Endotoxin-Induced Uveitis in Cyclooxygenase-2–Deficient Mice

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Purpose. Endotoxin-induced uveitis (EIU) is a model that mimics human acute anterior uveitis. Cyclooxygenase (COX)-2 is an enzyme that initiates the conversion of arachidonic acid (AA) into prostaglandins (PGs), whereas 5-lipoxygenase (5-LO) generates leukotrienes (LTs). The purpose of this study was to delineate the role of COX-2 in acute ocular inflammation.

Methods. EIU was induced in wild-type (WT), heterozygotic (COX-2+/−) and COX-2 null (COX-2−/−) mice by injection of lipopolysaccharide (LPS). Other mice were coinjected with LPS and IFNγ. Ocular histology, serum cytokines, and AA products determined by ELISA, and relevant ocular messengers determined by RT-PCR were compared among the different groups.

Results. Histology showed that the EIU score was significantly enhanced in COX-2−/− mice in comparison to WT and COX-2+/− mice but not in COX-2−/−/Eiu. LTB4, in serum and ocular 5-LO transcripts were increased in COX-2−/− Eiu mice in comparison with WT and COX-2−/− Eiu mice. IL-6 increased, whereas IFNγ decreased both in serum and ocular transcripts in COX-2−/− Eiu mice in comparison with WT and COX-2−/−. Furthermore, EIU was suppressed in mice treated with recombinant IFNγ, as shown by the decreased EIU scores, the presence of serum LTβ and IL-6 and ocular 5-LO and IL-6 mRNA, and the increases in serum IFNγ and ocular IFNγ, particularly in COX-2−/− mice.

Conclusions. These data suggest that disturbance of the AA pathway exacerbates EIU in COX-2−/− deficient mice. IFNγ moderately reverses this exacerbation and protects against EIU.

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Endotoxin-induced uveitis (EIU) is an animal model of acute ocular inflammation, produced by injection of endotoxin (lipopolysaccharide; LPS) to mimic human acute anterior uveitis.1–4 By inducing EIU in gene-knockout animals, the model can be used to elucidate the role of specific proteins in the pathogenesis of EIU, to test possible candidate factors that could alter the process of EIU and to offer clues to target molecules for possible intervention in cases of EIU.

Arachidonic acid (AA) is an unsaturated fatty acid that is a normal constituent of membrane phospholipids and is released by the actions of phospholipase A2 (PLA2).5,6 AA is converted to prostaglandins (PGs) by cyclooxygenase (COX) and to leukotrienes (LTs) by 5-lipoxygenase (5-LO), metabolites that are biologically very active and modulate cellular functions.7,8 The two isoforms of COX, COX-1 and -2, are encoded by two separate genes and exhibit distinct cell-specific expression, regulation, and subcellular localization.9 COX-1 is a constitutive enzyme and is associated with the endoplasmic reticulum (ER). Prostaglandins (PGs) are synthesized in the ER by COX-1, then exit the cells and bind to G-protein–coupled cell surface receptors to mediate homeostasis functions.9 In contrast, COX-2 is an inducible enzyme that is induced in a variety of cell types by diverse stimuli.10–12 COX-2 is primarily responsible for increased PG production during inflammation, and PGs are generally considered to be proinflammatory agents.13 However, studies show that COX-2 could play anti-inflammatory roles during certain situations.14–17 A study also indicates that prostanooids may exert a beneficial effect on retinal blood perfusion and may even act as neuroprotective agents.18

LT is derived directly from AA by 5-LO, which acts on AA to produce 5-hydroperoxyeicosatetraenoic acid (5-HPETE). 5-HPETE is converted to LTA4 first and then to LTβ, which induces inflammation by its adhesion, chemotaxis, chemokinesis, and degranulation actions on polymorphonuclear lymphocytes.5,6 LTβ also stimulates phospholipase A2 through a positive feedback loop.19 In general, LTs are considered strong proinflammatory factors.

In this study, we investigated the role of the COX-2 in murine EIU using COX-2 knockout mice. We found that loss of COX-2 allele(s) and subsequent aberrant metabolizing AA resulted in exacerbation of EIU in mice.

