Luteolin-7-*O*-glucoside Suppresses Leukotriene C₄ Production and Degranulation by Inhibiting the Phosphorylation of Mitogen Activated Protein Kinases and Phospholipase C γ 1 in Activated Mouse Bone Marrow-Derived Mast Cells

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In this study, luteolin-7-*O*-glucoside (L7G), an herbal medicine isolated from *Ailanthus altissima*, inhibited 5-lipoxygenase (5-LOX)-dependent leukotriene C₄ (LTC₄) production in bone marrow-derived mast cells (BMMCs) in a concentration-dependent manner with an IC₅₀ of 3.0 μ M. To determine the action mechanism of L7G, we performed immunoblotting for cytosolic phospholipase A₂ (cPLA₂) and mitogen-activated protein kinases (MAPKs) following *c-kit* ligand (KL)-induced activation of BMMCs with or without L7G. Inhibition of LTC₄ production by L7G was accompanied by a decrease in cPLA₂ phosphorylation, which occurred via the extracellular signal-regulated protein kinase-1/2 (ERK1/2) and p38 and c-Jun N-terminal kinase (JNK) pathways. In addition, L7G also attenuated mast cell degranulation in a dose-dependent manner (IC₅₀, 22.8 μ M) through inhibition of phospholipase C γ 1 (PLC γ 1) phosphorylation. Our results suggest that the anti-asthmatic activity of L7G may be mediated in part via the inhibition of LTC₄ generation and mast cell degranulation.

Key words luteolin-7-*O*-glucoside; bone marrow-derived mast cell; degranulation; phospholipase C γ 1; leukotriene C₄; mitogen activated protein kinase

Mast cells are one of the most important effector cells in inflammation and allergic diseases.¹⁾ When activated by various stimuli, mast cells release a number of lipid-derived chemical mediators, such as prostaglandin D₂ (PGD₂) and leukotriene C₄ (LTC₄) via activation of the cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) pathways, as well as various pre-formed and *de novo* synthesized cytokines.^{2,3)} Signaling pathways that lead to degranulation of mast cells through high-affinity immunoglobulin E (IgE) receptor (Fc ϵ RI) or *c-kit* ligand (KL) have been extensively characterized.^{4,5)} Such signaling pathways include phospholipase C γ (PLC γ)-mediated Ca²⁺ mobilization, which is an essential step in mast cell degranulation as well as PGD₂ and LTC₄ production.^{6,7)} 5-LOX catalyzes the conversion of arachidonic acid (AA) into an unstable epoxide, LTA₄, which can then be converted into the cysteinyl leukotrienes LTC₄, LTD₄, and LTE₄. Leukotrienes (LTs) are potent mediators of asthma as well as inflammatory mediators.^{8,9)} In addition, LTs are associated with atherosclerosis and osteoporosis.^{10–12)} Thus, inhibitors of LTs synthesis might be of interest for the treatment of asthmatic as well as cardiovascular diseases.¹³⁾ It is well known that LTC₄ is generated by eosinophils, mast cells, monocytes, and macrophages.¹⁴⁾ In mast cells, the synthesis of LTC₄ and PGD₂ is initiated by the 85 kDa protein cytosolic phospholipase A₂ (cPLA₂), which is specific to phosphoglycerides containing AA in the *sn*-2 position.¹⁵⁾ The enzymatic activity of cPLA₂ is increased through phosphorylation by mitogen-activated protein kinases (MAPKs), such as extracellular regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAP kinase.^{16,17)} Previously, some groups reported that luteolin-7-*O*-glucoside (3',4',5'-hydroxylflavone-7-*O*-glucoside, L7G) from dandelion flower sup-

