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European Journal of Pharmacology 511 (2005) 191–198

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Subconjunctivally administered celecoxib-PLGA microparticles sustain retinal drug levels and alleviate diabetes-induced oxidative stress in a rat model

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Received in revised form 28 January 2005; accepted 9 February 2005
Available online 17 March 2005

Abstract

We have previously reported that repeated oral doses of celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, reduced diabetes-induced retinal vascular endothelial growth factor (VEGF) expression [Ayalasomayajula, S.P., Kompella, U.B., 2003. Celecoxib, a selective cyclooxygenase-2 inhibitor, inhibits retinal vascular endothelial growth factor expression and vascular leakage in a streptozotocin-induced diabetic rat model. Eur J Pharmacol 458, 283–289] and that retinal celecoxib delivery can be improved by several-fold following subconjunctival administration [Ayalasomayajula, S.P., Kompella, U.B., 2004. Retinal delivery of celecoxib is several-fold higher following subconjunctival administration compared to systemic administration. Pharm Res 21, 1797–1804]. The objective of the current study was to determine whether polymeric microparticles of celecoxib sustain retinal drug levels following subconjunctival administration and alleviate diabetes-induced oxidative stress in a streptozotocin-induced diabetic rat model. Biodegradable poly (lactide-co-glycolide) (PLGA; 85:15) microparticles of celecoxib were prepared using solvent evaporation method and characterized for their size, morphology, encapsulation efficiencies, and in vitro release. The celecoxib-PLGA microparticles or solution containing 75 μg of celecoxib was administered subconjunctivally to one eye (ipsilateral) of Sprague Dawley rats and drug levels in the retina, vitreous, lens, and cornea of ipsilateral and contralateral eyes were determined on 1, 7, and 14 days using high-performance liquid chromatography (HPLC). The effect of subconjunctivally administered celecoxib-PLGA microparticles on oxidative stress in day 14 diabetic rat retinas was determined by measuring the retinal glutathione (reduced (GSH) and oxidized (GSSG)), thiobarbituric acid reactive substances, and 4-hydroxynonenal levels using spectrofluorometric and colorimetric methods. Solvent evaporation method produced spherical celecoxib-PLGA microparticles with mean diameters of 3.9±0.6 μm and 68.5% loading efficiency. These microparticles sustained celecoxib release during the 49-day in vitro release study. Subconjunctivally administered celecoxib-PLGA microparticles sustained retinal and other ocular tissue drug levels during the 14-day study in rats. No detectable celecoxib levels were observed in the contralateral eye. The celecoxib-PLGA microparticles significantly inhibited the diabetes-induced increases in thiobarbituric acid reactive substances (P=0.012) and 4-hydroxynonenal levels (P=0.029). The particles also inhibited the GSH depletion and the increase in GSSH/GSH ratio associated with diabetes but the effects were not statistically significant (P=0.12). Thus, following subconjunctival administration, celecoxib-PLGA microparticles sustained retinal celecoxib delivery and inhibited diabetes-induced retinal oxidative damage, indicating their potential usefulness in treating diabetes-induced retinal abnormalities.

Keywords: Celecoxib; Microparticle; Retinal delivery; Oxidative stress; Diabetes

1. Introduction

Diabetic retinopathy, a leading cause of blindness in the USA, is characterized by vascular leakage and angiogenesis (Klein et al., 1989). Currently several experimental

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doi:10.1016/j.ejphar.2005.02.019

approaches are aimed at retinal delivery of drugs capable of inhibiting factors responsible for the initiation and progression of diabetic retinopathy (Aukunuru et al., 2002; Carrasquillo et al., 2003; Kompella et al., 2003). The retinal delivery of drugs from the systemic circulation is inefficient because of the rate limiting nature of a tight endothelium lining the retinal blood vessels. To circumvent this limitation of the systemic mode of drug delivery to the retina, various localized retinal delivery approaches including intravitreal, subconjunctival, sub-tenon, and retrobulbar administrations are being investigated. Previously, we have demonstrated that subconjunctival administration of celecoxib, a selective cyclooxygenase-2 inhibitor, results in several-fold greater retinal celecoxib delivery compared to intraperitoneal administration (Ayalasomayajula and Kompella, 2004). In the current study, we have prepared and assessed biodegradable polymeric microparticles entrapping celecoxib for their ability to sustain retinal drug levels following subconjunctival administration. Such locally administered sustained release systems minimize fluctuations in drug levels observed with immediate release dosage forms such as solutions and improve the patient compliance by reducing dose, dosing frequency, and any systemic side effects.

