Expression of Cyclooxygenase-1 and -2 in Normal and Glaucomatous Human Eyes

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Purpose. Primary open-angle glaucoma (POAG) is the predominant form of chronic glaucoma, but the underlying pathologic mechanisms are largely unknown. Because prostaglandins (PGs) have been introduced into POAG treatment with remarkable success, this study was undertaken to investigate whether a change in the expression of the PG-synthesizing enzymes cyclooxygenase (COX)-1 and -2 might be involved in the pathogenesis of POAG.

Methods. Expression of COX-1 and -2 was assessed by confocal laser microscopy, immunohistochemistry, Western blot analysis, and real-time RT-PCR in human eyes with different forms of glaucoma (primary open-angle, angle-closure, congenital juvenile, and steroid-induced), as well as in age-matched control eyes. Additionally, PGE_2 was measured in aqueous humor by means of an enzyme-linked immunoassay as a product of COX activity.

Results. In normal eyes, ocular COX-1 and -2 expression were largely confined to the nonpigmented secretory epithelium of the ciliary body. By immunohistochemistry and real-time RT-PCR, COX-2 expression was completely lost in the nonpigmented secretory epithelium of the ciliary body of eyes with end-stage POAG, whereas COX-1 expression was unchanged. By immunohistochemistry, in the ciliary bodies of eyes in five patients with diagnosis of early POAG, eyes in two had complete loss of COX-2 expression and in three showed only a few remaining scattered COX-2–expressing cells. COX-2 expression in the ciliary body was also lost in patients with steroid-induced glaucoma and was reduced in patients receiving topical steroid treatment. Eyes of patients with either congenital juvenile or angle-closure glaucoma showed COX-2 expression indistinguishable from control eyes. Aqueous humor of eyes with POAG contained significantly less PGE_2 than control eyes.

Conclusions. Both cyclooxygenase isoforms are constitutively expressed in the normal human eye. Specific loss of COX-2 expression in the nonpigmented secretory epithelium of the ciliary body appears to be linked to the occurrence of POAG and steroid-induced glaucoma. (*Invest Ophthalmol Vis Sci.* 2001;42:2616–2624)

Primary open-angle glaucoma (POAG) is among the dominating ocular diseases in industrialized countries.[1,2] Intraocular pressure (IOP) increases in persons older than 50 years without any subjective symptoms until irreversible damage occurs.[1,2] If untreated, it leads to impaired vision and ultimately to blindness in approximately 2% of the elderly population.[1,2] The pathogenesis of POAG is still largely unknown, although in some patients there is evidence of genetic linkage to *GLC1A/B*.[3–5]

POAG shares several characteristics with corticosteroid hormone–induced secondary open-angle glaucoma. Prolonged topical administration of glucocorticoids to the eye causes increased IOP and a decay of vision in about 30% of the patients.[6,7] Glucocorticoids are well known to inhibit the expression of cyclooxygenase (COX)-2, a key enzyme in the formation of prostaglandins (PGs).[8] These agents play an important role in the regulation of IOP.[9] Therefore, reduction of PGs in the eye by inhibition of COX-2 expression appears to be a mechanism by which glucocorticoids cause secondary glaucoma. In fact, synthetic $PGF_{2\alpha}$ analogues such as latanoprost are remarkably effective in glaucoma therapy.[9–11]

PG production involves two isoenzymes, COX-1 and -2.[12–14] Today, it is generally assumed that COX-1 is constitutively expressed in most tissues and displays the characteristics of a "housekeeping" enzyme.[14] By contrast, COX-2 is the product of an immediate early gene that is rapidly inducible and tightly regulated.[12,13] Under normal conditions, COX-2 expression is highly restricted to certain organs including the central nervous system (CNS)[15,16] and the kidney,[17] but COX-2 expression can be dramatically increased in various tissues after initiation of transcription by activating factors including different proinflammatory cytokines,[18] sheer forces in the arterial wall,[19] or salt deprivation.[17]

In view of the potential importance of cyclooxygenases in glaucoma, the localization and expression of these enzymes in normal and glaucomatous human eyes was investigated.

Materials and Methods

Informed consent to eye and tissue donation was obtained and the study adhered to the tenets of the Declaration of Helsinki.

Immunofluorescence and Confocal Laser Microscopy

Anterior segments prepared from normal human donor eyes were embedded in optimal cutting temperature compound (OCT; Tissue-Tek; Miles Laboratories, Elkhart, IN) and snap frozen in isopentane-liquid nitrogen. Six-micrometer cryostat sections were thawed onto gelatin-coated glass slides, air dried, and fixed in paraformaldehyde (4%, 10 minutes) followed by incubation in Tris-buffered saline (TBS; pH 7.4) containing 10% normal donkey serum and 1% bovine serum

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albumin (BSA) for 60 minutes at room temperature (RT). After the slides were rinsed in TBS, incubation was continued with the polyclonal goat anti-human COX-1 or -2 antiserum (dilution, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) in TBS containing 1% BSA overnight at 4°C. After slides were again rinsed with TBS, binding sites were detected using donkey anti-goat IgG conjugated with cyanine-3 (diluted 1:200 in TBS, for 1 hour at RT; Santa Cruz Biotechnology). Sections were coverslipped with TBS-glycerol (1:1, pH 8.6) and examined by confocal laser scanning microscopy (MRC 1000; Bio-Rad, Richmond, CA).

Electron Microscopic Immunohistochemistry

Tissue specimens of normal human donor eyes were fixed in a solution of paraformaldehyde (4%) and glutaraldehyde (0.1%) in 0.1 M cacodylate buffer (pH 7.4; 1–2 hours at 4°C). After specimens were rinsed, they were dehydrated serially to 70% ethanol at −20°C, embedded in resin (LR White; Electron Microscopy Sciences, Fort Washington, PA), and polymerized for 24 hours at 45°C. Ultrathin sections were incubated successively in drops of TBS, 0.5% ovalbumin and 0.5% fish gelatin in TBS, anti-human COX-2 antibody (diluted 1:300 in TBS-ovalbumin overnight at 4°C), and 10 nm gold-conjugated secondary antibody (diluted 1:30 in TBS-ovalbumin; for 1 hour at RT; BioCell, Cardiff, UK). After a rinse, sections were stained with uranyl acetate and examined with an electron microscope (EM 906; Leo, Oberkochen, Germany).

