Regulation of Leukotriene B₄ Secretion by Human Corneal, Conjunctival, and Meibomian Gland Epithelial Cells

Afsun Sahin, MD; Wendy R. Kam, MSc; Raheleh Rahimi Darabad, MD; Kimberly Topilow, BA; David A. Sullivan, PhD

Objectives: To test the hypotheses that lipopolysaccharide (LPS) stimulates leukotriene B₄ (LTB₄) production in human ocular surface and adenexal cells, arachidonic acid duplicates the stimulatory effect of LPS, LPS-binding protein potentiates LPS-induced LTB₄ secretion, and dihydrotestosterone attenuates the immune effect of LPS.

Methods: Immortalized human corneal, conjunctival, and meibomian gland epithelial cells were cultured in the presence or absence of fetal bovine serum and were exposed to vehicle, LPS, LPS plus LPS-binding protein, arachidonic acid, or dihydrotestosterone. Culture media were processed for the LTB₄ analysis.

Results: Lipopolysaccharide stimulates time-dependent secretion of LTB₄ by human corneal, conjunctival, and meibomian gland epithelial cells. This effect, which we could not detect with arachidonic acid, is potentiated by exposure to LPS-binding protein. This potentiation, in turn, is significantly reduced by cellular treatment with dihydrotestosterone.

Conclusions: Ocular epithelial cells have the ability to generate LTB₄ in response to LPS exposure. This proinflammatory process is modulated by LPS-binding protein and by dihydrotestosterone.

Clinical Relevance: When induced by appropriate stimuli, LTB₄ production may have a role in the generation of inflammation in ocular surface disease.


Lipopolysaccharide (LPS), a glycolipid of gram-negative bacterial cell walls, is a potent inducer of inflammation. ¹,² The mechanism underlying this inflammatory response involves LPS transfer to CD14 by the lipid transferase LPS-binding protein (LBP), LPS and CD14 stimulation of the Toll-like receptor 4 and MD-2 complex on the cell surface, and consequent Toll-like receptor 4 activation of the innate immune system.³⁻¹¹ This system, in turn, is the first line of defense against bacterial infection.¹¹

Among the most important mediators of LPS-induced inflammation are the leukotrienes (LTs). These lipid compounds are synthesized from arachidonic acid (AA) by 5-lipoxygenase in various cells¹² and, once secreted, act to orchestrate the inflammatory response. The generation of LTs requires strict physiological control given that aberrant overproduction promotes several diseases, including ocular allergy,¹³ asthma,¹⁴ inflammatory bowel disease,¹⁵ and cancer.¹⁶

The most potent chemotactrant in this LT class of compounds is leukotriene B₄ (LTB₄). For example, following LPS stimulation, neutrophil LTB₄ attracts and activates leukocytes, induces the formation of reactive oxygen species, and causes the release of lysosomal enzymes.¹⁷ Researchers have speculated that LTB₄ may also be generated by corneal and conjunctival epithelial cells in response to bacterial products, such as LPS,¹⁸ and be involved in polymorphonuclear cell recruitment into the tear film during closed-eye sleep and tissue infiltration during inflammatory responses.¹⁹ If so, epithelial cell LTB₄ could have a critical role in ocular surface innate immunity. However, it is unknown whether human ocular surface epithelial cells synthesize and secrete LTB₄.

We hypothesize that human ocular surface and adenexal epithelial cells have the ability to produce and release LTB₄ in response to LPS exposure. We also hypothesize the following: (1) AA duplicates the stimulatory effect of LPS given that AA is reported to increase LTB₄ production by...
human SZ95 sebaceous gland epithelial cells\textsuperscript{19}, (2) LBP potentiates LPS-induced LT\textsubscript{B} secretion by ocular epithelial cells; and (3) dihydrotestosterone (DHT), a potent androgen, attenuates the immune effect of LPS. Androgens are known to modulate the function of ocular surface and adnexal epithelial cells,\textsuperscript{20-25} suppress LPS-induced proinflammatory responses,\textsuperscript{26-28} and decrease 5-lipoxygenase activation and LT synthesis\textsuperscript{29,30} in nonocular sites. The objective of this study was to test our hypotheses.