Ischemia in the retinal blood vessels, which results in local inflammatory response (Barouch et al., 2000), is the earliest event associated with the pathogenesis of diabetic retinopathy (Aiello et al., 1998). Indeed, pro-inflammatory genes are induced early in diabetes (Joussen et al., 2001). Cyclooxygenase-2 (COX-2) is an early gene product of inflammation (Dubois et al., 1998) and it is elevated in diabetic subjects with proliferative retinopathy (Sennlaub et al., 2003) and in experimental diabetic rat models (Ayalasomayajula and Kompella, 2003; Carmo et al., 2000). COX-2 along with constitutively expressed cyclooxygenase-1 (COX-1) is involved in the metabolism of arachidonic acid to prostaglandins. Prostaglandins, in turn, induce the expression of vascular endothelial growth factor (VEGF) (Hoper et al., 1997), which is responsible for the initiation and progression of diabetic retinopathy (Frank, 2004). Since COX-2 is overexpressed in diabetic retinas, COX-2 selective inhibitors are expected to reduce induced prostaglandin levels and VEGF expression (Joussen et al., 2002; Ozaki et al., 2002). We have previously reported that repeated high oral doses (50 mg/kg, bid) of celecoxib inhibited diabetes-induced retinal VEGF mRNA expression and vascular leakage on day 8 in diabetic rats (Ayalasomayajula and Kompella, 2003). The VEGF inhibitory effects of celecoxib were shown to be associated with a reduction in induced prostaglandin levels (Ayalasomayajula et al., 2004; Gallo et al., 2001). Our recent findings indicated that COX-2 inhibition but not COX-1 inhibition normalizes the elevated prostaglandin secretion in diabetic rat retinas (Ayalasomayajula et al., 2004).

During the synthesis of prostaglandins, cyclooxygenase enzymes (both COX-1 and COX-2) release oxygen free radicals. These free radicals aggravate oxidative stress in the fatty acid rich environment of the retina. In addition, the auto-oxidation of glucose (Wolff and Dean, 1987), advanced glycation end products (Masaki et al., 1999), and the metabolism of glucose through the aldose reductase pathway (Lee and Chung, 1999) contribute to the elevated oxidative stress levels in the retina with the progression of diabetes (Doly et al., 1992). Oxidative stress, in turn, can induce VEGF expression (Kuroki et al., 1996). Although the potential use of celecoxib to inhibit prostaglandin synthesis (Gallo et al., 2001) and VEGF expression (Ayalasomayajula and Kompella, 2003; Ozaki et al., 2002) is known, its effect on diabetes-induced retinal oxidative stress has yet to be determined. Since elevated oxidative stress due to inflammatory events is an early event in diabetic retinas, this study investigated whether subconjunctivally administered polymeric celecoxib microparticles inhibit oxidative stress in the streptozotocin-induced diabetic rat model.

2. Methods

2.1. Chemicals

Celecoxib was a gift from Pharmacia, St. Louis, MO (Pfizer). Polyvinyl alcohol was obtained from Sigma Chemical (St Louis, MO). Poly (lactide co-glycolide) (PLGA) with a lactide–glycolide ratio of 85:15 (intrinsic viscosity: 0.83 g/dl) was obtained from Birmingham Polymers (Birmingham, AL). The HPLC grade methylene chloride and acetonitrile were obtained from Fischer Scientific (Pittsburgh, PA). Reduced (GSH) and oxidized (GSSG) glutathione, malondialdehyde bis (dimethyl acetal) or 1,1,3,3,-tetramethoxy propane, O-pthalaldehyde, *N*-ethylmaleimide, thiobarbituric acid, and ethylene diamine tetra acetate (EDTA) were purchased from Sigma Chemical (St. Louis, MO). 4-Hydroxynonenal was purchased from Cayman Chemicals (Cambridge, MA).

