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Apoptosis signal-regulating kinase 1 in leukotriene D_4-induced activator protein-1 activation in airway smooth muscle cells

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Abstract

Cysteinyl leukotrienes (LTs) are involved in allergic disorders including bronchial asthma. Transcription factor activator protein-1 (AP-1) activation is essential for cell proliferation and differentiation. LTD_4 is shown to promote human airway smooth muscle cell proliferation; however, the effect of LTD_4 on AP-1 activation in airway smooth muscle cells and the molecular mechanism in regulating AP-1 activation have not been determined. We examined the effect LTD_4 on AP-1 activation in human airway smooth muscle cells and analyzed a role of apoptosis signal-regulating kinase1 (ASK1), an upstream kinase kinase of c-*Jun*-NH_2-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) in LTD_4-induced AP-1 activation to clarify the signaling molecule regulating AP-1 activation. The results showed that LTD_4 induced AP-1 activation determined by AP-1-dependent luciferase gene activity and ASK1 phosphorylation. Transient transfection of the dominant negative form of ASK1 attenuated LTD_4-induced AP-1 activation. In addition, LTD_4-induced AP-1 activity was depressed in the dominant negative form of ASK1-stably transfected porcine artery endothelial cells compared to that in the parental porcine artery endothelial cells. These results indicate that LTD_4 is capable of inducing AP-1 activation and ASK1 regulates AP-1 activation in LTD_4-stimulated airway smooth muscle cells.

Keywords: LTD_4; ASK1; AP-1; Airway smooth muscle cells; Remodeling

1. Introduction

Cysteinyl leukotrienes (LTs), LTC_4, LTD_4 and LTE_4, metabolites of arachidonate via the 5-lipoxygenase pathways, have various biological activities such as bronchial constriction, mucous hypersecretion, increase in microvascular permeability, bronchial hyperresponsiveness, and eosinophil chemotaxis (Peters-Golden, 2003; Panettieri et al., 1998). In addition, cysteinyl LTs are known to promote airway smooth muscle cell proliferation, induce the production of interleukin (IL)-4, granulocyte-macrophage colony stimulating factor (GM-CSF), IL-5, tumor necrosis factor-α (TNF-α) and regulated upon activation, normal T-cell expressed and secrete (RANTES), the synthesis of collagenase and proteoglycan and the induction of P-selectin expression (Parameswaran et al., 2002; Bandeira-Melo et al., 2002; Mellor et al., 2002; Kawano et al., 2003; Pedersen et al., 1997; Medina et al., 1994; Potter-Perigo et al., 2004). LT receptor antagonist attenuates airway remodeling in animal model of asthma (Henderson et al., 2002). Thus, cysteinyl LTs play an important role in the pathogenesis of airway inflammation and remodeling.

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The transcription factor activator protein-1 (AP-1) that is composed of members of the Jun and Fos families. AP-1 activation is essential for cell proliferation and differentiation. In addition, AP-1 is involved in the transcriptional control of various inflammatory mediators including inflammatory cytokines and chemokines (Karin et al., 1997). c-*fos* that is a component of AP-1 is up-regulated in the airway from bronchial asthma (Demoly et al., 1992). AP-1 is activated by various stimuli including growth factors, cytokines, Ultra violet irradiation and nitric oxide (Karin et al., 1997; Jibiki et al., 2003); however, the effect of cysteinyl LTs on AP-1 activation has not been determined.

Members of the mitogen-activated protein kinase (MAPK) family are involved in signal transduction of cytokine expression and apoptosis as well as cell growth and differentiation (Kyriakis and Avruch, 2001; Ichijo, 1999). Two different MAPK cascades that converge on c-*Jun* N-terminal kinase (JNK) and p38 MAPK are preferentially activated by environmental stresses such as hyperosmotic shock, cold shock, ultra violet irradiation, chemical mediators, inflammatory cytokines and virus infection (Kyriakis and Avruch, 2001; Ichijo, 1999; Hashimoto et al., 1999) and play an important role in apoptosis and cytokine expression (Kyriakis and Avruch, 2001; Ichijo, 1999; Hashimoto et al., 1999; Raingeaud et al., 1995). Extracellular signal-regulated kinase (ERK) is activated by mitogenic stimuli and plays a central role in cell proliferation and differentiation (Force and Bonventre, 1998). 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 (Kyriakis and Avruch, 2001). Apoptosis signal-regulating kinase1 (ASK1) was identified as a member of the MAPKKK family that activates two different MAPK cascades, SEK1/MKK7-JNK and MKK3/MKK6-p38 MAPK pathways (Ichijo et al., 1997). Overexpression of wild-type or the constitutively active form of ASK1 induces apoptosis in various cell types (Ichijo et al., 1997; Chen et al., 1999) and the kinase-inactive mutant of ASK1 inhibits apoptosis induced (Ichijo et al., 1997; Chen et al., 1999; Kanamoto et al., 2000). By contrast to the role of ASK1 in apoptosis, recent evidence has showed that ASK1 also functions the cell signaling molecule for regulating cell survival and differentiation (Sayama et al., 2001). ASK1 has thus a broad range of biological activities.

