

Original article

Leukotriene E₄ induces MUC5AC release from human airway epithelial NCI-H292 cells

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ABSTRACT

Background: Hypersecretion of mucin in the airway epithelium is an important feature of allergic airway diseases. Of the 3 cysteinyl leukotrienes (CysLTs; LTC₄ LTD₄ and LTE₄), only LTE₄ is sufficiently stable to be detectable in extracellular fluids. However, LTE₄ has received little attention because it binds poorly to the CysLT₁ and CysLT₂ receptors; therefore, little is known about the effects of LTE₄ on mucous secretion. Recently, studies have focused on the P2Y₁₂ receptor as a potential receptor for LTE₄, because this receptor is required for LTE₄-mediated pulmonary inflammation. In our previous study, we confirmed the expression of P2Y₁₂ receptor in human airway epithelial cells. To clarify the roles of LTE₄ in airway epithelial cells, we investigated mucus secretion by LTE₄ in vitro.

Methods: Confluent NCI-H292 cells were stimulated with LTE₄ (0.01–1 μM) for 24 h. The release and production of MUC5AC protein, a gel-forming mucin, were evaluated with an enzyme-linked immunosorbent assay.

Results: Western blot analysis revealed that NCI-H292 cells expressed P2Y₁₂ receptor protein. LTE₄ significantly induced the release of MUC5AC mucin in a dose-dependent manner. Th2 cytokines such as IL-4 (10 ng/mL) and IL-13 (10 ng/mL) accelerated the LTE₄-induced release of MUC5AC protein. MRS2935, a P2Y₁₂ receptor antagonist, partially inhibited the LTE₄-induced release of MUC5AC protein in the airway. In contrast, MK571, a CysLT₁ receptor antagonist, did not affect the release of MUC5AC protein elicited by LTE₄.

Conclusions: These results suggest that LTE₄ may play some important roles in allergic mucus secretion partially via activation of P2Y₁₂ receptor.

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Introduction

The allergic response is a complex process involving the interaction of many mediators. Cysteinyl leukotrienes (CysLTs) are one of the most important mediators in the pathogenesis of airway allergic diseases such as allergic rhinitis and asthma.¹ LTE₄ is the most stable CysLT and can remain active at the site of release for a prolonged period after its synthesis.² Although LTE₄ is a weak agonist of classical receptors for CysLTs (CysLT₁ and CysLT₂),^{3,4} administration of exogenous LTE₄ into the human airway causes airway obstruction and mucosal eosinophilia in patients with asthma.^{5–7} P2Y₁₂ is the major platelet receptor that mediates ADP-induced aggregation.⁸ An in silico study predicted that LTE₄ might

be a surrogate ligand for P2Y₁₂ receptor,⁹ and purinergic P2Y₁₂ receptor was shown to be required for LTE₄-mediated pulmonary inflammation.^{10,11} Recently, we detected expression of P2Y₁₂ receptor in epithelial cells and submucosal glands in human allergic and non-allergic nasal mucosa by western blotting and immunohistochemical analysis,¹² indicating its potential role in the allergic response.

Mucus hypersecretion from the airway epithelium is a feature of asthma and allergic rhinosinusitis, and airway mucin is synthesized by epithelial goblet cells and submucosal glands, which may express P2Y₁₂ receptor as shown in our previous study.¹² Moreover, little is known about the effects of LTE₄ on mucous secretion such as that of MUC5AC mucin, which is a major gel-forming mucin in the airway. In a preliminary study, MUC5AC protein in the medium was undetectable under conventional cell culture conditions. Previous reports showed that retinoic acid¹³ or epidermal growth factor (EGF)¹⁴ could alter MUC5AC protein in a human airway epithelial cell line, NCI-H292; therefore, we here evaluated the level of MUC5AC mucin in retinoic acid and EGF-pretreated cells. We

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further evaluated the effect of LTE₄ and a P2Y₁₂ antagonist on the release of MUC5AC protein in NCI-H292 cells.

