous was isolated. The contents of the vitreous were centrifuged at 13,000g for 10 minutes, and the supernatant was diluted appropriately and used for protein estimation. The protein estimations were performed with the protein assay kit.

FITC-Dextran Leakage—The blood–retinal barrier leakage was determined with an FITC-dextran leakage assay, as previously described, with some modifications.³⁸ Briefly, after induction of deep anesthesia, the animals were injected intravenously with FITC-dextran (4.4 kDa, 50 mg/mL in PBS, 50 mg/kg body weight). After 10 minutes, the chest cavity was opened, and the animals were perfused with PBS (500 mL/kg body weight). Blood samples were collected immediately before perfusion. Immediately after perfusion, the retinas were dissected and homogenized, and the FITC-dextran was extracted with 750 μ L of water. The extract was centrifuged at 7000 rpm for 10 minutes, and the supernatant (500 μ L) was used to measure the fluorescence. Corrections for the blank were made by subtracting the fluorescence obtained from eyes of rats not injected with FITC-dextran. The amount of FITC-dextran in the samples was quantified with a standard curve of FITC-dextran in water. The amount of FITC-dextran in the retina was normalized to the retinal weight and to the plasma concentration of FITC-dextran. The blood–retinal barrier breakdown was calculated by using the following formula, as previously described.³⁸

$$\frac{\text{Retinal FITC – dextran } (\mu\text{g})/\text{retinal weight (g)}}{\text{Plasma FITC – dextran } (\mu\text{g}/\mu\text{L}) \times \text{circulation time (min)}}$$

Drug Level Estimation in Ocular Tissues

The celecoxib levels in plasma and in various ocular tissues, including the sclera-choroid, retina, vitreous, and cornea, were measured at 2 months after administration of celecoxib-PLGA microparticles in diabetic rats, by using liquid–liquid extraction followed by an HPLC method described elsewhere.³¹

Safety of Periocular Microparticles

The safety of periocular microparticles was assessed by visual inspection, histology, and blood chemistry and blood count analysis between the normal rats injected with vehicle (PBS) and normal rats that were injected with celecoxib-PLGA microparticles. The visual inspection of the periocular tissue was performed to determine any overt inflammation of the tissue, including edema in the periocular area.

For the histologic analysis, the rats were deeply anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg) by intraperitoneal injection, the heart was exposed, and the rats were perfused with 500 mL/Kg of 4% paraformaldehyde solution. The eyes were enucleated, and the conjunctiva and periocular tissues were removed and immersed in 10% formalin for 24 hours. After rehydration, the tissues were embedded in paraffin blocks and 7- μ m-thick sections were cut. The sections were stained with H&E or Masson trichrome stain and

viewed with a microscope (Axioscope 40; Carl Zeiss Meditec, Inc., Dublin, CA). The images were taken with a digital camera (HV-D25 using EPIX-XCAP lite V2.2 for Windows; Hitachi, Tokyo, Japan).

For the blood metabolic parameter assessment, the animals were anesthetized with ketamine-xylazine, and blood was collected from the left ventricle into heparinized tubes and submitted to the clinical laboratory at the University of Nebraska Medical Center for the analysis of blood chemistry and blood cell counts.

Morphometric Analysis of the Retina

Morphometric analysis was performed on the H&E-stained retinal sections prepared as above. Every third section containing the optic nerve was used in the analysis, for a total of four sections per animal. The sections were photographed at a total magnification of 50 $\times$. The morphometric analysis was performed on the retina from 0.5 to 1.5 mm superior and inferior to the optic nerve. Ten measurements for the superior retina and 10 measurements for the inferior retina with a total of 20 measurements per section were made. The readings from all four sections for each animal were averaged to get an average retinal thickness for that animal. The thickness of the neural retina (from photoreceptor layer to ganglion cell layer), outer nuclear layer (ONL), and inner nuclear layer (INL) were compared between normal rats and normal rats that were given celecoxib-PLGA microparticles. Morphometric software (Image Pro Plus; Silver Springs, MD) was used.

Data Analysis

Unless otherwise stated, all data are expressed as mean $\pm$ SD. The in vitro and in vivo comparisons between the five groups were made by one-way ANOVA followed by the Tukey post hoc analysis. The comparisons between the ipsilateral and contralateral eyes were made by a dependent-samples *t*-test. The results were considered statistically significant at $P < 0.05$. The data were analyzed on computer (SPSS 11.5 for windows; SPSS Inc., Chicago, IL).

