Biochemical Pharmacology 82 (2011) 1700–1708

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Emodin, a naturally occurring anthraquinone derivative, suppresses IgE-mediated anaphylactic reaction and mast cell activation

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ARTICLE INFO

Article history:
Received 22 July 2011
Accepted 26 August 2011
Available online 3 September 2011

Keywords:
Emodin
Passive anaphylactic reaction
Mast cells
IgE
Syk kinase

ABSTRACT

The high-affinity receptor for IgE (FcεRI)-mediated activation of mast cells plays an important role in allergic diseases such as asthma, allergic rhinitis and atopic dermatitis. Emodin, a naturally occurring anthraquinone derivative in oriental herbal medicines, has several beneficial pharmacologic effects, such as anti-cancer and anti-diabetic activities. However, the anti-allergic effect of emodin has not yet been investigated. To assess the anti-allergic activity of emodin, *in vivo* passive anaphylaxis animal model and *in vitro* mouse bone marrow-derived mast cells were used to investigate the mechanism of its action on mast cells. Our results showed that emodin inhibited degranulation, generation of eicosanoids (prostaglandin D_2 and leukotriene C_4), and secretion of cytokines (TNF-α and IL-6) in a dose-dependent manner in IgE/Ag-stimulated mast cells. Biochemical analysis of the FcεRI-mediated signaling pathways demonstrated that emodin inhibited the phosphorylation of Syk and multiple downstream signaling processes including mobilization of intracellular Ca^{2+} and activation of the mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and NF-κB pathways. When administered orally, emodin attenuated the mast cell-dependent passive anaphylactic reaction in IgE-sensitized mice. Thus, emodin inhibits mast cell activation and thereby the anaphylactic reaction through suppression of the receptor-proximal Syk-dependent signaling pathways. Therefore, emodin might provide a basis for development of a novel anti-allergic drug.

1. Introduction

Anaphylaxis is a life-threatening, rapid-onset allergic reaction with an estimated frequency of 50–2000 episodes per 100,000 people [1]. Asthma, rhinitis and atopic dermatitis are more complex, chronic forms of Th2-dependent allergic inflammation, affecting as many as 300 million people worldwide [2,3]. Approaches for the treatment of these allergic diseases include allergen-specific immunotherapy, DNA vaccination, administration of humanized anti-IgE antibody, treatment with soluble IL-4 receptor, use of antagonists of the leukotriene and histamine receptors, and administration of glucocorticoids [4]. Since it is projected that the number of patients with allergy will increase as more countries become urbanized, it will be important to develop a novel anti-allergic agent that is easy to use and can improve the quality of life of affected patients.

Mast cells are a central player in immediate-type allergic reactions. Aggregation of the high-affinity IgE receptor (FcεRI) on mast cells triggers release of preformed mediators (e.g. histamine and proteases) and newly synthesized inflammatory mediators (e.g. lipid mediators, such as prostaglandin D_2 (PGD_2) and leukotriene C_4 (LTC_4), and various cytokines) [5–7]. FcεRI engagement by cognate antigen (Ag) initiates signaling cascades that include activation of receptor-proximal tyrosine kinases, such as Syk, Lyn, Fyn and Btk, and phosphorylation of various adapter molecules. Syk plays an essential role in the initiation of IgE-dependent activation of mast cells: once activated, Syk phosphorylates adapter proteins such as linker for activation of T cells (LAT), resulting in the formation of a macromolecular signaling complex, which allows the diversification of downstream signaling that is required for the release of various pro-inflammatory mediators [8–11]. Such signaling pathways include phospholipase Cγ (PLCγ)-mediated Ca^{2+} mobilization, which is a prerequisite step for subsequent degranulation and

Abbreviations: BMMC, bone marrow-derived mast cell; Syk, spleen tyrosine kinase; LAT, linker of activated T cells; PLCγ1, phospholipase Cγ1; $cPLA_2α$, cytosolic phospholpase $A_2α$; COX-2, cyclooxygenase-2; 5-LO, 5-lipoxygenase; PCA, passive cutaneous anaphylaxis.

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doi:10.1016/j.bcp.2011.08.022

LTC_4 generation [8]. Therefore, blockade of Syk kinase could inhibit the allergen-induced release of multiple granule-stored and newly synthesized mediators [9]. FcεRI crosslinking also induces activation of the mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3K)/Akt, and NF-κB signaling pathways, which ultimately contribute to the inducible expression of multiple pro-inflammatory genes, such as those for cytokines and cyclooxygenase (COX)-2, for propagation of inflammation [11]. In addition, phosphorylation of cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) by MAPKs is essential for the release of arachidonic acid (AA), a common precursor of eicosanoids [12].

The herbaceous plants *Rheum officinale* Bail, *Polygonum multiflorum* Thunberg, *Polygoni cuspidati* (*P. cuspidati*) radix and *Cassia obtusifolia* seed have been used safely for many centuries as traditional medicines in Eastern Asia. These oriental herbs display multiple pharmacologic effects, such as anti-allergic and anti-inflammatory activities among others [13–16]. Emodin (1,3,8-trihydroxy-6-methylanthraquinone), an active component commonly present in these herbs, has been shown to display a number of biological activities such as anti-microbial, immunosuppressive, anti-inflammatory, and anti-atherosclerotic activities [17–20]. Emodin also has anti-diabetic activity [21] and is also used as a nutritional supplement to regulate bowel motility. Moreover, emodin suppresses the oncogenic transformation of human breast and lung cancer cells through attenuation of HER2/neu tyrosine kinase activity, suggesting its potential chemotherapeutic application as an anti-cancer agent to target HER2/neu-dependent types of cancer [22,23]. However, the effect of emodin on allergic reactions has not yet been addressed.

Here we describe for the first time the anti-allergic activity of emodin and the mechanism of its action on mouse bone marrow-derived mast cells (BMMCs). Our findings suggest that emodin stabilizes mast cells by preventing Syk phosphorylation *in vitro* and attenuates the IgE-mediated anaphylactic reaction *in vivo*, suggesting the prophylactic and therapeutic potential of this natural product for allergic diseases.

2. Material and methods

2.1. Plant material

Emodin was isolated using the previously published method by Lee et al. [24]. Emodin was prepared by dissolving in 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.

