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Luteolin Inhibits Extracellular Signal-Regulated Kinase Pathway Through Protease-Activated Receptors (-2 and -4) and Their Agonist Activity

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Abstract – Luteolin is a major flavonoid of *Lonicera japonica* and has anti-inflammatory effect. The activation of proteinase-activated receptor (PAR)-2 and -4 by trypsin appears to play a role in inflammation. In the present study, we examined the inhibitory effects of luteolin on activation of trypsin-induced human leukemic mast cells (HMC-1). HMC-1 cells were stimulated with trypsin, PAR-2 and PAR-4 agonist, in the presence or absence of luteolin. The level of TNF-α secretion was measured by enzyme-linked immunosorbent assay (ELISA). The expression of tryptase and phosphorylated-extracellular signal-regulated kinase (ERK) were assessed by Western blot analysis. Moreover, trypsin activity was measured by the substrate Bz-DL-Arg-p-nitroanilide (BAPNA). TNF-α secretion and tryptase expression in trypsin-stimulated HMC-1 cells were markedly inhibited by pretreatment of luteolin. Furthermore, the pretreatment of luteolin resulted in the reduction of ERK phosphorylation and trypsin activity. These results suggest that luteolin might has the inhibitory effects on the PAR-2 and -4-dependent inflammation.
Keywords – Protease-activated receptor, luteolin, trypsin, mast cells, tryptase, TNF-α, ERK

Introduction

Trypsin is one of proteases derived from digestive glands and inflammatory cells by bacterial and viral pathogens and activates protease-activated receptor-2 (PAR-2) and PAR-4 (Macfarlane *et al.*, 2001). It has been shown that the activation of PARs by trypsin induces nitric oxide-dependent vasodilation, extravasation of plasma proteins, infiltration of neutrophils, and colonic inflammation (Kawabata *et al.*, 1998; Cenac *et al.*, 2002) and that PAR-2 (Bohm *et al.*, 1996; Xu *et al.*, 1998) and PAR-4 (Mule F *et al.*, 2004) are highly expressed in the gastrointestinal tract.

Human mast cells are well known to mediate the immediate type allergic reactions. In addition, there is increasing evidence that mast cells are involved in colonic inflammatory processes such as inflammatory bowel disease (IBD). It was reported that mast cell hyperplasia in the gut is a feature of IBD (Stoyanova and Gulubova, 2002). Mast cells play an important role in the development of inflammation by releasing various mediators such as histamine, neutral protease, and proinflammatory cytokines (Metcalfe *et al.*, 1997). We previously demonstrated that trypsin induced TNF-α secretion by HMC-1 cells via extracellular signal-regulated kinase (ERK) signaling pathway (Kang *et al.*, 2003).

Luteolin is a major flavonoid of *Lonicera japonica.* It is thought to play an important role in the human body as an antioxidant, a free radical scavenger, an agent in the prevention of inflammation. Our previous results showed that luteolin has the inhibitory effects on TNF-α-induced IL-8 production in the intestinal epithelial cells through blockade in the phosphorylation of MAPKs, following IkappaB degradation and NF-kappaB activation. The present study thus aimed to determine whether luteolin inhibits the PAR-2 and -4-mediated activation of human mast cells.

Experimental

Materials – Human trypsin, substrate Bz-DL-Arg-p-nitroanilide (BAPNA) was purchased from Sigma (St. Louis, MO). Anti-ERK antibody, anti-phospho-ERK antibody, and anti-β-actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-IgG-horseradish peroxidase (HRP) conjugate was from DAKO (High Wycomde, Bucks,UK). Anti-tumor necrosis factor (TNF)-α antibody and recombinant human TNF-α

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were obtained from R&D Systems (Minneapolis, MN). Luteolin (Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO, 0.01%). DMSO (0.01%) used as vehicle did not affect HMC-1 cells.

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

Enzyme-linked immunosorbent assay for TNF-α – Cells were seed at 5×10^5 cells per well in 24 well tissue culture plates and pretreated with various concentration of luteolin (1, 10, and 100 μM) 30 min before trypsin (100 nM) stimulation. Eight hours after trypsin stimulation, TNF-α concentrations in the supernatant were measured as commercial instruction (Pharmingen assay, SanDiego, CA). Briefly, ELISA plates (Falcon, Becton Dickinson Labware, USA) 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 assay diluent (PBS containing 10% FBS, pH 7.0) 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 (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 stop solution (2N H_2SO_4) and absorbance was read at 450 nm. All subsequent steps took place at room temperature, and all standards and samples were assayed in triplicate.

