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15,16-Dihydrotanshinone I suppresses IgE-Ag stimulated mouse bone marrow-derived mast cell activation by inhibiting Syk kinase

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ABSTRACT

Ethnopharmacological relevance: 15,16-Dihydrotanshinone I (DHT-I), isolated from the dried root of *Salvia miltiorrhiza* Bunge, which is traditionally used to treat cardiovascular and inflammatory diseases agent in Chinese medicine. DHT-I has been reported to have a broad range of biological activities, including antibacterial activity, and has been used to treat circulatory disorders, hepatitis, inflammation, cancer, and neurodegenerative diseases.

Aim of the study: The aim of this study was to evaluate the anti-allergic inflammatory effects of DHT-I on degranulation and on the generation of eicosanoids, such as, prostaglandin D₂ (PGD₂) and leukotriene C₄ (LTC₄), in IgE/Ag-stimulated bone marrow-derived mast cells (BMMCs).

Materials and methods: The anti-allergic inflammatory activity of DHT-I was evaluated using BMMCs. The effects of DHT-I on mast cell activation were investigated by following degranulation and eicosanoid generation using ELISA and immunoblotting and immunoprecipitation techniques.

Results: DHT-I at a concentration of 20 μM markedly inhibited degranulation and the generation of PGD₂ and LTC₄ in IgE/Ag-stimulated BMMCs (about 90% inhibitions, respectively). Analyses of FcεRI-mediated signaling pathways demonstrated that DHT-I inhibited the phosphorylations of spleen tyrosine kinase (Syk) and linker for activation of T cells (LAT), and inhibited downstream signaling process, including [Ca²⁺]_i mobilization induced by the phosphorylation of phospholipase Cγ1 (PLCγ1), and the activations of mitogen-activated protein kinases (MAPKs) and the Akt-nuclear factor-κB (NF-κB) pathway.

Conclusions: DHT-I inhibits the release of allergic inflammatory mediators from IgE/Ag-stimulated mast cells by suppressing a FcεRI-mediated Syk-dependent signal pathway. This result suggests DHT-I offers a novel developmental basis for drugs targeting allergic inflammatory diseases.

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Abbreviations: AA, arachidonic acid; BMMCs, bone marrow-derived mast cells; COX-1,-2, cyclooxygenase-1, -2; cPLA₂, cytosolic phospholipase A₂; DMSO, dimethyl sulfoxide; DNP, dinitrophenol; DTT, dithiothreitol; EIA, enzyme immunoassay; ERK1/2, extracellular signal regulated kinase1/2; FBS, fetal bovine serum; Gab2, Grb2-associated binder 2; HSA, human serum albumin; β-Hex, β-hexosaminidase; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; LAT, linker for activation of T cells; LTC₄, leukotriene C₄; 5-LO, 5-lipoxygenase; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; NF-κB, nuclear factor-κB; PI3K, phosphatidylinositol 3-kinase; PGD₂, prostaglandin D₂; PLCγ1, phospholipase Cγ1; PMSF, phenylmethanesulfonylfluoride; SLP-76, SH2 domain-containing leukocyte protein of 76 kDa; Syk, spleen tyrosine kinase

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1. Introduction

Salvia miltiorrhiza Bunge (*S. miltiorrhiza*), is a perennial member of the lamiaceae, which are known to contain tanshinones as main constituents and are widely used in traditional Chinese medicine. *S. miltiorrhiza* has many therapeutic uses to treatment of cardiovascular diseases, hepatitis, inflammation, and cancer (Zhou et al., 2005; Wang, 2010). 15, 16-Dihydrotanshinone I (DHT-I) is a constituent of *S. miltiorrhiza*, and has been reported to have a broad range of biological activities, such as, anti-platelet aggregation (Park et al., 2008), anti-inflammatory (Choi et al., 2004; Lee et al., 2006), anti-tumor (Tsai et al., 2007), and antibacterial activities against a broad range of Gram positive bacteria (Lee et al., 1999).

Mast cells are granulated cells that play a central role in inflammatory and allergic reactions. The crosslinking of IgE bound to its high-affinity receptor, FcεRI, on mast cells by antigen leads to release of potent inflammatory mediators, such as, histamine, proteases, and *de novo* synthesized lipid mediators, such as, prostaglandin D₂ (PGD₂), leukotriene C₄ (LT₄), platelet-activating factor, and various cytokines and chemokines (Murakami et al., 1995; Yamaguchi et al., 1999). The allergen-induced aggregation of FcεRI on mast cells initiates the activations of tyrosine kinases, such as, Syk, Lyn, Fyn, and BTK, and the phosphorylations of various adaptor molecules (Siraganian, 2003; Gilfillan and Rivera, 2009; Kambayashi and Koretzky, 2007). Syk is essential for the activation of downstream signal molecules, such as, phospholipase Cγ1 (PLCγ1), linker for activation of T cells (LAT), SH2 domain-containing leukocyte protein of 76 kDa (SLP-76), Grb2-associated binder 2 (Gab2), and phosphoinositide 3-kinase (PI3K), which are all essential for intracellular calcium mobilization and degranulation (Siraganian et al., 2010). Previously, we and others groups have suggested the inactivation of Syk kinase could suppress IgE/Ag-stimulated degranulation and the synthesis of eicosanoids and pro-inflammatory cytokines (Rossi et al., 2006; Lu et al., 2011, 2012; Li et al., 2014; Lu et al., 2014).

FcεRI signaling also triggers the activation of mitogen-activated protein kinases (MAPKs), such as, extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNKs) and p38, PI3K/Akt, and NF-κB signaling pathways, which eventually contribute to the release of various granule-derived mediators, such as, histamine, serotonin, and serine proteases, and induce the expressions of several pro-inflammatory genes, such as, COX-2 and proinflammatory cytokines, which are required for the propagation of inflammation (Tak and Firestein, 2000; Lawrence et al., 2001). In addition, MAPKs play crucial roles in the activation of cytosolic phospholipase A₂α (cPLA₂α), which is essential for the release of arachidonic acid from membrane phospholipid, a common precursor of PGD₂ and LTC₄ (Lin et al., 1993; Lu et al., 2011, 2012; Li et al., 2014; Lu et al., 2014). However, the effect of DHT-I on the IgE/Ag-stimulated mast cells has not been studied.

In this study, we examined the effect of DHT-I on mast cell mediator release after IgE-Ag activation in bone marrow-derived mast cells (BMMCs).