2.2. Induction of IgE-mediated passive cutaneous anaphylaxis (PCA) and passive systemic anaphylaxis (PSA) in mice

Nine ICR mice (Hyochang Science, Daegu, Korea) were placed in each cage within a laminar airflow cabinet and were fed with standard laboratory chow (Purina, Seoul, Korea) and water ad libitum. The mice were kept at a temperature of 22 ± 1 °C and at a relative humidity of 55 ± 10% and 12 h/12 h (light/dark) cycle for at least 7 days prior to the experiments throughout the study. For PCA, 80 ng of mouse anti-dinitrophenyl (DNP) IgE (Sigma–Aldrich, St. Louis, MO, USA) were intradermally injected into one ear of 7-week-old male mice, followed 24 h later by oral administration of 5–40 mg/kg emodin or 50 mg/kg fexofenadine-HCl, a histanime H1 receptor antagonist (Korea Pharma, Seoul). One hour later, the mice were intravenously challenged with 60 μg of Ag (DNP-human serum albumin (HSA); Sigma–Aldrich, St. Louis, MO, USA) in 200 μl of PBS containing 1% (w/v) Evans blue. The mice were euthanized 1 h after treatment with Ag, and their ears were removed and dissolved with 400 μl of formamide at 63 °C overnight. The amount of dye extravasation was determined colorimetrically at 630 nm. For PSA, mice were sensitized by intravenous injection of 2 μg of IgE in 100 μl of saline or treated with saline alone. After 24 h, the mice were challenged intravenously with 4 mg of DNP-HSA in 200 μl of saline after oral administration of 5–40 mg/kg emodin or 50 mg/kg fexofenadine-HCl for 1 h, and blood was collected by cardiac puncture 5 min after Ag challenge [25]. Serum histamine, LTC_4 and PGD_2 concentrations were determined by respective assay kits (Cayman Chemicals, Ann Arbor, MI, USA). Experiments using mice were approved by the Institutional Animal Care and Use Committee of Yeungnam University.

2.3. Culture and activation of mouse bone marrow-derived mast cells (BMMCs) and HMC-1 cells

BMMCs isolated from male Balb/cJ mice (Sam Taco, INC, Seoul, Korea) were cultured in RPMI 1640 media (Thermo Scientific, Utah, USA) containing 10% FBS, 100 U/ml penicillin (Thermo Scientific, Utah, USA), 10 mM HEPES buffer (Sigma–Aldrich Company, UK), 100 μM MEM non-essential amino acid solution (Invitrogen, NY, USA) and 20% PWM-SCM (pokeweed mitogen-spleen cell conditioned medium) as a source of IL-3. HMC-1 cells, a human mast cell line, were cultured in Iscove's Modified Dulbecco's Medium (IMDM) with 10% FBS, 100 U/ml penicillin, 10 mM HEPES buffer, and 100 μM MEM non-essential amino acid solution. For cell stimulation, 10^6 cells/ml cells were sensitized overnight with 500 ng/ml anti-DNP and then stimulated for 15 min with 100 ng/ml DNP-HSA. When the effects of emodin and PP2 (Calbiochem, La Jolla, CA, USA) were examined, they were added 1 h prior to the addition of DNP-HSA. β-Hexosaminidase (β-Hex), a marker of mast cell degranulation, was quantified by spectrophotometric method, as described previously [26]. LTC_4 was quantified using LTC_4 assay kit. To assess COX-2-dependent PGD_2 synthesis, BMMC were preincubated with 1 μg/ml aspirin for 2 h to irreversibly inactivate preexisting COX-1. After washing, BMMC were activated with DNP-HSA for 7 h with or without emodin or inhibitors. PGD_2 in the supernatants were quantified using PGD_2 assay kit. IL-6 and TNF-α released from BMMCs activated for 6 h with Ag were quantified using respective immunoassay kits (R&D Systems, Minneapolis, MN, USA).

2.4. Measurement of intracellular Ca^{2+} level

IgE-sensitized BMMCs on coverslips were loaded for 60 min with the fluorescent Ca^{2+} indicator fura-2/AM (5 μM) (Invitrogen, NY, USA) in HEPES buffer (pH 7.4). Then, the cells were washed and stimulated with Ag. Fura-2 fluorescence images were obtained using ARGUS-50 image analyzer (Hamamatsu Photonics, Shizuoka, Japan) with excitation at 340 nm (F340) and 380 nm (F380) at 5-s interval. The ratio (F340/F380) was calculated by ImageJ software (NIH, Bethesda, MD, USA).

2.5. Preparation of nuclear and cytosolic extracts

Cells were suspended in wash buffer containing 10 mM HEPES buffer (pH 8.0), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride (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 resuspended in a buffer containing 20 mM HEPES (pH 8.0), 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and the protease inhibitor cocktail. This suspension was incubated for 30 min at 4 °C followed by centrifugation at 10,000 × *g*, and the resultant supernatants were used as a nuclear fraction. The purity

of the resultant cytosolic and nuclear fractions was confirmed by proper distributions of GAPDH (a cytosolic marker) and lamin B (a nuclear marker), respectively [27].

2.6. Membrane fraction extraction

Cell were washed with cold PBS, resuspended in 100 μl of lysis buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 M dithiothreitol, 200 mM NaF, 200 mM Na_3VO_4, and protease inhibitor cocktail) and sonicated. The cell sonicates were then centrifuged at 700 × *g* for 10 min to remove intact cells and nuclei. The recovered supernatants were centrifuged at 20,000 × *g* for 30 min. Proteins in the pellet fractions were solubilized with 1% (v/v) Triton X-100, 1% (v/v) NP40, and 0.1% (w/v) SDS in lysis buffer for 30 min on ice, followed by centrifugation at 15,000 × *g* for 15 min. The recovered supernatants were used as membrane fractions.

2.7. Western blotting

IgE-sensitized mast cells were stimulated with Ag for indicated times with or without emodin or other inhibitors. The cells (2×10^6 cells equivalent) were then applied to 8% (w/v) SDS-PAGE under reducing conditions, transfer 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 (ERK) 1/2, p38, JNK, PI3K (p85/p55), Akt (Ser473), IKKα/β, IκBα, Syk, PLCγ1 (Tyr783) and LAT (Tyr171), those against total PI3K, Akt, IκBα, ERK1/2, JNK and p38 (Cell Signaling Technology, Danvers, MA, USA), or those against NF-κB, β-actin, lamin B, $cPLA_2\alpha$, phospho-$cPLA_2\alpha$ (Ser505), 5-lipoxygenase (5-LO), IKKα/β, Syk, PLCγ1, LAT (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and COX-2 (Cayman Chemicals, Ann Arbor, MI, USA). After incubation for 1 h with horseradish peroxide-conjugated goat anti-rabbit IgG (1:3000 dilution), the proteins were visualized using an ECL system (Pierce Biotechnology, Rockford, IL, USA).

