

Imperatorin Suppresses Degranulation and Eicosanoid Generation in Activated Bone Marrow-Derived Mast Cells

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Abstract

Imperatorin has been known to exert many biological functions including anti-inflammatory activity. In this study, we investigated the inhibitory effects of imperatorin on the production of inflammatory mediators in mouse bone marrow-derived mast cells (BMMC). Imperatorin inhibited degranulation and the generation of eicosanoids (leukotriene C₄ (LTC₄) and prostaglandin D₂ (PGD₂)) in IgE/antigen (Ag)-stimulated BMMC. To elucidate the molecular mechanism involved in this process, we investigated the effect of imperatorin on intracellular signaling in BMMC. Biochemical analyses of the IgE/Ag-mediated signaling pathway demonstrated that imperatorin dramatically attenuated degranulation and the production of 5-lipoxygenase-dependent LTC₄ and cyclooxygenase-2-dependent PGD₂ through the inhibition of intracellular calcium influx/phospholipase C γ 1, cytosolic phospholipase A₂/mitogen-activated protein kinases and/or nuclear factor- κ B pathways in BMMC. These results suggest that the effects of imperatorin on inhibition of degranulation and eicosanoid generation through the suppression of multiple steps of IgE/Ag-mediated signaling pathways would be beneficial for the prevention of allergic inflammation.

Key Words: Leukotriene C₄, Prostaglandin D₂, Cytosolic phospholipase A₂, Mitogen-activated protein kinases, Phospholipase C γ 1

INTRODUCTION

Mast cells are major effector cells in allergic inflammation, and they are being increasingly recognized for their roles in innate and adaptive immune responses. When mast cells are activated through IgE-dependent or IgE-independent ways, they release preformed mediators, including histamine, proteases, and proteoglycans from their granules. Their activation also triggers rapid synthesis and release of lipid mediators derived from arachidonic acid (AA) metabolism such as prostaglandin D₂ (PGD₂) and leukotriene C₄ (LTC₄). Activated mast cells synthesize and secrete chemokines and pro-inflammatory cytokines over a period of several hours (Boyce, 2003; Kalesnikoff and Galli, 2008).

Multiple receptors are expressed on the surface of mast cells. The high affinity receptor for IgE (Fc ϵ RI) is the most well known receptor in antigen (Ag)-mediated responses, whereas signaling induced by stem cell factor (SCF) and its receptor

c-Kit also plays an important role in mast cell development and function (Roskoski, 2005). Binding of Ag recognized by bound IgE on mast cells results in dimerization of the receptor followed by phosphorylation of phospholipase C γ 1 (PLC γ 1), which triggers release of calcium (Ca²⁺) from internal stores. Ca²⁺ is an important intracellular messenger in mast cells because it plays a major role not only in mast cell degranulation but also in eicosanoid generation (Gilfillan and Tkaczyk, 2006; Gilfillan and Rivera, 2009).

Binding of Ag to Fc ϵ RI on mast cells also triggers the activation of three families of mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)- κ B signaling pathways, which contribute to the inducible expression of inflammatory genes including cyclooxygenase (COX)-2 (Kambayashi and Koretzky, 2007). In addition, activated MAPKs lead to the phosphorylation of cytosolic phospholipase A₂ (cPLA₂) and release of AA, a common precursor of eicosanoids (Soberman and Christmas, 2003).

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Imperatorin, belonging to a group of furanocoumarins and found in *Angelicae Dahuricae* Radix, is known to have various biological functions, including anti-inflammatory activity. It showed inhibitory activity on the LPS-induced NO and PGE₂ production by suppressing elevated iNOS and COX-2 protein expression as well as the release of pro-inflammatory cytokines through the inactivation of MAPKs and NF- κ B in RAW 264.7 cells (Ban *et al.*, 2003; Guo *et al.*, 2012; Huang *et al.*, 2012; Kang *et al.*, 2010). In addition, imperatorin reduced the development of carrageenan-induced paw edema *in vivo* (Huang *et al.*, 2012). Furthermore, imperatorin exerted inhibitory effects in an allergic rhinitis model and in an *in vitro* assay by regulating caspase-1 activity (Oh *et al.*, 2011).