Methods

Mice

Breeding pairs of mice heterozygous for the COX-2 (also known as Ptg2(lof/het)) targeted mutation were purchased from the Jackson Laboratory (Bar Harbor, ME) with the strain name B6.129S-Ptgs2tm1Jed. The establishment and basic characteristics of the strain have been described previously.20,21 The mice were kept in a pathogen-free environment. COX-2–deficient mice were generated by gene targeting.20 Absence of COX-2 resulted in female infertility in mice. Therefore, heterozygous breeding pairs were used in this study to generate COX-2 null (COX-2−/−), COX-2+/−, and wild-type mice for use. All newborns were genotyped by PCR using DNA isolated from tail pieces, as described in the genotyping protocol supplied by the Jackson Laboratory (Fig. 1).22,23 Experiments were performed when mice were 6 to 10 weeks old. Mice were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Experimental Design

Each COX-2 strain of mice was grouped as EIU, EIU+IFNγ, or control (PBS) with three to eight mice in each group. Experiments were repeated seven times with similar results. EIU was induced by a single intraperitoneal injection of 0.1 mg Salmonella typhimurium LPS endotoxin (Difco Laboratories, Detroit, MI) in 0.1 mL PBS.24 Clinical assessment of EIU was based on redness and discharge of the eye.
cloudy anterior chamber, and lack of papillary reactivity to the light. We have reported the role of IFNγ in protection against innate immune responses in the eye, including EIU and toxoplasmosis.24,25 To test whether IFNγ was protective against EIU in COX-2-deficient mice, mouse recombinant IFNγ (Endogen, Woburn, MA) was given intraperitoneally simultaneously with LPS at a dose of 2 × 10^5 U/mouse. Control mice were injected with 0.1 mL PBS intraperitoneally. Mice were killed at 24 hours after injection. Right eyes were enucleated and used for histopathology. Left eyes were enucleated and used for ocular gene expression assays. Serum was collected for cytokine determination. IL-6 was measured as an indicator of EIU response.24

Histopathology
Right eyes were immersed in 4% glutaraldehyde for 30 minutes, transferred to and fixed in 10% buffered formalin for at least 24 hours, dehydrated in a series of alcohol solutions, and embedded in methacrylate. Four- to 6-μm vertical sections were cut through the pupillary optic nerve axis and stained with hematoxylin and eosin (H&E). An ocular pathologist counted inflammatory cells in six sections per eye were averaged and recorded.

Enzyme-Linked Immunosorbent Assay
Levels of serum PGE2, LTB4, IL-6, and IFNγ were determined by ELISA. PGE2 and LTB4 ELISA kits were purchased from Cayman Chemical Company (Ann Arbor, MD), and IL-6 ELISA kits from BioSource International (Camarillo, CA), and IFNγ ELISA kits from R&D Systems (Minneapolis, MN). Assays were performed as described by the suppliers.

Reverse Transcription–Polymerase Chain Reaction
Ocular expression of IL-6, IFNγ, and 5-LO was determined by RT-PCR.25,26 Left eyes from a group with the same treatment were pooled for RNA preparation (TRizol reagent; Invitrogen-Gibco, Gaithersburg, MD). Briefly, eyes were homogenized in the reagent, and RNA was extracted by phenol-chloroform and treated with DNase I.25 Ten micrograms of RNA was used in the reverse transcription reaction with commercially available reverse transcriptase (Superscript II; Invitrogen-Gibco, Grand Island, NY) and random hexamers (Promega, Madison, WI). The 10-μL PCR amplification of 2 μL single-strand cDNA was performed by 40 cycles of 45 seconds of denaturation (94°C), 1.5 minutes of annealing, and 2 minutes of elongation (72°C), using 0.5 U gold polymerase (AmpliGold; Applied Biosystems, Foster City, CA). The final cycle was completed by 7 minutes of elongation at 72°C. Three picomoles of the 32P end-labeled sense primer and the unlabeled antisense oligonucleotides were used as appropriate. PCR parameters are shown in Table 1. PCR products were size fractionated using 15% polyacrylamide TBE gels (Bio-Rad, Hercules, CA). For those gels with weak signals under UV light, images were documented by a gel documentation system. The band density was quantified on computer (Labworks 3.02; UVP Laboratory Product, Upland, CA). For those gels with weak signals under UV light, images were captured by autoradiography, and the band densities were quantified on a phosphorescence imager (Image Quant; PhosphorImager; Molecular Dynamics, Sunnyvale, CA). The relative band densities were expressed as arbitrary units.

Statistical Analysis
Groups were compared by one-way ANOVA. The Duncan multiple-range test was used for post hoc comparison of means. Differences were considered significant when P < 0.05.

