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Inhibition of Trypsin-Induced Mast Cell Activation by Water Fraction of *Lonicera japonica*

Ok-Hwa Kang, Yeon-A Choi, Hye-Jung Park, Joo-Young Lee, Dae-Ki Kim[1], Suck-Chei Choi[2], Tae-Hyun Kim[2], Yong-Ho Nah[2], Ki-Jung Yun[2], Suck-Jun Choi[2], Young-Ho Kim[3], Ki-Hwan Bae[3], and Young-Mi Lee

Department of Oriental Pharmacy, College of Pharmacy; Wonkwang University, Iksan, Jeonbuk 570-749, Korea, [1]Department of Immunology, Chonbuk National University Medical School, Jeonju, Jeonbuk, 561-182, Korea, [2]Institute of Digestive Disease, Wonkwang University School of Medicine, Iksan, Jeonbuk 570-749, Korea, and [3]College of Pharmacy, Chungnam National University, Daejeon 305-764, Korea

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Lonicera japonica Thunb.(Caprifoliaceae) has long been known as an anti-inflammatory. In the present study, the effect of water fraction of *Lonicera japonica* (LJ) on trypsin-induced mast cell activation was examined. HMC-1 cells were stimulated with trypsin (100 nM) in the presence or absence of LJ (10, 100, and 1000 μg/mL). TNF-α and tryptase production were measured by enzyme-linked immunosorbent assay (ELISA) and reverse transcription-PCR. Extracellular signal-regulated kinase (ERK) phosphorylation was assessed by Western blot. Trypsin activity was measured by using Bz-DL-Arg-p-nitroanilide (BAPNA) as substrate. LJ (10, 100, and 1000 μg/mL) inhibited TNF-α secretion in a dose-dependent manner. LJ (10, 100, and 1000 μg/mL) also inhibited TNF-α and tryptase mRNA expression in trypsin-stimulated HMC-1. Furthermore, LJ inhibited trypsin-induced ERK phosphorylation. However, LJ did not affect the trypsin activity even 1000 μg/mL. These results indicate that LJ may inhibit trypsin-induced mast cell activation through the inhibition of ERK phosphorylation than the inhibition of trypsin activity.

Key words: *Lonicera japonica*, Trypsin, Tryptase, TNF-α, ERK

INTRODUCTION

Lonicera japonica Thunb.(Caprifoliaceae) has long been used for treatment of inflammation and infectious disease in East Asian countries. It is supposed that *Lonicera japonica* dispels noxious heat from blood and neutralizes the poisonous effects (Wang, 1989). *Lonicera japonica* was reported to have a possibility as a therapeutic agent for inflammatory disease through a selective regulation of NF-κB activation (Lee *et al.*, 2001). We previously also showed that *Lonicera japonica* have anti-inflammatory effect in proteinase-activated receptor 2-mediated paw edema (Tae *et al.*, 2003).

Mast cells are found in all layers of gut wall throughout the gastrointestinal tract. Mast cells play an important role in regulation of inflammation by functioning as a source of histamine, neutral protease, and proinflammatory cytokines (Metcalfe *et al.*, 1997). The mast cells are involved in chronic inflammatory processes such as inflammatory bowel disease (IBD) (Raithel *et al.*, 2001).

The gastrointestinal tract is exposed to high level of proteinases both physiologically and during diseases. Serine protease trypsin derives from digestive glands, inflammatory cells, and bacterial and viral pathogens. It has been shown that trypsin plays an important role in inflammation. Trypsin induces nitric oxide-dependent vasodilation, extravasation of plasma proteins, infiltration of neutrophils, and colonic inflammation (Kawabata *et al.*, 2001). Intracolonic injection of trypsin induced an inflammatory reaction characterized by granulocyte infiltration, increased wall thickness, tissue damage, and elevated T-helper cell type 1 cytokine (Cenac *et al.*, 2002). Trypsin also stimulates inflammatory mediator release from peritoneal macrophages (Lundberg *et al.*, 2000). We previously showed that trypsin induced TNF-α secretion from HMC-1 cells through extracellular signal-regulated kinase (ERK) cascade without any detectable phospho-rylation of *c*-Jun *N*-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAP kinase) (Kang *et al.*, 2003).

Correspondence to: Young-Mi Lee, Department of Oriental Pharmacy, College of Pharmacy, Wonkwang University, Iksan 570-749, Korea
Fax: 82-63-855-6807
E-mail: ymlee@wonkwang.ac.kr.