Protein Extraction and Western Blot Analysis

Ciliary processes from normal donor eyes (<4 hours after death) were homogenized in lysis buffer, containing 0.15 M NaCl, 0.1 M TBS, 50 mM diethyldithiopyrocarbonate, 1% (vol/vol) Tween-20, and 10 mM phenylmethylsulfonyl fluoride. For Western blot analysis, protein (50 μg/lane) was loaded on a 10% SDS-polyacrylamide gel and electroblotted onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Membranes were probed with polyclonal goat anti-human COX-2 antiserum (diluted 1:1000), followed by a horseradish peroxidase (HRP)–linked donkey anti-goat IgG secondary antibody (diluted 1:1000; Santa Cruz Biotechnology). Blots were developed with enhanced chemiluminescence (ECL) detection reagents (Amersham, Arlington Heights, IL) and exposed to film for 2 to 10 minutes.

RT-PCR Analysis

RNA was prepared using a kit (RNeasy; Qiagen, Hilden, Germany) from the ciliary processes of normal human donor eyes, which were homogenized with a Dounce homogenizer (Bellco Glass Co., Vineland, NJ) in lysis buffer (RLT; Qiagen).

For real-time RT-PCR, the following primers and probes were selected. For COX-2 (accession number M90100): 5′-CACAATCTGGCTGAGGGAACA-3′ (upstream) and 5′-ACTGGTATTTCATCTGCCTGCTCT-3′ (downstream), 5′-(FAM)CCTGCCAGCAA (TAMRA)TTTGCCTGGTGAAT-3′ (internal oligonucleotide probe). Human β-actin (accession number M12481) was used for quantification, and the following primers and probes were used: 5′-TCACCCACACTGTGCCCATCTACGA-3′ (upstream), 5′-GGATGCCACAGGATTCCATACCCA-3′ (downstream), and 5′-(FAM)TATGCTC(TAMRA)TCCCTCACGCCATCCTGCGT-3′ (internal oligonucleotide probe).

PCR reagents were purchased from Perkin Elmer-Applied Biosystems (Weiterstadt, Germany), primers and probes from TIB Molbiol (Berlin, Germany), and rTth-DNA-polymerase from Diagonal (Münster-Roxel, Germany). The PCR mixture (25 μl total volume) consisted of 200 nM primer, 100 nM probe, 300 nM dNTPs, 3 mM $Mn(OAc)_2$, 1 μl total RNA, 0.02 U rTth-DNA polymerase, and 5× *Taq* buffer (*Taq*Man EZ; containing 250 mM Bicine buffer, 575 mM potassium acetate, 0.05 mM EDTA, 40% [wt/vol] glycerol [pH 8.2]; Perkin Elmer-Applied Biosystems). Amplification and detection were performed with a sequence detection system (Prism 7700; Perkin Elmer-Applied Biosystems) with the following profile: 50°C for 2 minutes, 60°C for 30 minutes, and 95° for 5 minutes and 45 cycles of 94°C for 15 seconds and 60°C for 1 minute. Quantification was performed by determining the threshold cycle (C_T). C_T is proportional to the amplified starting copy number of cDNA or RNA.[20] All reactions were controlled by standards (no-template control and standard positive control). The quantity of mRNA was calculated by normalizing the C_T of genes of interest to the C_T of the housekeeping protein β-actin of the same RNA probe, according to the following formula: $\Delta C_T = C_T^{\text{COX-2 mRNA}} - C_T^{\beta\text{-actinmRNA}}$.

RNA Isolation and RT-PCR Analysis from Paraffin-Embedded Tissue

To investigate COX-2-mRNA expression in glaucomatous eyes, total-RNA was isolated from paraffin-embedded eye sections fixed with 4% paraformaldehyde (fixation time, <12 hours). Ten-micrometer-thick slices of respective eyes were cut. Afterward, ciliary processes were separated by means of microdissection and transferred into cups (Eppendorf, Fremont, CA). All isolation steps were performed at room temperature in the same cup. First, paraffin was dissolved in Roticlear (Carl Roth GmbH, Karlsruhe, Germany). The solution was then centrifuged at full speed for 5 minutes, and the supernatant was removed. This procedure was followed by three intensive washing steps with ethanol (96%) to remove the Roticlear agent. After the last wash, ethanol was totally removed and the pellet was air dried. All steps were performed in detail, according to the manufacturer's protocol (Mini RNeasy; Qiagen) Protocol. After adding 350 μl of lysis buffer, the tissue samples were shredded with a microshredder.

For RT-PCR, 1 μg eye tissue RNA was reverse transcribed into cDNA (Superscript II Polymerase; Life Technologies GmbH, Eggenstein, Germany). Special primers were designed for the detection of potential fragmented RNA, according to reports in the respective literature.[21,22] In particular, they should amplify very short target sequences. The sequences of the oligonucleotide primers (Eurogentec, Liege, Belgium) were COX-1 (79 bp): 5′-CAC AGT GCG CTC CAA CCT TA-3′ (upstream), 5′-TGG AGA AAG ACT CCC AGC TGA-3′ (downstream), and 5′-(FAM) CTT ATC CCC AGT CCC CCC ACC TAC AAC TC (TAMRA)-3′ (internal oligonucleotide probe); COX-2 (88 bp): 5′-GCT GGA ACA TGG AAT TAC CCA-3′ (upstream), 5′-CTT TCT GTA CTG CGG GTG GAA-3′ (downstream), and 5′-(FAM) CCT GCC AGC AA (TAMRA) T TTG CCT GGT GAAT-3′ (internal oligonucleotide probe); and β-actin (79 bp): 5′-AGT ACT CCG TGT GGA TCG GC-3′ (upstream), 5′-GCT GAT CCA CAT CTG CTG GA-3′ (downstream). PCR was conducted in a real-time thermal cycler (ABI 7700; Perkin Elmer-Applied Biosystems) using a kit (Ampli*Taq* Gold; Perkin Elmer-Applied Biosystems).

