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Conjunctival Goblet Cell Secretion Stimulated by Leukotrienes Is Reduced by Resolvins D1 and E1 To Promote Resolution of Inflammation

Darlene A. Dartt[*,†], **Robin R. Hodges**[*,†], **Dayu Li**[*,†], **Marie A. Shatos**[*,†], **Kameran Lashkari**[*,†], and **Charles N. Serhan**[‡,§]

[*]Schepens Eye Research Institute, Harvard Medical School, Boston, MA 02114

[†]Department of Ophthalmology, Harvard Medical School, Boston, MA 02114

[‡]Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115

[§]Department of Anesthesia, Perioperative and Pain Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115

Abstract

The conjunctiva is a mucous membrane that covers the sclera and lines the inside of the eyelids. Throughout the conjunctiva are goblet cells that secrete mucins to protect the eye. Chronic inflammatory diseases such as allergic conjunctivitis and early dry eye lead to increased goblet cell mucin secretion into tears and ocular surface disease. The purpose of this study was to determine the actions of the inflammatory mediators, the leukotrienes and the proresolution resolvins, on secretion from cultured rat and human conjunctival goblet cells. We found that both cysteinyl leukotriene (CysLT) receptors, $CysLT_1$ and $CysLT_2$, were present in rat conjunctiva and in rat and human cultured conjunctival goblet cells. All leukotrienes LTB_4, LTC_4, LTD_4, and LTE_4, as well as PGD_2, stimulated goblet cell secretion in rat goblet cells. LTD_4 and LTE_4 increased the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and LTD_4 activated ERK1/2. The $CysLT_1$ receptor antagonist MK571 significantly decreased LTD_4-stimulated rat goblet cell secretion and the increase in $[Ca^{2+}]_i$. Resolvins D1 (RvD1) and E1 (RvE1) completely reduced LTD_4-stimulated goblet cell secretion in cultured rat goblet cells. LTD_4-induced secretion from human goblet cells was blocked by RvD1. RvD1 and RvE1 prevented LTD_4- and LTE_4-stimulated increases in $[Ca^{2+}]_i$, as well as LTD_4 activation of ERK1/2. We conclude that cysteinyl leukotrienes stimulate conjunctival goblet cell mucous secretion with LTD_4 using the $CysLT_1$ receptor. Stimulated secretion is terminated by preventing the increase in $[Ca^{2+}]_i$ and activation of ERK1/2 by RvD1 and RvE1.

An increase in goblet cell mucin production causes pathogenesis in several tissues. In the lung, an increase in mucin production occurs in asthma, chronic obstructive pulmonary

Address correspondence and reprint requests to Dr. Darlene A. Dartt, Schepens Eye Research Institute, 20 Staniford Street, Boston, MA 02114. darlene.dartt@schepens.harvard.edu.

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Disclosures

Most of the experiments and results reported in this article were initiated by the authors before licensing of patents for clinical development. Resolvins are biotemplates for stable analogs, and patents on these are awarded and assigned to the Brigham and Women's Hospital, of which C.N.S. is the inventor. These analog patents are licensed for clinical development. C.N.S. retains founder stock in Resolvyx Pharmaceuticals.

disease, and cystic fibrosis (1). In the upper airways, an increase in goblet cell mucin production occurs in chronic rhinosinusitis (2). Goblet cells and their mucin are also upregulated in the intestine in the setting of inflammatory bowel disease, Crohn's disease, and ulcerative colitis (3). In the ocular surface (cornea and conjunctiva), an excess in goblet cell mucin production is deleterious to the ocular surface and occurs in early dry eye and allergic conjunctivitis (4).

Conjunctival goblet cell secretion is induced by stimulation of the afferent sensory nerves in the cornea and conjunctiva that by a reflex arc activate efferent parasympathetic and sympathetic nerves that surround the goblet cells (5). Activation of parasympathetic nerves releases acetylcholine and vasoactive intestinal peptide (VIP) to cause goblet cell secretion, whereas the role of sympathetic nerves is unknown. Acetylcholine and VIP interact with muscarinic acetylcholine receptors type 2 and 3 ($MAchR_2$ and $MAchR_3$) (6) and VIP receptors type 2 ($VIPAC_2$) (7), respectively. Activation of these receptors increases the intracellular $[Ca^{2+}]$ concentration ($[Ca^{2+}]_i$) and activates ERK1/2, also known as p44/p42 MAPK (8, 9). Activation of ERK1/2 causes fusion of secretory granules and release of their contents into the tear film. Cholinergic agonists activate protein kinase C in addition to increasing the $[Ca^{2+}]_i$, and both of these cellular mediators transactivate the epidermal growth factor (EGF) receptor that initiates the signaling cascade culminating in the stimulation of ERK1/2 activity.

Production of cysteinyl leukotrienes LTC_4, LTD_4, and LTE_4 from arachidonic acid initiates the inflammatory response (10). Inflammation is actively terminated with the production of resolvins. Resolvins are products of omega-3 fatty acids that are biosynthesized by switching of the enzymes that produce leukotrienes to synthesize the proresolving resolvins. Resolvins possess potent actions in actively controlling the resolution of inflammatory exudates in multiple different models of inflammation (for recent reviews, see Refs. 11, 12). Resolvin E1 (RvE1) is derived from eicosapentaenoic acid (13) and is a potent mediator that: 1) reduces polymorphonuclear neutrophil infiltration; 2) promotes resolution; 3) reduces colitis; 4) regulates dendritic cell function and IL-12 production; 5) protects from osteoclast-mediated bone destruction (reviewed in Ref. 14); and 6) regulates IL-23, IFN-γ, and lipoxin A_4 to resolve allergic airway inflammation (14, 15). Resolvin D1 (RvD1) is derived from docosahexaenoic acid (16) and is another powerful compound that: 1) stops neutrophil recruitment in peritonitis and the dorsal skin air pouch of mice (16), 2) protects from ischemia-reperfusion–induced kidney and lung damage and loss of function (17, 18), and 3) protects against neovascularization in retinopathy (12, 14). In the eye, RvE1 and RvD1 protect against retinal and corneal neovascularization (19, 20).

In this study, we have investigated the action of the chemical mediators cysteinyl leukotrienes and resolvins on conjunctival goblet cell secretion using rat and human conjunctival goblet cells in primary culture. We report that cysteinyl leukotrienes stimulated goblet cell secretion that is regulated by resolvins.

Materials and Methods

Animals

Male Sprague Dawley rats weighing between 125 and 150 g were obtained from Taconic Farms (Germantown, NY). Rats were anesthetized with CO_2 for 1 min, decapitated, and the nictitating membranes and fornix removed from both eyes. All experiments conformed to the U.S. Department of Agriculture Animal Welfare Act (2007) and were approved by the Schepens Eye Research Institute Animal Care and Use Committee.

Human material

Human conjunctival tissue was obtained from patients during ocular surgery using a protocol that adhered to the tenets of the Declaration of Helsinki. The protocol was approved by the Schepens Eye Research Institute Human Studies Internal Review Board. The tissue, which was normally discarded during surgery, was donated by three patients (two male and one female patient; age range, 58–73 y; average age, 65 y). The surgeries performed on the patients were pars plana vitrectomy, pars plana vitrectomy with membrane peeling, and scleral buckle. Tissue was placed in PBS solution containing penicillin-streptomycin (300 μg/ml).

Cell culture

Goblet cells from rat and human conjunctiva were grown in organ culture as described previously (21–23). Pieces of minced tissue were placed in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, and 100 μg/ml penicillin-streptomycin. The tissue plug was removed after nodules of cells were observed. As described previously, cells were identified as goblet cells by the following characteristics: 1) morphology by light microscopy both bright-field and histochemical staining with Alcian blue/periodic acid–Schiff's reagent (indicates secretory product); 2) positive staining with the lectin Ulex europaeus agglutinin type I (UEA-I) and Abs to MUC5AC (both stain secretory product) and cytokeratin 7 (stains cell body); and 3) negative staining with Ab to cytokeratin 4 and lectin Griffonia Bandeiraea simplicifolia (indicates stratified squamous cells). First-passage goblet cells were used in all experiments.

Secretion

First-passage, cultured goblet cells were plated in 24-well plates and grown to confluence. Cells were serum starved for 2 h before use, preincubated with antagonists for 30 min, and then stimulated with agonists for 0–4 h in the presence of serum-free RPMI 1640 supplemented with 0.5% BSA. Goblet cell secretion was measured using an enzyme-linked lectin assay (ELLA) with the lectin UEA-I that detects rat conjunctival goblet cell mucins. The media were collected and analyzed for the amount of lectin-detectable glycoconjugates, which include the mucin MUC5AC and indicate goblet cell secretion. The standards and supernatant were spotted onto Nunc microplates and dried overnight at 60°C. The ELLA was performed according to a protocol from Pierce, using UEA-I conjugated to HRP. The UEA-I was then detected using Amplex Red (Invitrogen, Carlsbad, CA), which when oxidized by peroxidase in the presence of hydrogen peroxide produces a highly fluorescent molecule. The fluorescence was quantified on a fluorescent ELISA reader (model FL600; Bio-Tek, Winooski, VT) with an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The cells were removed and sonicated, and the cell homogenate analyzed for the total amount of protein by using the Bradford protein assay. Glycoconjugate secretion was normalized to total protein in the homogenate. Bovine submaxillary mucin was used for the standard curve. Glycoconjugate secretion was expressed as fold increase over basal that was set to 1.

