Mitogen-activated protein kinase (MAPK) regulates leukotriene D$_4$-induced HB-EGF and ADAM12 expression in human airway smooth muscle cells

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Summary

Background: Cysteinyl leukotriene (LT) induces bronchoconstriction as well as airway inflammation and remodeling. Heparin-binding EGF-like growth factor (HB-EGF) is associated with remodeling in airway smooth muscle (ASM) cells in bronchial asthma. A disintegrin and metalloproteinase (ADAM) 12 is an enzyme implicated in the ectodomain shedding of membrane-anchored proHB-EGF and release of HB-EGF.

Objective: To determine the role of LTD$_4$ in HB-EGF and ADAM12 expression and the regulatory mechanism in human ASM cells, we analyzed a functioning signaling molecule in LTD$_4$-induced HB-EGF and ADAM12 expression in human ASM cells by focusing on the role of mitogen-activated protein kinase (MAPK) cascades.

Method: Human ASM cells were stimulated LTD$_4$ in a time-dependent manner. We observed phosphorylation of MAPK by western blot analysis and the expression of HB-EGF and ADAM12 by quantitative PCR analysis of mRNA. Furthermore, we pretreated with specific inhibitors of MAPK and LTD$_4$.

Results: LTD$_4$ induced an extracellular-signal regulated kinase (ERK), p38 MAPK and c-Jun-NH$_2$-terminal kinase (JNK) phosphorylation in human ASM cells. LTD$_4$ induced HB-EGF and ADAM12 mRNA expression. Furthermore, the regulation of LTD$_4$-induced HB-EGF and ADAM12 mRNA expression is associated with ERK and p38 MAPK, not but JNK.

Conclusion: we conclude that p38 MAPK and ERK are capable of regulating LTD$_4$-induced HB-EGF and ADAM12 expression in human ASM cells. In bronchial asthma, the specific inhibitor of p38 MAPK and ERK may produce beneficial effects in controlling airway remodeling and inflammation. *(Asian Pac J Allergy Immunol 2013;31:58-66)*

Key words: ADAM12, HB-EGF, LTD$_4$, MAPK, remodeling

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Introduction

Cysteinyl leukotrienes (LTs), LTC$_4$, LTD$_4$ and LTE$_4$, which are metabolites of arachidonate via the 5-lipoxygenase pathway, have various biological activities such as bronchial constriction, mucous hypersecretion, increase in microvascular permeability, bronchial hyper-responsiveness, and eosinophil chemotaxis.[1,2] In addition, cysteinyl LTs are known to promote airway smooth muscle (ASM) cell proliferation, induce the production of granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin (IL)-4, IL-5, tumor necrosis factor-alpha (TNF-alpha) and are regulated upon activation of normal T-cell expression and secretion is equal to RANTES, the synthesis of collagenase and proteoglycan and the induction of P-selectin expression.[3-7] In animal models of asthma, LT receptor antagonist attenuates airway remodeling.[8] Thus, cysteinyl LTs play important roles in the pathogenesis of airway inflammation and remodeling.

Heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) was originally purified from conditioned medium in which macrophage like U-937 cells had been cultured.[9] As a member of the EGF family, HB-EGF binds to EGF receptor (EGFR) directly and thereby enhances its phosphorylation, resulting in cell growth and differentiation.[10] HB-EGF is synthesized as a

membrane-anchored form (proHB-EGF) and then proteolytically processed to become a bioactive soluble form by ectodomain shedding.[11] The ectodomain shedding of proHB-EGF is an important post-translational modification that converts a tethered insoluble juxtacrine growth factor into a soluble ligand leading to the autocrine or paracrine activation of EGFR. In this EGFR transactivation signaling, the ectodomain shedding of proHB-EGF is required to generate specific metalloproteinases, such as ADAM12.[12,13] ADAM12 belongs to the ADAM family; more than 30 ADAMs have been identified in flies, worms, and humans. ADAM has emerged as a major proteinase family involved in various biological functions. For example, ADAM10 regulates Notch signaling in neurogenesis,[14] and ADAM33 is associated with airway remodeling and hyperresponsiveness in bronchial asthma.[15,16]

Members of the mitogen-activated protein kinase (MAPK) family are involved in signal transduction of cytokine expression and apoptosis as well as cell proliferation growth and differentiation.[17,18] Three subgroups of mammalian MAPKs have been molecularly characterized: c-Jun N-terminal kinase (JNK), p38 MAPK, and extracellular signal-regulated kinase (ERK). They are preferentially activated by environmental stresses such as hyperosmotic shock, cold shock, ultraviolet irradiation, chemical mediators, inflammatory cytokines and virus infections,[17,18] and play an important role in apoptosis and cytokine expression.[17-19] In the MAPK signaling cascades, MAPK kinase kinase (MAPKKK) activates MAPK kinase (MAPKK), which subsequently activates MAPK. Each MAPK is activated by distinct upstream kinases.[17]