Results

Cytotoxicity of Celecoxib on ARPE-19 and RF6A Cells

Celecoxib did not decrease the viability of ARPE-19 and RF6A cells up to 25- μ M concentration when compared with the control (Fig. 1). The cell viability decreased by 63% and 64% for ARPE-19 cells and by 75% and 74% for RF6A cells with 50 and 100 μ M celecoxib, respectively.

Effect of Celecoxib on VEGF and VEGF Receptor mRNA Expression and VEGF Secretion in ARPE-19 Cells

There was a statistically significant inhibition of the VEGF mRNA expression in ARPE-19 cells with 10 nM celecoxib (Fig. 2B). With celecoxib concentrations of 0.01, 0.1, 1, and 10 μ M, there was a 15% $\pm$ 6%, 20.5% $\pm$ 5%, 25.1% $\pm$ 3%, and 40.3% $\pm$ 2% reduction, respectively, in normalized VEGF₁₆₅ mRNA and a 21.2% $\pm$ 2%, 28% $\pm$ 2%, 34.6% $\pm$ 1%, and 48% $\pm$ 1% reduction in the VEGF₁₂₁ mRNA, respectively. For VEGF₁₆₅ mRNA, the data was confirmed with a real-time PCR study. There was a statistically significant dose-dependent decrease in VEGF₁₆₅ mRNA in ARPE-19 cells with celecoxib concentrations in the range of 0.1 to 10 μ M with 32% $\pm$ 19%, 67% $\pm$ 5%, and 91% $\pm$ 8% reduction in VEGF mRNA with 0.1, 1, and 10 μ M celecoxib, respectively (Fig. 2C). VEGF receptor mRNA studies using ARPE-19 cells revealed that there is no statistically significant difference in the VEGF R2 mRNA expression in ARPE-19 cells incubated with 0.1 to 10 μ M celecoxib

(data not shown). VEGF R1 signal could not be quantified in the ARPE-19 cells by real-time PCR, probably because of very low levels of expression.

In terms of the VEGF protein, the protein-normalized VEGF secretion was reduced by $30.45\% \pm 12.95\%$, $38.56\% \pm 11.54\%$, and $44.28\% \pm 15.08\%$, and $45.18\% \pm 13.83\%$ with 0.01, 0.1, 1, and 10 μM celecoxib, respectively (Fig. 3).

Particle Size, ζ Potential, and Drug Encapsulation Efficiency of Celecoxib PLGA Particles

The mean particle size of the celecoxib-PLGA particles obtained by dynamic light scattering was $1.11 \pm 0.08 \mu\text{m}$. The ζ potential was $-38 \pm 0.56 \text{ mV}$. The celecoxib loading into the microparticles was $14.93\% \pm 0.12\%$, with a loading efficiency of $51.48\% \pm 0.42\%$.

In Vitro Drug Release from Celecoxib-PLGA Microparticles

The celecoxib microparticles released the drug in a biphasic manner over a period of 60 days. There was an initial burst release lasting for 3 days followed by a sustained release of celecoxib over the next 60 days. Approximately 50% of the entrapped drug was released in vitro at the end of 60 days (Fig. 4). With the plain drug suspension, 100% celecoxib was released in vitro in 7 days with an average release rate of $2.65 \mu\text{g/d}$ or $13.25\%/d$ (Fig. 4, inset). In contrast, celecoxib-PLGA microparticles sustained drug levels during the 9-week in vitro release study with an average postburst release rate of $0.07 \mu\text{g/d}$ or $0.35\%/d$. The release rate was thus ~38-fold lower for the microparticles than with the suspension dosage form. Thus, the polymer matrix plays a role in slowing the drug release rate, much beyond what is feasible based on the low aqueous solubility ($3\text{--}7 \mu\text{g/mL}$) of celecoxib.

Effect of Sterilization with γ -Irradiation on Drug Release from Celecoxib-PLGA Microparticles

The sterilization process did not affect the release of celecoxib from the celecoxib-PLGA microparticles. The release was slightly lower at a few intermediate time points with the unsterilized microparticles. However, the differences were not statistically significant (Fig. 4).

Effect of Diabetes and Microparticles on Body Weight and Blood Sugar Levels of Rats

With the induction of diabetes, there was a significantly lower weight gain in the diabetic rats than in the normal rats. The weight gain was 71% at the end of 60 days in the normal rats and was reduced to a 7% weight gain at the end of 60 days in the diabetic rats. The treatment with celecoxib microparticles or placebo microparticles did not affect the weight gain. The blood sugar levels were significantly higher in the diabetic, diabetic+celecoxib, and diabetic+placebo groups than in the normal or normal+celecoxib groups (Table 1).