2.2. Fabrication of celecoxib-PLGA microparticles

Polymeric celecoxib microparticles were formulated using a solvent evaporation method (Kompella et al., 2003). Briefly, celecoxib and PLGA (85:15) were mixed in a ratio of 1:10 and dissolved in 1 ml of dichloromethane. This solution was added slowly to 10 ml of an aqueous polyvinyl alcohol (2% w/v) solution and the mixture was sonicated for 1.5 min at 20 W (Ultrasonic processor, Misonix, NY) to obtain an O/W emulsion. This emulsion was immediately added drop-wise to 125 ml of an aqueous polyvinyl alcohol (2% w/v) solution. The contents were stirred overnight at room temperature to evaporate the methylene chloride, allowing the formation of a turbid particulate suspension. The microparticles were separated by centrifuging the suspension at 1000 *g* for 30 min. The pellet was washed twice with phosphate-buffered saline

(PBS), re-suspended in de-ionized water, and freeze-dried to obtain lyophilized microparticles entrapping celecoxib.

2.3. Characterization of celecoxib-PLGA microparticles

2.3.1. Morphological analysis

The morphology of the celecoxib-PLGA microparticles was observed using a scanning electron microscope. Briefly, a small quantity of microparticles were layered on the scanning electron microscope stubs and coated with gold–palladium under an argon atmosphere using a gold sputter module in a high vacuum evaporator. Samples were then observed for their surface morphology with a Phillips SEM 51S scanning electron microscope set at 15 kV.

2.3.2. Drug loading measurement

The loading efficiency of celecoxib-PLGA microparticles was determined by extracting and quantifying the encapsulated celecoxib. Briefly, celecoxib-PLGA microparticles (2 mg) were taken in a glass tube and 2 ml of methylene chloride was added and mixed thoroughly at room temperature for 16 h to dissolve the polymer and the drug. The resulting solution was evaporated to dryness under nitrogen and the dried residue was reconstituted with 1000 μl of acetonitrile: water mixture (70:30). This reconstituted solution was mixed on a vortex for 1 min, centrifuged at 12,000 *g* for 5 min, and celecoxib levels were measured by injecting 100 μl of the supernatant onto HPLC as described in the methods. The loading efficiency was estimated as the amount entrapped×100/initial amount.

2.3.3. In vitro drug release

The in vitro release of celecoxib from the PLGA particles was carried out at 37 °C using dialysis membrane bags (Molecular weight cut off: 10,000, Spectrum Laboratories) as described earlier (Kompella et al., 2001). Briefly, a 0.5 ml suspension of the celecoxib-PLGA microparticles containing 20 μg of celecoxib was taken into dialysis membrane bags and the units were allowed to float in 50 ml of release medium (phosphate-buffered saline (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 using a HPLC method.

2.4. Subconjunctival administration of celecoxib-PLGA microparticles

Sprague Dawley rats weighing 180–200 g were purchased from SASCO, Wilmington, MA. All the animals in this study were maintained and treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the treatment of animals in ophthalmic and vision research. Subconjunctival administration of celecoxib-PLGA microparticles were performed as described earlier (Kompella et al., 2003). Briefly, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). Following this, 75 μl of either solution (10% ethyl alcohol in 20% w/v solution of hydroxylpropyl-β-cyclodextrin) or microparticle suspension containing 75 μg of celecoxib was administered into the posterior subconjunctival space of one eye (ipsilateral) using a 27G needle. The other eye (contralateral) served as a control. At the end of 1, 7, and 14 days, the animals were euthanized, eyes were enucleated and the eyes were frozen immediately and stored at −80 °C. The ocular tissues including retina, vitreous, lens, and cornea were isolated and the drug levels were estimated using an HPLC method described below.

2.5. HPLC determination of celecoxib

Celecoxib levels in the ocular tissues, in vitro release samples, and samples for loading efficiency were determined as described previously (Ayalasomayajula and Kompella, 2003). Budesonide (2.5 μl of 40 μg/ml solution) was used as the internal standard. The extracted drugs were reconstituted in 150 μl of acetonitrile:water (70:30) mixture and 100 μl was injected onto a Waters HPLC system that included a pump (Waters TM 616), a controller (Waters 600 S), an autoinjector (Waters 717 plus), and a PDA detector (Waters 996). The peak areas were integrated using Millennium software (Version 2.15.01). The drugs were separated with a 25-cm long Discovery C-18 column (Supelco, Emeryville, CA) with a particle diameter of 5 μm and a pore size of 100 Å. The mobile phase for the assay consisted of acetonitrile and aqueous buffer mixture (70:30 v/v). The buffer was 0.1% acetic acid in water at pH 3. The run time for the assay was 10 min and the retention times for internal standard and celecoxib were 4.9 and 7.1 min, respectively. The limit of detection of celecoxib was 1 ng in the lens and 0.5 ng in the retina, vitreous, and cornea.