Immunohistochemistry

For immunofluorescence microscopy of intact conjunctiva, the eyes were enucleated with the lids intact and fixed in 4% formaldehyde in PBS for overnight at 4°C. Eyes were embedded in paraffin. Sections (6 μm) were placed on slides and kept at −20°C until use. For immunohistochemistry of cultured cells, first-passage cells were grown on glass coverslips and then fixed in methanol before use. Tissue sections and cultured cells were processed and viewed for immunofluorescence as described previously. Abs used included anti-cysteinyl leukotriene $(CysLT)_1$ receptor (rabbit polyclonal Ab used at 1:200, sc-25448;

Santa Cruz Biotechnology, Santa Cruz, CA), anti-$CysLT_2$ receptor (goat polyclonal Ab used at 1:200, sc-27097; Santa Cruz Biotechnology), anti-human ChemR23 (rabbit polyclonal Ab used at 1:100, sc-66829; Santa Cruz Biotechnology), and anti-human GPR32 (rabbit polyclonal Ab used at 1:50, GTX71225; Gene Tex, Irvine, CA). UEA-I conjugated to FITC (Sigma-Aldrich, St. Louis, MO) was used at a dilution of 1:500 and identified goblet cell secretory product. DAPI was in the mounting medium and indicated cell nuclei. Secondary Abs were conjugated to either Cy2 or Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA) and were used at a dilution of 1:150. Negative control experiments included use of isotype controls (sc-3888 [anti-rabbit] and sc-3887 [anti-goat]; Santa Cruz Biotechnology).

Western blotting

Pieces of rat conjunctiva and goblet cells cultured in eight-well plates were homogenized in RIPA buffer. The homogenate was centrifuged at 2000 × *g* for 30 min at 4°C. Sample buffer (4X) was added to the homogenate and protein separated by $NaDodSO_4$-PAGE (SDS-PAGE) using a 10% gel and processed for Western blotting as described previously. Primary Abs were the same as those used for immunofluorescence experiments and were diluted to 1:500. Secondary Ab was from Millipore (Billerica, MA) and was used at a dilution of 1:5000. Immunoreactive bands were visualized by the ECL method. Negative control experiments included omission of the primary Ab.

Measurement of $[Ca^{2+}]_i$

Cultured goblet cells were seeded onto glass-bottom 35-mm petri dishes (MatTek, Ashland, MA) and allowed to attach overnight at 37°C. Cells were then incubated for 1 h with 8 μM fura 2-AM (Invitrogen, Carlsbad, CA) in buffer containing 119 mM NaCl, 4.8 mM KCl, 1.0 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, and 25 mM $NaHCO_3$ supplemented with 10 mM HEPES, 5.5 mM glucose, 250 μM sulfinpyrazone, and 0.5% BSA (KRB-HEPES). Cells were stimulated with agonists alone or preincubated with RvD1 or RvE1 for 0.5 h and then stimulated with agonist. Fluorescent images of cells were recorded and analyzed with a digital fluorescence imaging system (InCyte Im2, Intracellular Imaging). Peak $[Ca^{2+}]_i$ was calculated by subtracting the basal values (before the addition of agonist) from the peak calcium value.

Measurement of ERK1/2 activity

Goblet cells were grown in 12-well plates. Cells were serum starved for 2 h and then stimulated with LTD_4 (10^{-11} M) for 0–10 min. In other experiments, cells were preincubated with RvD1 or RvE1 for 0.5 h before stimulation with LTD_4 (10^{-11} M) for 5 min. The reaction was terminated by removal of buffer and addition of ice-cold RIPA buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, and protease inhibitors). The cells were scraped and proteins were separated by SDS-PAGE. Western blot was performed with Abs directed against phosphorylated (phospho-; active) ERK1/2 (Tyr204) and total ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were digitally scanned and analyzed using ImageJ (National Institutes of Health). The amount of phospho-ERK1/2 in each sample was standardized to the amount of total ERK2. Basal values were set to 1.

Solutions and chemicals

PBS used for immunofluorescence microscopy contained 145 mM NaCl, 7.3 mM Na_2HPO_4, and 2.7 mM NaH_2PO_4 (pH 7.2). RIPA used for Western blotting contained 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, and 1 mM EDTA. Leukotrienes, PGD_2, montelukast, MK571, and MK886 were purchased from

Cayman Chemical (Ann Arbor, MI). RvD1 and RvE1 were obtained from Cayman Chemical, and their physical properties were monitored according to published criteria (24, 25). Chemerin was obtained from R&D Systems (Minneapolis, MN).

Statistical analysis

Results were expressed as the fold increase above basal or the percentage of inhibition of the net stimulation by agonist alone. Results are presented as mean ± SEM. Data were analyzed by Student *t* test. A p value <0.05 was considered statistically significant.

Results

Cysteinyl leukotrienes LTD_4 and LTE_4 stimulate conjunctival goblet cell secretion

One of the hallmarks of inflammation is the generation of LTD_4 and LTE_4 by activated mast cells. Cultured rat goblet cells were incubated for 0.5 h with increasing concentrations of LTD_4. LTD_4 significantly increased secretion at 10^{-14} to 10^{-9} M, and 10^{-11} M caused a maximum increase in secretion of 4.5 ± 2.4-fold that decreased with increasing stimulus concentration (Fig. 1*A*). A second incubation time of 2 h was chosen because this is the time that a known agonist of conjunctival goblet cell secretion, the cholinergic agonist carbachol, caused a significant increase in secretion (26). When incubated for 2 h, LTD_4 at only 10^{-10} M stimulated secretion, which was a 2.8 ± 0.6 increase (Fig. 1*B*). When the time dependency of goblet cells was examined, LTD_4 at 10^{-11} M induced a rapid peak of secretion at 0.5 h that decreased by 1 h but remained elevated for 2 and 4 h (Fig. 1*C*). The time course of secretion is consistent with results from the two concentration-response experiments in which secretion was decreased at almost all concentrations of LTD_4 used at 2 compared with 0.5 h. The effect of LTD_4 on human conjunctival goblet cells was investigated. LTD_4 was incubated for 0.5 h with cultured human goblet cells. LTD_4 at 10^{-11} M caused a maximum increase in secretion of 2.2 ± 0.5-fold that decreased with increasing concentration (Fig. 1*D*). LTD_4 is a potent and effective stimulus of conjunctival goblet cell secretion in both human and rat conjunctival goblet cells.

LTD_4 produced a rapid burst of secretion that declined to a lower constant level for 4 h. Because LTD_4 is rapidly metabolized to LTE_4, LTE_4 could be a less effective stimulus than LTD_4, and this could account for the decrease in secretion with time. LTE_4 at 10^{-12} M significantly stimulated secretion with a maximum 1.9 ± 0.3-fold, whereas LTE_4 at 10^{-11} M also significantly increased secretion 2.9 ± 0.3-fold, both at 1 h of stimulation (Fig. 1*E*). Secretion then decreased to plateau for 3 and 4 h of stimulation. When incubated for 0.5 h, maximum secretion of 3.0 ± 0.4-fold was induced by 10^{-9} M LTE_4 (Fig. 1*F*). The cholinergic agonist carbachol at 10^{-4} M stimulated secretion to the same extent as LTE_4 (Fig. 1*E*, 1*F*). Thus, LTE_4 induces conjunctival goblet cell secretion but is less effective and followed a slower time course than LTD_4. Therefore, the metabolism of LTD_4 to LTE_4 did not account for the decrease in secretion obtained with LTD_4 at longer incubation times. Both LTD_4 and LTE_4 are potent and effective stimuli of conjunctival goblet cell secretion.

LTB_4, LTC_4, and PGD_2 stimulate conjunctival goblet cell mucous secretion

To determine the effect of the inflammatory mediators LTB_4, LTC_4, and PGD_2 on conjunctival goblet cell secretion, cultured goblet cells were incubated for 2 h in the presence of increasing concentrations (10^{-12} to 10^{-6} M) of each mediator. A 2-h incubation time was chosen because this is the time that the cholinergic agonist carbachol caused a significant increase in secretion (26). LTB_4 stimulated secretion a maximum of 2.3 ± 0.9 at 10^{-10} M (Fig. 2*A*). LTC_4 similarly induced secretion a maximum of 2.6 ± 0.6-fold at 10^{-8} M, a 100-fold greater concentration than LTB_4 (Fig. 2*B*). For both compounds, secretion remained increased as the concentration of LT increased. PGD_2 stimulated secretion by 2.2

± 0.8-fold at 10^{-11} M, but secretion decreased as the concentration of PGD_2 increased (Fig. 2*C*). These data suggest that the proinflammatory products of both neutrophils and mast cells cause goblet cells to secrete. Because LTD_4 and LTE_4 were the most effective stimuli of secretion, these compounds were further characterized.

