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Time Course of Rabbit Ocular Inflammatory Response and Mediator Release after Intravitreal Endotoxin

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An inflammatory response was elicited in the rabbit eye by intravitreal injection of endotoxin. The appearance in aqueous humor of selected metabolites of arachidonic acid metabolism at various times was correlated with the influx of protein and myeloperoxidase activity in the iris–ciliary body. After intravitreal injection of endotoxin, aqueous humor protein levels increased substantially within 2 hr. This aqueous humor protein increase occurred before a significant appearance of prostaglandin E_2 (PGE_2) in the aqueous humor. Myeloperoxidase activity in the iris–ciliary body, a measure of polymorphonuclear leukocyte (PMN) infiltration, showed little elevation until 6 hr after endotoxin injection and then increased rapidly through 24 hr. The appearance of the leukotriene B_4 (LTB_4) followed a similar time course: levels in the aqueous humor were partially elevated until 6 hr after endotoxin injection, when levels begin to rise rapidly. These findings are interpreted to demonstrate the dependence of PMN infiltration on the release and accumulation of LTB_4; the initial breakdown of the blood–aqueous barrier and influx of protein appears to be independent of significant release of PGE_2.
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The ability of ocular tissues to metabolize arachidonate and to generate inflammatory mediators has been demonstrated adequately.[1–4] It has been shown also that after an inflammatory stimulus in vivo, ocular tissues can release both the lipoxygenase and cyclooxygenase products of arachidonate metabolism.[5–7] The ocular inflammatory response includes both the breakdown of the blood–aqueous barrier, resulting in protein extravasation into the aqueous humor, and also the influx of polymorphonuclear leukocytes (PMNs).

Breakdown of the blood–aqueous barrier has been associated with the release of certain prostaglandins,[8–10] whereas the influx of inflammatory cells is considered to be the result of the release of potent chemoattractants, such as leukotriene B_4 (LTB_4), generated by the lipoxygenase pathway of arachidonic acid metabolism.[11–13] Although there is ample evidence to support the relationship between protein extravasation and prostaglandins, and between PMN infiltration and LTB_4 release, little is known of the time course of these events during the initiation, continuation, and resolution of an inflammatory episode.

The current study was directed towards clarifying the temporal fluctuation in levels of two major arachidonic acid metabolites, prostaglandin E_2 (PGE_2) and LTB_4 after endotoxin injection into the vitreous body. The intent was to determine the extent of correlation between temporal release of arachidonic acid (AA) metabolites and coincident or subsequent perturbations of normal physiologic function associated with the inflammatory response.

Materials and Methods

All experiments were performed with New Zealand strain albino rabbits (Clerco, Cinncinati, OH), weighing 2–2.5 kg. The animals were anesthetized with 37.5 mg/kg ketamine (Bristol Laboratories, Syracuse, NY) and 5 mg/kg xylazine HCl (Miles Laboratories, Shawnee, KS) administered by intramuscular injection. Animals were euthanized before tissue collection by an intravenous overdose of sodium pentobarbital (100 mg/kg; Butler, Columbus, OH). Rabbits were treated in accordance with the ARVO Resolution on the Use of Animals in Research.

Endotoxin Injection

Under anesthesia, the eyelids were retracted and the globe gently proptosed. Each control eye received 10 μl sterile saline, through a 30-gauge needle, inserted posterior to the limbus into the vitreous body.

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The contralateral eye received 10 μl sterile saline containing 1 μg lipopolysaccharide (*E. coli* 055a:B5; Sigma, St. Louis, MO).

