

Emodin Isolated from *Polygoni cuspidati* Radix Inhibits TNF- α and IL-6 Release by Blockading NF- κ B and MAP Kinase Pathways in Mast Cells Stimulated with PMA Plus A23187

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Abstract

Emodin, a naturally occurring anthraquinone derivative isolated from *Polygoni cuspidati* radix, has several beneficial pharmacologic effects, which include anti-cancer, anti-diabetic, and anti-inflammatory activities. In this study, the authors examined the effect of emodin on the production of proinflammatory cytokines, such as, tumor necrosis factor (TNF)- α and interleukin (IL)-6, in mouse bone marrow-derived mast cells (BMMCs) stimulated with phorbol 12-myristate 13-acetate (PMA) plus the calcium ionophore A23187. To investigate the mechanism responsible for the regulation of pro-inflammatory cytokine production by emodin, the authors assessed its effects on the activations of transcriptional factor nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinases (MAPKs). Emodin attenuated the nuclear translocation of (NF)- κ B p65 and its DNA-binding activity by reducing the phosphorylation and degradation of I κ B α and the phosphorylation of I κ B kinase B (IKK). Furthermore, emodin dose-dependently attenuated the phosphorylations of MAPKs, such as, extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAP kinase, and the stress-activated protein kinases (SAPK)/c-Jun-N-terminal kinase (JNK). Taken together, the findings of this study suggest that the anti-inflammatory effects of emodin on PMA plus A23187-stimulated BMMCs are mediated via the inhibition of NF- κ B activation and of the MAPK pathway.

Key Words: Emodin, Pro-inflammatory cytokine, NF- κ B, Mitogen-activated protein kinase, Bone marrow-derived mast cells, PMA plus A23187

INTRODUCTION

Mast cells are effector cells that display various functions during immune response and regulate allergic inflammation, such as, those associated with asthma, atopic dermatitis, and sinusitis. Activated mast cells release pro-inflammatory cytokines, such as, tumour necrosis factor (TNF)- α , interleukin (IL)-6, IL-8, IL-13, and inflammatory mediators, including histamine, leukotrienes, serotonin, prostaglandin (PG) E_2 , and PGD $_2$ (Barrett and Austen, 2009; Mukherjee and Zhang, 2011). The expressions of proinflammatory cytokines depend on the activations of the transcription factor, nuclear factor- κ B (NF- κ B), and mitogen-activated protein kinases (MAPKs), such as, extracellular signal-regulated kinase (ERK), p38,

stress-activated protein kinases (SAPK)/c-Jun-N-terminal kinase (JNK) (Jeong *et al.*, 2003; Rasheed *et al.*, 2009). The MAPK signaling pathway is activated by various extracellular stimuli, and these activations result in a wide range of cellular responses, such as, apoptosis, proliferation, and allergic and inflammatory responses (Lu *et al.*, 2011)

In most cell types, NF- κ B is present in the cytosol in an inactive form due to its association with inhibitory proteins, called inhibitor of NF- κ B (I κ Bs); the I κ B family includes I κ B α , I κ B β , I κ B ϵ , and Bcl-3, and I κ B α is regarded as being prototypic (Suh and Rabson, 2004). The activation of NF- κ B by extracellular stimuli follows the rapid phosphorylation, ubiquitination, and proteolytic degradation of I κ B α , which leads to the nuclear translocation and phosphorylation of NF- κ B, and

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binding of NF- κ B to a specific sequence in the promoter region of its target genes, triggers its transcriptional activation (Amit and Ben-neriah, 2003; Takada *et al.*, 2003; Xu *et al.*, 2005).

Root extracts of *Polygonum cuspidatum* (*P. cuspidatum*) are used as an analgesic and to treat allergic inflammation in Korea, Japan, and China (Lim *et al.*, 2007). Many groups have reported that *P. cuspidatum* contains a large number of anthraquinones, naphthoquinones, flavonoids, and stilbenes. In particular, anthraquinone derivatives, such as, emodin, physcion, citreoselin, emodin-8-O- β -D-glucoside, physcion-8-O- β -D-glucoside, and cis- and trans-resveratrol have also been isolated from this plant (Hwangbo *et al.*, 2012). Furthermore, emodin has been shown to have a number of biological activities, such as, anti-microbial, immunosuppressive, anti-inflammatory, and anti-atherosclerotic activities (Heo *et al.*, 2008; Lee *et al.*, 2010; Lin *et al.*, 2010; Meng *et al.*, 2010), and anti-diabetic and anti-obesity activities (Tzeng *et al.*, 2012; Song *et al.*, 2013).