In the present study, the anti-inflammatory effect of imperatorin and its mechanism were evaluated and the results suggest that imperatorin inhibits Ca²⁺ influx and degranulation through the inhibition of PLC γ 1 phosphorylation in activated mast cells. In addition, imperatorin strongly suppresses the generation of 5-lipoxygenase (LO)-dependent LTC₄ and COX-2-dependent PGD₂ through the regulation of several signaling molecules by phosphorylation.

MATERIALS AND METHODS

Chemicals and reagents

RPMI-1640, Modified Eagle Medium (MEM), non-essential amino acid solution, and penicillin-streptomycin were acquired from Hyclone (Logan, Utah, USA). Imperatorin was purchased from ChromaDex (Irvine, CA, USA). Mouse anti-dinitrophenyl (DNP) IgE and DNP-human serum albumin (HAS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The recombinant mouse SCF was purchased from STEMCELL Technologies Inc (Vancouver, BC, Canada). The primary antibodies used in the experiments were as follows: rabbit polyclonal antibodies specific for phospho-ERK1/2, ERK1/2, phospho-p38, p38, phospho-JNK, JNK, phospho-PLC γ 1, phospho-Akt, Akt, phospho-I κ B α , I κ B α , phospho-IKK α/β , and β -actin from Cell Signaling Technology, Inc. (Beverly, MA, USA); rabbit polyclonal antibodies for 5-LO, and phospho-cPLA₂ from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Specific inhibitors for NF- κ B (pyrrolidine dithiocarbamate, PDTC) and MAPKs (SB203580, PD98059, and SP600125) were obtained from Sigma. The horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was from Cell Signaling. 1 \times RIPA buffer, NE-PER Nuclear Protein Extraction kit, phosphatase/protease inhibitor cocktail, and enhanced chemiluminescence (ECL) detection reagent were from Pierce (Rockford, IL, USA). The enzyme immunoassay (EIA) kits for LTC₄ and PGD₂, and COX-2 antibody were purchased from Cayman Chemicals (Ann Arbor, MI, USA).

Preparation of pokeweed mitogen-stimulated spleen condition medium (PWM-SCM) and bone marrow-derived mast cells (BMMC)

Spleen cells (2 \times 10⁶ cells) from male BALB/c mice (Koatek, Seoul, Korea) were incubated for 5 days in 30 ml of enriched medium (RPMI 1640 containing 100 U/ml of penicillin, 100 μ g/ml of streptomycin, 2 mM L-glutamine and 10% FBS) with 2.5 μ g/ml of PWM (Sigma) and 48 μ M of 2-mercaptoethanol. The culture supernatant was collected and named PWM-SCM as a source of interleukin-3 (IL-3). Bone marrow cells from male

BALB/c mice were cultured for up to 10 weeks in enriched medium containing 20% PWM-SCM and BMMC were used for assays after 3 weeks (Jeong *et al.*, 2014).

Cell viability

CellTiter 96 Aqueous One kit (Promega, Madison, WI, USA) was used to assess cell viability. Briefly, BMMC were seeded onto a 96-well plate at 2 \times 10⁵ cells/well. After incubation with different concentrations of imperatorin for 7 h, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) was added to each well. After 2 h of incubation, the optical densities at 490 nm were measured using a microplate reader (Tecan System, San Jose, CA, USA).

Assay of β -hexosaminidase (β -HEX)

The release of β -HEX was quantified by spectrophotometric method as described previously (Jeong *et al.*, 2014). Briefly, after harvesting supernatant, cells were lysed in the same volume of medium by freeze and thaw three times. Twenty five μ L of BMMC lysate or supernatant was 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, Sigma) in each well of 96-well plates and then incubated at 37°C for 60 min. The reaction was stopped by adding 175 μ L of 0.2 M Glycin/NaOH (pH 10.7). The absorbance at 405 nm was measured in a microplate reader. The percentage of β -HEX released into the supernatant was calculated by the following formula: [S/(S+P)] \times 100, where S and P are the β -HEX contents of supernatant and cell pellet, respectively.