2. Materials and Methods

2.1. Chemicals

Mouse anti-dinitrophenyl (DNP) IgE and DNP-human serum albumin (HSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The rabbit polyclonal antibodies specific for phospho-IκBα, IKKα/β, ERK1/2, JNK, p38, Akt, β-actin and total form for IκBα, ERK1/2, JNK, p38, and Akt, and 5-LO were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Rabbit polyclonal antibodies for phospho-cPLA₂ (Ser505), cPLA₂, 5-LO, PLCγ1, IKKα/β, lamin B, NF-κB

p65, secondary goat anti-rabbit IgG-HRP, rabbit anti-goat IgG-HRP antibodies, total Syk and LAT, and Bay 61-3606 were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Antibody for phosphotyrosine was purchased from Millipore (Millipore, Billerica, MA, USA). The antibody-reactive bands were visualized with an enhanced chemiluminescence (ECL) system (Pierce Biotechnology, Rockford, IL, USA). The enzyme immunoassay (EIA) kits for PGD₂, LTC₄ and the antibody for COX-2 were purchased from Cayman Chemicals (Ann Arbor, MI, USA). All other reagents were of the highest analytical grade commercially available.

2.2. Plant material

DHT-I (Fig. 1A) was isolated from the roots of *S. miltiorrhiza* Bunge (Lamiaceae) and structure of DHT-I was verified by comparing NMR data with those reported in the literature (Ryu et al., 1997). DHT-I was prepared by dissolving it in dimethyl sulfoxide (DMSO) diluted with RPMI 1640 medium. The final concentration of DMSO in culture media was adjusted to 0.1% (v/v). DMSO alone was run as a control in all cases. Control experiments showed that DMSO at this concentration had no effect on mast cell activation.

2.3. Culture and activation of bone marrow derived mast cells (BMMCs)

BMMCs were isolated from bone marrow of Balb/cJ mice (Sam Taco, INC, Seoul) and differentiated as described previously (Lu et al., 2011). Briefly, BMMCs were cultured in RPMI 1640 medium (Thermo Scientific, Utah, USA) containing 10% fetal bovine serum (FBS, Thermo Scientific, Utah, USA), 100 U/ml penicillin, 10 mM HEPES, 100 μM MEM non-essential amino acid solution (Invitrogen, Grand Island, NY) and 20% pokeweed mitogen-spleen cell conditioned medium as a source of IL-3. For stimulation, 10⁶ cells/ml were sensitized overnight with 500 ng/ml anti-DNP IgE (Sigma), pretreated with indicated concentration of DHT-I or Bay 61-3606 for 1 h at 37 °C, and stimulated for appropriate periods with 100 ng/ml DNP-human serum albumin (HSA; Sigma). The reactions were terminated by centrifugation of the cells at 1000 × g for 5 min at 4 °C.

2.4. Cell viability

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay. Briefly, BMMCs were seeded onto 96 well culture plates at 2 × 10⁴ cells/200 μl/well. After incubation with various concentrations of DHT-I for 8 h, 20 μl of MTT (5 mg/ml) was added to each well. After 4 h incubation, 150 μl of culture medium was removed, and cells were dissolved in 0.4 N HCl/isopropyl alcohol. The optical densities (OD) at 570 nm and 630 nm were measured using a microplate reader (Sunrise, Tecan, Switzerland).

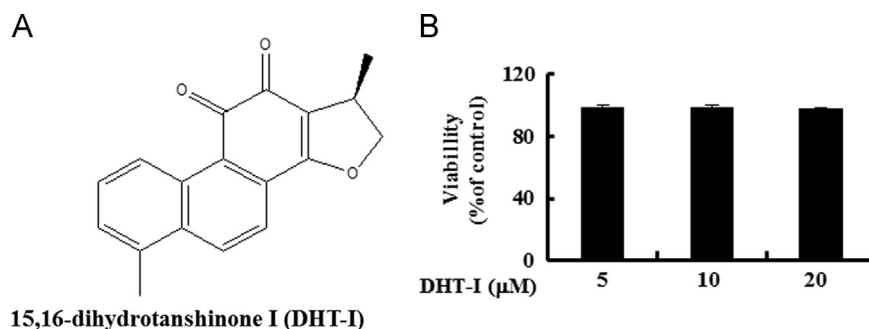


Fig. 1. Chemical structures of 15,16-dihydrotanshinone I (DHT-I).

2.5. Measurement of β -hexosaminidase (β -Hex) release, LTC₄ and PGD₂ generation

BMMCs suspended in enriched medium at a cell density of 1×10^6 cells/ml were pretreated with DHT-I or Bay 61-3606 for 1 h at 37 °C. After 15 min of stimulation, the supernatants were isolated for further analysis by EIA. LTC₄ was determined using an EIA kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instruction. Degranulation was determined by measuring the release of β -Hex, a marker for mast cell degranulation. In brief, 25 μ l cell-free supernatants were mixed with 50 μ l of β -Hex substrate solution (1.3 mg/ml p-nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside in 100 mM sodium 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 a percentage of intracellular β -Hex that was released in the medium. To determine COX-2-dependent PGD₂ generation, BMMCs were preincubated with 1 μ g/ml of aspirin for 2 h to irreversibly inactivate preexisting COX-1. After washing, BMMCs were incubated with 100 ng/ml DNP-HSA at 37 °C for 7 h in the presence of DHT-I or Bay 61-3606. PGD₂ in the supernatants were quantified using PGD₂ EIA kit and cells were used for immunoblots analysis. Under the conditions employed, LTC₄ reached 23.4 ng/10⁶ cells and PGD₂ generation reached 2.08 ng/10⁶ cells, respectively. All data were the arithmetic mean of triplicate determinations.

2.6. Measurement of intracellular Ca²⁺ level

Intracellular Ca²⁺ levels were determined using FluoForte™ Calcium Assay Kit (Enzo Life Sciences, Ann Arbor, MI), as described previously (Hwang et al., 2013). Briefly, BMMCs (1×10^6 cells) were sensitized overnight with 500 ng/ml anti-DNP IgE. Sensitized BMMCs were preincubated with FluoForte™ Dye-Loading Solution for 1 h at room temperature. After washing the dye from cell surface with HBSS, cells (5×10^4) were seeded into 96-well microplates and pretreated with DHT-I or Bay 61-3606 for 1 h before adding DNP-HSA. Fluorescence was measured using a fluorometric imaging plated reader at an excitation wavelength of 485 nm and an emission wavelength of 520 nm on a BMG Labtechnologies FLUOStar OPTIMA platereader (Offenburg, Germany).