2.8. Statistic analysis

All experiments described were performed three or more times. Average values were expressed as means ± s.e.m. The Student's *t*-test was used for the comparison of two independent groups. For all tests, a *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. Emodin suppresses anaphylactic reaction in mice

Anaphylaxis is a profound allergic reaction initiated by allergen-induced cross-linking of specific IgE bound to FcεRI. IgE/FcεRI-stimulated mediator release from mast cells is a major determinant of anaphylaxis [25,28,29]. To assess the anti-allergic activity of emodin, we performed PCA and PSA models in mice. PCA was examined by i.v. challenge with Ag (DNP-HSA in 1% Evans blue dye) of sensitized mice after oral administration of 25 mg/kg and 50 mg/kg emodin as well as 50 mg/kg fexofenadine-HCl for 1 h. Emodin significantly attenuated the mast cell-dependent PCA reaction in a dose-dependent manner ($n = 9$), exhibiting 48% ($P < 0.001$) and 55% ($P < 0.001$) suppression at 25 and 50 mg/kg of Evans blue exudation (Fig. 1A). PSA was assessed in mice sensitized by i.v. injection of IgE or control saline and challenged 24 h later with an i.v. injection of DNP-HSA. Emodin reduced serum histamine, LTC_4 and PGD_2 levels in a dose-dependent manner ($n = 9$), exhibiting 38% ($P < 0.05$) and 70% ($P < 0.05$) suppression of LTC_4 generation, 41% ($P < 0.01$) and 48% ($P < 0.01$) suppression of PGD_2 generation, and 13.6% ($P < 0.05$) and 34.7% ($P < 0.01$) suppression of histamine release at 25 and 50 mg/kg of emodin, respectively (Fig. 1B). The suppressive effect of 50 mg/kg of emodin was similar to that of 50 mg/kg of fexofenadine-HCl [30], a histamine H1 receptor antagonist that is also capable of stabilizing mast cells used as a positive control [31].

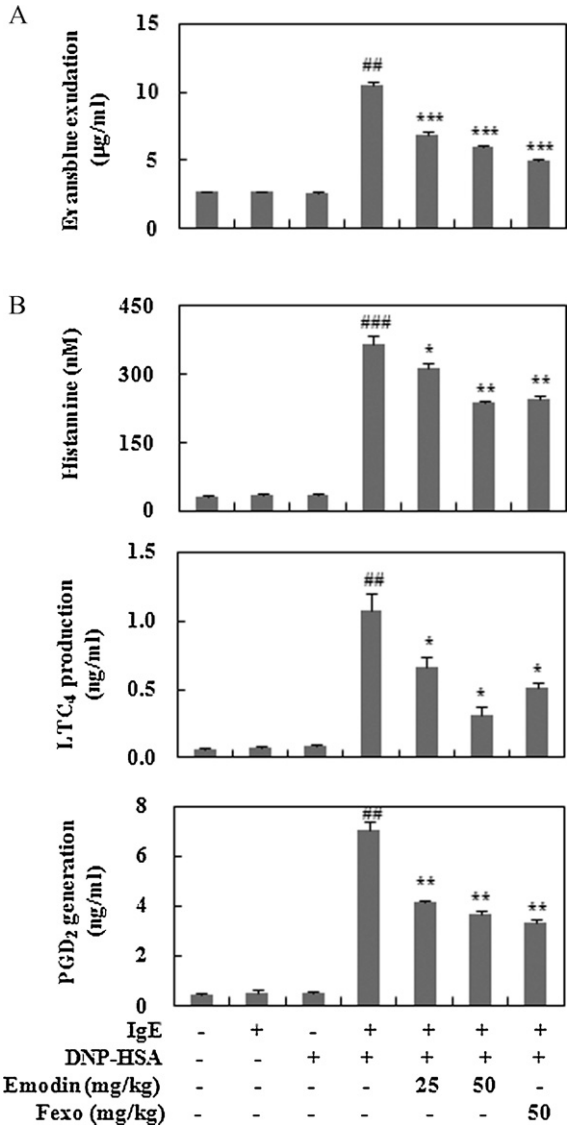

Fig. 1. Emodin inhibits IgE/Ag induced PCA and PSA reaction. (A) In a PCA test, ICR mice were injected intradermally with 80 ng of anti-DNP into one ear. After 24 h, the mice were intravenously injected with 60 μg of DNP-HSA in 200 μl of PBS containing 1% Evans blue. To evaluate the effect of emodin, it was orally administered 1 h before Ag administration. The dye extravasation in ears were quantified ($n = 9$ animals in three independent experiments, mean ± s.e.m., $^{\#\#}P < 0.01$ was compared to non-treated mice. $^{***}P < 0.001$ were compared to IgE/DNP-HSA sensitized mice). (B) In PCA test, Mice were sensitized by i.v. injection of 2 μg of IgE in 100 μl saline or treated with saline alone. Alter 24 h, the mice were challenged i.v. with 4 mg of DNP-HSA in 200 μl saline after oral administration of 25 mg/kg and 50 mg/kg emodin or 50 mg/kg fexofenadine-HCl for 1 h. Blood was collected 5 min after Ag challenge, and serum histamine, LTC_4 and PGD_2 concentrations were determined by respective assay kits ($n = 9$ animals in three independent experiments, mean ± s.e.m., $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ were compared to non-treated mice. $^{*}P < 0.05$ and $^{**}P < 0.01$ were compared to IgE/Ag sensitized mice). Fexo (50 mg/kg) was used as an atypical anti-histamine control drug.