Effect of Celecoxib Microparticles on Retinal PGE_2 Secretion

The PGE_2 secretion in normal rat retinas isolated from the rats after 60 days was $6.9 \pm 0.8 \text{ pg/mg tissue}$ (Fig. 5A). Diabetes increased the PGE_2 secretion to $21.3 \pm 2.3 \text{ pg/mg tissue}$. The placebo particles had no effect on this elevated PGE_2 secretion. In the diabetic animals that received celecoxib-PLGA microparticles, the PGE_2 secretion was $12.2 \pm 1 \text{ pg/mg}$ in the ipsilateral eye, which was significantly lower than the diabetic or diabetic+placebo groups. The levels in the ipsilateral eye in the animals that received the celecoxib microparticles were significantly lower than the levels in the contralateral eyes. The celecoxib microparticles did not affect the PGE_2 levels in the normal rats.

Effect of Celecoxib-PLGA Microparticles on Retinal VEGF

With the induction of diabetes, the retinal VEGF levels were elevated from 314 ± 66 to 515 ± 102 pg/mg protein (Fig. 5B). The VEGF levels in the diabetic+celecoxib microparticles in the ipsilateral eye were reduced by 40% to 287 ± 92 pg/mg protein. The VEGF levels in the contralateral eye were higher (431 ± 174 pg/mg protein) and the difference was marginally significant ($P=0.053$). The levels in the ipsilateral eye with diabetic+celecoxib microparticles were significantly different from those in the diabetic+placebo group (545 ± 81 pg/mg protein). With the diabetic+celecoxib group, the VEGF levels in the ipsilateral eye were marginally significantly lower than those in the contralateral eye ($P=0.053$).

Effect of Celecoxib-PLGA Microparticles on Blood–Retinal Barrier Leakage

With 60 days of diabetes, there was a greater than threefold elevation in the vitreous-plasma protein ratio from 0.35 ± 0.07 to 1.17 ± 0.21 (Fig. 5C). There was a ~40% inhibition in the elevated ratio with the celecoxib microparticles (0.67 ± 0.08). The placebo microparticles did not have any effect. There was no difference in the vitreous/plasma protein ratio between normal and normal+celecoxib groups. The blood–retinal barrier leakage assessed using 4.4-kDa FITC-dextran indicated an increase in leakage from 12.9 ± 2.1 to 34.8 ± 4.4 $\mu\text{L/g}$ per minute of plasma (Fig. 5D). In the diabetic+placebo group the barrier breakdown was similar to the diabetic group with the leakage being 28.7 ± 6.9 $\mu\text{L/g}$ per minute. There was a ~50% inhibition of the vascular leakage in the ipsilateral eyes of the celecoxib-PLGA microparticle-treated group, but there was no significant decrease in the contralateral eyes.

Drug Levels in the Ocular Tissues

At the end of 60 days, celecoxib could be quantified in the diabetic, treated eye tissues including sclera-choroid, retina, vitreous, and the cornea. The drug levels in the ipsilateral eye were 0.66 ± 0.33 , 0.79 ± 0.34 , 0.23 ± 0.1 , and 3.68 ± 3.01 ng/mg tissue, respectively, in the sclera-choroid, retina, vitreous, and the cornea. The total tissue drug levels in the retina correspond to approximately 2 μM celecoxib. The celecoxib levels were below quantification limits in the plasma and the contralateral eye tissues.

Safety of Periocular Microparticles

The visual inspection of the periocular tissue (site of injection) did not reveal the presence of any inflammation, including redness and edema for the rats that were injected with celecoxib-PLGA microparticles.

The retina and the choroid appear normal in the histologic sections without any cellular infiltration, inflammation or atrophy of the retina (Fig. 6). The Masson trichrome staining of the periocular tissue including the conjunctiva revealed no difference in collagen staining pattern between the control rats and rats that were injected with periocular celecoxib-PLGA micro-particles (Fig. 6).

There was no difference in the blood cell counts and the blood chemistry profiles of the rats that were injected with the celecoxib-PLGA microparticles and the control rats (Table 2).

The morphometric analysis of the retina revealed no significant differences in the thickness of the neural retina, ONL, or INL between the celecoxib-PLGA microparticle group and the control group indicating no degeneration or atrophy of the retinal tissue (Fig. 7). The mean thickness of the neural retina, ONL, and INL, respectively, were 304 ± 12 , 67 ± 4 , and 35 ± 3 μm in the control group and 302 ± 5 , 67 ± 2 , and 35 ± 2 μm in the celecoxib microparticle group.