2.7. Preparation of nuclear and cytosolic extracts

The nuclear and cytoplasmic extracts were prepared as described previously (Hwang et al., 2013). Briefly, BMMCs were pretreated with DHT-I or Bay 61-3606 for 1 h. Cultured BMMCs were collected by centrifugation, washed with PBS and lysed in a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 0.1% NP40 by incubation on ice for 10 min. After centrifugation at 1000g for 4 min, the supernatants were used as the cytosolic fraction. The nuclear pellets were washed and lysed in a buffer containing 20 mM HEPES (pH 7.9), 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and a protease inhibitor cocktail.

2.8. Immunoblotting

IgE-sensitized BMMCs were stimulated with DNP-HSA for indicated times with or without DHT-I or Bay 61-3606. Total cell lysates were prepared in RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethanesulfonylfluoride (PMSF), 1 M dithiothreitol (DTT), 200 mM NaF, 200 mM Na₃VO₄, and protease inhibitor Cocktail). Cell debris was removed by centrifugation at 14,000 g for 15 min at 4 °C, and the resulting supernatant was western blotted. Protein concentration was measured using a

Qubit Fluorometer (Invitrogen, USA). Samples were separated by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). The primary antibodies (1:1000–3000 dilution) were incubated at 4 °C for overnight. The immunoreactive proteins were incubated with the use of HRP-coupled secondary antibodies diluted 1:3000-fold for 1 h at room temperature, which were subsequently washed three times for 3 min each in TBS-T buffer and then developed using the enhanced chemiluminescence (ECL) detection kit (Pierce Biotechnology, Rockford, IL, USA).

2.9. Immunoprecipitation (IP)

Immunoprecipitation was performed as described previously (Hwang et al., 2013). Briefly, cell lysates were obtained using modified lysis buffer [0.1% Nonidet P-40, 50 mM HEPES (pH 7.0), 250 mM NaCl, 5 mM EDTA, 1 mM PMSF, and 0.5 mM dithiothreitol]. Total cell lysates (1 mg protein equivalent) were incubated with anti-Syk or anti-LAT antibodies for 2 h at 4 °C and immunocomplexes were precipitated with 20 μ l of protein A-Sepharose and washed 3 times with ice-cold lysis buffer. Precipitates and total cell lysates were subjected to SDS-PAGE and immunoblotted with appropriate antibodies.

2.10. Statistical analysis

All experiments were performed three or more times. Average values are expressed as means $\pm$ S.D. Statistical analyses were performed using SPSS 19.0 (SPSS, Chicago, IL, USA). Student's *t*-test was used to compare pairs of independent groups. Statistical significance was accepted for *p* values < 0.05.

3. Results

3.1. DHT-I inhibited IgE/Ag-stimulated degranulation and Ca²⁺ influx in BMMCs

Before investigating the effect of DHT-I (Fig. 1) on mast cell activation, we examined the cytotoxic effect of DHT-I on BMMCs using an MTT assay. DHT-I did not affect cell viability at concentrations up to 20 μ M (data not shown), and thus, concentrations of < 20 μ M were used in subsequent experiments. The effect of DHT-I on the degranulation of IgE/Ag-stimulated BMMCs was determined by measuring the release of β -hexosaminidase (β -Hex). As shown in Fig. 2A, DHT-I significantly and dose-dependently inhibited β -Hex release ($P < 0.01$). Since an increase in cytosolic Ca²⁺ concentration is essential for the degranulation of activated mast cells (Aketani et al., 2001), we investigated the effect of DHT-I on [Ca²⁺]_i in activated BMMCs. DHT-I dose-dependently reduced [Ca²⁺]_i ($P < 0.01$) (Fig. 1B), and under the same conditions, Bay 61-3606 (a Syk inhibitor) strongly inhibited degranulation ($P < 0.01$) and [Ca²⁺]_i increases ($P < 0.01$) in IgE/Ag-stimulated BMMCs.

3.2. Effects of DHT-I on cPLA₂ phosphorylation and on the translocations of p-cPLA₂ and 5-LO in IgE/Ag-stimulated BMMCs

LTC₄ is synthesized from arachidonic acid (AA) by 5-lipoxygenase (5-LO) in mast cells. To examine the effects of DHT-I on LTC₄ generation, BMMCs were pretreated with different concentrations of DHT-I or Bay 61-3606. As shown in Fig. 3A, BMMCs stimulated with IgE/Ag promptly produced LTC₄ and this response was markedly inhibited by DHT-I or Bay 61-3606 ($P < 0.001$). Several reports show that rapid LTC₄ production involves two steps, that is, the release of AA from membrane phospholipids by 85-kDa cPLA₂ α and the oxygenation of free AA by 5-LO (Murphy and Gijon, 2007; Werz, 2002; Lu