RESULTS
Increased Ocular Inflammation in COX-2–Deficient Mice with EIU
After LPS injection, mice exhibited conjunctival chemosis (edema), erythema (redness), and ocular discharge. Although all strains of mice had these signs, the clinical response was reproducibly more intense in COX-2-deficient mice than in the wild-type control. Histopathologically, EIU is characterized by the infiltration of inflammatory cells in the eye, mainly in the anterior chamber and posterior vitreous. The main inflammatory cells are neutrophils and macrophages. On average, threefold more inflammatory cells per ocular section (mainly neutrophils, but also macrophages) were detected in the eyes of COX-2−/− mice than that in the wild-type control after LPS injection (Figs. 2, 3). The ratio of infiltrating neutrophils to macrophages was similar in the eyes among the three genotypes. The EIU score of COX-2−/− mice was between that of COX-2+/− and COX-2+/+ mice (Figs. 2, 3), indicating a gene dosage effect between COX-2 and EIU.

Table 1. PCR Parameters

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FIGURE 2. Histopathology of eye sections from mice 1 day after systemic LPS injection. (A) More infiltrating inflammatory cells (arrows) in an eye of a COX-2-deficient mouse compared with that of a WT and heterozygous mice; (B) Higher manifestation showing the greater number of neutrophils in the anterior angles of the EIU eyes. Hematoxylin and eosin; original magnification: (A) top, ×400; bottom, ×200; (B) ×600.
Recombinant IFN γ administration of recombinant IFN γ resulted in increased serum IFN γ levels. The gene expression assay also showed that the ocular 5-LO and IL-6 mRNA level decreased in all three strains of mice after receiving recombinant IFN γ, whereas levels of IFN γ mRNA increased in comparison with EIU mice who were not treated with IFN γ. The densities of the 5-LO bands in all three strains treated with IFN γ are approximately five times lower in WT and COX-2+/− and three folds lower in COX-2−/− than those of the corresponding strains without IFN γ treatment (Fig. 5). The densities of the IL-6 bands in the mice treated with IFN γ were approximately 50% less than those detected in the mice without IFN γ treatment in all three strains (Fig. 5). In contrast, the densities of IFN γ bands increased 50% in WT and COX-2+/− and increased from undetectable to a clearly visible band in COX-2−/− mice that received IFN γ compared with the EIU controls without IFN γ treatment (Fig. 5).

**Discussion**

In the present study, we found that COX-2-deficient mouse present more severe ocular inflammation after systematic LPS injection compared with its wild-type and heterozygous littermate controls. In addition, serum levels of LTB4 and ocular expression of 5-LO were increased in EIU when the COX-2 gene is knocked out; thus, LTB4 and 5-LO levels are associated with the severity of EIU in mice. This observation has not been reported previously. We also showed that IL-6 increased and IFN γ decreased in COX-2−/− mice with EIU when compared with WT and COX-2+/− EIU mice, further confirming previous reports that upregulated IL-6 and downregulated IFN γ are associated with higher scores of EIU.24,25,27 Furthermore, EIU was suppressed, particularly in COX-2−/− mice, if mice were treated with recombinant IFN γ.

Our data suggest that a COX-2 deficiency may exacerbate EIU through multiple mechanisms. Alterations of LTB4 and 5-LO levels in COX-2−/− mice during EIU may be indicators of aberrant processing of AA metabolism that diverts into the LT cascade. It is a common phenomenon in biochemistry that metabolism is shunted to an alternative pathway when a certain pathway is blocked, which can cause a physiologic imbalance. The RT-PCR data indicated an increase of intraocular 5-LO transcripts in COX-2−/− mice after LPS challenge. The mechanism remains to be clarified. The result suggests that in addition to the possible accumulated 5-LO substrate when the COX-2 AA metabolism is impaired, elevated 5-LO expression could be another factor in the overflow of LTB4. The production and expression of 5-LO leading to LT formation, has long been recognized as an inflammatory cascade. LTs are made predominantly by inflammatory cells such as PMNs, macrophages, and mast cells.20 LTB4 promotes neutrophil chemotaxis and adhesion to vascular endothelium. The cysteinyl leukotrienes cause plasma leakage from postcapillary venules and enhance mucus secretion. LTB4 and another 5-LO-derived eicosanoid, 5-oxo-ETE, are eosinophil chemoattractants.29-32 Numerous studies have documented the effects of LT on eyes. Injection of LTB4 into the anterior chamber of rabbit eyes caused leukocyte accumulation in the intraocular fluid and tissues. LTB4 also was a more potent chemotactic agent in the rabbit eye than the chemotactic peptide F-Met-Leu-Phe.33-35

**Expression of 5-LO, IL-6, and IFNγ Transcripts in EIU Model in COX-2-Deficient Mice**

As shown in Figure 5, in accordance with the patterns found in serum, intraocular IL-6 transcripts increased the most in COX-2−/− EIU mice, twofold higher than that of WT and COX-2+/−. In contrast, ocular IFN γ transcripts were not detectable in COX-2−/− mice. In accordance with the pattern of LTB4 in serum, intraocular 5-LO transcripts in COX-2−/− mice were two times higher than that of WT and one time higher than that of COX-2+/− with EIU. This implies that in addition to the possible increase of 5-LO substrate due to the blockage of COX-2 pathway in AA metabolism, elevated 5-LO expression might also contribute to the increase of LTB4.

