Cyclooxygenase-2 Gene Expression and Regulation in Human Retinal Pigment Epithelial Cells

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Purpose. Cyclooxygenases (COX) orchestrate a variety of homeostatic processes and participate in various pathophysiological conditions. The retinal pigment epithelium (RPE) cell performs a variety of regulatory functions within the retina. The conditions under which COX-1 and COX-2 are expressed and upregulated in human RPE (HRPE) cells were determined.

Methods. COX gene expression was examined using RT-PCR analysis of untreated HRPE cultures or cultures exposed to bacterial lipopolysaccharide or various cytokines. COX proteins were detected by immunohistochemistry and Western blot analysis. Prostaglandin (PG) production was analyzed by EIA.

Results. Examination of untreated RPE cells revealed the presence of COX-2 mRNA and the absence of COX-1 mRNA. Moreover, cytokine stimulation more readily enhanced COX-2 gene expression than COX-1 gene expression. IL-1β, the most potent inducer of COX-2, also resulted in detection of COX-2 protein by immunocytochemical staining and Western blot analysis. There was a direct relationship between both the appearance and amount of COX-2 mRNA and protein synthesis and the degree of PG synthesis by RPE cells. Furthermore, COX inhibitors significantly decreased PG production. Pretreatment of RPE cells with a NF-κB inhibitor, PDTC, resulted in dose-dependent decrease in IL-1β–induced COX-2 gene expression and PG production.

Conclusions. COX-2 was the predominant isoform of cyclooxygenase in untreated HRPE cells. When HRPE cells were treated with proinflammatory cytokines, COX-2 gene expression and synthesis of PGs were enhanced. NF-κB mediated the induction of COX-2 gene expression in HRPE cells. These studies indicate that RPE cells may participate in normal and pathologic retinal conditions through the induction of COX-2. (*Invest Ophthalmol Vis Sci.* 2001;42:2338–2346)

Cyclooxygenase (COX) is a bifunctional enzyme that catalyzes the first two steps in the biosynthesis of eicosanoids from arachidonic acid (or other 20 carbon fatty acids). Two isoforms of the enzyme have been identified, COX-1 and COX-2. Both are identical in length, with molecular weights of 71 kDa, and their amino acid sequences are approximately 60% identical.[1] COX-1 is constitutively expressed in almost all tissues[1] and is believed to be responsible for maintenance levels of prostaglandins for various housekeeping functions. COX-2 expression is normally absent from most tissues and is induced by pathologic stimuli, such as bacterial lipopolysaccharide (LPS) and proinflammatory cytokines (interleukin [IL] 1, IL-2, and TNF-α).[1] Increases in COX-2 expression have been found in vivo associated with inflammation, rheumatoid arthritis, seizures, ischemia, and various cancers.[1–4] Inhibition of COX-2 by nonsteroidal anti-inflammatory drugs (NSAIDs) has been shown to be effective in controlling rheumatoid arthritis[5] as well as in reducing the risk and spread of some cancers.[4]

Products of the cyclooxygenase reaction include prostaglandins and thromboxanes. Prostaglandins are found in a broad range of human tissues and act as paracrine and autocrine mediators in regulating physiological and pathologic functions. These diverse functions include regulating renal blood flow, platelet aggregation, ovulation, parturition, bone metabolism, nerve growth and development, wound healing, immune responses, febrile response, inflammation, and cytoprotection.[1,2,6–8] In the eye, the concentration of prostaglandins increases after corneal injury.[9] In addition to ocular inflammation,[10] these molecules regulate intraocular pressure,[11] influence corneal neovascularization,[12] play a role in corneal edema,[13] affect retinal blood flow,[14] and disrupt the blood–retinal/blood–aqueous barrier.[15] Prostaglandin E_2 (PGE_2) is also released by fibroblasts in proliferative vitreoretinopathy, a severe sight-threatening disease that also involves retinal pigment epithelium (RPE) cells.[16]

The RPE is a single layer of cells located between the rod and cone photoreceptors and the highly vascularized choroid. RPE cells are responsible for (1) the phagocytosis of photoreceptors, (2) vitamin A metabolism, (3) providing nutrients to and removing wastes from the photoreceptors, (4) maintaining retinal adhesion, (5) acting as part of the blood–retinal barrier, and (6) absorbing light and dissipating heat energy derived from incident light.[17,18] The RPE cell has also been shown to perform immunologic functions in the retina. For example, it can express major histocompatibility complex (MHC) class I and II molecules and intercellular adhesion molecule-1 (ICAM-1) as well as process antigen and present it to helper T cells.[19–23] RPE cells can respond to proinflammatory cytokines by regulating ocular immune responses as previously described, in addition to secreting IL-6, IL-8, and monocyte chemotactic protein.[24–28]

RPE cells play a critical role in degenerative, inflammatory, and infectious diseases of the eye because of their various functions and strategic location. Previous studies of the role of prostaglandins in the retina have focused on their effect on retinal blood flow.[14] Less is known about the physiological and pathologic roles of COX-2 in the retina. Bazan and coworkers[29] demonstrated that rod outer segment phagocytosis and growth factors induced COX-2 gene expression in rat RPE cells. Hanna et al.[30] found that the light-activated production of reactive oxygen species by COX leads to photic-induced retinal injury. Because RPE cells are important during inflammatory and infectious diseases of the eye, we wanted to examine COX gene and protein expression in these cells. We treated human RPE

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(HRPE) cells with cytokines and found that proinflammatory cytokines such as IL-1β and TNF-α increase COX-2 gene expression and synthesis of prostaglandins. We have also found that the NF-κB signaling pathway has a role in the upregulation of COX-2 gene expression in RPE cells.