Collection of Samples

Animals were sacrificed at either 2, 6, 14, or 24 hr after the intravitreal injection of endotoxin. Immediately after euthanasia, aqueous humor was recovered by aspiration through a 25-gauge needle attached to a standard vein infusion set. Aqueous humor samples were analyzed for levels of PGE_2 and LTB_4 by radioimmunoassay (see below) and for protein, by the method of Lowry et al.[14]

After aspiration of aqueous humor, the anterior segment was removed by radial excision approximately 2 mm posterior to the limbal region. The iris–ciliary body was then dissected from the anterior segment and processed for myeloperoxidase activity analysis. Aqueous humor and iridial samples were placed in chilled 2-ml vials and weighed; each vial contained 20 μl 3.8% Na-citrate solution (anticoagulant) containing the AA metabolism inhibitors BW4AC (32 ng) and indomethacin (1 μg) to prevent further lipoxygenase or cyclooxygenase metabolism.

Myeloperoxidase Assay

Myeloperoxidase (MPO) activity was measured in the iris–ciliary body as an index of PMN infiltration.[15] The activity of MPO, found concentrated in granules within PMNs, was determined in iris–ciliary body homogenized in 2 ml hexadecyltrimethyl ammonium bromide, and freeze–thawed three times by immersion in liquid nitrogen alternated with tap water. The samples were centrifuged at 40,000 *g* for 15 min at 4°C. Aliquots (100 μl) of the resulting supernatant were then analyzed according to the method of Williams et al.[15]

PGE_2 and LTB_4 Radioimmunoassays (RIAs)

In trial experiments it was determined that the presence of protein in the aqueous humor could affect the accurate RIA determination of AA metabolites. Therefore, samples of aqueous humor used for analysis were solvent-extracted to recover the AA metabolites and to remove protein and other cellular material. Samples were acidified to pH 4.0 with formic acid. One milliliter ethyl acetate then was added to each sample, which then was vortexed and centrifuged at 10,000 *g* for 1 min in an Eppendorf Model 5416 centrifuge. Eight hundred fifty microliters of the resulting supernatant was dried under nitrogen and reconstituted in RIA buffer for analysis of PGE_2 and LTB_4. The procedure for analysis of samples by RIA were identical to those described in the commercially prepared kits from Amersham (Arlington Heights, IL). Unlabeled AA standards were purchased from Sigma. Tritiated PGE_2 and LTB_4 were purchased from Amersham. Antisera were obtained from Advanced Magnetics (Cambridge, MA). The cross reactivities of these antisera with other AA metabolites were less than 5%. All RIA tubes were prepared as duplicates and incubated for 18 hr. Levels of PGE_2 and LTB_4 in sample tubes were calculated from standard curves which were generated for each assay, and were corrected for losses due to extraction.

Statistics

Statistical analysis was performed using the student paired t-test. Comparison between control and experimental groups was done at identical time intervals.

Results

The clinical signs of a response to intravitreal endotoxin, including iridial hyperemia and flare in the anterior chamber, were evident at approximately 6 hr. The degree of iridial hyperemia and flare as well as of conjunctival hyperemia continued to develop through 24 hr, when clinical signs were maximal. After 24 hr, the clinical signs were stable, and subsided slowly through 5–7 days, after which the eyes were again quiescent. Control eyes were generally quiescent throughout the entire period, with an occasional instance of mild iridial hyperemia after 24 hr.

Aqueous Humor Protein

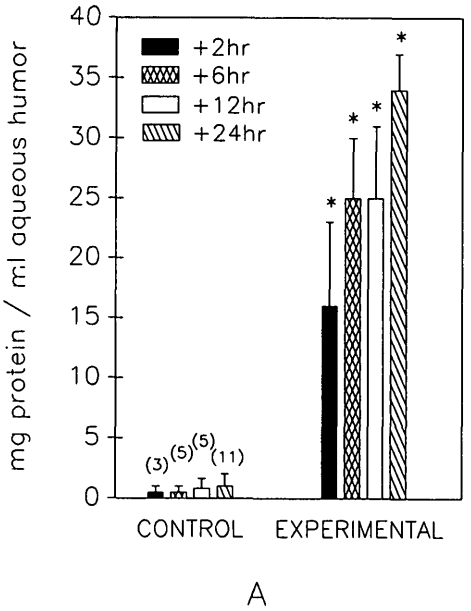
Protein concentrations in saline-injected (control) eyes were not significantly increased at any time interval after endotoxin injection into the contralateral (experimental) eye. Protein levels in experimental eyes were significantly greater than control eye values at all time intervals ($P < 0.05$; Fig. 1A). The minimal protein increase in the control eye is assumed to be due to the injection procedure itself.