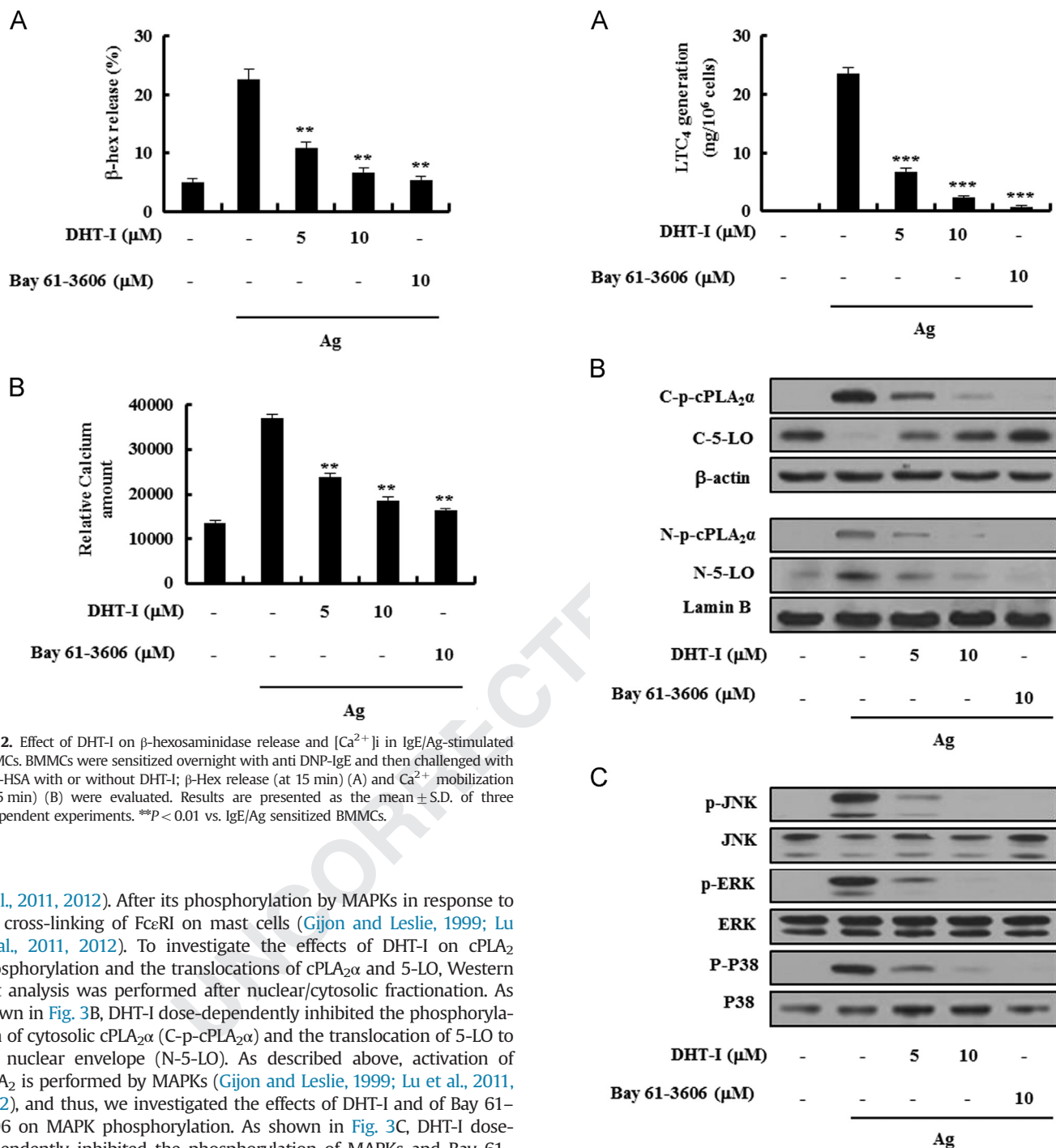


Fig. 2. Effect of DHT-I on β -hexosaminidase release and $[Ca^{2+}]_i$ in IgE/Ag-stimulated BMMCs. BMMCs were sensitized overnight with anti DNP-IgE and then challenged with DNP-HSA with or without DHT-I; β -Hex release (at 15 min) (A) and Ca^{2+} mobilization (at 5 min) (B) were evaluated. Results are presented as the mean $\pm$ S.D. of three independent experiments. ** $p < 0.01$ vs. IgE/Ag sensitized BMMCs.

et al., 2011, 2012). After its phosphorylation by MAPKs in response to the cross-linking of Fc ϵ R1 on mast cells (Gijon and Leslie, 1999; Lu et al., 2011, 2012). To investigate the effects of DHT-I on cPLA₂ phosphorylation and the translocations of cPLA₂ α and 5-LO, Western blot analysis was performed after nuclear/cytosolic fractionation. As shown in Fig. 3B, DHT-I dose-dependently inhibited the phosphorylation of cytosolic cPLA₂ α (C-p-cPLA₂ α) and the translocation of 5-LO to the nuclear envelope (N-5-LO). As described above, activation of cPLA₂ is performed by MAPKs (Gijon and Leslie, 1999; Lu et al., 2011, 2012), and thus, we investigated the effects of DHT-I and of Bay 61-3606 on MAPK phosphorylation. As shown in Fig. 3C, DHT-I dose-dependently inhibited the phosphorylation of MAPKs and Bay 61-3606 almost completely inhibited MAPKs phosphorylation. Under these conditions, β -actin and lamin B were used as internal controls for the cytosolic and nuclear fractions, respectively (Fig. 3B).

3.3. Effects of DHT-I on COX-2 dependent PGD₂ generation and Akt-NF- κ B activation

PGD₂ is a major prostanoid that is generated by mast cells, and has long been implicated in the etiologies and manifestations of allergic diseases (Moore and Peebles, 2006). In a previous study, we found that SCF or IgE/antigen plus IL-10 and lipopolysaccharide (LPS) stimulated PGD₂ generation by BMMCs in a biphasic manner, that is, by immediate COX-1 dependent PGD₂ generation (within 2 h) followed by inducible COX-2-dependent PGD₂ generation (during 2–10 h) (Ashraf et al., 1996; Moon et al., 1998; Lu et al., 2011). In the

Fig. 3. Effects of DHT-I on LTC₄ generation, the nuclear translocations of cPLA₂ α and 5-LO, and on MAPKs activation. IgE-sensitized BMMCs were pre-incubated for 1 h with the indicated concentrations of DHT-I or Bay 61-3606 and then stimulated with DNP-HSA for 15 min. (A) LTC₄ release into supernatants were quantified using an EIA kit. (B) Cytosolic and nuclear fractions were immunoblotted with antibodies for phospho-cPLA₂ α (Ser505) and 5-LO (A), and cell lysates were immunoblotted for the total and phosphorylated forms of ERK1/2, JNK, and p38 (B). β -Actin and lamin B were used as controls for cytosol and nuclear fractions, respectively. *** $p < 0.001$ vs. IgE/Ag sensitized BMMCs.

present study, we assessed the effects of DHT-I and of Bay 61-3606 on COX-2-dependent delayed PGD₂ generation and COX-2 expression. BMMCs were pre-treated with aspirin to inactivate preexisting COX-1 activity, washed, and then stimulated with Ag for 7 h with or without DHT-I or Bay 61-3606. As shown in Fig. 4A, PGD₂ generation and COX-2 expression were not detected in unstimulated BMMCs,