3.2. Emodin inhibits mast cell degranulation and Ca^{2+} mobilization in mast cells

The *in vivo* results described above led us to evaluate the mechanism of the anti-allergic activity of emodin, by placing our

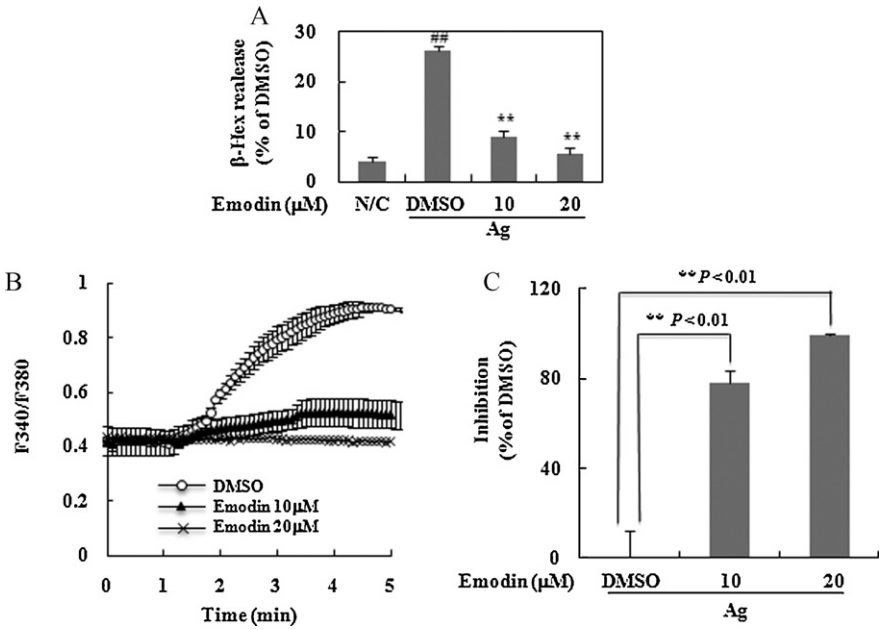

Fig. 2. Emodin inhibits degranulation and Ca^{2+} mobilization in IgE/Ag-activated BMMCs. After BMMCs were sensitized overnight with anti DNP-IgE and challenged with DNP-HSA with or without emodin, β-Hex release (at 15 min) (A) and Ca^{2+} mobilization (by 5 min) (B) were evaluated. % inhibition of intracellular Ca^{2+} level was determined (C). N/C means non-treated BMMCs. The values indicate the mean ± s.e.m. from three independent experiments. $^{\#\#}P < 0.01$ was compared to non-treated BMMCs, and $^{**}P < 0.01$ was compared to IgE/Ag sensitized BMMCs.

main focus on its effects on mast cells, a central player of anaphylactic reaction. We first examined the cytotoxic effect of emodin on BMMCs using MTT assay and found that it did not affect cell viability at 40 μM (data not shown). Therefore, emodin at concentrations of <20 μM were used for subsequent experiments. To investigate the effect of emodin on degranulation of IgE/Ag-activated BMMCs, we measured the release of β-Hex in the presence or absence of emodin. We found that emodin strongly suppressed β-Hex release in a dose-dependent manner ($P < 0.01$) (Fig. 2A). Since the increase in the concentration of cytosolic Ca^{2+} is essential for degranulation of mast cells [32], we investigated the effect of emodin on Ca^{2+} influx. Indeed, 20 μM emodin completely inhibited IgE/Ag-stimulated Ca^{2+} influx ($P < 0.01$) (Fig. 2B and C).

3.3. Emodin inhibits LTC_4 generation by mast cells

IgE/Ag stimulation of BMMCs promptly elicited LTC_4 generation, which was blocked by emodin in a dose-dependent manner (data from three independent experiments, $P < 0.05$ and $P < 0.01$ *versus* without emodin) (Fig. 3A). Generation of LTC_4 is regulated by two steps: liberation of AA from membrane phospholipids by $cPLA_2\alpha$ and oxygenation of free AA by 5-LO [32]. Both 5-LO and $cPLA_2\alpha$ translocate from the cytosol to the perinuclear membrane in response to an increase in the cytosolic Ca^{2+} level [33,34]. Additionally, $cPLA_2\alpha$ is phosphorylated by MAPKs, a process that is necessary for maximum AA release. In order to determine the action of emodin, we performed immunoblotting of $cPLA_2\alpha$, 5-LO and MAPKs after IgE/Ag treatment with or without emodin. Although the majority of $cPLA_2\alpha$ resided in the cytosol regardless of IgE/Ag stimulation, a pool of phosphorylated $cPLA_2\alpha$ was detected in both the cytosol (C-p-$cPLA_2$) and nuclear (N-p-$cPLA_2$) fractions in activated cells, a period when LTC_4 generation proceeded. Under these conditions, the levels of β-actin and lamin B (internal controls for the cytosolic and nuclear fractions, respectively) were unchanged, indicating successful fractionation and equal sample loading. The IgE/Ag-dependent appearance of C-p-$cPLA_2$ and N-p-$cPLA_2$ was suppressed by emodin (Fig. 3B), indicating that emodin blocked MAPK (likely ERK1/2)-dependent phosphorylation and Ca^{2+}-dependent translocation of $cPLA_2\alpha$. 5-LO was detected mainly in the cytosol (C-5-LO), and a portion of the 5-LO pool translocated to the nuclear fraction (N-5-LO) after cell activation, which again paralleled LTC_4 generation. Emodin as well as each MAPK inhibitor efficiently inhibited the nuclear translocation of 5-LO (Fig. 3B and Supplementary Fig. 1A). Densitometric analyses of the immunoblots in Fig. 3B confirmed that emodin inhibited the Ag-dependent translocation of $cPLA_2\alpha$ and 5-LO from the cytosolic to nuclear fractions (Fig. 3D–F). Several lines of evidence have indicated that intracellular Ca^{2+} influx is involved in the regulation of 5-LO translocation [33,34], while other groups have reported Ca^{2+}-independent 5-LO translocation into the nucleus [35]. Although MAPKs can phosphorylate and activate 5-LO [36], it is at present difficult to explain how the MAPK inhibitors blocked 5-LO translocation in IgE/Ag-activated BMMC. However, our preliminary results suggested that both p38 and ERK inhibitors blocked intracellular Ca^{2+} influx in IgE/Ag-activated BMMCs (data not shown). Thus, we speculate that the inhibition of 5-LO translocation into the nuclear fraction by the MAPK inhibitors might be in part through their suppression of intracellular Ca^{2+} influx. Furthermore, IgE/Ag-elicited activation of all MAPKs was attenuated by emodin (Fig. 3C), as confirmed by densitometric analysis (Fig. 3G–I).

