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Anti-Inflammatory Effects of Specific Cyclooxygenase 2,5-Lipoxygenase, and Inducible Nitric Oxide Synthase Inhibitors on Experimental Autoimmune Anterior Uveitis (EAAU)

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Abstract

Purpose—Inflammation, in general, causes the release of a variety of inflammatory mediators that in turn induce cyclooxygenase (COX) 2, nitric oxide synthase (iNOS) and 5-lipoxygenase (LP) synthesis, producing large amounts of inflammatory prostaglandins (PG), nitric oxide (NO), and leukotriene (LT) B₄. Therefore, inhibition of these enzymes may abrogate intraocular inflammation in experimental autoimmune anterior uveitis (EAAU).

Methods—Lewis rats were immunized with melanin-associated antigen (MAA) isolated from bovine iris and ciliary body. These animals were divided into three groups. The first group of rats received subcutaneous injection of COX 2 inhibitor CS 236 at different time points. The second and third groups of animals received subcutaneous aminoguanidine (AG), an iNOS inhibitor, and nordihydroguaiaretic acid (NDGA), a 5-LP inhibitor, respectively. Control animals received vehicle. Rat eyes were examined daily by slit-lamp biomicroscopy from Day 7 to 30 post injection for uveitis. Animals were also sacrificed at various time points for histologic analysis.

Results—Control animals developed severe EAAU in both eyes. The disease started in these animals on Day 12 post immunization and lasted for ten days. Interestingly, CS 236, a potent COX 2 inhibitor, completely abrogated EAAU when the animals were treated daily from the Day 0 to 14 or Day 0 to 20 after MAA injection. Furthermore, daily CS 236 treatment after the onset of EAAU (Day 14–20) significantly reduced the severity (both clinical and histologic) of EAAU and shortened the duration of disease. iNOS inhibitor (AG) and 5-LP inhibitor (NDGA) partially attenuated EAAU.

Conclusions—Our results show that EAAU was partially attenuated by AG and NDGA. Interestingly, CS 236, a potent COX 2 inhibitor, completely inhibited EAAU in male Lewis rats most likely by inhibiting the initial phase and onset of the disease.

Keywords

COX 2 inhibitor; EAAU; eicosanoids; iNOS inhibitor; 5-LP inhibitor; nitric oxide

INTRODUCTION

In the normal eye, nitric oxide (NO), prostaglandins (PG), and leukotrienes (LT) are reported to be locally synthesized by various ocular tissues.^{1,2} NO is synthesized from L-arginine (L-ARG) by nitric oxide synthase (NOS), while PG and LT are synthesized from arachidonic acid (AA) by cyclooxygenase (COX) and 5-lipoxygenase (LP) enzymes, respectively.^{1–3} The

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physiologic effects of NO, PG, and LT include vasodilation, resulting in improved blood supply to various ocular tissues, and reduction of intraocular pressure.¹⁻⁴

In the 1970s and 80s when most studies on PG including ocular effects were conducted, it was assumed that a single ubiquitous enzyme, a constitutive COX, was responsible for the formation of all PG from a common precursor AA. Furthermore, since AA is released from the membrane phospholipid pool by phospholipase A₂ (PLA₂), it was assumed that PLA₂ was the main, and possibly the only, enzymatic step that determined the rate of PG production. Similarly, it was assumed that the sole therapeutic effect of aspirin (ASA) and indomethacin (INDO) was the result of inhibition of COX activity.¹

A new era in anti-inflammatory drug therapy began with the discovery of two COX enzymes. COX 1 is present at all times in most tissues and is responsible for the basal PG synthesis required for normal homeostasis. In contrast, COX 2, an inducible enzyme which is rapidly expressed by mononuclear cells as well as possibly other cells within the eye, contributes to local inflammation.¹

Three distinct isoforms of NOS have been identified. These include inducible (iNOS), endothelial (eNOS), and neuronal (nNOS) isoenzymes.^{5,6} iNOS has been reported to play an important role in various inflammatory conditions.⁶ Thus, iNOS and COX 2 have a synergistic effect during the development of the inflammatory response.^{3,7,8}