Methods

Culture of NCI-H292 cells

Cells of a human lung mucoepidermoid carcinoma cell line (NCI-H292) were purchased from the American Type Culture Collection. Cell suspensions were plated onto collagen type I-coated 6-well culture plates (Asahi Glass Co., Ltd., Funabashi, Japan) for a MUC5AC enzyme-linked immunosorbent assay (ELISA) or on collagen type 1 coated 4-well culture slides (Becton Dickinson Biosciences Discovery Labware, Bedford, MA, USA) for immunocytochemistry. The cells were cultured in RPMI-1640 medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics (50 U/mL penicillin and 50 µg/mL streptomycin) and cultured in a 5% carbon dioxide humidified atmosphere at 37 °C. The culture medium was changed at day 1 and every 2 days thereafter. To overexpress mucin, confluent cells were pretreated with 20 ng/mL EGF (Sigma) and 100 nM retinoic acid (Sigma) for 48 h.

Primary cultured human tracheal and nasal epithelial cells

Primary cultured human tracheal epithelial cells (HTECs) and primary cultured human nasal epithelial cells (HNECs) were used for western blot analyses of P2Y₁₂ receptor expression. HTECs were purchased from Lonza Japan Ltd (Tokyo, Japan), and the cells were cultured according to the manufacturer instructions. To obtain nasal epithelial cells, we used surgical specimens. Human inferior turbinates were obtained after turbinectomy from 3 patients with nasal obstruction that were refractory to medical therapy. Informed consent was obtained from the patients, and this study was approved by the ethics committee of Sapporo Medical University (approval number: 24-14). HNECs were isolated from human nasal mucosa specimens according to a previously described protocol.¹⁵ Cell suspensions were plated onto collagen type-I-coated 6-well culture plates in Ham's HD medium and cultured in a 5% carbon dioxide humidified atmosphere at 37 °C. The culture medium was changed at day 1 and every 2 days thereafter. Monolayer cell confluence was achieved after 6–8 days of culture. Morphological observations using a phase contrast microscope showed that these cultured cells consisted primarily of epithelial cells. More than 95% of these cells showed positive reactions for anti-human cytokeratin antibody (Dako, Glostrup, Denmark). HTECs and HNECs grown to 80% confluence were used for western blot analysis.

Immunohistochemistry for MUC5AC mucin

For immunohistochemistry of MUC5AC mucin, a mouse anti-human MUC5AC monoclonal antibody against the MUC5AC peptide corresponding to the core of gastric mucin (Acris Antibodies, Inc., San Diego, CA, USA) was used at 1:150 dilutions. NCI-H292 cells on the culture slides were initially washed with phosphate-buffered saline (PBS), and the cells were fixed by 100% methanol and 100% acetone each for 5 min at –20 °C. After air-drying for 5 min, the sections were incubated in blocking solution (10% normal goat serum in PBS) for 30 min before incubation in primary antibody. Then, the sections were incubated with anti-MUC5AC monoclonal antibody for 2 h at room temperature. Sections were washed in PBS and were incubated for 30 min with Alexa Fluor 594-labeled goat anti-mouse IgG (diluted 1:50; Molecular Probes, Eugene, OR, USA). Sections were mounted with Vectashield® mounting medium with 4',6-diamidino-2-phenylindole (Vector

Laboratories Inc., Burlingame, CA, USA) and examined under an Olympus BX51 microscope, attached to a DP70 CCD camera (Olympus Optical Co., Ltd., Tokyo, Japan). All images were processed with DP Controller and DP Manager Software (Olympus Optical Co., Ltd.) for image analysis. Negative controls were obtained by replacing primary antibodies with mouse IgG1 (Dako).

Western blot analysis

For western blot of P2Y₁₂ receptor, rabbit anti-human P2Y₁₂ receptor polyclonal antibody (product number P4871, Sigma) was used at 4 µg/ml concentration. For western blot analysis, NCI-H292 cells, HTECs, or HNECs were homogenized in T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA), supplemented with a proteinase inhibitor cocktail (Sigma). The protein extracts were purified by using PAGEprep Advance Kit (Thermo Fisher Scientific Inc.). Protein concentration in the homogenates was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific Inc.). Protein extracts (20 µg protein each) were separated on a 4–12% sodium dodecyl sulfate-polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane (Invitrogen Corporation, Carlsbad, CA, USA). Membranes were then blocked and probed with rabbit anti-human P2Y₁₂ receptor polyclonal antibody (Sigma). The bands were visualized using a WesternBreeze chromogenic western blot immunodetection kit (Invitrogen).

MUC5AC mucin analysis using ELISA

MUC5AC protein levels in the cellular secretions and lysates were evaluated by ELISA. Confluent NCI-H292 cells pretreated with EGF (20 ng/ml) and retinoic acid (100 nM) for 48 h were stimulated with 0.01–1 µM LTE₄ (Cayman Chemical Co., Ann Arbor, MI, USA) for 24 h. To determine the effects of Th2 cytokines on the LTE₄-induced release of MUC5AC protein, the cells were exposed to 10 ng/mL IL-4 (Sigma) and 10 ng/mL IL-13 (Sigma). To ascertain whether LTE₄ could mediate signal transduction through the P2Y₁₂ receptor or CysLT₁ receptor, the inhibitory effects of a receptor-specific antagonist for P2Y₁₂ receptor or a CysLT₁ receptor antagonist on LTE₄-induced mucous secretion were also examined. The cells were stimulated with LTE₄ alone or LTE₄ in combination with a P2Y₁₂ receptor antagonist, MRS2395 (1–10 µM; Sigma) or a CysLT₁ receptor antagonist, MK571 (10 µM; Cayman Chemical Co.) for 24 h. The culture medium was collected at 24 h after the stimulations with LTE₄ or control vehicle. Cells were washed with PBS, and the cells were lysed in T-PER Tissue Protein Extraction Reagent. Protein concentration in the cell lysates was determined using a BCA Protein Assay Kit. The levels of MUC5AC protein in the culture medium and cell lysates were evaluated by using ELISA kits (USCN Life Science Inc., Wuhan, China). The detection range of the kits is 0.312–20 ng/mL.

Statistical analysis

MUC5AC levels were determined in triplicate from 5 separate experiments and are presented as mean ± SD. Where appropriate, statistical differences were assessed by a non-parametric Mann–Whitney *U* test. *P*-values ≤0.05 were considered statistically significant.

Results

Immunohistochemical analyses for MUC5AC mucin

We evaluated the level of MUC5AC mucin in retinoic acid and EGF-pretreated cells by immunohistochemistry. As shown in

Fig. 1A, the pretreatment with EGF and retinoic acid increased MUC5AC protein expression in NCI-H292 cells relative to untreated control cells (Fig. 1B). Specificity of the staining was confirmed by absence of labeling with normal mouse immunoglobulin (Fig. 1C).

Western blotting for P2Y₁₂ receptor

In order to confirm expression of P2Y₁₂ receptor in human airway epithelial cells, western blot analysis was performed. As shown in Fig. 2, expression of a single band for P2Y₁₂ receptor protein in human airway epithelial cells was clearly demonstrated.

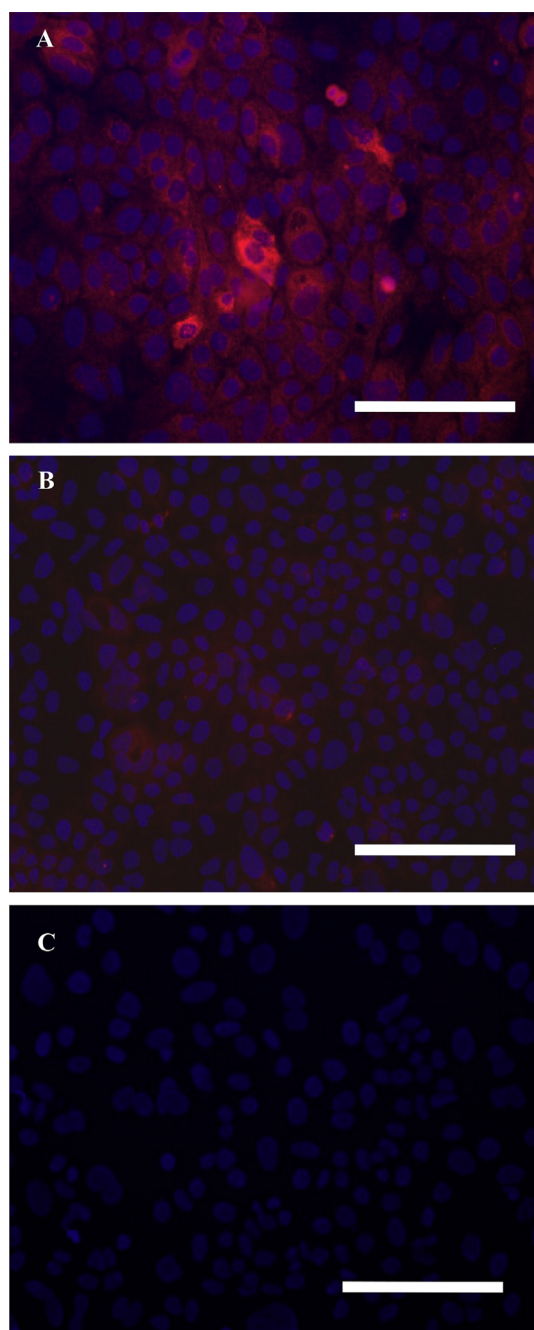


Fig. 1. Immunofluorescence staining for MUC5AC mucin in NCI-H292 cells. (A) Pretreatment with EGF and retinoic acid for 48 h increased MUC5AC protein expression. (B) Appearance of cells under conventional cell culture conditions without EGF and retinoic acid. (C) Negative control staining with normal mouse immunoglobulin. Scale bar = 100 μ m.

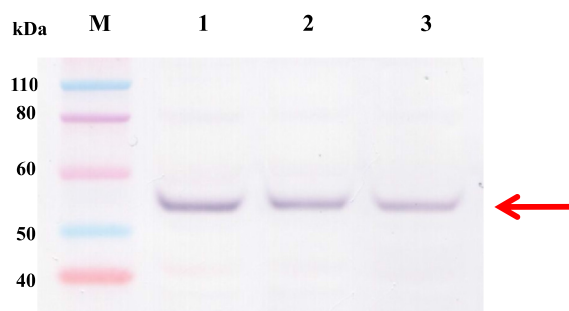


Fig. 2. Representative western blots for the P2Y₁₂ receptor protein in human airway epithelial cells (lane 1: primary cultured human nasal epithelial cells, lane 2: primary cultured human tracheal epithelial cells, lane 3: NCI-H292 cells). The antibody recognized a single protein of 55 kDa referred to as P2Y₁₂ receptor.

Therefore, these primary cultured human airway epithelial cells and NCI-H292 cells expressed P2Y₁₂ receptor protein.

LTE₄ induced the release of MUC5AC mucin

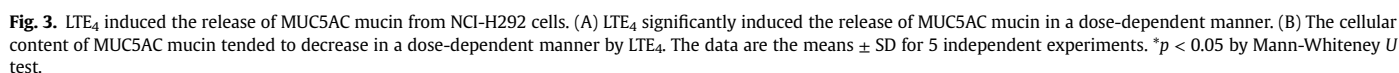
MUC5AC protein levels in the cellular secretions and lysates were evaluated by ELISA. As shown in Fig. 3A, LTE₄ significantly induced the release of MUC5AC mucin in a dose-dependent manner. After the addition of 1 μ M of LTE₄, there was a 3-fold increase in MUC5AC concentration (control, 1.3 ± 0.3 ng/mL; LTE₄ 1 μ M, 3.8 ± 0.7 ng/mL). By contrast, the cellular content of MUC5AC mucin tended to decrease in a dose-dependent manner (Fig. 3B).

To determine the effects of Th2 cytokines on the LTE₄-induced release of MUC5AC protein, the cells were exposed to Th2 cytokines. As shown in Fig. 4A, Th2 cytokines such as IL-4 and IL-13 accelerated the LTE₄-induced release of MUC5AC protein (LTE₄ 1 μ M alone, 3.4 ± 0.5 ng/mL; L-4 10 ng/mL and IL-13 10 ng/mL, 3.4 ± 0.6 ng/mL; LTE₄ 1 μ M plus IL-4 10 ng/mL and IL-13 10 ng/mL, 4.3 ± 0.5 ng/mL). As shown in Fig. 4B, Th2 cytokines tended to increase the cytoplasmic MUC5AC concentration even in LTE₄-stimulated cells (LTE₄ 1 μ M alone, 17.1 ± 3.5 ng/mL; L-4 10 ng/mL and IL-13 10 ng/mL, 29.1 ± 6.3 ng/mL; LTE₄ 1 μ M plus IL-4 10 ng/mL and IL-13 10 ng/mL, 21.9 ± 4.8 ng/mL).

To clarify whether LTE₄ could mediate signal transduction through the P2Y₁₂ receptor or CysLT₁ receptor, the inhibitory effects of a receptor-specific antagonist for P2Y₁₂ receptor or a CysLT₁ receptor antagonist on LTE₄-induced mucous secretion were examined. As shown in Fig. 5, MRS2395, a P2Y₁₂ receptor antagonist, could partially inhibit the LTE₄-induced release of MUC5AC protein (control, 0.8 ± 0.2 ng/mL; LTE₄ 1 μ M alone, 3.5 ± 0.6 ng/mL; LTE₄ 1 μ M plus MRS2395 10 μ M, 2.0 ± 0.3 ng/mL). By contrast, MK571, a CysLT₁ receptor antagonist, did not affect the release of MUC5AC protein elicited by LTE₄. (LTE₄ 1 μ M alone, 3.5 ± 0.6 ng/mL; LTE₄ 1 μ M plus MK571 10 μ M, 3.7 ± 0.5 ng/mL).

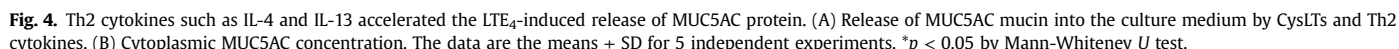
Discussion

In the present study, we used retinoic acid and EGF-pretreated NCI-H292 cells to study mucin secretion. NCI-H292 is an epithelial cell line that has been frequently used as an in vitro model system to study gene regulation and signal transduction.¹⁶ We also conducted in vitro experiments using BEAS-2B cells, primary cultured HTECs, and primary cultured HNECs to detect MUC5 mucin secretion at protein level. Unfortunately, we could not detect any mucins secreted from these epithelial cells by ELISA and western blot analysis. Therefore, retinoic acid and EGF-pretreated NCI-H292 cells may be useful for studies of MUC5AC secretions in airway epithelial cells.



receptors as determined through western blotting and RT-PCR analyses (data not shown). Therefore, it seems possible that LTD₄-induced mucous secretion from NCI-H292 cells occurred via activation of LTE₄ receptor that was simulated by stable LTE₄ converted from unstable LTD₄.

In this study, the release of MUC5AC mucin from NCI-H292 cells was observed following stimulation with LTE₄ at concentration greater than 0.1 μM (44 ng/mL). Because LTE₄ is sufficiently stable to be prominent in biologic fluids, urinary LTE₄ is an appropriately sensitive biomarker for the aspirin-induced asthma.¹⁹ Although many studies concerning the levels of urinary LTE₄ have been reported,¹⁹ there are few reports about the concentration of LTE₄ in the airway. We have previously reported that the median concentration of CysLTs (LTE₄/LTD₄/LTE₄) in nasal lavage fluid after nasal