Light Microscopic Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded eye sections from the archives of the Department of Ophthalmology of the University of Erlangen-Nürnberg and on donor eyes from the Glaucoma Research Foundation (San Francisco, CA), both fixed with 4% paraformaldehyde. The labeled streptavidin-biotin method performed with a kit (LSAB Plus; Dako, Glostrup, Denmark) was applied according to the manufacturer's instructions. Briefly, after endogenous peroxidase activity of the tissue was quenched with 3% hydrogen peroxide in distilled water for 5 minutes, sections were incubated with proteinase K (0.02 mg/ml in TBS; pH 8) for 20 minutes for antigen unmasking. After sections were rinsed, they were incubated with a polyclonal goat anti-human COX-1 or -2 antibody (diluted 1:100 in TBS), with the biotinylated link antibody and the HRP-conjugated streptavidin, respectively, for 30 minutes each. 3-Amino 9-ethyl carbazole (10 minutes; Dako) was used as a chromogenic substrate. The sections were counterstained with Mayer's hemalun (Chroma, Köngen, Germany) and mounted (Aquatex; Merck, Darmstadt, Germany). After staining procedures, sections were coded and assessed by blinded observers (CM, USS) for presence of COX-1 and -2 immunoreactivity (IR).

Negative controls included incubation of ocular tissues with nonimmunized goat serum, omitting the primary antibody, and incubation

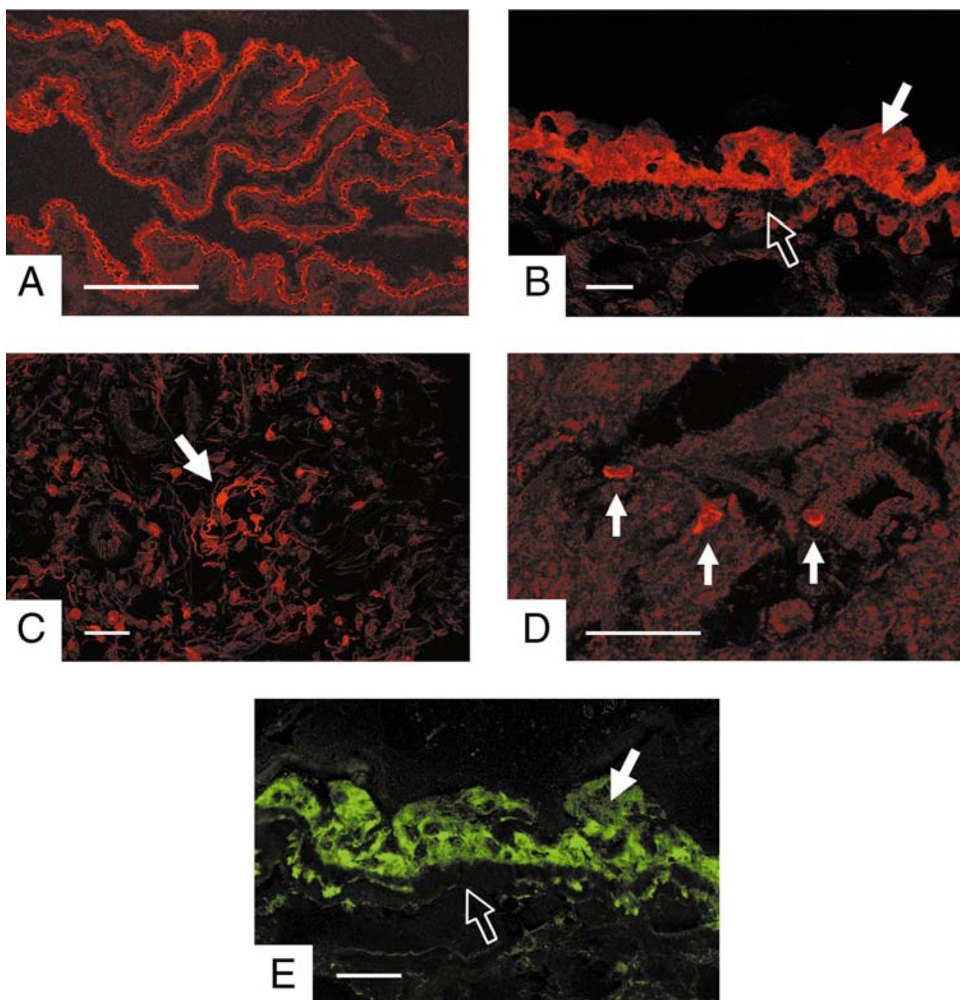

FIGURE 1. Indirect immunofluorescence and confocal laser scanning microscopy for COX-1 and -2 protein in normal human donor eyes. (**A**) COX-2 IR (*bright red*) was constitutively found in the epithelium of the ciliary body of normal human donor eyes. (**B**) Differential expression of COX-2 in the two layers of the ciliary epithelium. COX-2 protein stained intensely in the secretory nonpigmented epithelial layer (*filled arrow*) in contrast to the lightly stained pigmented epithelial layer (*open arrow*). The ciliary body could be seen below the epithelial layers. (**C**) COX-2 IR in connective tissue cells of the iris stroma (*arrow*). (**D**) Stromal cells located in the ciliary connective tissue between the ciliary muscle and the posterior chamber showing COX-2 IR (*arrows*). (**E**) Localization of COX-1 IR in the nonpigmented epithelial layer of the ciliary body (*filled arrow*). The pigmented epithelial layer showed no COX-1 IR (*open arrow*). Scale bar, (**A**) 250 μm; (**B**, **E**) 25 μm; (**C**, **D**) 50 μm.

of negative control tissues (muscle). Positive tissue controls included sections of brain, kidney, and uterus (data not shown).

Measurements of PGE_2 in Aqueous Humor

Aqueous humor obtained at eye surgery was immediately snap frozen in liquid nitrogen. PGE_2 concentrations were determined using a commercially available enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI). The reliable limit of quantification for PGE_2 was 15 pg/ml, and the coefficient of variation was less than 14% within the calibration range (15–1000 pg/ml).

RESULTS

Expression of COX-1 and -2 in Normal Human Eyes

The expression of COX-1 and -2 at different sites in the human eye was investigated with immunofluorescence and confocal laser microscopy (Fig. 1). In eight apparently normal eyes of human organ donors (no known ocular diseases; age range, 38–85 years), which were enucleated shortly after death (<8 hours) COX-1 and -2 IR were most prominent in the cells of the nonpigmented epithelial layer of the ciliary body (Figs. 1A, 1B, 1E). By contrast, only minor expression of both cyclooxygenases was observed in other areas of the anterior eye segments. Some COX-2 IR was observed in connective tissue cells scattered in the iris stroma (Fig. 1C), particularly in the pupillary and iris root area, as well as in some stromal cells between the ciliary muscle and the anterior chamber (Fig. 1D).