MAPK cascades are connected with the activation of various transcription factors (Karin et al., 1997). AP-1 is activated by MAPK, JNK and p38 MAPK (Karin et al., 1997); however, little is known about a role of their upstream kinase, ASK1 in LTD_4-induced AP-1 activation in airway smooth muscle cells. In the present study, we firstly examined whether LTD_4 could induce AP-1 activation and analyzed the molecular mechanism in LTD_4-induced AP-1 activation by focusing on the role of ASK1.

2. Materials and methods

2.1. Reagents and cell cultures

LTD_4 [5S-hydroxy-6R-(S-cysteinylglycinyl)-7E, 9E, 11Z, 14Z-eicosatetraenoic acid] (LTD_4) and the selective cysteinyl LT1 receptor antagonist MK571 (Lynch et al., 1999) were obtained from Cayman Chemical Company (Ann Arbor, MI). LTD_4 and were dissolved in ethanol. Human airway smooth muscle cells derived from normal healthy subjects used as airway smooth muscle cells in this study were obtained from Clonetics (San Diego, CA). The cells were placed onto tissue culture plate (Falcon 1007, Oxnard, CA) and cultured using cell growth medium (SmBM; Clonetics) containing 5% fetal bovine serum (FBS), gentamycin-amphotericin B, epidermal growth factor (EGF), fibroblast growth factor (FGF) and dexamethasone at 37 °C in humidified 5% CO_2 atmosphere. Porcine aortic endothelial cells (American Type Culture Collection, Rockville, MD) and ASK1-stably transfected porcine aortic endothelial cells (Jibiki et al., 2003) were grown in Ham's F12 medium (Nissui Co. Ltd.) supplemented with 10% heat-inactivated FCS, streptomycin and penicillin. When the cells were grown in subconfluent conditions, the culture medium was replaced with growth factor and serum-free medium and the cells were cultured for 16 h.

2.2. Transfection and luciferase reporter assay

Serum-starved airway smooth muscle cells were transiently transfected with the pcDNA3-ASK1-dominant negative expression vectors using FuGENE6 (Roche Diagnostics Corp., Indianapolis, IN). The total amount of cDNA was kept constant by supplementation with empty vector, pcDNA3 (Invitrogen, Carlsbad, CA). Every transfection included 500 ng of pAP-1-Luc reporter plasmid, together with either 5 ng of pRL-SV40-Renilla for normalization of transfection efficiency. After incubation for 24 h, cells were stimulated with LTD_4. Then the cells were lysed in a luciferase lysis buffer (Promega, Madison, WS), and luciferase activity was determined using an assay kit (Promega) with TD-20/20 Luminometer (Promega). For controlling the efficiency of the transfection, the Renilla luciferase gene expression was monitored using pRL-SV40 and a dual luciferase system (Promega). Assays were performed in triplicate. The relative fold activation of luciferase was calculated.

2.3. Western blot analysis of ASK1

ASK1 phoshorylation was analyzed using rabbit polyclonal antibody (Ab) to phosphorylated ASK1 (Phospho-ASK1) directed against a phosphorylated peptide fragment of mouse ASK1. Characterization of rabbit polyclonal Ab to ASK1 has been described previously (Tobiume et al., 2001). ASK1 phosphorylation was analyzed using an anti-phosphorylated ASK1 Ab described previously (Maruoka et al., 2003).

2.4. 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.

