

Original article

Cysteinyl leukotrienes synergize with growth factors to induce proliferation of human bronchial fibroblasts

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Background: Cysteinyl leukotrienes (cys-LTs) are potent asthma-related mediators that function through their G protein-coupled receptors, cys-LT receptor type 1 (CysLT1R) and cys-LT receptor type 2 (CysLT2R).

Objective: Because many G protein-coupled receptors transactivate the epidermal growth factor receptor (EGFR) through metalloprotease-mediated ligand shedding, we investigated the effects of cys-LTs on signal transduction and proliferation of bronchial fibroblasts.

Methods: Human bronchial fibroblasts were grown from biopsy specimens of healthy subjects. Mitogenesis was assessed on the basis of tritiated methylthymidine incorporation.

Results: Leukotriene (LT) D₄ alone did not increase mitogenesis but dose-dependently increased thymidine incorporation and cell proliferation in the presence of epidermal growth factor (EGF). The enhancement was not prevented by CysLT1R antagonists (MK-571 and montelukast) or by a dual antagonist (BAY u9773), which is consistent with the lack of detectable mRNA for CysLT1R and CysLT2R in bronchial fibroblasts. LTD₄ did not cause EGFR transphosphorylation nor was the synergism blocked by the metalloprotease inhibitor GM6001. The EGFR-selective kinase inhibitor AG1478 suppressed the synergy between LTD₄ and EGF but had no effect on synergistic interactions of LTD₄ with other receptor tyrosine kinase growth factors. The effect of LTD₄ involved a pertussis toxin-sensitive and protein kinase C-mediated intracellular pathway, leading to sustained growth factor-dependent phosphorylation of extracellular signal-regulated kinase 1/2 and protein kinase B (PKB/Akt).

Conclusion: Cys-LTs do not transactivate EGFR but have a broader capability to synergize with receptor tyrosine kinase pathways.

Clinical implications: This study implies a critical role of cys-LTs in airway fibrosis in asthma and other chronic airway diseases, which might not be blocked by therapy with current LT receptor antagonists. (J Allergy Clin Immunol 2006;117:1016-1028.)

Key words: Cysteinyl leukotriene, bronchial asthma, airway remodeling, fibroblast, epidermal growth factor, G protein-coupled receptor

Leukotriene (LT) C₄, LTD₄, and LTE₄, collectively referred to as cysteinyl leukotrienes (cys-LTs), are lipid mediators generated through the 5-lipoxygenase pathway in activated eosinophils, basophils, mast cells, and macrophages. Cys-LTs are not only powerful bronchoconstrictor agents but also promote mucus secretion, tissue edema, and leukocyte infiltration, all of which are observed in chronic asthma.¹

Cys-LTs are known to function through the CysLT receptor type 1 (CysLT1R)² and the CysLT receptor type 2 (CysLT2R),^{3,4} both being typical G protein-coupled receptors (GPCRs). These receptors are variably expressed on bronchial smooth muscle and on peripheral blood leukocytes, suggesting that the 2 receptors might mediate diverse effects of cys-LTs at these sites. The order of potency of agonist activation for CysLT1R is LTD₄ > LTC₄ > LTE₄, and that for CysLT2R is LTD₄ = LTC₄ > LTE₄.

Potent and specific antagonists for CysLT1R have been developed as oral controller drugs for asthma, both as monotherapies⁵⁻⁷ and in combination with other controllers.^{8,9} Clinical trials demonstrate beneficial effects on lung function, symptom scores, and exacerbation rates and a moderate antieosinophilic effect. In an ovalbumin (OVA)-challenged allergic mouse model, the CysLT1R antagonist montelukast not only reduced bronchoconstriction, mucus production, and expression of eosinophilopoietic cytokines but also markedly reduced airway fibrosis.⁶

Although the contractile activity of cys-LTs on airway smooth muscle is their most well-known attribute, a

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Abbreviations used

ADAM:	A disintegrin and metalloproteinase
bFGF:	Basic fibroblast growth factor
cys-LT:	Cysteinyl leukotriene
CysLT1R:	Cysteinyl leukotriene receptor type 1
CysLT2R:	Cysteinyl leukotriene receptor type 2
DMEM:	Dulbecco's modified Eagle's medium
EGF:	Epidermal growth factor
EGFP:	Enhanced green fluorescent protein
EGFR:	Epidermal growth factor receptor
Erk:	Extracellular signal-regulated kinase
Gi/o:	G protein α inhibitory subunit
GPCR:	G protein-coupled receptor
LT:	Leukotriene
MAPK:	Mitogen-activated protein kinase
OVA:	Ovalbumin
PDGF:	Platelet-derived growth factor
PKC:	Protein kinase C
PTX:	Pertussis toxin
RTK:	Receptor tyrosine kinase
RT-qPCR:	Reverse transcription quantitative PCR
TBST:	Tris-buffered saline with 0.1% Tween 20

mitogenic effect of cys-LTs has also been reported. In sensitized Brown Norway rats the LT synthesis inhibitor SB210661 and the CysLT1R antagonist pranlukast attenuated the increases in airway smooth muscle cell DNA synthesis and smooth muscle thickness caused by repeated challenges of OVA.¹⁰ In an *in vitro* system LTD₄ (0.1 to 10 μ mol/L) synergistically augmented human tracheal smooth muscle cell proliferation induced by epidermal growth factor (EGF), whereas proliferation was not stimulated by LTD₄ alone.¹¹ Similarly, pretreatment with TGF- β or IL-13 markedly increased the expression of CysLT1R in human bronchial smooth muscle cells, inducing a capability to proliferate in response to LTD₄.¹² These studies suggest that cys-LTs cooperate with growth factors, cytokines, or both to stimulate proliferation of lung smooth muscle cells.

Synergistic proliferative activity of GPCR ligands and receptor tyrosine kinase (RTK)-dependent growth factors has been reported not only in airway smooth muscle cells but also in human fetal lung fibroblasts (HFL-1 cells).¹³ We therefore hypothesized that cys-LTs might increase the mitogenic activity of bronchial fibroblasts in the presence of EGF and other growth factors. Recent reports have shown that signaling from some GPCRs activates a disintegrin and metalloproteinase (ADAM) proteins, which cleave membrane-bound forms of EGF receptor (EGFR) ligands, thereby transactivating EGFRs.^{14,15} Therefore we postulated that cys-LTs acting on CysLT1R, CysLT2R, or both might transactivate EGFR through the activity of ADAM sheddases. Here we report that cys-LTs synergistically augment fibroblast mitogenesis and proliferation evoked by different RTK growth factors through intracellular transactivation and that this augmentation is not blocked by CysLT1R and CysLT2R antagonists.

METHODS**Cell cultures**

Primary human bronchial fibroblasts were grown from bronchial biopsy specimens obtained from healthy subjects, as previously reported.¹⁶ The detailed methods are described in the [Methods](#) section in the Online Repository at www.jacionline.org.

Reagents

LTD₄, LTC₄, and MK-571 were from Cayman Chemical (Ann Arbor, Mich). Montelukast sodium was a kind gift from Dr Jilly Evans (Merck, West Point, Pa). Other reagents are described in the [Methods](#) section in the Online Repository at www.jacionline.org.

Mitogenesis analyses

Bronchial fibroblasts were seeded into 96-well trays (Packard, Downers Grove, Ill) at a density of 5×10^3 cells per well in Dulbecco's modified Eagle's medium (DMEM)/10% FBS and grown to achieve confluence; the cells were then placed in serum-free medium for 24 hours to render them quiescent. Cells were stimulated with cys-LTs or with the same volume of vehicle (ethanol) in 1% fatty acid-free BSA/DMEM and incubated for 2 hours before addition of EGF in DMEM containing 1% BSA (Fraction V) and ITS (10 μ g/mL insulin, 5.5 μ g/mL transferrin, and 5 ng/mL sodium selenite; Sigma, Poole, United Kingdom). After 24 hours of stimulation, the cells were pulsed with 0.5 μ Ci tritiated methylthymidine (Amersham Biosciences, Amersham, United Kingdom) for 2 hours. The supernatant was removed, and cells were fixed with 5% trichloroacetic acid. Acid-insoluble material was dissolved in 40 μ L of 0.2 mol/L NaOH before adding 150 μ L of Microscint 40 (PerkinElmer, Boston, Mass). Radioactivity was quantified by using a TopCount scintillation counter (Packard). Conditions were tested in triplicate, and each experiment was performed in at least 3 independent assays.

Assessment of cell proliferation

The methylene blue assay was based on the method of Oliver et al.¹⁷ The detailed methods are described in the [Methods](#) section in the Online Repository at www.jacionline.org.

Reverse transcription quantitative PCR

The methods for a SYBR green reverse transcription quantitative PCR (RT-qPCR) assay and primer sequences for CysLT1R and CysLT2R are described in the [Methods](#) section in the Online Repository at www.jacionline.org.

Western blotting

The methods for Western blotting are described in the [Methods](#) section in the Online Repository at www.jacionline.org.

Statistical analyses

Data were analyzed with SPSS software (SPSS, Inc, Chicago, Ill). Paired *t* tests were used for data sets following a normal distribution, and the Wilcoxon signed-rank test was used for nonparametric data (SPSS). *P* values of less than .05 were considered significant.

RESULTS**LTD₄ synergizes with EGF to induce mitogenesis**

To assess whether cys-LTs have mitogenic activity, we analyzed the incorporation of tritiated thymidine into primary cultures of bronchial fibroblasts. LTC₄ or LTD₄

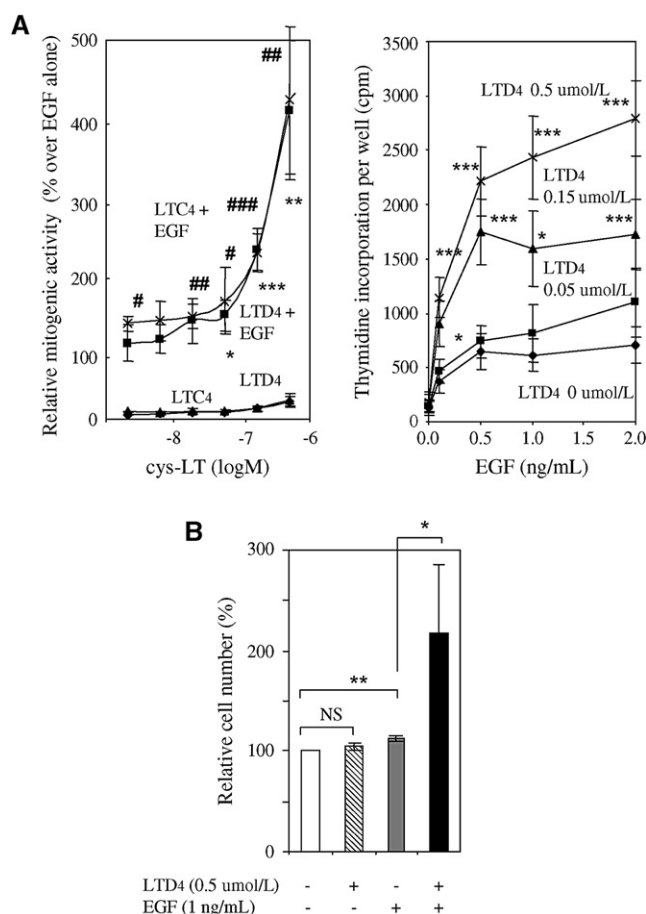


FIG 1. A, Effect of LTD₄ on fibroblast mitogenesis. Cells were stimulated with LTD₄, LTC₄, or vehicle and EGF. After 24 hours, mitogenic activities were determined. Values are presented as relative mitogenic activities over EGF alone (*left*) or radioactivity (in counts per minute) per well (*right*). **P* < .05, ***P* < .01, and ****P* < .001 for LTD₄ and #*P* < .05, ##*P* < .01, and ###*P* < .001 for LTC₄ versus EGF alone. **B**, Effect of LTD₄ on fibroblast proliferation. Cells were stimulated with LTD₄, EGF, or both, and then fixed at day 3. **P* < .05, ***P* < .01.

alone had little mitogenic effect. However, when cocultured with EGF, both LTC₄ and LTD₄ dose-dependently augmented EGF-induced mitogenesis. Thus 0.5 μmol/L of LTD₄ or LTC₄ increased mitogenesis by 408% ± 93% (*P* = .002) or 421% ± 97% (*P* = .002), respectively, compared with EGF (1 ng/mL) alone. The dose-response curves show very similar potency and efficacy for LTC₄ and LTD₄ (Fig 1, A). This increased mitogenic activity was shown to lead to increased cell proliferation, as determined by methylene blue uptake experiments; costimulation with EGF and LTD₄ significantly increased cell numbers by 193% ± 61% compared with EGF alone (*P* = .026; Fig 1, B). The mitogenesis experiments were repeated in cells from 3 other healthy subjects, showing that LTD₄ had a synergistic effect on EGF-stimulated proliferation in each cell culture tested (data not shown).

Effect of CysLT receptor antagonists

Fibroblasts were pretreated with 3 different receptor antagonists before stimulation with LTD₄ and EGF to

investigate the involvement of CysLT1R and CysLT2R in the enhanced proliferation induced by LTD₄. Neither MK-571 nor montelukast, both of which are potent and specific CysLT1R antagonists, prevented the synergistic effect of LTD₄. The involvement of CysLT2R was investigated by using BAY u9773, the only CysLT2R antagonist presently available. In the presence of a full agonist, such as LTD₄, BAY u9773 acts as an effective competitive antagonist of both CysLT1R and CysLT2R.^{3,4} However, in the absence of a full agonist, BAY u9773 might express partial agonist activity at CysLT2R (but not CysLT1R).³ Fig 2, A, shows that in the presence of LTD₄ (0.5 μmol/L), BAY u9773 (1 or 5 μmol/L) failed to inhibit the synergism, suggesting that neither CysLT1R nor CysLT2R is involved. Fig 2, B, shows that when LTD₄ was either absent or present only at low concentrations (<0.2 μmol/L), 1 μmol/L of BAY u9773 showed a significant agonistic effect on proliferation with EGF, but this confirms that BAY u9773 neither enhanced nor inhibited the synergy of LTD₄ and EGF when LTD₄ was present at the higher concentration (0.5 μmol/L).

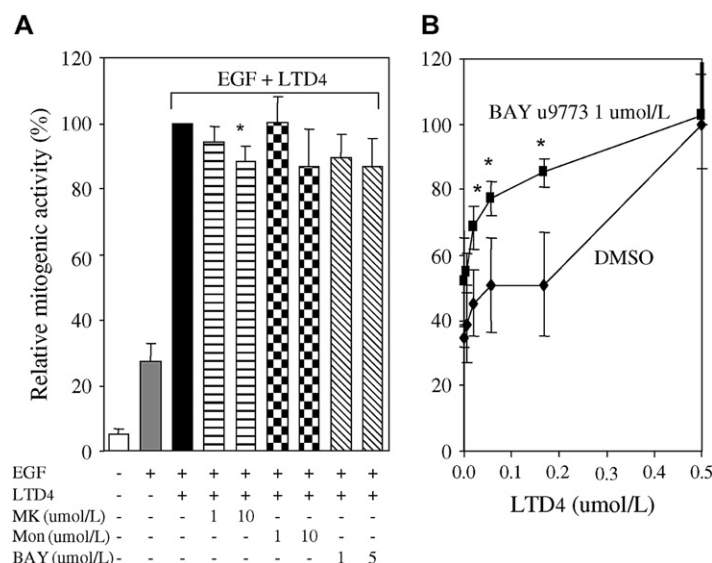


FIG 2. **A**, Effect of CysLT receptor antagonists. Fibroblasts were treated with indicated concentrations of MK-571 (MK), montelukast (Mon), BAY u9773 (BAY), or dimethyl sulfoxide (DMSO) for 1 hour before stimulation with LTD₄ (0.5 μmol/L) and EGF (1 ng/mL). **P* < .05 versus costimulation. **B**, The partial agonistic effect of BAY u9773. Fibroblasts were pretreated with DMSO or BAY u9773 (1 μmol/L) for 1 hour before stimulation with LTD₄ and EGF. **P* < .05 versus DMSO treatment.

Analyses of expression of CysLT1R and CysLT2R on human bronchial fibroblasts

Because the pharmacology suggested that neither CysLT1R nor CysLT2R was involved in the synergistic effect by LTD₄ with EGF, we analyzed the expression of these receptors in human bronchial fibroblasts using RT-qPCR. Fig 3 shows that the amount of mRNA for both CysLT1R and CysLT2R is very small in cultured bronchial fibroblasts (cycle threshold value of around 35), which is comparable with the expression level in mock-transfected HEK293 cells, which are thought not to express either CysLT1R or CysLT2R.^{3,4} On the other hand, whole lung resulted in a reasonable level of expression (cycle threshold of around 27), probably because of expression in smooth muscle cells and leukocytes. Although fibroblasts showed distinct immunofluorescence for CysLT1R and CysLT2R by using antibodies supplied by Cayman Chemical in flow cytometric analyses, control studies with HEK293 cells expressing recombinant CysLT receptors suggested that these signals were nonspecific because similar signals were observed in HEK293 cells regardless of transfection with recombinants CysLT1R or CysLT2R (see Figs E1–E3 in the Online Repository at www.jacionline.org). We conclude from these results that CysLT1R and CysLT2R are not expressed or are expressed at very low levels in human bronchial fibroblasts. This was also confirmed by the fact that in contrast with the transfected cells there was no calcium flux observed in response to LTD₄ in bronchial fibroblasts (compare Figs E4 and E5 in the Online Repository at www.jacionline.org).

Synergy is not mediated by transactivation of EGFR

To further characterize the synergistic responses of the fibroblasts to LTD₄ and EGF, we investigated the involvement of the EGFR. Agonists for a variety of GPCRs stimulate activation of ADAM sheddases that liberate membrane-bound forms of growth factors, such as heparin-binding EGF-like growth factor, leading to transactivation of EGFR.^{14,15} Although both an EGFR neutralizing antibody, which competes for ligand binding, and an EGFR-selective tyrosine kinase inhibitor, AG1478, significantly prevented the synergy of LTD₄ and EGF (Fig 4, A), these inhibitors are unable to discriminate between direct activation of the EGFR by EGF or transactivation by the GPCR. Thus we investigated the effect of GM6001, a broad-spectrum metalloprotease inhibitor, on the synergy. Fig 4, A, shows that GM6001 (5 and 20 μmol/L) failed to prevent the synergy. Similarly, (3R)-(+)-(2-[4-methoxybenzenesulfonyl]-1,2,3,4-tetrahydroisoquinoline-3-hydroxamate), which we have previously found to significantly block EGFR ligand shedding at 1 and 10 μmol/L,¹⁸ did not prevent the LTD₄-evoked effect (data not shown). A neutralizing antibody for heparin-binding EGF-like growth factor also did not affect the synergy. Furthermore, Western blot analyses consistently showed that stimulation of fibroblasts with LTD₄ was unable to induce phosphorylation of EGFR, nor did it augment the phosphorylation of EGFR induced by EGF (Fig 4, B). These results indicated that LTD₄ does not activate growth factor shedding leading to transactivation of EGFR but that an EGF-stimulated signal is essential for the LTD₄-induced synergistic effect.

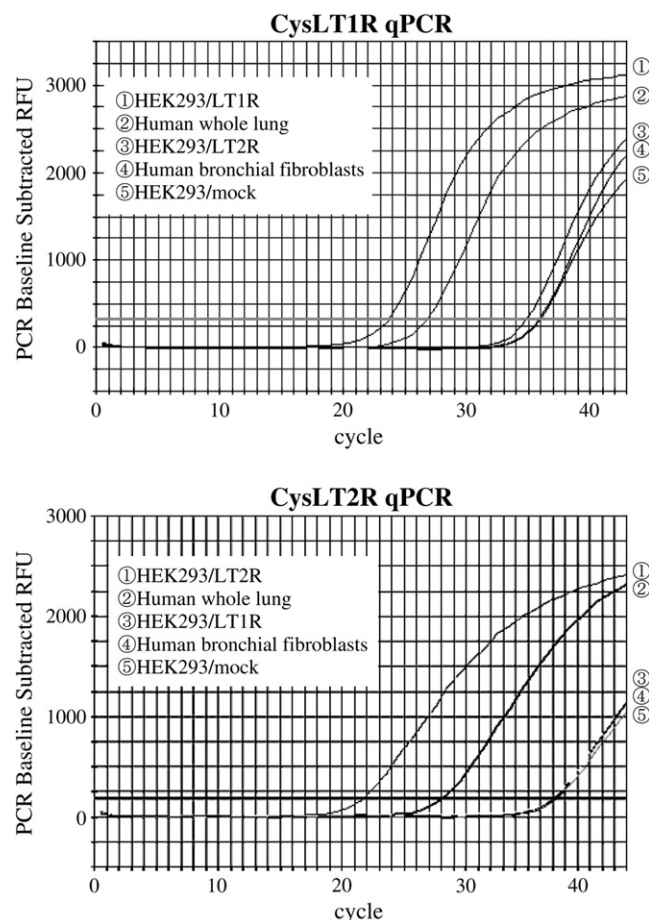


FIG 3. Expression analyses of CysLT1R and CysLT2R in human bronchial fibroblasts. cDNA samples from HEK293 cells transiently transfected with a recombinant plasmid encoding CysLT1R or CysLT2R or vector (pcDNA3.1, *mock*), human whole lung, or cultured human bronchial fibroblasts were subjected to RT-qPCR analyses with SYBR green for detection.

Synergy with other RTK growth factors

The data suggest that the interaction between CysLT GPCRs and EGFR might occur inside the cells rather than through transactivation of EGFR by ligand liberation. We therefore postulated that LTD₄ might also enhance the mitogenesis of bronchial fibroblasts by synergizing with other RTK-mediated pathways. LTD₄ significantly augmented DNA synthesis elicited by platelet-derived growth factor (PDGF)-BB or by basic fibroblast growth factor (bFGF). Dual stimulation resulted in a 5.59 ± 1.38 -fold induction of thymidine incorporation for LTD₄ and PDGF-BB ($P = .005$) and a 4.30 ± 1.79 -fold induction for LTD₄ and bFGF ($P = .006$) compared with the growth factor alone ($n = 4$, data not shown). In these cases the synergistic effect was insensitive to AG1478 (data not shown), suggesting that the EGFR had not been transactivated by the LTD₄ to augment the proliferative signals from the other RTKs. Methylene blue uptake experiments also showed that cell number was significantly increased by means of coinubation

with LTD₄ ($154\% \pm 25\%$) when compared with PDGF-BB alone ($P = .005$, data not shown).

Synergy is prevented by inhibitors for mitogen-activated protein kinase and phosphatidyl inositol 3-kinase

To explore the signaling mechanisms, we investigated the effect of several inhibitors of intracellular signaling pathways. U0126, an inhibitor of mitogen-activated protein kinase (MAPK) kinase 1 acting upstream of extracellular signal-regulated kinase (MAPK/Erk) 1/2, significantly prevented the synergy, as did 2 p38 MAPK inhibitors (SB203580 and SB202190). Also preventing the synergism were LY294002, a phosphatidyl inositol 3-kinase inhibitor; rapamycin, an inhibitor of p70S6 kinase; and GF109203X, a general protein kinase C (PKC) inhibitor. However, these drugs inhibited the mitogenesis elicited by EGF alone to the same extent as they inhibited the synergistic mitogenic effect (Fig 5), suggesting that their inhibition of the synergy was dependent on blocking EGF-induced signaling.

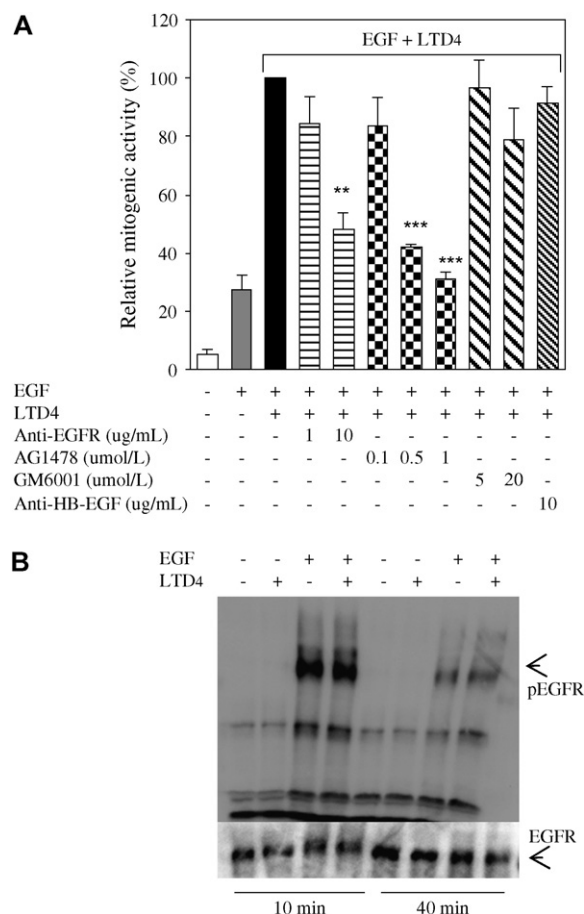


FIG 4. Synergism by LTD₄ requires an EGF signal but not growth factor shedding. **A**, Effect of EGFR antagonists and inhibitors of EGFR transactivation on mitogenesis. ***P* < .01 and ****P* < .001 versus costimulation with EGF (1 ng/mL) and LTD₄ (0.5 μmol/L). **B**, EGFR phosphorylation. Fibroblasts were stimulated with LTD₄ (0.5 μmol/L) or vehicle for 2 hours before stimulation with EGF (1 ng/mL), and then the cells were incubated for another 10 or 40 minutes. Cell lysates were analyzed by means of immunoblotting with the indicated antibodies. HB-EGF, Heparin-binding EGF-like growth factor.

Involvement of G protein α inhibitory subunit (Gi/o)

Given that CysLT₂R is reported to be coupled with Gi/o in human mast cells,¹⁹ we examined the effect of pertussis toxin (PTX) on LTD₄ and EGF synergism in fibroblasts. Pretreatment with PTX significantly inhibited the potentiating effects of LTD₄ on fibroblast mitogenesis induced not only by EGF (60.7% ± 4.3% inhibition at 200 ng/mL) but also by PDGF-BB (70.7% ± 0.1% inhibition) and bFGF (71.4% ± 2.4% inhibition), without preventing the mitogenesis elicited by each growth factor alone (Fig 6).

Upregulation of MAPK and phosphatidylinositol 3-kinase by means of coinubation with EGF and LTD₄

To investigate further the involvement of signaling molecules in the LTD₄-enhanced mitogenesis, we

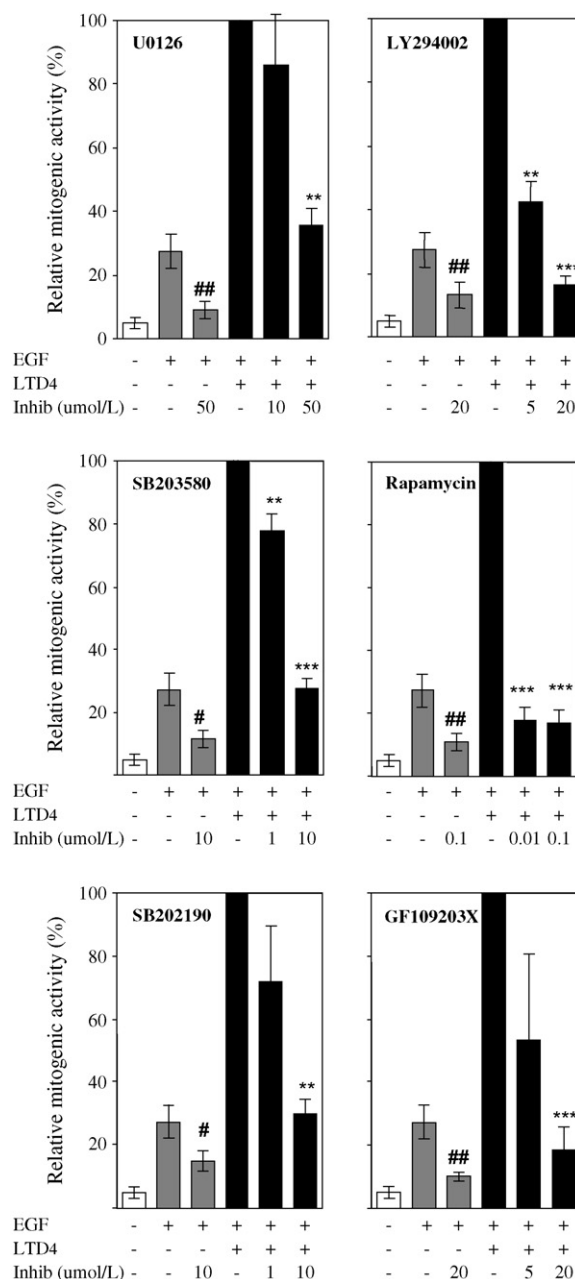


FIG 5. Dependence of the synergistic mitogenesis on an EGF-stimulated signaling pathway. Fibroblasts were treated with indicated inhibitors or vehicle (dimethyl sulfoxide) for 1 hour before stimulation with EGF (1 ng/mL) alone or costimulation with EGF and LTD₄ (0.5 μmol/L). ***P* < .01 and ****P* < .001 versus costimulation. #*P* < .05 and ##*P* < .01 versus EGF alone.

analyzed the phosphorylation of PKC, MAPK, and Akt. Phosphorylation of PKC was upregulated after treatment with LTD₄ alone, and this was blocked by pretreatment with PTX. In contrast, LTD₄ did not cause increased phosphorylation of Erk-1/2 and Akt, whereas EGF provided a clear upregulation of both Erk-1/2 and Akt (Fig 7, A). However, when cells were stimulated with EGF for extended periods, the addition of LTD₄ resulted in sustained

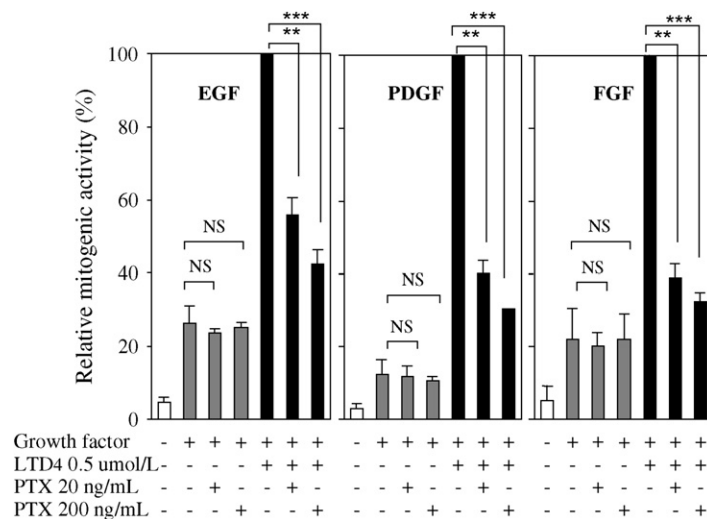


FIG 6. The effect of PTX on LTD₄ and growth factor–induced mitogenesis. Cells were treated with PTX for 2 hours before stimulation with growth factor alone or costimulation with LTD₄ (0.5 μ mol/L) and EGF (1 ng/mL), PDGF-BB (10 ng/mL), or bFGF (1 ng/mL). ** P < .01 and *** P < .001 versus costimulation.

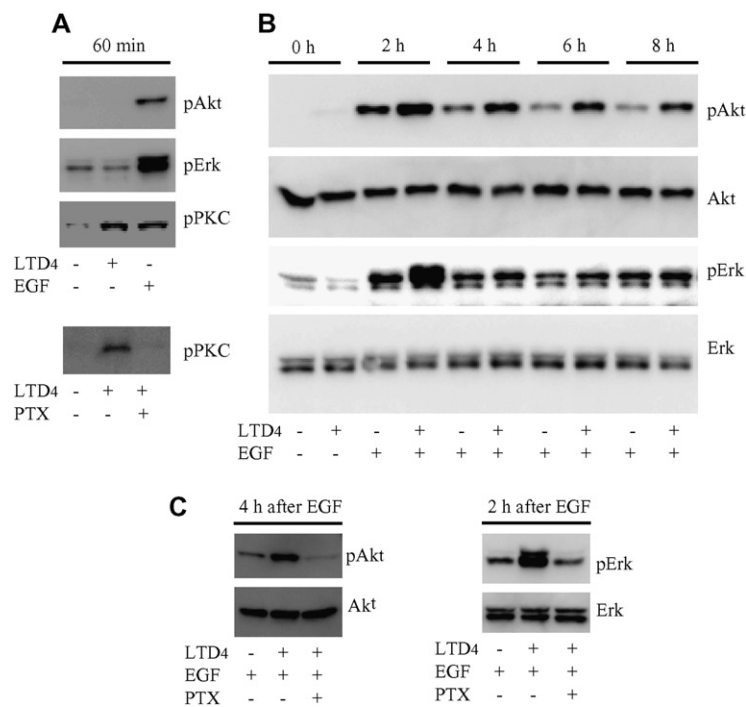


FIG 7. **A**, LTD₄ stimulates PKC. Fibroblasts were stimulated with vehicle, LTD₄ (0.5 μ mol/L), or EGF (1 ng/mL; upper panel) or incubated with or without PTX (200 ng/mL) for 2 hours before addition of LTD₄ (lower panel). Cell lysates were analyzed by means of immunoblotting. **B**, Sustained phosphorylation of Erk and Akt by means of costimulation with LTD₄ and EGF. **C**, Enhanced phosphorylation is dependent on Gi/o. Cells were incubated with or without PTX before costimulation with LTD₄ and EGF. *pAkt*, Phospho-Akt; *pErk*, phospho-Erk.

phosphorylated Akt at 2, 4, 6, and 8 hours after costimulation, whereas a transient increase of phospho-Erk-1/2 was seen only at 2 hours (Fig 7, B). The upregulation of both kinases was prevented by pretreatment with PTX (Fig 7, C), indicating the involvement of Gi/o in the increased phosphorylation.

DISCUSSION

Airway remodeling is an important component of chronic asthma involving proliferation and activation of airway fibroblasts.²⁰ Previous studies have shown that LTC₄ and LTD₄ stimulate mitogenesis of human skin fibroblasts

in vitro.²¹ Furthermore, targeted deletion of the CysLT1R gene unexpectedly augmented bleomycin-induced lung fibrosis and alveolar septal thickening,²² whereas deletion of CysLT2R prevented these effects.²³ These data suggest that CysLT1R and CysLT2R have distinct functions *in vivo* and that CysLT2R might have a role in pulmonary fibrosis. In this study we have shown that although cys-LTs alone are unable to stimulate proliferation, they potentially synergize with EGF, PDGF, and fibroblast growth factor to promote proliferation of normal bronchial fibroblasts.

We could not assign the response to CysLT1 or CysLT2 receptors based on agonist potency, receptor antagonist pharmacology, and lack of expression of CysLT1R and CysLT2R mRNA and proteins. Thus the equivalent potency and efficacy of LTC₄ and LTD₄ and the inability of MK-571 or montelukast to block the synergy argue strongly against the involvement of CysLT1R in this fibroblast response. It has been reported that the synergy between LTD₄ and EGF on smooth muscle cell proliferation was not prevented by montelukast.²⁴ In contrast, Panettieri et al¹¹ found that the synergistic effect of LTD₄ on smooth muscle cell mitogenesis was inhibited by CysLT1R receptor antagonists with differing efficacies. Pranlukast at 1 μ M prevented the effect, whereas a higher concentration (30 μ mol/L) was required for pobelukast, and zafirlukast was without effect at 1 μ mol/L.

As predicted by the CysLT2R gene deletion mouse model²³ and studies with skin fibroblasts,²¹ the comparable potency and efficacy of LTC₄ and LTD₄ might implicate CysLT2R as the transducer of fibroblast proliferation. Recent reports with human mast cells have shown that CysLT2R couples with a PTX-sensitive Gi/o protein or proteins, leading to p38 MAPK activation, independently of calcium flux.¹⁹ Furthermore, stimulation of IL-8 production from mast cells by LTC₄ is prevented by PTX but is resistant to MK-571, implying that LTC₄-induced IL-8 generation depends on CysLT2R. In our studies we also observed that the synergistic effect of cys-LTs was sensitive to PTX, and we observed no obvious calcium flux after administration of LTD₄ to the fibroblasts, whereas histamine efficiently evoked a calcium flux. Although these data implicate CysLT2R in fibroblast proliferation, this conclusion is contradicted by the inability of the dual antagonist BAY u9773 to inhibit the synergism and by the absence of detectable CysLT2R expression in bronchial fibroblasts analyzed by means of RT-qPCR. Because CysLT1R and CysLT2R are structurally similar and phylogenetically related to the purinergic receptors, we also tested the effect of pyridoxal phosphate-6-azo (benzene-2,4-disulfonic acid), a selective antagonist for P2 purinoreceptors.²⁵ However, pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) (10 and 50 μ mol/L) did not inhibit the synergistic effect by LTD₄ with EGF (not shown), suggesting that P2 receptors at least are not involved in the LTD₄-mediated mitogenic effect in human bronchial fibroblasts.

Thus it is likely that the response to LTC₄ and LTD₄ is mediated by a distinct and as yet uncharacterized CysLT receptor. Existence of a third CysLT receptor has been

proposed previously, based largely on evidence showing insensitivity of LTC₄-induced vasoconstriction of human and porcine pulmonary artery to inhibition by CysLTR antagonists, including BAY u9773.^{26,27} The availability of more selective CysLT2R antagonists might help to resolve this unanswered question. Nevertheless, despite the efficient blockade of airway fibrosis shown by montelukast in an OVA-challenged mouse model,⁶ the inability of antagonists that selectively target CysLT1R to block the synergistic effect of cys-LTs and growth factors on human bronchial fibroblast proliferation suggests that these asthma therapies might be ineffective in suppressing the fibrotic changes that occur in asthmatic airways.

The expression and function of CysLT1R and CysLT2R on lung fibroblasts have not been explored in detail previously. In HFL-1 cells LTC₄ synergistically augmented eotaxin production stimulated by IL-13, which was caused by upregulation of CysLT1R by IL-13; the effect of LTC₄ was prevented by montelukast.²⁸ It is also reported that although LTD₄ alone had no effect, in combination with TGF- β , it significantly augmented collagen production from HFL-1 cells compared with TGF- β alone. This synergy was also caused by upregulation of CysLT1R by TGF- β .²⁹ These results imply that human airway fibroblasts, like airway smooth muscle cells, have the ability to respond to cys-LTs through upregulation of CysLT1R when coincubated with some cytokines. Although we also show here that bronchial fibroblasts respond to cys-LTs only in the presence of RTK growth factors, it is unlikely that this is caused by the upregulation of CysLT1R or CysLT2R by RTK growth factors based on the failure of the receptor antagonists to inhibit the response. This difference might be due to the source of the fibroblasts; we established cells from adult human bronchial tissue, whereas HFL-1 cells are from fetal lung tissue.

Despite our initial expectations, LTD₄ did not cause transactivation of EGFR in bronchial fibroblasts. However, with hindsight, this might not have been surprising because the cys-LTs were unable to induce mitogenesis in the absence of exogenous growth factor. In contrast, GPCR agonists, such as thrombin, that can directly transactivate EGFR^{30,31} are mitogens in their own right. This suggests that, at least in bronchial fibroblasts, cys-LTs activate intracellular cascades that potentiate RTK signals rather than feeding directly into an RTK pathway by means of transactivation. Whether the EGFR is directly transactivated by cys-LTs in other cell types has not been investigated to date. Although thrombin phosphorylates EGFR in keratinocytes, astrocytes,^{30,31} and aortic smooth muscle cells,³² it does not transactivate EGFR in human airway smooth muscle cells.³³ This suggests that cross-talk between GPCR and RTK pathways differs between cell types, even for the same GPCR agonist.

The molecular mechanism of the synergy by thrombin, histamine, and carbachol on airway smooth muscle cells has been investigated. As observed in the present study examining the effect of cys-LTs on bronchial fibroblasts, none of the 3 agonists caused phosphorylation of EGFR or augmentation of EGF-induced EGFR phosphorylation in smooth muscle cells. Furthermore, whereas only thrombin

increased EGF-stimulated phospho-Erk1/2, all the GPCR agonists consistently increased the activity of p70S6 kinase at late phases (1-12 hours).³³ The sustained activation of Akt and Erk-1/2 by costimulation with EGF and LTD₄ suggests that the interaction or interactions between the GPCR and RTK pathways for LTD₄ and thrombin are similar.

In conclusion, cys-LTs synergize with EGF, PDGF, and fibroblast growth factor to promote fibroblast proliferation through a PTX-sensitive and PKC-mediated intracellular pathway, leading to sustained growth factor-dependent phosphorylation of Erk-1/2 and Akt. The effect is unlikely to be mediated by CysLT1R or CysLT2R, as defined by classical receptor pharmacology, but might be mediated by a novel receptor. Although cys-LTs are unable to transactivate the EGFR, they have a broader capability to synergize with RTK pathways. These findings have clinical implications for the therapeutic use of CysLT1R antagonists in asthma and other allergic diseases, as well as for understanding the role of cys-LTs in chronic fibrotic diseases of the airways.

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