2.6. Effect of celecoxib-PLGA microparticles on diabetes-induced retinal oxidative stress

The effect of celecoxib-PLGA microparticles on diabetes-induced oxidative stress parameters was determined on day 14 following subconjunctival administration. Diabetes was induced in Sprague Dawley rats with a single intraperitoneal administration of streptozotocin (60 mg/kg) prepared in 10 mM citrate buffer after fasting animals for 24 h. The animals administered with equal volume of citrate buffer served as controls. Following the treatment, the animals were given free access to food and water and the blood glucose levels were measured using a glucometer (OneTouch Ultra, LifeScan, Milpitas, CA). The animals with blood glucose>250 mg/dl were deemed diabetic. The animals were grouped as follows: Group 1: non-diabetic untreated rats; Group 2: non-diabetic rats administered with celecoxib-PLGA microparticles subcon-

junctivally; Group 3: diabetic untreated rats; and Group 4: diabetic rats administered with celecoxib-PLGA microparticles subconjunctivally. The microparticles containing 75 μg of celecoxib were administered 1 day after inducing diabetes in rats. On day 14, the animals were sacrificed, eyes were enucleated, and retinas were isolated and homogenized in sodium phosphate-EDTA buffer (pH 7.4). The homogenates were centrifuged at 10,000 *g* for 10 min at 4 °C and the supernatant was taken to measure thiobarbituric acid reactive substances, 4-hydroxynonenal, and both reduced (GSH) and oxidized (GSSG) forms of glutathione.

2.6.1. Thiobarbituric acid reactive substances and 4-hydroxynonenal estimations

For the estimation of thiobarbituric acid reactive substances, 200 μl of the supernatant was mixed with an equal volume of thiobarbituric acid (29 mM in glacial acetic acid) and incubated at 95 °C. After 1 h, the reactants were cooled to room temperature and acidified with 2.5 μl of 5 M HCl. The thiobarbituric acid reactive substances were extracted with 700 μl of *n*-butanol and the fluorescence of the butanol layer was determined at 547-nm emission wavelength after an initial excitation at 525 nm. The concentrations were determined by using standards (1,1,3,3,-tetramethoxy propane or malonaldehyde bismethyl acetal) processed similarly. 4-Hydroxynonenal levels were measured in 200 μl of the supernatant using phenylindole method (Oxford Biomedical research, MI).

2.6.2. GSH and GSSG estimations

Reduced and oxidized forms of glutathione (GSH and GSSG) were estimated using a spectrofluorometric method described earlier (Ayalasomayajula and Kompella, 2002). For GSH estimation, 50 μl of supernatant was mixed with 900 μl of sod. phosphate-EDTA buffer. This mixture was incubated with 50 μl of O-phthalaldehyde (1 mg/ml in methanol) for 15 min at room temperature and fluorescence was measured at 420 nm after exciting at 350 nm. For GSSG estimation, 50 μl of the supernatant was incubated with 20 μl of 0.04 M *N*-ethylmaleimide for 30 min at room temperature to block the reactivity of GSH with O-phthalaldehyde. Then the solution pH was made basic by adding 880 μl of 0.1 N NaOH to convert GSSG to GSH, which was estimated by incubating the sample with 50 μl of O-phthalaldehyde as mentioned above. The concentrations of GSH and GSSG were calculated by using GSH and GSSG standards processed similarly.

2.7. Statistical analysis

The data were represented as mean±S.D. for $n=4$. The groups were compared using one way analysis of variance (ANOVA) followed by Tukey's post-hoc analysis. The differences were considered significantly different at $P<0.05$.

3. Results

3.1. Particle morphology, particle size, and celecoxib encapsulation efficiency

The emulsion solvent evaporation technique resulted in celecoxib-PLGA microparticles with spherical morphology (Fig. 1A). The mean particle size and encapsulation efficiency of the celecoxib-PLGA microparticles were 3.9±0.6 μm and 68.5%, respectively.

3.2. In vitro drug release

The celecoxib-PLGA microparticles sustained drug release in vitro. At the end of 7 weeks, 30.4±3% of the entrapped celecoxib was released from the particles.