In bronchial asthma, HB-EGF is a potent mitogen and chemotactic factor with multiple potential roles in airway remodeling in ASM cells.[20] However, the regulatory mechanisms of HB-EGF and ADAM12 in ASM cells are largely unknown. We therefore analyzed a functioning signaling molecule in LTD$_4$-induced HB-EGF and ADAM12 expression in human ASM cells by focusing on the role of MAPK cascades. To this end, we first examined p38 MAPK, JNK, and ERK phosphorylation in LTD$_4$-stimulated ASM cells and the effect of MK571 as an LT receptor antagonist of LTD$_4$. Next, we examined HB-EGF and ADAM12 mRNA expression in LTD$_4$-stimulated ASM cells and the effect of MK571, SB203580 as a specific

inhibitor of the p38 MAPK-mediated signaling pathway, SP600125 as a specific inhibitor of JNK-mediated signaling pathway, and PD98059 as a specific inhibitor of MAPK-1 (MEK-1), which is upstream of ERK, in LTD$_4$-stimulated ASM cells.

Methods

Reagents and Cell cultures

LTD$_4$ [5S-hydroxy-6R-(S-cysteinylglycinyl)-7E,9E,11Z,14Z-eicosatetraenoic acid] was obtained from the Cayman Chemical Company (Ann Arbor, MI). LTD$_4$ was dissolved in ethanol. Human ASM cells derived form normal healthy subjects were used as ASM cells in this study and were obtained from Clonetics (San Diego, CA). The cells were placed on a tissue culture plate (Falcon 1007, Oxnard, CA) and cultured using ASM cell growth medium (SmBM; Clonetics) containing 5% fetal bovine serum (FBS), gentamycin-amphotericin B, EGF, fibroblast growth factor (FGF) and dexamethasone (DEX) at 37 °C in a humidified 5% CO$_2$ atmosphere. When the cells had grown in subconfluent conditions, the culture medium was replaced with growth factor and serum free medium and the cells were cultured for 16 h. SB203580 of p38 MAPK inhibitor, SP600125 of JNK inhibitor, and PD98059 of MEK-1 inhibitor were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). The cells that had been incubated with various concentrations of SB203580 (0.1, 1, 10μM), SP600125 (0.2, 2, 20μM), or PD98059 (0.5, 5, 50μM) for 30 minutes were stimulated with LTD$_4$.

Western blot analysis of threonine and tyrosine phosphorylation of MAPK

Analysis of threonine and tyrosine phosphorylation of p38 MAPK was performed using an anti-phosphorylated threonine and tyrosine of p38 MAPK antibody (Ab) (anti-phospho-specific p38 MAPK Ab, New England Biolabs ,Inc., Beverly, MA, USA) that is specific for active p38 MAPK and does not cross react with JNK and ERK. Analysis of threonine and tyrosine phosphorylation of JNK was performed using an anti-phosphorylated threonine and tyrosine of JNK Ab (anti-phospho-specific JNK kinase Ab, New England Biolabs, Inc., Beverly, MA, USA) that is specific for active JNK and does not cross react with p38 MAPK and ERK. Analysis of threonine and tyrosine phosphorylation of ERK was performed using an anti-phosphorylated threonine and tyrosine of ERK Ab (anti-phospho-specific ERK Ab, New England Biolabs, Inc., Beverly, MA, USA) that is specific for active ERK

and does not cross react with p38 MAPK and JNK. Analysis of threonine and tyrosine phosphorylation of p38 MAPK, JNK, or ERK was performed according to the manufacturer's instructions, as described previously.[18] Briefly, after separating proteins from cell lysate by 15% SDS-page, 10μg of cell lysate of protein was electrophoretically transferred to a nitrocellulose membrane, and the membrane was incubated with specific Ab to phosphorylated threonine and tyrosine of p38 MAPK (affinity-purified rabbit polyclonal IgG), specific Ab to phosphorylated threonine and tyrosine of JNK (affinity-purified rabbit polyclonal IgG), or specific Ab to phosphorylated threonine and tyrosine of ERK (affinity-purified rabbit polyclonal IgG). It was incubated with the horseradish peroxidase-conjugated anti-rabbit Ab horseradish peroxidase-conjugated antibiotin Ab to detect biotinylated protein markers. Blots were incubated in ECL (enhanced chemiluminescence) solution for 1 min and exposed on KODAK XAR films. In order to show the amounts of p38 MAPK, JNK, and ERK precipitated, blots were stripped and reprobed with phosphorylation-state independent p38 MAPK-specific Ab (affinity-purified rabbit polyclonal IgG) to determine total p38 MAPK levels, phosphorylation-state independent JNK-specific Ab (affinity-purified rabbit polyclonal IgG) to determine total JNK levels, or phosphorylation-state independent ERK-specific Ab (affinity-purified rabbit polyclonal IgG) to determine total ERK levels, respectively.