Determination of LTC₄ and PGD₂

For LTC₄ determination, BMMC at a cell density of 1 \times 10⁶ cells/ml were sensitized with anti-DNP IgE for overnight (500 ng/ml) and seeded in 96 well plate. After pre-incubated with imperatorin for 1 h, BMMC were stimulated with DNP-HSA (Ag, 100 ng/ml) for 15 min and all reactions were stopped by centrifugation at 120 g for 5 min at 4°C, and then the supernatants were immediately used for LTC₄ determination. The level of LTC₄ was determined using EIA kit (Cayman Chemical) accordance with the manufacturer's protocols. To measure COX-2-dependent PGD₂ generation, BMMC (1 \times 10⁶ cells/ml) were pre-incubated with aspirin (10 μ g/ml) for 2 h to irreversibly inactivate pre-existing COX-1. After washing, BMMC were incubated with Ag for 7 h at 37°C in the presence of imperatorin and the supernatants were measured using PGD₂ EIA kit (Cayman Chemical).

Measurement of intracellular Ca²⁺ level

Intracellular Ca²⁺ levels were determined with FluoForte™ Calcium Assay Kit (Enzo Limperatorin Sciences, Ann Arbor, MI, USA). Briefly, BMMC were sensitized with anti-DNP IgE (500 ng/ml) for overnight and sensitized BMMC were pre-incubated with FluoForte™ Dye-Loading Solution at room temperature for 1 h. After washing to remove the dye from cell surface with PBS, BMMC (5 \times 10⁴) were seeded into a 96-well microplate and pre-treated with imperatorin for 1 h followed by stimulation with Ag. The fluorescent was monitored with a plated reader at an Ex 485 nm/Em 535 nm (Perkin Elmer's VICTOR™ Multilable Plate Reader, Waltham, MA, USA).

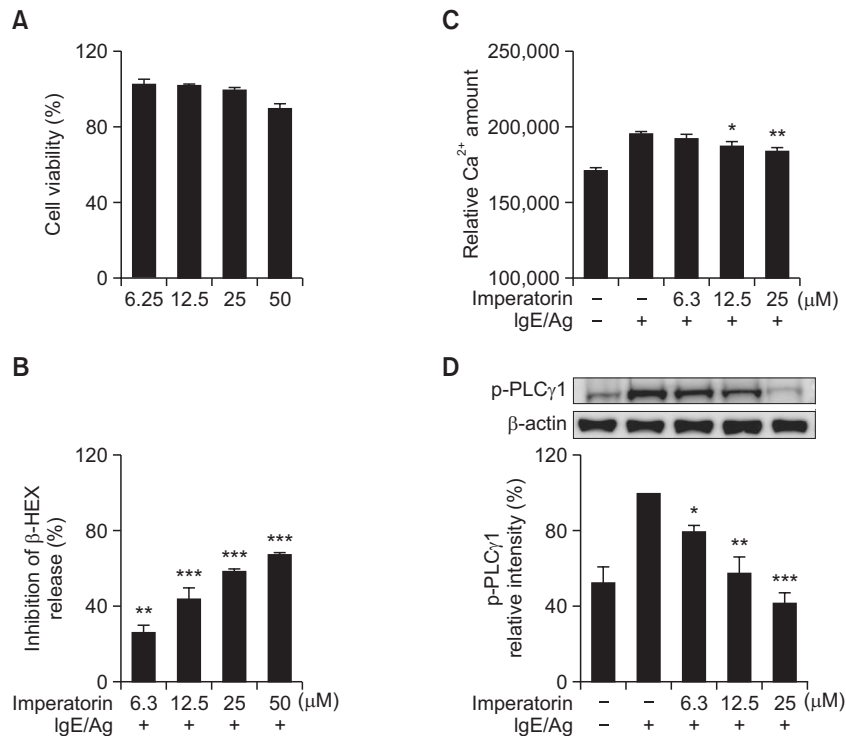


Fig. 1. Effect of imperatorin on cell viability (A), degranulation (B), intracellular Ca²⁺ level (C), and PLCγ1 phosphorylation (D) in IgE/Ag-stimulated BMMC. Cell viability was measured using an MTS assay (A). BMMC sensitized overnight with IgE were pre-incubated with the indicated concentrations of imperatorin for 1 h and stimulated with DNP-HAS (Ag, 100 ng/ml) for 15 min. β-HEX release into the supernatant was determined (B) and BMMC were used for Western blotting (D). IgE-sensitized BMMC pretreated with FluoForte™ Dye-Loading Solution were seeded into 96-well microplates, pre-incubated with imperatorin, and stimulated with DNP-HAS. Relative Ca²⁺ levels were measured using Multilabel Plate Reader (C). The values are expressed as the means ± S.E.M. of three different samples. **p*<0.05, ***p*<0.01, ****p*<0.001, significantly different from the control as determined by one-way ANOVA.