Western blot analysis for tryptase or ERK – Luteolin-pretreated HMC-1 cells were stimulated with trypsin (100 nM) for 8 h or15 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 μg of protein) was separated by SDS-PAGE with 12% acrylamide gel and transferred on PVDF membrane (Millipore). After blocking with 5% skim milk, membranes were blotted with anti-tryptase, anti-phospho-ERK, or anti-ERK antibody for 12 h at 4 °C. HRP-conjugated antibody against rabbit IgG was used as a secondary antibody. Finally, Epitopes on proteins recognized specifically by antibodies were visualized by using enhanced chemiluminescence (ECL) detection kit (Amersham, Milan). After stripping, the membranes were reprobed with anti-β-actin or 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 20mM 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 luteolin and B is a trypsin activity with luteolin.

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 luteolin on trypsin-induced TNF-α secretion and tryptase expression – To study the effect of luteolin on trypsin-induced TNF-α secretion and tryptase expression in HMC-1 cells, HMC-1 cells were pretreated with various concentrations of luteolin (1, 10, or 100 μM) for 30 min and then stimulated with trypsin (100 nM) for 8 h. The levels of TNF-α secreted from HMC-1 cells were measured by ELISA. Treatment of trypsin appears to induce a 10 fold increase in TNF-α secretion compared to trypsin-nontreated cells. Luteolin significantly inhibited TNF-α secretion induced by trypsin in high concentration (10 μM or 100 μM) but little in low concentration (Fig. 1). Luteolin showed 76.4 % inhibition for TNF-α in concentration of 100 μM. Furthermore, trypsin led to an increase in tryptase expression in HMC-1 cells. Pretreatment of luteolin (10 μM or 100 μM) attenuated tryptase expression similar to control level (Fig. 2). These results indicate that luteolin modulates TNF-α and tryptase production in trypsin-stimulated HMC-1 cells.

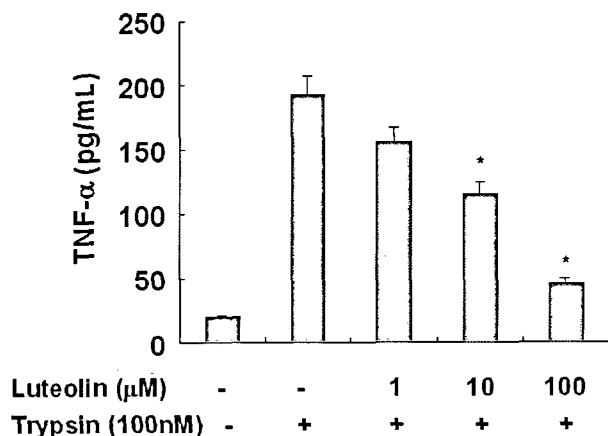

Fig. 1. Effect of luteolin on TNF-α secretion in trypsin-stimulated HMC-1 cells.

The cells (5×10^5 cells/ well) were pre-incubated with luteolin (1, 10, or 100 μM) 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).

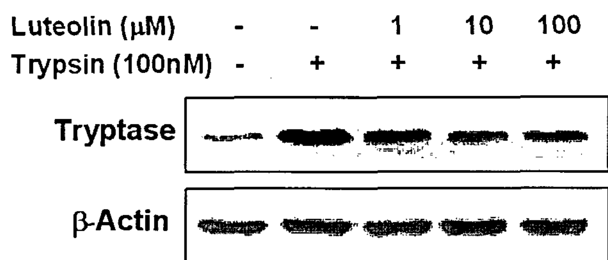

Fig. 2. Effect of luteolin on tryptase expression in trypsin-stimulated HMC-1 cells.

The cells were pre-incubated with luteolin (1, 10, or 100 μM) before stimulation with trypsin (100 nM) for 8h. Total protein (50 μg) per lane was loaded. Tryptase was detected as a band with a molecular mass of about 32-36 kDa. β-actin was carried out in parallel to confirm equivalency of protein preparation (42 kDa).

Effect of luteolin on trypsin-induced ERK phosphorylation – To examine the effect of luteolin on trypsin-induced ERK phosphorylation, luteolin-pretreated HMC-1 cells were stimulated with trypsin for 15 min. Major immunoreactive band was identified with phosphorylated ERK by Western blot analysis (Fig. 3). Trypsin (100 nM) stimulation (Fig. 3, lane 2) induced increase in the phosphorylation of ERK compared to that of trypsin-nontreated cells (Fig. 3, lane 1). Luteolin pretreatment (1, 10 or 100 μM) resulted in the blockade of trypsin-induced ERK phosphorylation, without affecting the levels of whole ERK (Fig. 3, lane 3, 4, and 5). The result suggests that the inhibitory effect of luteolin on production of inflammatory mediators might be caused through the suppression of ERK activation pathway.

