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Celecoxib, a selective cyclooxygenase-2 inhibitor, inhibits retinal vascular endothelial growth factor expression and vascular leakage in a streptozotocin-induced diabetic rat model

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Abstract

Overexpression of vascular endothelial growth factor (VEGF) is implicated in the development of vascular leakage and retinal neovascularization in diabetic subjects. The objective of this study was to determine whether celecoxib, a selective cyclooxygenase-2 enzyme inhibitor, reaches ocular tissues following oral administration and inhibits the retinal VEGF expression and vascular leakage in a streptozotocin-induced diabetic rat model. After administering a single intraperitoneal injection of streptozotocin (60 mg/kg) to Sprague–Dawley rats and ensuring the induction of diabetes at the end of 24 h, celecoxib was administered b.i.d. by oral gavage (50 mg/kg). On day 8, the animals were sacrificed and the retinal VEGF and cyclooxygenase-2 mRNA levels, ocular tissue celecoxib concentrations, and the vitreous/plasma protein ratio were determined. In diabetic rats, the retinal VEGF mRNA expression was 2.3-fold compared to controls, with a corresponding increase in cyclooxygenase-2 mRNA expression. Celecoxib treatment inhibited VEGF mRNA expression without any significant reduction in cyclooxygenase-2 mRNA. Furthermore, the retinal vascular leakage estimated as vitreous to plasma protein ratio increased in diabetic animals from 0.35 ± 0.1 to 1.1 ± 0.1 and celecoxib treatment significantly decreased this ratio to 0.4 ± 0.1. Celecoxib levels were 24.8 ± 6.6, 1.9 ± 1, 1.7 ± 0.8, and 6.9 ± 0.9 ng/mg in the retina, vitreous, lens, and cornea, respectively. The plasma celecoxib levels were 85 ± 24 ng/ml. Thus, celecoxib reaches the retina after oral administration and reduces diabetes-induced retinal VEGF mRNA expression and vascular leakage by inhibiting the activity of cyclooxygenase-2 enzyme.

Keywords: Cyclooxygenase-2; VEGF (vascular endothelial growth factor); Celecoxib; Diabetes

1. Introduction

Vascular leakage and neovascularization are pathologies of the diabetic retinas that lead to retinal detachment and blindness (Garner, 1993). These two vascular changes in diabetic retinopathy are associated with elevated retinal and vitreal vascular endothelial growth factor (VEGF) (Adamis et al., 1994; Aiello et al., 1994; Boulton et al., 1998), a potent inducer of vascular hyper-permeability and neovasculization (Ferrara, 2000; Miller et al., 1997). Macromolecules including VEGF neutralizing soluble receptor construct (VEGF TrapA) (Qaum et al., 2001), VEGF neutralizing antibody (Sone et al., 1999), and VEGF-receptor chimeric proteins (Aiello et al., 1995) inhibited vascular hyperpermeability and/or neovascularization in diabetic rat and neovascular mouse models, suggesting that VEGF inhibition is useful in treating diabetic retinopathy. However, macromolecules suffer from poor stability and limited permeability (Kompella, 1999; Kompella and Lee, 1999), prompting the need to identify and develop small molecular drugs that can inhibit VEGF expression. To this end, we are investigating small molecular drugs capable of inhibiting VEGF expression (Aukunuru et al., 2002; Bandi and Kompella, 2001).

The production of prostaglandins, the products of cyclooxygenase pathway that are capable of inducing VEGF expression (Cheng et al., 1998), is increased by 40% in diabetic rat retinas (Johnson et al., 1999), suggesting that cyclooxygenase plays a role in VEGF induction. There are