**METHODS**

**CELL CULTURES**

Human breast cancer cells (MCF-7) were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 IU/mL), and streptomycin sulfate (100 µg/mL). Immortalized human conjunctival\textsuperscript{13} and corneal\textsuperscript{12} epithelial cells were cultured in keratinocyte serum-free medium supplemented with bovine pituitary extract (25 µg/mL), epidermal growth factor (50 ng/mL), penicillin, and streptomycin. Immortalized human meibomian gland epithelial cells\textsuperscript{21} were cultured in keratinocyte serum-free medium supplemented with bovine pituitary extract (30 µg/mL), epidermal growth factor (50 ng/mL), penicillin, and streptomycin. Cell viability was evaluated with trypan blue. Cells were maintained in 75-cm\textsuperscript{2} flasks and were plated for experimentation in 6-well culture dishes (Corning). At confluence, the cell numbers varied from 3.8 to 4.9 × 10\textsuperscript{3} cells/well; the number of cells depended on the cell type. All cell culture reagents described were purchased commercially (Invitrogen Corporation), except for DMEM (Mediatech, Inc).

On reaching confluence, cells were rinsed twice with a phosphate-buffered saline solution and exposed to a stratification medium consisting of DMEM and F12 (Mediatech, Inc) with 10% fetal bovine serum, epidermal growth factor (10 ng/mL), penicillin, and streptomycin for 2 days. After this period, cells were incubated in serum-free DMEM and F12 and were treated with vehicle, LPS (15 µg/mL, unless otherwise noted; Escherichia coli strain 0127:B8, lot 050M4094; Sigma-Aldrich), or LPS plus LBP (150 ng/mL; R&D Systems, Inc), as described in the “Results” section. The LPS was dissolved in DMEM and the LBP was dispersed in 1% bovine serum albumin (Sigma-Aldrich) in phosphate-buffered saline (Mediatech, Inc). For AA experiments, cells were exposed to the ethanol vehicle or AA (100nM). For androgen-related studies, the stratification medium contained 10% charcoal and dextran-treated fetal bovine serum (Invitrogen Corporation) with ethanol or DHT (10nM; Steraloids); the poststratification medium also contained ethanol or DHT. Following experimental completion, culture media were collected and frozen at −80°C until analysis.

**SAMPLE PURIFICATION AND IMMUNOAASSAY**

Supernatant samples were purified over C-18 columns (Cayman Chemical), and LT\textsubscript{B} enzyme immunosassays (Cayman Chemical) were performed according to the manufacturer’s recommendations. The primary antibody in this assay, as reported by Cayman Chemical (http://www.caymanchem.com/app/template/Product.vm/catalog/520111), has 100.0% specificity for LT\textsubscript{B} and cross-reacts between 2.7% and 6.6% with 5(5)-hydroxyeicosatetraenoic acid, 5(5)-hydroxyeicosatetraenoic acid, and 20-hydroxy LT\textsubscript{B}. The latter products are intermediates in the LT synthesis pathway. Cross-reactivities ranging from less than 0.01% to 0.98% may be found with other LT metabolites, as noted by Cayman Chemical. For our assay, a standard curve (in duplicate) was run in parallel with each experiment and ranged from 3.5 to 500 pg/mL of LT\textsubscript{B}. In addition, experimental samples were evaluated for potential interference in the LT\textsubscript{B} enzyme-linked immunosorbent assay (ELISA). The results demonstrated that dilution of various samples yielded values that were parallel to the standard curve. Moreover, unless otherwise noted, when known amounts of LT\textsubscript{B} were added to samples, no significant interference in the measurement of the standard was identified. All data shown are representative of at least 3 independent experiments. To confirm these data, selected supernatants from corneal, conjunctival, and meibomian gland epithelial cells were evaluated using LT\textsubscript{B} enzyme immunoassay kits (GE Healthcare). The results all showed analogous LT\textsubscript{B} responses to LPS exposure.