Cysteinyl leukotriene receptors are present on conjunctival goblet cells

The cysteinyl leukotrienes LTD_4 and LTC_4 interact with their receptors $CysLT_1$ and $CysLT_2$. LTD_4 binds $CysLT_1$ receptor more effectively than LTC_4, and both LTC_4 and LTD_4 bind $CysLT_2$ receptor equally effectively (27). To determine the presence of $CysLT_1$ and $CysLT_2$ receptors, homogenates prepared from rat conjunctiva and cultured conjunctival goblet cells were subjected to Western blotting analysis using Abs specific to these receptors. In rat conjunctiva and goblet cells, $CysLT_1$ receptor appeared as a single band at 41 kDa and $CysLT_2$ receptor at 35 kDa (Fig. 3*A*). Rat conjunctival sections were analyzed by immunohistochemistry with anti-$CysLT_1$ receptor Ab and the lectin UEA-I that stains goblet cell secretory granules. In the rat, conjunctival goblet cells occur as clusters with the cell body reaching to the basement membrane and the secretory granules abutting the tear film. Multiple layers of stratified squamous cells surround the goblet cells. In the rat, $CysLT_1$ receptor was detected on the basolateral membranes of clusters of filled goblet cells (Fig. 3*B*). $CysLT_1$ receptor was also present on the plasma membranes of stratified squamous cells. $CysLT_2$ receptor immunoreactivity was found diffusely distributed in the cytoplasm of both goblet and stratified squamous cells (Fig. 3*C*). Incubation with the isotype control Abs showed no apparent immunoreactivity (Fig. 3*D*, 3*E*).

Immunoreactivity to $CysLT_1$ and $CysLT_2$ receptors was observed with punctate staining in the cytoplasm of all cultured rat goblet cells labeled with UEA-I (Fig. 3*F–K*). When cells were incubated in the absence of Triton X-100 to prevent permeabilization of cells and demonstrate cell surface binding, both $CysLT_1$ and $CysLT_2$ receptors were present on the cell surface (Fig. 3*L–Q*). Incubation with the isotype control Abs showed no apparent immunoreactivity (Fig. 3*R*, 3*S*).

Localization of $CysLT_1$ and $CysLT_2$ receptors in human cultured goblet cells was similar to that seen in rat cultured goblet cells where it was detected in plasma membranes and stress fibers (Supplemental Fig. 1A–F). Incubation with isotype control Abs showed no apparent immunoreactivity (Supplemental Fig. 1G, 1H).

$CysLT_1$ receptor antagonists block LTD_4-stimulated conjunctival goblet cell secretion

Cultured rat goblet cells were preincubated for 0.5 h with the $CysLT_1$ receptor antagonists MK571 and montelukast, and then stimulated for 0.5 h with 10^{-11} M LTD_4. LTD_4 significantly stimulated secretion by 2.1 ± 0.4-fold, respectively (data not shown). Secretion stimulated by 10^{-11} M LTD_4 was decreased in a concentration-dependent manner by 10^{-10} to 10^{-7} M MK571. The action of MK571 was significant at 10^{-8} and 10^{-7} M MK571, with MK571 at 10^{-7} M completely blocking secretion (Fig. 4*A*). Montelukast also significantly inhibited secretion stimulated by LTD_4 10^{-11} M (Fig. 4*B*). Both concentrations of montelukast were effective, with a maximum inhibition of 55 ± 22% and 76 ± 14% obtained with 10^{-10} and 10^{-9} M montelukast, respectively. LTD_4 uses $CysLT_1$ receptors to stimulate conjunctival goblet cell secretion.

LTD_4 and LTE_4 increase $[Ca^{2+}]_i$ in conjunctival goblet cells

Rat conjunctival goblet cells containing the Ca^{2+}-sensitive dye fura 2 were stimulated with LTD_4. LTD_4 increased the $[Ca^{2+}]_i$ in a concentration-dependent manner with a maximum increase of 104 ± 19 nM occurring at 10^{-9} M, a 100-fold greater concentration than stimulates secretion (Fig. 5). LTE_4 also increased the $[Ca^{2+}]_i$ to a maximum of 514 ± 58 nM

at 10^{-9} M, the same concentration that was maximal for secretion (Fig. 5). Comparison of the Ca^{2+} responses to LTD_4 and LTE_4 demonstrated that the response to LTE_4 was significantly greater than the response to LTD_4. The cholinergic agonist carbachol at 10^{-4} M increased $[Ca^{2+}]_i$ to 328 ± 60 nM, a value between those obtained in response to LTD_4 and LTE_4 (Fig. 5, *inset*), though not significantly different from either one.

Because chemerin, in addition to RvE1, can activate the ChemR23 receptor, we determined whether chemerin increases the $[Ca^{2+}]_i$ in cultured goblet cells. Chemerin from 10^{-9} to 10^{-7} M did not increase the $[Ca^{2+}]_i$ in the same experiments in which both LTD_4 and LTE_4 were effective (Fig. 5).

To determine whether the leukotriene-induced increase in $[Ca^{2+}]_I$ was mediated by the $CysLT_1$ receptor, cultured goblet cells were preincubated for 30 min with MK571. LTD_4 at 10^{-9} M increased the $[Ca^{2+}]_i$ by 104 ± 19 nM (Supplemental Fig. 2A). The presence of MK571 at 10^{-9} to 10^{-7} M blocked the LTD_4 increase in $[Ca^{2+}]_i$. MK571 at 10^{-8} M was most effective, almost completely blocking the response. LTE_4 at 10^{-7} M increased $[Ca^{2+}]_i$ by 493 ± 45 nM (Supplemental Fig. 2B). MK571 was less effective in blocking the LTE_4 Ca^{2+} response. Only 10^{-8} M MK571 was effective, inhibiting the Ca^{2+} response by ~60%.

Both LTD_4 and LTE_4 increase $[Ca^{2+}]_i$ in conjunctival goblet cells; LTD_4 works through the $CysLT_1$ receptor, and a component of the LTE_4 response could be through this receptor as well.

LTD_4 activates ERK1/2 in conjunctival goblet cells

Conjunctival goblet cells were stimulated for 0–10 min with LTD_4 and phospho-ERK1/2 activity measured. LTD_4 at 10^{-11} M activated ERK1/2 a maximum of 2.0 ± 0.3-fold at 5 min of stimulation (Fig. 6*A*, 6*B*). LTD_4 activates ERK1/2 in addition to increasing the $[Ca^{2+}]_i$ in conjunctival goblet cells; both of these signaling components are the major mechanism by which goblet cell secretion is stimulated in the conjunctiva.

Cholinergic agonists do not generate leukotrienes, but their stimulation of secretion is blocked by resolvins

Cholinergic agonists mediate parasympathetic stimulation of conjunctival goblet cell secretion (7). To determine whether cholinergic agonists cause secretion by producing leukotrienes, cultured rat goblet cells were incubated with MK886, an inhibitor of 5-lipoxygenase–activating protein (FLAP), the accessory protein of 5-lipoxygenase that is responsible for leukotriene synthesis (28). Cells were incubated for 0.5 h with MK886 (10^{-6} and 10^{-5} M) and then stimulated with carbachol for 2 h. MK886 alone did not significantly alter goblet cell secretion (data not shown). Carbachol 10^{-5} and 10^{-4} M stimulated goblet cell secretion 1.8 ± 0.5- and 1.9 ± 0.5-fold, respectively. Neither concentration of MK886 altered secretion stimulated by carbachol at any concentration (data not shown).

Carbachol at 10^{-5} and 10^{-4} M significantly increased secretion 2.1 ± 0.2- and 2.5 ± 0.3-fold, respectively (data not shown). RvD1 decreased cholinergic agonist-stimulated secretion in a concentration-dependent manner. RvD1 at 10^{-9} M caused a maximum inhibition of 62 ± 13 and 72 ± 3% at carbachol concentrations of 10^{-5} and 10^{-4} M, respectively (Fig. 7*A*). In separate experiments, carbachol increased secretion by 1.5 ± 0.3-fold, which was decreased by a maximum of 83 ± 10% with RvE1 at 10^{-8} M (Fig. 7*B*).

Cholinergic agonists do not produce leukotrienes in conjunctival goblet cells, but both resolvins terminated secretion stimulated by this agonist.

RvE1 and RvD1 reduce LTD_4-stimulated conjunctival goblet cell secretion

Cultured goblet cells were preincubated with no addition or increasing concentrations of the resolvins RvE1 and RvD1 for 0.5 h and then stimulated with LTD_4 at 10^{-11} M for 0.5 h. Neither RvE1 nor RvD1 alone caused goblet cell secretion during the 0.5-h stimulation (data not shown). LTD_4 significantly induced secretion 2.7 ± 0.4-fold (data not shown). RvE1 and RvD1 blocked LTD_4-stimulated secretion in a concentration-dependent manner (Fig. 8*A*, 8*B*). RvE1 and RvD1 at 10^{-9} and 10^{-8} M completely blocked secretion.

Experiments analogous to those performed on rat goblet cells were carried out with human conjunctival goblet cells. LTD_4 significantly stimulated secretion 2.0 ± 0.3-fold (data not shown). In the human cells, RvD1 inhibited LTD_4-stimulated secretion a maximum of 74 ± 15% at 10^{-9} M (Fig. 8*C*).

Goblet cell mucin secretion stimulated by LTD_4 can be completely blocked by both types of resolvins in rat goblet cells. In human goblet cells, RvD1 terminates LTD_4-stimulated secretion.