In the present study, we have investigated whether LJ inhibits trypsin-induced mast cell activation.

MATERIALS AND METHODS

Materials

Human trypsin (M.W. 23.8 kDa), soybean trypsin inhibitor (SBTI), Bz-DL-Arg-*p*-nitroanilide (BAPNA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). Anti-human ERK antibody and anti-human-phospho-ERK antibody were purchased from Santa Cruz Biotechnology Inc. (CA, USA), and anti-IgG-horseradish peroxidase (HRP) was purchased from DAKO (High Wycomde, Bucks, UK). Anti-human tumor necrosis factor (TNF)-α antibody and recombinant human TNF-α were obtained from R&D Systems (Minneapolis, MN).

Plant material and fraction preparation

Lonicera japonica (LJ) was obtained from an oriental drug store (Iksan, Korea). LJ was prepared by decocting for about 2 h with distilled water (100 g/L). The extract was filtered through a 0.45 μm filter, lyophilized, and kept at 4°C. The dried extract was dissolved in sterile saline before use. LJ has been deposited at the herbarium at the College of Pharmacy, Wonkwang University.

Cell culture

The human leukemic mast cell line HMC-1 (kindly provided by Dr. Y. Kitamura, Department of Pathology, Osaka University Medical School, Japan) was cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/mL penicillin, 50 μg/mL streptomycin, and 1.2 mM α-thioglycerol at 37°C under 5% CO_2 in air.

Cell viability

To determine the cell viability, MTT assay was performed. HMC-1 cells were seeded at 5×10^4/mL densities in 96-well plates in 100 μL cell suspension per well. Each group had three wells with a non-treated group as control. LJ (10, 100, and 1000 μg/mL) was added to each well and incubated at 37°C under 5% CO_2. After 48 h of incubation, 10 μL of MTT (5 mg/mL) was added to each well and cultured for another 4 h. The supernatant was discarded and 100 μL of dimethyl sulphoxide (DMSO, Sigma, St. Louis) was added. When the formazan crystals were dissolved, the optical density was measured using an ELISA reader at 540 nm. The optical density of formazan formed in control (untreated) cells was taken as 100% of viability.

Enzyme-linked immunosorbent assay for TNF-α

Cells were seed at 1×10^6 cells/mL in 24-well tissue culture plates and pretreated with various concentration of LJ (10, 100, and 1000 μg/mL) for 30 min before trypsin (100 nM) stimulation. Eight hours after trypsin stimulation, TNF-α concentrations in the supernatant were measured as commercial instruction (Pharmingen, San Diego, CA). Briefly, ELISA plates were coated overnight at 4°C with anti-human TNF-α antibody diluted in coating buffer (0.1 M carbonate, pH 9.5) and then washed four times with PBS containing 0.05% tween 20 (PBS-T). Non-specific protein binding sites were blocked with PBS containing 10% FBS, pH 7.0 (assay diluent) for at least 1 h, and 100 μL of each sample or TNF-α standards diluted in assay diluent were applied to wells. After incubation for 2 h, 100 μL of working detector (biotinylated anti-TNF-α monoclonal antibody and avidin-HRP reagent) was added and incubated for 1 h. Consequently, 100 μL of substrate solution containing tetramethylbenzidine (TMB) and H_2O_2 was added to wells and incubated for 30 min in the dark before stopping the reaction by 50 μL of stop solution (2N H_2SO_4) and the absorbance was read at 450 nm. All subsequent steps took place at room temperature and all standards and samples were assayed in triplicate.