3.4. Emodin inhibits delayed PGD_2 generation and cytokine production in mast cells

In mast cells, free AA could also be metabolized to PGD_2 through the COX pathway. Unlike LTC_4 synthesis, PGD_2 synthesis occurs in a biphasic manner. The immediate phase of PGD_2 production, which depends on preexisting COX-1, occurs in parallel with LTC_4 production within a few minutes. This initial phase is followed by the second phase of PGD_2 production that is sustained over 2–10 h and is dependent on *de novo*-induced COX-2 [37,38]. To assess COX-2-mediated, delayed PGD_2 generation, IgE-sensitized BMMC were pre-treated with aspirin to abolish any preexisting COX-1 activity, followed by a brief wash, and then stimulated with Ag for 7 h with or without emodin. We found that delayed PGD_2

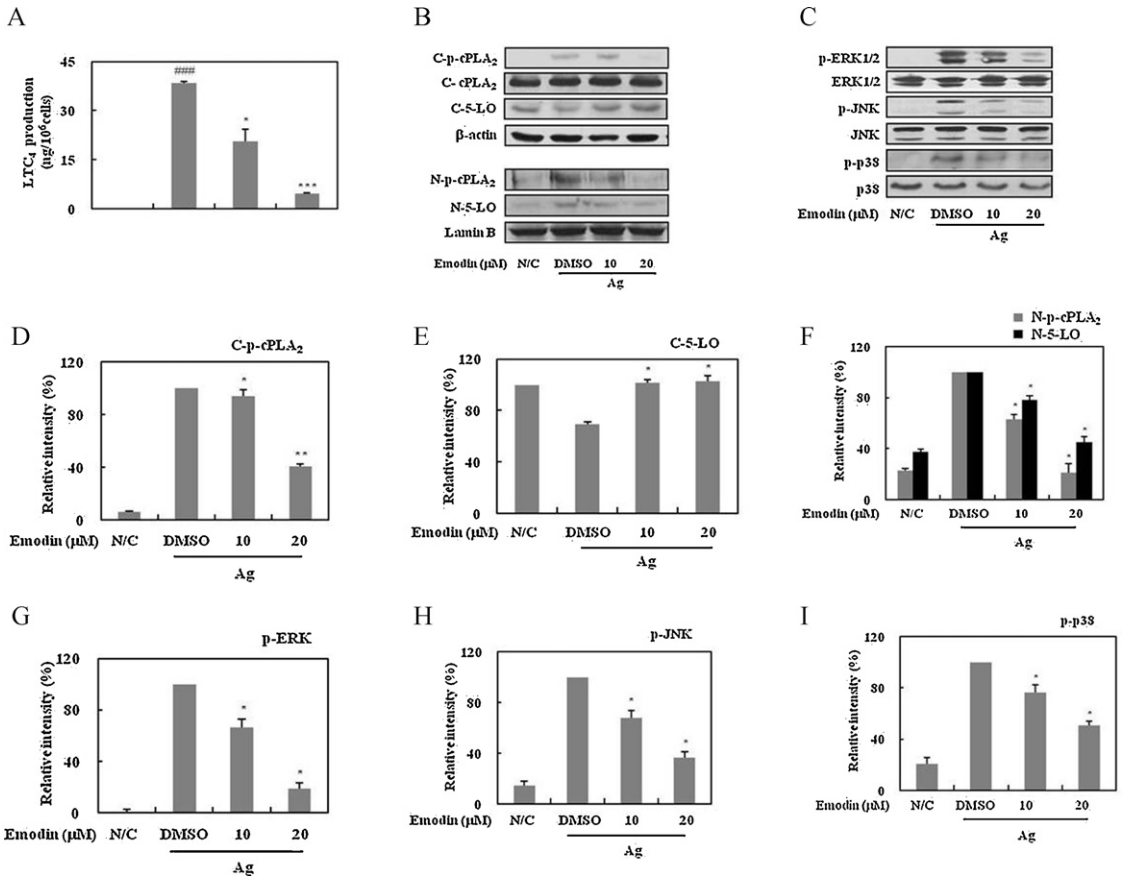

Fig. 3. Emodin suppresses LTC_4 generation by IgE/Ag-activated BMMCs. (A) After pre-incubation with emodin for 30 min, IgE-sensitized BMMCs were challenged with DNP-HSA for 15 min, and LTC_4 generation was measured. N/C means non-stimulated control. The values indicate the mean ± s.e.m. from three independent experiments, $^{\#\#\#}P < 0.001$ was compared to non-treated BMMCs, $^{*}P < 0.05$ and $^{***}P < 0.001$ were compared to IgE/Ag sensitized BMMCs. (B and C) After preincubation with emodin for 1 h, IgE-sensitized BMMCs were challenged with DNP-HSA for 15 min, cytosolic and nuclear lysates were immunoblotted with anti-phospho $cPLA_2\alpha$ and anti-5-LO antibodies (B), and cell lysates were subjected to immunoblotting for total and phosphorylated forms of ERK1/2, JNK and p38 (C). Immunoblots of β-actin and lamin B were used as controls for cytosol and nuclear fractions, respectively. In (B) and (C), representative results of three independent experiments are shown. (D–I) The relative ratios of C-p-$cPLA_2$/p-$cPLA_2$ (D), C-5-LO/β-actin (E), N-p-$cPLA_2$, or N-5-LO/lamin B (F), p-ERK/ERK (G), p-JNK/JNK (H) and p-p38/p38 (I) protein levels were determined by measuring immunoblot band intensities by scanning densitometry, respectively ($^{*}P < 0.05$ and $^{**}P < 0.01$). The results from three separate experiments as relative ratios (%) are represented.