RvD1 blocks LTD_4-stimulated secretion at all times tested

Conjunctival goblet cells were preincubated with RvD1 at 10^{-9} M (Supplemental Fig. 3A) and 10^{-8} M (Supplemental Fig. 3B) for 0.5 h and then stimulated with LTD_4 (10^{-11} M) for 0.5, 1, and 4 h. RvD1 itself slightly, but significantly, stimulated goblet cell secretion at almost all times and concentrations used (Supplemental Fig. 3A, 3B). As demonstrated in Fig. 1*C*, LTD_4-stimulated secretion was maximum at 0.5 compared with 1 and 4 h. RvD1 at 10^{-9} and 10^{-8} M almost completely blocked LTD_4-stimulated secretion for up to 4 h (Supplemental Fig. 3A, 3B).

RvD1 and RvE1 receptors are present on rat conjunctival goblet cells

The receptors mediating the proresolution actions of RvD1 and RvE1 were recently identified. RvD1 can activate the orphan receptor GPR32 and ALX/FPR2, a lipoxin A_4 receptor (29). Using immunohistochemistry, we detected GPR32 on both goblet and stratified squamous cells in the intact conjunctiva. Notably, GPR32 was localized on the basal and lateral membranes of the goblet cells (Supplemental Fig. 4A). In cultured rat goblet cells, GPR32 was localized in a distinct area of the cytoplasm (Supplemental Fig. 4D). This localization does not correspond to the secretory vesicles (identified by binding of the lectin UEA-I; Supplemental Fig. 4E) and was not in the nucleus (Supplemental Fig. 14F).

RvE1 binds the receptor ChemR23 to activate specific signaling pathways that resolve inflammation (30). ChemR23 is an orphan receptor that can also be activated by the polypeptide chemerin (31). ChemR23 was detected in rat conjunctiva by immunohistochemistry. This receptor, similarly to GPR32, was located on both stratified squamous and goblet cells (Supplemental Fig. 4B). In particular, GPR32 localized to the lateral membranes of the goblet cells. In cultured rat goblet cells, ChemR23 was found in a punctuate pattern within the cytosol (Supplemental Fig. 4G–I). In the presence of isotype control Ab, there was minimal diffuse staining in the conjunctival epithelium and goblet cells in culture (Supplemental Fig. 4C, 4J).

RvD1 and RvE1 block LTD_4- and LTE_4-stimulated increase in $[Ca^{2+}]_i$

Fura 2-containing cultured goblet cells were preincubated for 0.5 h with RvD1 (10^{-9} and 10^{-8} M) before stimulation with LTD_4 at 10^{-9} M, the concentration that gave a maximum increase in $[Ca^{2+}]_i$. LTD_4 increased the $[Ca^{2+}]_i$ by 104 ± 19 nM (Fig. 9*A*). RvD1 at both concentrations almost completely attenuated the LTD_4 increase in $[Ca^{2+}]_i$. Similar results

were obtained for LTE_4 (10^{-7} M), whose increase in $[Ca^{2+}]_i$ of 474 ± 92 nM was substantially prevented by both concentrations of RvD1 (Fig. 9*B*). In similar experiments, goblet cells were preincubated with RvE1 (10^{-10} and 10^{-9} M) before stimulation with LTD_4. LTD_4 increased the $[Ca^{2+}]_i$ by 66 ± 13 nM (Fig. 9*C*). RvD1 at 10^{-9} M blocked the increase in $[Ca^{2+}]_i$ by almost 70%. Similar results were obtained for LTE_4 (10^{-7} M), whose increase in $[Ca^{2+}]_i$ of 87 ± 13 nM was inhibited by 85% by the RvE1 at 10^{-9} M (Fig. 9*D*).

RvD1 and RvE1 attenuate LTD_4 activation of ERK1/2

Western blotting analysis was used to determine the effect of RvD1 on LTD_4 stimulation of ERK1/2 activation. Goblet cells were preincubated for 0.5 h with RvD1 from 10^{-10} to 10^{-8} M before stimulation for 5 min with LTD_4 at 10^{-11} M, the concentration that gave maximum stimulation of secretion. RvD1 did not increase phospho-ERK1/2 activity, whereas LTD_4 increased it by 2.1 ± 0.3-fold (Fig. 10*A*, 10*B*). RvD1 at 10^{-9} and 10^{-8} M almost completely decreased the activation of ERK1/2 by LTD_4. Similar experiments were performed with RvE1. RvE1 did not increase phospho-ERK1/2 activity, whereas LTD_4 increased it by 2.0 ± 0.2-fold (Fig. 10*C*, 10*D*). RvE1 at 10^{-9} and 10^{-8} M decreased by ~80% the activation of ERK1/2 by LTD_4.

Thus, RvD1 and RvE1 each block LTD_4-stimulated goblet cell secretion by preventing the increase in the $[Ca^{2+}]_i$ and the activation of ERK1/2, the two major mechanisms by which conjunctival goblet cell secretion is stimulated.

Discussion

A hallmark of conjunctival inflammation that occurs in ocular allergy is an influx of mast cells and neutrophils that release histamine, leukotrienes, proteases, PGs, and cytokines (32, 33). Similarly, in dry eye disease, inflammation of the ocular surface includes cytokine production and infiltration of T cells and neutrophils (34). We now show that cysteinyl leukotrienes LTC_4, LTD_4, and LTE_4 produced in the conjunctiva during ocular allergy, dry eye disease, or other inflammatory diseases of the ocular surface stimulate goblet cell mucous secretion that can contribute to the excess mucous seen in these diseases. The chronic inflammation in these ocular surface diseases damages the cornea and conjunctiva, causing chronic pain from exposed nerve endings. In this study, we demonstrated that cysteinyl leukotriene-stimulated goblet cell secretion was completely blocked by the proresolution resolvins RvD1 and RvE1. Thus, resolution of inflammation by the production of proresolution mediators, namely, RvD1 and RvE1, can terminate excess goblet cell mucous secretion allowing the ocular surface to repair. These results also support the hypothesis that resolution of inflammation is an active process.

Dry eye and allergic conjunctivitis are chronic inflammatory diseases of the cornea and conjunctiva (ocular surface). Dry eye affects 5 million individuals in the United States alone and is more prevalent in women than men (35). Allergy including seasonal allergic conjunctivitis, vernal keratoconjunctivitis, giant papillary conjunctivitis (also known as contact lens-induced papillary conjunctivitis), and atopic keratoconjunctivitis affects ~20% of the population (36). Hallmarks of these diseases are symptoms of ocular pain and discomfort, and signs of ocular surface inflammation that generate inflammatory cytokines and matrix metalloproteinases. These inflammatory mediators lead to death of the surface cells of the corneal and conjunctival epithelia (4). Conjunctival goblet cells are potential targets of the inflammatory mediators produced during dry eye disease and allergic conjunctivitis as an increase in tear mucus accompanies these diseases (4). In the initial stages of dry eye and in allergic conjunctivitis, ocular surface damage and irritation of the afferent sensory nerves leads to the neural reflex stimulation of conjunctival goblet cell secretory product that includes the large gel forming mucin MUC5AC.

An increase in ocular surface mucus appears to be correlated with damaging inflammation of the ocular surface in the setting of early dry eye and allergic conjunctivitis, and the proinflammatory leukotrienes play a pivotal role in allergic and hypersensitivity reactions and goblet cell mucous production in the airway (10). Yet, there is limited information on the effect of inflammatory mediators on conjunctival goblet cell mucin secretion. In the only published study, an in vivo one in the guinea pig conjunctiva, platelet-activating factor and histamine, but not LTD_4 or LTE_4, increased goblet cell secretion (37). Until now goblet cells in culture have not been available; thus, in this report, we are able to address the role of goblet cell secretion uncomplicated by effects on the other cell type of the conjunctiva and independently of changes in proliferation and differentiation. Goblet cells cultured from the upper airways or lung have not been used for investigation of inflammation. In vivo studies on the effect of leukotrienes and resolvins on goblet cells in these two tissues cannot differentiate between acute and chronic effects, and cannot distinguish between effects on cell proliferation, secretion, and differentiation as can the present studies in the conjunctiva.

Use of rat goblet cells is an excellent model for human conjunctiva. In previous studies, we compared human and rat goblet cells, and found that they were similar in their stimulation of proliferation by EGF, activation of ERK1/2 by EGF and cholinergic agonists, and the signaling pathways activated by EGF to stimulate proliferation (21, 38). The cysteinyl leukotriene LTD_4 is also an effective mucin stimulus in human goblet cells as in rat goblet cells, and the proresolution mediator RvD1 blocks secretion in both human and rat goblet cells.

Cysteinyl leukotrienes work by activating $CysLT_1$ or $CysLT_2$ receptors. We found that both receptors were present in conjunctival goblet cells. LTD_4 works by activating the $CysLT_1$ receptors as two $CysLT_1$ receptor antagonists blocked induced mucous secretion. The role of $CysLT_2$ receptor was not tested. Strategies that block $CysLT_1$ receptors such as topical or systemic application of $CysLT_1$ receptor antagonists should be beneficial in treatment of ocular allergy. In one 12-patient clinical trial of oral montelukast in patients with vernal conjunctivitis and asthma, a significant and persistent reduction of ocular signs and symptoms was reported (39). Further evaluation of the use of $CysLT_1$ receptor antagonists in ocular allergy is warranted.