Discussion

This study for the first time has demonstrated the safety and effectiveness of a periocular microparticulate delivery system of celecoxib in inhibiting diabetes-induced blood–retinal barrier leakage. There has been a revived interest in the use of anti-inflammatory drugs for the prevention and treatment of diabetic retinopathy based on the findings about the role of inflammation in diabetic retinopathy.^{39,40} In addition, anti-inflammatory drugs have been shown to have beneficial effects in rat and dog models of diabetic retinopathy.^{41,42} Even though earlier studies have demonstrated the effectiveness of systemic high-dose Cox-2 inhibitors in the suppression of early diabetic retinopathy in rat models,^{10,41} this study has demonstrated that effective inhibition of blood–retinal barrier leakage can be achieved with a periocular delivery system over a 60-day period at a much lower dose than that used previously. This study also demonstrated the effectiveness of celecoxib in inhibiting diabetes-induced retinal PGE₂ secretion and VEGF protein expression. Periocular microspheres have been shown to be effective delivery systems in choroidal neovascularization in a porcine model.⁴³ The duration of this study was much longer (60 days) than in the porcine study (20 days) with choroidal neovascularization, indicating that microparticles can be designed to sustain delivery to the retina and achieve therapeutic efficacy for prolonged periods.

The blood–retinal barrier breakdown is a major cause of macular edema during the progression of diabetic retinopathy.⁴⁴ Macular edema is associated with a loss of visual acuity.⁴⁴ The retinas of human as well as diabetic rats show similarities in the early pathophysiological changes of diabetic retinopathy.^{45–47} Blood–retinal barrier leakage is thus common in the human disease and the streptozotocin rat model. Current therapies targeted toward macular edema, a consequence of the breakdown of the blood–retinal barrier, have not been satisfactory. Hence, there is a need to look for alternative therapies. The evaluation of blood–retinal barrier breakdown can be achieved through several experimental techniques. These techniques include the isotope-dilution method, the Evans blue assay, and measurement of extravasation of FITC-albumin.⁴⁸ In addition, the measurement of the ratio of vitreal endogenous protein concentration to plasma protein concentration can be used to measure the extent of blood–retinal barrier breakdown.⁴⁹ The information obtained about blood–retinal barrier breakdown can differ, based on the technique used.⁴⁸ We have used two methods in the assessment of blood–retinal barrier breakdown: the vitreous/plasma protein ratio and the leakage of 4.4-kDa FITC-dextran. FITC-dextran has been used in the assessment of the molecular size of the retinal vascular leakage in patients with posterior uveitis⁵⁰ and in animal models of experimental choroidal neovascularization.⁵¹ In their study, Atkinson et al.⁵⁰ report that the 4.4-kDa dextran did not penetrate the healthy blood–retinal barrier but leaked out from areas of macular edema, new retinal vessels, and swollen optic discs. This method has been used in previous studies with normal and streptozotocin diabetic rats^{38,52,53} and has been shown to have good sensitivity and quantitative predictability comparable to the Evans blue assay.³⁸ Our results indicate that there is a breakdown of the blood–retinal barrier, evidenced by both the methods used. The extent of blood–retinal barrier breakdown is threefold and 2.7-fold, respectively, based on the vitreous-plasma protein ratio and the dextran leakage assay. The slightly higher blood–ocular barrier breakdown indicated by the vitreous-plasma protein ratio could be because this method measures the protein concentration in the vitreous and not the retina, possibly accounting leakage from and blood–aqueous barriers. A limitation of using the vitreous to plasma protein ratio to indicate vascular permeability is highlighted by the observation that significant increases in vitreous protein have been observed in 1-month streptozotocin diabetic rats without any discernible eye disease.⁵⁴ The FITC-dextran leakage measurement does not have this drawback. This study demonstrated the efficacy of the sustained-release celecoxib-PLGA microparticulate system in inhibiting vascular leakage,

using both the methods mentioned herein, with the percentage of inhibition being 40% and 50%, respectively, for vitreous-plasma protein ratio and FITC-dextran leakage for assessment of barrier breakdown.