3.3. Celecoxib-PLGA microparticles sustain celecoxib levels in the ocular tissues

Ocular tissue levels of celecoxib were compared following a single subconjunctival injection of either celecoxib solution or celecoxib-PLGA microparticles (containing 75 μg of celecoxib) at the end of days 1, 7, and 14 (Fig. 2). At all these time points, detectable levels of celecoxib were not present in the ocular tissues of the contralateral eye. On day

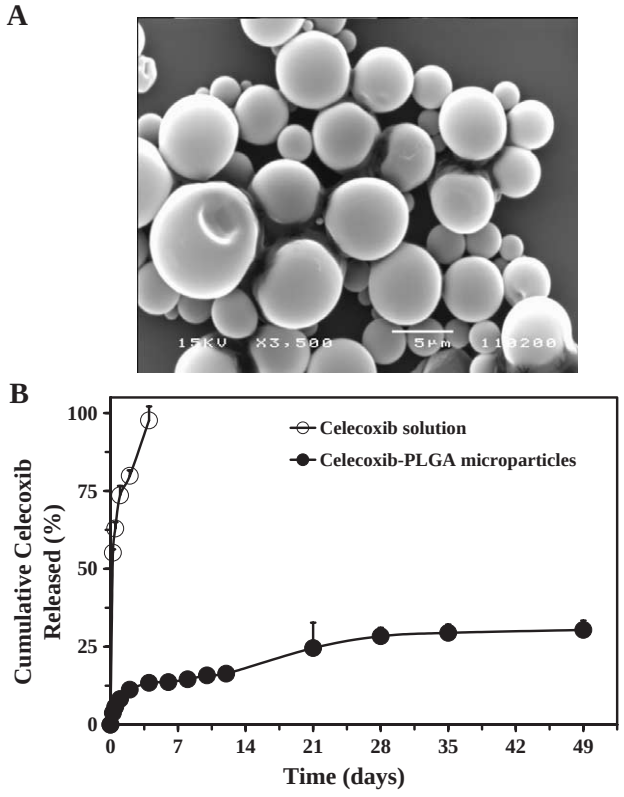

Fig. 1. (A) Scanning electron microscopy pictures of celecoxib-PLGA microparticles. Bar indicates 5 μm. (B) Cumulative amount (%) of celecoxib released in vitro from the PLGA microparticles and solution containing 20 μg of celecoxib.