RNA extraction and reverse-transcription PCR (RT-PCR) for TNF-α and tryptase

LJ-pretreated HMC-1 cells were stimulated with trypsin (100 nM) for 2 h. Total RNA was isolated from the harvested cells using the easy-BLUE™ RNA extraction kit (iNtRON Biotech, Korea). Total RNA (5 μg) was converted to cDNA by reverse transcriptase at 37°C for 90 min using first-strand cDNA synthesis kit (Amersham Pharmacia Biotech). PCR amplification was performed as follows: tryptase (94 °C for 45 sec, 50°C for 1 min, 72°C for 1 min: 30 cycles), TNF-α (94°C for 1 min, 60°C for 1 min, 74°C for 1 min: 30 cycles), GAPDH (94°C for 1 min; 60°C for 2 min; 72°C for 1 min: 30 cycles). Primers used in this study were as follows: tryptase (forward primer 5'-AGGATGCTGAATC TGCTGCTGCTG-3' and reverse primer 5'-TCACGGCTT TTTGGGGACATAGTG-3': 831 bp) (Vanderslice *et al.*, 1990), TNF-α (forward primer 5'-CAAAGTAGACCTGCCCAGA C-3' and reverse primer 5'-GACCTCTCTCTAATCAGCC C-3': 490bp), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer 5'-CCATGTTCGTCATGGGTG TGAACCA-3' and reverse primer 5'-GCCAGTAGAGGCA GGGATGATGTTC-3': 251 bp) (Lee *et al.*, 2000). Final PCR products were separated on 1% agarose gels and photographed under UV light.

Western blot analysis for ERK

LJ-pretreated HMC-1 cells were stimulated with trypsin (100 nM) for 15 min. The cells were lysed with ice-cold lysis buffer (iNtRON Biotech, Korea). Western blot analysis was performed according to a standard procedure.

Lysates (50 mg of protein) was separated by SDS-PAGE with 12% polyacrylamide gel and transferred on PVDF membrane (Millipore). After blocking with 5% skim milk, membranes were blotted with anti-human phospho-ERK for 12 h at 4°C. HRP-conjugated antibody against rabbit IgG was used as a secondary antibody. Finally, proteins recognized by specific antibodies were visualized by using enhanced chemiluminescence (ECL) detection kit (Amersham, Milan). After stripping, the membranes were reprobed with anti-ERK antibody as respective loading controls.

Trypsin activity assay

Trypsin activity was assessed by the amidolytic ability of trypsin on a substrate BAPNA (Smith *et al.*, 1984). Samples were added to each tube containing 2.85 ml of 0.1 M Tris-HCl (pH 8.0) and 1 M glycerol, and the assay was started with the addition of 20 mM substrate in DMSO. After 30 min incubation at 37°C, the reactions were stopped by the addition of 50 μL of glacial acetic acid, and the absorbance was measured at 405 nm with spectrophotometer and compared to those given by standard curves of *p*-nitroanilide to determine the concentration of product released. Inhibition percentages of trypsin activity were calculated using the following equation:

$$\% \text{ of inhibition} = \frac{(A - B)}{A} \times 100$$

where A is a trypsin activity without LJ and B is a trypsin activity with LJ.

Statistical analysis

The results were expressed as mean±S.E. for a number of experiments. Statistical significance was compared between each treated group and control by the Student's *t*-test. Each experiment was repeated at least three times and yielded comparable results. Values with $p<0.05$ were considered significant.

RESULTS

Effect of LJ on cell viability of HMC-1 cells

To examine the direct cytotoxic effect of LJ, cell viability was examined after treatment of HMC-1 cells with three concentrations of LJ (10, 100, and 1000 μg/mL) for 48 h. LJ did not affect the viability of HMC-1 cells even up to 1000 μg/mL (Fig. 1).

Effect of LJ on trypsin- induced TNF-α secretion

To study the effect of LJ on trypsin-induced TNF-α secretion from HMC-1 cells, HMC-1 cells were pretreated with three concentrations of LJ (10, 100, and 1000 μg/mL) for 30 min and then stimulated with trypsin (100 nM) for 8 h. TNF-α secreted from HMC-1 cells were measured by ELISA. Trypsin (100 nM) induced a 10 fold increase in TNF-α secretion compare to trypsin-nontreated cells. LJ (100 and 1000 μg/mL) significantly inhibited TNF-α secretion in a dose-dependent manner (Fig. 2). LJ showed 71% inhibition for TNF-α in concentration of 1000 μg/mL. This result demonstrates that LJ could down-regulate TNF-α secretion in trypsin-stimulated HMC-1 cells.

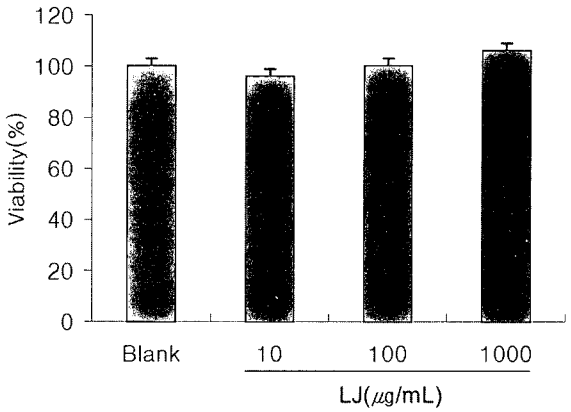

Fig. 1. Effect of LJ on cell viability of HMC-1 cells. The cells were incubated with various concentration of LJ (10, 100, and 1000 μg/mL). Cell viability was evaluated by MTT assay (expressed as percent of control). Values are mean ± S.E. of three independent experiments.