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two distinct isoforms of the enzyme cyclooxygenase—cyclooxygenase-1 and cyclooxygenase-2 (Kujubu et al., 1991). While cyclooxygenase-1 is ubiquitous, cyclooxygenase-2 is induced under inflammatory conditions (Needleman and Isakson, 1997). Administration of high dose aspirin, a non-selective inhibitor of cyclooxygenase-1 and cyclooxygenase-2, reduced the incidence of retinopathy in diabetic human subjects (Powell and Field, 1966), retinal vascular leakage in diabetic rats (Qaum et al., 2001), and retinal vascular abnormalities in diabetic dogs (Kern and Engerman, 2001), suggesting that inhibition of cyclooxygenase might be beneficial in ameliorating diabetic retinopathy. However, the retinal expression of cyclooxygenase-2 (Carmo et al., 2000) but not cyclooxygenase-1 (Fang et al., 1997) is elevated during diabetes, suggesting that inhibition of cyclooxygenase-2 activity might be responsible for aspirin effect. Thus, selective inhibition of cyclooxygenase-2 activity is likely to inhibit diabetes-induced retinal vascular changes. In addition, cyclooxygenase-2 selective inhibition is claimed to be devoid of side effects such as gastric ulcers seen with nonselective inhibitors due to cyclooxygenase-1 inhibition (Goldstein et al., 2000). Therefore, in the current study, we have investigated the role of celecoxib, a selective cyclooxygenase-2 inhibitor, in inhibiting retinal vascular leakage and VEGF expression in the streptozotocin-induced diabetic rat model.

Celecoxib (4-[5-(4-methylphenyl)-3-(trifluoro-methyl)-1H-pyrazol-1-yl] benzene sulfonamide) is a potent (IC_{50} for cyclooxygenase-2 and cyclooxygenase-1 are 0.003–0.006 and 4–19 μM, respectively, measured as the inhibition of prostaglandin E2 production) low molecular weight (M.Wt.: 385) cyclooxygenase-2 inhibitor with a cyclooxygenase-2/cyclooxygenase-1 selectivity ratio of 1333–3167 (Gierse et al., 1999; Penning et al., 1997). Celecoxib is currently approved for the treatment of osteoarthritis, rheumatoid arthritis and is indicated as an adjuvant in the treatment of familial adenomatous polyposis. Based on its anti-angiogenic properties (Koki and Masferrer, 2002), celecoxib is currently being investigated for its use in colon cancer treatment. Celecoxib has a plasma elimination half-life of 2.8–3.73 h and is well absorbed following oral administration (~ 60%) (Paulson et al., 2000).

Since elevated retinal VEGF and cyclooxygenase-2 expression are associated with increased retinal vascular leakage in diabetes, this study tested the hypothesis that celecoxib reaches retinal tissue following oral administration and reduces diabetes-induced VEGF expression and associated vascular leakage.

2. Materials and methods

2.1. Chemicals

Celecoxib was a gift from Pharmacia, St. Louis, MO. High performance liquid chromatography (HPLC) grade acetonitrile was obtained from Fisher Scientific, Illinois. Streptozotocin was purchased from Spectrum Chemicals, New Jersey. Reverse trasncriptase polymerase chain reaction (RT-PCR) kit was purchased from Promega, Madison, WI, and the VEGF primers were custom synthesized. Cyclooxygenase-2 primers were bought from Oxford Biomedical Research, Oxford, MI. The BCA protein assay kit was purchased from Pierce, Rockford, IL.

2.2. Animal study

Sprague–Dawley rats weighing 150–160 g were purchased from SASCO, Wilmington, MA. After fasting the animals for 24 h, they were injected with a single intraperitoneal dose of streptozotocin (60 mg/kg) dissolved in 10 mM citrate buffer. The animals intraperitoneally injected with an equal volume of citrate buffer served as controls. Following the injections, the animals were given free access to food and water. The blood glucose levels were measured 24 h later using a glucometer (OneTouch Ultra, LifeScan, Milpitas, CA). The animals with blood glucose >250 mg/dl were divided into two groups—one receiving celecoxib by oral gavage at a dose of 50 mg/kg two times a day and the other receiving vehicle (0.5% carboxymethylcellulose) alone. Streptozotocin treated animals with blood glucose levels <120 mg/dl were regarded as "streptozotocin-treated nondiabetic controls". The animals were grouped as follows: Group 1: Citrate buffer treated control rats; Group 2: Streptozotocin-treated nondiabetic rats; Group 3: Streptozotocin-treated diabetic rats; and Group 4: Streptozotocin-treated diabetic rats receiving celecoxib treatment (50 mg/kg, b.i.d., p.o.). The body weights of all the groups were monitored daily and blood glucose levels were measured on days 1, 4, and 7. On day 8, animals were sacrificed and eyes were enucleated and frozen for further analysis. All the animals 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.