The ultrastructural localization of COX-2 was revealed by immunoelectron microscopy. The strongest staining was found in the basolateral membranes of the nonpigmented secretory epithelial cells (Fig. 2A), which secret the eye's aqueous humor. Additionally, COX-2-protein was found in association with intercellular contacts (maculae and zonulae adherentes, Figs. 2B, 2C), and in the rough endoplasmic reticulum including the perinuclear cistern (Fig. 2D). By contrast, no COX-2 IR was detected in the pigmented epithelial layer of the ciliary body. A schematic overview of the distribution of COX-2 in the ciliary epithelium based on immunoelectron microscopy is shown in Figure 2E.

Constitutive expression of COX-2-protein was further confirmed by Western blot analysis using protein extracts from human ciliary body tissue. The antibody raised against human COX-2 protein detected a double band of approximately 68 to 75 kDa, corresponding to the molecular weight of COX-2 (Fig. 3). The detected bands were also in the same range as a parallel-run positive control.

Expression of COX-1 and -2 in Glaucomatous Eyes

Paraffin-embedded sections of surgically enucleated blind and painful glaucomatous eyes were obtained from the archives of the Department of Ophthalmology at the University of Erlangen, Germany. Five eyes with absolute (end-stage) POAG (72 ± 6 years), five with secondary angle-closure glaucoma due to rubeosis iridis (78 ± 7 years), and three with congenital juve-

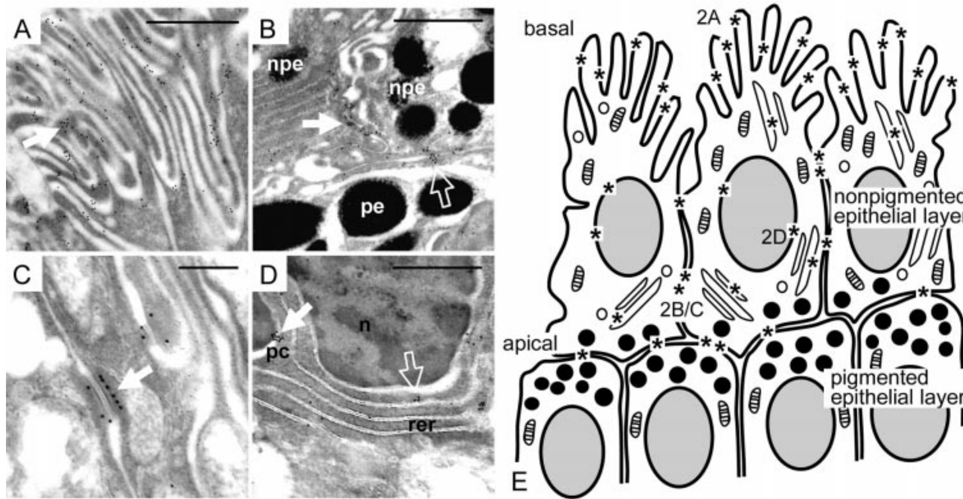

FIGURE 2. Electron microscopic immunogold labeling for COX-2 protein in the ciliary epithelium of normal human donor eyes. (**A**) A strong immunogold reaction for COX-2 was localized to the basolateral membranes of the nonpigmented secretory epithelial cells (*arrow*). (**B**) Association of gold labeling for COX-2 with intercellular contacts between cells of the nonpigmented epithelial layer (*filled arrow*) and between cells of the nonpigmented epithelial layer and the pigmented epithelial layer (*open arrow*). (**C**) Presence of gold labeling indicating COX-2 protein along a macula adherens type of intercellular contact (*arrow*). (**D**) Localization of COX-2 protein in the perinuclear cistern (*filled arrow*) and rough endoplasmic reticulum (*open arrow*). (**E**) Overview of the ultrastructural distribution of COX-2 protein (*stars*) in the two epithelial layers of the ciliary body. The anatomic regions shown in (**A**) through (**D**) are indicated. npe, nonpigmented epithelial layer; pe, pigmented epithelial layer; n, nucleus; rer, rough endoplasmic reticulum; pc, perinuclear cistern. Scale bar, (**A**) 1 μm; (**B**) 0.8 μm; (**C**) 0.2 μm; (**D**) 0.5 μm.

nile glaucoma (44 ± 2 years) were analyzed. In the end-stage POAG eyes, COX-2 expression was completely lost in the nonpigmented epithelium of the ciliary body (Figs. 4C, 4D). This loss was very specific in two respects. First, loss of COX-2 expression was restricted to the nonpigmented ciliary epithelium and did not affect COX-2 expression in the connective tissue cells of the ciliary body and iris stroma (4D, inset). Second, COX-1 expression remained unchanged, both in the ciliary body and in other sites of ocular COX-1 expression (Figs. 4J, 4K). By contrast, all eyes with congenital juvenile and angle-closure glaucoma exhibited an expression of COX-2 very similar to that in normal control eyes (Figs. 4F, 4G).

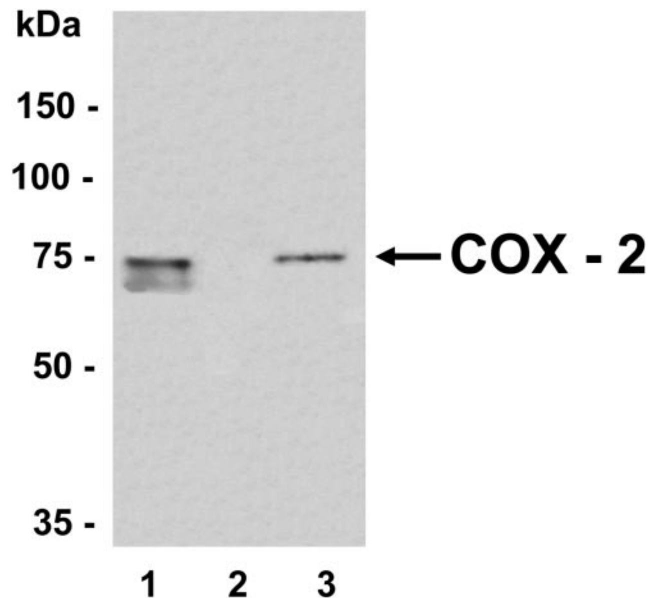

FIGURE 3. Western blot analysis demonstrating the expression of COX-2 protein in the ciliary body of normal human donor eyes. *Lane 1*: Total cell lysates prepared from ciliary body tissue (50 μg protein); *lane 2*: purified COX-1 (0.1 μg); and *lane 3*: purified COX-2 protein (0.1 μg) were electrophoresed, transferred onto nitrocellulose membrane, and immunostained with polyclonal antiserum against human COX-2. The antibody used was highly specific for COX-2 and did not detect COX-1. Unpurified COX-2 proteins derived from cell lysates or tissues typically produced a double or triple band in Western blot analysis at 68 to 75 kDa (*lane 1*). Similar results were obtained in three independent experiments.