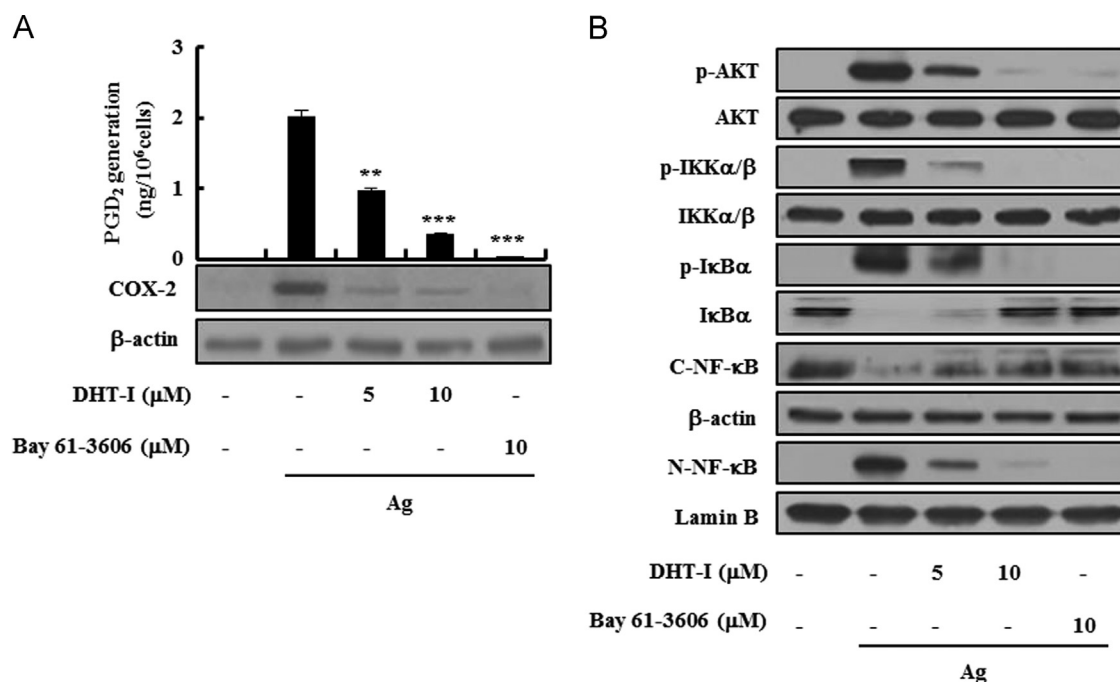


Fig. 4. Effect of DHT-I on COX-2 dependent PGD₂ generation and Akt-NF-κB activation. IgE-sensitized BMMCs were pre-incubated with the indicated concentrations of DHT-I or Bay 61-3606 for 1 h and then stimulated with DNP-HSA for 7 h. (A) PGD₂ released into supernatant was quantified using an EIA kit and cell lysates were immunoblotted for COX-2 protein. IgE-sensitized BMMCs were pre-incubated with DHT-I or Bay 61-3606 for 1 h and then stimulated with DNP-HSA for 15 min. Cells lysates were then immunoblotted for Akt, IKKα/β, IκBα, cytosolic NF-κB (p65), nuclear NF-κB (p65), β-actin, and lamin B. Nuclear extracts were immunoblotted for NF-κB (p65). Results are presented as the means ± S.D. of three independent experiments. ***p* < 0.01 and ****p* < 0.001 vs. IgE/Ag-treated BMMCs.

whereas DHT-I dose-dependently inhibited PGD₂ generation (*P* < 0.01 or 0.001 vs. without DHT-I) and COX-2 protein expression in IgE/Ag-stimulated BMMCs (Fig. 4A, lower). It has been reported that Syk downstream PI3K/Akt pathway affect transcription factor NF-κB activation in gastric cancer cells and mast cells (Lu et al., 2011; Siraganian et al., 2010; Chen et al., 2013). Furthermore, NF-κB has been reported to be a critical transcription factor for the induction of several inflammatory mediators including, COX-2, inducible NO synthase, TNF-α, and IL-6 (Lawrence et al., 2001; Tak and Firestein, 2000; Lu et al., 2011). Thus, we investigated the effects of DHT-I and of Bay 61-3606 on Akt and NF-κB pathways. As shown in Fig. 4B, phosphorylated Akt, IKK complex (p-IKKα/β), and IκBα (p-IκBα) were detected in stimulated BMMCs, and DHT-I or Bay 61-3606 strongly suppressed the phosphorylations of Akt, IKKα/β, and IκBα, the degradation of IκBα, and the concomitant translocation of the p65 subunit of NF-κB (cytosolic-p65) to nuclear fractions (nuclear-p65) in IgE/Ag-stimulated BMMCs. Based on these results, we speculated that inhibitions of the productions of PGD₂ and LTC₄ and of degranulation by DHT-I might be due to suppression of the Syk pathway during the FcεRI-mediated activation of BMMCs.

3.4. DHT-I inhibited the Syk pathway in IgE/Ag-stimulated BMMCs

It has been reported that tyrosine kinase Syk plays an essential role in the initiation of the activation of FcεRI-mediated mast cells (Rossi et al., 2006; Siraganian et al., 2010). Subsequently, Syk activates multiple downstream signals including the LAT, PLCγ1, SLP-76, and PI3K pathways, which are crucial for calcium mobilization and degranulation (Siraganian et al., 2010; Lu et al., 2011). We examined the effect of DHT-I or Bay 61-3606 on the phosphorylations of Syk, LAT, and PLCγ1 in IgE/Ag-stimulated BMMCs. As shown in Fig. 5A, DHT-I significantly and dose-dependently inhibited the phosphorylations of Syk, LAT and PLCγ1, and Bay 61-3606 completely inhibited the phosphorylations of all three proteins. Densitometric analyses of the immunoblots in Fig. 5B–D confirmed that DHT-I inhibited the IgE/Ag-stimulated the

phosphorylations of Syk, LAT, and PLCγ1. These results suggest that DHT-I inhibits PGD₂ and LTC₄ generation and degranulation by suppressing Syk-dependent signaling pathway.

4. Discussion

The root of *S. miltiorrhiza* is used in Chinese medicine for treating vascular diseases, particularly atherosclerosis and blood clotting abnormalities (Wang, 2010). However, the mechanism responsible for the anti-allergic inflammatory effects of DHT-I is not fully understood. The aim of this study was to identify the effect and the mode of action of DHT-I on mediator release in IgE/Ag-stimulated mast cells. We found that DHT-I inhibited 5-LO dependent LTC₄ and COX-2 dependent PGD₂ generation and the degranulation of IgE/Ag-stimulated BMMCs. In order to identify the mechanism underlying the anti-allergic inflammatory effect of DHT-I, we investigated; (i) the phosphorylation of cPLA₂α and translocation of 5-LO to the nuclear envelope, (ii) the MAPKs and AKT-mediated IKK/IκBα/NF-κB pathways, and (iii) the FcεRI-associated proximal tyrosine kinase Syk pathway.