**Ameliorated EIU in COX-2−/− Mice after Administration of Recombinant IFNγ**

EIU was suppressed in the mice treated with mouse recombinant IFN γ as shown by the decrease in the number of infiltrating inflammatory cells (Figs. 3, 6), particularly in COX-2−/− mice (P < 0.05). However, the proportion of infiltrating neutrophils to macrophages remained the same compared with the mice that did not receive IFN γ. Levels of LTB4 and IL-6 were lowered after administration of recombinant IFN γ in comparison with LPS alone, particularly in COX-2−/− mice (P < 0.05, Figs. 4B, 4C). Injection of recombinant IFN γ resulted in increased serum IFN γ levels. The gene expression assay also showed that the ocular 5-LO and IL-6 mRNA level decreased in all three strains of mice after receiving recombinant IFN γ, whereas levels of IFN γ mRNA increased in comparison with EIU mice who were not treated with IFN γ. The densities of the 5-LO bands in all three strains treated with IFN γ are approximately five times lower in WT and COX-2+/− and three folds lower in COX-2−/− than those of the corresponding strains without IFN γ treatment (Fig. 5). The densities of the IL-6 bands in the mice treated with IFN γ were approximately 50% less than those detected in the mice without IFN γ treatment in all three strains (Fig. 5). In contrast, the densities of IFN γ bands increased 50% in WT and COX-2+/− and increased from undetectable to a clearly visible band in COX-2−/− mice that received IFN γ compared with the EIU controls without IFN γ treatment (Fig. 5).

**Altered Serum Profiles of Relevant Cytokines in COX-2-Deficient Mice with EIU**

PGE2 is increased in WT and COX-2+/− but not in COX-2−/− mice with EIU (Fig. 4A), indicating that PGE2 is induced in EIU, whereas this induction is blocked in the absence of COX-2. LTB4 and IL-6 were increased, with the highest level in COX-2−/− mice (Figs. 4B, 4C, respectively). Among the three murine strains, COX-2−/− mice had the lowest level of IFN γ (Fig. 4D).

**Table 1.** Summary of the infiltrating inflammatory cell counts in the eyes from seven experiments. There were more infiltrating inflammatory cells in COX-2-deficient mice than WT and heterozygous mice. Recombinant IFN γ was capable of lowering the number of infiltrating cells in eyes with EIU, especially in COX-2−/− mice. The total number of mice in each group was: with LPS treatment, WT, 45; COX-2−/−, 35; COX-2+/−, 21; and with a combination of LPS and IFN γ treatment, WT, 40; COX-2−/−, 52; COX-2+/−, 22. *P < 0.05 in comparison with the same strains treated with recombinant IFN γ and lymphocytes (Lym) are shown in the table.

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**Figure 3.** Summary of the infiltrating inflammatory cell counts in the eyes from seven experiments. There were more infiltrating inflammatory cells in COX-2-deficient mice than WT and heterozygous mice. Recombinant IFN γ was capable of lowering the number of infiltrating cells in eyes with EIU, especially in COX-2−/− mice. The total number of mice in each group was: with LPS treatment, WT, 45; COX-2−/−, 35; COX-2+/−, 21; and with a combination of LPS and IFN γ treatment, WT, 40; COX-2−/−, 52; COX-2+/−, 22. *P < 0.05 in comparison with the same strains treated with recombinant IFN γ and lymphocytes (Lym) are shown in the table.

In the present study, we found that COX-2-deficient mouse present more severe ocular inflammation after systematic LPS injection compared with its wild-type and heterozygous littermate controls. In addition, serum levels of LTB4 and ocular expression of 5-LO were increased in EIU when the COX-2 gene is knocked out; thus, LTB4 and 5-LO levels are associated with the severity of EIU in mice. This observation has not been reported previously. We also showed that IL-6 increased and IFN γ decreased in COX-2−/− mice with EIU when compared with WT and COX-2+/− EIU mice, further confirming previous reports that upregulated IL-6 and downregulated IFN γ are associated with higher scores of EIU.24,25,27 Furthermore, EIU was suppressed, particularly in COX-2−/− mice, if mice were treated with recombinant IFN γ.
contrast, PGE$_2$ did not cause significant accumulation of leukocytes under the same conditions. The infiltration of inflammatory cells into the ocular chambers is one of the principle pathologic characteristics of EIU. Therefore, the exacerbation of EIU in COX-2-deficient mice may result from a predominance of chemoattractant factors in the inflammatory mediator profile after administration of LPS.