Although earlier studies have demonstrated that celecoxib is distributed in the eye after oral administration^{10,55} and have also demonstrated the efficacy of celecoxib in inhibiting diabetes-induced blood-retinal barrier leakage,¹⁰ the doses required to achieve therapeutic concentrations in the retina were very high. This is especially important because the use of selective Cox-2 inhibitors has been a recent matter of controversy due to their cardiovascular side effects.²⁷ We have demonstrated previously that the dose-normalized AUC of celecoxib in the retina is 54-fold higher after periocular administration than after systemic administration.²⁹ Thus, peri-ocular delivery of celecoxib can provide higher retinal levels with a concurrent reduction in the plasma levels. The dose of celecoxib used in this study is low (750 $\mu\text{g}/\text{rat}$) and also involves only a one-time administration of the celecoxib-PLGA microparticulate delivery system. At this dose, with approximately 50% of drug expected to be released in 60 days, the measured concentration of celecoxib in the retina of the treated eye 60 days after administration is approximately 2 μM , which, even if largely bound to tissue, is likely to achieve therapeutic concentrations based on its ability to inhibit VEGF secretion and Cox-2 at nanomolar concentrations.

VEGF, an endothelium-specific mitogen, is known to play an important role in the pathogenesis of several retinal disorders, including diabetic retinopathy. Preclinical and clinical studies have demonstrated that VEGF is an important factor in the pathologic course of both background and proliferative diabetic retinopathy.^{15,56–58} Because VEGF is elevated in diabetic retinopathy, one purpose of this study was to assess the efficacy of celecoxib in inhibiting VEGF secretion from the retina. We investigated the effect of celecoxib on VEGF secretion in ARPE-19 cells, a human RPE cell line, in which we have demonstrated VEGF expression.⁵⁹ The RPE cells form the outer barrier and constitutively express VEGF.⁶⁰ Celecoxib at nano-molar concentrations inhibited the VEGF protein secretion (Fig. 3) as well as the VEGF mRNA expression (Fig. 2) in ARPE-19 cells. The inhibition of VEGF was at concentrations similar to the median inhibitory concentration IC_{50} of celecoxib in inhibiting Cox-2 (40–90 nM).^{61,62} The inhibition of VEGF mRNA and protein secretion was not due to a cytotoxic effect, because celecoxib had no effect on cell viability up to a concentration of 25 μM (Fig. 1). Cox-2 enzyme products like PGE_2 are known to stabilize HIF1 α ,⁶³ and translocate it to the nucleus. HIF1 α acts as a potent transcription factor for several genes, including VEGF.⁶⁴ Thus, at least in part, the inhibition of VEGF mRNA and protein secretion could be accounted for by the inhibition of Cox-2.

In our in vitro cytotoxicity studies, we observed no cytotoxicity up to 25- μM concentrations of celecoxib in both ARPE-19 and RF-6A cells. There was a dramatic increase in cytotoxicity at 50 μM and no difference between 50- and 100- μM concentrations. This cytotoxic effect is probably compound-solubility limited. The reported solubility of celecoxib in water at 37°C and pH 7.0 ranges from 3 to 7 $\mu\text{g}/\text{mL}$.^{65,66} Because the pKa of celecoxib is 11.2, a similar solubility is expected in physiological buffers at this temperature. This corresponds to a celecoxib concentration of 8 to 18 μM . In our studies, the solutions of celecoxib were made in ethanol at a concentration of 10 mM and then diluted to the required concentrations, which led to a maximum of 1% ethanol in the solution prepared. Cosolvents can increase the solubility of poorly soluble organic compounds. Indeed, celecoxib solubility has been shown to be increased by the cosolvents ethanol, glycerol and dimethyl sulfoxide (DMSO) up to 25 to 50 μM celecoxib.⁶⁷ Around this concentration range, celecoxib may precipitate out of solution. This could be the reason for the observed lack of increase in cytotoxicity at 100 μM compared with 50 μM .