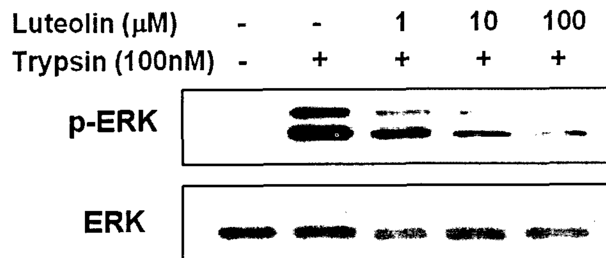

Fig. 3. Effect of luteolin on ERK1/2 phosphorylation in trypsin-stimulated HMC-1 cells.

HMC-1 cells (5×10^6 cells/mL) pretreated with luteolin (1, 10, or 100 μM) were cultured for 15min with trypsin (100 nM), and ERK1/2 phosphorylation were analyzed by Western blot using specific antibodies against ERK1/2 and phospho-ERK1/2 (42/44 kDa).

Table 1. Inhibitory effect of luteolin on trypsin activity

Treatment		Inhibition (%)
SBTI	0.1 1	9.0 ± 0.3
(μM)	10	80.0 ± 4.7
	1	92.0 ± 5.9
luteolin	10	1.9 ± 0.2
(μM)	100	16.2 ± 0.7*
		52.0 ± 2.5*

SBTI : Soybean trypsin inhibitor, Trypsin concentration is 20 μg/mL. Values are the mean ± S.E. of duplicate determinations from three separate experiments (*p < 0.05).

Effect of luteolin on trypsin activity – There is a possibility that the inhibitory effect of luteolin was caused by the direct blockage of the trypsin activity. Thus, the trypsin activity assay was performed using BAPNA substrate. Trypsin (20 μg/mL) was incubated in the presence or absence of luteolin. Luteolin inhibited trypsin activity in a concentration-dependent manner and showed 52.0% inhibition in trypsin activity in concentration of 100mM. In contrast, SBTI used as a positive control almost inhibited the trypsin activity in the concentration of 10 μM (Table 1). These results suggest that luteolin might inhibit the production of TNF-α and tryptase through both blockage of ERK signaling pathway and inhibition of trypsin activity.

Discussion

Mast cells might play a role in the pathogenesis of IBD (Raithel *et al.*, 2001, Stoyanova and Gulubova, 2002). The tissue of patients with IBD reveals an increase of mast cell number and accumulation of the mast cell-derived mediators, such as histamine, cytokines, serine proteases, leukotrienes, and prostaglandins. Our previous study have showed that the levels of tryptase and tumor

necrosis factor (TNF)-α in intestinal mast cells were significantly elevated in tissue of UC patients compared to normal tissues (Kim *et al.*, 2003). Furthermore, we reported that TNF-a and tryptase production in mast cells was induced by trypsin, which lead the activation of PAR-2 and PAR-4 (Kang *et al.*, 2003). Mast cell-derived TNF-α and tryptase by trypsin play a role in recruitment of inflammatory cells like neutrophil during inflammation. (Tae *et al.*, 2003). Ultimately, screening of trypsin inhibitors, PAR-2 and PAR-4 antagonists may be a pharmacological strategy for the treatment of intestinal inflammation. It has been reported that SBTI, a trypsin inhibitor, significantly inhibited the inflammatory reaction induced by the implantation of dry sponges in rats (Damas *et al.*, 1990). SBTI also inhibited collagenase-induced oedema in the rat paw (Souza *et al.*, 1995). In our previous study, SBTI (10 μM) inhibited makedly TNF-a and tryptase production, and trypsin activity (Kang *et al.*, 2005). In this study, luteolin also significantly inhibited trypsin-induced TNF-α and tryptase production by inhibition of ERK phosphorylation in HMC-1 cells. Furthermore, luteolin (100 μM) showed 52% inhibition on the trypsin activity. Some researchers reported that luteolin inhibited chemical mediator-induced ear edema in mice (Ueda *et al.*, 2002) and lipopolysaccharide (LPS)-induced TNF-α, IL-6, and inducible nitric oxide production (Xagorari *et al.*, 2001)

To examine mechanism on inhibition of trypsin-induced TNF-α and tryptase production, ERK phosphorylation was investigated. Our previous results showed that trypsin induced the ERK phosphorylation but not JNK or p38 MAP kinase (Kang *et al.*, 2003). In the present study, we have also observed that luteolin reduced the production of TNF-α and tryptase in trypsin-stimulated mast cells through inhibition of ERK phosphorylation. Xagorari et al showed that luteolin inhibited LPS-induced TNF-α release by inhibiting the extracellular-regulated kinases (ERK) and p38 MAPK pathways in macrophages (Xagorari *et al.*, 2002).

Taken together, luteolin inhibited the production of inflammatory mediators such as TNF-α and tryptase in trypsin-stimulated HMC-1, and the inhibitory effects of luteolin could be caused by the blockage of ERK pathway as well as the reduction of trypsin activity. Luteolin may be a candidate of therapeutic agent for intestinal inflammation induced by PAR-2 and PAR-4-mediated mast cell activation.

Acknowledgements

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