It has been well established that the eicosanoids, PG and LT, are important mediators of intraocular inflammation.¹ Increased levels of PG and LT were demonstrated in inflamed tissue as well as in intraocular fluid using various experimental models such as para-centesis, corneal denudation, and endotoxin-induced uveitis. Moreover, these increased levels correlated with the breakdown of the blood-aqueous barrier, hyperemia, edema, and chemotaxis.⁹⁻¹⁹ However, COX inhibitors like INDO, flurbiprofen (FLURBI), and 5-LP inhibitors BWA4C and BW218C only partially attenuated the inflammation.¹²

Nitric oxide (NO) has been shown to play an important role in various animal models of intraocular inflammation.^{2,20-28} NOS inhibitors such as NG-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine (AG) significantly inhibited iris hyperemia, aqueous flare, and cellular infiltration in endotoxin-induced uveitis.²⁰⁻²⁵

In guinea pigs, the breakdown of the blood-aqueous barrier has been reported to correlate with increased aqueous PLA₂ during s-antigen-induced uveitis. Furthermore, PLA₂ inhibitors (EDTA, KLM-583, and KLM-538) and COX inhibitors (INDO and FLURBI) in high doses partially attenuated the intraocular inflammation.^{9,29} In the Lewis rat, uveitis induced by the footpad injection of melanin from the RPE and uvea was suppressed by Tricyclodecan-9-yl-xanthogenate, an inhibitor of phosphatidylcholine-specific phospholipase C, which also inhibits iNOS.²⁶

Experimental autoimmune anterior uveitis (EAAU) is an organ-specific autoimmune disease of the eye, originally described by Broekhuysen and co-workers in 1991.³⁰ EAAU serves as an animal model of idiopathic human anterior uveitis.³¹ We have extensively characterized this model and have shown that severe inflammation occurs in the anterior segment of the eye of Lewis rats after the foot-pad injection of melanin-associated antigen (MAA) isolated from bovine iris and ciliary body.³²⁻³⁴ The present study was undertaken to investigate the role of AA metabolites and NO in EAAU by using selective inhibitors of COX 2, iNOS, and 5-LP.

MATERIALS AND METHODS

CS 236, a specific COX 2 inhibitor, was a gift from Pharmacia (St. Louis, MO, USA). Aminoguanidine (iNOS inhibitor) and nordihydroguaiaretic acid (5-LP inhibitor) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals

Pathogen-free male Lewis rats (5–6 wks old) were obtained from Harlan Sprague-Dawley (Indianapolis, IN, USA) and the animals were cared for in accordance with the guidelines of the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

Induction of EAAU

Insoluble melanin-associated antigen (MAA) was prepared as previously described by us.^{32–34} For the induction of uveitis, male Lewis rats were immunized with 50 μ l stable emulsion containing 100 μ g MAA emulsified (1:1) in complete Freund's adjuvant (CFA, Difco Laboratories, Inc., Detroit, MI, USA) using a single-dose induction protocol in the hind footpad.^{32–34} Purified pertussis toxin (PTX; 1 μ g/animal) was used as an additional adjuvant.

Effect of Specific COX 2, 5-LP, and iNOS Inhibitors on EAAU

The MAA-immunized rats were injected subcutaneously with specific COX 2, 5-LP, and iNOS inhibitors separately as described below. Animals were examined daily between Days 7 and 30 post injection for clinical signs of uveitis using slit-lamp biomicroscopy. EAAU was graded in a masked fashion using the criteria previously reported.^{32–34} Eyes were also harvested on Days 10, 12, 14, 18, 22, and 30 for histologic analysis to assess the course and severity of inflammation. Freshly enucleated eyes were fixed immediately for 24 hours in 2.5% buffered glutaraldehyde and transferred into 10% neutral formalin. Fixed and dehydrated eyes were then embedded in paraffin. Six-micron sections were stained with hematoxylin and eosin and examined by light microscopy. The intensity of uveitis was histologically scored in a masked fashion on an arbitrary scale of 0–4.^{32–34} The minimal criterion for scoring an animal as positive by histopathology was the presence of inflammatory cell infiltration of the iris, ciliary body, and anterior chamber. All experiments were terminated on Day 30 after antigen administration.