In agreement with our in vitro studies using celecoxib, we found that the microparticulate system of celecoxib used in this study was effective in reducing retinal VEGF protein levels. The reduction of the blood–retinal barrier breakdown can be partly explained by the reduction in the retinal VEGF expression. Several lines of evidence suggest an important role for VEGF in the breakdown of the blood–retinal barrier.^{15,57,68} It is a potent vasopermeabilizing agent,^{69–71} with efficacy 4 to 5 orders of magnitude higher than histamine.⁷² How hyperglycemia leads to increased VEGF is still open to investigation. There is some evidence of direct elevation of VEGF by hyperglycemia in culture systems. However, the major stimuli are considered to be inflammatory cytokines and oxidative stress that is generated due to the hyperglycemia. Our present and previous studies demonstrate that the celecoxib or celecoxib-PLGA microparticulate systems do not have an effect on the hyperglycemia or body weights in rats.¹⁰ Thus, reduction in VEGF is an outcome of other mechanisms. These could involve a reduction in the production of inflammatory prostaglandins, which is a direct consequence of the Cox-2 inhibitory effect of celecoxib. PGE₂, a major prostaglandin produced by the Cox enzyme is known to stimulate VEGF in several cancer cells as well as in certain normal cells, including the retina.^{16–22} In addition, PGE₂ has a direct effect on vascular permeability and can cause a breakdown of the blood–retinal barrier in rats and rabbits.^{8,73,74} In addition, studies from our laboratory have demonstrated that celecoxib can cause a reduction in diabetes-induced retinal oxidative stress.³¹ Thus, multiple pathways could be involved in the inhibition of VEGF by celecoxib. A recent important discovery of antiangiogenic isoforms of VEGF has brought to light a reason for possible failure of long-term anti-VEGF therapies.⁷⁵ It is hypothesized that these antiangiogenic isoforms of VEGF may be inhibited by anti-VEGF therapies.^{75–77} If celecoxib inhibits antiangiogenic isoforms of VEGF, it could be a potential limitation of this drug. However, the present study demonstrated a 60% inhibition in PGE₂, a 40% inhibition in VEGF, and a 40% to 50% inhibition of vascular leakage. Thus, the inhibition of vascular leakage might be due to the inhibition of VEGF and other possible pathways, including the direct vasopermeability effect of PGE₂. Our studies to date suggest that, in diabetic conditions, the Cox-2 expression and activity, oxidative stress, VEGF expression, and vascular leakage are elevated (Fig. 8) and that all these parameters except Cox-2 expression can be reduced by celecoxib, a Cox-2 inhibitor.

In the present study, we observed that there was no reduction in elevated PGE₂ and VEGF or in the blood–retinal barrier leakage in the contralateral eye. These findings are consistent with our previous observations, which showed the contralateral ocular tissue levels of celecoxib to be undetectable after administration of celecoxib PLGA microparticles.³¹ In previous pharmacokinetic studies, we have demonstrated that the plasma-normalized AUC in the retina is more than 50-fold higher in the ipsilateral eye than in the contralateral eye.²⁹ The concentrations of celecoxib in the contralateral eye were probably subtherapeutic.

Safety is an important concern with any new dosage form. The safety of periocular microparticles of biodegradable polymers has not been extensively investigated. Saishin et al.⁴³ observed that microparticles of PLGA-glucose containing PKC412, a protein kinase C inhibitor, or blank PLGA-glucose microparticles did not cause any discernible signs of infection or inflammation in the conjunctiva 20 days after periocular administration of the microspheres in pigs. Kimura et al.,⁷⁸ have evaluated the effects of periocular PLA microparticles on retina and surrounding tissues in a rabbit model. At 1 week after administration, they observed a few inflammatory cells and some fibrous tissue at the site of administration. At the injection site, the microspheres were degraded and phagocytosed by several multinucleated giant cells. The inflammatory reaction lasted for approximately 2 weeks, and at 12 weeks after administration, there were pieces of microparticles at the site of administration along with few inflammatory cells. However, the retina and the ciliary body were found to be normal on histopathologic examination. We observed no inflammation in

the retina at 60 days after administration of the celecoxib-PLGA microparticles, indicating that the approach is safe without any cytotoxic damage to the retina. This was confirmed by our morphometric analyses, which showed no differences in the thickness of the retinal layers between untreated normal rats and normal rats injected with celecoxib microparticles. We have reported that, at 2 months after periocular injection in rats, nonbiodegradable polystyrene microparticles (2 μm) and nanoparticles (200 nm) are almost completely retained at the site of administration, whereas 20-nm particles disappear completely.³² With the polystyrene particles, we observed a few inflammatory cells in the periocular tissue, but not in the retina or other ocular tissues at the end of 2 months.³² However, with the biodegradable microparticles used in the present study, we did not observe any inflammatory cells or fibrous tissue response in the periocular tissue at the site of injection. Although no quantitative comparison of the inflammatory response can be made between the polystyrene and PLGA particles in these studies, the inflammatory response appears less for the PLGA particles, possibly due to the biodegradable nature of PLGA. Also, we observed that polystyrene particles have a greater tendency to aggregate in vivo, potentially influencing their clearance and inflammatory response. Further, celecoxib-PLGA particles did not induce systemic toxicity, as evidenced by no changes in blood cell counts or the clinical chemistry parameters in the plasma (Table 2).