2.3. Semi-quantitative analysis of VEGF mRNA

The retinas were isolated, weighed and total RNA was extracted using RNA STAT-60™ RNA isolation kit (TEL-TEST, Friendswood, TX). The RNA pellet was dissolved in autoclaved water, quantified for RNA and a volume equivalent to 3 μg was taken to amplify VEGF mRNA using access RT-PCR kit as previously described (Aukunuru et al., 2002). The primers used can detect all the five known splice variants of VEGF. The cyclooxygenase-2 mRNA was amplified by using corresponding primers (Oxford Biomedical Research) following reverse transcriptase reaction for 60 min at 48 °C. The amplification was done for 35 cycles of incubation at 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 2 min. Finally an extension step at 72 °C was carried out for 7 min. The VEGF and cyclooxygenase-2 mRNA

expressions were normalized to 18S rRNA expression. The products were separated using electrophoresis on a 2% agarose gel and $VEGF_{164}$ mRNA (462 bp), $VEGF_{120}$ mRNA (330 bp), COX-2 (724 bp), and 18S rRNA (495 bp) were identified and quantified using densitometric analysis (Nucleovision™ Imaging System, Nucleotech, San Mateo, CA).

2.4. Estimation of VEGF protein

The retinas were homogenized in phosphate buffered saline (PBS) containing protease inhibitor (Boehringer Mannhem, Germany) and a volume equivalent to 50 μg protein was taken for VEGF protein estimation. The VEGF protein in the retinal lysates was estimated using an enzyme-linked immunosorbant assay (ELISA). A mouse VEGF ELISA kit capable of detecting both VEGF164 and VEGF120 isoforms was obtained from R&D Systems, Minneapolis. The antibodies in the kit have >95% cross-reactivity with rat VEGF. This kit has been previously used to detect VEGF in rat tissues (Gloddek et al., 1999).

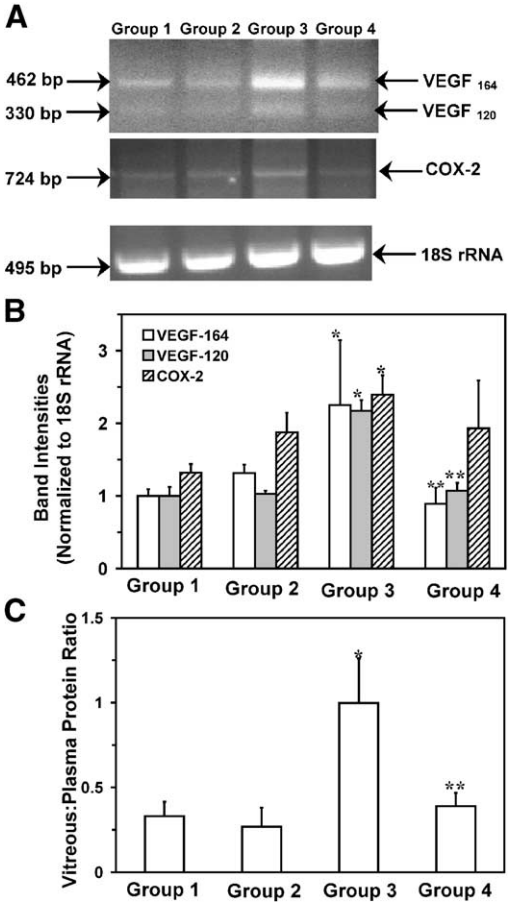

Fig. 1. The effect of celecoxib (50 mg/kg; b.i.d., p.o.) treatment on diabetes-induced retinal VEGF and cyclooxygenase-2 mRNA expression and vascular leakage. (A) RT-PCR analysis of retinal VEGF mRNA, cyclooxygenase-2 mRNA, and 18S rRNA. (B) The band intensities of VEGF and cyclooxygenase-2 mRNAs normalized to 18S rRNA. (C) Vitreous/plasma protein ratio. Data is expressed as a mean ± S.D. for $n=4$. *Group 3 vs. Group 1 and **Group 4 vs. Group 3 at $P<0.05$. Key: Group 1—Citrate buffer treated control rats; Group 2—Streptozotocin-treated nondiabetic rats; Group 3—Streptozotocin-treated diabetic rats; and Group 4—Streptozotocin-treated diabetic rats receiving celecoxib treatment.

Table 1
The ocular tissue concentrations of celecoxib on day 8

Tissue	Celecoxib levels
Plasma	84.99 ± 24.33
Retina	24.8 ± 6.6
Vitreous	1.93 ± 1.2
Cornea	6.9 ± 0.93
Lens	1.71 ± 0.86

Data is expressed as mean ± S.D. for $n=4$. The concentration is expressed as ng/ml in plasma and as ng/mg in other tissues.