ADAM12 and HB-EGF mRNA expression

For quantitative PCR analysis of mRNA, total mRNA was isolated from ASM cells using an RNAgent total RNA isolation system (Promega, Madison WI) and reverse transcribed using Primer Express 2.0 software (Applied Biosystems, Foster City CA). The following oligonucleotides were used:

Human ADAM12 forward primer; 5'-CTGGGC ACCTCCCTTCTGT-3'

Human ADAM12 reverse primer; 5'-TGCTTC TGCTTGCCGGA-3'

Human HB-EGF sense; 5'-TCGCTTATATACC TATGACCACACAAC-3'

Human HB-EGF anti-sense; 5'-CATAACCTCCT CTCCTATGGTACCTAAA-3'

Human GAPDH sense; 5'-GTCGGAGTCAA CGGATTTGG-3'; and

Human GAPDH anti-sense; 5'-GGCAACAATAT CCACTTTACCAGAG-3'.

Quantitative RT-PCR was carried out by an ABI PRISM 7300 sequence detection system using SYBR Green PCR Master Mix (Applied Biosystems).

Statistical analysis

Statistical significance was analyzed by using analysis of variance (ANOVA). P value less than 0.05 was considered significant. When statistical significance was reached, post hoc tests (Fischer's protected least significant difference, Scheff's F) were performed.

Results

LTD$_4$ induces MAP kinase phosphorylation in ASM cells

First, we examined the threonine and tyrosine phosphorylation of p38 MAPK, JNK, and ERK in LTD$_4$-stimulated ASM cells which were immunoblotted at the desired times as indicated. The amounts of phosphorylated threonine and tyrosine of p38 MAPK, JNK, and ERK increased at 5 min, were maximal at 10 min, and thereafter returned to the basal level (Figure 1a, upper panel). Pretreatment with MK571 resulted in the inhibition of LTD$_4$-induced p38 MAPK, JNK, and ERK phosphorylation in a dose-dependent manner (Figure 1b, upper panel). These results confirmed that LTD$_4$-induced p38 MAPK, JNK, and ERK phosphorylation is a leukotriene receptor-mediated event. The lower panels of Figure 1a and 1b show that equal amounts of p38 MAPK, JNK, and ERK protein were immunoblotted with phosphorylation-independent p38 MAPK, JNK, and ERK-specific Ab regardless of the length of the culture period. These results indicate that LTD$_4$-stimulated increases in the threonine and tyrosine phosphorylation of p38 MAPK, JNK, and ERK occurred in the absence of changes in p38 MAPK, JNK, and ERK protein levels.

LTD$_4$ induces HB-EGF and ADAM12 mRNA expression in ASM cells

In the next experiment, we examined the kinetics of the effects of LTD$_4$ on HB-EGF and ADAM12 mRNA expression in ASM cells. HB-EGF and ADAM12 mRNA expression in 100nM of LTD$_4$-stimulated ASM cells increased in a time-dependent manner (Figure 2a). Both HB-EGF and ADAM12 mRNA increased after 6 h, were maximal at 8 h and thereafter returned to the basal level. ASM cells were stimulated with various concentrations of LTD$_4$, and then HB-EGF and ADAM12 mRNA expression was determined at 8 h after stimulation
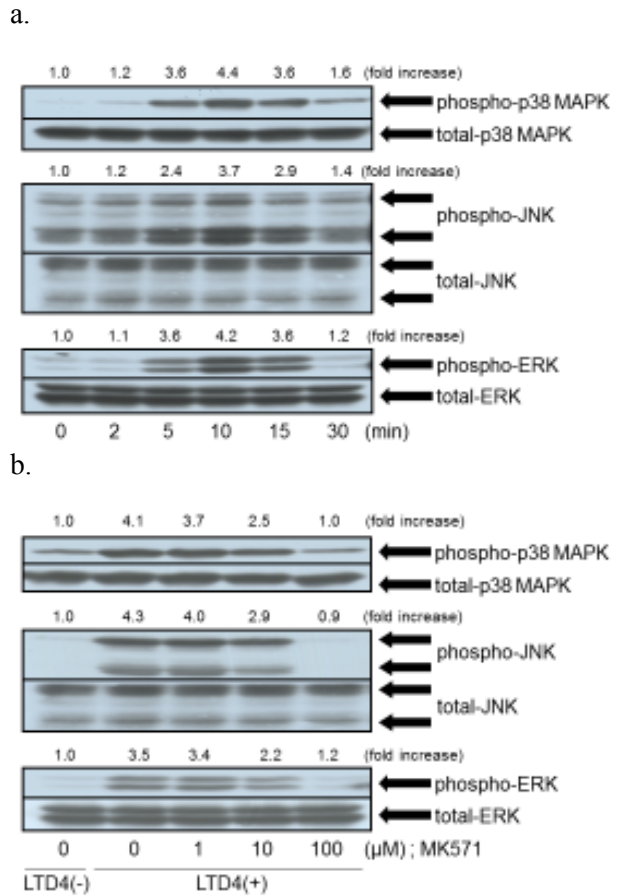
a.