Materials and Methods

Salmonella typhosa LPS, piroxicam, Triton X-100, pyrrolidine dithiocarbamate (PDTC), glycine, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human interferon (IFN) γ, recombinant human tissue necrosis factor (TNF) α, RT-PCR kit, DIG oligonucleotide labeling kit, the Genius Random priming kit, digoxigenin-alkaline phosphatase Fab fragments, and CSPD were acquired from Roche Molecular Biochemicals (Indianapolis, IN). Recombinant human IL-1β was obtained from Collaborative Research Inc. (Bedford, MA). RNA STAT-60 was purchased from Tel-Test (Friendswood, TX). Human COX-1 primers (upstream, 5′-TGCCCAGCTCCTGGCCCGCCGCTT-3′ and downstream, 5′-GTGCATCAACACAGGCGCCTCTTC-3′), human COX-2 primers (upstream, 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′ and downstream, 5′-AGATCATCTCTGCCTGAGTATCTT-3′), human COX-1 probe (5′-CTTGGGCCATGGGGTAGACCTCGGCC-3′) and human COX-2 probe (5′-GCGAGGGCCAGCTTTCACCAACGGGC-3′) were custom synthesized by Bio-Synthesis, Inc. (Louisville, TX). Actin primers were bought from Research Genetics, Inc. (Huntsville, AL). Aspirin, NS-398, PGE_2 Enzyme Immunoassay kit, $PGF_{2\alpha}$ Enzyme Immunoassay kit, rabbit anti-human COX-2 polyclonal antibody, monoclonal antibody to human COX-2 and monoclonal antibody to ovine COX-1 were from Cayman Chemical Co., Inc. (Ann Arbor, MI). Rabbit anti-human NF-κB p65 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PGE_1 Enzyme Immunoassay kit was from R&D Systems (Minneapolis, MN). NuPAGE gels were from Invitrogen (Carlsbad, CA). Minimal essential media (MEM), fetal bovine serum (FBS), penicillin/streptomycin/fungizone, nonessential amino acids, and normal goat serum were purchased from Life Technologies/Gibco (Gaithersburg, MD). Rabbit Vectastain ABC kit, 3,3′-diaminobenzidine substrate kit and VectaMount were procured from Vector Laboratories (Burlingame, CA). Horseradish peroxidase–conjugated goat anti-mouse IgG was supplied by Kirkegaard & Perry Laboratories (Gaithersburg, MD).

Cell Culture

Human RPE (HRPE) cultures were prepared from human donor eyes[28] and grown in MEM containing 10% heat-inactivated FBS, nonessential amino acids, and penicillin/streptomycin/fungizone in a humidified 37°C incubator. The cells are an untransformed primary cell line that has not been treated with retinoic acid. Characterization of these cells has been described previously.[23,28,31] Briefly, these cells demonstrated a hexagonal morphology when grown to confluence and formed monolayers with distinct intercellular boundaries.[28] Homogeneity of the cultures was established by positive immunostaining with monoclonal antibodies to cytokeratin, an epithelial cell–specific cytoskeletal protein.[28] The characterized cells were expanded and cryopreserved at passage 6. For these experiments the characterized HRPE cells were revived from frozen storage, passaged, grown to confluence, and then used between passages 8 and 14.

Analysis of COX Gene Expression

Confluent monolayers of RPE cells in 24-well plates were washed with serum-free media (SFM) and incubated in SFM for 1 hour. Next the cells were treated with various stimulants in SFM for 24 hours. The effect of COX inhibitors was examined by washing the cells with SFM after IL-1β treatment and then incubating with inhibitors in SFM. Stimulants used include 10 μg/ml LPS (*S. typhosa*), and the following cytokines either alone or in various combinations: 100 U/ml IFN-γ, 10 ng/ml TNF-α, and 0.001 to 10 ng/ml IL-1β. Total RNA was prepared from the cell monolayers using the RNA STAT-60 protocol, and RNA from triplicate wells was pooled. RT-PCR analysis of COX-1 and COX-2 gene expression was carried out using 0.5 μg total RNA per reaction. Actin, human COX-1, and human COX-2 primers yield products 289, 304, and 329 bp, respectively. Ten microliters of the PCR products were separated by electrophoresis on a 4% agarose gel and transferred to a nylon membrane following the alkaline denaturation method. Hybridization was then carried out with digoxigenin-labeled probes for actin, COX-1, or COX-2. COX-1 and COX-2 probes were labeled by oligonucleotide tailing. The actin probe was labeled by random priming. Detection of the probes was accomplished using anti–digoxigenin-alkaline phosphatase Fab fragments and the chemiluminescent alkaline phosphatase substrate CSPD. Quantitative analysis of the RT-PCR products detected by chemiluminescence was performed on a Power Macintosh G3 computer (Apple Computer, Cupertino, CA) using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Immunohistochemical Staining of RPE Cells for COX-2

Confluent RPE cells, seeded onto chambered slides were washed with SFM and then incubated in SFM for 1 hour before stimulation with various agents. Twenty-four hours after treatment, media were removed, and the cells were fixed in ice-cold methanol/acetone (1:1) for 10 minutes. Slides were rinsed in phosphate-buffered saline (PBS), treated with a 0.6% hydrogen peroxide solution to quench endogenous peroxidases, and again washed with PBS. After incubating slides in blocking solution (PBS with 0.4% Triton X-100, 1% glycine, 2% BSA, and 10% normal goat serum) for 90 minutes at room temperature to block nonspecific binding, the slides were incubated overnight at 4°C with either rabbit anti-human COX-2 antibody or normal rabbit IgG diluted in blocking solution. Slides were washed in PBS with 1% normal goat serum and developed according to the Vectastain ABC kit instructions with 3,3′-diaminobenzidine as the peroxidase substrate.

Western Blotting

Confluent HRPE cells in 10-cm dishes were washed with SFM and then incubated in SFM for 1 hour before treatment with 10 ng/ml of IL-1β in SFM or SFM alone for 24 hours. The cells were scraped off the plates, transferred to centrifuge tubes, and centrifuged at 1000 rpm. The medium was removed, and the cells were resuspended in protein extraction buffer (50 mM Tris-Cl, pH 7.5, 10% glycerol, 5 mM magnesium acetate, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF). The cells were lysed by freeze-thawing, and the membrane fraction was separated from the soluble fraction by centrifuging at 14,000 rpm for 30 minutes. The soluble fraction was mixed with sample buffer and boiled for 10 minutes. The membrane fraction was resuspended in 2 volumes of protein extraction buffer, mixed with sample buffer, and boiled for 10 minutes. Electrophoresis was performed using 10% NuPAGE Bis-Tris gel (1.5 hours, 150 V, 80 mA, 2 μg protein per lane). Separated proteins were transferred to nitrocellulose membranes (1.6 hours, 30 V). Membranes were incubated in PBS containing 0.1% Tween 20, 5% dry milk, and 1% cold water fish gelatin to block nonspecific IgG binding, followed by treatment with either COX-1– or COX-2–specific antibodies for 2 hours. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG for 1 hour. The Western blot analysis was developed using Luminol as a substrate.