2.5. Estimation of vitreous protein

Frozen rat eyes were cut along the corneo-scleral junction. The posterior portion was placed in an eppendorff tube and maintained at room temperature until the frozen vitreous liquefied. The liquefied vitreous was isolated and centrifuged at $13{,}000 \times g$ for 10 min. The supernatant was collected and diluted ten times. The protein content was estimated and the vitreous: plasma protein ratio was taken as a measure of the blood–tissue barrier integrity.

2.6. Estimation of celecoxib in ocular tissue samples

The ocular tissues were homogenized in PBS (200 μl). Internal standard (budesonide, 100 ng) was spiked and 2 ml of methylene chloride was added and mixed well for 3 min. The contents were centrifuged and the organic layer was collected and dried. The pellet was reconstituted in 150 μl of mobile phase (70:30 mixture of acetonitrile and water (0.06% acetic acid)), vortexed for 2 min, centrifuged and 100 μl of the supernatant was injected onto a HPLC column (Microsorb, C18, 5 μm, 25 cm). The mobile phase was maintained at a flow rate of 0.8 ml/min (Waters, 616 pump). Celecoxib was detected using a photo diode array detector (λ: 250 nm) (Waters, PDA 996). Celecoxib was quantified using standards processed similarly.

2.7. Statistical analysis

Data is expressed as mean ± S.D. Comparison of mean values was done using a paired Student's *t*-test. Differences were considered statistically significant at $P<0.05$.

3. Results

3.1. Celecoxib treatment inhibits diabetes-induced retinal VEGF mRNA expression and vascular leakage

After short time induction of diabetes with streptozotocin, retinal VEGF mRNA expression was increased and this increase was inhibited by celecoxib treatment (Fig. 1A). The VEGF mRNA expression was 2.3 ± 0.8-fold in diabetic retinas compared to 0.93 ± 0.3-fold with celecoxib treatment (Fig. 1B). Cyclooxygenase-2 mRNA expression in diabetic retinas was increased by 2.5 ± 0.3-fold (Fig. 1B). However, this expression was not significantly reduced by celecoxib (Fig. 1B). The vitreous/plasma protein ratio was determined as a measure of blood–ocular barrier integrity. This ratio was increased in diabetic rats to 1.1 ± 0.1 compared to control rats of 0.35 ± 0.1 (Fig. 1C). Treatment of diabetic animals with celecoxib reduced this ratio to 0.4 ± 0.1. The VEGF protein levels in the retinal lysates of rats in control, streptozotocin-treated non-diabetic, streptozotocin-treated diabetic, and streptozotocin-treated diabetic with celecoxib treatment groups were 32.5 ± 2.8, 29.6 ± 3.8, 34.8 ± 5.8, and 27.6 ± 4.2 pg/ml, respectively.

3.2. Celecoxib reaches retina following oral administration

Following oral administration, celecoxib levels were detected in ocular tissues as follows: retina>cornea>vitreous>lens (Table 1). Plasma celecoxib levels were 84.99 ± 24.3 ng/ml.

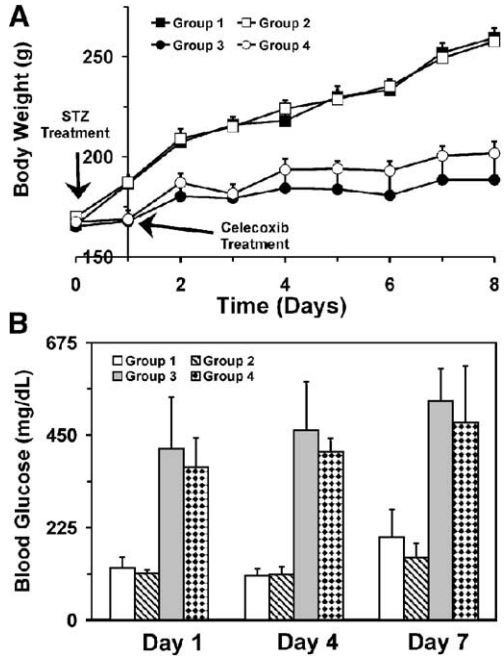

Fig. 2. Effect of celecoxib treatment (50 mg/kg, b.i.d., p.o.) on (A) body weights and (B) blood glucose levels in streptozotocin-treated diabetic rats. Data is expressed as mean ± S.D. for $n = 4$. Key: Group 1—Citrate buffer treated control rats; Group 2—Streptozotocin-treated nondiabetic rats; Group 3—Streptozotocin-treated diabetic rats; and Group 4—Streptozotocin-treated diabetic rats receiving celecoxib treatment.