MPO Assay of Iris–Ciliary Body Tissue

MPO activity from control (saline-injected) eyes remained at background levels for up to 6 hr postendotoxin injection (Fig. 1B); at 14 and 24 hr the levels were slightly elevated. At 2 and 6 hr the experimental eyes responded similarly to the control eyes; however, at 14 and 24 hr MPO activity was significantly elevated over that found in control eyes ($P < 0.05$, Fig. 1B).

LTB_4 Levels in Aqueous Samples

LTB_4 levels ranged between 0.5 and 2.5 ng/ml aqueous humor through 24 hr (Fig. 2A) in both con-



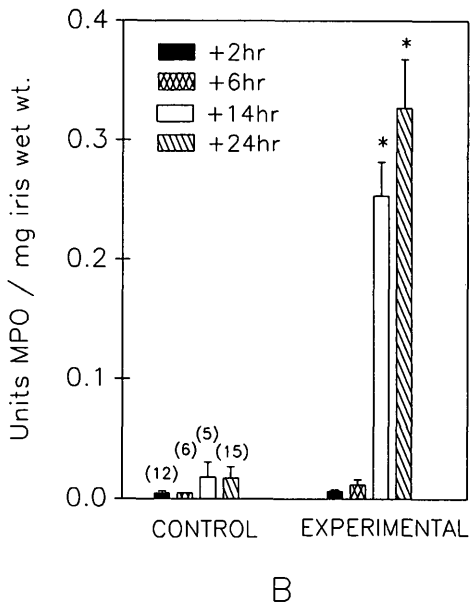


Fig. 1. Protein levels **(A)** measured in aqueous humor samples and MPO activity **(B)** from iris-ciliary body homogenates. Control eyes received 10 μl intravitreal saline, and experimental eyes received 1 μg endotoxin in 10 μl saline by intravitreal injection. The time intervals indicate time elapsed after intravitreal injection, and the bar values represent the mean (±SEM, indicated by the line segments). The number of animals in each group is given in parentheses. A significant difference ($P < 0.05$) between an experimental and control group is indicated by an asterisk.

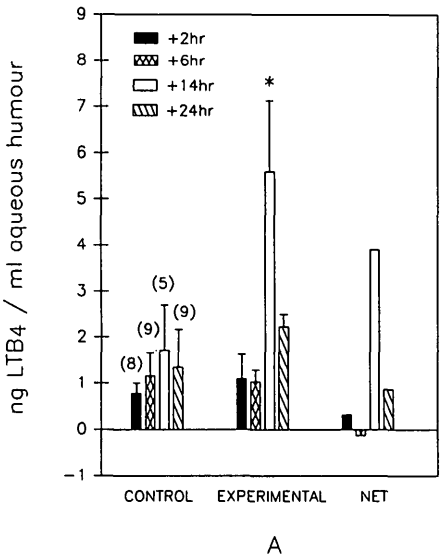
trol and endotoxin-treated eyes. The only exception was a several-fold increase in the LTB_4 level in the experimental eye, to 5.6 ng/ml at 14 hr. At 24 hr, the LTB_4 level in the endotoxin-treated eye had decreased significantly but remained elevated over the control eye values.

PGE_2 Levels in Aqueous Samples

PGE_2 levels in control eyes ranged from 2 to 4 ng/ml aqueous humor through 24 hr (Fig. 2B). After endotoxin injection, values remained at control levels until the 14-hr interval, when there was an 8-fold increase in PGE_2. The PGE_2 levels peaked at 14 hr and then declined slightly at 24 hr. The PGE_2 values in saline-injected eyes exhibited a time course in aqueous humor similar, but attenuated in comparison, to that seen in experimental values.