Crosslinking the high affinity IgE receptor FcεRI with multivalent antigen activates the Syk/LAT axis in mast cells, which in turn phosphorylates PLCγ1 and increases [Ca²⁺]_i, and thus, triggers degranulation and the activations of AA metabolizing enzymes (Siraganian, 2003). The present results show that DHT-I significantly inhibits the phosphorylations of Syk, LAT, and PLCγ1 and intracellular Ca²⁺ influx, and in so doing results inhibits degranulation. These results suggest that the inhibition of degranulation by DHT-I occurs via attenuation of the Syk-mediated PLCγ1-Ca²⁺ pathway. On the other hand, it is well known that cPLA₂α is the major regulator of the AA release required for eicosanoid production (Murakami et al., 1995; Ueno et al., 2011; Lu et al., 2011, 2012). In order to the release AA from the sn-2 position of cell membrane phospholipids, the phosphorylation of cPLA₂ by MAPKs, the nuclear translocation of cPLA₂α, and

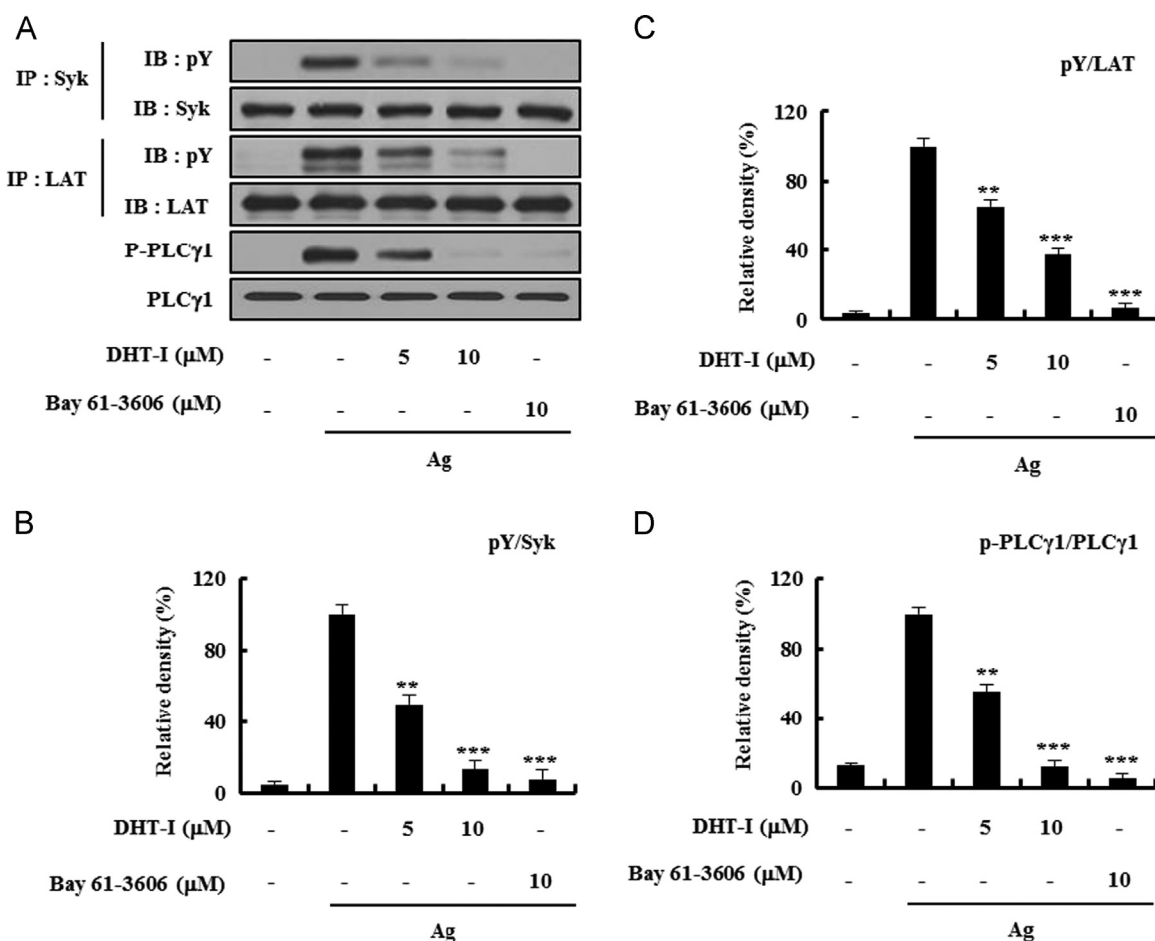


Fig. 5. Effect of DHT-I on the Syk pathway. IgE-sensitized BMMCs were preincubated with DHT-I or Bay 61-3606 for 1 h, and then stimulated with DNP-HSA for 5 min. (A) Cell lysates were immunoprecipitated and immunoblotted for the phosphorylated forms of Syk, LAT, and PLCγ1. Bay 61-3606 was used as a positive control for suppression of the Syk-mediated pathway. Relative ratios of p-Syk/Syk (B), p-LAT/LAT (C), and p-PLCγ1/PLCγ1 (D) proteins were determined by measuring immunoblot band intensities by scanning densitometry ($n=3$, ** $p < 0.01$ and *** $p < 0.001$). The results shown are representative of three independent experiments.

increases in $[Ca^{2+}]_i$ are required (Nakatani et al., 1994; Gijon and Leslie, 1999; Lu et al., 2011). When mast cells are activated by the cross-linking of FcεRI, 5-LO is activated by Ca^{2+} -dependent translocation to the nuclear envelope, where 5-LO-activating protein (FLAP) presents AA for sequential conversion to LTA_4 , which is then converted into LTC_4 by LTC_4 synthase (Dixon et al., 1990; Murakami et al., 1995). Here, we investigated the effects of DHT-I on cPLA₂ phosphorylation by MAPKs and translocation of p-cPLA₂α and 5-LO to nuclear envelope. DHT-I was found to suppress the translocations of p-cPLA₂α and 5-LO, and to dose-dependently inhibit 5-LO dependent LTC_4 production (Fig. 3). In addition, DHT-I significantly inhibited COX-2 expression and concomitant PGD₂ generation. We and other groups have reported the PI3K/Akt pathway lies upstream of the NF-κB pathway, and that NF-κB is an essential transcription factor for the expressions of COX-2 and proinflammatory cytokines in IgE/Ag-stimulated BMMCs (Tak and Firestein, 2000; Lawrence et al., 2001; Lu et al., 2011). Furthermore, in Akt phosphorylation is enhanced in IgE/Ag-stimulated BMMCs and the subsequent activation of IKK in turn phosphorylates and promotes the degradation of IκB, which enables the nuclear translocation of NF-κB (Lu et al., 2011). Therefore, we investigated the effects of DHT-I and Bay 61-3606 on COX-2 expression and PGD₂ generation via the blockade of Akt and NF-κB activation in IgE/Ag-stimulated BMMCs. As we reported previously (Lu et al., 2013), DHT-I significantly and dose-dependently suppressed the phosphorylations of Akt, IKK, and IκBα, the degradation of IκBα, and the nuclear translocation of NF-κB. Taken together, our results suggest that reduced COX-2-dependent PGD₂