3.3. Celecoxib treatment has no effect on hyperglycemia or body weights

Streptozotocin-induced hyperglycemia is associated with a reduction in body weights. It was previously shown that pretreating animals with a cyclooxygenase-2 specific inhibitor prevented hyperglycemia and reduction in body weights (Tabatabaie et al., 2000). However, we did not observe a reduction in the hyperglycemia (Fig. 2B) or a reversal of reduction in body weights following celecoxib treatment (Fig. 2A).

4. Discussion

The onset of diabetes in rats is associated with an increase in the pancreatic levels of inflammatory mediators such as prostaglandins and thromboxanes (Gonzalez et al., 1999). Retinas of experimental diabetic rats as well as human diabetic subjects exhibit vasodilatation, vascular leakage, and the accumulation and migration of platelets and leukocytes (Gallin and Snyderman, 1999; Garner, 1994; Miyamoto et al., 1999), factors associated with various inflammatory conditions (Adamis, 2002). Exposure to high glucose concentration induces the production of inflammatory mediators via the cyclooxygenase pathway in rat retinal endothelial cells (Sone et al., 1996), human retinal pericytes, and human retinal microvascular endothelial cells (Rymaszewski et al., 1992). Products of arachidonic acid metabolism via cyclooxygenase (prostaglandings, prostacyclin, and thromboxane), known to play a major role in the intraocular inflammation process (Eakins, 1977), are capable of inducing VEGF expression (Cheng et al., 1998; Hata et al., 2000; Nie et al., 2000). Thus, anti-inflammatory agents capable of inhibiting cyclooxygenase will likely be useful in alleviating retinal vascular leakage and neovascularization in diabetes. Joussen et al. (2002) demonstrated the ability of aspirin and meloxicam to reduce retinal VEGF expression and vascular leakage in day 8 diabetic rats. However, both these agents can inhibit cyclooxygenase-1 and cyclooxygenase-2, with low cyclooxygenase-2/cyclooxygenase-1 selectivity ratios of 0.15 and 0.56–23, respectively (Gierse et al., 1999; Giuliano and Warner, 1999). In this study we demonstrated that celecoxib, a highly selective and potent inhibitor of cyclooxygenase-2, inhibits diabetes-induced retinal vascular leakage and VEGF expression.

We observed a significant increase in the retinal expression of VEGF mRNA in diabetic rats when compared to normal rats, with a corresponding increase in the expression of cyclooxygenase-2 mRNA (Fig. 1A). This is consistent with the previous observations that the retinal cyclooxygenase-2 (Carmo et al., 2000) and VEGF (Qaum et al., 2001) expressions are elevated within 7 days of inducing diabetes

in rats. This elevated cyclooxygenase-2 expression results in the increased production of prostaglandins and oxidative stress, which can induce VEGF expression (Ayalasomayajula and Kompella, 2002; Cheng et al., 1998; Kuroki et al., 1996). Also, cyclooxygenase-2 activity is known to inhibit apoptosis either by inducing anti-apoptotic genes such as Bcl-2, par-4, and H-ras (Sheng et al., 1997, 1998; Zhang and DuBois, 2000) or by inhibiting pro-apoptotic protease caspase-3 (McGinty et al., 2000). Since VEGF is known to inhibit apoptosis (Pidgeon et al., 2001), it can be hypothesized that the anti-apoptotic activity of cyclooxygenase-2 might also be mediated via the induction of VEGF expression. In addition, unlike cyclooxygenase-1, which is located in the cytoplasm, cyclooxygenase-2 is located in the nuclear envelope (Parfenova et al., 2001), allowing the products of cyclooxygenase-2 to possibly activate transcriptional factors such as nuclear factor kappa beta (NF-κβ), activator protein (AP)-1, and peroxisomal proliferator activator receptor (PPAR)-γ (Straus and Glass, 2001), which are known to induce the expression of VEGF (Ferrara, 2000). In the current study, we observed that celecoxib treatment reduces diabetes-induced retinal VEGF mRNA expression (Fig. 1A) without any significant decrease in the cyclooxygenase-2 expression (Fig. 1B), suggesting that celecoxib inhibits VEGF expression by inhibiting the cyclooxygenase-2 enzyme activity.