Equally processed and age-matched normal human eyes obtained from organ donors and eyes enucleated for small juxtapapillary malignant melanomas of the choroid not affecting anterior segment tissues served as control eyes (Figs. 4A, 4B). The latter eyes are regarded as otherwise normal and have the advantage of a very short time until fixation of the tissue can begin. Therefore, postmortem changes can be largely excluded. These eyes showed the same COX-2 expression as observed in normal human donor eyes.

Because glucocorticoids are known to inhibit COX-2 expression in various cell types,[8,14,16] we investigated whether topical ocular steroid treatment affects the COX-2 expression in the ciliary epithelium. Four paraffin-embedded eyes were examined from patients (75 ± 12 years) who had been treated topically with glucocorticoids preceding perforation in ulcerative herpetic keratitis. COX-2 expression was almost completely absent in glucocorticoid-treated eyes (Fig. 4H). Because the analysis was performed on paraffin-embedded sections from the archives of the Department of Ophthalmology at the University of Erlangen, the specimens were not suitable for quantitative investigations, such as Western blot analysis or RT-PCR.

To investigate COX-2 expression in earlier stages of POAG, eyes were obtained from the Glaucoma Research Eye Donor Network (GREDN) of the Glaucoma Research Foundation (time from death to dissection <8 hours). Patients with clinically diagnosed glaucoma in various stages of the disease were registered in their lifetimes by the foundation. All donors agreed that at death their eyes could be immediately enucleated and committed to glaucoma research. After evaluation of the clinical data, five eyes (80.5 ± 4 years) with earlier stages of POAG were found. All patients had typical glaucomatous visual field loss: one had already undergone glaucoma filtration surgery 2 years before his death. The cup-to-disc ratio ranged between 0.7 and 0.95. In addition, we had access to two eyes with ocular hypertension without any clinical signs of glaucoma and one eye with steroid-induced glaucoma. Of the five eyes of patients with POAG, two were completely devoid of COX-2 IR, and three still showed a few scattered COX-2–positive cells in the ciliary epithelium (Fig. 4E). COX-2-protein

was completely absent in the eye with steroid-induced glaucoma (Fig. 4J).

To add further proof to our histologic and biochemical findings (described later), we tried to assess the expression of COX-2 mRNA in paraffin-embedded tissue specimens. We had access to two specimens obtained during eye surgery that were