$CysLT_1$ or $CysLT_2$ receptors were also detected on conjunctival stratified squamous cells. Stratified squamous cells secrete electrolytes and water in response to β-adrenergic or purinergic P2Y agonists. Activation of cysteinyl leukotrienes receptors could stimulate electrolyte and water secretion or modify agonist-stimulated secretion, thereby increasing tear production. Conjunctival epithelium differs from the other goblet cell-containing epithelia including upper and lower airways, intestine, and colon, in that conjunctival epithelium expresses abundant cysteinyl leukotriene receptors, whereas the other epithelia contain little or no expression of these receptors (40, 41).

Both LTD_4 and LTE_4 stimulated conjunctival goblet cell mucous secretion. Of interest, LTE_4 appeared to be a potent stimulus for $[Ca^{2+}]_i$ mobilization and secretion in these cells. Hence the potential and capacity for LTC_4 and LTD_4 conversion in this tissue to LTE_4 is of interest for further studies along these lines. LTE_4 was also recently reported to be a potent agonist for mast cell activation (42). Together, these results and those of this study document the potent activity of LTE_4, which was considered earlier to be an inactive member of the SRS-A/leukotriene family (10).

Active resolution of inflammation is mediated by poly-unsaturated fatty acids (PUFA), the precursors of the resolvins. Results from several clinical studies have shown that systemic or topical PUFA are important for the health of the ocular surface and can improve dry eye

disease (14–17). The mechanisms underlying the improvement of dry eye with omega-3 fatty acids have not been investigated. PUFA are also important in the retina because increased dietary intake of PUFA reduces pathological retinal angiogenesis (19). These studies suggest that resolvins might be beneficial to treat ocular surface disease.

The proresolution mediators RvD1 and RvE1 both reduced LTD_4-stimulated conjunctival goblet cell secretion, which occurs by exocytosis. Goblet cell secretion is stimulated by increasing the $[Ca^{2+}]_i$ and activating ERK1/2 (9). Both LTD_4 and LTE_4 increase the $[Ca^{2+}]_i$, and LTD_4 activated ERK1/2 and likely stimulated goblet cell secretion by these mechanisms. RvD1 activates the GPR32 receptor that is present in conjunctival goblet cells and prevents the increase in $[Ca^{2+}]_i$ stimulated by LTD_4 and LTE_4, and the activation of ERK1/2 by LTD_4. It is also possible that RvD1 works through the ALX/FPR2 receptor, but this was not determined in the present system. We suggest that these inhibitory actions prevent stimulation of goblet cell secretion by the leukotrienes. RvE1 acts via the ChemR23 receptor (28) that is also present in conjunctival goblet cells to block the increase in $[Ca^{2+}]_i$ and inhibit the activation of ERK1/2.

The proresolution mediators RvD1 and RvE1 inhibited cholinergic agonist-stimulated goblet cell secretion in addition to their actions on leukotriene-induced secretion. Cholinergic agonists play a role in the normal goblet cell secretory response to changes in the external environment such as mechanical, thermal, chemical, and microbial stimuli. The neural regulation of goblet cell secretion functions to protect the ocular surface from the environment. The neural regulation of secretion could also play a role in the inflammatory response as histamine released during allergic inflammation can interact with histamine receptors (usually H_4 receptors) on sensory nerves and activate them to stimulate the efferent parasympathetic nerves. Furthermore, leukotrienes and cholinergic agonists use the same signaling pathways to stimulate goblet cell secretion as both types of agonists increase $[Ca^{2+}]_i$ and activate ERK1/2. Thus, resolvins appear to block the same cellular signaling pathway to prevent cholinergic and leukotriene-induced goblet cell secretion.

Mucin production is regulated by controlling the number of goblet cells (proliferation), the rate of mucin synthesis, and the rate of mucin secretion. In the lung, the leukotrienes appear to increase mucin production by increasing the number of goblet cells by stimulating goblet cell proliferation and differentiation. Resolvins decreased the number of filled goblet cells, but the mucin production mechanism used by resolvins was not investigated (43). In contrast, in our study, we found that, in the conjunctiva, leukotrienes increase mucin production by stimulating mucin secretion. Using cultured goblet cells, we were able to study secretion, without change in goblet cell number or other chronic effects that could alter the steady-state mucin production. We showed that leukotrienes stimulate conjunctival goblet cell secretion and resolvins block the stimulation. Similarly in the colon, omega-3 PUFA restored the number of filled goblet cells and the maturity of their mucins in a rat model of experimental ulcerative colitis (44). Thus, resolvins affect different cellular processes to block mucin production in the lung compared with the conjunctiva and return the tissue to its normal predisease state.

Resolvins block many physiological processes including reducing corneal angiogenesis, decreasing neutrophil infiltration, blocking cytokine production, inhibiting osteoclast bone growth and reabsorption, and decreasing airway mucous production (15, 45, 46). Based on these cellular actions, resolvins were able to reduce inflammation in animal models of a variety of chronic inflammatory diseases including corneal angiogenesis, asthma, colitis, obesity-induced insulin resistance, periodontal disease, atherosclerosis, and kidney reperfusion injury (15, 18, 45, 47, 48). Thus, resolvins should reduce allergic inflammation in ocular allergy by blocking goblet cell secretion as demonstrated in this study, and by

reducing the accompanying neutrophil infiltration, increase vascular permeability and cytokine production.

We conclude that the cysteinyl leukotrienes acting via $CysLT_1$ receptors are potent stimuli of conjunctival goblet cell mucous secretion, and the proresolution mediators RvE1 and RvD1 terminate this action by preventing the increase in $[Ca^{2+}]_i$ and activation of ERK1/2. Because resolvins stimulate resolution of inflammation and are not immunosuppressive (14), they may be useful alternatives to prolonged steroid use in the eye. Our results support the notion that goblet cell mucous secretion is an important component of ocular allergy and early dry eye, and termination of this secretion occurs by active resolution of inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article

$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
CysLT	cysteinyl leukotriene
EGF	epidermal growth factor
ELLA	enzyme-linked lectin assay
LTB_4	leukotriene B_4
LTC_4	leukotriene C_4
LTD_4	leukotriene D_4
LTE_4	leukotriene E_4
PUFA	polyunsaturated fatty acid
RvD1	resolvin D1
RvE1	resolving E1
UEA-I	Ulex europaeus agglutinin type I

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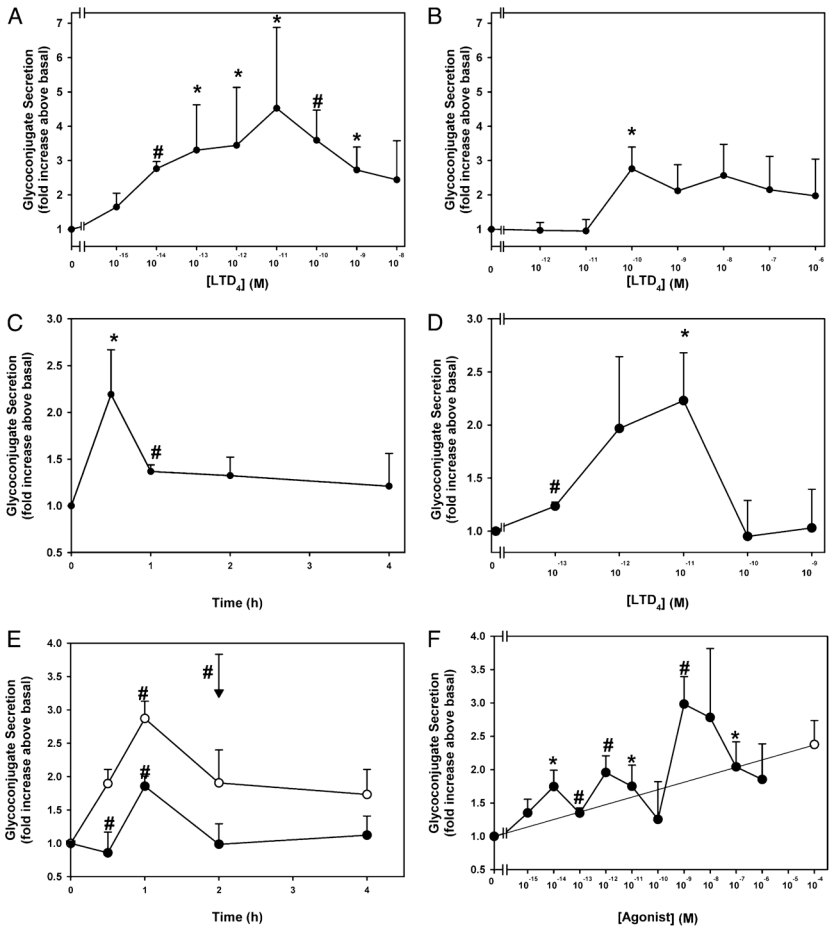

FIGURE 1.