Preparation of nuclear and cytoplasm extracts

The nuclear and cytoplasmic extracts were prepared using NE-PER Nuclear Protein Extraction kit (Pierce) according to the manufacture's instructions.

Western blotting

Western blotting was performed as described previously (Park *et al.*, 2013). Whole cell protein lysates were prepared in RIPA lysis buffer in the presence of protease inhibitors (Pierce). The proteins were separated via 10% SDS-PAGE, transferred to nitrocellulose membranes in 20% methanol, 25 mM Tris, and 192 mM glycine (Schleicher and Schull, Dassel, Germany), and then blocked via incubation in TTBS (25 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween 20) containing 5% non-fat milk. The membranes were subsequently incubated with a variety of first antibodies for overnight, washed, and finally incubated for 1 h with a secondary antibody conjugated to horseradish peroxidase. The protein bands were then visualized with an ECL system (Pierce). The densities of the bands were measured with the ImageQuant LAS 4000 luminescent image analyzer and ImageQuant TL software system (GE Healthcare, Little Chalfont, UK).

Statistical analysis

All values are represented as arithmetic means ± S.E.M. One-way ANOVA by Duncan's multiple range tests was utilized to determine the statistical significance. A *p*<0.05 was consid-

ered as significance. Linear regression analysis was conducted to calculate the IC₅₀ value.

RESULTS

Inhibitory effects of imperatorin on degranulation, intracellular Ca²⁺ level, and PLCγ1 phosphorylation

For assessing the cytotoxic activity, BMMC were treated with various concentrations of imperatorin for 7 h and cell viability was not affected upto 50 μM of imperatorin (Fig. 1A). When mast cells are activated, one of the primary released mediators is histamine, which is stored in preformed granules. Because the release of histamine by activated mast cells parallels β-HEX release, we first examined the inhibitory effect of imperatorin on β-HEX release. IgE-sensitized BMMC were pre-treated with different concentrations of imperatorin for 1 h followed by 15 min of stimulation with Ag. The supernatants were collected for determination of β-HEX. As shown in Fig. 1B, Ag treatment significantly increased β-HEX release and pre-treatment with imperatorin resulted in a dose-dependent suppression of β-HEX release, with an IC₅₀ value of 15.9 μg/ml. Because elevated intracellular Ca²⁺ concentration is known to cause mast cell degranulation and eicosanoid generation (Fischer *et al.*, 2005), we examined whether imperatorin affects intracellular Ca²⁺ levels in activated BMMC. The activation of BMMC induced an increase in the intracellular Ca²⁺