Comparison of Time Courses in Endotoxin-Injected Eyes

After intravitreal injection of endotoxin in sterile saline into one eye and sterile saline alone into the control contralateral eye, there was occasional evidence of a mild inflammatory response in the control



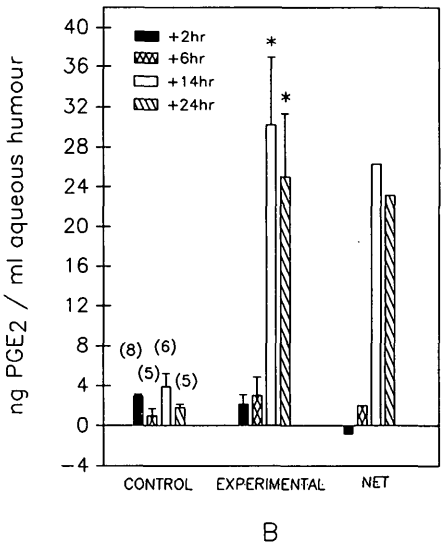


Fig. 2. LTB_4 **(A)** and PGE_2 **(B)** levels measured by RIA in aqueous humor samples. Control eyes received 10 μl intravitreal saline, and experimental eyes received 1 μg endotoxin in 10 μl saline by intravitreal injection. Net levels represent the numeric difference between the values measured in the experimental and control eyes. The time intervals indicate time elapsed after intravitreal injection, and the bar values represent the mean (±SEM, indicated by the line segments). The number of animals in each group is given in parentheses. A significant difference ($P < 0.05$) between an experimental and control group is indicated by an asterisk.

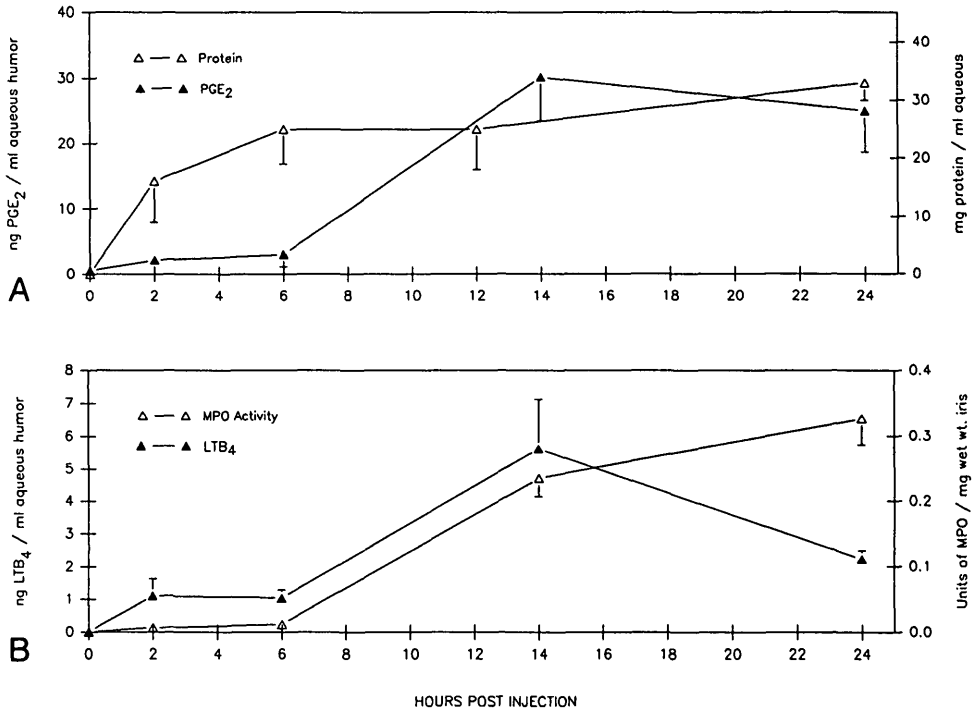


Fig. 3. The temporal relationship between aqueous humor protein and PGE_2 **(A)** and between iris–ciliary body MPO activity and aqueous humor LTB_4 levels **(B)** in the experimental eye during the 24-hr period after intravitreal injection of endotoxin. The intervals on the abscissa represent time elapsed after intravitreal injection, and the values represented are the mean (±SEM, indicated by the vertical line segments).