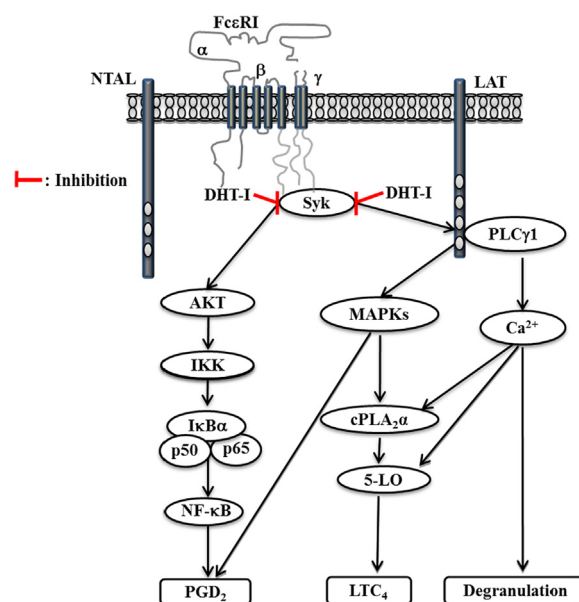


Fig. 6. Putative mechanism for FcεRI-mediated mast cell activation by DHT-I. Aggregation of FcεRI triggers Syk activation, and activated Syk regulates the PI3K pathway via the adaptor protein NTAL, which plays a key role in NF-κB-mediated expression of COX-2. Syk also phosphorylates LAT, which facilitates the phosphorylations of downstream signal pathways, such as the PLCγ1, MAPKs and Akt-NF-κB pathways. DHT-I inhibits degranulation and eicosanoid generation by primarily suppressing Syk-dependent signal pathways in IgE/Ag-stimulated BMMCs.

production by DHT-I and by Bay 61-3606 is due to the inhibition of both MAPKs pathway leading to the activation of cPLA₂ α , which supplies AA to COX-2, and to the activation of the Akt-NF- κ B pathways leading to the expression of inducible COX-2 protein. As described above, Syk plays critical roles in the initiation of the Fc ϵ RI-mediated expressions of various inflammatory genes and in the release of multiple mediators (Siraganian, 2003; Masuda and Schmitz, 2008). Based on our results, we propose action mechanisms for the inhibition of mast cell activation by DHT-I (Fig. 6), as follows; (1) DHT-I inhibits degranulation by suppressing the PLC γ 1-Ca²⁺ pathway; (2) DHT-I inhibits COX-2 dependent PGD₂ generation via two distinct pathways, that is, via the Syk-AKT-IKK and MAPK pathways; and (3) DHT-I inhibits LTC₄ generation via Syk-MAPKs-cPLA₂ pathways.

In conclusion, anti-allergic inflammatory activity of DHT is mediated by the inhibition of Fc ϵ RI-mediated Syk phosphorylation in IgE/Ag-stimulated mast cells.

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References

- Aketani, S.I., Teshima, R., Umezawa, Y., Sawada, J., 2001. Correlation between cytosolic calcium concentration and degranulation in RBL-2H3 cells in the presence of various concentrations of antigen-specific IgEs. *Immunol. Lett.* 75, 185–189.
- Ashraf, M., Murakami, M., Kudo, I., 1996. Cross-linking of the high-affinity IgE receptor induces the expression of cyclooxygenase 2 and attendant prostaglandin generation requiring interleukin 10 and interleukin 1 beta in mouse cultured mast cells. *Biochem. J.* 320, 965–973.
- Chen, L., Monti, S., Juszczynski, P., Ouyang, J., Chapuy, B., Neuberg, D., Doench, J.G., Bogusz, A.M., Habermann, T.M., Dogan, A., Witzig, T.E., Kutok, J.L., Rodig, S.J., Golub, T., Shipp, M.A., 2013. SYK inhibition modulates distinct PI3K/AKT-dependent survival pathways and cholesterol biosynthesis in diffuse large B cell lymphomas. *Cancer Cell* 23, 826–838.
- Choi, H.S., Cho, D.I., Choi, H.K., Im, S.Y., Ryu, S.Y., Kim, K.M., 2004. Molecular mechanisms of inhibitory activities of tanshinones on lipopolysaccharide-induced nitric oxide generation in RAW 264.7 cells. *Arch. Pharmacol. Res.* 7, 1233–1237.
- Dixon, R.A., Diehl, R.E., Opas, E., Rands, E., Vickers, P.J., Evans, J.F., Gillard, J.W., Miller, D.K., 1990. Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. *Nature* 343, 282–284.
- Gijon, M.A., Leslie, C.C., 1999. Regulation of arachidonic acid release and cytosolic phospholipase A₂ activation. *J. Leukoc. Biol.* 65, 330–336.
- Gilfillan, A.M., Rivera, J., 2009. The tyrosine kinase network regulating mast cell activation. *Immunol. Rev.* 228, 149–228.
- Hwang, S.L., Li, X., Lu, Y., Jin, Y., Jeong, Y.T., Kim, Y.D., Lee, I.K., Taketomi, Y., Sato, H., Cho, Y.S., Murakami, M., Chang, H.W., 2013. AMP-activated protein kinase negatively regulates Fc ϵ RI-mediated mast cell signaling and anaphylaxis in mice. *J. Allergy Clin. Immunol.* 132, 729–736.
- Kambayashi, T., Koretzky, G.A., 2007. Proximal signaling events in Fc epsilon RI-mediated mast cell activation. *J. Allergy Clin. Immunol.* 119, 544–552 (quiz 53–54).
- Lawrence, T., Gilroy, D.W., Colville-Nash, P.R., Willoughby, D.A., 2001. Possible new role for NF- κ B in the resolution of inflammation. *Nat. Med.* 7, 1291–1297.
- Lee, P., Hur, J., Lee, J., Kim, J., Jeong, J., Kang, I., Kim, S.Y., Kim, H., 2006. 15,16-Dihydrotanshinone I suppresses the activation of BV-2 cell, a murine microglia cell line, by lipopolysaccharide. *Neurochem. Int.* 48, 60–66.
- Lee, D.S., Lee, S.H., Noh, J.G., Hong, S.D., 1999. Antibacterial activities of cryptotanshinone and dihydrotanshinone I from a medicinal herb, *Salvia miltiorrhiza* Bunge. *Biosci., Biotechnol., Biochem.* 63, 2236–2239.