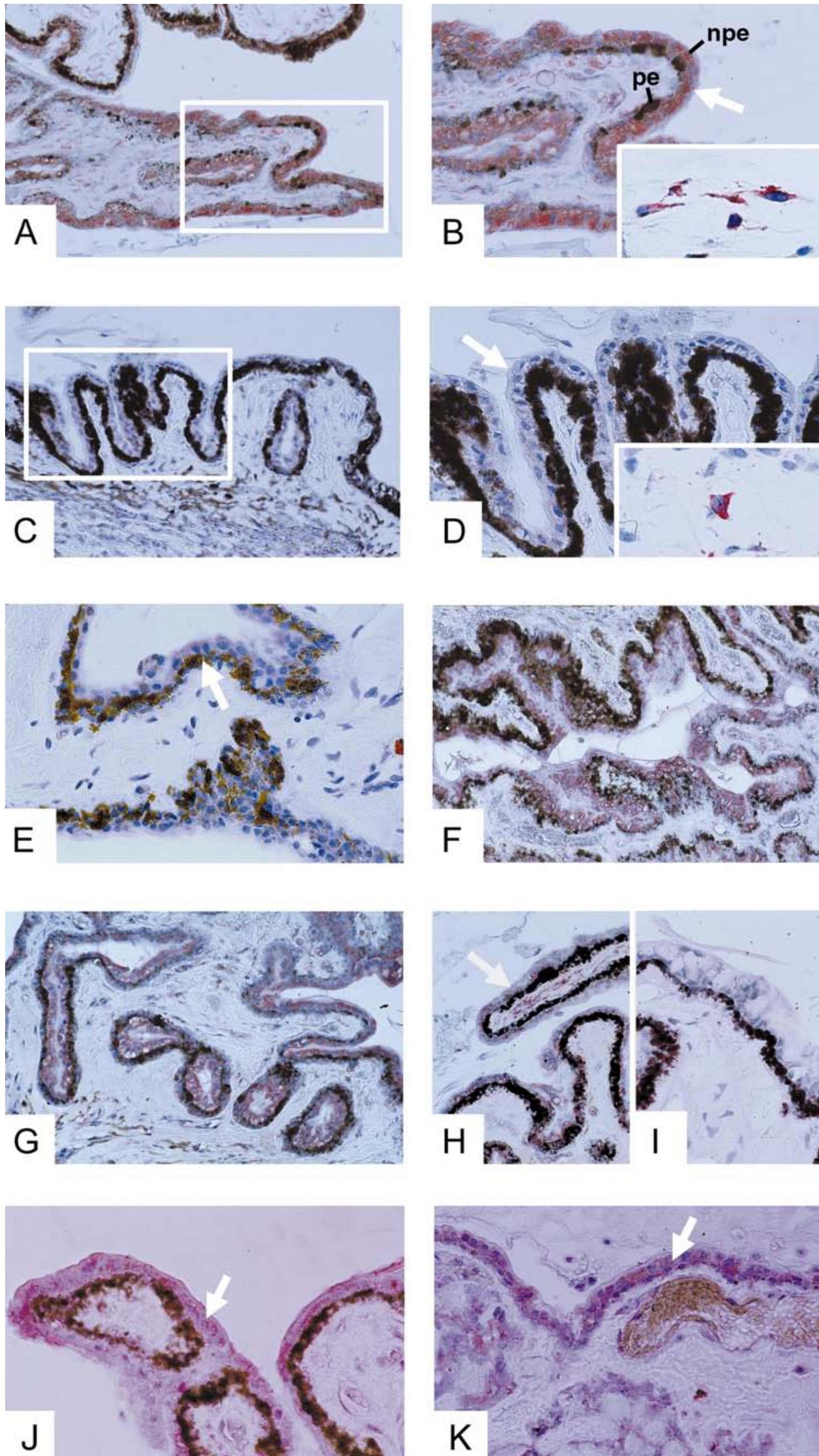

FIGURE 4. Light micrographs of immunohistochemistry for COX-2 protein on paraffin-embedded eye sections. Positive cells show *reddish-brown* staining (in contrast to the *black* melanin pigment of the pigmented epithelial layer of the ciliary body). (**A**) Control eye enucleated for a small juxtapapillary malignant melanoma of the choroid not affecting anterior segment tissues. (**B**) Higher magnification of section in (**A**) shows intense staining of the nonpigmented epithelial layer of the ciliary body (*arrow*). COX-2–positive cells were also found in the connective tissue between the ciliary body and the posterior chamber (*inset*). The staining pattern exactly corresponded to the distribution of COX-2 IR in Figures 1A, 1B, and 1C. (**C**) Ciliary body of an eye with absolute POAG. (**D**) Higher magnification of section in (**C**) shows absence of COX-2 IR in the nonpigmented epithelial layer (*arrow*). COX-2–positive cells were still found in the connective tissue between the ciliary body and the posterior chamber (*inset*). Cell nuclei are stained *blue*. (**E**) Eye with an early form of POAG. IOP was elevated (24 mm Hg) and there were visual field defects. COX-2 IR was clearly reduced compared with control eyes but was still detectable in some cells of the nonpigmented ciliary epithelium (*arrow*). (**F**, **G**) Approximately normal content of COX-2 IR in ciliary epithelia of eyes with congenital juvenile (**F**) and secondary angle-closure (**G**) glaucoma. (**H**) Ciliary body of an eye pretreated with glucocorticoids showed markedly decreased content of COX-2 protein (*arrow*) compared with the control in (**A**). (**I**) Eye with steroid-induced glaucoma (IOP, 25 mm Hg). COX-2 IR was completely absent in this eye. (**J**) Section of control eye shown in (**A**) and (**B**) stained for COX-1 protein showed intense staining of the nonpigmented epithelial layer of the ciliary body (*arrow*). (**K**) Eye with absolute POAG shown in (**C**) and (**D**) stained for COX-1 protein. In sharp contrast to COX-2 IR, no change in COX-1 IR was detected (*arrow*) compared with the control eye (**J**). npe, nonpigmented epithelial layer; pe, pigmented epithelial layer. Original magnification, (**A**, **C**, **F–H**) ×55; (**B**, **D**, **E**, **J**, **K**) ×140; (**B**, **D**, *inset*) ×200; (**I**) ×150.