Effect of time and concentration of LTD_4 and LTE_4 on glycoconjugate secretion from cultured rat and human conjunctival goblet cells. Cultured rat conjunctival goblet cells were serum starved for 2 h before stimulation with increasing concentrations of LTD_4 for 0.5 (*A*) ($n = 5$) or 2 h (*B*) ($n = 5$), or LTD_4 (10^{-11} M) for 0–4 h (*C*) ($n = 4$). Cultured human conjunctival goblet cells were incubated with increasing concentrations of LTD_4 for 0.5 h (*D*) ($n = 3$). Cultured rat conjunctival goblet cells were serum starved for 2 h before stimulation with LTE_4 (10^{-11} M, open circles, and 10^{-12} M, closed circles) for 0–4 h or the cholinergic agonist carbachol (10^{-4} M, inverted triangle) for 2 h (*E*) ($n = 5$), or increasing concentrations of LTE_4 (closed circles) for 1 h or carbachol (10^{-4} M, open circles) for 2 h (*F*) ($n = 5$). The amount of glycoconjugate secretion was measured by ELLA. Data are mean ± SEM. $^{*}p < 0.05$ from no addition, $^{\#}p < 0.01$ from no addition.

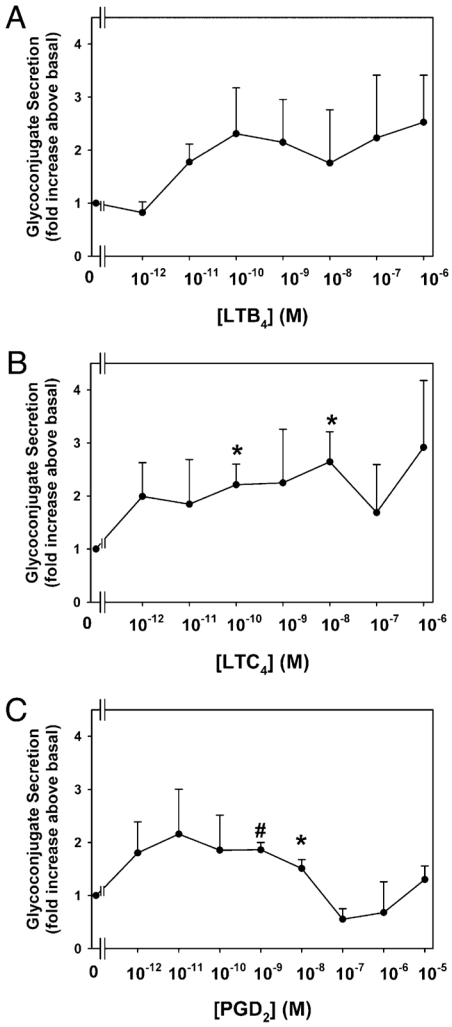

FIGURE 2.

Effect of leukotrienes and PGD_2 on glycoconjugate secretion from cultured rat conjunctival goblet cells. Cultured rat conjunctival goblet cells were serum starved for 2 h before stimulation with LTB_4 (*A*), LTC_4 (*B*), or PGD_2 (*C*) for 2 h. The amount of glycoconjugate secretion was determined with ELLA. Data are mean ± SEM from four to five individual experiments. $^{*}p < 0.05$ from no addition, $^{\#}p < 0.01$ from no addition.

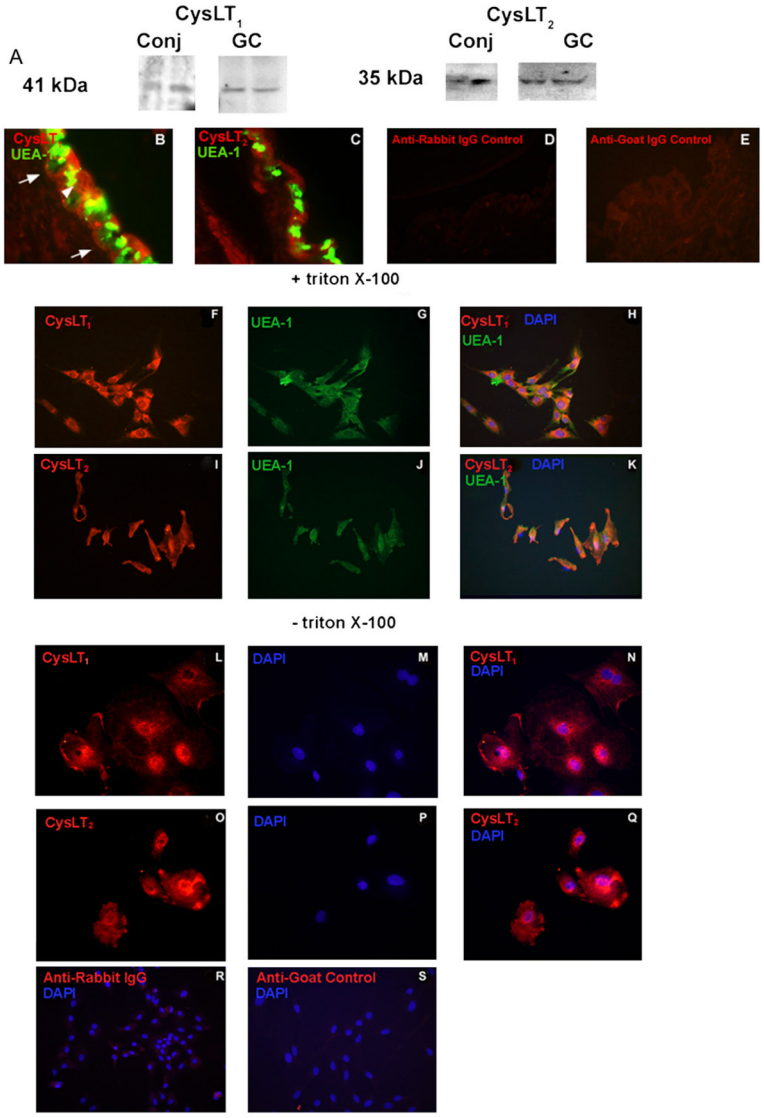

FIGURE 3.

Identification of cysteinyl leukotriene receptors in rat conjunctival epithelium and cultured rat conjunctival goblet cells. The presence of $CysLT_1$ and $CysLT_2$ receptors was determined by Western blot analysis and is shown in *A*. Each lane represents a separate animal. Fluorescence micrographs show localization of $CysLT_1$ and $CysLT_2$ receptors (*B*, *C*). CysLT receptors are shown in red, and UEA-I, which indicates goblet cell secretory granules, is shown in green. Arrows indicate basolateral membranes of goblet cells. Arrowhead indicates stratified squamous cells. Anti-rabbit isotype control is shown in *D*, whereas anti-goat isotype control is shown in *E*. Fluorescence micrographs show localization of $CysLT_1$ and $CysLT_2$ in cultured rat goblet cells in the presence (*F–K*) and absence (*L–Q*) of a permeabilization compound Triton X-100. Anti-rabbit isotype control is shown in *R*, whereas anti-goat isotype control is shown in *S*. CysLT receptors are shown in red, UEA-I indicating goblet cell secretory granules is shown in green, and DAPI indicating cell nuclei is shown in blue. Original magnification ×200. Micrographs are representative of three separate animals.

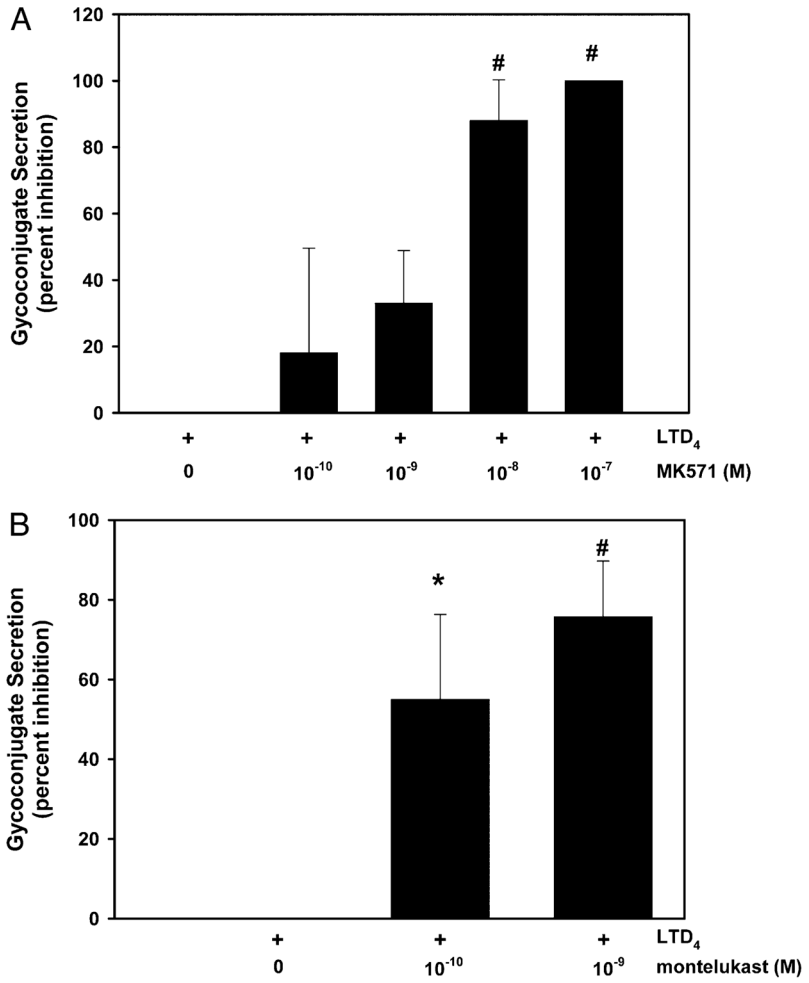

FIGURE 4.