3. Results

3.1. LTD_4 induces AP-1 promoter activity in airway smooth muscle cells

Firstly, we examined the kinetics of the effects of LTD_4 on AP-1 activation in airway smooth muscle cells. To this end, the cells that had been transiently transfected by AP-1-luciferase reporter plasmid were stimulated with various concentrations of LTD_4 and then AP-1-dependent-Luciferase gene activity was determined at 4 h after stimulation with LTD_4 (Fig. 1A). Increases in AP-1 reporter activity in airway smooth muscle cells were observed at 10 and 100 nM of LTD_4, but not at 1 nM. Next, we examined the kinetics of the effects of LTD_4 on AP-1 reporter activity. To this end, the cells that had been transiently transfected by AP-1-Luciferase reporter plasmid were stimulated with 100 nM of LTD_4 and then AP-1-dependent-Luciferase gene activity was determined at 4 h stimulation with LTD_4. AP-1 reporter activity in LTD_4-stimulated cells increased in a time-dependent manner (Fig. 1B). Pretreatment with MK571 of LT receptor antagonist resulted in the inhibition of LTD_4-induced AP-1 activation (Fig. 2). These results verified that LTD_4-induced AP-1 activation is LT receptor-mediated event. An optimal concentration for pharmacological effect of MK571 is reported to be 10 μM (Mellor et al., 2002). This study showed that the maximal inhibition of AP-1 activity and ASK1 phosphorylation were seen at 1 and 10 μM, and at 10 μM of MK571, respectively. These results are consistent with the previous study (Mellor et al., 2002).

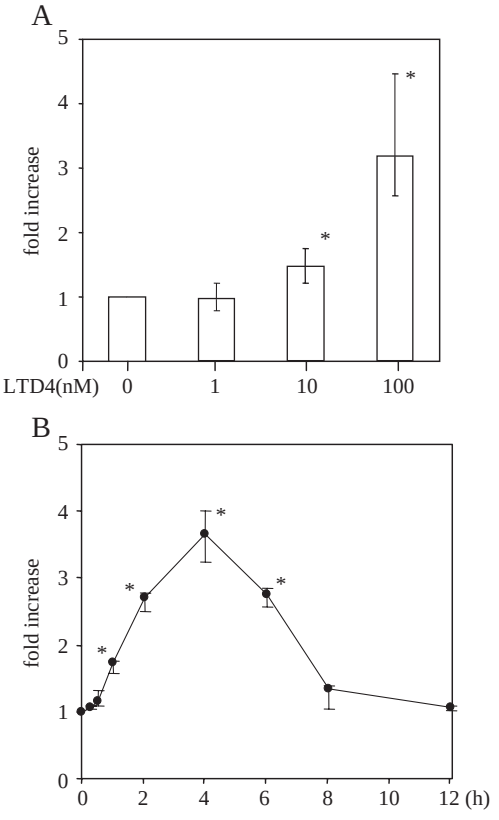

Fig. 1. LTD_4 induces AP-1 activity in human airway smooth muscle cells. (A) Airway smooth muscle cells were stimulated with various concentrations with LTD_4 and AP-1 reporter activity was determined at 4 h after stimulation. (B) Airway smooth muscle cells were stimulated with 100 nM LTD_4 and AP-1 reporter activity was determined at the desired times as indicated. The results are expressed as means±S.D. of six different experiments. *Indicates $p<0.01$ compared with the LTD_4-unstimulated cells.

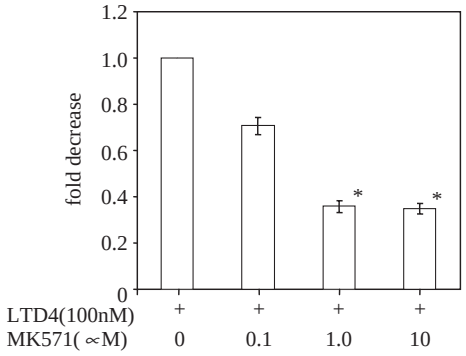

Fig. 2. Luekotriene receptor antagonist inhibits LTD_4-induced AP-1 activation. Airway smooth muscle cells that had been pretreated with MK571 for 30 min were stimulated with LTD_4 for 4 h. AP-1 activation was determined at 4 h after stimulation as described in Materials and Methods. The results are expressed as means±S.D. of six different experiments. *Indicates $p<0.05$ compared with the LTD_4-stimulated Airway smooth muscle cells without MK571.