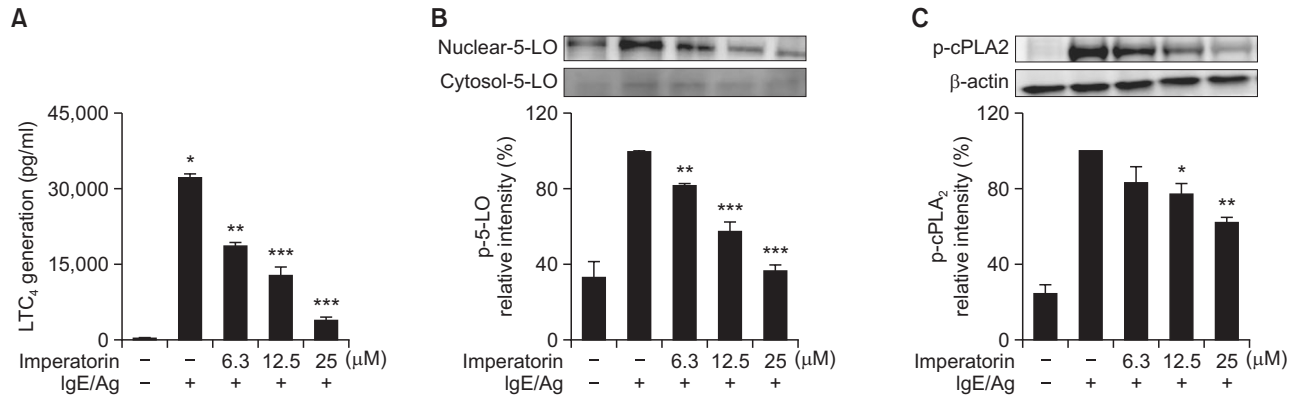


Fig. 2. Effect of imperatorin on LTC₄ generation (A), 5-LO (B), and cPLA₂ phosphorylation (C). BMMC sensitized overnight with IgE were pre-incubated with the indicated concentrations of imperatorin for 1 h and stimulated with DNP-HAS (Ag, 100 mg/ml) for 15 min. LTC₄ release into the supernatant was determined using an enzyme immunoassay kit (A) and the cells were used for Western blot analysis to detect the cytosolic and nuclear fraction of 5-LO (B), and total cell lysates for cPLA₂ (C). The values are expressed as the means ± S.E.M. of three different samples. **p*<0.05, ***p*<0.01, ****p*<0.001, significantly different from the control as determined by one-way ANOVA.