Effect of $CysLT_1$ antagonists on LTD_4-stimulated glycoconjugate secretion from cultured rat conjunctival goblet cells. Cultured rat conjunctival goblet cells were preincubated for 0.5 h with increasing concentrations of MK571 (*A*) or montelukast (*B*) before stimulation with LTD_4 (10^{-11} M) for 0.5 h. The amount of glycoconjugate secretion was measured by ELLA. Data are mean ± SEM from three (MK571) or four (montelukast) independent experiments. $^{*}p < 0.05$ from LTD_4 alone, $^{\#}p < 0.01$ from LTD_4 alone.

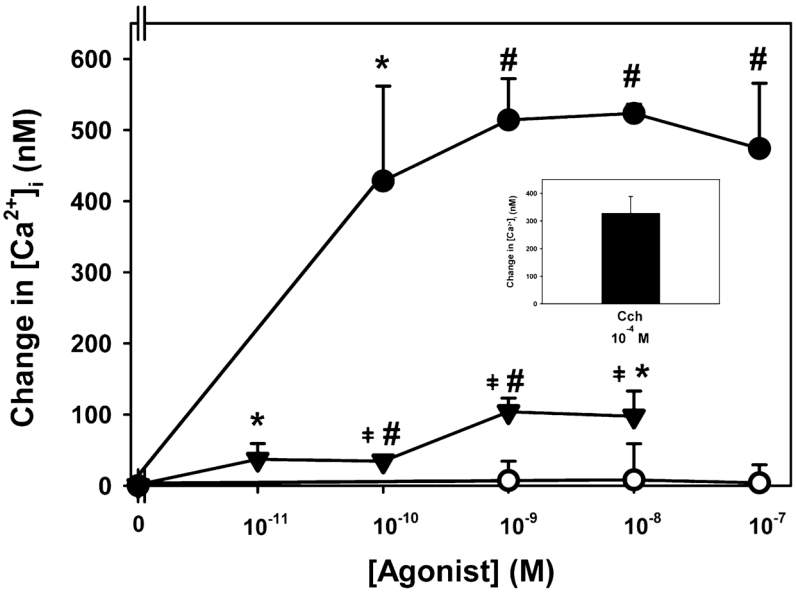

FIGURE 5.

Effect of LTD_4. LTE_4 and chemerin on peak $[Ca^{2+}]_i$ in cultured rat conjunctival goblet cells. Cultured rat conjunctival goblet cells containing the calcium-sensitive dye fura 2 were stimulated with increasing concentrations of LTE_4 (closed circles), LTD_4 (inverted triangles), and chemerin (open circles). The effect of the cholinergic agonist carbachol (Cch) used at a concentration (10^{-4} M) maximum for increasing $[Ca^{2+}]_i$ is shown in the *inset*. Data are mean ± SEM from three independent experiments. $^{*}p < 0.05$ from no addition, $^{\#}p < 0.01$ from no addition, $^{\ddagger}p < 0.01$ from LTE_4.

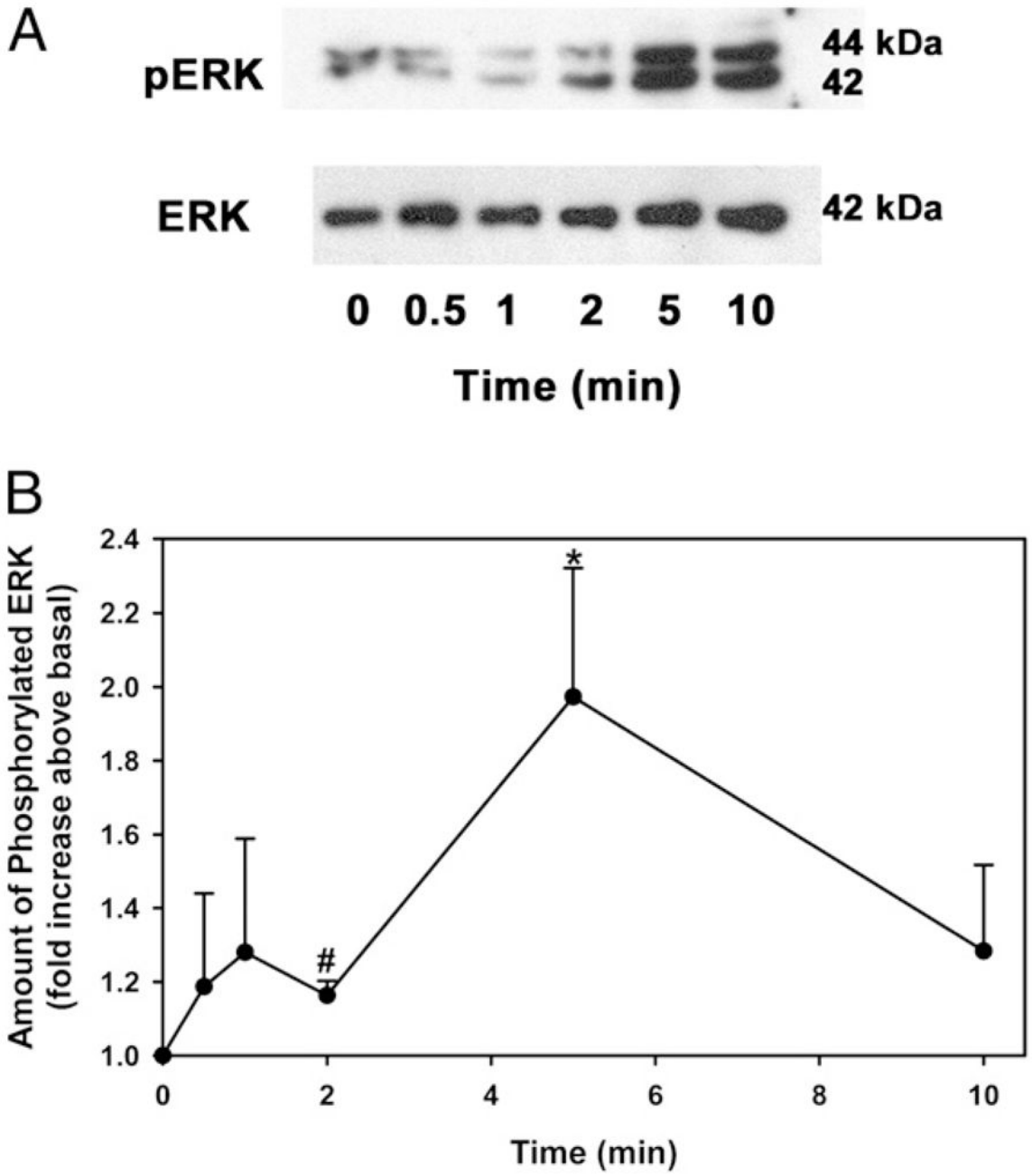

FIGURE 6.

Effect of LTD_4 on activation ERK1/2 in cultured rat conjunctival goblet cells. Cultured rat conjunctival goblet cells were stimulated for 0–10 min with LTD_4 (10^{-11} M), and the amount of phospho-ERK1/2 (pERK1/2) and total ERK2 was determined by Western blotting analysis. One representative Western blot is shown in *A*. Three independent experiments were analyzed by densitometry, and the mean ± SEM is shown in *B*. $^{*}p < 0.05$ from no additions, $^{\#}p < 0.01$ from no addition.

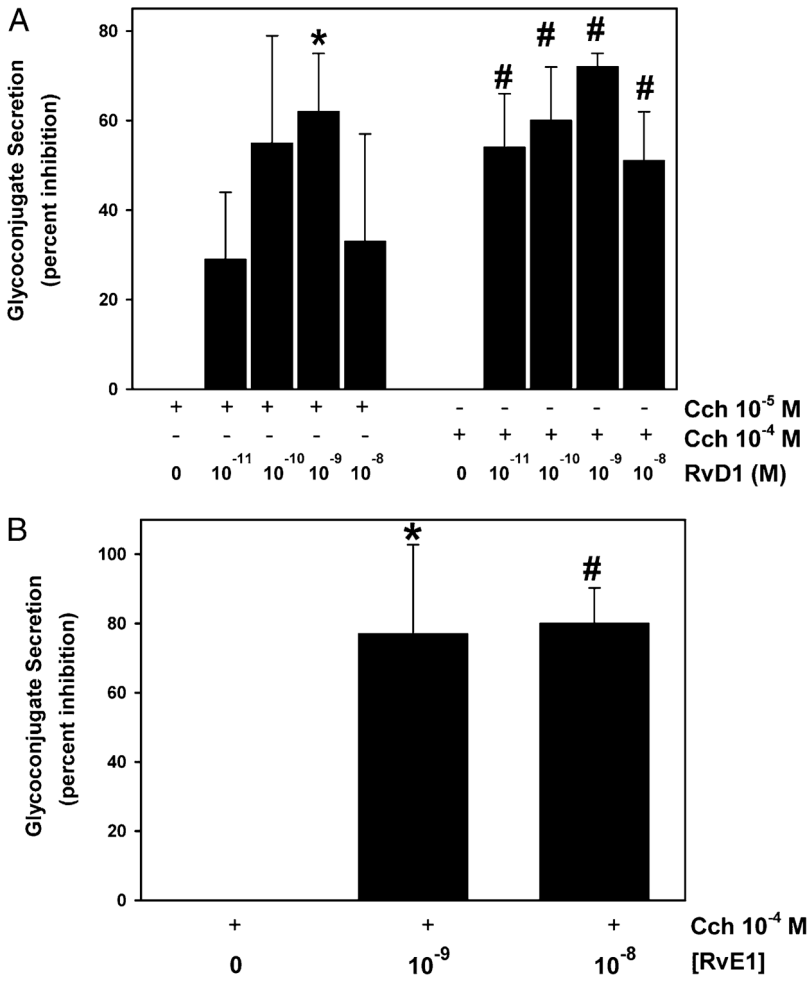

FIGURE 7.