This study demonstrated that a microparticulate system of celecoxib can be used periocularly for therapy in early background diabetic retinopathy. Potentially, such particulate systems can be used for the therapy of other disorders of the posterior segment. Even with the present controversy, celecoxib could be beneficial in the treatment of diabetic retinopathy if we use local routes to minimize plasma concentrations. The efficacy, however, must be shown on a long-term basis, with no toxicity. Future studies thus should be designed to evaluate the efficacy of celecoxib in the actual lesions of diabetic retinopathy (pericyte loss, microaneurysms) and to investigate its use in proliferative diabetic retinopathy. This involves administration of celecoxib after the disease has set in and not the chemopreventive approach used in the present study.

Conclusions

Celecoxib can effectively inhibit VEGF mRNA and protein secretion in RPE cells. A sustained drug delivery system of celecoxib, in vivo, has beneficial effects in a rat model of early background diabetic retinopathy without any overt toxicity. Celecoxib microparticles may have potential as a locally administered preventive measure to delay the development or progression of the early pathophysiological changes in the retina as a result of diabetes.

Acknowledgments

The authors thank Devender S. Dhanda for assistance with the preparation of celecoxib-PLGA microparticles and some of the in vivo studies, and Tom Bargar at the UNMC electron microscope core facility for help with the morphometric analysis of the retina.

Supported by National Institute of Digestive and Kidney Diseases Grant DK064172 and National Eye Institute Grant EY013842. ACA and NPSC are recipients of graduate fellowships from the University of Nebraska Medical Center.

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Figure 1.

Celecoxib is not toxic to ARPE-19 and RF6A cells up to a concentration of 25 μM . After a 12-hour quiescence period, ARPE-19 cells and RF6A cells were exposed to celecoxib ranging in concentration from 1 nM to 100 μM . Data are expressed as the mean $\pm$ SD ($n = 8$). *Significantly different from the control.

Figure 2.

Celecoxib inhibits VEGF mRNA expression in ARPE-19 cells. After a 12-hour quiescence period, ARPE-19 cells were exposed to celecoxib ranging in concentration from 1 nM to 10 μM. (A) Agarose gel electrophoresis of RT-PCR products for VEGF₁₆₅, VEGF₁₂₁, and 18S rRNA. (B) The densitometric band intensities normalized to 18S rRNA. Data are expressed as the mean ± SD of results in three experiments. Significantly different from the control for *VEGF₁₆₅ or †VEGF₁₂₁. (C) Real-time quantitative RT-PCR for VEGF₁₆₅. Data are expressed as the mean ± SD of results in four experiments. ‡Significantly different from the control ($P < 0.05$).

Figure 3. Celecoxib inhibits VEGF secretion from ARPE-19 cells. After a 12-hour quiescence period, ARPE-19 cells were exposed to celecoxib ranging in concentration from 100 pM to 10 μ M. Secreted VEGF was measured at the end of 12 hours of treatment. Data are expressed as the mean $\pm$ SD of results in eight experiments. *Significantly different from the control.

Figure 4.

In vitro release of celecoxib from drug suspension and sterilized or unsterilized celecoxib-PLGA (85/15) microparticles. Particles equivalent to 20 µg of celecoxib were suspended in a dialysis bag and immersed in 50 mL of PBS. There was no statistically significant difference in celecoxib release at any time point between the sterilized and unsterilized particles. *Inset:* release of celecoxib from celecoxib suspension (20 µg). Data are expressed as the mean ± SD of three experiments.

Figure 5.

Inhibitory effects of celecoxib-PLGA microparticles on diabetes induced elevations in (A) retinal PGE₂ ($n = 4$); (B) retinal VEGF ($n = 8-9$); (C) vitreous-plasma protein ratio ($n = 4$); and (D) blood-retinal barrier leakage ($n = 4$). The parameters were estimated 60 days after periocular administration of the placebo or celecoxib-PLGA microparticles to rats. Data are expressed as the mean $\pm$ SD. Significantly different from the *diabetic group, the †diabetic +placebo group, or the ‡contralateral eye.

Figure 6.

Celecoxib-PLGA microparticles are well tolerated and do not have any significant adverse effects in normal rats. Representative histologic sections depicting the health of the retina and the periocular tissues in rats injected with periocular celecoxib-PLGA microparticles. *Top*: normal rats injected with PBS; *bottom*: normal rats injected with celecoxib PLGA microparticles. (A, D) Retinal sections stained with hematoxylin and eosin (H&E). (B, E) Periocular tissue (site of administration) stained with H&E. (C, F) Periocular tissue stained with Masson trichrome stain. Magnification: (A, D) $\times 200$; (B, C, E, F) $\times 100$.