b.

a.

b.

Figure 1. LTD$_4$ induces phosphorylation of MAPK in airway smooth muscle cells. (a)ASM cells were stimulated with 100nM of LTD$_4$ for the desired times as indicated. (b)ASM cells that had been pretreated with various concentrations MK571 for 30 min were stimulated with 100nM of LTD$_4$ at 10 min after stimulation. The cell lysate containing 10μg of protein separated by 15 % SDS-PAGE was electophoretically transferred to a nitrocellulose membrane and the membrane was blotted with an anti-phosphorylated threonine and tyrosine of MAPK Ab [p-MAPK; upper panel]. Then it was incubated with the HRP-conjugated anti-rabbit IgG Ab and HRP-conjugated anti-biotin Ab to detect biotinylated protein markers. Blots were incubated with ECL solution for 1 min and exposed on KODAK XAR film. Blots were stripped and reprobed using phosphorylation-state independent MAPK Ab [MAPK; lower panel]. The amounts of phosphorylated MAPK were quantified by an NIH image analyzer and are presented as the amounts of phosphorylated MAPK proteins relative to control cells treated without LTD$_4$. The increases in phosphorylated MAPK proteins are indicated below. Three identical experiments independently performed gave similar results.
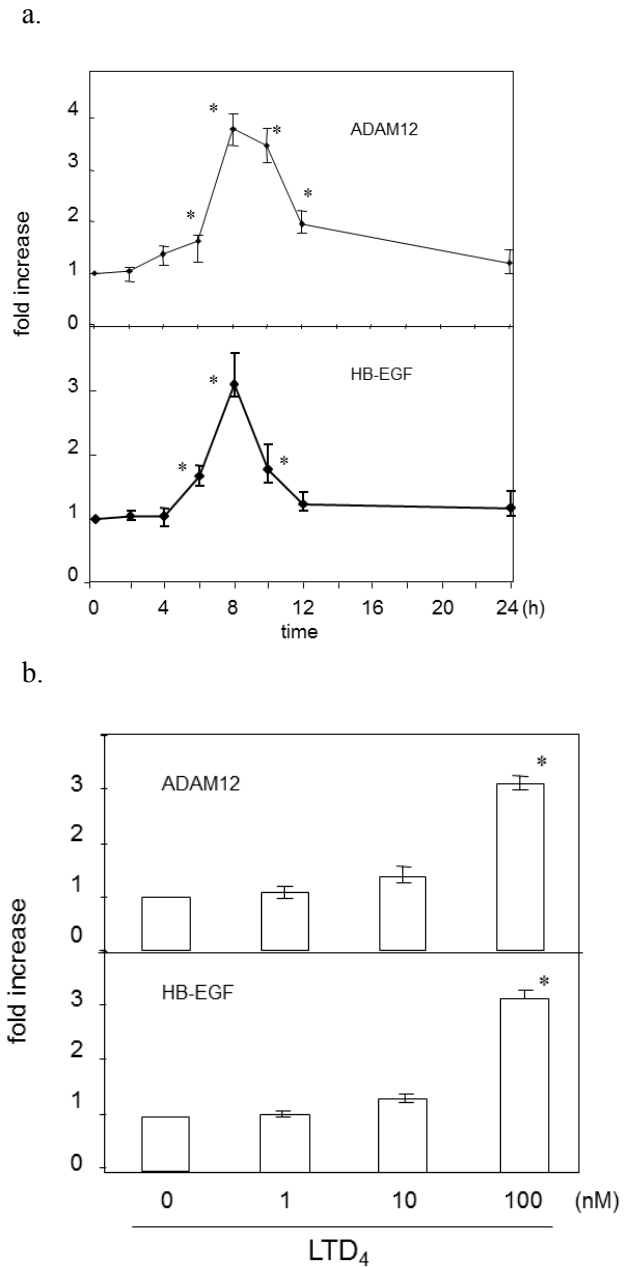
Figure 2. LTD$_4$ induces HB-EGF and ADAM12 mRNA expression. (a)ASM cells were stimulated with 100nM of LTD$_4$ and mRNA expression of HB-EGF / ADAM12 were determined at the desired times as indicated. (b)ASM cells were stimulated with various concentrations with LTD$_4$ and mRNA expression of HB-EGF / ADAM12 were determined at 8 h after stimulation. The results are expressed as means ± S.D. of six different experiments. *indicates $P < 0.01$ compared with the LTD$_4$-unstimulated cells.