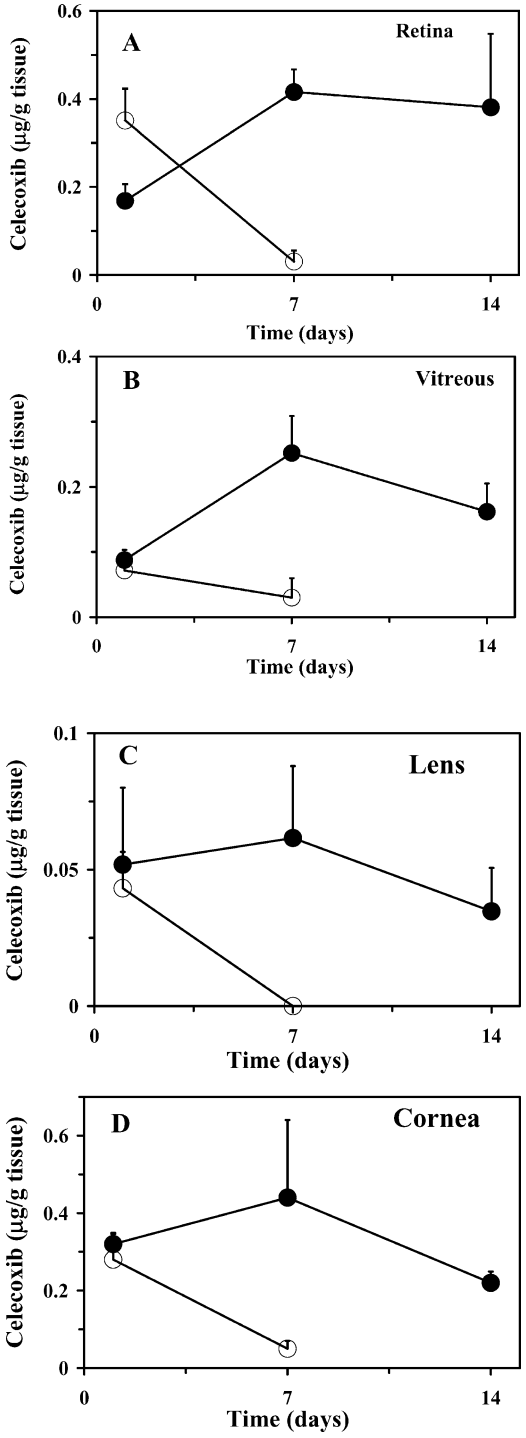

Fig. 2. Celecoxib-PLGA microparticles sustain ocular tissue levels of celecoxib following subconjunctival administration at a dose of 75 μg/rat. Rats were administered with celecoxib (75 μg/eye) either in the form of solution (○) or microparticles (●) to one eye and drug levels were estimated in (A) retina, (B) vitreous, (C) cornea, and (D) lens as described in the Methods. Data are expressed as mean±S.D. for n=4. Data are shown for the ipsilateral (dosed) eye. Drug levels were below the detection limits in the contralateral (undosed) eye at all the time intervals studied. Also, celecoxib levels were below the detection limits on day 14 in the solution group.

1, in the ipsilateral eye, celecoxib levels in the solution group were 2.6-, 1.4-, 2.3-, and 1.9-fold higher in the retina, vitreous, lens, and cornea, respectively, when compared to microparticles group (P<0.05). On day 7, drug levels in the microparticle group were 10.5-, 25.2-, and 11.5-fold higher in the retina, vitreous, and cornea, respectively, compared to the solution group (P<0.05). No detectable levels of celecoxib were observed in the lens of solution group on day 7 and in all the ocular tissues on day 14. However, celecoxib-PLGA microparticles sustained drug levels even on day 14 in the retina, vitreous, lens, and the cornea.

3.4. Celecoxib-PLGA microparticles inhibit diabetes-induced retinal oxidative stress

The effect of single dose subconjunctival administration of celecoxib-PLGA microparticles on diabetes-induced oxidative stress is shown in Fig. 3. The retinal thiobarbituric acid reactive substances (P<0.0009) and 4-hydroxynonenal levels (P=0.012) were significantly increased in day 14 diabetic rats compared to age matched control rats. Treatment with celecoxib-PLGA microparticles significantly reduced the thiobarbituric acid reactive substances

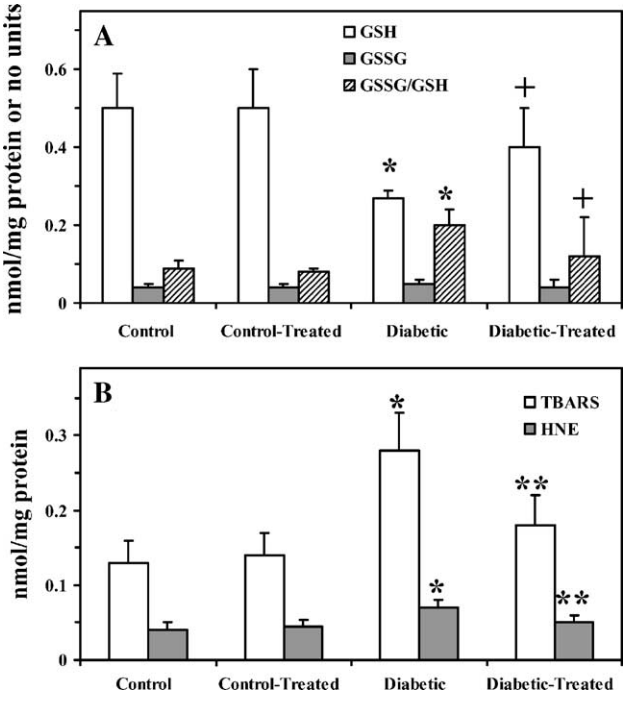

Fig. 3. Subconjunctivally administered celecoxib-PLGA microparticles inhibit diabetes-induced retinal oxidative stress. Following single dose (75 μg celecoxib/rat) subconjunctival administration of celecoxib-PLGA microparticles, the retinal (A) GSH and GSSG levels and (B) thiobarbituric acid reactive substances (TBARS) and 4-hydroxynonenal (HNE) levels were measured as described in the Methods. Data are expressed as mean±S.D. for n=5. The units for all parameters are nmol/mg protein for all parameters except GSSG/GSH ratio, which has no units. *Significantly different compared to controls and control-treated groups at P<0.05. **Significantly different compared to diabetic group at P<0.05. +Indicates difference compared to diabetic group at P=0.12.