Measuring PGE_2, $PGF_{2\alpha}$, and PGE_1 Production by RPE Cells

Confluent monolayers of RPE cells were treated with different combinations of stimulants as described above. After 24 hours the medium was removed and stored at −70°C until it could be assayed for PGE_2, $PGF_{2\alpha}$, and PGE_1 production. The effect of COX inhibitors on PGE_2 production was examined by treating the cells as described above but with 0.1 ng/ml IL-1β. After 24 hours the cells were washed with SFM and incubated with COX inhibitors in SFM for 30 minutes, followed by SFM with COX inhibitors and arachidonic acid for 15 minutes. Quan-

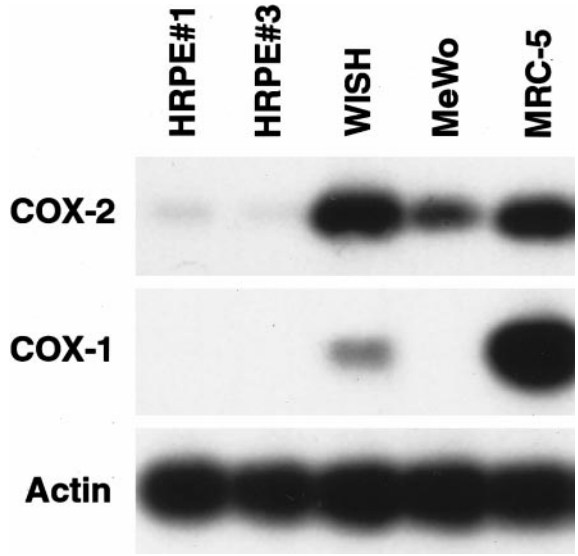

FIGURE 1. Cyclooxygenase-2 and -1 (COX-2 and -1) gene expression in HRPE cells, amnionic epithelial (WISH) cells, melanoma (MeWo) cells, and lung fibroblasts (MRC-5). Total RNA was prepared from cells and analyzed for COX-2, COX-1, and β-actin mRNA by RT-PCR and Southern blot hybridization.

tification of PGE_2 was accomplished using the PGE_2 Enzyme Immunoassay Kit from Cayman Chemicals.

Analysis of the Effect of Pyrrolidine Dithiocarbamate on COX-2 Gene Expression and PGE_2 Production

Confluent monolayers of RPE cells were rinsed with SFM and incubated in SFM for 1 hour before treatment of the cells with PDTC for 30 minutes. Then IL-1β was added to a final concentration of 0.5 ng/ml. COX gene expression and PGE_2 production were analyzed as described above.

Confluent RPE cells, seeded onto chambered slides were washed with SFM and then incubated in SFM for 1 hour before treatment with PDTC for 30 minutes followed by addition of IL-1β to a final concentration of 0.5 ng/ml. After 5 minutes, 15 minutes, 30 minutes, and 1 hour slides were fixed in ice cold methanol/acetone (1:1) for 10 minutes and then stored at −20°C until they could be stained for NF-κB. Slides were rinsed twice in PBS and incubated in blocking solution for 90 minutes at room temperature. The slides were incubated overnight at 4°C with rabbit anti-human NF-κB antibody diluted in blocking solution. Slides were washed in PBS with 1% normal goat serum, and localization of NF-κB was accomplished using goat anti-rabbit-FITC.

RESULTS

COX Gene Expression in RPE Cells and Other Cells

COXs are the enzymes catalyzing the rate-limiting step in the conversion of arachidonic acid to prostanoids. Of the two isozymes, COX-2 is the inducible form responsible for the production of inflammatory mediators. Examination of COX expression in untreated HRPE cells by RT-PCR found that COX-2 mRNA was weakly expressed and COX-1 mRNA was not detected (Fig. 1), and this is true for both HRPE cell lines examined. Figure 1 shows COX-2 and COX-1 gene expression in HRPE, WISH, MeWo, and MRC-5 cells by RT-PCR. COX-2 mRNA was quantitated from the x-ray film record of the Southern blot with COX-2 as the probe. The amount of COX-2 mRNA in HRPE cells was approximately threefold less than in the other cell lines examined. Under these conditions, the signal of the actin controls for all cells examined was of the same intensity. COX-2 mRNA expression was more abundant than COX-1 expression for HRPE, WISH, and MeWo. COX-1 gene expression was greater than COX-2 gene expression in MRC-5 cells.

Cytokine-induced Upregulation of COX Gene Expression

Experiments were conducted to determine whether the treatment of HRPE cells with LPS or proinflammatory cytokines altered COX gene expression. HRPE cells were incubated for 24 hours with media alone or media with LPS or cytokines (IL-1β, IFN-γ, and TNF-α). COX gene expression was analyzed by RT-PCR of isolated total RNA (Fig. 2). Treatment of HRPE cells with 10 μg/ml LPS, 100 U/ml IFN-γ, and 10 ng/ml TNF-α resulted in very minute increases in COX-2 mRNA (less than a 10% increase). However, the combination of 100 U/ml IFN-γ and 10 ng/ml TNF-α or any cytokine combination containing 10 ng/ml IL-1β caused an increase of approximately 30% in COX-2 mRNA detected by RT-PCR. Only treatment of HRPE cells with the combination of 10 ng/ml IL-1β and 10 ng/ml TNF-α showed upregulation in COX-1 gene expression.

Detection of COX-1 and COX-2 in HRPE Cells

Because cytokines increased COX gene expression in HRPE cells, we next examined these cells by immunocytochemistry for COX protein production. COX-1 protein was not detected in either SFM or cytokine-treated HRPE cells (data not shown). Reaction of SFM-treated HRPE cells with the polyclonal antibody against COX-2 also showed no staining for COX-2 protein (Fig. 3B). Both SFM and IL-1β–treated cells did not react with normal rabbit IgG (data not shown). Treatment of HRPE cells with any cytokine combination that included IL-1β showed staining for COX-2 protein as demonstrated by the cytoplasmic and perinuclear staining shown in Figure 3A and the appearance of the 71-kDa protein on the Western blot (Fig. 3C). LPS, IFN-γ, and TNF-α treatments did not increase production of COX-2 protein (data not shown). Expression of COX-1 protein did not increase when HRPE cells were stimulated with LPS or various cytokine combinations (data not shown). These data indicate that COX-2 protein expression mimics COX-2 mRNA expression in cytokine-treated RPE cells as demonstrated by