with LTD$_4$ (Figure 2b). Increases in HB-EGF and ADAM12 mRNA expression in ASM cells were observed at 100nM of LTD$_4$, but not at 1nM and 10nM. Next, we found that pretreatment with MK571 of LT receptor antagonist resulted in the inhibition of LTD$_4$-induced HB-EGF and ADAM12 mRNA expression (Figure 3a). These results verified that LTD$_4$-induced HB-EGF and ADAM12 mRNA expression is a LT-receptor-mediated event. The optimal concentration for the pharmacological effect of MK571 has been reported to be 100μM.[5] The study showed that the dose-dependent inhibition of HB-EGF and ADAM12 mRNA expression, and phosphorylation of p38 MAPK, JNK, and ERK were seen at 1 and 10μM, and at 100μM of MK571, respectively. These results are consistent with the previous study.[5]

MAPK inhibitors inhibit LTD$_4$-induced MAPK phosphorylation in ASM cells

Next, to examine the role of p38 MAPK, JNK, and ERK in LTD$_4$-medicated MAPK phosphorylation, ASM cells pretreated with various concentrations of SB203580, SP600125, and PD98059 were stimulated with LTD$_4$. In 100nM of LTD$_4$-stimulated ASM cells for 10 min, MAPK inhibitors resulted in the inhibition of LTD$_4$-induced p38 MAPK, JNK, and ERK phosphorylations in dose-dependent manner (Figure 3b). We found that the threonine and tyrosine phosphorylations of p38 MAPK, JNK, and ERK in LTD$_4$-stimulated ASM cells were immunoblotted at the desired concentrations as indicated. The amounts of phosphorylated threonine and tyrosine of p38 MAPK, JNK, and ERK decreased from concentrations of 0.1μM, 0.2μ and 0.5μM, and phosphorylation of p38 MAPK, JNK, and ERK was almost entirely inhibited for 10μM, 20μ and 50μM. (Figure 3b, upper panel). These results verified that concentrations of MAPK inhibitors were observed that LTD$_4$-induced p38 MAPK, JNK, and ERK phosphorylation inhibited. The lower panels of Figure 3b show that equal amounts of p38 MAPK, JNK, and ERK protein were immunoblotted with phosphorylation-independent p38 MAPK, JNK, and ERK-specific Ab regardless of the length of the culture period, indicating that LTD$_4$-stimulated increases in the threonine and tyrosine phosphorylation of p38 MAPK, JNK, and ERK occurred in the absence of changes in p38 MAPK, JNK, and ERK protein levels.

MEK-1 and p38 MAPK inhibitors inhibit LTD$_4$-induced HB-EGF and ADAM12 mRNA expression in ASM cells

Next, to examine the role of p38 MAPK, JNK, and ERK in LTD$_4$-medicated HB-EGF and ADAM12 mRNA expression, ASM cells pretreated with SB203580, SP600125, and PD98059 were stimulated with LTD$_4$. Using DMSO as a negative control, ASM cells pretreated with various concentrations of DMSO, SB203580, SP600125, and PD98059 for 30 min were stimulated with 100nM of LTD$_4$, and then mRNA expression of HB-EGF and ADAM12 was determined 8 h after stimulation (Figure 3c). Decreases in HB-EGF and ADAM12 mRNA expression in ASM cells were observed at 10μM of SB203580 and at 5 and 50μM of PD98059. Decreases in HB-EGF and ADAM12 mRNA expression in ASM cells were not observed at any concentrations of SP600125.

Discussion

In the present study, we examined the effect of LTD$_4$ in enhancing MAPK phosphorylation and inducing HB-EGF and ADAM12 expression in human ASM cells. The results showed that 1) LTD$_4$ induced increases in MAPK phosphorylation and MAPK phosphorylation were inhibited by a specific inhibitor of MAPK, and 2) LTD$_4$ induced HB-EGF and ADAM12 expression. To further characterize the role of MAPK, a specific inhibitor of MAPK was utilized. HB-EGF and ADAM12 expressions were depressed in specific inhibitors of p38 MAPK or MEK-1-treated ASM cells, but these expressions were not depressed in specific inhibitors of JNK-treated ASM cells. In the MAPK cascade, MEK-1 is upstream of ERK. These results indicate that LTD$_4$ is capable of inducing HB-EGF and ADAM12 expression and enhancing MAPK phosphorylation. Thus, at least in part, p38 MAPK and ERK regulate HB-EGF and ADAM12 expressions in LTD$_4$-stimulated ASM cells.