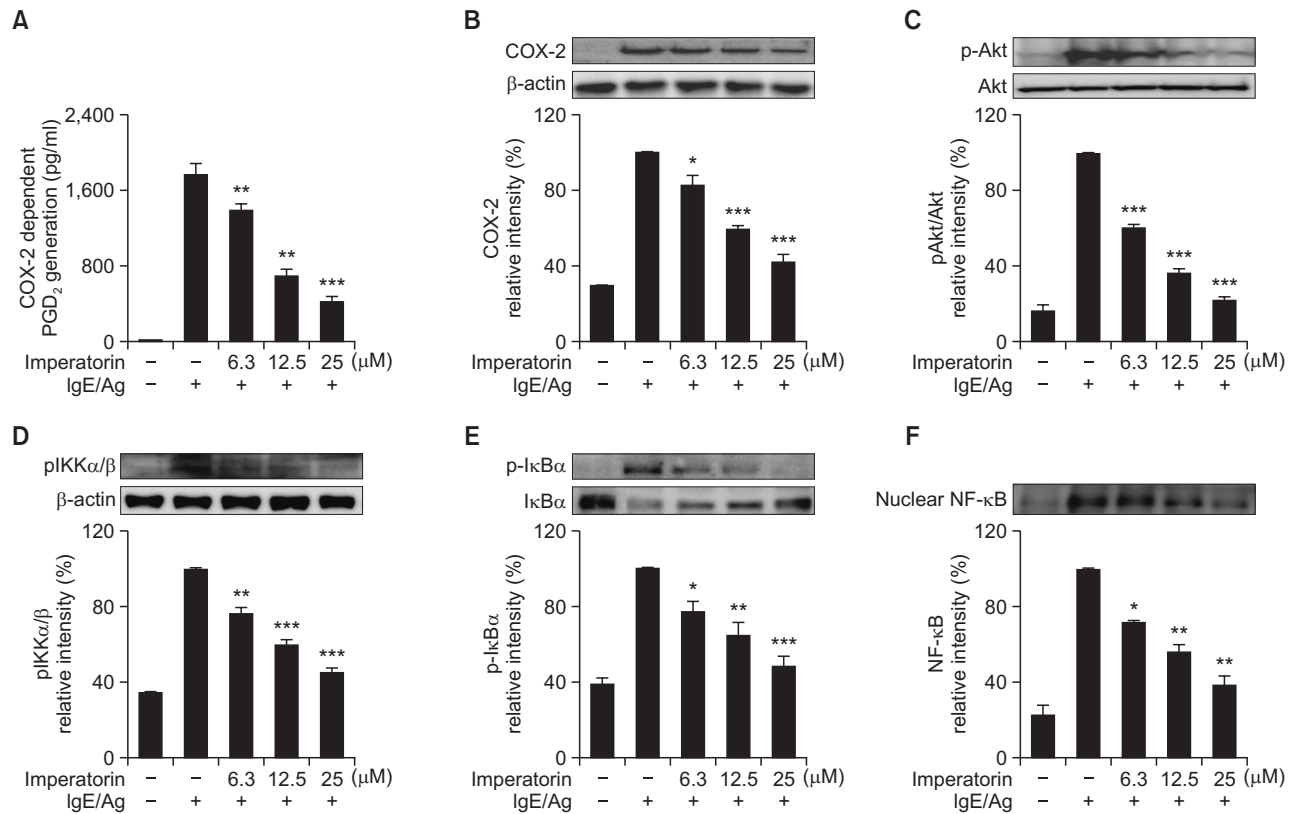


Fig. 3. Suppressive effect of imperatorin on COX-2-dependent PGD₂ production (A, B) and Akt/IKK/IκBα/NF-κB activation (C-F). BMMC sensitized overnight with IgE were pre-incubated with aspirin for 2 h and then stimulated with DNP-HAS (Ag, 100 mg/ml) for 7 h with the indicated concentrations of imperatorin. PGD₂ release into the supernatant was determined using an enzyme immunoassay kit (A) and the cells were used for Western blotting to detect COX-2 protein (B). BMMC were pre-incubated with the indicated concentrations of imperatorin for 1 h and stimulated with DNP-HAS (Ag, 100 mg/ml) for 30 min. Total cell lysates and nuclear fractions were prepared and used for Western blot analysis (C-F). The values are expressed as the means ± S.E.M. of three different samples. **p*<0.05, ***p*<0.01, ****p*<0.001, significantly different from the control as determined by one-way ANOVA.

level and this increase was inhibited by imperatorin treatment (Fig. 1C). To further study the inhibitory effect of imperatorin on degranulation, we examined PLCγ1 activation in BMMC.

The result showed that phosphorylation of PLCγ1 was up-regulated by Ag treatment of IgE-sensitized BMMC. However, pre-treatment of BMMC with imperatorin suppressed PLCγ1

phosphorylation in a dose-dependent manner (Fig. 1D), indicating that the inhibitory effect of imperatorin on degranulation was mediated through inhibition of PLC γ 1 phosphorylation.

Effect of imperatorin on LTC $_4$ generation, 5-LO, and cPLA $_2$ phosphorylation

The lipid mediator such as LT can initiate and amplify inflammatory responses (Harizi *et al.*, 2008). In order to determine whether imperatorin inhibits LTC $_4$ generation, BMMC were pre-treated with different concentrations of imperatorin for 1 h and then stimulated with Ag for 15 min. As shown in Fig. 2A, we observed that the LTC $_4$ level was significantly increased to 32 ng/ml, but imperatorin consistently inhibited LTC $_4$ generation in a dose-dependent manner, with an IC $_{50}$ value of 7.6 μ g/ml. LTC $_4$ generation is regulated by the liberation of AA from membrane phospholipids by cPLA $_2$ and 5-LO, which translocate from the cytosol to the nuclear membrane in the presence of 5-LO activating protein in response to the increased intracellular Ca $^{2+}$ level (Fischer *et al.*, 2005; Flamand *et al.*, 2006). In this study, we investigated the effect of imperatorin on phosphorylation and translocation of cPLA $_2$ and 5-LO. As shown in Fig. 2B, 5-LO was mainly localized in the cytosol in unstimulated BMMC, but it was translocated to the nuclear fraction by Ag treatment. However, imperatorin inhibited the translocation of 5-LO. The p-cPLA $_2$ level was dramatically increased in Ag-activated BMMC but it was suppressed by imperatorin treatment in a dose-dependent manner (Fig. 2C). These results indicate that imperatorin suppressed LTC $_4$ generation by inhibiting phosphorylation and translocation of cPLA $_2$ and 5-LO.

Suppressive effect of imperatorin on COX-2-dependent PGD $_2$ production and Akt/IKK/I κ B α /NF- κ B activation

To evaluate COX-2 dependent PGD $_2$ generation, BMMC were pre-treated with aspirin to eliminate pre-existing COX-1 activity, and then stimulated with Ag for 7 h with or without imperatorin treatment. COX-2-dependent PGD $_2$ generation was suppressed by imperatorin (IC $_{50}$ =13.4 μ g/ml) with decreased COX-2 protein expression (Fig. 3A, B). Since NF- κ B has been recognized as a transcription factor for the induction of inflammatory mediators including COX-2, we examined the effect of imperatorin on the Akt/I κ B kinase (IKK)/I κ B α /NF- κ B signaling pathway. After stimulation with Ag, the phosphorylation of Akt, IKK α/β , and I κ B α was increased with a concomitant decrease in total I κ B α and NF- κ B nuclear proteins. However, imperatorin suppressed the phosphorylation of Akt, IKK α/β , and I κ B α , degradation of I κ B α , and the translocation of cytosolic p65 to the nucleus (Fig. 3C-F), indicating that the Akt-mediated NF- κ B signaling pathway regulates the reduction of COX-2-dependent PGD $_2$.