eye. The assumption was made that the control eye response resulted from manipulation of the globe and from the intravitreal injection procedure. In Figure 3, the time course of PGE_2 and LTB_4 appearance in aqueous humor is compared with protein extravasation into aqueous humor and PMN infiltration into iris–ciliary body, respectively. Comparisons were made between endotoxin-treated eyes for the stated parameters; values for control eyes were not subtracted from the experimental values.

Aqueous humor protein levels began to rise almost immediately in the endotoxin-injected eye, but PGE_2 levels did not rise significantly in the first 6 hr. Protein levels in aqueous were nearly maximal at 2 hr, whereas PGE_2 levels did not peak until 14 hr (Fig. 3A). LTB_4 levels in aqueous humor closely paralleled the early time course of appearance of MPO activity (an index of tissue PMN infiltration) in the iris–ciliary body. MPO activity in experimental eyes was minimal between 2 and 6 hr but increased markedly between 6 and 14 hr after endotoxin injection (Fig. 3B). LTB_4 levels in both control and experimental eyes were elevated above levels found in naive uninjected eyes between 2 and 6 hr, suggesting a response to the injection procedure or a contralateral effect in the control eye, due to endotoxin injection in the experimental eye. In either case, experimental eye levels were not elevated above control eye levels. In the ensuing 8-hr interval, experimental eye LTB_4 levels were observed to increase rapidly until they peaked at 14 hr. At 24 hr, LTB_4 levels were declining, while MPO activity remained elevated.

Discussion

The involvement of AA metabolism in inflammation is well recognized. Products of the cyclooxygenase pathway, the prostaglandins, are principally implicated in the vascular events of inflammation, while certain leukotrienes, derived from the lipoxygenase pathway, attract PMNs to the site of inflammation. There is ample evidence that AA metabolism plays a central role in ocular inflammation. Ocular tissues can metabolize AA by way of both the cyclooxygenase and the lipoxygenase pathways;[2-4,7] administration of individual AA metabolites can induce segments of the inflammatory response;[13,16-18] and certain prostaglandins and leukotrienes can be identified in the eye during an inflammatory episode.[5-8]

In the current study, we have used an endotoxin-induced model of ocular inflammation to examine in greater detail the time course of appearance of two principal AA metabolites in aqueous humor and have compared this with the time course of two principal physiologic events of ocular inflammation. Generally speaking, there appears to be a good correlation between the appearance in aqueous humor of the chemoattractant LTB_4 and the infiltration of PMNs, as measured by MPO activity in the tissue. However, extravasation of protein into the aqueous

humor, a measure of vascular permeability and blood–aqueous barrier integrity, appears to occur several hours prior to the identification of significant levels of PGE_2 in aqueous humor. These physiologic and biochemical events parallel our clinical observation of anterior chamber cells and flare, iris hyperemia and morphologic demonstration of PMN infiltration, as demonstrated previously.[15] Our results are supported also by the study of Herbort et al,[19] in which the ocular responses of rats to a footpad injection of endotoxin resulted in a series of events similar to that chronicled in the current study. Leukocyte influx was found to parallel the rise in aqueous LTB_4 levels, and breakdown of the blood–aqueous barrier occurred between 1 and 6 hr postinjection. However, PGE_2 levels did not register a detectable increase until 6 hr postinjection.[19]