The role of CysLTs on airway inflammation and remodeling by promoting ASM cell proliferation and inducing cytokine expression and extracellular matrix synthesis has been previously documented.[1-7] The role of HB-EGF on airway inflammation and remodeling by promoting ASM cell growth and inducing cytokine expression has also been documented.[10,21] In human bronchial epithelial cells, LTD$_4$ induces increases in the release of HB-EGF.[22] As previously defined, since the HB-EGF promoter involves activator protein-1

a.

ADAM12

HB-EGF

fold increase

3
2
1
0

3
2
1
0

*

*

0   0   1   10   100   (MK571;μM)

LTD$_4$(-)   LTD$_4$(+)

b.

1   0.8   0.3   0.1   (fold increase)

phospho-p38 MAPK

total-p38 MAPK

0   0.1   1   10   (μM) ; SB203580

1   0.7   0.2   0.0   (fold increase)

phospho-JNK

total-JNK

0   0.2   2   20   (μM) ; SP600125

1   0.6   0.2   0.1   (fold increase)

phospho-ERK

total-ERK

0   0.5   5   50   (μM) ; PD98059

c.

ADAM12

HB-EGF

fold increase

3
2
1
0

3
2
1
0

*   *   *

*   *   *

0.01 0.1   1   0.1   1   10   0.2   2   20   0.5   5   50

DMSO (%)   SB203580(μM)   SP600125(μM)   PD98059(μM)
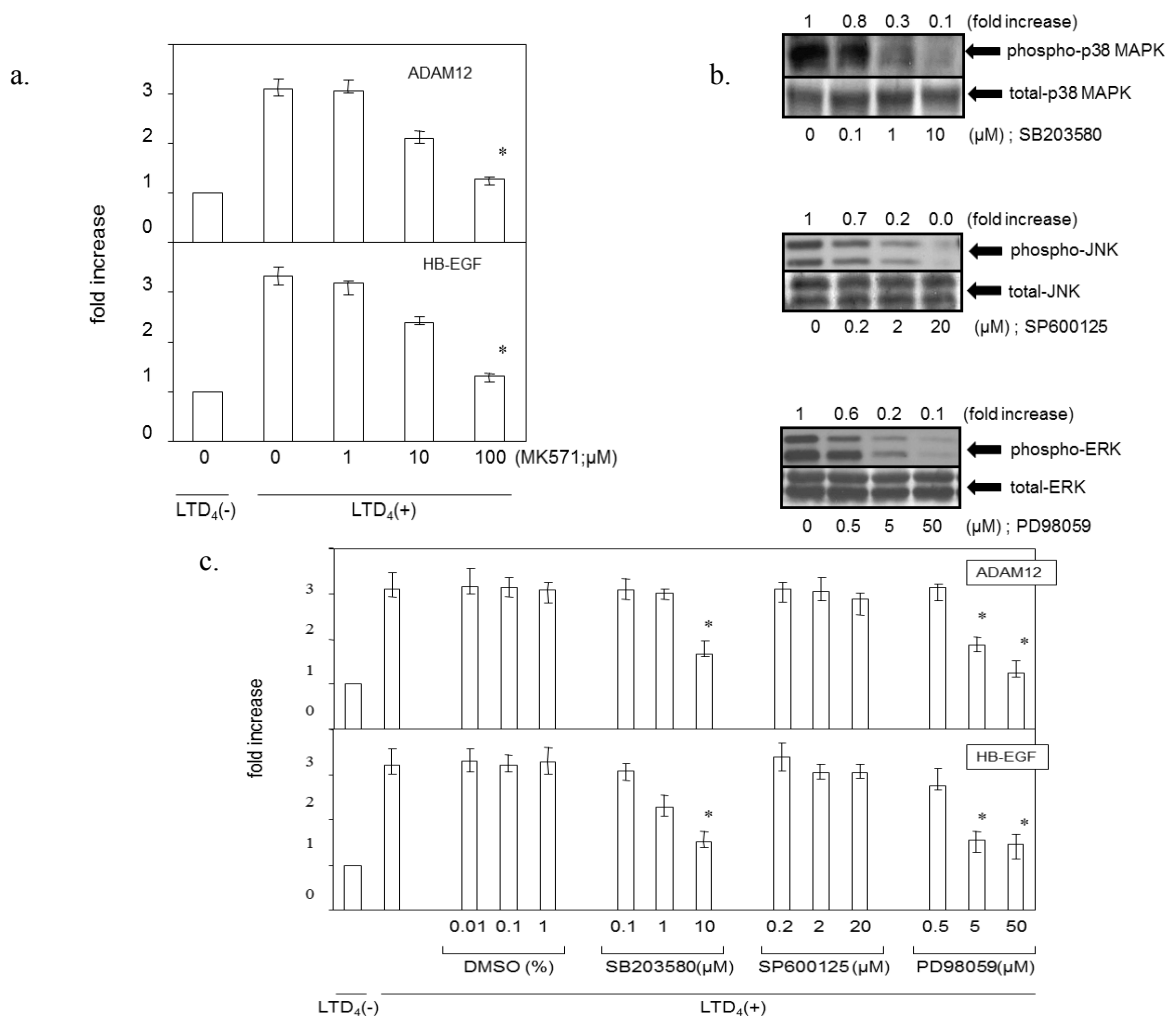
LTD$_4$(-)   LTD$_4$(+)