**RESULTS**

**LPS STIMULATION OF LT\textsubscript{B} SECRETION BY HUMAN OCULAR SURFACE AND ADNEXAL EPITHELIAL CELLS**

To determine whether LPS stimulates LT\textsubscript{B} secretion by human ocular surface and adnexal epithelial cells, cells were cultured in varying concentrations of LPS for 6, 24, and 48 hours. As shown in Figure 1, exposure to LPS (15 µg/mL) led to a significant increase in LT\textsubscript{B} release by human corneal, conjunctival, and meibomian gland epithelial cells. The magnitude of this LPS response reached 2.0-fold to 4.8-fold after 6 hours of treatment, rose to 4.3-fold to 7.3-fold by 48 hours, and was analogous to that found in the positive control MCF-7 cells.

Higher LPS concentrations promoted ocular epithelial cell death. For example, after 6 hours of treatment of meibomian gland epithelial cells with LPS at 15 and 75 µg/mL, the cell viabilities equaled 98% and 50%, respectively (Figure 2); similarly, following 48 hours of LPS exposure, the cell viabilities were 71% and 4%, respectively. When cell viability fell below 65%, it was im-

**Figure 1.** Effect of lipopolysaccharide on leukotriene B\textsubscript{4} (LT\textsubscript{B}) secretion by human conjunctival, corneal, meibomian gland, and MCF-7 epithelial cells. Lipopolysaccharide was dissolved in Dulbecco modified Eagle medium, which served as the vehicle. Columns and bars represent means (SEs). *Significantly (P < .05) greater than vehicle; †, significantly (P < .05) greater than vehicle; and ‡, significantly (P < .05) greater than vehicle.
possible to detect LTB₄ in cell culture media. The toxic levels of LPS were lot dependent (data not shown).

**DOES AA DUPLICATE THE EFFECT OF LPS ON CELLULAR LTB₄ OUTPUT?**

To examine whether AA duplicates the stimulatory effect of LPS on cellular LTB₄ output. Our studies with MCF-7 cells indicated that AA (100mM) induced a dramatic increase in the generation of LTB₄ (Figure 3) and that media LTB₄ levels were maintained for 24 and 48 hours (data not shown). However, our assay interference experiments demonstrated that this putative AA response was an artifact. The addition of AA to cell-free DMEM resulted in apparently high levels of LTB₄. These values were clearly not due to LTB₄ secretion but rather reflected a cross-reaction of the immunoassay antibody with AA.

**LBP EFFECT ON LPS-INDUCED LTB₄ SECRETION BY HUMAN OCULAR EPITHELIAL CELLS**

To assess whether LBP potentiates LPS-induced LTB₄ secretion by human ocular surface and adnexal epithelia, cells were cultured for 6 hours with LPS (15 µg/mL) in the presence or absence of LBP (150 µg/mL). As shown in Figure 4, LBP supplementation significantly enhanced the LPS stimulation of LTB₄ production by human corneal, conjunctival, meibomian gland, and MCF-7 cells. In contrast, treatment of MCF-7 cells with LBP alone had no effect on LTB₄ levels (data not shown).

**DHT EFFECT ON LPS-INDUCED LTB₄ SECRETION BY HUMAN OCULAR EPITHELIAL CELLS**

To determine whether DHT suppresses LPS plus LBP–induced LTB₄ output by human ocular epithelia, cells were cultured in media containing LPS (15 µg/mL) and LBP (150 µg/mL) with or without DHT (10nM). Our results show that androgen treatment significantly decreased the release of cellular LTB₄ (Figure 5). The extent of this hormonal effect ranged from 6.3% to 45.0% in conjunctival (45.0%, 11.9%, and 6.3% decreases), corneal (34.4%, 11.6%, and 24.5% decreases), and meibomian gland (19.1%, 31.2%, and 13.9% decreases) epithelial cells in 3 separate experiments.
The present study demonstrates that LPS stimulates time-dependent secretion of LTB4 by human corneal, conjunctival, and meibomian gland epithelial cells. This effect, which we could not detect with AA administration, is potentiated by exposure to LBP. This potentiation, in turn, is significantly reduced by cellular treatment with DHT. These results support our hypotheses that human ocular surface and adnexal epithelial cells have the ability to generate LTB4 in response to LPS exposure and that this proinflammatory process is modulated by LBP and by DHT.