The model of endotoxin-induced inflammation in the eye is well established in the literature.[19–21] In our laboratory, the response of the eye to intravitreal endotoxin injection followed a predictable course in terms of classical signs of inflammation and consistent changes in aqueous humor protein levels and tissue levels of MPO. MPO has been found at a characteristically high level in PMNs[15,22] and its activity in tissue has been used as an index of PMN infiltration.[15,21]

The discovery that LTB_4 levels in both control and experimental eyes were elevated between 2 and 6 hr above levels found in naive uninjected eyes suggests a minimal response to the injection procedure or a contralateral effect in the control eye, due to endotoxin injection in the experimental eye. However, evidence exists that intravitreal saline injection alone does not influence leukocyte chemotactic activity in aqueous humor.[21] Rosenbaum and Raymond[21] identified chemotactic factors in aqueous humor extracts from rabbits injected with endotoxin. LTB_4 was identified as a component of the extracts by both high-pressure liquid chromatography and RIA. The relationship between the appearance of the chemoattractant LTB_4 in aqueous humor and the influx of PMNs in our model appeared to be rather straightforward. LTB_4, one of the most potent chemotactic agents,[12] appeared early in the aqueous humor and appeared to precede the influx of PMNs into the ocular tissue. Concomitant with the later influx of PMNs, a further increase in levels of LTB_4 supports the idea that infiltrating PMNs themselves release LTB_4. Latanza et al[24] observed elevated LTB_4 levels in aqueous humor after blunt trauma; the increase in LTB_4 preceded the appearance of PMNs by 6 hr.[24]

The significant decline in LTB_4 observed in the current study after 14 hr may indicate a negative feedback mechanism that ultimately leads to a reduction in PMN infiltration. The fall in LTB_4 could result from its metabolism by PMNs,[25] an interaction possibly playing a role in modulation of the inflammatory response. There have been suggestions also that PGE_2 can play a regulatory role in LTB_4 release.[26,27] Bhattacherjee et al[13] demonstrated that PGE_2 injected into the anterior chamber concurrently with LTB_4 resulted in a decrease in the expected level of PMN infiltration. PGE_2 at sustained ocular levels might therefore result in modulation of LTB_4 release.

Comparison of the time course of PGE_2 appearance in aqueous humor and the extravasation of protein may indicate that the initial influx of protein is independent of PGE_2 release. PGE_2 levels in aqueous humor are elevated only minimally above control levels at 2 and 6 hr, while protein influx has increased substantially at the same time intervals. In fact, PGE_2 levels do not rise markedly until after 6 hr, when protein levels have reached a plateau. Therefore, it may be necessary to invoke another mechanism for the initial stimulus for blood–aqueous barrier breakdown. However, our observations may be explained also by the fact that PGE_2 levels measured in aqueous humor are not an accurate reflection of its tissue levels. In other words, while the early release of PGE_2 into aqueous humor might not be substantial, the local levels of PGE_2 at the site of action in the tissue might be more than adequate to incite vascular changes. Other possible mechanisms responsible for the early breakdown of the blood–aqueous barrier and the extravasation of protein may include complement activation, histamine release from mast cells, and release of kinins,[28,29] as well as the vascular effects of released lipoxygenase products.[30–32] The delayed appearance of substantial levels of PGE_2 in aqueous humor appeared to correlate with the time course of PMN infiltration into the ocular tissue. It is possible that another source of PGE_2 measured in aqueous humor may derive from activated PMNs present in the inflamed tissues. Nevertheless, the continued rise in PGE_2 in aqueous humor is not associated with further influx of protein in the aqueous humor.

This study of the relative time courses of mediator appearance and physiologic response is expected to provide a foundation for more detailed evaluation of the impact of specific antiinflammatory agents and mediator antagonists on the time course of events associated with the initiation, continuation, and resolution of ocular inflammation.

Key words: prostaglandins, leukotrienes, endotoxin-induced inflammation, rabbit eye, time course

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