Effect of LJ on trypsin-induced TNF-α and tryptase mRNA expression

To study the effect of LJ on TNF-α synthesis in trypsin-

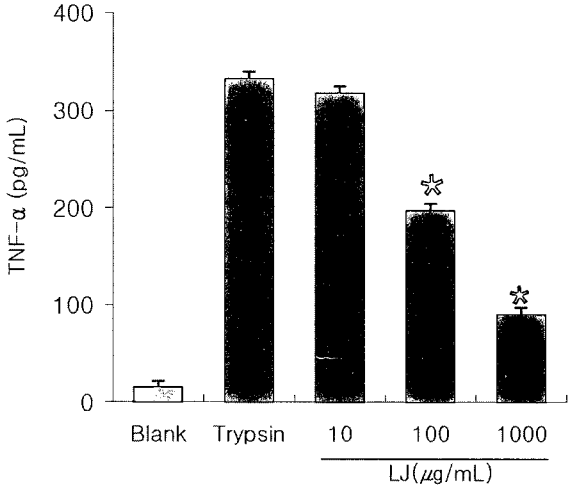

Fig. 2. Effect of LJ on TNF-α secretion in trypsin-stimulated HMC-1 cells. The cells (1 x 10^6 cells/mL) were pre-incubated with three concentrations of LJ (10, 100, and 1000 μg/mL) before stimulation with trypsin (100 nM) for 8 h. TNF-α levels in supernatant were measured by ELISA. Values are the mean±S.E. of duplicate determinations from three separate experiments (*$p < 0.05$).

stimulated HMC-1 cells, LJ pretreated HMC-1 cells were stimulated with trypsin for 2 h. Tryptase and TNF-α mRNA expression levels in intracellular of HMC-1 cells activated by trypsin were determined by RT-PCR. Trypsin led to an increase of TNF-α and tryptase mRNA expression in HMC-1 cells. LJ (100 and 1000 μg/mL) significantly inhibited TNF-α (Fig. 3) and tryptase (Fig. 4) mRNA expression in a dose-dependent manner. Especially, LJ inhibited to near control level at concentrations of 1000 μg/mL. These results indicate that LJ modulates TNF-α and tryptase synthesis in trypsin-stimulated HMC-1 cells.

Effect of LJ on trypsin-induced ERK phosphorylation

To examine the effect of LJ on trypsin-induced ERK phosphorylation, LJ-pretreated HMC-1 cells were stimulated with trypsin for 15 min. Major immunoreactive band was identified with phosphorylated ERK by Western blot analysis and the level of phosphorylation was described as the relative ratio of band density against that of whole ERK (Fig. 5). Trypsin (100 nM) stimulation (Fig. 5, lane 2) induced 4.5-fold increase in the phosphorylation of ERK compared to that of trypsin-nontreated cells (Fig. 5, lane 1). LJ pretreatment (100 and 1000 μg/mL) resulted in the blockade of trypsin-induced ERK phosphorylation, without affecting the levels of whole ERK (Fig. 5, lane 4, and 5). The result suggests that the inhibitory effect of LJ could be caused through the suppression of ERK activation pathway.

Effect of LJ on trypsin activity

To determine whether LJ can modulate just trypsin activity, trypsin activity was measured by using BAPNA substrate. Assay was performed with three concentrations of LJ (10, 100, and 1000 μg/mL). Trypsin activity was inhibited to 89.0±8.8% at 1 μM SBTI and to 94.0±9.1% at 10 μM SBTI, whereas LJ did not affect even 100 μg/mL (Table I). The result suggests that effect of LJ on trypsin-induced TNF-α secretion is not by inhibition of trypsin activity.