(P=0.012) and 4-hydroxynonenal (P=0.029) levels in diabetic rats. The retinal GSH levels were significantly decreased (P=0.007) in diabetic rats and celecoxib-PLGA microparticle treatment increased GSH levels in diabetic rats to 80% of control values but this increase was not statistically significant (P=0.12). No significant changes in the GSSG levels were observed in any of the groups in this study. The ratio of GSSG to GSH, an indicator for oxidative stress, significantly increased in diabetic rat retinas (P=0.013). Although celecoxib-PLGA microparticles reduced this ratio, the effect was not statistically significant. None of the parameters were significantly altered by celecoxib microparticle treatment in the normal animals, indicating that the effects of celecoxib microparticles are limited to diabetic conditions in this study.

4. Discussion

Clinical management of diabetic retinopathy requires chronic treatment. Thus, development of drug delivery systems that reduce the frequency of administration and dose is desirable. To this end, there is a growing interest in developing various sustained-release formulations including gels, microparticles, nanoparticles, and liposomes for ocular drug delivery (Assil et al., 1988; Das et al., 1995; Kompella et al., 2003). In addition, the route of administration of these delivery systems needs to be selected carefully. Although intravitreal administration of microparticles sustain vitreal drug levels (Chowdhury and Mitra, 2000), the suspended microparticles in the vitreous increase polymeric burden in the eye and are likely to interfere with vision. We have demonstrated previously that subconjunctivally injected microparticles of a corticosteroid sustained drug delivery for longer periods compared to nanoparticles administered by the same route (Kompella et al., 2003). Also, we have demonstrated that the microparticles are better retained at the site of administration compared to nanoparticles (Amrite and Kompella, 2004). Thus, subconjunctivally administered microparticles are of potential value in sustaining retinal drug delivery.

We have previously demonstrated that subconjunctival administration facilitates local drug delivery and results in 54-fold higher retinal drug levels compared to the intraperitoneal route in rats (Ayalasomayajula and Kompella, 2004). In this study, we investigated the ability of celecoxib polymeric microparticles in sustaining retinal drug delivery following subconjunctival administration. The polymer used in this study for drug encapsulation is PLGA (85:15), a biodegradable polymer. Such polymers are used in the fabrication of several FDA-approved sustained release injectable products including Lupron Depot®, Nutropin Depot®, Zoladex®, and Sandostatin LAR Depot ®, in addition to their use in surgical sutures. Our studies indicated that PLGA-celecoxib microparticles are spherical (Fig. 1A) and sustain celecoxib release during the 7-week in vitro study. Very low or no burst release was observed from these microparticles. Therefore, we have evaluated celecoxib-PLGA microparticles in vivo for their ability to sustain retinal drug levels following subconjunctival administration. Since celecoxib is a low molecular weight drug, it can readily permeate the sclera (Ambati et al., 2000). However, the microparticles, while sustaining the drug release, cannot permeate the sclera due to their large size. For this reason, we observed that celecoxib-PLGA microparticles better sustained drug levels in the retina as well as the vitreous, lens, and the cornea during the 14-day study compared to solution (Fig. 2). The celecoxib levels were below the detection limit in the contralateral eye, suggesting that local delivery is responsible for the higher ocular tissue levels in the ipsilateral eyes. Assuming that a gram of tissue is equivalent to 1 ml, the day 14 concentrations of celecoxib in the microparticle group are 1.0 ± 0.4, 0.4 ± 0.1, 0.1 ± 0.04, and 0.6 ± 0.1 μM, respectively, in the retina, vitreous, lens, and cornea. These concentrations are much above the IC50 value of celecoxib for COX-2 (0.003–0.006 μM). Thus, subconjunctivally administered celecoxib microparticles deliver therapeutic concentrations to the retina. This was further confirmed by the beneficial effects observed in this study.

Even though local diffusion across sclera and underlying choroid and retinal pigment epithelial layers is primarily responsible for the drug delivery to the retina from the subconjunctival space, the delivery does not necessarily correlate with drug lipophilicity. We have previously shown that the retinal tissue levels of celecoxib (log P: 3.82) at 15 min post-dosing were lower compared to more hydrophilic budesonide (log P: 3.2) and sodium fluorescein (log P: −0.67) following subconjunctival administration in a rat model (Cheruvu et al., 2003). Consistent with this observation, subconjunctival administration of celecoxib-PLGA microparticles resulted in days 1, 7, and 14 drug levels in the retina that were lower compared to budesonide levels attained following subconjunctival administration of an equal dose of budesonide in polymeric particles with similar drug release rates (Kompella et al., 2003). These differences could be attributed to differences in either drug absorption or clearance of the drugs.