Suppressive effect of imperatorin on activation of MAPKs

The phosphorylation and activation of MAPKs are crucial for NF- κ B and the subsequent activation of inflammatory mediators (Kaminska, 2005). In addition, cPLA $_2$ activity and COX-2-dependent PGD $_2$ generation are increased by phosphorylation of MAPKs (Lin *et al.*, 1993; Lu *et al.*, 2011). Therefore, we examined the effect of imperatorin on phosphorylation of MAPKs in IgE-sensitized BMMC. BMMC were pre-treated with imperatorin and inhibitors of MAPKs for 1 h before stimulation with Ag. As shown in Fig. 4, Ag stimulation in IgE-sensitized BMMC induced the expression of JNK, ERK1/2, and p38 with-

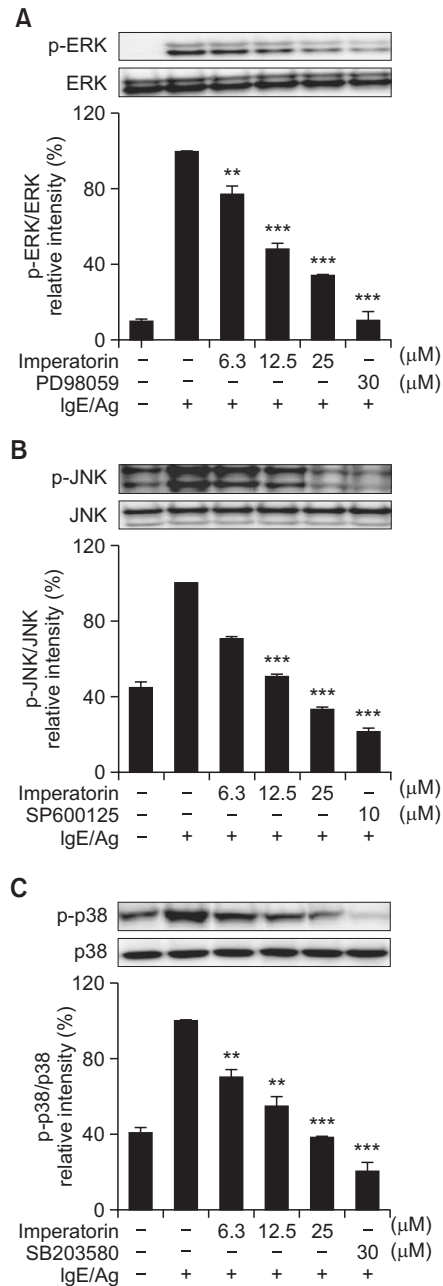


Fig. 4. Suppressive effect of imperatorin on activation of MAPKs. BMMC sensitized overnight with IgE were pre-incubated with the indicated concentrations of imperatorin for 1 h and stimulated with DNP-HAS (Ag, 100 mg/ml) for 15 min. The cells were harvested and cell lysates were prepared for measurement of total or phosphorylated ERK1/2 (A), JNK (B), and p38 (C) by Western blot analysis. Results are representative of three separate experiments. The values are expressed as the means \pm S.E.M. of three different samples. ** p <0.01, *** p <0.001, significantly different from the control as determined by one-way ANOVA.

out altering total protein levels and imperatorin suppressed the phosphorylation of all three MAPKs. The specific inhibitors of MAPKs (SB203580, SP600125, and PD98059) inhibited the phosphorylations of their respective MAPKs.

DISCUSSION

In our previous study, we identified imperatorin from *Angelica Dahuricae Radix* (Jeong *et al.*, 2014), but the detailed mechanism for alleviating anti-inflammatory and anti-allergic effects in BMMC was not elucidated. The present study demonstrated that imperatorin suppressed degranulation and eicosanoid production, and these effects of imperatorin were mediated through the inhibition of 5-LO, cPLA₂, MAPKs, PLCγ1, and NF-κB/IKK/ Akt activation in BMMC.