Figure 3. p38 MAPK and ERK are capable of regulating LTD$_4$–induced HB-EGF and ADAM12 expression in ASM cells. (a) ASM cells that had been pretreated with various concentrations with MK571 for 30 min were stimulated with LTD$_4$ and mRNA expression of HB-EGF / ADAM12 were determined at 8h after stimulation. The results are expressed as means ± S.D. of six different experiments. *indicates P <0.05 compared with the LTD$_4$-stimulated ASM cells without MK571. Leukotriene receptor antagonist inhibits LTD$_4$-induced HB-EGF and ADAM12 mRNA expression. (b) ASM cells pretreated with various concentrations of SB203580, SP600125, and PD98059 were stimulated with 100nM of LTD$_4$ for 10 min, MAPK inhibitors resulted in the inhibition of LTD$_4$-induced p38 MAPK, JNK, and ERK phosphorylations in a dose-dependent manner. We examined the threonine and tyrosine phosphorylation of p38 MAPK, JNK, and ERK in LTD$_4$-stimulated ASM cells which were immunoblotted at the desired concentrations as indicated. The amounts of phosphorylated threonine and tyrosine of p38 MAPK, JNK, and ERK decreased from concentrations of 0.1μM, 0.2μ and 0.5μM, and phosphorylation of p38 MAPK, JNK, and ERK were almost complete inhibited for 10μM, 20μ and 50μM (upper panel). These results verified that concentrations of MAPK inhibitors were observed that LTD$_4$-induced p38 MAPK, JNK, and ERK phosphorylation inhibited. Data in thelower panels show that equal amounts of p38 MAPK, JNK, and ERK protein were immunoblotted with phosphorylation-independent p38 MAPK, JNK, and ERK-specific Ab, regardless of length of the culture period, indicating that LTD$_4$-stimulated increases in the threonine and tyrosine phosphorylation of p38 MAPK, JNK, and ERK occurred in the absence of changes in p38 MAPK, JNK, and ERK protein levels. The increase in phosphorylated proteins is indicated below. Three identical experiments independently performed gave similar results. MAPK inhibitors inhibit LTD$_4$-induced MAPK phosphorylation in ASM cells. (c) ASM cells that had been pretreated with various concentrations with SB203580 / SP600125 / PD98059 (p38 inhibitor / JNK inhibitor / ERK inhibitor) for 30 min were stimulated with LTD$_4$ and the mRNA expression of HB-EGF / ADAM12 were determined at 8h after stimulation. The results are expressed as means ± S.D. of six different experiments. *indicates P<0.05 compared with the LTD$_4$-stimulated ASM cells without MAP kinase inhibitors. ERK and p38 MAP kinase inhibitors inhibit LTD$_4$-induced mRNA expression of HB-EGF and ADAM12.