- Li, X., Lu, Y., Jin, Y., Son, J.K., Lee, S.H., Chang, H.W., 2014. Curcumin inhibits the activation of immunoglobulin E-mediated mast cells and passive systemic anaphylaxis in mice by reducing serum eicosanoid and histamine levels. *Biomol. & Ther.* 22, 27–34.
- Lin, L.L., Wartmann, M., Lin, A.Y., Knopf, J.L., Seth, A., Davis, R.J., 1993. cPLA₂ is phosphorylated and activated by MAP kinase. *Cell* 72, 269–278.
- Lu, Y., Li, Y., Seo, C.S., Murakami, M., Son, J.K., Chang, H.W., 2012. Saucerneol D inhibits eicosanoid generation and degranulation through suppression of Syk kinase in mast cells. *Food Chem. Toxicol.* 50, 4382–4388.
- Lu, Y., Li, X., Park, Y.N., Kwon, O., Piao, D., Chang, Y.C., Kim, C.H., Lee, E., Son, J.K., Chang, H.W., 2014. Britanin suppresses IgE/Ag-induced mast cell activation by inhibiting the Syk pathway. *Biomol. Ther.* 23, 193–199.
- Lu, Y., Yang, J.H., Li, X., Hwangbo, K., Hwang, S.L., Taketomi, Y., Murakami, M., Chang, Y.C., Kim, C.H., Son, J.K., Chang, H.W., 2011. Emodin, a naturally occurring anthraquinone derivative, suppresses IgE-mediated anaphylactic reaction and mast cell activation. *Biochem. Pharmacol.* 82, 1700–1708.
- Masuda, E.S., Schmitz, J., 2008. Syk inhibitors as treatment for allergic rhinitis. *Pulm. Pharmacol. Ther.* 21, 461–467.
- Moon, T.C., Murakami, M., Ashraf, M.D., Kudo, I., Chang, H.W., 1998. Regulation of cyclooxygenase-2 and endogenous cytokine expression by bacterial lipopolysaccharide that acts in synergy with c-kit ligand and Fc epsilon receptor I crosslinking in cultured mast cells. *Cell. Immunol.* 185, 146–152.
- Moore, M.L., Peebles Jr., R.S., 2006. Update on the role of prostaglandins in allergic lung inflammation: separating friends from foes, harder than you might think. *J. Allergy Clin. Immunol.* 117, 1036–1039.
- Murakami, M., Austen, K.F., Arm, J.P., 1995. The immediate phase of c-kit ligand stimulation of mouse bone marrow-derived mast cells elicits rapid leukotriene C₄ generation through posttranslational activation of cytosolic phospholipase A₂ and 5-lipoxygenase. *J. Exp. Med.* 182, 197–206.
- Murphy, R.C., Gijon, M.A., 2007. Biosynthesis and metabolism of leukotrienes. *Biochem. J.* 405, 379–395.
- Nakatani, Y., Murakami, M., Hara, S., Kudo, I., Inoue, K., 1994. Dual regulation of cytosolic phospholipase A₂ in mast cells after crosslinking of Fc epsilon-receptor. *J. Immunol.* 153, 796–803.
- Park, J.W., Lee, S.H., Yang, M.K., Lee, J.J., Song, M.J., Ryu, S.Y., Chung, H.J., Won, H.S., Lee, C.S., Kwon, S.H., Yun, Y.P., Choi, W.S., Shin, H.S., 2008. 15,16-dihydrotanshinone I, a major component from *Salvia miltiorrhiza* Bunge (Dansham), inhibits rabbit platelet aggregation by suppressing intracellular calcium mobilization. *Arch. Pharmacol. Res.* 31, 47–53.
- Rossi, A.B., Herlaar, E., Braselmann, S., Huynh, S., Taylor, V., Frances, R., Issakani, S.D., Argade, A., Singh, R., Payan, D.G., Masuda, E.S., 2006. Identification of the Syk kinase inhibitor R112 by a human mast cell screen. *J. Allergy Clin. Immunol.* 118, 749–755.
- Ryu, S.Y., No, Z., Kim, S.H., Ahn, J.W., 1997. Two novel abietane diterpenes from *Salvia miltiorrhiza*. *Planta Medica* 63, 44–46.
- Siraganian, R.P., 2003. Mast cell signal transduction from the high-affinity IgE receptor. *Curr. Opin. Immunol.* 15, 639–646.
- Siraganian, R.P., de Castro, R.O., Barbu, E.A., Zhang, J., 2010. Mast cell signaling: the role of protein tyrosine kinase Syk, its activation and screening methods for new pathway participants. *FEBS Lett.* 584, 4933–4940.
- Tak, P.P., Firestein, G.S., 2000. NF-kappaB: a key role in inflammatory diseases. *J. Clin. Invest.* 107, 7–11.
- Tsai, S.L., Suk, F.M., Wang, C.I., Liu, D.Z., Hou, W.C., Lin, P.J., Hung, L.F., Liang, Y.C., 2007. Anti-tumor potential of 15,16-dihydrotanshinone I against breast adenocarcinoma through inducing G1 arrest and apoptosis. *Biochem. Pharmacol.* 74, 1575–1586.
- Ueno, N., Taketomi, Y., Yamamoto, K., Hirabayashi, T., Kamei, D., Kita, Y., Shimizu, T., Shinzawa, K., Tsujimoto, Y., Ikeda, K., Taguchi, R., Murakami, M., 2011. Analysis of two major intracellular phospholipase A₂s in mast cells reveals crucial contribution of cPLA₂ α , not iPLA₂ β , to lipid mobilization in proximal mast and Distal Fibroblasts. *J. Biol. Chem.* 286, 37249–37263.
- Wang, B.Q., 2010. *Salvia miltiorrhiza*: Chemical and pharmacological review of a medicinal plant. *J. Med. Plants Res.* 4, 2813–2820.
- Werz, O., 2002. 5-lipoxygenase: cellular biology and molecular pharmacology. *Curr. Drug Targets. Inflamm. Allergy* 1, 23–44.
- Yamaguchi, M., Sayama, K., Yano, K., Lantz, C.S., Noben-Trauth, N., Ra, C., Costa, J.J., Galli, S.J., 1999. IgE enhances Fc epsilon receptor I expression and IgE-dependent release of histamine and lipid mediators from human umbilical cord blood-derived mast cells: synergistic effect of IL-4 and IgE on human mast cell Fc epsilon receptor I expression and mediator release. *J. Immunol.* 162, 5455–5465.
- Zhou, L., Zuo, Z., Chow, M.S., 2005. Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. *J. Clin. Pharmacol.* 45, 1345–1359.