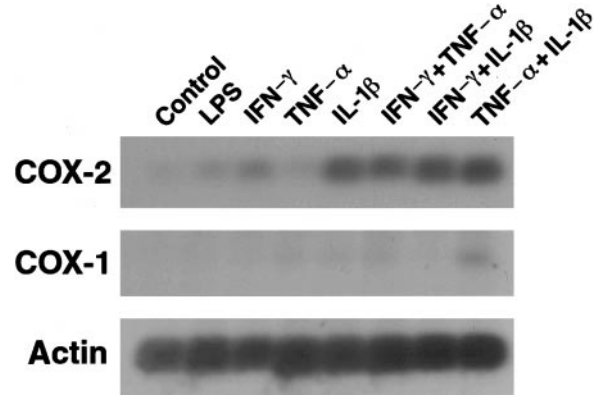

FIGURE 2. Effect of LPS, IFN-γ, TNF-α, and IL-1β on COX-2 and -1 expression in HRPE cells. Cells were treated with LPS or various combinations of cytokines or were untreated for 24 hours. Total RNA was isolated from treated and untreated cells and analyzed for COX-2, COX-1, and β-actin mRNA by RT-PCR and Southern blot hybridization.

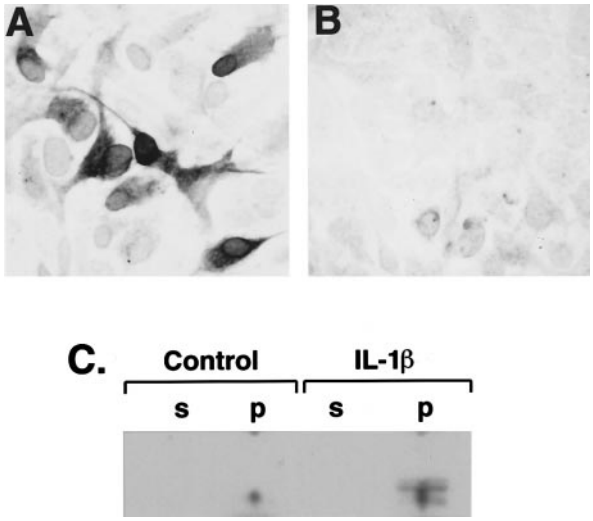

FIGURE 3. Immunocytochemical staining of COX-2 in HRPE cells and in cell extracts of HRPE cells. After treating cells with (**A**) 10 ng/ml of IL-1β in SFM or (**B**) serum-free media for 24 hours, cells were fixed and permeabilized with acetone/methanol and reacted with a rabbit anti–COX-2 polyclonal antibody. (**C**) Western immunoblot of COX-2 protein induced by IL-1β in HRPE cells. Cells were treated as described above and then scraped off the tissue culture dish, lysed, separated into supernatant (s) and pellet (p) fractions by centrifugation, and boiled in sample buffer. Two micrograms of protein was loaded in each lane and subjected to SDS-PAGE electrophoresis and then transferred to a nitrocellulose membrane. COX-2 was detected using rabbit anti–COX-2 polyclonal antibody.

immunohistochemical staining of COX-2 in IL-1β–treated HRPE cells and no staining for COX-1 in IL-1β–treated HRPE cells.

Effect of Cytokines on COX Activity

To determine whether the COX gene expression and protein production was associated with COX activity, PGE_2, $PGF_{2\alpha}$, and PGE_1 production was evaluated by enzyme immunoassay (EIA) for two HRPE cell lines. The data presented in Table 1 are representative of data obtained from both cell lines. Treatment of HRPE cells with TNF-α resulted in a 9-fold increase in PGE_2 production ($P < 0.05$) and a 2.5-fold increase in $PGF_{2\alpha}$ production ($P < 0.1$; Table 1). Treatment of HRPE cells with IL-1β resulted in a greater than 250-fold increase in PGE_2 production ($P < 0.00{,}005$) and a 33-fold increase in $PGF_{2\alpha}$ ($P < 0.005$). Treatment with LPS or IFN-γ did not result in an increase in PGE_2 or $PGF_{2\alpha}$. Stimulation with any cytokine combination including IL-1β produced levels of PGE_2 and $PGF_{2\alpha}$ that were greater than the levels produced by treatment with IL-1β alone (data not shown).

In addition to analyzing the production of prostaglandins involved in inflammation, we also examined the production of PGE_1, a cytoprotective prostaglandin. PGE_1 synthesis was increased in IL-1β–treated cells (Fig. 4) for both HRPE cell lines examined. Cells treated simultaneously with IL-1β and TNF-α resulted in a significant increase in PGE_1 production ($P < 0.01$). Treatment with LPS, IFN-γ, TNF-α, and a combination of IFN-γ and TNF-α did not increase PGE_1 production.

We next examined the effect of dose and the kinetics of IL-1β induction on COX-2 gene expression and PGE_2 production. Treatment of RPE cells with IL-1β (0.001–10 ng/ml) resulted in a dose-dependent increase in COX-2 gene expression (Fig. 5A) and PGE_2 production (Fig. 5B). An increase in COX-2 gene expression was achieved with a dose of 0.05 ng/ml of IL-1β, and maximal upregulation of COX-2 gene expression occurred with a dose of 0.5 ng/ml IL-1β. A dose of 0.1 ng/ml of IL-1β resulted in an increase in PGE_2 production, and a dose of 1 ng/ml yielded maximal PGE_2 production.

Exposure of RPE cells to IL-1β resulted in a time-dependent increase of COX-2 gene expression (Fig. 5C) and PGE_2 production (Fig. 5D). An increase in COX-2 gene expression was noted after treating cells for 1 hour with IL-1β, and maximal gene expression occurred between 6 and 12 hours of treatment with IL-1β. PGE_2 production started to increase 1 hour after treatment with IL-1β, and maximum production of PGE_2 occurred between 12 and 24 hours.