3.2. LTD_4 induces ASK1 phosphorylation in airway smooth muscle cells

SEK1/MKK7-JNK and MKK3/MKK6-p38MAPK pathways regulate AP-1 activation (Karin et al., 1997). ASK1 regulates the activation of SEK1/MKK7-JNK and MKK3/MKK6-p38MAPK pathways (Ichijo, 1999; Ichijo et al., 1997). We examined the role of ASK1 in LTD_4-induced AP-1 activation in airway smooth muscle cells. First, we examined the threonine and tyrosine phosphorylation of ASK1 in LTD_4-stimulated airway smooth muscle cells. ASK1 phosphorylation in LTD_4-stimulated airway smooth muscle cells were immunoblotted at the desired times as indicated. Amounts of phosphorylated threonine and tyrosine of ASK1 were increased 2 min, were maximal at 5 min and thereafter returned to the basal level (Fig. 3A, upper panel). Pretreatment with MK571 resulted in the inhibition of LTD_4-induced ASK1 phosphorylation in a dose-dependent manner (Fig. 3B). These results verified that LTD_4-induced ASK1 phosphorylation is LT receptor-mediated event. Lower panels of Fig 3A and B showed that equal amounts of ASK1 protein were immunoblotted with phosphorylation-independent ASK1-specific Ab regardless of time culture periods, indicating that LTD_4-stimulated increases in the threonine and tyrosine phosphorylation of ASK1 occurred in the absence of changes in ASK1 protein levels. An optimal concentration for pharmacological effect of MK571 is 10 μM (Mellor et al., 2002). This study showed that the maximal inhibition of AP-1 activity and ASK1 phosphorylation were seen at 1 and 10 μM (Fig. 2B), and at 10 μM of MK571 (Fig. 3), respectively. These results are consistent with the previous study (Mellor et al., 2002).

3.3. ASK1 regulates LTD_4-induced AP-1 activation

In the next, to examine a role of ASK1 in LTD_4-medicated AP-1 activation, the airway smooth muscle cells were transiently transfected by the dominant negative form of ASK1. Transient transfection of the dominant negative form of ASK1 attenuated

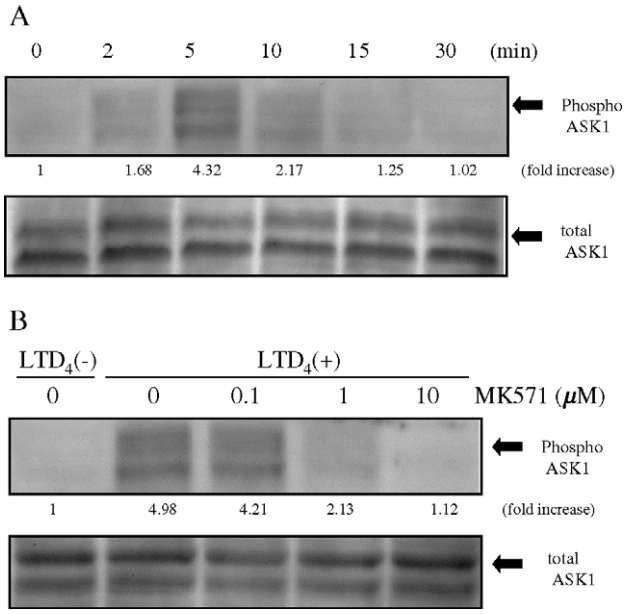

Fig. 3. LTD_4 induces the threonine and tyrosine phosphorylation of ASK1 in human airway smooth muscle cells. (A) Airway smooth ells were stimulated with 100 nM of LTD_4 for the desired times as indicated. (B) Airway smooth muscle cells that had been pretreated with MK571 for 30 min were stimulated with LTD_4 for the desired times as indicated. The cell lysate containing 10 μg of protein separated by 15% SDS-PAGE was electophoretically transferred to nitrocellulose membrane and the membrane was blotted with an anti-phosphorylated threonine and tyrosine of ASK1 Ab [p-ASK1; 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 ASK1 Ab [ASK1; lower panel]. The amounts of phosphorylated ASK1 were quantified by a National Institutes of Health (NIH) image analyzer (National Institute of Health, Bethesda, MD, USA) and are presented as the amounts of phosphorylated ASK1 proteins relative to control cells treated without LTD_4 (1.0). The fold increases in amounts of phosphorylated ASK1 proteins are indicated below. Three identical experiments independently performed gave similar results.