The doses of the specific COX 2,^{8,35,36} 5-LP,²⁹ and iNOS^{25–27} inhibitors were selected from the previous reports in which these drugs were effective in inhibiting their respective enzymes. The animals were divided into following three groups.

Group A: Cyclooxygenase 2 Inhibitor—Lewis rats in Group A were divided into seven subgroups and received subcutaneous injections of CS 236 (4[5-(4-chlorophenyl)-3-(trifluoromethyl)-1 H-pyrazole-1-yl] benzenesulfonamide (3 mg/kg)), a COX 2 inhibitor, at various time points. The first group of rats (n = 3) received only one subcutaneous injection of CS 236 in Dimethyl Sulfoxide 24 hours prior to MAA sensitization. The second group (n = 3) received CS 236 one hour prior to MAA sensitization and the treatment was continued daily for 19 days. The third group of rats (n = 3) received CS 236 one hour prior to MAA injection and the treatment was continued daily for 13 days. The fourth group of rats (n = 3) was injected only once on Day 7 post immunization and animals in the fifth group (n = 3) received a single injection of CS 236 on Day 14 post immunization. The sixth group of rats (n = 3) received a daily single injection of CS 236 from Days 14 to 20 after MAA injection. Animals in the control (7th) group (n = 3) received an equal volume of DMSO for 20 days. The experiment was repeated three times.

Group B: Inducible Nitric Oxide Synthase Inhibitor—Aminoguanidine (AG) was injected subcutaneously (100 mg/kg) in Lewis rats ($n = 3$) one hour prior to immunization. Subsequently, AG was administered daily for 19 days. Control rats ($n = 3$) received PBS. The experiment was repeated three times.

Group C: 5-Lipoxygenase Inhibitor—Nordihydroguaiaretic acid (NDGA) was administered subcutaneously in Lewis rats ($n = 3$) one hour prior to immunization (10 mg/kg). Subsequently, injections were given daily for 19 days. Control rats ($n = 3$) received PBS. The experiment was repeated three times.

Statistical Analysis

Differences between experimental (drug-treated) and control (vehicle-treated) groups were analyzed using the Mann-Whitney U test. The probability value $p \leq 0.05$ was considered statistically significant.

RESULTS

Effect of Cyclooxygenase 2 Inhibitor, CS 236, on EAAU

Animals were divided into seven groups and CS 236 (3 mg/kg) treatment was performed at different time points during the disease process (Table 1). DMSO-treated control rats (Group 7) developed severe EAAU in both eyes after MAA sensitization. In these animals, the disease started at Day 12 post immunization and lasted for ten days. Maximum inflammation occurred between Days 16 and 18. Twelve days after immunization, mononuclear cells started to infiltrate the center of the ciliary body stroma with a few cells in the iris and stroma (data not shown). In the severe stage of the disease, the ciliary body and iris became swollen and heavily infiltrated with inflammatory cells (Fig. 1B). An exudate of proteinaceous material with inflammatory cells appeared in the anterior chamber with spillover into the anterior vitreous (Fig. 1B). The clinical and histopathological features of EAAU in rats in Groups 4 and 5 (injected once with CS 236 on the Day 7 or 14, respectively) were similar to control DMSO-treated animals (Table 1). In contrast, the COX 2 inhibitor, CS 236, when injected once 24 hours prior to MAA sensitization (Group 1) completely prevented the development of EAAU (Table 1). Furthermore, daily subcutaneous injections of CS 236 in rats up to 20 days in Group 2 (Fig. 1A, Table 1) and up to 14 days in Group 3 completely abrogated EAAU (Table 1). Interestingly, when the animals in Group 6 were treated daily with CS 236 from Day 14 to 20 (after the onset of EAAU), the EAAU symptoms were very mild with a clinical and histologic scores of 0.5. This mild EAAU lasted only for six days in these animals (Table 1). The experiment was repeated three times with similar results and there was no apparent side effect observed in rats treated with CS236.