Inhibitors of COX Activity

To further characterize COX gene expression and PGE_2 production, inhibition of COX activity was evaluated. HRPE cells were incubated with IL-1β for 24 hours, followed by treatment with COX inhibitors for 30 minutes, and then arachidonic acid was added for 15 minutes. As seen in Figure 6, NS-398, a selective COX-2 inhibitor, dramatically decreased the production of PGE_2 from arachidonic acid in HRPE cells. At a concentration of 0.1 μM NS-398, PGE_2 production decreased by 38% compared with cells not treated with inhibitor ($P < 0.01$). Piroxicam, a nonselective COX-1 inhibitor, and aspirin, an inhibitor of COX-1 and COX-2, also inhibited the production of PGE_2. However, at twice the lowest concentration of NS-398 used, piroxicam decreased PGE_2 production by 11%, and aspirin did not decrease PGE_2 production. The inhibitory effect of aspirin and piroxicam on PGE_2 production was only significant at higher concentrations of these compounds ($P < 0.01$). These studies demonstrate that NS-398, a COX-2 selective inhibitor, was more effective in the inhibition of COX-2 activity than were a nonselective COX-1 or a nonspecific COX inhibitor.

Effect of an Inhibitor of NF-κB Activation on COX-2 Gene Expression and PGE_2 Production

The COX-2 gene has many putative transcription factor sites in the 5′-promoter region, including NF-κB. Subsequently, we wanted to evaluate the role of NF-κB on COX-2 gene expression and PGE_2 production in HRPE cells. PDTC inhibits the translocation of NF-κB from the cytoplasm to the nucleus and thereby inhibits activation of NF-κB. Immunofluorescent staining for NF-κB p65 in HRPE cells incubated with only IL-1β shows cells in which the fluorescent signal is localized to the nucleus (Fig. 7A). In cells treated with PDTC alone or PDTC before incubation with IL-1β, NF-κB was present throughout the whole cell and was not only limited to the nucleus as demonstrated by the fluorescent signal. Treatment of HRPE cells with IL-1β resulted in translocation of NF-κB from the cytoplasm to the nucleus. If the cells were pretreated with PDTC, an inhibitor of NF-κB activation, NF-κB was not transported to the nucleus.

TABLE 1. PGE_2 and $PGF_{2\alpha}$ Production by HRPE Cells Treated with LPS, IFN-γ, TNF-α, or IL-1β

Treatment	[PGE_2] (pg/ml)	[$PGF_{2\alpha}$] (pg/ml)
Control	18.7 ± 9.2	74.2 ± 45.6
LPS (10 μg/ml)	19.7 ± 5.6	56 ± 7.5
IFN-γ (100 U/ml)	18 ± 4.1	55.8 ± 8.4
TNF-α (10 ng/ml)	164 ± 33.1*	194.3 ± 35.2
IL-1β (10 ng/ml)	>5000†	>2500†

Cells were treated with LPS, various cytokines, or SFM for 24 hours. Culture media were analyzed for PGE_2 and $PGF_{2\alpha}$ production by EIA. Values indicated (*$P < 0.05$, †$P < 0.005$) are significantly different from the control group.

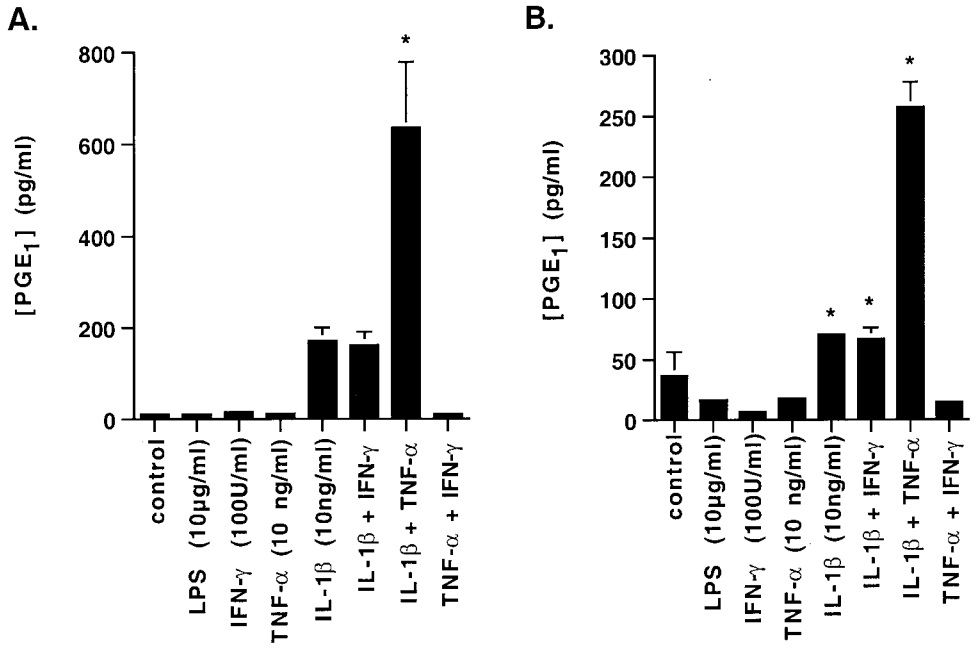

FIGURE 4. Production of PGE_1 in HRPE1 (**A**) and HRPE3 (**B**) cells. Cells were untreated or treated with LPS or cytokines for 24 hours. Culture media were collected and analyzed for PGE_1 production by EIA. Values indicated ($^*P < 0.01$) are significantly different from the untreated group.

Cells treated with PDTC before treatment with IL-1β showed a dose-dependent decrease in IL-1β–induced COX-2 gene expression (Fig. 7B). PDTC concentrations of 10 and 25 μM caused a 20% decrease in COX-2 mRNA expression, and 50 μM resulted in a 40% decrease in COX-2 mRNA expression. As seen in Figure 7C, 10 μM of PDTC caused a threefold reduction in PGE_2 production ($P < 0.05$). Furthermore, 25 and 50 μM PDTC were able to abolish PGE_2 production ($P < 0.01$).

These studies demonstrate that cytokines induced COX activity in HRPE cells, and this activity was blocked with COX inhibitors. These studies also indicated that NF-κB acted as a transcription factor mediating IL-1β–induced COX-2 gene expression in HRPE cells.