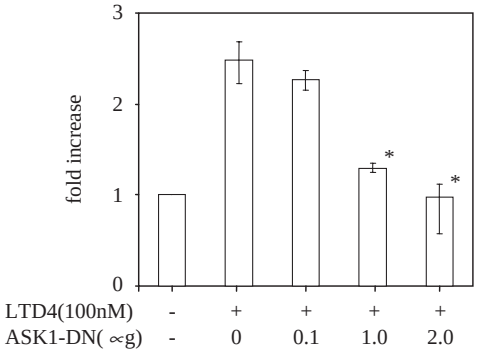

Fig. 4. AP-1 activity is depressed in the dominant negative form of ASK1-transfected human airway smooth muscle cells. Airway smooth muscle cells were transiently co-transfected with the pcDNA3-ASK1-dominant negative expression vectors or empty pcDNA3 vector and pAP-1-Luc reporter plasmid. After 24 h with the transfection, airway smooth muscle cells were stimulated with 100 nM and AP-1 activity was determined at 6 h after the stimulation with LTD_4. The results are expressed as means ± S.D. of six different experiments. *Indicates $p < 0.01$ compared with the LTD_4-stimulated dominant negative form of ASK1-untransfected cells.

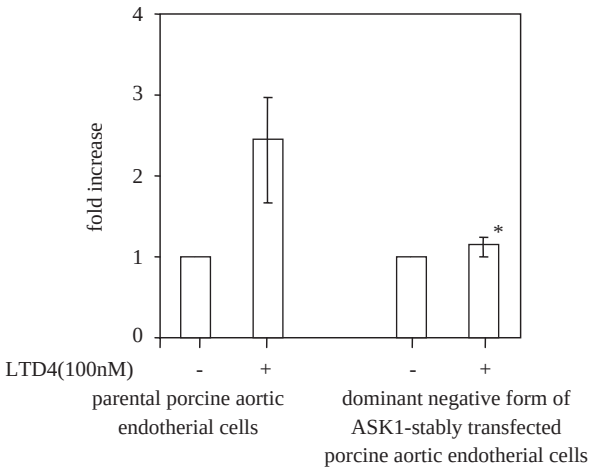

Fig. 5. AP-1 activity is depressed in the dominant negative form of ASK1-stably transfected porcine aortic endothelial cells. After 24 h with the transient transfection of pAP-1-Luc reporter plasmid or empty pcDNA3 vector, the parental porcine aortic endothelial cells and the dominant negative form of ASK1-stably transfected porcine aortic endothelial cells were stimulated with 100 nM LTD_4 and AP-1 activity was determined at 12 h after the stimulation with LTD_4. The results are expressed as means ± S.D. of six different experiments *Indicates $p < 0.01$ compared with the LTD_4-stimulated parental porcine aortic endothelial cells.

LTD_4-medicated AP-1 activation in airway smooth muscle cells (Fig. 4). AP-1 activation was depressed in the dominant negative form of ASK1-transfected airway smooth muscle cells in a concentration-dependent manner. To further characterize the role of ASK1 in LTD_4-induced AP-1 activation, we utilize the dominant negative form of ASK1-stably transfected porcine aortic endothelial cells (Jibiki et al., 2003). The dominant negative form of ASK1-stably transfected porcine aortic endothelial cells and the parental porcine aortic endothelial cells were stimulated with LTD_4 and AP-1 activity was determined at 24 h after in LTD_4 stimulation. AP-1 reporter activity is lower in the dominant negative form of ASK1-stably transfected porcine aortic endothelial cells than those in the parental porcine aortic endothelial (Fig. 5), showing that AP-1 activity was depressed in the dominant negative form of ASK1-stably transfected porcine aortic endothelial cells. These results indicate that ASK1 regulates LTD_4-induced AP-1 activation. The total number of the cells, cell viability determined by trypan blue exclusion dye and total cell protein at the end of the culture period of each experiment indicated in Figs. 1–5 did not differ with culture conditions (data not shown).

4. Discussion

In the present study, we examined the effect of LTD_4 in inducing AP-1 activation in human airway smooth muscle cells and analyzed the intracellular signal regulating LTD_4-mediated AP-1 activation. The results indicate that LTD_4 is capable of inducing AP-1 activation and ASK1, at least in part, regulates AP-1 activation in LTD_4-stimulated airway smooth muscle cells.