presses expression of inducible nitric oxide synthase (iNOS) and COX-2 protein in lipopolysaccharide (LPS)-stimulated RAW264.7 cells,¹⁸⁾ whereas others reported that L7G from leaves of *Salix matsudana* inhibits COX-2 activity.¹⁹⁾ L7G from *Buddleja globosa* also shows *in vivo* anti-inflammatory activity and protective effects against gastritis, esophagitis,^{20,21)} and cardiovascular diseases.²²⁾ Quite recently, our group reported anti-asthmatic activity for L7G from *Ailanthus altissima* in an ovalbumin (OVA)-induced murine asthmatic model.²³⁾ However, the molecular action mechanism of L7G in inflammatory cells such as mast cells has not been elucidated sufficiently. Therefore, the objective of this study was to characterize the action mechanism underlying the inhibition of LTC₄ generation and degranulation in activated bone marrow-derived mast cells (BMMCs).

MATERIALS AND METHODS

Material Luteolin-7-*O*-glucoside (L7G) was isolated from a leaf and branch of *Ailanthus altissima* according to our previous report.²³⁾ L7G was prepared by dissolving in dimethyl sulfoxide (DMSO), and final concentrations of DMSO were adjusted to 0.1% (v/v) in culture media. Control with DMSO alone was run in all cases.

Reagents Recombinant mouse KL was purchased from STEMCELL Technologies Inc. (Vancouver, BC, Canada). The rabbit polyclonal antibodies specific for phospho-ERK1/2, ERK1/2, phospho-Akt, phospho-p38, p38, phospho-JNK, JNK, phospho-PLC γ 1, β -actin and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody were purchased from Cell Signaling Technology, Inc. (Danvers, MA, U.S.A.). Rabbit polyclonal antibody for p-cPLA₂, PLC γ 1 and cPLA₂ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The ECL Western blot detection reagent was purchased from Amersham Biosciences,

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Inc. (Piscataway, NJ, U.S.A.). RPMI-1640, Modified Eagle medium (MEM), non-essential amino acid solution, and penicillin-streptomycin were acquired from Hyclone (Logan, Utah, U.S.A.) and GIBCO-BRL (Carlsbad, California, U.S.A.).

Preparation and Activation of Bone Marrow-Derived Mast Cells (BMMCs) Bone marrow cells from male Balb/c mice were cultured for up to 10 weeks in 50% enriched medium (RPMI 1640 containing 100 U/ml of penicillin, 100 μ g/ml of streptomycin, 100 μ M MEM non-essential amino acid solution and 10% fetal bovine serum (FBS)) and 20% pokeweed mitogen-stimulated spleen condition medium (PWM-SCM) as a source of IL-3. After 3 weeks more than 99% of the cells were found to be BMMCs when checked by the previously described procedure.²⁴⁾

Determination of LTC₄ The BMMCs suspended in enriched medium at a density of 1×10^6 cells/ml were seeded in 96 well plate and cells were pretreated for 1 h with L7G at various concentration prior to 15 min at 37 °C of stimulation with KL (30 ng/ml). After 15 min stimulation, the supernatants were immediately utilized for LTC₄ determination. LTC₄ was determined using enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical, Ann Arbor, MI, U.S.A.) accordance with the manufacturer's protocols. Under the conditions employed, LTC₄ reached approximately 1.32 ng/ 10^6 cells. All data was the arithmetic mean of triplicate determinations.