involved in the transcriptional control of various inflammatory mediators including inflammatory cytokines and chemokines.[25] AP-1 is activated in hypertrophied heart and in vascular smooth muscle cells in hypertensive and balloon-injured arteries.[26] In contrast, it has been recently reported that HB-EGF down-regulates the expression of AP-1.[27] We previously reported that AP-1 is activated in LTD_4-stimulated human ASM cells,[28] but the effect of LTD_4 on HB-EGF and ADAM12 expression in human ASM cells has not been determined. The present study showed that LTD_4 induced HB-EGF and ADAM12 expression in human ASM cells. Increases in ASM cell mass are one of characteristic features of the airway remodeling occurring in asthma,[20] and LTD_4 can promote ASM cell proliferation.[3] HB-EGF is essential for cell growth and differentiation,[29] but HB-EGF activation is also associated with cardiac hypertrophy and remodeling in vascular smooth muscle cells.[30,31] In mice with cardiac hypertrophy, a specific inhibitor of ADAM12 inhibited the shedding of HB-EGF and the induction of cardiac hypertrophy.[30] Thus, it is essential to clarify the key mechanism in LTD_4-induced HB-EGF expression in ASM cells. The functioning and regulation of MAPK have been extensively documented. JNK, p38 MAPK and ERK are involved in MAPKs, which are activated by various environmental stresses and are involved in signal transduction of cytokine expression and apoptosis, as well as cell proliferation growth and differentiation.[17,18,32,33] These results indicate that p38 MAPK and ERK regulate HB-EGF and ADAM12 expression in LTD_4-stimulated human ASM cells. Furthermore, we consider that the transcription factors of HB-EGF and ADAM12 may not only be p38 MAPK and ERK but also proHB-EGF, the carboxyl-terminal fragment (CTF) HB-EGF and AP-1. The blockade of the LT pathway by inhibiting synthesis of LT and/or the binding of LTs to their receptors resulting in the inhibition of HB-EGF and ADAM12 expression is potentially important in attenuating the progression of airway remodeling.

The JNK and p38 MAPK signaling pathways are activated by pro-inflammatory cytokines such as TNF-α and IL-1β or in response to cellular stresses.[17,18] The JNK pathway consists of JNK, an MAPKK, such as stress-activated protein kinases (SAPK)/ERK kinase 1 (SEK1) or MAPK kinase (MKK) 7, and a MAPKKK, such as apoptosis signal-regulating kinase 1 (ASK1), MAPK/ERK

kinase kinase 1 (MEKK1), mixed-lineage kinase (MLK), or transforming growth factor-β-activated kinase 1 (TAK1). In the p38 MAPK signaling pathway, distinct MAPKKs such as MKK3 and MKK6 activate p38 MAPK and are themselves activated by the same MAPKKKs (such as ASK1 and TAK1) that function in the JNK pathway.[17-19] In the ERK signaling pathway, ERK1 or ERK2 (ERK1/2) is activated by MEK1/2, which in turn is activated by a Raf isoform such as A-Raf, B-Raf, or Raf-1. The kinase Raf-1 is activated by the small GTPase Ras. Members of the Ras family of proteins play important roles in transmission of extracellular signals to cells. Among the MAPKKKs, MEKK1 possesses not only a kinase activity for phosphorylation of a MAPKK in the JNK or ERK signaling pathways but also an E3 ubiquitin ligase activity. MEKK1 regulates cell adhesion and migration through binding to several proteins such as RhoA, Rac, and actin. ASK1 in both the JNK and p38 signaling pathways, is activated in response to a variety of stressors including reactive oxygen species (ROS), lipopolysaccharide (LPS), endoplasmic reticulum (ER) stress, and Ca^{2+} influx.[17,18] Therefore, these MAPKs can play both roles in inducing cell proliferation and apoptosis. According to the kind of the cells, LTD_4 may stimulate cell proliferation or apoptosis. The $CysLT_1$ receptor mediates human ASM cells proliferation.[34] The $CysLT_1$ receptor mediates human bronchial epithelial cells proliferation, too.[22] However, the $CysLT_1$ receptors do not mediate human umibilical vein endothelial cell proliferation.[35] We consider that the LTD_4-induced HB-EGF activation is determined by the concentration of LTD_4 or the LTD_4-stimulated time, according to the kind of the cell. In airway remodeling, our experiments suggested that LTD_4-induced p38 MAPK and ERK phosphorylation might play role on mediating ASM cells proliferation with the activation of HB-EGF and ADAM12. On the other hand, LTD_4-induced JNK phosphorylation might play role in mediating ASM cells apoptosis, therefore JNK cannot be associated with the activation of them. We suggest that therapeutic intervention inhibiting ERK and p38 MAPK activation may be beneficial in reducing HB-EGF and ADAM12 activity in ASM cells for prevention of airway remodeling.

From the data presented here, we conclude that p38 MAPK and ERK are capable of regulating LTD_4-induced HB-EGF and ADAM12 expression in human ASM cells. Our results for the role of p38

MAPK, and ERK-mediated LTD$_4$-induced HB-EGF and ADAM12 expression in human ASM cells are important for understanding the molecular mechanism of HB-EGF and ADAM12 expression that is seen in the airways of bronchial asthmatics, and a strategy of attenuating airway remodeling as well as inflammation by the specific inhibition of p38 MAPK and ERK may produce beneficial effects in controlling airway remodeling and inflammation.

Acknowledgements

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