DISCUSSION

The present study showed that the cyclooxygenase isoform preferentially expressed in HRPE cells was COX-2. Several lines of evidence indicate that COX-2 was the primary form. First,

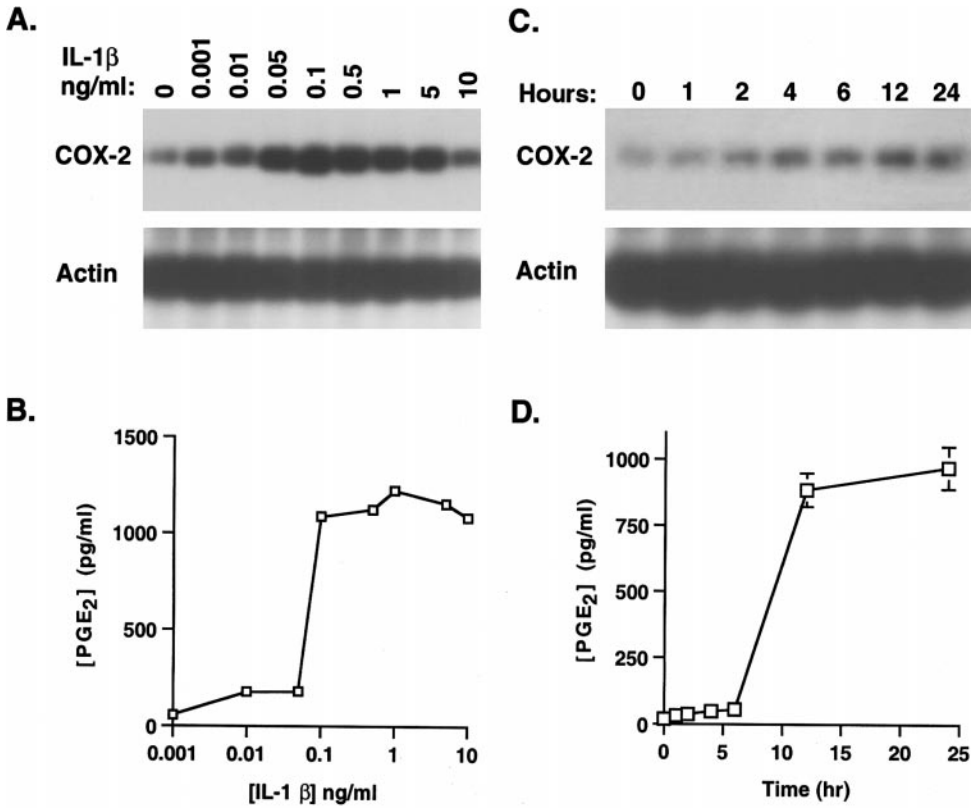

FIGURE 5. Concentration- and time-dependent increase in COX-2 gene expression and PGE_2 production caused by IL-1β in HRPE cells. Cells were incubated with various concentrations of IL-1β for 24 hours (**A**) or with IL-1β (0.5 ng/ml) for various time intervals (**C**); total RNA was recovered from cells and analyzed for COX-2, COX-1, and β-actin mRNA by RT-PCR and Southern blot hybridization. Culture media were also collected and PGE_2 production was measured by EIA (**B** and **D**).

examination of untreated RPE cells revealed the presence of COX-2 mRNA and the absence of COX-1 mRNA. Second, cytokine stimulation more readily enhanced COX-2 gene expression than COX-1 gene expression. Third, treatment of RPE cells with IL-1β resulted in the detection of COX-2 protein by immunocytochemical staining and Western blot analysis. In contrast, COX-1 protein remained undetectable. Fourth, there was a direct relationship between both the appearance and the amount of COX-2 gene expression and protein synthesis and the degree of prostaglandin synthesis by RPE cells. Fifth, NS-

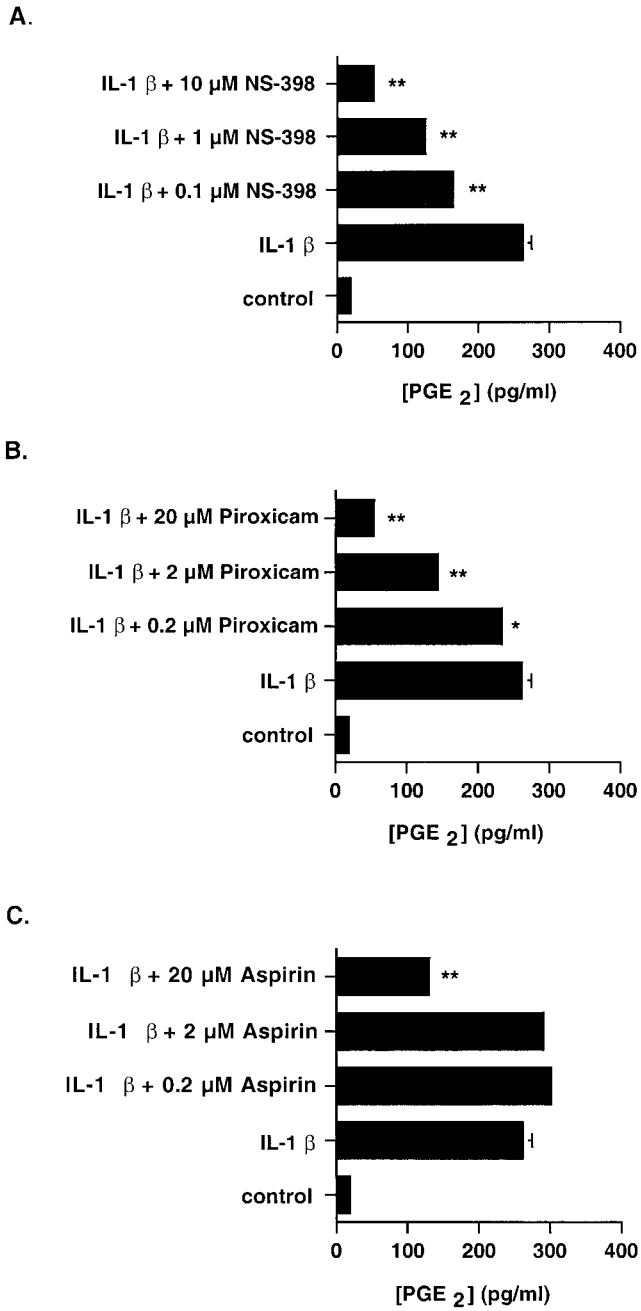

FIGURE 6. Dose-dependent inhibition of PGE_2 production by HRPE with NS-398 (**A**), Piroxicam (**B**), and aspirin (**C**). Cells were incubated with 0.1 ng/ml IL-1β for 24 hours, followed by treatment with COX inhibitors for 30 minutes and then treated with COX inhibitors and arachadonic acid for 15 minutes. Culture media were collected, and PGE_2 was measured by EIA. Values indicated ($^*P < 0.05$, $^{**}P < 0.01$) are significantly different from the group incubated with IL-1β but not treated with NSAIDs.