Effect of addition of resolvins on cholinergic agonist-stimulated glycoconjugate secretion from cultured rat conjunctival goblet cells. Cultured rat conjunctival goblet cells were serum starved for 2 h and preincubated for 0.5 h with increasing concentrations of RvD1 before stimulation with the cholinergic agonist carbachol (Cch; 10^{-5} and 10^{-4} M) for 2 h (*A*, $n = 3$). Cultured rat conjunctival goblet cells were serum starved for 2 h and preincubated for 0.5 h with increasing concentrations of RvE1 before stimulation with the cholinergic agonist Cch (10^{-4} M) for 2 h (*B*) ($n = 5$). The amount of glycoconjugate secretion was measured by ELLA. Data are mean ± SEM. $^{*}p < 0.05$ from Cch alone, $^{\#}p < 0.01$ from Cch alone.

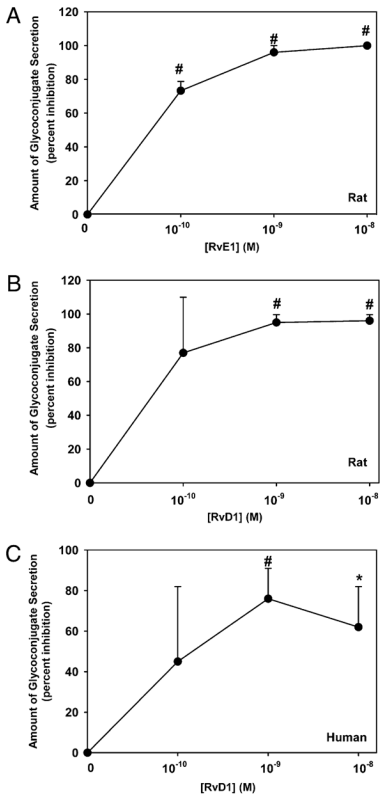

FIGURE 8.

Effect of RvE1 and RvD1 on LTD_4-stimulated glyco-conjugate secretion from cultured rat and human conjunctival goblet cells. Cultured rat conjunctival goblet cells were serum starved for 2 h and preincubated for 0.5 h with increasing concentrations of RvE1 (*A*) or RvD1 (*B*) before stimulation with LTD_4 (10^{-11} M) for 0.5 h. Cultured human conjunctival goblet cells were serum starved for 2 h and preincubated for 0.5 h with increasing concentrations of RvD1 (*C*) before stimulation with LTD_4 (10^{-11} M) for 0.5 h. The amount of glycoconjugate secretion was measured by ELLA. Data are mean ± SEM from six (rat) or three (human) independent experiments. $^{*}p < 0.05$ from agonist alone, $^{\#}p < 0.01$ from agonist alone.

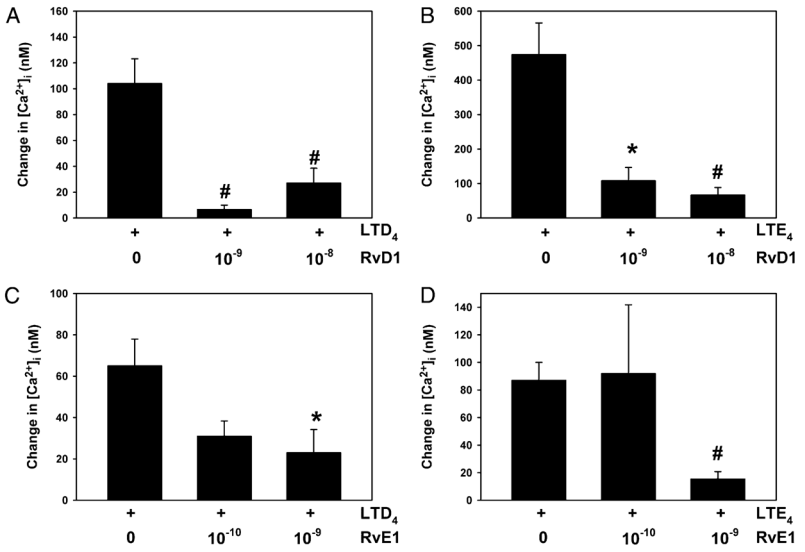

FIGURE 9.

Effect of RvD1 and RvE1 on LTD_4- and LTE_4-simulated increase in peak $[Ca^{2+}]_i$ in cultured rat conjunctival goblet cells. Cultured rat conjunctival goblet cells containing the calcium-sensitive dye fura 2 were preincubated for 0.5 h with RvD1 (10^{-9} and 10^{-8} M) before stimulation with LTD_4 (10^{-9} M, *A*) or LTE_4 (10^{-7} M, *B*). Cultured rat conjunctival goblet cells were preincubated for 0.5 h with RvE1 (10^{-10} and 10^{-9} M) before stimulation with LTD_4 (10^{-9} M, *C*) or LTE_4 (10^{-7} M, *D*). Data are mean ± SEM from three independent experiments. $^{*}p < 0.05$ from leukotriene alone, $^{\#}p < 0.01$ from leukotriene alone.

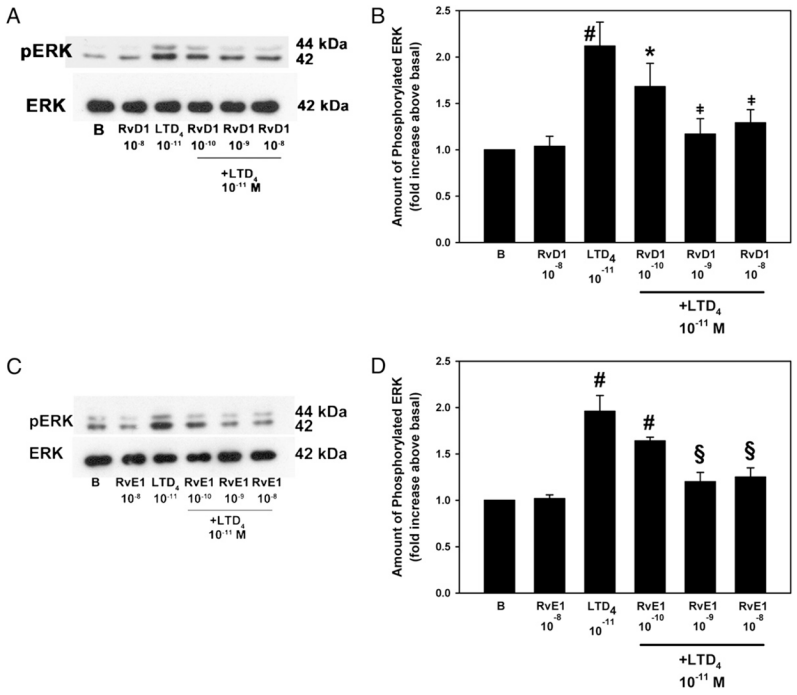

FIGURE 10.

Effect of RvD1 and RvE1 on leukotriene D_4-simulated activation of ERK1/2 in cultured rat conjunctival goblet cells. Cultured rat conjunctival goblet cells were preincubated for 0.5 h with RvD1 (10^{-10} to 10^{-8} M) before stimulation with LTD_4 (10^{-11} M) for 5 min, and the amount of phospho-ERK1/2 (pERK) and total ERK2 was determined by Western blotting analysis. One representative Western blot is shown in *A*. Three independent experiments were analyzed by densitometry, and the mean ± SEM is shown in *B*. Cultured rat conjunctival goblet cells were preincubated for 0.5 h with RvE1 (10^{-10} to 10^{-8} M) before stimulation with LTD_4 (10^{-11} M) for 5 min, and the amount of phospho-ERK1/2 and total ERK2 was determined by Western blotting analysis. One representative Western blot is shown in *C*. Three independent experiments were analyzed by densitometry, and the mean ± SEM is shown in *D*. $^{*}p < 0.05$ from no additions, $^{\#}p < 0.01$ from no addition, $^{\ddagger}p < 0.05$ from leukotriene alone, $^{\S}p < 0.01$ from no addition.