In our previous studies, we described the anti-allergic activity of emodin and the mechanism of its action on Fc ϵ RI-mediated mouse bone marrow-derived mast cells (BMMCs) (Lu *et al.*, 2011). Because, the effects of emodin on proinflammatory cytokine production in Fc ϵ RI independent stimulated mast cells has not been determined, we evaluated its inhibitory effect on the productions of IL-6 and TNF- α and sought to determine the mechanism responsible for its inhibitory effect in phorbol myristate acetate (PMA) plus A23187-stimulated BMMCs.

MATERIALS AND METHODS

Plant material

Emodin was isolated from the ethyl acetate fraction of a methanol extract of *P. cuspidatum* radix as described previously (Lee *et al.*, 2003). The emodin used in this study showed a single spot on TLC plates and had a purity exceeding 99.5% by HPLC analysis. Prior to use, emodin was dissolved in dimethyl sulfoxide (DMSO); final concentrations of DMSO in culture media were adjusted to 0.1% (v/v). DMSO alone as added to controls in all cases.

Chemicals and reagents

PMA, A23187 (calcium ionophore), and pyrrolidinedithiocarbamate (PDTC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits for TNF- α and IL-6 were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Rabbit polyclonal antibodies for phospho-I κ B, IKK α / β , ERK1/2, JNK, p38, β -actin, and the total forms of I κ B, ERK1/2, JNK, p38 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Rabbit polyclonal antibodies for p65 and IKK α / β , goat polyclonal antibodies for lamin B, rabbit anti-goat IgG-HRP and goat anti-rabbit IgG-HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody-reactive bands were visualized with an enhanced chemiluminescence (ECL) system (Pierce Biotechnology, Rockford, IL, USA).

Preparation of mouse bone marrow-derived mast cells (BMMCs)

BMMCs isolated from male Balb/cJ mice (Sam Taco, Seoul) were cultured in RPMI 1640 media (Thermo Scientific, Utah, USA) containing 10% FBS, 100 U/ml penicillin (Thermo Sci-

entific, Utah, USA), 10 mM HEPES buffer (Sigma-Aldrich, St. Louis, MO, USA), 100 μ M MEM non-essential amino acid solution (Invitrogen, Carlsbad, USA) and 20% PWM-SCM (pokeweed mitogen-spleen cell conditioned medium) as a source of IL-3 (Lu *et al.*, 2013). After 3 weeks, >98% of the cells were found to be BMMCs (Murakami *et al.*, 1994).

Determination of proinflammatory cytokine levels

BMMCs suspended in enriched medium at a cell density of 10^6 cells/ml were pretreated with the indicated concentrations of emodin for 1 h at 37°C and then stimulated with PMA (50 nM) plus A23187 (1 μ M) for 6 h. Concentrations of IL-6 and TNF- α in supernatants were then determined using enzyme immunoassay kits (R&D Systems Inc. Minneapolis, MN, USA).

Immunoblotting

Immunoblotting was performed as previously described (Lu *et al.*, 2011). Briefly, BMMCs were lysed in RIPA lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM PMSF, 1M DTT, 200 mM NaF, 200 mM Na₃VO₄, protease inhibitor cocktail). Cell debris was removed by centrifugation at 14,000 g for 15 min at 4°C and resulting supernatants were immunoblotted. Samples were separated by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA), which were then blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 and incubated with individual antibodies. Primary antibodies were diluted 1:1,000-fold (unless otherwise mentioned), and after treatment, membranes were incubated at 4°C overnight. Membranes were then washed three times for 10 minutes with TBS-T buffer, treated with HRP-coupled secondary antibodies (diluted 1:3000-fold) for 1 h at room temperature, washed three times for 3 min in TBS-T buffer, and developed using enhanced chemiluminescence (ECL) detection kits (Pierce Biotechnology, Rockford, IL, USA).