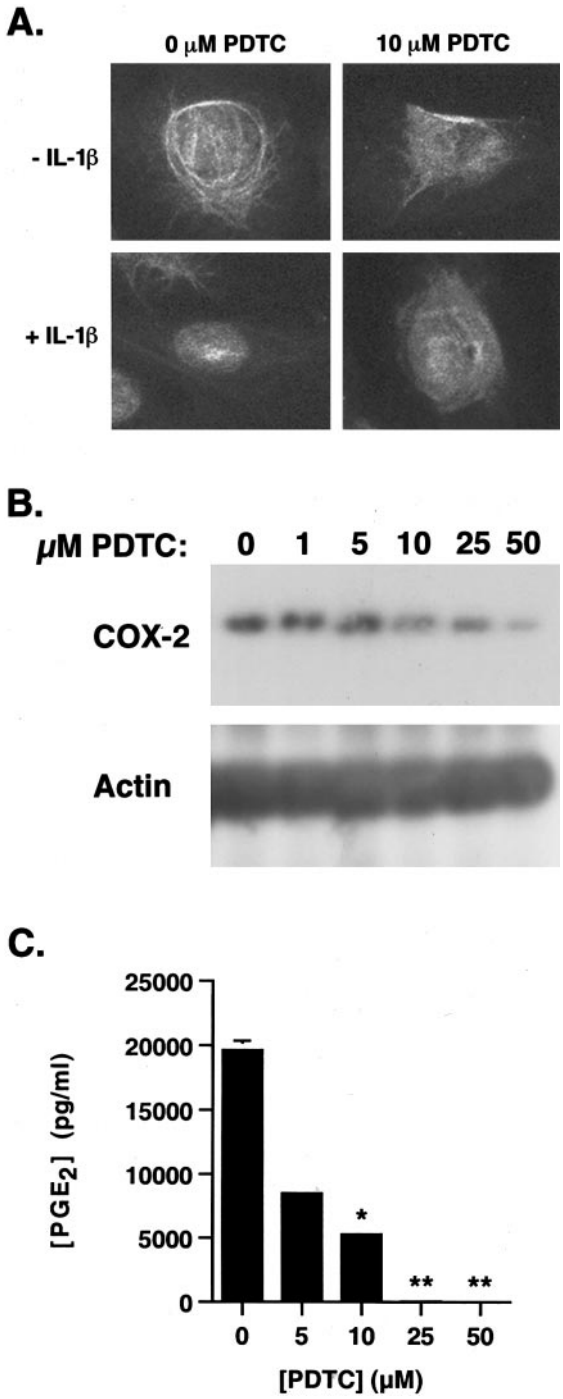

FIGURE 7. The effect on COX-2 gene expression and PGE_2 production by PDTC, an inhibitor of NF-κB. (**A**) Immunofluorescent staining for NF-κB p65 in HRPE cells incubated with SFM, PDTC, IL-1β, and PDTC + IL-1β. Cells were treated with PDTC for 30 minutes, followed by treatment with IL-1β for 15 minutes. Then cells were fixed and permeabilized with acetone/methanol and reacted with a rabbit anti–NF-κB IgG. Dose-dependent inhibition of COX-2 gene expression (**B**) and PGE_2 production (**C**) by HRPE cells with PDTC. Cells were treated with PDTC for 30 minutes, followed by treatment with 0.5 ng/ml IL-1β for 24 hours. Total RNA was recovered from cells and analyzed for COX-2 and β-actin mRNA by RT-PCR and Southern blot hybridization, and media were collected and PGE_2 was measured by EIA. Values indicated ($^*P < 0.05$, $^{**}P < 0.01$) are significantly different from the control group that was not treated with PDTC.

398, a COX-2 selective inhibitor, was more effective in the inhibition of prostaglandin synthesis than were a nonselective COX-1 or a non-specific COX inhibitor. Using PDTC, an inhibitor of NF-κB translocation, we also demonstrated that NF-κB acts as a transcription factor mediating IL-1β–induced COX-2 gene expression in HRPE cells.

COX catalyzes the first two steps in the conversion of arachidonic acid to prostaglandin H_2 that is further metabolized to other prostaglandins and thromboxanes.[1,2] COX-1 is ubiquitously expressed and believed to be necessary for maintaining essential physiological functions, and COX-2 is induced rapidly under pathophysiological conditions.[1] COX-2 mRNA and protein have been found to be upregulated in epithelial cells,[32,33] fibroblasts,[34,35] smooth muscle cells,[36–38] and T cells stimulated with proinflammatory cytokines, growth factors, and/or PMA.[39,40]

We examined COX-2 and COX-1 gene expression by RT-PCR in a number of human cell lines and found COX-2 mRNA expressed in HRPE, WISH, MeWo, and MRC-5 cells. With this method, COX-1 gene expression was detected in WISH and MRC-5 cells, but not in MeWo or HRPE cells. The level of COX-2 gene expression in HRPE cells may be low compared with the other cells examined because the HRPE cells are primary cell lines and/or because transcriptional regulation of COX-2 is tightly regulated in HRPE cells. It was remarkable that COX-2 was the predominant isoform of COX expressed in the HRPE cells and not COX-1. However, it has been recently demonstrated that COX-2 appears to be constitutively expressed in certain cells and tissues such as nasal mucosa,[41] bronchial epithelial cells,[42,43] tracheal epithelial cells,[44] gastric mucosal cells,[45] macula densa and medullary cells of the kidney,[46] neuronal cells,[1] the choroid plexus of the developing brain,[47] smooth muscle cells,[48] and vascular endothelial cells.[49] COX-2 mRNA and protein have also been detected in rabbit corneal epithelium[50] and rat RPE cells.[29] In airway and gastric cells, constitutive expression of COX-2 may be important in the function of these cells as protective barriers to the environment.[41–43,45] COX-2 plays a role in brain and kidney development.[46] The expression of COX-2 in rat retinal pigment epithelial cells has been proposed necessary for photoreceptor cell renewal.[29] Work by Langenbach et al.[51] demonstrated that the ablation of COX-2 in mice produces more severe effects than a deficiency in COX-1. These findings suggest that COX-2 may have physiologic roles as well as pathophysiologic roles. It is also possible that the detection of COX-2 gene expression in cultured cells is a byproduct of the nonphysiologic environment of the in vitro situation. For example, vascular smooth muscle cells and endothelial cells grown in vitro were found to express COX-2 mRNA, but no COX-2 mRNA was present in arterial and venous biopsies.[49]

Many different effectors can stimulate COX-2 gene expression. These include pathologic stimuli such as bacterial LPS and proinflammatory cytokines as well as peptide growth factors[29,34,50,52] and polyunsaturated fatty acids.[53] IL-1β increases COX-2 gene expression in human pulmonary epithelial cells, human thyroid epithelial cells, and human amnionic epithelial cells.[32,33,54–56] TNF-α also increases COX-2 gene expression in human thyroid epithelial cells.[54] The proinflammatory cytokines, IL-1β, TNF-α, and IFN-γ, were found to have no effect on COX-1 gene expression in the above-mentioned cells.