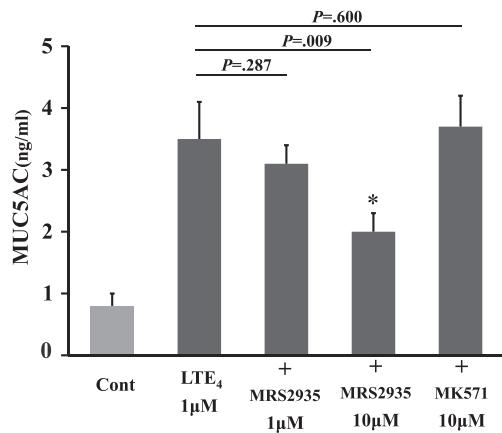


Fig. 5. The effects of a P2Y₁₂ receptor antagonist on LTE₄-induced MUC5AC secretion. MRS2935, a P2Y₁₂ receptor antagonist, partially inhibited the LTE₄-induced release of MUC5AC protein. In contrast, MK571, a CysLT₁ receptor antagonist, did not affect the release of MUC5AC protein elicited by LTE₄. The data are the means $\pm$ SD for 5 independent experiments. * $p < 0.05$ by Mann-Whitney U test.

allergen challenge was about 0.5 ng/mL.²⁰ O'Sullivan et al. reported that the median concentrations of LTE₄ in nasal lavage fluid in healthy volunteers was 60 pg/mL following exposure to swine house dust.²¹ Because these nasal lavage fluid samples were diluted with saline, it might be possible that the density of local LTE₄ was close to the density required to cause MUC5AC secretion.

The studies of Parruchuri et al. and Maekawa et al. have demonstrated that LTE₄ signaling can occur independently of the classical CysLT receptors, CysLT₁ and CysLT₂, indicating that LTE₄ preferentially signals via another as-yet-unidentified CysLT receptor.^{10,11} As shown in Fig. 2, we found that P2Y₁₂ receptor is expressed in primary cultured human airway epithelial cells and NCI-H292 cells. Our previous immunohistochemical studies, western blotting and RT-PCR analysis clearly showed the expression of P2Y₁₂ receptor not only in nasal vascular endothelial cells but also in nasal epithelial cells at the mRNA and protein levels.¹² A previous study also showed that P2Y₁₂ mRNA was expressed in human primary cultured HNECs by using RT-PCR analysis.²¹ The extracellular purines (adenosine, ADP, and ATP) and pyrimidines (UDP, UTP) regulate various physiological responses via cell-surface receptors termed purinoreceptors and exert autocrine or paracrine effects on ion transport,²² fluid transport,²³ ciliary beat frequency,^{22,24} and mucin secretion.²⁵ Thus the purinoreceptors in airway epithelial cells may play an important role in mucous secretion under inflammatory conditions.

Because the P2Y₁₂ antagonist was able to only partially control LTE₄-induced MUC5AC secretion, other pathways may be involved in this process. Recently, GPR99, previously reported as an oxyglutarate receptor,²⁶ was identified as a potential LTE₄ receptor.²⁷ LTE₄ binds to and activates GPR99 at low nanomolar-range concentrations in transfected cells, and resists blockage by MK571, a CysLT₁ receptor antagonist.²⁷ Using immunohistochemical analysis, we recently confirmed that GPR99 protein was present as a dimer in human nasal mucosa by western blot analysis, and was localized in epithelial cells, submucosal glands, and inflammatory leukocytes such as eosinophils and macrophages in the nasal mucosa (data not shown). Unfortunately, there has not been any report about GPR99-specific antagonists, although studies using small interfering RNA against GPR99 are currently in progress in our laboratory to clarify the role of GPR99 on the LTE₄-induced mucous secretion from epithelial cells.

In conclusion, this study provides important information about the role of LTE₄ on airway mucous secretion. The present findings

should be of considerable interest for understanding the potential roles of LTE₄ in allergic airway diseases characterized by hypermucous secretion such as asthma and eosinophilic rhinosinuitis.

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Conflict of interest

The authors have no conflict of interest to declare.

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