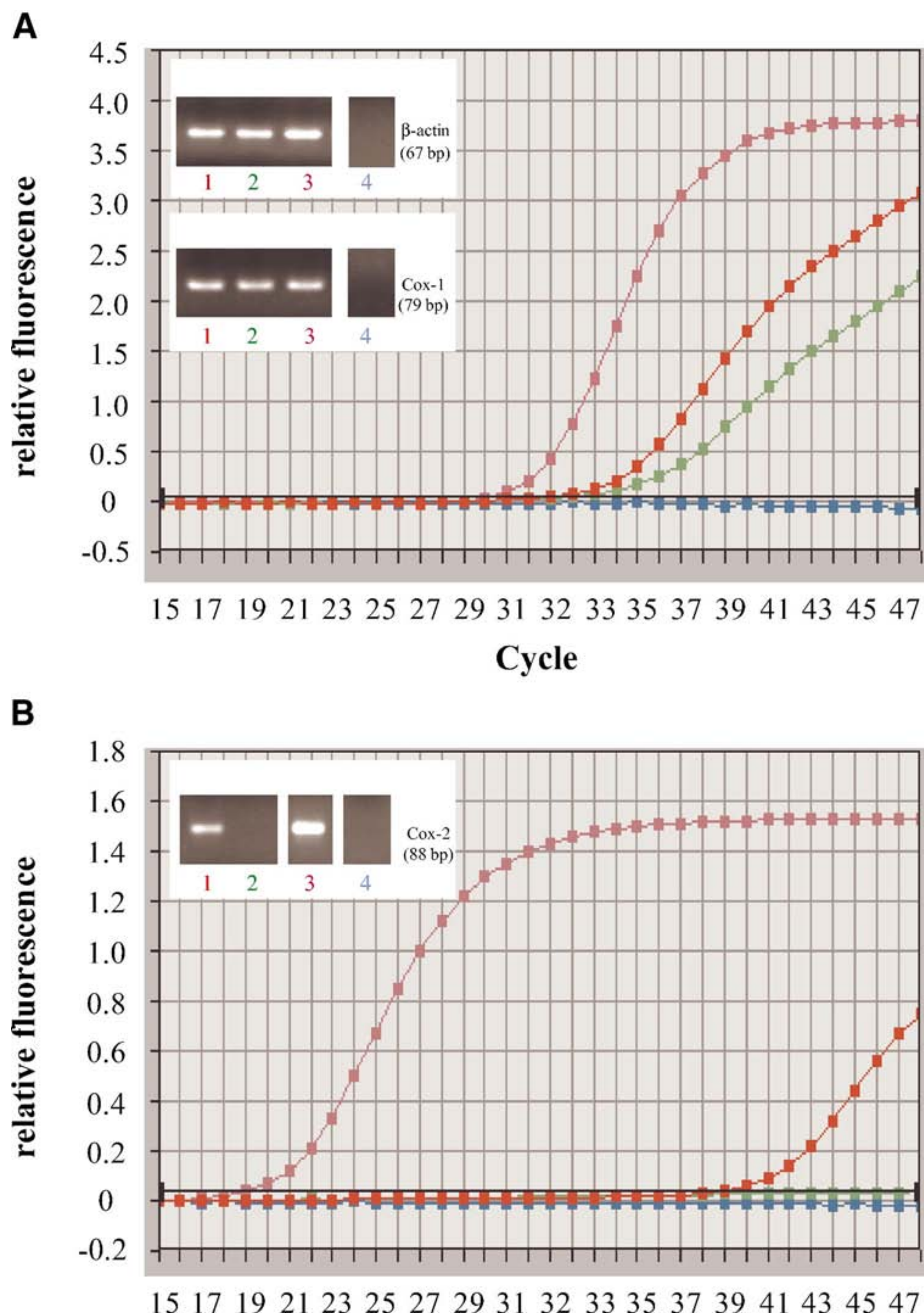

FIGURE 5. Real-time RT-PCR experiments comparing the expression of mRNA for COX isoforms in ciliary bodies of an eye enucleated due to absolute POAG and a small juxtapapillary malignant melanoma, respectively. (**A**) Original amplification plot for COX-1 mRNA. A prominent fluorescence signal was obtained from lipopolysaccharide (LPS) stimulated human monocytes (*pink*). Basal COX-1 mRNA expression was found in both eyes: control eye (*orange*); POAG (*green*); no-template control (*blue*). *Inset*: electrophoresis of the specimens after real-time RT-PCR for β-actin (*top*) and COX-1 (*bottom*). *Lane 1*: control eye; *lane 2*: POAG; *lane 3*: human monocytes; and *lane 4*: no-template control. The obtained bands were of the expected 79-bp (COX-1) and 67-bp (β-actin) sizes. (**B**) Original amplification plot for COX-2 mRNA. A prominent fluorescence signal was obtained from LPS-stimulated human monocytes (*pink*). Basal COX-2 mRNA expression was found in the eye with malignant melanoma (*orange*). In contrast, COX-2 mRNA was not detectable in the eye with POAG (*green*); no-template control (*blue*). After amplification, the specimens were electrophoresed to assess the size of the respective fragments (*inset*). *Lane 1*: amplification product of eye with malignant melanoma of the expected 88-bp size; *lane 2*: no signal detectable in the eye with POAG; *lane 3*: signal obtained from LPS-stimulated human monocytes of the expected size; and *lane 4*: no amplification for no-template control.

carefully prepared with the intention of using them for the analysis of mRNA expression. As far as possible, the specimens were processed in RNase-free conditions after enucleation of the eye. Because the specimens were fixed in paraformaldehyde and degradation of RNA could be expected, we designed special primers for the detection of small fragments (COX-1, 79 bp; COX-2, 88 bp; β-actin, 67 bp), according to reports in the literature.[21,22] The fragments were supposed to be within the approximately 200 nucleotide fragments resulting from fixation. One eye with absolute (end-stage) POAG (from an 80-year-old woman) was compared with an eye enucleated due to a small juxtapapillary malignant melanoma (from a 70-year-old man), which served as a control. The respective ciliary processes were separated by microdissection, and afterward, RNA-extraction was performed. As seen in Figure 5B, the control eye showed a clear expression of COX-2 mRNA. In contrast, no COX-2 mRNA was detectable in the eye enucleated due to absolute POAG. No difference in β-actin and COX-1 content was observed between both eyes (Fig. 5A).

PGE_2 Concentrations in Aqueous Humor

PGE_2 content was analyzed in the ocular aqueous humor in patients with different ocular diseases (Fig. 6). PGE_2 concentrations were significantly less in patients with POAG (34.3 ± 17.6 ng/ml [SEM]; $n = 19$) than in control patients (48.9 ± 7.6 ng/ml; $n = 13$) who underwent eye surgery for cataract ($P < 0.02$; ANOVA followed by the Bonferroni post hoc test), but comparable with patients who received topical steroids (25.5 ± 2.5 ng/ml; $n = 3$) for treatment of anterior uveitis or

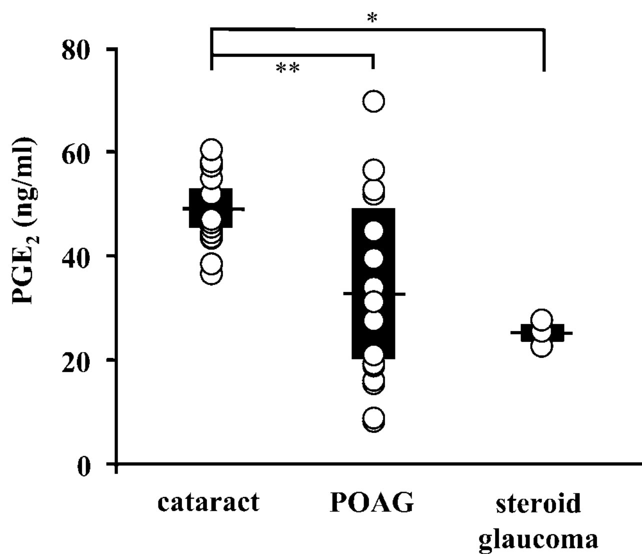

FIGURE 6. Measurement of PGE_2 in the aqueous humor of patients with various ocular diseases. PGE_2 was determined by enzyme-linked immunoassay. (○) PGE_2 levels in individual patients. *Straight line*: median level; *shaded area*: 25% to 75% range. PGE_2 concentrations of the ocular aqueous humor of eyes of patients with POAG or of patients pretreated with glucocorticoids were significantly lower than in the aqueous humor of eyes of control patients with cataract. $^*P < 0.05$; $^{**}P < 0.02$.

corneal herpes virus infection. $PGF_{2\alpha}$ content in the same samples was comparatively low (<4 pg/ml) and showed no significant differences between patients with POAG and cataract (data not shown).

DISCUSSION

Reduced COX-2-Expression in POAG

This study demonstrated a complete and selective loss of COX-2 expression in the ciliary nonpigmented epithelial layer of eyes with end-stage POAG. This loss was anatomically highly selective. The changes in COX-2 expression were restricted to the secretory epithelium of the ciliary body, whereas COX-2 expression remained unchanged in other parts of the eye. COX-1 expression was unchanged throughout the eye. The loss of COX-2 expression appears to be associated with POAG and is not a consequence of increased IOP, in that COX-2 was present in normal amounts in eyes with congenital juvenile and angle-closure glaucoma that had long-standing elevations of IOP. Furthermore, COX-2 expression was already clearly reduced at earlier stages of POAG development (compare Fig. 4E). Two of five eyes with early stages of POAG showed complete COX-2 loss, and three of five eyes showed COX-2 IR only in some scattered cells. Therefore, the selective loss of ciliary COX-2 expression appears to be linked to the occurrence of POAG. Although the results obtained from our specimens were very consistent, further investigations are needed to elucidate whether reduced COX-2 expression is a common characteristic of POAG or occurs only in a particular subtype of this disease.

In addition, this study showed that COX-2 expression in the ciliary epithelium was lost in steroid-induced glaucoma and was reduced in patients treated topically with glucocorticoids. Moreover, aqueous humor of eyes treated topically with glucocorticoids contained significantly less PGE_2 than did control eyes.