Although COX-2-derived PGE$_2$ has inflammatory properties, multifaceted roles of eicosanoids have been extensively reported. An in vitro study showed that endogenous PGE$_2$ may modulate inflammation by suppressing macrophage-derived chemokine production through the EP4 receptor. Anti-inflammatory properties of COX-2 were also demonstrated in carrageenin-induced pleurisy in rats. During the later phase of this animal model, COX-2 expressed by migrating mononuclear cells may regulate the resolution of acute inflammation by generating an alternate set of prostaglandins such as those of the cyclopentenone family. These responses could be another explanation for why COX-2 deficiency led to severe EIU in our study.

Numerous data indicate that cytokines play an essential role in the development of EIU. In this study, serum levels and ocular transcripts of IL-6 were significantly increased in COX-2$^{-/-}$ mice and associated with severity of EIU, which agrees with previous reports suggesting that IL-6 was concomitant with maximum uveitis. IL-6 is a multifunctional cytokine that plays important roles in host defense, acute phase reactions, immune responses, and hematopoiesis. Its production is upregulated by various factors, including LPS and cytokines. IL-6 is a crucial cytokine in neonatal sepsis and in the biphasic ocular inflammatory response to LPS in C3H/HeN mice. Reports regarding the effects of IL-6 on EIU pathogenesis were paradoxical. Current data remain incapable of addressing whether IL-6 functions as a bystander or participant in EIU. Intravitreal injection of endotoxin-free human recombinant IL-6 in rats resulted in uveitis, resembling the ocular response to endotoxin. However, results in a study using IL-6 gene-deficient mice indicate that IL-6 may not be essential in the pathogenesis of EIU. The increased levels of IL-6 may be important as an innate immune response to enhance the adaptive immune response to microorganisms but may not have a pathologic role in the simplified disease model of EIU.
IFNγ was decreased both in serum and in ocular transcripts in COX-2−/− EIU in comparison with WT and COX-2+/− EIU mice. We have reported that MCP-1−/− mice are less susceptible to EIU than their wild-type counterparts and have increased levels of IFNγ in both serum and ocular transcripts during EIU. This consistency suggests that LPS-induced IFNγ may have a protective effect against EIU. Furthermore, EIU was suppressed if mice were treated with recombinant IFNγ, indicated by a decreased number of infiltrating inflammatory cells in the eye, decreased LTB4 in serum and 5-LO mRNA levels in the eye, decreased IL-6 serum and ocular mRNA levels, and increased IFNγ both in serum and ocular mRNA levels, particularly in COX-2−/− mice. Although the details of the interaction between IFNγ and EIU remain unknown in the present study, it is unlikely that the exogenous IFNγ with a very short half-life in vivo could contribute to the increases in IFNγ detected in serum 24 hours after injection. Furthermore, the increases of ocular IFNγ transcripts after the exogenous IFNγ injection suggest the endogenous production of serum IFNγ. The interactions among IFNγ and LPS, and their effects on AA metabolism in COX-2−deficient mice are complicated and require further investigation. However, the studies of the exact mechanism of IFNγ in EIU and innate immunity are beyond the scope of the present experiments.

In summary, the present study suggests that COX-2 deficiency exacerbates EIU in mice. Elevation of LTB4 and 5-LO in COX-2−deficient mice during EIU indicates an enhanced alternative metabolism of AA through lipoxgenase pathway, which causes more severe EIU. It is unlikely that exacerbation of EIU in COX-2−deficient mice is due to the failure of inducible PG synthesis. Instead, our data support the notion that COX-2 may have anti-inflammatory properties. The severity of EIU in COX-2−deficient mice is associated with an increase of IL-6 and a decrease of IFNγ, which could be partly overcome by giving exogenous recombinant IFNγ. These data...
demonstrate interactions among certain important inflammatory mediators and cytokines in the eye and thus suggest the potential utilization of more specific anti-inflammatory medications. Manipulation of cytokines and inflammatory mediators are useful strategies for the treatment of ocular inflammation.

References

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