generation was dose-dependently inhibited by emodin (data from three independent experiments, $P < 0.01$ *versus* without emodin) (Fig. 4A upper), with a concomitant reduction of COX-2 protein (Fig. 4A lower). The suppression of COX-2 induction by emodin may be due to its inhibitory effect on MAPKs, since all MAPK inhibitors ablated COX-2 expression (Supplementary Fig. 1B). Furthermore, emodin dose-dependently inhibited the production of the proinflammatory cytokines TNF-α and IL-6 (data from three independent experiments, $P < 0.05$ and $P < 0.01$ *versus* without emodin) (Fig. 4B). Since NF-κB has been identified as a central regulator of the expression of both COX-2 and cytokines [39–41], we examined whether emodin suppresses the NF-κB pathway, in which IKK-dependent phosphorylation and degradation of the inhibitory IκBα results in the nuclear translocation of NF-κB. After IgE/Ag activation, phosphorylation of the IKK complex (p-IKKα/β) and IκBα (p-IκBα) was increased, with a concomitant decrease in total IκBα protein and nuclear translocation of NF-κB (N-NF-κB). Emodin suppressed the increases of p-IKKα/β and p-IκBα, the reduction of IκBα, and the appearance of N-NF-κB (Fig. 4C and E–G). Since the PI3K/Akt pathway also affects gene transcription in activated mast cells, we next examined the effect of emodin on this pathway. IgE/Ag stimulation resulted in increase of the phosphorylation forms of Akt, and emodin suppressed these responses (Fig. 4C and D). Notably, treatment with wortmannin potently reduced the phosphorylation of IKK and IκBα, the degradation of IκBα and the nuclear translocation of NF-κB (Supplementary Fig. 2A) as well as the secretion of TNF-α and IL-6 (Supplementary Fig. 2B), suggesting that the PI3K/Akt pathway lies upstream of the NF-κB pathway. Thus, sequestration of the PI3K/Akt/NFκB axis by emodin culminates in reduction of COX-2-dependent PGD_2 generation and cytokine expression.

3.5. Emodin inhibits Syk activation

The fact that multiple effector functions of mast cells were all suppressed evenly by emodin indicates that it may block an earlier regulatory step of FcεRI signaling. Since the tyrosine kinase Syk plays an essential role in the initiation of FcεRI-dependent signaling [9–11], and since emodin has a tyrosine kinase inhibitor activity [14,42–44], we examined whether emodin affects phosphorylation of Syk. We also detected the phosphorylated forms of LAT and PLCγ1, which lie immediately downstream of Syk [45]. We found that tyrosine phosphorylation of Syk, LAT and PLCγ1 was significantly inhibited by emodin (Fig. 5A). PP2, a general Src family kinase inhibitor used as a positive control, potently inhibited the phosphorylation of Syk, PLCγ1 and LAT (Fig. 5A). Considering the obligatory role of PLCγ1 phosphorylation in the inositol phospholipid turnover and subsequent Ca^{2+} signaling [30], it is likely that the observed inhibition of Ca^{2+} influx by emodin (Fig. 2B) relies on its inhibitory effect on Syk-dependent PLCγ1 activation. Finally, to assess whether emodin could also block the activation of human mast cells, we examined