Mast cells represent a major source of histamine, proteases, and other potent chemical mediators implicated in a wide variety of inflammatory and immunologic processes (Boyce, 2003). Activated mast cells degranulate and release preformed mediators such as histamine or AA metabolites. Among the preformed mediators, β-HEX, an acid hydrolase, is a marker of mast cell degranulation. In this study, we investigated the role of Ca²⁺ influx and PLCγ1 phosphorylation in the modulation of degranulation by imperatorin because PLCγ1-mediated Ca²⁺ signal is essential for mast cell degranulation (Metcalfe *et al.*, 2009). The results show that imperatorin suppresses PLCγ1 phosphorylation and intracellular Ca²⁺ influx, resulting in the inhibition of degranulation of IgE/Ag-stimulated BMMC (Fig. 1).

The newly synthesized lipid-derived mediators such as LTC₄ and PGD₂ after mast cell activation are obtained from the AA release from membrane phospholipids by cPLA₂. LTC₄ generation is sequentially regulated by cPLA₂ and 5-LO (Fischer *et al.*, 2005). Both 5-LO and cPLA₂ translocate from the cytosol to the perinuclear membrane in response to an increased intracellular Ca²⁺ level (Flamand *et al.*, 2006). In addition, cPLA₂ is phosphorylated by MAPKs (Soberman and Christmas, 2003). To determine the effect of imperatorin on these serial events, we examined the phosphorylation of 5-LO, cPLA₂, and MAPKs after IgE/Ag activation. The results show that the phosphorylation of cPLA₂ and 5-LO was suppressed by imperatorin (Fig. 2), indicating that imperatorin inhibited phosphorylation of MAPKs (Fig. 4), thus leading to reduced production of LTC₄.

PGD₂, a major prostaglandin, is produced by the COX-2 pathway in mast cells. Because it has been reported that PGD₂ production results from COX-2 expression via activation of the NF-κB and MAPK pathways (Lu *et al.*, 2011; Lu *et al.*, 2014), we elucidated the effects of imperatorin on the NF-κB and MAPK pathways in IgE/Ag-stimulated BMMC. In this study, imperatorin decreased the phosphorylation of Akt/IKK/IκBα as well as the degranulation of IκBα, consequently reducing the translocation of NF-κB subunit p65 from the cytosol into the nucleus (Fig. 3). Thus, the reduction in PGD₂ production by imperatorin depends on the suppression of NF-κB-induced COX-2 expression. Furthermore, the inhibition of phosphorylation of MAPKs by imperatorin suppresses COX-2-dependent PGD₂ generation because MAPKs play important roles in cytokine production and eicosanoid generation (Lu *et al.*, 2011; Lu *et al.*, 2012).

In conclusion, we propose the possible inhibitory mechanisms of imperatorin involved in inflammatory response of mast cells. Imperatorin inhibited degranulation through the PLCγ-Ca²⁺ pathway, LTC₄ generation through the cPLA₂/5-LO pathway, and PGD₂ production through Akt/IKK/IκBα/NF-κB/COX-2 along with the inactivation of MAPKs in IgE/Ag-stimulated BMMC (Fig. 5), suggesting a possible approach to the

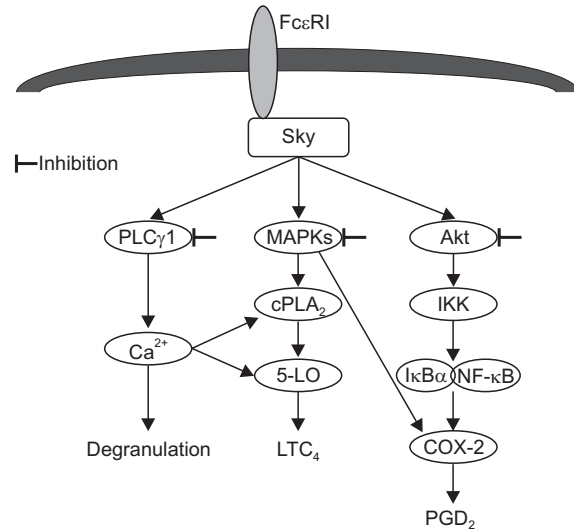


Fig. 5. Possible inhibitory mechanisms of imperatorin on degranulation and eicosanoid generation in mast cells.

treatment of inflammatory diseases.

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