Effect of Inducible Nitric Oxide Synthase Inhibitor, AG, on EAAU

We next studied the effect of daily AG treatment (100 mg/kg) for 20 days on EAAU. The results presented in Table 2 show that the severity of EAAU (both clinical and histologic) was significantly ($p < 0.001$) reduced in the AG-treated rats compared to control animals. However, the duration of EAAU was only slightly reduced in the AG-treated animals compared to controls (Table 2). The experiment was repeated three times with similar results and there was no apparent side effect observed in rats treated with AG.

Effect of 5-Lipoxygenase Inhibitor, NDGA, on EAAU

As shown in Table 3, daily subcutaneous injections of NDGA (10 mg/kg) for 20 days decreased both the clinical and histologic severity of anterior uveitis induced by MAA in Lewis rats. The incidence and the duration of EAAU, however, were not altered in these animals (Table 3).

The experiment was repeated three times with similar results and there was no apparent side effect observed in rats treated with NDGA.

DISCUSSION

Acute anterior uveitis (AAU), which includes iritis and/or iridocyclitis, is the most common form of intraocular inflammation, is usually of unknown etiology, and is a major cause of visual disability in our society. Inflammation occurs in either the iris or ciliary body, with spillover of inflammatory cells into the vitreous space behind the lens.³¹ Experimental autoimmune anterior uveitis (EAAU) is an organ-specific inflammatory disease affecting the anterior segment (iris and ciliary body) of the eye, which closely resembles human idiopathic acute anterior uveitis (AAU).^{30–34} The effects of CS 236, aminoguanidine (AG), and nordihydroguaiaretic acid (NDGA), the selective inhibitors of COX 2, iNOS, and 5-LP, respectively, on EAAU was investigated in the current study.

The results presented here show that CS 236 completely abrogated EAAU induced by bovine MAA in Lewis rats. CS 236 is a highly selective inhibitor of COX 2.³⁵ It is similar to the commercially available COX 2 inhibitor celebrex, but four times more potent with a half-life of 15 days in vivo.³⁵ When animals were treated with CS 236 24 hours prior to MAA injection or for 14 or 20 days daily after sensitization with MAA, the Lewis rats did not develop EAAU. In contrast, control rats, which received only DMSO vehicle, developed severe EAAU. Interestingly, very mild uveitis was observed both clinically and histologically in Lewis rats treated with CS 236 after the onset of EAAU and the duration of the disease was also shortened. This observation has important clinical significance because EAAU serves as an animal model for human AAU.^{30, 32–34}

COX inhibitors like indomethacin, flurbiprofen, and diclofenac have been reported to partially inhibit the inflammatory reactions in various animal models of ocular inflammation, including endotoxin-induced uveitis,¹² s-antigen-induced EAU,^{9, 37} and corneal denudation.^{15, 16} However, the effects of specific COX 2 inhibitor(s) were not explored in any of these studies. In a controlled clinical trial of nonsteroidal and steroidal anti-inflammatory treatment, indomethacin attenuated AAU.³⁸ To the best of our knowledge, this is the first study demonstrating the effect of a specific COX 2 inhibitor on EAAU. The results reported in this manuscript suggest that PGs of the COX 2 origin may play a significant role in EAAU.

Our results demonstrate that NDGA, a 5-LP inhibitor, partially attenuated EAAU. AA is also metabolized to leukotrienes (LTs) by means of the 5-lipoxygenase (LP) pathway. LTC₄, D₄, and E₄ are the mediators of asthma and allergic reactions, while LTB₄ is a potent chemotactic agent.^{9–14} The latter has been reported to stimulate the production of a number of inflammatory cytokines and mediators from infiltrating cells.³⁹ Chen and co-workers reported that NDGA partially attenuated s-antigen-induced uveitis.²⁹ These researchers suggested LTB₄ might play a role in mediating cellular infiltration in anterior uvea and aqueous humor.