Assay of β -Hexosaminidase Release β -Hexosaminidase (β -HEX), a marker of mast cell degranulation, was quantified by spectrophotometric analysis of the hydrolysis of substrate (*p*-nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside, Sigma-Aldrich). Briefly, 25 μ l of cell-free supernatants were mixed with 50 μ l of substrate in 0.1 M citrate, pH 4.5. After incubation for 1 h at 37 °C, 175 μ l of stop solution (0.2 M NaOH-glycine) was added to stop the reaction, and absorbance was measured at 405 nm. Values were expressed as percentage release relative to total β -HEX in the cells. All data was the arithmetic mean of triplicate determinations.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Immunoblot Analysis BMMCs were seeded on 1.5 ml micro E-tubes (1×10^6 cells/tube) and pretreated with various concentration of L7G or MAPKs inhibitors for 1 h and more treated with KL for 10 min. The cells were then washed and scraped into cold phosphate-buffered saline (PBS) and centrifuged at $500 \times g$ at 4 °C. The cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 1 mM PMSF, 1% NP40, 0.5% protease inhibitor cocktail (Merck, Darmstadt, Germany), 1 mM DTT, 1 mM NaF, 1 mM Na₃VO₄) and centrifuged to yield whole-cell lysates. The proteins (30 μ g of lysates) were then separated via 10% SDS-PAGE and transferred to nitrocellulose membranes in 20% methanol, 25 mM Tris, and 192 mM glycine (Schleicher and Schull, Dassel, Germany). The nitrocellulose membranes were then blocked via incubation in TTBS (25 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween 20) containing 5% nonfat milk. The membranes were subsequently incubated first with a variety of antibodies overnight, washed, and finally incubated for 1 h with a secondary antibody conjugated to horseradish peroxidase. The protein bands were then visualized with an en-

hanced chemiluminescence (ECL) system (Amersham Biosciences, Piscataway, NJ, U.S.A.).

Statistical Analysis All values are presented as arithmetic means $\pm$ S.D. from 3 separate experiments. One-way ANOVA was utilized to determine the statistical significance. Linear regression analysis was conducted to calculate the IC₅₀ value.

RESULTS

Effect of L7G on Degranulation in KL-Induced BMMCs We first measured the cytotoxicity of L7G in BMMCs by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay and found that cell viability was not significantly affected by L7G up to 100 μ M. Therefore, L7G at concentrations lower than 50 μ M were used for subsequent experiments. To investigate the effect of L7G on degranulation in KL-induced BMMCs, we measured the release of β -HEX, which is a degranulation marker in mast cells. BMMCs were pre-treated with various concentrations of L7G for 1 h, followed by incubation for 15 min with KL. As shown in Fig. 1, L7G caused the dose-dependent inhibition of β -HEX release with an IC₅₀ value of 17.7 μ M. Under the same conditions, the rate of β -HEX release was about 20–25% without L7G pretreatment.

Effect of L7G on Phosphorylation of PLC γ 1 in KL-Induced BMMCs Phosphorylation of PLC γ 1 triggers the release of inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DG) which leads to increase of cytosolic Ca²⁺ concentration. Since this event is essential for the release of various mediators, including histamine, eicosanoids and cytokines from mast cells.²⁵⁾ Thus we investigate the effect of L7G on the phosphorylation of PLC γ 1 by KL-induced BMMCs. BMMCs were pre-incubated with L7G for 1 h and stimulated with KL for 5 min. As shown in Fig. 2, L7G dose dependently inhibited the phosphorylation of PLC γ 1 level, which is downstream signaling via membrane Kit. It is therefore that the inhibition of degranulation by L7G might be through the suppression of PLC γ 1 phosphorylation.

Effect of L7G on LTC₄ Generation in KL-Induced BMMCs Arachidonic acid can be converted into leukotrienes (LTs) by the action of 5-LOX in BMMCs. Inhibition of 5-LOX is believed to be the ideal treatment for al-

Fig. 1. Effects of L7G on β -HEX Release in KL-Treated BMMCs

Cells were pretreated with various concentrations of L7G for 1 h and then incubated for 15 min with KL (30 ng/ml). After incubation, β -HEX release in the medium was determined. The values are expressed as the means $\pm$ S.D. of three independent experiments. *** $p < 0.001$ versus without L7G.

Fig. 2. Effects of L7G on KL-Induced PLC- γ Phosphorylation in BMMCs

The BMMCs were pretreated with L7G (2, 10, 50 μ M) and wortmannin (1 μ M) for 5 h and then the cells were incubated for an additional 5 min with KL (30 ng/ml). The cells were harvested for measurements of total or phosphorylated PLC- γ by Western blot analysis.