The transcription factors, AP-1 and nuclear factor-κB (NF-κB), participate to various extents in the inducible expression of the genes regulating cell proliferation,

cytokine expression and extracellular matrix synthesis (Karin et al., 1997; Pahl, 1999). Cysteinyl LTs have been shown to induce NF-κB activation in human mononuclear cells and human bronchial epithelial cells (Aoki et al., 1998) and AP-1 activation in human monocytes (Stankova and Rola-Pleszczynski, 1992). However, the effect of LTD_4 in AP-1 activation in human airway smooth muscle cells has not been determined. The present study showed that LTD_4 induced increases in AP-1 activity in human airway smooth muscle cells. Increases in airway smooth muscle cell mass are one of characteristic feature of airway remodeling of asthma (Elias et al., 1999). LTD_4 can promote airway smooth muscle cell proliferation (Parameswaran et al., 2002). AP-1 activation is essential for cell proliferation and differentiation (Shaulian and Karin, 2001). AP-1 is activated in hypertrophied heart and vascular smooth muscle cells in hypertensive and balloon-injured artery (Kim and Iwao, 2003). Taken together, it is important to clarify the mechanism in LTD_4-induced AP-1 activation in airway smooth muscle cells.

MAPK cascades are connected with the activation of AP-1. Phosphorylation of c-*Jun*, JunD and ATF-2 by JNK and phosphorylation of ATF-2 by p38 MAPK result in an increased transactivation potential of the factors (Karin et al., 1997). In the present study, we analyzed the role of ASK1 in LTD_4-induced AP-1 activation in airway smooth muscle cells. ASK1 is an up-stream kinase kinase of JNK and p38 MAPK (Kyriakis and Avruch, 2001; Ichijo et al., 1997). JNK and p38 MAPK are activated through ASK1 in response to various extracellular stimuli including hydrogen peroxide, TNF-α, and microtubule-disrupting agents (Force and Bonventre, 1998; Ichijo et al., 1997; Chen et al., 1999). We examined the role of ASK1 in LTD_4-induced AP-1 activation by transient transfection of a dominant negative form of ASK1 in airway smooth muscle cells. The results showed that transient transfection of the dominant negative form of ASK1 attenuated LTD_4-mediated AP-1 activation in airway smooth muscle cells. To further characterize the role of ASK1 in LTD_4-mediated AP-1 activation, we utilize the dominant negative form of ASK1-stably transfected porcine artery endothelial cells. AP-1 activation was depressed in the dominant negative form of ASK1-stably transfected porcine artery endothelial cells. These results indicate that ASK1 regulates AP-1 activation in LTD_4-stimulated airway smooth muscle cells.

ASK1 was originally identified as a kinase that regulates cell apoptosis. Recently, Izumiya et al. have shown that angiotensin II-induced cardiac hypertrophy and remodeling is depressed in ASK1-knockout mice, indicating that ASK1 is a critical signaling molecule for angiotensin II-induced cardiac hypertrophy (Izumiya et al., 2003). Collectively, ASK1 is a potential therapeutic target molecule for airway remodeling.

AP-1 activation in smooth muscle cells may imply the remodeling responses as shown in hypertrophied heart and vascular smooth muscle cells in balloon-injured artery (Kim and Iwao, 2003). AP-1 decoy and dominant negative c-*Jun* have been shown to inhibit vascular smooth muscle cell proliferation (Ahn et al., 2002; Zhan et al., 2002). Therefore, the regulation of AP-1 activity may attenuate the progression of airway remodeling. The blockade of LT pathway by inhibiting LT synthesis and/or by inhibiting the binding of LTs to their receptors is potentially important to attenuate the progression of airway remodeling via the attenuation of AP-1 activation. In addition, therapeutic intervention in inhibiting ASK1 activation may be beneficial in reducing AP-1 activity in airway smooth muscle cells.

From the data presented here, we conclude that ASK1 regulates LTD_4-induced AP-1 activation in human airway smooth muscle cells. Our results with the role of ASK1-mediated LTD_4-induced AP-1 activation in human airway smooth muscle cells is important in understanding the molecular mechanism in AP-1 activation seen in airway of bronchial asthmatics, and a strategy of attenuating airway remodeling as well as inflammation by the specific inhibitor of ASK1 cascade may produce beneficial effects in controlling airway remodeling and inflammation.

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