Celecoxib is known to selectively inhibit COX-2, which is constitutively expressed in the retina (Ju and Neufeld, 2002) and further induced in diabetes (Ayalasomayajula and Kompella, 2003; Carmo et al., 2000). We have shown previously that repeated high oral doses of celecoxib inhibited diabetes-induced retinal VEGF mRNA expression and vascular leakage (Ayalasomayajula and Kompella, 2003). We have also demonstrated that oxidative stress induces VEGF expression in retinal pigment epithelial cells and that this can be countered by antioxidants (Ayalasomayajula and Kompella, 2002). Since oxidative stress is a principal stimulus for the induction of VEGF in various cell types (Silacci and Hayoz, 1998), this study investigated celecoxib encapsulating polymeric microparticles for their

effect on retinal oxidative stress in diabetic rats. Following streptozotocin treatment, inflammatory changes are expected in diabetic retinas, which in turn lead to elevated oxidative stress (Van Reyk et al., 2003). As the diabetic condition progresses, advanced glycation products will also contribute to oxidative stress (Vlassara and Palace, 2002). We hypothesized that in the early stages of diabetes induction in streptozotocin-treated rat models, the oxidative stress is primarily in response to the inflammatory events and hence treatment with a COX-2 inhibitor such as celecoxib should inhibit the oxidative stress. Oxidative stress in tissues can be measured by estimating the levels of oxidative stress related products such as 4-hydroxynonenal, thiobarbituric acid reactive substances such as malondialdehyde, and cellular levels of antioxidants such as glutathione. We measured all these parameters in response to celecoxib-PLGA microparticles administered subconjunctivally.

In diabetic rat retinas, we observed oxidative stress induction estimated as an increase in thiobarbituric acid reactive substances and 4-hydroxynonenal levels (Fig. 3A) and decrease in GSH levels (Fig. 3B). Apart from being an oxidative stress marker, 4-hydroxynonenal has several bioactive properties including the induction of growth factor expression (Ayalasomayajula and Kompella, 2002; Comporti, 1998; Parola et al., 1999). While GSH is responsible for scavenging oxidants and for facilitating the elimination of toxic products including 4-hydroxynonenal from the cells, action of glutathione reductase on GSSG is responsible for the supply of intracellular GSH. Evidence exists for the inhibition of glutathione reductase activity in the diabetic retinas (Obrosova et al., 2000), possibly resulting in the accumulation of GSSG. Interestingly, we observed that single dose subconjunctival administration of celecoxib-PLGA microparticles inhibited increase in retinal thiobarbituric acid reactive substances and 4-hydroxynonenal levels while partly normalizing GSH levels (Fig. 3). These observations can be explained on the basis of anti-inflammatory effects of celecoxib in the streptozotocin-induced diabetic rat model. This is the first evidence demonstrating the ability of celecoxib to inhibit retinal oxidative stress in a diabetic model.

In this study, we assessed drug delivery in the normal but not diabetic rats. Very little is known about drug metabolism in the eye, especially under disease conditions such as diabetes. There is one report indicating that cytochrome oxidase levels are elevated in the retinal pigment epithelium of diabetic rats (Caldwell and Slapnick, 1989). To better design the therapeutic regimens, future studies should assess differences in retinal drug disposition including metabolism under disease conditions.

In conclusion, single dose subconjunctival administration of celecoxib-PLGA microparticles sustained retinal celecoxib levels during the 14-day study in rats and inhibited diabetes-induced retinal oxidative stress. Also, celecoxib levels can be sustained in other ocular tissues including the vitreous, lens, and the cornea following subconjunctival administration of celecoxib-PLGA microparticles, indicating that this approach can be potentially employed for the treatment of other ocular disorders including vitreo-retinitis, corneal inflammation, and choroidal neovascularization.

Acknowledgements

This work was supported by National Institutes of Health grants DK064172 and EY013842. The authors are thankful to the University of Nebraska Medical Center for the graduate student research fellowship awarded to Mr. Ayalasomayajula.

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