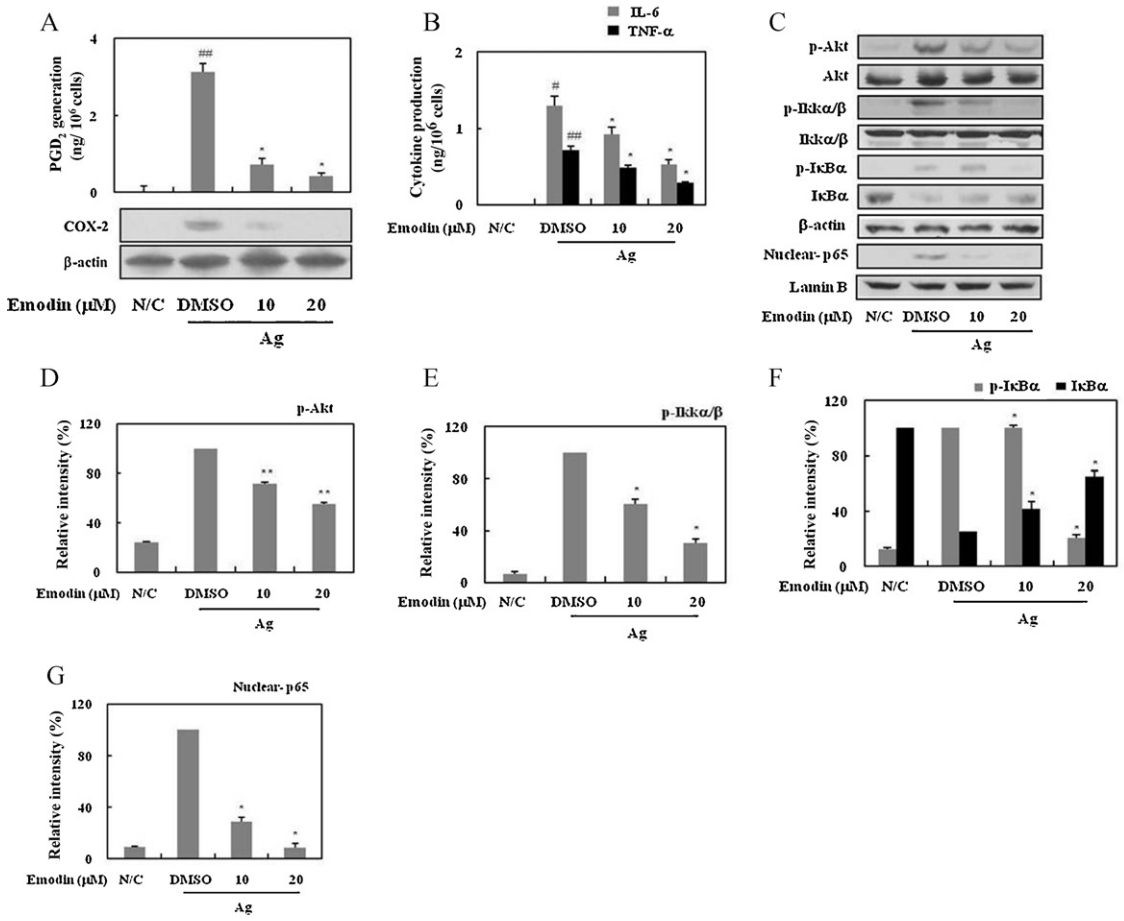

Fig. 4. Emodin suppresses PGD_2 generation and cytokines through the PI3K and NFκB pathways. (A) Activated BMMCs were preincubated with 1 μg/ml asprin for 2 h to abolish pre-existed COX-1 activity followed by a brief washing and the stimulated with DNP-HSA for 7 h. PGD_2 released into the supernatant was quantified by PGD_2-MOX enzyme immunoassay kit, cells were used for immunoblotting of COX-2 protein. The values indicate the mean ± s.e.m. from three independent experiments, $^{\#\#}P < 0.01$ was compared to non-treated BMMCs, $^{*}P < 0.05$ was compared to IgE/Ag sensitized BMMCs. (B) Effects of emodin on TNF-α and IL-6 production were measured after stimulated of BMMCs with DNP-HSA for 6 h. N/C means non-stimulated control. The values indicate the mean ± s.e.m. from three independent experiments, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ were compared to non-treated BMMCs, $^{*}P < 0.05$ was compared to IgE/Ag sensitized BMMCs. (C) Activated BMMCs were preincubated with emodin for 1 h and stimulated with DNP-HSA for 10 min. Cell lysates were subjected to immunoblotting with antibodies for phospho-Ser473 Akt (p-Akt), phospho-IKKα/β (p-IKKα/β) and phospho-IκBα (p-IκBα) followed by stripping and reprobing with antibodies against their respective total proteins. Nuclear extracts from BMMCs that were preincubated with emodin for 1 h and stimulated with DNP-HSA for 30 min were taken for immunoblotting of NF-κB-p65 to assess its nuclear translocation. Immunoblots of β-actin and lamin B were used as controls for cytosol and nuclear fractions, respectively. In (C), representative results of three independent experiments are shown. (D–G) The relative ratios of p-Akt/AKt (D), p-IKKα/β/IKKα/β (E), p-IκBα, IκBα/β-actin (F) and nuclear-p65/lamin B (G) protein levels were determined by measuring immunoblot band intensities by scanning densitometry, respectively ($^{*}P < 0.05$ and $^{**}P < 0.01$). The results from three separate experiments as relative ratios (%) are represented.

its effect on the IgE/Ag-dependent phosphorylation of Syk, PLCγ1 and LAT in HMC-1 cells, a human mast cell line. As shown in Fig. 5B, these receptor-proximal events were efficiently suppressed by emodin in HMC-1 cells, with the maximal inhibition being equivalent to that by PP2.

4. Discussion

In IgE/Ag-stimulated BMMCs, the Lyn/Syk/LAT axis activates the Ca^{2+}-dependent pathways. Activation of this pathway results in phosphorylation and thereby activation of PLCγ1, which

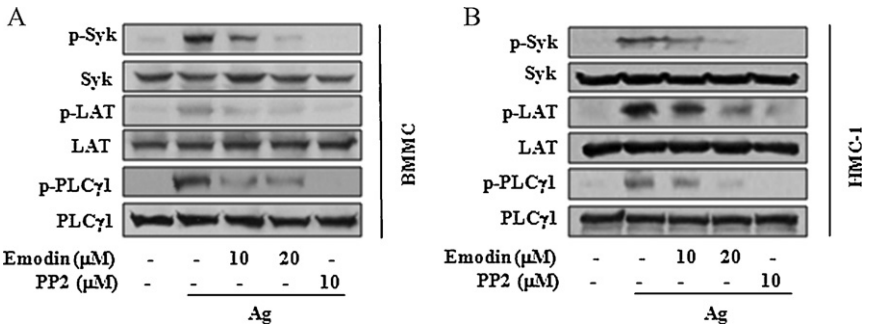

Fig. 5. Effect of emodin on the Syk-associated pathway. After preincubated with emodin or PP2 for 1 h, IgE-sensitized BMMCs (A) or HMC-1 cells (B) were challenged with DNP-HSA for 5 min, membrane fraction were immunoblotted with antibodies against phospho-Syk (p-Syk), phospho-LAT (p-LAT), phospho-PLCγ1 (p-PLCγ1), phospho-PI3K (p-PI3K) and phospho-Akt (p-Akt). PP2, a well known Src family inhibitor, was used as a positive control that suppresses the Syk-mediated pathway. All experiments were repeated three times, and representative results are shown.

promotes an increase of cytosolic Ca^{2+} leading to degranulation and eicosanoid production [46]. Our data show that emodin markedly decreased the degranulation reaction (Fig. 2A). Emodin also suppressed the phosphorylation of Syk, PLCγ1 and LAT (Fig. 5A) and influx of Ca^{2+} (Fig. 2B and C), suggesting that emodin influences degranulation through attenuation of the Syk-mediated PLCγ1-Ca^{2+} pathway. Importantly, these effects of emodin were recapitulated in the human mast cell line HMC-1 (Fig. 5B), suggesting that emodin acts on the common regulatory step that is essential for FcεRI signaling in mast cells.

$cPLA_2\alpha$ is a central regulator of AA release for eicosanoid production. The ability of $cPLA_2\alpha$ to generate free AA requires MAPK-mediated phosphorylation [47,48] and Ca^{2+}-dependent membrane translocation that allows access to its phospholipid substrate [49]. Emodin attenuated these responses in parallel with the reduction of LTC_4 release. Ca^{2+} binds to the N-terminal C2 domains of $cPLA_2\alpha$ and 5-LO and regulates their translocation to the perinuclear membrane [50,51]. Emodin suppressed the translocation of both enzymes, in agreement with its inhibitory effect on Ca^{2+} influx.

Emodin also inhibited the PI3K/Akt pathway (Fig. 4C). The Fyn/Gab2/PI3K axis, which is Ca^{2+}-independent, also contributes to mast cell activation. Early studies showed that Fyn kinase is a key regulator of mast cell activation, especially phosphorylation of the adapter protein Gab2, whose phosphorylation is critical for membrane targeting of protein tyrosine phosphatase [52]. However, the role of Fyn in this pathway still remains controversial, since a recent study has shown that siRNA knockdown of Gab2, but not Fyn, reduced the activation of PI3K [53]. Furthermore, other studies using a Syk inhibitor and Syk-deficient mast cells have shown that Syk can also activate Gab2 in a critical manner [54,55]. Thus, although the two Src-family tyrosine kinases, Lyn and Fyn, have been proposed to participate in phosphorylation of Gab2, they are capable of acting through activation of Syk. This idea fits with our observation that PP2, which inhibits Src-family kinases (including Fyn and Lyn), prevented phosphorylation of Syk and PI3K/Akt. It is therefore likely that the suppression of PI3K/Akt phosphorylation by emodin occurs through inhibition of Syk.