We did not measure cyclooxygenase-2 protein levels. However, a previous report indicated an elevation of cyclooxygenase-2 protein in diabetic rat retinas (Carmo et al., 2000) and another report indicated a direct relationship between cyclooxygenase-2 mRNA levels and the protein synthesis in retinal pigment epithelial cells (Chin et al., 2001). We did not determine the effect of celecoxib in non-diabetic rats. However, it should be noted that previous studies indicated that in normal rats, the retinal expression of cyclooxygenase-2 (Carmo et al., 2000), retinal vascular permeability and VEGF expression (Joussen et al., 2002; Qaum et al., 2001) are not altered upon treatment with anti-inflammatory agents.

The elevation in the retinal VEGF expression is associated with the induction of retinal vascular leakage (Ozaki et al., 1997; Qaum et al., 2001). The blood–ocular vascular leakage can be determined by normalizing vitreal or retinal levels of exogenously administered radio-labeled tracers, fluorescein isothiocyanate-bovine serum albumin, or Evans blue to those in the plasma (Vinores, 1995). Alternatively, such leakage can be estimated by comparing vitreal endogenous protein concentration to plasma protein concentration (Carmo et al., 2000), which was used in this study. However, this measurement, which measures protein concentration only in the vitreous but not the retina, is indicative of blood-retinal and blood-aqueous barrier leakage. In 1 month diabetic rats, the vitreous protein content increased from 0.7 to 3.9 (Shires et al., 1993). We observed a significant increase in the normalized vitreal protein concentration in 1 week in diabetic rats compared to control rats (Fig. 1C). Treatment with celecoxib normalized this ratio to control levels, suggesting that cyclooxygenase-2 activity is responsible for the vascular leakage. Since vitreous/plasma protein ratio is reflective of vascular leakage from both blood-aqueous and blood-retinal barriers and because blood-retinal barrier breakdown has been reported within a week in diabetic rats (Qaum et al., 2001), our results are consistent with the inhibition of vascular leakage from both these barriers by celecoxib. Consistent with previous observations (Joussen et al., 2002), we did not observe any change in VEGF protein levels in the diabetic rat retinal lysates. This is possibly because the measured VEGF isoforms, $VEGF_{165}$ and $VEGF_{121}$, are secreted upon formation (Leung et al., 1989), and the cellular levels may not reflect the effective levels of these isoforms.

Cycloxygenase-2 is constitutively expressed in the islets of pancreas (Sorli et al., 1998) and its expression is elevated during early stage of diabetes, suggesting that cyclooxygenase-2 has a role in diabetic pathology. Possibly for this reason, pretreatment (15 min prior to streptozotocin treatment) with cyclooxygenase-2 inhibitors prevented the destruction of β-cells and development of diabetes upon low dose streptozotocin injection in rats (Tabatabaie et al., 2000). However, we did not observe any reduction of blood glucose levels with celecoxib treatment in diabetic rats (Fig. 2B). This is possibly because of the higher dose of streptozotocin (60 mg/kg) used and 1-day delay in the initiation of cyclooxygenase-2 inhibitor administration following streptozotocin treatment. Since hyperglycemia and diabetic state are essential for this study, pretreatment with cyclooxygenase-2 inhibitors is inappropriate for the purpose of this study. With celecoxib treatment, we did not observe any reversal of reduced body weights in diabetic rats (Fig. 2A), suggesting that other metabolic factors mediated by hyperglycemia may contribute to weight loss.

For any drug to be effective, sufficient concentrations should reach the target tissues upon administration. Although a previous study indicated ocular distribution of celecoxib (Paulson et al., 2000), its distribution to individual ocular tissues including retina is not known. In the present study, we observed that celecoxib distribution to the retina is the highest followed by cornea, vitreous, and lens (Table 1). Thus, celecoxib reaches the retina following oral administration.

In conclusion, for the first time, we observed that oral cyclooxygenase-2 inhibitor (celecoxib) treatment delivered drug to the retina and decreased retinal VEGF mRNA expression and vascular leakage in streptozotocin-induced diabetic rats. The findings of this study indicate the potential value of celecoxib in treating diabetic retinal changes.

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