Figure 7.

Morphometric analysis of the retina in normal rats. The thickness of the different retinal layers was compared between control and celecoxib PLGA microparticle-treated groups. Data are expressed as the mean $\pm$ SD of results in four experiments. *Inset*: representative photomicrographs of the retina showing the different retinal layers. The thickness of the neural retina (*double-headed arrow*), outer nuclear layer (ONL), and inner nuclear layer (INL) was compared between the two groups. No statistically significant differences were found in the thickness of the layers measured between the two groups. GCL, ganglion cell layer.

Figure 8. Probable pathway for the reduction of diabetes-induced elevation in retinal PGE₂, VEGF, and vascular leakage by periocular celecoxib-PLGA microparticles.

Table 1

Blood Sugar Levels and Change in Body Weight in the Study Groups

Rat Group	Blood Sugar Level (mg/dL)	% Weight Change
Normal	130 ± 5 [*] [†]	71.5 ± 3.6 [*] [†]
Diabetic	442 ± 40 [†]	7.1 ± 1.7
Normal+celecoxib	120 ± 3 [*] [†]	65.7 ± 3.5 [*] [†]
Diabetic+celecoxib	501 ± 40	0.3 ± 5.2
Diabetic+placebo	563 ± 27	-5.9 ± 4.6

Data were collected from rats at the end of 60 days after doses of celecoxib-PLGA, placebo-PLGA microparticles, or PBS solution. The data are expressed as the mean ± SD for $n = 18-20$.

^{*} Significantly different from diabetic group.

[†] Significantly different from diabetic + placebo group.

Table 2**Blood Chemistry Analysis in Normal Rats**

Parameter	Control Group	Celecoxib-PLGA Microparticle Group	Units
AST	165.86 ± 38.84	181.42 ± 78.89	U/L
Alkaline phosphatase	190.57 ± 41.04	184.43 ± 37.30	U/L
ALT	61.00 ± 14.23	62.14 ± 17.92	U/L
Bilirubin (total)	0.50 ± 0.46	0.56 ± 0.54	mg/dL
Protein (total)	5.84 ± 0.41	6.28 ± 0.65	g/dL
Urea nitrogen (BUN)	20.71 ± 3.20	19.14 ± 1.86	mg/dL
Creatinine	0.61 ± 0.09	0.51 ± 0.13	mg/dL
BUN/Creatinine	34.44 ± 7.42	39.31 ± 10.34	mmol/L
Sodium	143.42 ± 2.37	139.00 ± 1.00	mmol/L
Potassium	7.13 ± 1.92	6.64 ± 3.29	mmol/L
Calcium	9.03 ± 0.64	8.90 ± 0.26	mmol/L
Chloride	103.71 ± 1.98	102.28 ± 1.11	mmol/L
Anion gap	13.29 ± 4.72	11.00 ± 3.21	mmol/L
Osmolality	295.86 ± 3.34	293 ± 3.70	mOsm/kg
WBC Count*	10.65 ± 1.56	11.05 ± 1.81	X10 ³ /μl
RBC Count*	8.63 ± 0.25	9.18 ± 0.12	X10 ⁶ /μl
Platelet Count*	795 ± 119	909 ± 162	X10 ³ /μl
Hemoglobin*	15.5 ± 0.41	15.88 ± 0.05	g/dL
Hematocrit*	75.56 ± 2.03	78.4 ± 1.51	%
MCV*	87.62 ± 1.44	85.45 ± 1.58	fL
MCHC*	20.28 ± 0.36	20.25 ± 0.31	%
% Neutrophils*	8.5 ± 1.2	8.25 ± 3	%WBC
% Eosinophils*	1.5 ± 0.58	1.75 ± 0.5	%WBC
% Basophils*	0	0	%WBC
% Lymphocytes*	87 ± 0.82	85.5 ± 4.7	%WBC
% Monocytes*	3 ± 1.63	4.5 ± 2.65	%WBC

Blood samples were collected 60 days after administration of celecoxib-PLGA microparticles by periocular injection. The parameters in the rats injected with celecoxib microparticles were compared with the parameters of normal rats injected with PBS. The data are expressed as the mean ± SD of $n = 7$ (or $n = 4$, wherever indicated by *). The chemistry parameters were estimated in the plasma, whereas the cell counts were estimated using whole blood. AST: aspartate amino transferase; ALT: alanine amino transferase MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration.