

Pinusolide Isolated from *Biota orientalis* Inhibits 5-Lipoxygenase Dependent Leukotriene C₄ Generation by Blocking c-Jun N-Terminal Kinase Pathway in Mast Cells

Ye Jin,^a Hyun Ok Yang,^b Jong Keun Son,^a and Hyeun Wook Chang^{*,a}

^a College of Pharmacy, Yeungnam University; Gyeongsan 712–749, Republic of Korea; and ^b Natural Medicine Center, Korea Institute of Science and Technology; Gangneung, Gangwon-do 210–340, Republic of Korea.

Received March 22, 2012; accepted May 11, 2012

Pinusolide, an herbal medicine isolated from *Biota orientalis* L. (*B. orientalis*), inhibited 5-lipoxygenase (5-LO)-dependent leukotriene C₄ (LTC₄) generation in immunoglobulin E (IgE)/Ag-induced bone marrow-derived mast cells (BMMCs) in a concentration-dependent manner. To clarify the action mechanism of pinusolide on the inhibition of LTC₄ generation, we examined the effect of pinusolide on phosphorylation of cytosolic phospholipase A₂ (cPLA₂), as well as translocation phospho-cPLA₂ and 5-LO to nucleus. Inhibition of LTC₄ generation by pinusolide was accompanied by a decrease in cPLA₂ phosphorylation which occurred via a decrease in intracellular Ca²⁺ influx and blocking the c-Jun N-terminal kinase (JNK) pathways. However, pinusolide had no effect on extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein (MAP) kinase phosphorylation. Taken together, the present results suggest that potent inhibition of 5-LO dependent LTC₄ generation by pinusolide requires both suppression of calcium influx and JNK phosphorylation.

Key words *Biota orientalis*; pinusolide; bone marrow-derived mast cell; leukotriene C₄; cytosolic phospholipase A₂; c-Jun N-terminal kinase

Mast cells are one of the most important effector cells in immediate-type allergic reactions. Aggregation of the high-affinity immunoglobulin E (IgE) receptor (FcεRI) on mast cells triggers release of preformed mediators such as histamine and proteases as well as newly synthesized inflammatory mediators (e.g. lipid mediators, such as prostaglandin D₂ (PGD₂) and leukotriene C₄ (LTC₄), and various cytokines).^{1–3} The synthesis of prostaglandins (PGs) and leukotrienes (LTs) in mast cells is initiated by release of free arachidonic acid (AA) from membrane phospholipid by cytosolic phospholipase A₂ (cPLA₂). 5-Lipoxygenase (5-LO) catalyzes the conversion of free arachidonic acid (AA) into LTA₄ and it is further converted to LTB₄ by LTA₄ hydrolase, and LTC₄ by LTC₄ synthase. LTB₄ is considered as a potent chemoattractant for neutrophils and LTC₄, LTD₄ and LTE₄ are potent mediators of asthma, inflammation and atherosclerotic disease.⁴ In resting mast cells, 5-LO exists in both the cytosol and nucleus. Upon mast cells activation by IgE–antigen complexes or other stimuli, both cytosolic and soluble 5-LO can translocate to the nuclear envelope, leading to colocalization with cytosolic phospholipase A₂ (cPLA₂) and 5-LO activating protein (FLAP). For generation of LTC₄ in mast cells, it requires two regulation steps. One is the release of AA from phospholipids by cPLA₂ and oxygenation of AA by 5-LO. The other is the modulation of phosphorylation of cPLA₂ by MAP kinase and translocation of both cPLA₂ and 5-LO to nuclear membrane.⁵

Dried leaves of *Biota orientalis* L. (Cupressaceae) have been used as Korean folk medicine for treating gout, rheumatitis, and diarrhea, all implying anti-inflammatory functions. Previous chemical studies of *B. orientalis* have reported a large number of terpenes, flavonoids and phenolics.^{6,7} Isolated diterpene such as pinusolide have been reported to exert the neuroprotective activity and platelet activating factor (PAF)

antagonistic activity.^{8–10} However, the effect of pinusolide on the production of LTC₄ generation, a potent inflammatory mediator in activated mast cells has not been studied to date. In this study, we evaluated the effect of pinusolide on IgE/Ag-induced 5-LO dependent LTC₄ generation and clarified its mode of action in bone marrow-derived mast cells (BMMCs).

MATERIALS AND METHODS

Reagents The rabbit polyclonal antibodies specific for phospho-extracellular signal-regulated kinase (ERK)1/2, ERK1/2, phospho-p38, p38, phospho-c-Jun N-terminal kinase (JNK), JNK, phospho-Akt, Akt and β-actin were purchased from Cell Signaling Technology, Inc. (Danvers, MA, U.S.A.). Rabbit polyclonal antibody for phospho-cPLA₂ (Ser505), cPLA₂ and 5-LO as well as secondary goat anti-rabbit IgG-horse-radish peroxidase (HRP) and rabbit anti-goat IgG-HRP antibodies, lamin B, total Syk, total LAT were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and antibodies for phosphotyrosine was purchased from Millipore (Millipore, Billerica, MA, U.S.A.). U0126, SB203580 and SP600125 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The antibody-reactive bands were visualized with an enhanced chemiluminescence (ECL) system (Pierce Biotechnology, Rockford, IL, U.S.A.).

Plant Materials Pinusolide (Fig. 1A) was isolated from the methanol extract of the leaves of *B. orientalis* and structurally identified according to the previous report. This compound was prepared by dissolving it in dimethyl sulfoxide (DMSO) and the final concentration of DMSO was adjusted to 0.1% (v/v) in culture media. Control experiments indicated that DMSO at this concentration had no effect on LTC₄ generation.

Preparation and Activation of Mouse BMMCs BMMCs isolated from male Balb/cJ mice (Sam Taco, Inc., Seoul,

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: hwchang@yu.ac.kr

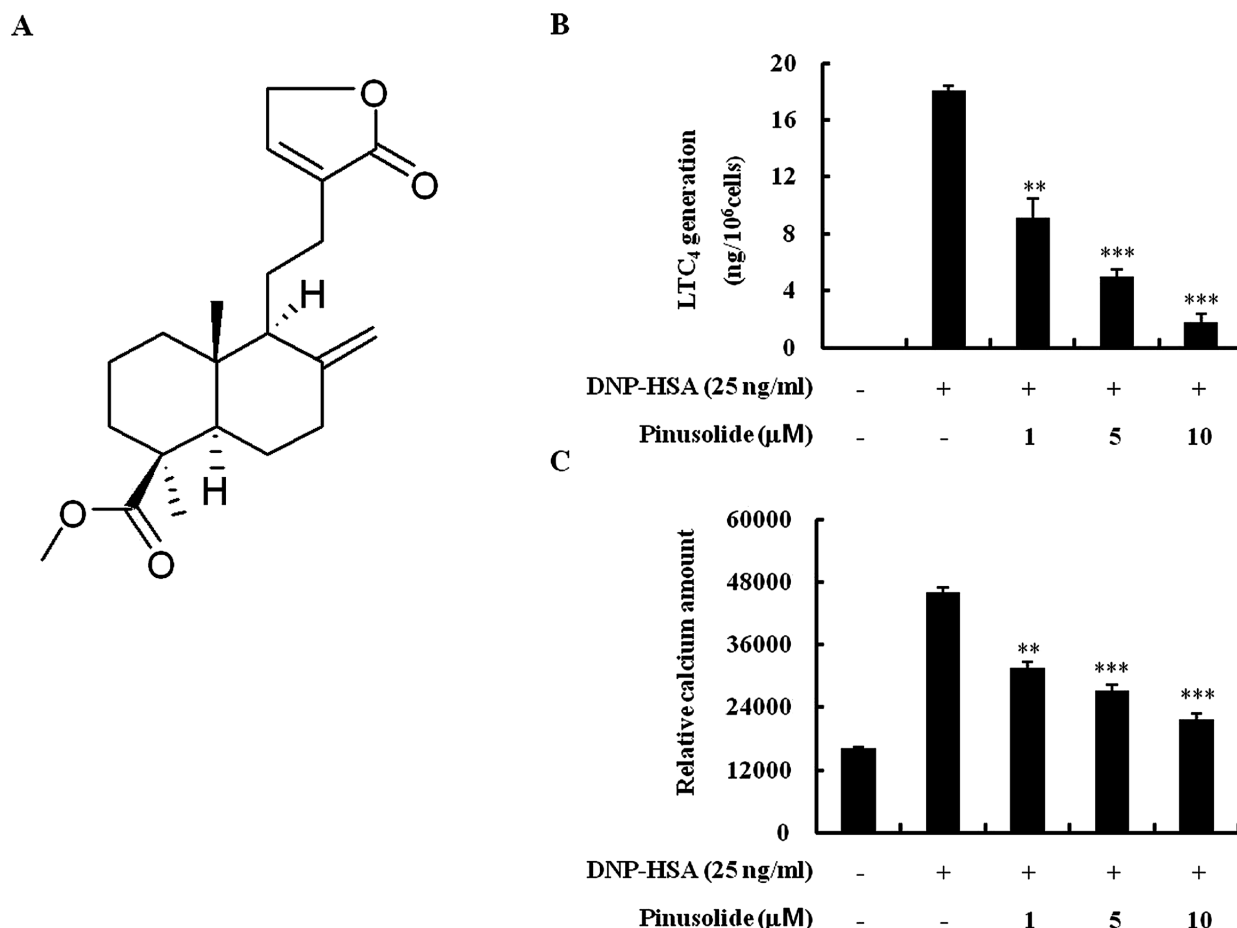


Fig. 1. Chemical Structure of Pinusolide and the Effect of Pinusolide on LTC₄ Generation and Ca²⁺ Mobilization in IgE/Ag-Activated BMMCs

DNP-IgE sensitized BMMCs were pre-incubated with the indicated concentration of pinusolide (A) for 1 h, after then were stimulated with DNP-HSA for 15 min (B) LTC₄ released into the supernatant was quantified using an enzyme immunoassay kit. (C) Relative intracellular Ca²⁺ levels were determined (at 5 min). All data are the arithmetic mean of triplicate determinations. ** $p < 0.01$ and *** $p < 0.001$ indicate significant differences compared to the IgE/Ag-treated group.

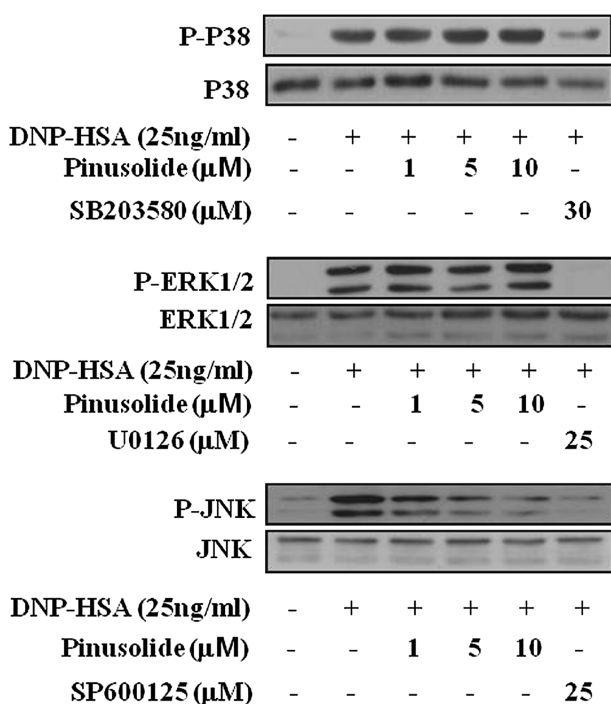
Korea) were cultured in RPMI 1640 media (Thermo Scientific, Utah, U.S.A.) containing 10% FBS, 100 U/mL penicillin (Thermo Scientific, Utah, U.S.A.), 10 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (Sigma-Aldrich, St. Louis, MO, U.S.A.), 100 μM MEM non-essential amino acid solution (Invitrogen, NY, U.S.A.) and 20% pokeweed mitogen-spleen cell conditioned medium (PWM-SCM) as a source of IL-3. After 4 weeks, >98% of the cells were found to be BMMCs. For cell stimulation, 10⁶ cells/mL cells were sensitized overnight with 100 ng/mL of mouse anti-dinitrophenyl (DNP) IgE (Sigma-Aldrich, St. Louis, MO, U.S.A.) and then stimulated for 15 min with 25 ng/mL of DNP-HSA (human serum albumin, Sigma-Aldrich).

Determination of Leukotriene C₄ (LTC₄) IgE-sensitized BMMCs suspended in enriched medium at a cell density of 1×10⁶ cells/mL were pretreated with pinusolide or three mitogen activated protein kinases (MAPKs) inhibitors for 1 h at 37°C and stimulated with or without 25 ng/mL DNP-HSA. After 15 min of stimulation, the supernatants were isolated for further analysis by EIA. LTC₄ was determined using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, U.S.A.) according to the manufacturer's instructions. Under the conditions employed, LTC₄ reached approximately 18 ng/10⁶ cells. All data were the arithmetic mean of triplicate determinations.

Measurement of Intracellular Ca²⁺ Level Intracellular Ca²⁺ levels were determined with FluoForte™ Calcium assay kit (Enzo Life Sciences, Ann Arbor, MI, U.S.A.). IgE-sensitized BMMCs were preincubated with FluoForte™ Dye-Loading solution for 1 h at room temperature. After washing the dye from the cell surface with phosphate buffered saline (PBS), BMMCs (5×10⁴) were seeded into 96-well microplates. Then the cells were pretreated with pinusolide for 30 min before adding DNP-HSA. The fluorescence was measured using a fluorometric imaging plate reader at an excitation of 485 nm and an emission of 520 nm (BMG Labtechnologies FLUOStar OPITIMA plate reader, Offenbourg, Germany). All assay were independently repeated at least three times.

Immunoprecipitation (IP) Cell lysates were collected using modified lysis buffer (0.1% Nonidet P-40, 50 mM HEPES (pH 7.0), 250 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 mM dithiothreitol (DTT)). One milligram protein of total cell lysate was incubated with anti-Syk or LAT antibody for 2 h at 4°C and the immunocomplexes were precipitated with 20 μL of protein A-Sepharose. These precipitates were extensively washed 3 times with ice-cold lysis buffer. These precipitates or total cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with indicated antibodies.

A



B

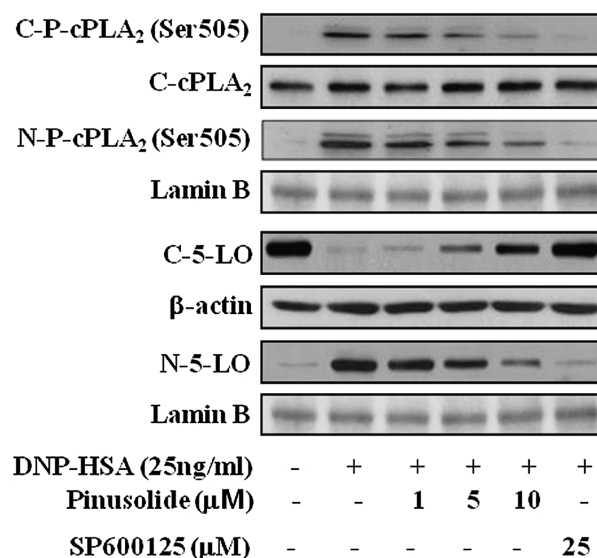


Fig. 2. Effects of Pinusolide or JNK Inhibitor on cPLA₂ Phosphorylation, and Translocation of Phospho-cPLA₂ and 5-LO in IgE/Ag-Induced BMMCs

(A) BMMCs were pre-incubated with indicated concentration of pinusolide or 30 μM SB203580 (p38 inhibitor), 25 μM SP600125 (JNK inhibitor) and 25 μM U0126 (ERK1/2 inhibitor) for 1 h and then stimulated with DNP-HSA for 15 min. Cells were harvested for measurements of total or phosphorylated ERK1/2, JNK, and p38 by Western blot analysis. (B) After preincubation with pinusolide or SP600125 for 1 h, IgE-sensitized BMMCs were challenged with DNP-HSA for 15 min, then cytosolic and nuclear lysates were immunoblotted with anti-phospho cPLA₂ anti-5-LO antibody. The results from three separate experiments are presented.

Preparation of Nuclear and Cytosolic Extracts Cells were suspended in wash buffer containing 10 mM HEPES buffer (pH 8.0), 1.5 mM MgCl₂, 10 mM KCl, 1 mM PMSF and 1 mM protease inhibitor cocktail (Merck Biosciences, Darmstadt, Germany) and then lysed in wash buffer containing 0.1% (v/v) NP40 by incubating on ice for 10 min. After centrifugation at 1000×g for 4 min, supernatants were used as a cytosolic fraction. Nuclear pellets were washed and re-suspended in a buffer containing 20 mM HEPES (pH 8.0), 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and the protease inhibitor cocktail. This suspension was incubated for 30 min at 4°C followed by centrifugation at 10000×g, and the resultant supernatants were used as a nuclear fraction.

Western Blot Analysis IgE-sensitized mast cells were stimulated with Ag for indicated times with or without pinusolide or other inhibitors. BMMCs (2×10⁶) were then applied to an 8% (w/v) SDS-PAGE under reducing conditions, transferred onto nitrocellulose membranes, and were subjected to immunoblotting. Primary antibodies used (1:1000–3000 dilution) were as follows: antibodies against phosphorylated forms of extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun NH₂-terminal kinase (JNK), p38, those against ERK1/2, JNK and p38, or those against β-actin, lamin B, cPLA₂, phospho-cPLA₂ and 5-LO. The immunoreactive proteins were incubated with the use of HRP-coupled secondary antibodies diluted 1:3000 for 1 h at room temperature. The antibody-reactive bands were visualized with an ECL system (Pierce Biotechnology, Rockford, IL, U.S.A.).

Statistical Analysis All values are presented as the

arithmetic means±S.D. from 3 separate experiments. One-way analysis of variance (ANOVA) was utilized to determine the statistical significance.

RESULTS AND DISCUSSION

Several reports have documented that pinusolide, a plant diterpene, has potent anti-inflammatory properties both in *in vitro* and *in vivo*.^{6–10} However, little is known about the inhibitory mechanisms of pinusolide on the generation of the lipid inflammatory mediators, LTC₄. The aims of this study was to identify the effect of pinusolide on IgE/Ag-induced 5-LO dependent LTC₄ generation in BMMCs. At first, we determined the cytotoxicity of pinusolide on BMMCs using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and found that cell viability was not affected by this compound up to 20 μM (data not shown). Therefore, the concentration of pinusolide used in the experiments was below 20 μM and used for all subsequent experiments. We investigated the inhibitory activity of pinusolide on the generation of LTC₄ in BMMCs. As shown in Fig. 1B, pinusolide markedly inhibited LTC₄ biosynthesis in a dose-dependent manner. It has been reported that an increase in intracellular Ca²⁺ potentially stimulates leukotriene biosynthesis in purified human leukocytes.¹¹ We next investigated the effect of pinusolide on the intracellular Ca²⁺ influx in BMMCs. As shown in Fig. 1C, pinusolide markedly inhibited Ca²⁺ influx in a dose-dependent manner. This result suggests that the inhibition of LTC₄ by pinusolide may through the involvement of Ca²⁺ influx.

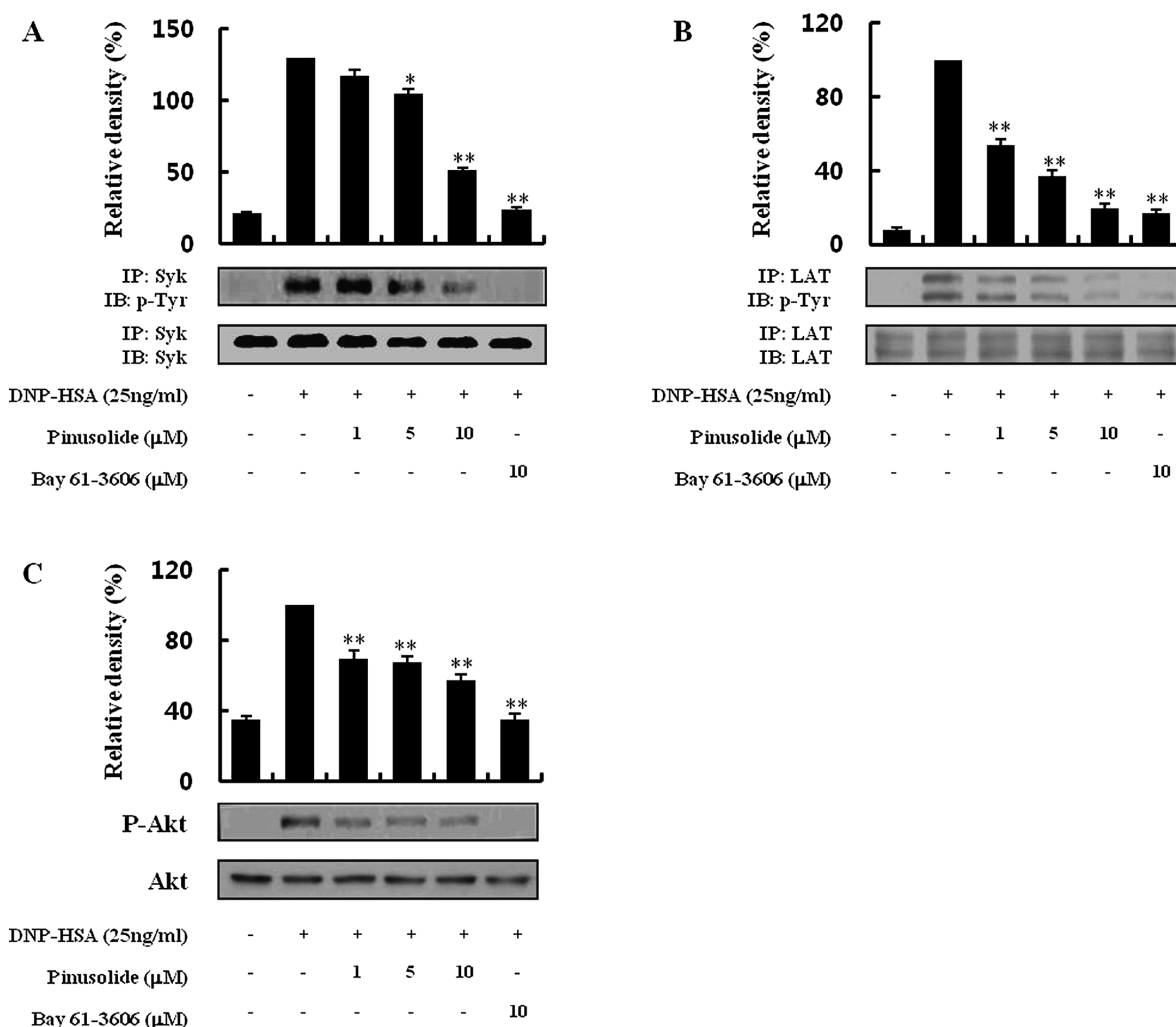


Fig. 3. Effect of Pinusolide and Bay61-3606 on Syk-Associated Pathway

After preincubated with pinusolide or Bay61-3606 1h, IgE sensitized BMMCs were stimulated with DNP-HSA for 15 min. The cell lysates were used in immunoprecipitation and immunoblotting analysis to detect the phosphorylated forms of Syk, LAT and Akt. The relative ratios of p-Syk/Syk, p-LAT/LAT and p-Akt/Akt protein levels were determined by measuring immunoblot band intensities by scanning densitometry (* $p < 0.05$ and ** $p < 0.01$). The results from three separate experiments as relative ratios (%) are represented.

Recently, we documented that three MAP kinases were phosphorylated within 10–15 min in IgE/Ag-induced BMMCs.¹²⁾ To explore the effect of pinusolide on phosphorylation of three MAP kinase families in IgE/Ag-induced BMMCs, cells were pretreated with indicated concentration of pinusolide or MAP kinase inhibitors such as U0126, SP600125 and SB203580 for 1h prior to stimulation with DNP-HSA for 15 min. As shown in Fig. 2A, all three inhibitors strongly inhibited MAP kinase phosphorylation, respectively. However, pinusolide attenuated only phosphorylation of JNK, but did not affect ERK1/2 and p38. These results indicate that the inhibition of JNK phosphorylation was correlated with the suppression of 5-LO dependent LTC₄ generation in BMMCs. To further investigate 5-LO dependent LTC₄ generation, we next performed the effects of pinusolide or SP600125 on the phosphorylation of cPLA₂, in addition to translocation of phospho-cPLA₂ and 5-LO to nucleus using Western blot analysis. As shown in Fig. 2B, the majority of cPLA₂ resided in the cytosol regardless of IgE/Ag stimulation, while the level phosphorylated cPLA₂ was detected in both the cytosol (C-p-cPLA₂) and nuclear fraction (N-p-cPLA₂) in IgE/Ag-induced BMMCs.

Pinusolide suppressed the level of C-p-cPLA₂ and N-p-cPLA₂ in a dose-dependent manner, whereas 25 μ M SP600125 almost completely inhibited this reaction. This result demonstrated that pinusolide inhibited LTC₄ generation through the phosphorylation of cPLA₂ and its translocation to the nucleus was associated with JNK activation. Next, we investigated whether pinusolide or SP600125 affected translocation of 5-LO into the nucleus. As shown in Fig. 2B, 5-LO was detected mainly in the cytosol (C-5-LO), and a large amount of C-5-LO translocated to the nucleus (N-5-LO) after IgE/Ag stimulation. Pinusolide clearly inhibited translocation of 5-LO to nucleus in a dose-dependent manner. Under the same condition, SP600125 completely suppressed 5-LO translocation.

It is well known that the tyrosine kinase Syk plays an essential role in the initiation of IgE-dependent mast cells activation and mediates linker for activation of T cells (LAT), resulting in activation of downstream signaling including MAPKs, PI3K and phospholipase C γ (PLC γ). PLC γ -mediated Ca²⁺ mobilization is essential for subsequent degranulation and LTC₄ generation.¹²⁾ Thus we examined whether pinusolide or Syk inhibitor, affects phosphorylation of Syk and

downstream signal molecules such as LAT and Akt. As shown in Fig. 3, pinusolide significantly inhibited phosphorylation of Syk, LAT and Akt in a dose-dependent manner. Bay 61-3606, the Syk inhibitor used as a positive control, almost completely inhibited the phosphorylation of Syk, LAT and Akt.

In summary, we have demonstrated for the first time that inhibition of FcεRI-mediated LTC₄ generation by pinusolide in BMMCs might be attributed mainly to blocking the influx of intracellular Ca²⁺ and thus this inhibition was associated with the suppressive effect of cPLA₂ phosphorylation, and translocation of cPLA₂ and 5-LO to the nucleus *via* modulation of Syk dependent pathway.

Acknowledgements This work was supported by the National Research Foundation of Korea Grant funded by the Korea government (NRF-2010-616-E00011).

REFERENCES

- 1) Murakami M, Kudo I. Diversity and regulatory functions of mammalian secretory phospholipase A2s. *Adv. Immunol.*, **77**, 163–194 (2001).
- 2) Kimura Y, Okuda H, Baba K. Histamine-release effectors from *Angelica dahurica* var. *dahurica* root. *J. Nat. Prod.*, **60**, 249–251 (1997).
- 3) Lewis RA, Austen KF, Soberman RJ. Leukotrienes and other products of the 5-lipoxygenase pathway. Biochemistry and relation to pathobiology in human diseases. *N. Engl. J. Med.*, **323**, 645–655 (1990).
- 4) Werz O, Steinhilber D. Therapeutic options for 5-lipoxygenase inhibitors. *Pharmacol. Ther.*, **112**, 701–718 (2006).
- 5) Werz O. 5-Lipoxygenase: cellular biology and molecular pharmacology. *Curr. Drug Targets Inflamm. Allergy*, **1**, 23–44 (2002).
- 6) Yang HO, Kang YH, Suh DY, Kim YC, Han BH. Biological and pharmacological effects of pinusolide, a novel platelet activating factor antagonist. *Planta Med.*, **61**, 519–522 (1995).
- 7) Zhu JX, Wang Y, Kong LD, Yang C, Zhang X. Effects of *Biota orientalis* extract and its flavonoid constituents, quercetin and rutin on serum uric acid levels in oxonate-induced mice and xanthine dehydrogenase and xanthine oxidase activities in mouse liver. *J. Ethnopharmacol.*, **93**, 133–140 (2004).
- 8) Yang HO, Suh DY, Han BH. Isolation and characterization of platelet-activating factor receptor binding antagonists from *Biota orientalis*. *Planta Med.*, **61**, 37–40 (1995).
- 9) Kim KA, Moon TC, Lee SW, Chung KC, Han BH, Chang HW. Pinusolide from the leaves of *Biota orientalis* as potent platelet activating factor antagonist. *Planta Med.*, **65**, 39–42 (1999).
- 10) Koo KA, Sung SH, Kim YC. A new neuroprotective pinusolide derivative from the leaves of *Biota orientalis*. *Chem. Pharm. Bull.*, **50**, 834–836 (2002).
- 11) Rådmark O, Werz O, Steinhilber D, Samuelsson B. 5-Lipoxygenase: regulation of expression and enzyme activity. *Trends Biochem. Sci.*, **32**, 332–341 (2007).
- 12) Lu Y, Yang JH, Li X, Hwangbo K, Hwang SL, Taketomi Y, Murakami M, Chang YC, Kim CH, Son JK, Chang HW. Emodin, a naturally occurring anthraquinone derivative, suppresses IgE-mediated anaphylactic reaction and mast cell activation. *Biochem. Pharmacol.*, **82**, 1700–1708 (2011).