DISCUSSION

Recent studies have suggested that mast cells may play a role in the pathogenesis of IBD (Raithel *et al.*, 2001). The tissues of patients with IBD reveal an increase of mast cell number and accumulation of the mast cell-derived

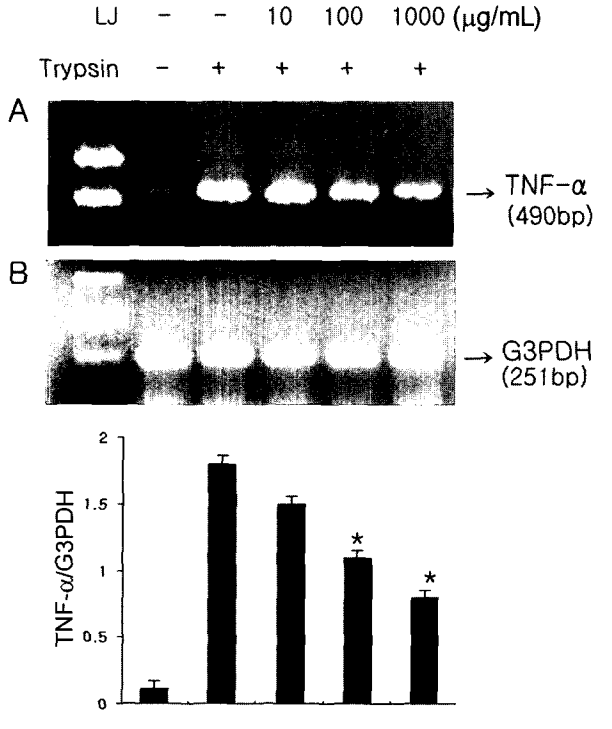

Fig. 3. Effect of LJ on TNF-α mRNA expression in trypsin-stimulated HMC-1 cells. The cells were pre-incubated with three concentrations of LJ (10, 100, and 1000 μg/mL) before stimulation with trypsin (100 nM) for 2 h. Total RNA (5 μg) was converted to cDNA by reverse transcriptase. GAPDH mRNA was carried out in parallel to confirm equivalency of cDNA preparation (B). Size marker is λ DNA/HaeIII. *Significantly different ($P < 0.05$) from positive control.

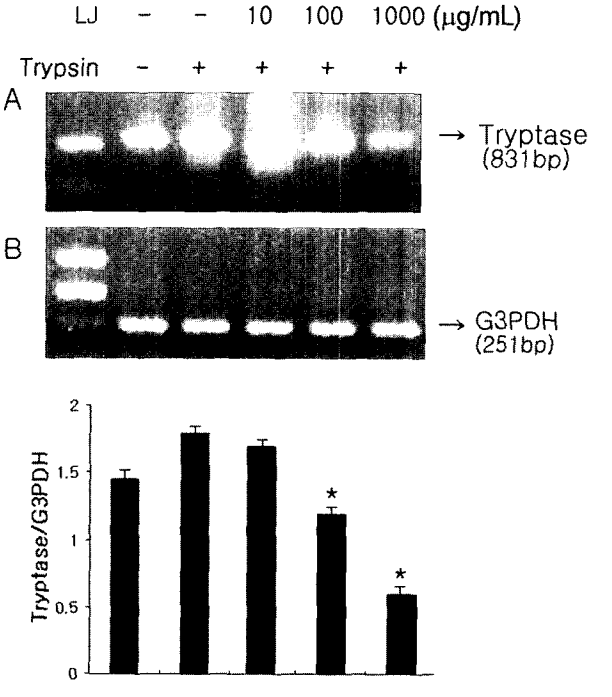

Fig. 4. Effect of LJ on tryptase mRNA expression in trypsin-stimulated HMC-1 cells. The cells were pre-incubated with three concentrations of LJ (10, 100, and 1000 μg/mL) before stimulation with trypsin (100 nM) for 2 h. Total RNA (5 μg) was converted to cDNA by reverse transcriptase. GAPDH mRNA was carried out in parallel to confirm equivalency of cDNA preparation (B). Size marker is λ DNA/HaeIII. *Significantly different ($P < 0.05$) from positive control.