PI3K/Akt is a major regulator for the expression of transcription factors including NF-κB, A P-1 and NF-AT. The functional connection of PI3K/Akt to the NF-κB pathway, in which Akt phosphorylates and activates IKK that in turn phosphorylates and promotes the degradation of IκB, allowing the free NF-κB to translocate into the nucleus [56,57], can operate in macrophages and other cell types [40,41]; however, little is known about whether this signal crosstalk occurs in mast cells. Herein, we showed that the PI3K inhibitor wortmannin suppressed not only phosphorylation of PI3K and Akt, but also phosphorylation of IKK and IκB, degradation of IκB, and nuclear translocation of NF-κB (Supplementary Fig. 2A), suggesting that PI3K/Akt is located upstream of IKK/IκB/NF-κB in Ag-activated BMMCs. Emodin inhibited COX-2 expression and attendant PGD_2 generation, most likely because it blocked NF-κB, a major transcription factor for COX-2 [39]. Likewise, our finding that MAPK inhibitors suppressed PGD_2 generation and COX-2 expression (Supplementary Fig. 1B) suggests that MAPKs are also important for up-regulation of COX-2. These results, together with the view that PGD_2 generation is also controlled by MAPK-dependent phosphorylation of $cPLA_2\alpha$, which supplies AA to COX-2, suggest that emodin may inhibit COX-2-dependent PGD_2 generation in two distinct ways: the MAPK pathway for post-transcriptional activation of $cPLA_2\alpha$, and the MAPK and PI3K-Akt-NF-κB pathways for transcriptional induction of COX-2.

Transcriptional up-regulation of the pro-inflammatory cytokines TNF-α and IL-6, which play important roles in mast cell-dependent chronic inflammation, such as the late-phase reaction of the IgE-dependent response, is regulated by various signaling modules, including the PI3K/Akt [56,57], NF-κB [41] and MAPK/AP-1 [58] pathways. Emodin, as did wortmannin, decreased the secretion of TNF-α and IL-6 from Ag-activated BMMCs (Fig. 4B and Supplementary Fig. 2B), implying that it affects the expression of these cytokines through inhibition of these signaling pathways. Most importantly, besides its mast cell-stabilizing effect *in vitro*, emodin suppressed IgE-dependent PCA and PSA, which are mast cell-dependent *in vivo* models of local and systemic allergic reaction [59] with a potency equivalent to that of an H1 histamine antagonist (Fig. 1A and B). Thus, emodin is a potent agent that can ameliorate the anaphylactic reaction by inhibiting multiple effector functions of mast cells.

Emodin is a pluripotent pharmacologic agent, having anti-viral, immunosuppressive, anti-cancer and anti-diabetic activities, among others. It is well known that Japanese knotweed is a concentrated source of emodin, and this is used as a nutritional supplement to regulate bowel motility. In addition, an extract of *P. cuspidati* radix, a traditional Chinese, Korea and Japanese herbal medicine, is used as a natural laxative. The active component responsible for the laxative effect is emodin. Thus, various herbaceous plants containing emodin have been used safely for many centuries in Eastern Asia. Syk inhibition results in the reduction of allergic airway inflammation [60,61], and is being evaluated for use in nasal sprays for treatment of seasonal allergic rhinitis [9,61]. In this respect, emodin has a similar potential for

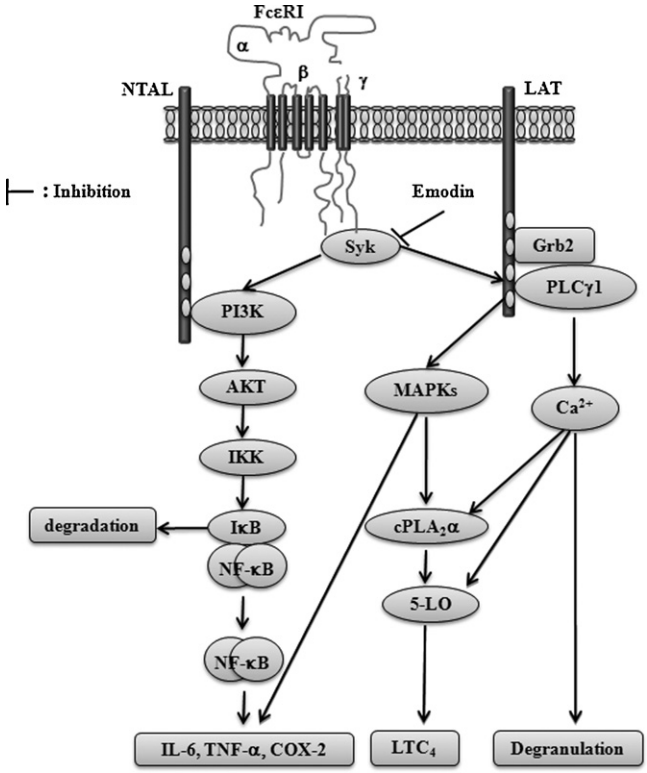

Fig. 6. Possible inhibitory mechanisms of emodin on FcεRI-induced activation of mast cells. Engagement of FcεRI with cognate antigen triggers the activation of Syk, a receptor-proximal tyrosine kinase. Activated Syk regulates the PI3K pathway through the adaptor protein NTAL (11), which plays a key role in NF-κB-mediated expression of COX-2 and pro-inflammatory cytokines. Syk also phosphorylates LAT, resulting in the formation of a macromolecular signaling complex that allows the diversification of downstream signaling, such as PLCγ1 and Grb2. PLCγ1 thus activated is essential for Ca^{2+} responses (through generation of inositol triphosphate) and for activation of PKCs (through generation of diacylglycerol), which are pivotal for degranulation as well as for translocation of $cPLA_2\alpha$ and 5-LO to the perinuclear membrane. The Grb2-mediated pathway is crucial for optimal activation of $cPLA_2\alpha$, leading to eicosanoid generation.

topical therapy of various allergic diseases, especially in view of its low toxicity in humans.

In summary, emodin is a potent inhibitor of Ag-stimulated degranulation, eicosanoid synthesis, and cytokine secretion in mast cells, and of mast cell-mediated PCA and PSA in mice. The results of this study demonstrate that emodin exhibits remarkable similarity in its potency for quenching various signaling pathways that all share a common requirement for upstream activation of Syk pathway (Fig. 6). Thus, emodin acts primarily on Syk to suppress downstream signaling events and mast cell activation, although additional inhibitory actions on downstream signals cannot be ruled out. These and the known anti-inflammatory properties of emodin suggest that further evaluation of its utility in the treatment of immediate and delayed allergic diseases is warranted.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) Grant (NRF-2010-616-E00011) and by grant-in aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Makoto Murakami and Hyeun Wook Chang by the JSPS-NRF Bilateral Joint Projects FY2010 and in part by Fisheries Technology Development Program funded by Ministry for Food, Agriculture, Forestry and Fisheries of Korean Government.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.08.022.

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