In HRPE cells, we found that IL-1β and TNF-α increase COX-2 gene expression. IFN-γ did not increase either COX-1 or COX-2 gene expression in HRPE cells. When IL-1β and TNF-α were used simultaneously, COX-1 gene expression was upregulated in HRPE cells. Because COX-1 is believed to be the COX isozyme responsible for housekeeping functions, its expression is purported to be stable. However, recent studies demonstrate that COX-1 mRNA expression can be upregulated in various cells by physiological agonists.[41] The expression of COX proteins in HRPE cells mirrors the expression of COX mRNA, with only the COX-2 isozyme detected on IL-1β–stimulated HRPE cells and Western blot analysis of IL-1β–stimulated HRPE cells. COX-1 protein was not detected on IL-1β–stimulated HRPE cells or on Western blot analysis of IL-1β–stimulated HRPE cells (data not shown). The focus of our experiments was on COX expression in HRPE cells in vitro, but we are also interested in COX expression in HRPE cells in vivo. We examined one human donor eye for the presence of COX proteins by immunohistochemistry but did not detect either COX-1 or COX-2 proteins in the RPE layer of this specimen (data not shown). The in vivo results reflect the in vitro results of unstimulated HRPE cells in that COX-1 and COX-2 proteins were not detected by immunocytochemical methods. We did not examine this eye for the presence of COX-2 mRNA and cannot say at this time whether COX-2 mRNA is expressed in low levels in the in vivo situation.

The eicosanoid products of the cyclooxygenase reaction include prostaglandins and thromboxanes. These molecules are lipid mediators that regulate pathologic effects as well as many critical physiological functions in various tissues.[1,2,57] For example, PGE_2 is involved in fever generation[58] and inflammation and inflammatory pain,[1,2] but it also has a cytoprotective role in the gastrointestinal tract.[59,60] PGE_1 has been shown to have cytoprotective effects in cultured HRPE cells under conditions of oxidative stress[61] as well as in gastric mucosa.[62,63] In addition to determining the effect of cytokines on COX gene expression, we also examined their effect on some of the products of the reaction, PGE_2, $PGF_{2\alpha}$, and PGE_1. Cytokines that stimulated COX-2 gene expression, such as TNF-α and IL-1β, also caused an increase in PGE_2 and $PGF_{2\alpha}$ production. PGE_1 production was only increased in HRPE cells incubated with any cytokine combination that included IL-1β. IL-1β and TNF-α seem to act synergistically to increase PGE_1 production. Treatment of HRPE with IL-1β resulted in a dose- and time-dependent increase in both COX-2 mRNA and PGE_2 release.

The increased PGE_2 release in IL-1β–treated HRPE cells was a direct result of the upregulation in COX-2 expression as demonstrated by the dose-dependent inhibition of PGE_2 production by NS-398, a COX-2 selective inhibitor. Aspirin and piroxicam were also able to inhibit PGE_2 production, but higher doses were required to achieve inhibition levels similar to that of NS-398. However, at these higher concentrations, the isozyme specificity of these drugs are lost.

Regulation of COX-2 gene expression appears complex because various substances induce COX-2 expression. Examination of the promoter region of the COX-2 gene reveals several putative transcription factor sites, such as NF-κB, SP1, CRE, ETS-1, AP1, AP2, and CEBP/NF-IL6 as well as a TATA box.[64,65] Several of these transcription factors have been demonstrated to regulate COX-2 gene expression in various cells. Transcription of COX-2 in human chondrocytes requires CRE.[66] Rat granulosa cells,[67] rat aortic smooth muscle cells,[68] and mouse skin carcinoma cells JWF2[69] use NF-IL6 as a transcription factor. AP1 is necessary for COX-2 gene expression on amnion epithelial cells (WISH).[32] Bronchial epithelial cells,[56] WISH cells,[32] immortalized human myometrial cells,[70] human gingival fibroblasts,[71] human neuroblastoma cells,[72] and rheumatoid synoviocytes[73] are some of the various cells using NF-κB to regulate COX-2 transcription.

We demonstrated that in HRPE cells IL-1β–induced activation of NF-κB was responsible for increased COX-2 gene expression and increased PGE_2 production. However, NF-κB may not be the only transcription factor involved in the IL-1β–induced upregulation of the COX-2 gene in HRPE cells because PDTC treatment of cells incubated with IL-1β did not abso-

lutely inhibit production of COX-2 transcripts. It was recently demonstrated in WISH cells that both AP-1 and NF-κB were both necessary for significant upregulation of COX-2.[32] Additional studies examining the role of other transcription factors as well as signaling pathways activated in the IL-1β–mediated COX-2 gene expression will further clarify the regulation of COX-2 transcription in IL-1β–treated HRPE cells.

The demonstration here that HRPE cells express COX-2 and produce prostaglandins in response to cytokines represents another mechanism by which HRPE cells can participate in physiologic and pathologic processes within the retina. Evidence from several laboratories indicates that COX-2 can no longer be thought of as having only proinflammatory activities. COX-2 has been shown to be important for development and normal functioning of various organ systems. Experiments by Gilroy et al.[74] imply that COX-2 may also have anti-inflammatory properties, depending on when it is expressed during an inflammatory disease. Recent evidence also indicates that PGE_1 may act as a cytoprotective molecule, protecting RPE cells from oxidative damage. The role of COX-2 in health and disease is becoming more complex. Further characterization of COX-2 expression could lead to a better understanding of pathogenesis and to novel therapeutic approaches for immune-mediated retinal pathology. Moreover, additional studies are warranted to evaluate the possible physiological role of COX-2 within the retina.

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