Fig. 3. Effects of L7G on LTC₄ Production in KL-Induced BMMCs

Cells were pretreated with various concentrations of L7G for 1 h and then incubated for 15 min with KL (30 ng/ml). After incubation, the LTC₄ production in the medium was determined. The values are expressed as the means $\pm$ S.D. of three independent experiments. ** p < 0.01, *** p < 0.001 versus without L7G.

lergic diseases and asthma.²⁶⁾ We therefore examined the inhibitory activity of L7G on the generation of LTC₄ in BMMCs. As shown in Fig. 3, BMMCs were pre-incubated with L7G for 1 h and stimulated with KL for 15 min. As shown in Fig. 3, L7G inhibited LTC₄ production in a dose-dependent manner with an IC₅₀ value of 3.0 μ M.

Effect of L7G on Phosphorylation of cPLA₂ in KL-Induced BMMCs The formation of PGD₂ and LTC₄ was initiated by 85-kDa calcium dependent cytosolic phospholipase A₂ (cPLA₂) which release arachidonic acid (AA) in mast cells.¹⁵⁾ Thus, we investigated whether or not L7G affects phosphorylation of cPLA₂ by Western blotting. BMMCs were pre-incubated for 1 h with different concentrations of L7G and the cells were incubated for an additional 10 min with KL (100 ng/ml). As shown in Fig. 4, phospho-cPLA₂ level was significantly increased after stimulate with KL. When BMMCs were pretreated with the L7G or MAPKs inhibitors such as U0126 (ERK inhibitor), SP600125 (JNK inhibitor) and SB203580 (p38 inhibitor), both L7G and MAPKs inhibitors attenuated cPLA₂ phosphorylation. The amount of β -actin protein which was used as an internal control, remained unchanged.

Fig. 4. Effects of L7G on KL-Induced cPLA₂ Phosphorylation in BMMCs

BMMCs were pre-incubated with L7G (2, 10, 50 μ M) or MAPK inhibitors for 1 h and then cells were incubated for 10 min with KL (30 ng/ml). The phosphorylation of cPLA₂ was evaluated via Western blot analysis. Data are representative of results from three independent experiments. * p < 0.05, ** p < 0.01 versus L7G.

Fig. 5. Effects of L7G on KL-Induced MAPKs Phosphorylation in BMMCs

BMMCs were pretreated with L7G (2, 10, 50 μ M), U0126 (50 μ M), SP600125 (25 μ M) and SB203580 (30 μ M) for 1 h and then stimulated with KL (30 ng/ml) for 10 min. The phosphorylation of MAPKs were determined by Western blot analysis. Similar results were observed in three independent experiments.

Effect of L7G on Phosphorylation of MAPKs in KL-Induced BMMCs The enzymatic activity of cPLA₂ is increased through phosphorylation by MAPKs.^{16,17)} We therefore investigated the effects of L7G and the three MAPK inhibitors on the KL-induced phosphorylation of ERK1/2, JNK, and p38 in BMMCs by Western blotting. As shown in Figs. 5A, B, and C, BMMCs pre-incubated with various concentrations of L7G and MAPK inhibitors for 1 h followed by stimulation with KL for 10 min revealed suppressed MAPK phosphorylation. This result clearly demonstrates that L7G inhibited the phosphorylation of ERK1/2, JNK, and p-38 MAPKs. Therefore, it was postulated that L7G inhibits cPLA₂ phosphorylation via MAPK activation.

DISCUSSION

Numerous studies have demonstrated that certain flavonoids may contribute to anti-inflammatory activity by inhibiting arachidonic acid (AA) cascade-related enzymes such as PLA₂, COX, and LOXs. Previously, we reported that the anti-asthmatic activity of L7G from *Ailanthus altissima* is mediated through the down-regulation of T helper 2 cytokine expression and inhibition of PGE₂ production in an OVA-induced asthma model.²³ However, the underlying mechanisms for the anti-asthmatic activity of L7G have not been elucidated sufficiently at the cellular level. Therefore, we investigated the effects of L7G on degranulation and LTC₄ production in KL-induced BMMCs. In the present study, we found that L7G attenuated both degranulation and LTC₄ production in KL-induced BMMCs. c-Kit and its ligand KL are critical regulate mast cell migration, growth, survival and differentiation. Activation of Kit receptor in mast cells stimulates cellular responses such as proliferation, survival, differentiation, migration and increase serotonin and histamine release.^{27–29} KL induces Kit dimerization, autophosphorylation, and transphosphorylation of Kit at specific tyrosine residues within the receptor chains, thus allowing recruitment of SH2 domain-containing signaling molecules comprising PLC γ . This results in the activation of IP₃, followed by an increase in the intracellular Ca²⁺ level. This Ca²⁺ influx is followed by degranulation and production of inflammatory mediators such as PGD₂ and LTC₄.^{6,7} Our present data show that L7G markedly decreased β -HEX release (Fig. 1) and significantly inhibited tyrosine phosphorylation of PLC γ 1 induced by KL (Fig. 2). We, therefore, conclude that L7G reduced β -HEX release through inhibition of PLC γ 1 activation.

Leukotrienes (LTs) are important lipid mediators that are involved in asthma, inflammation, and cardiovascular diseases and osteoporosis.^{8–13} When activated by KL or IgE/Ag stimulation, mast cells release AA through cPLA₂, rapidly generating both PGD₂ and LTC₄.^{2,3} Consistent with a previous report,¹⁸ we found that COX-2-dependent PGD₂ production was inhibited by L7G, with concomitant suppression of COX-2 protein expression in activated mast cells (data not shown). As shown in Fig. 3, L7G inhibited 5-LOX-dependent LTC₄ production in a dose-dependent manner.

Production of LTC₄ in mast cells is regulated in two steps. The first step is the liberation of AA from membrane phospholipids by cPLA₂ while the other is the oxygenation of free AA by 5-LOX/LTC₄ synthase.^{30,31} Both 5-LOX and cPLA₂ are translocated from the cytosol to the perinuclear membrane in response to increased cytosolic Ca²⁺, which allows access to phospholipids.³² Additionally, cPLA₂ is phosphorylated by MAPKs, a process that is necessary for maximum AA release.³³ Phosphorylation of cPLA₂ by MAPKs (at a S505 site) can augment activity 3- to 4-fold but is not required for membrane association.^{17,34,35} The role of MAPKs in cPLA₂ phosphorylation was confirmed by concurrent KL-induced increases in the phosphorylation of ERKs, JNK, and P38. As shown in Fig. 4, both L7G and MAPK inhibitors attenuated the phosphorylation of cPLA₂. This result clearly demonstrated that L7G inhibited LTC₄ production *via* a phosphorylation of cPLA₂ and this activation are associated with MAPKs activation. Next, we investigated whether or not L7G affects MAPK phosphorylation in KL-induced

BMMCs. As shown in Fig. 5, all three MAPKs were markedly phosphorylated after KL activation. Remarkably, L7G blocked the phosphorylation of all MAPKs in a dose-dependent manner. The suppressive effect of the highest concentration of L7G on ERK1/2, JNK, and p38 phosphorylation was comparable to those of U0126, SP600125, and SB203580. Degranulation and LTC₄ production are associated with intracellular Ca²⁺ influx and translocation of phospho-cPLA₂ and 5-LOX, respectively. Therefore, the effect of L7G on these responses should be investigated in the near future.

In conclusion, our findings have shown that the anti-asthmatic activity of L7G is mediated in part *via* the inhibition of degranulation and LTC₄ production in activated mast cells.

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