The results presented in the current manuscript show that EAAU was partially inhibited by aminoguanidine, a selective inhibitor of iNOS. Our observations are consistent with those reported in the literature. The anti-inflammatory action of different NOS inhibitors has been tested in animal models of intraocular inflammation. In those studies, NOS inhibitors have been shown to partially inhibit the inflammatory responses, suggesting some involvement of induced NO in ocular inflammation.^{11, 20–28} Using immunohistochemical analysis, Smith and co-workers demonstrated the presence of iNOS-positive cells in the anterior uvea and in the inflammatory exudates only during the early stages of experimental melanin-induced uveitis.²⁸ Furthermore, mice deficient in iNOS have been shown to retain susceptibility to endotoxin-induced uveitis. Smith and co-workers reported that C57BL/6 × 129 mice lacking

the gene encoding iNOS developed endotoxin-induced uveitis identical to that observed in wild type (iNOS producer) controls.⁴⁰

In summary, our results suggest that LTs and inducible NO may play some role in the pathogenesis of EAAU. Moreover, PCs of COX 2 origin may serve as important mediators of uveitis in EAAU. The mechanism of action of CS 236 in EAAU is currently being explored in our laboratory.

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FIGURE 1.

Inhibition of experimental autoimmune anterior uveitis (EAAU) by CS 236. Lewis rats were immunized with 100 μ g bovine melanin-associated antigen. The experimental group (A) received COX 2 inhibition, CS 236 (3 mg/kg), while the control animals (B) received an equal volume of DMSO (vehicle) daily for 20 days.

TABLE 1

Effect of CS 236 on EAAU in Lewis rats.

Group	CS 236 (3 mg/kg) injection	EAAU			
		Incidence	Duration (days)	Clinical score	Histologic score
1	Once 24 h before MAA injection	0/18	0	0	0
2	Daily for 20 days	0/18	0	0	0
3	Daily for 14 days	0/18	0	0	0
4	Once on Day 7	18/18	10	4	4
5	Once on Day 14	18/18	10	4	4
6	Daily from Day 14 to Day 20	18/18	6	0.5 [*]	0.5 [*]
7	Control Demethyl Sulfoxide (vehicle) injected daily for 20 days	18/18	10	4	4

EAAU, experimental autoimmune anterior uveitis; MAA, melanin-associated antigen. Incidence of EAAU given as positive/total eyes following clinical examination. The clinical and histologic scores presented in this table represent the scores obtained on Day 16 post MAA injection.

*
 $p < 0.001$.

TABLE 2

Effect of aminoguanidine (AG) on EAAU in Lewis rats.

Parameters	Control	Aminoguanidine (100 mg/kg)
Incidence	18/18	18/18
Duration of disease (days)	10	7
Uveitis (clinical score)	4	2*
Uveitis (histologic score)	4	1.5*

Aminoguanidine was injected daily for 20 days. EAAU, experimental autoimmune anterior uveitis. Incidence of EAAU given as positive/total eyes following clinical examination. The clinical and histologic scores presented in this table represent the scores obtained on Day 16 post MAA injection.

*
 $p < 0.001$.

TABLE 3

Effect of nordihydroguaiaretic acid (NDGA) on EAAU in Lewis rats.

Parameters	Control	Nordihydro- guaiaretic acid (10 mg/kg)
Incidence	18/18	18/18
Duration of disease (days)	10	10
Uveitis (clinical score)	4	2*
Uveitis (histologic score)	4/18	2*

NDGA was injected daily for 20 days. EAAU, experimental autoimmune anterior uveitis. Incidence of EAAU given as positive/total eyes following clinical examination. The clinical and histologic scores presented in this table represent the scores obtained on Day 16 post MAA injection.

*
 $p < 0.001$.