Our finding that LPS induces LTB4 production by human corneal, conjunctival, and meibomian gland epithelial cells is not unexpected. Investigators have shown that alveolar and bronchial epithelial cells also have the capacity to respond to LPS with an upregulation of 5-lipoxygenase pathway enzymes and LTB4 synthesis. Similarly, other researchers have reported that human skin and gastrointestinal epithelial cells contain 5-lipoxygenase enzymes and react to inflammatory stimuli with LT generation. However, another group was unable to detect an LTB4 response to various Serratia marcescens strains in SV40-immortalized human corneal epithelial cells. It may be that this inability was due to excessive levels of LPS, which (as we report) are toxic to ocular surface epithelial cells. Consistent with this explanation is the observation by Hume et al that the S marcescens strains reduced viability of or killed the epithelial cells. Furthermore, our results demonstrate that, when cell viability is decreased below 65%, it is impossible to detect LTB4 in the culture media. This LTB4 disappearance may be due to catabolism given that lysosomal enzymes that break down LTs would be released into media after epithelial cell death.

We found that the addition of LBP to LPS significantly increased LTB4 secretion by ocular surface and adnexal epithelial cells. This finding is analogous to that observed by Blais et al, who reported that LBP facili-
tated the LPS-induced release of the inflammatory cytokines interleukin 6 and interleukin 8 by human SV40-immortalized corneal epithelial cells. It is also possible that LBP, which has been identified in human tears,\textsuperscript{19} promotes the LPS-stimulated migration of polymorphonuclear cells onto the ocular surface during adverse responses to contact lens wear.\textsuperscript{18} In contrast to these findings, using the ELISA from Cayman Chemical, we were unable to determine whether AA increases epithelial cell LTB\textsubscript{4} production. This assay had been used by others to show striking AA stimulation of LTB\textsubscript{4} release by SZ95 sebaceous gland epithelial cells.\textsuperscript{13} Similarly, our results with this assay indicated significant AA-induced LTB\textsubscript{4} secretion by MCF-7 cells. However, these data were an artifact because we also discovered that AA cross-reacts with this ELISA's antibody to LTB\textsubscript{4}. Consequently, alternate procedures are necessary to evaluate whether AA has the ability to modulate LTB\textsubscript{4} release by human epithelial cells.

Of particular note was our finding that DHT attenuates the immune effect of LPS in ocular surface and adnexal epithelial cells. This observation is consistent with the findings by several investigators who reported that androgens suppress LPS-associated inflammatory responses, as well as LT generation, in nonocular sites.\textsuperscript{26-30} These results suggest that androgens are generally anti-inflammatory. In support of this proposal, it has been discovered that androgener exposure significantly downregulates genes related to the immune response and innate immunity in human meibomian gland and conjunctival epithelial cells, respectively,\textsuperscript{31} and dramatically reduces inflammation in autoimmune lacrimal glands.\textsuperscript{24,25} Such anti-inflammatory effects could account, at least in part, for the ability of androgens to alter the development of allergic conjunctivitis.\textsuperscript{22} Androgen action might also have a role in the absence of inflammation in the meibomian gland in health and in disease.\textsuperscript{38} Overall, our study provides new insight into the underlying mechanisms and physiological regulation of inflammation in the human ocular surface and adnexa.

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Correspondence: David A. Sullivan, PhD, Schepens Eye Research Institute, Massachusetts Eye and Ear, 20 Stanford St, Boston, MA 02114 (david_sullivan@mee.harvard.edu).

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REFERENCES


Ophthalmic Images

Anterior Segment Spectral-Domain Optical Coherence Tomography Findings in Cystinosis

Benoit Guignier, MD
Ines Samet, MD
Tristan Bourcier, MD, PhD

A 45-year-old woman was referred for a 10-year history of blurry vision and photophobia. Slitlamp examination showed bilateral corneal deposits (Figure, A and B). Best-corrected visual acuity was 20/25 in both eyes. Spectral-domain optical coherence tomography with an anterior segment module revealed hyperreflective deposits disseminated in the corneal stroma and endothelium that might correspond to cystine crystals (Figure, C). Systemic workup findings (kidney, thyroid, and liver) were negative. To our knowledge, this is the first report of spectral-domain optical coherence tomography performed in cystinosis.