The proposed mechanism of corticosteroid-induced glaucoma includes morphologic and functional changes in the trabecular meshwork system. These changes are thought to be similar to those in POAG.[6] In particular, trabecular cells of human eyes treated with glucocorticoids show endoreplication of nuclei,[23] an increase in cell size,[23] and excessive production of an approximately 56-kDa glycoprotein, termed trabecular meshwork glucocorticoid response protein (TIGR) and transcribed by the *GLC1A* gene.[24–27] This may indicate that loss of COX-2 expression is secondary to changes in the trabecular meshwork system, because COX-2 expression is normal in other parts of the eye. Furthermore, the outflow resistance is markedly increased in eyes with POAG and steroid-induced glaucoma.[28–35] Because PGs are known to facilitate outflow,[9,10,36–38] the reduction of PGs in aqueous humor, as reported in this study, may contribute to the increased outflow resistance in POAG and steroid-induced glaucoma.

PGE_2 Concentration in the Aqueous Humor in POAG

The PGE_2 concentration in the aqueous humor of glaucomatous eyes was found to be significantly lower than in control eyes. The decrease in endogenous PGs may contribute to the elevation of IOP in the glaucomatous eyes. This is consistent with the utility of PG derivatives in the treatment of POAG. For example, $PGF_{2\alpha}$ analogues have been introduced into the therapy of POAG with remarkable success.[10,11,39,40]

$PGF_{2\alpha}$ is thought to influence uveoscleral outflow through interaction with PGF receptors.[28] These receptors are located in proximity to the localization of COX-1 and -2 described in this study.[41–43] In addition, at least part of the effects of $PGF_{2\alpha}$ occur through $PGF_{2\alpha}$-induced PGE_2 release.[44] This PG has been shown to decrease IOP, as well.[36–38] It is not known which of the various PGs is the most relevant in the human eye. However, the predominant prostanoid receptor in the human ciliary body is EP3.[36] Stimulation of this receptor seems to be more important for reduction of IOP than stimulation of the FP receptor.[36] This is consistent with our observation that the PGE_2 content of the aqueous humor in human eyes exceeded that of $PGF_{2\alpha}$ several times.

PGE_2 concentrations in aqueous humor of patients with POAG showed considerable interindividual variation, although overall the data demonstrate significantly lower concentrations of PGE_2 in POAG. The PG-concentration in the ocular fluid is the sum of all COX-activity in anterior eye segments. We suggest there would be a more dramatic reduction of PGE_2 if PG production in the ciliary epithelium alone could be measured.

Nevertheless, our PG measurements agree with our immunohistochemical and molecular biological data and provide supportive evidence for a decreased COX-2-expression in POAG.

Constitutive Expression of COX-1 and -2 in the Human Eye

This is the first study that describes a constitutive expression of both COX isoforms in the anterior segments of normal human eyes. Neufeld et al.[45] have investigated the expression of COX isoforms in the human optic nerve head of normal and glaucomatous eyes. They found no basal COX-2 expression in the optic nerve and only a slight increase of COX-2 IR in POAG. It was argued that COX-2-derived PGs might play a role in the pathogenesis of glaucomatous optic neuropathy. In the anterior segments of the eye, the ability of the ciliary epithelium and the ciliary body to produce PGs has been previously described.[36,46] Yousufzai et al.[44] reported that release of PGE_2 is inhibited by dexamethasone in a dose-dependent manner.

They suggested that this inhibition may be due to inhibition of phospholipase A_2. However, inhibition of phospholipase A_2 by glucocorticoids occurs only at very high dosages.[47] An alternative and more likely mechanism is the hormonal regulation of constitutively expressed COX-2.[12,14,16] The glucocorticoid-dependent COX-2 expression, and therefore PG production, may comprise a mechanism by which IOP is regulated under physiological conditions. Circadian oscillations of endogenous glucocorticoid plasma levels may well contribute to the circadian rhythm of IOP.

In addition, ocular COX-2 expression appears to be regulated by glucocorticoids. Our findings further extend the concept[14] that COX-2 contributes constitutively to the physiological regulation in highly differentiated organ systems such as the central nervous system (CNS),[15,16] the kidney,[17] and, now, the eye. One explanation for this finding may be that the eye ontogenetically originates from the neuroepithelium and shares many characteristics with the CNS.

It is at present not known what factors lead to the observed decrease in COX-2 expression in POAG. These patients are not generally without COX-2, for the absence of expression is confined to the ciliary epithelium of the eye (e.g., stromal cells in the ciliary body and iris of eyes with POAG exhibit normal COX-2 IR). This specific loss may be explained either by the existence of COX-2 inductors specific for the ciliary body or by a failure within the ciliary epithelium. It is interesting to note that the *TIGR* gene, in which several gene mutations have been reported in familial forms of POAG,[3,5] is located in close proximity to the human *COX-2* gene. The *TIGR* gene has been mapped to chromosomal region 1q24.3-q25.2.[48] Tay et al.[49] assigned the human COX-2 gene to 1q25. Furthermore, promotors of both genes have glucocorticoid response elements. Thus, the *COX-2* and *TIGR* genes share similarities regarding regulation of expression. Interactions of both genes and their products may be possible, although chromosomal linkage of two different genes does not necessarily imply any interactions.

The implications of decreased COX-2 expression are unknown. In particular, it remains unclear whether decreased COX-2 expression contributes to the increased IOP in POAG or is just an epiphenomenon of the pathogenesis of POAG. Unlike most other organ systems, the eye has no PG-metabolizing enzymes. PGs are removed from the aqueous humor by a specific transporter.[50,51] Therefore, PGs derived from the nonpigmented ciliary epithelial layer could interact with cells of the ciliary body or trabecular meshwork, releasing metalloproteinases.[52–54] These enzymes are known to degrade proteins of the extracellular matrix and thereby facilitate uveoscleral or trabecular meshwork outflow.[52–54] This mode of PG action is thought to contribute to the IOP-decreasing effect of PGs. Lower levels of PGs in aqueous humor as reported in this study may then give rise over time to an increased outflow resistance.

In summary, this study reports for the first time that both COX isoforms are constitutively expressed in anterior segments of normal human eyes. Unlike in congenital juvenile or angle-closure glaucoma, COX-2 expression was strongly reduced or abolished in the ciliary epithelium of eyes with POAG and steroid-induced glaucoma. Therefore, this study may provide new insights in the pathogenesis of POAG. The functional implications of reduced COX-2 expression will be the subject of future investigations.

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