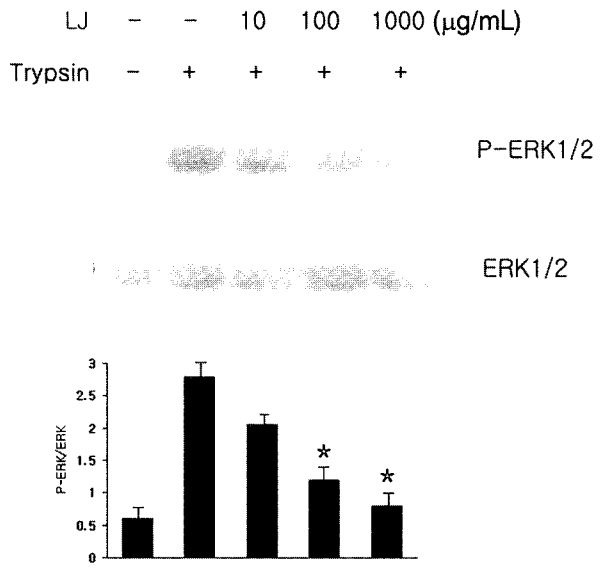

Fig. 5. Effect of LJ on ERK1/2 phosphorylation in trypsin-stimulated HMC-1 cells. HMC-1 cells (5×10^6 cells/mL) pretreated with LJ (10, 100, and 1000 μg/mL) were cultured for 15 min with trypsin (100 nM), and ERK1/2 phosphorylation were analyzed by Western blot using specific antibodies against ERK1/2 and phospho-ERK1/2. *Significantly different ($P< 0.05$) from positive control.

Table I. Inhibitory effect of LJ on trypsin[a] activity.

Treatment (μM)	Inhibition (%)
SBTI 1	89.0 ± 8.8
10	94.0 ± 9.1
100	ND[b]
LJ 10	0.5 ± 0.2
100	1.5 ± 0.5
1000	1.2 ± 0.4

[a]Trypsin concentration is 20 μg/mL.
[b]ND: not determined

mediators, such as histamine, proteases, leukotrienes, and prostaglandin. We also previously showed that tryptase and tumor necrosis factor (TNF)-α expression in intestinal mast cells are significantly elevated in UC tissues compared to normal tissues, indicating that mast cells may be involved in the inflammatory features of UC (Kim *et al.*, 2003). Furthermore, the development of dextran sulphate sodium-induced experimental colitis is suppressed in genetically mast cell-deficient *Ws/Ws* rats (Araki *et al.*, 2000). Therefore, agents that are non-toxic and can inhibit the production of TNF-α or tryptase by intestinal mast cells can be useful for the inhibition of intestinal inflammation (Gratz *et al.*, 2002; Tremaine *et al.*, 2002). Furthermore, blockers of signal pathway or trypsin inhibitors can be effective as anti-inflammatory agents. A report showed that FceRI-mediated TNF-α production in mast cells is regulated by mitogen-activated protein kinase (MEK) kinase (Ishizuka *et al.*, 1997). We previously showed that trypsin induced TNF-α secretion from HMC-1 cells *via* phosphorylation of ERK without any detectable phosphorylation of JNK and p38 MAP kinase (Kang *et al.*, 2003). Moxifloxacin exerts anti-inflammatory effects in THP-1 cells by inhibiting NF-κB, ERK, and JNK activation (Weiss *et al.*, 2004). Mitogen-activated protein kinase kinase/ERK inhibitor U0126 markedly inhibited OVA-induced lung tissue eosinophilia, airway mucus production, and expression of vascular cell adhesion molecule-1 (VCAM-1) in lung tissues of an asthma mouse (Duan *et al.*, 2004). Thus, ERK inhibitors have anti inflammatory effects. In present study, our results showed that LJ significantly inhibited trypsin-induced TNF-α and tryptase production by block of ERK pathway.

In order to examine another inhibitory mechanism of LJ on inflammatory mediator production in trypsin-stimulated HMC-1, we tested the effect of LJ on trypsin activity. Soybean trypsin inhibitor (SBTI) and Bowman-Birk inhibitor are trypsin inhibitors. SBTI inhibited collagenase-induced edema in the rat paw (Souza Pinto *et al.*, 1995). We previously showed that SBTI completely inhibited TNF-α secretion from trypsin-stimulated HMC-1 cells (Kang *et al.*, 2003). Moreover, Bowman-Birk inhibitor reduced colon inflammation in mice with dextran sulfate sodium-induced colitis (Ware *et al.*, 1999). Thus, trypsin inhibitors have anti-inflammatory effects. In this study, However, LJ did not affect the trypsin activity.

Taken together, LJ might inhibit trypsin-induced TNF-α and tryptase production through blocking of ERK pathway than inhibition of trypsin activity. This study provides a strong rationale to investigate the effect of LJ in an *in vivo* experimental model of trypsin-induced intestinal inflammation.

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