Preparation of nuclear and cytosolic extracts

Cell lysates were prepared as previously described (Lu *et al.*, 2011). Briefly, cells were suspended in wash buffer containing 10 mM HEPES buffer (pH 8.0), 1.5 mM MgCl₂, 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM protease inhibitor cocktail (Merck Biosciences, Darmstadt, Germany) and lysed in wash buffer containing 0.1% (v/v) NP40 by incubation on ice for 10 min. Supernatants (cytosolic fractions) were obtained by centrifugation at 1,000 g for 4 min. Nuclear pellets were washed and re-suspended in a buffer containing 20 mM HEPES (pH 8.0), 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and protease inhibitor cocktail. These suspensions were then incubated for 30 min at 4°C and centrifuged at 10,000 g. The resulting supernatants were defined as nuclear fractions.

RNA isolation, reverse transcription and quantitative polymerase chain reaction (qPCR)

Real time PCR analysis was performed as described previously (Lu *et al.*, 2012). IgE-sensitized BMMCs were preincubated with emodin for 1 h, and then stimulated with DNP-HSA (100 ng/ml) for 4 h. Total RNA was extracted from BMMCs using the easy-BLUE™ Total RNA Extraction Kit (iNtRON Biotechnology, Inc, Sungnam, Korea). Five hundred nanogram of total RNA was reverse transcribed for the synthesis of cDNA

using a one-step RT-PCR kit (Enzynomics, Daejeon, Korea). Real time PCR amplification was then carried out over 40 cycles using the following conditions: denaturation at 95°C for 5 s, annealing at 57°C for 10 s, and elongation at 72°C for 20 s. The primers used for SYBR Green real-time PCR were as follows: for TNF- α , sense primer, 5'-AGC ACA GAA AGC ATG ATC CG-3' and antisense primer, 5'-CTG ATG AGA GGG AGG CCA TT-3'; for IL-6, sense primer, 5'-GAG GAT ACC ACT CCC AAC AGA CC-3', and antisense primer, 5'-AAG TGC ATC ATC GTT GTT CAT ACA-3'; and for β -actin, sense primer, 5'-ATC ACT ATT GGC AAC GAG CG-3', and antisense primer, 5'-TCA GCA ATG CCT GGG TAC AT-3'. A dissociation curve for analysis of TNF- α , IL-6, and β -actin showed a single peak for each reaction. Mean Ct values of genes of interest were calculated from triplicate measurements and normalized versus the mean Ct of β -actin, which was used as a housekeeping gene.

NF- κ B activation assay

Nuclear fractions were prepared as described previously (Lu *et al.*, 2013). NF- κ B transactivation ability was assessed using a NF- κ B (p65) transcription factor assay kit (Cayman Chemicals, Ann Arbor, MI, USA). The results obtained are presented as percent activations with respect to the stimulated control.

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). The Student's *t*-test was used to compare pairs of independent groups. Statistical significance was accepted for *p* values <0.05

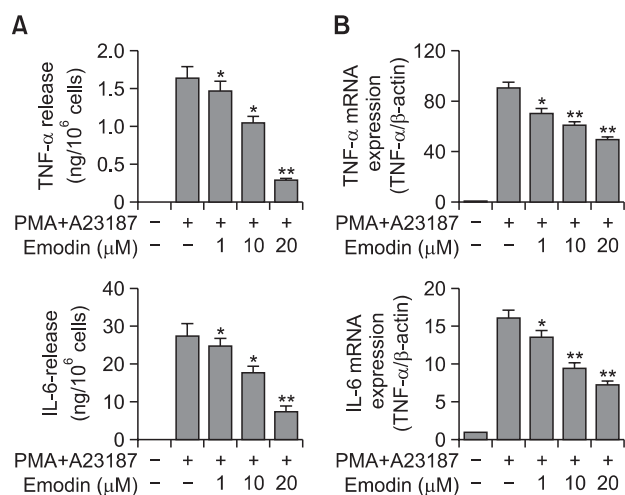


Fig. 1. Effect of emodin on PMA plus A23187-stimulated cytokine production and gene expression. BMMCs (10⁶ cell/ml) were pre-treated with the indicated concentrations of emodin for 1 h and then incubated for 6 h with PMA (50 nM) plus A23187 (1 μ M). TNF- α and IL-6 released into the supernatants were quantified by ELISA (A). The expression levels of IL-6 and TNF- α mRNAs were determined by real-time RT-PCR (B). Expression levels were normalized using expression of the housekeeping gene β -actin. Data are expressed as the means \pm S.D. of three independent experiments. **p*<0.05 and ***p*<0.01 versus PMA plus A23187-stimulated BMMCs.

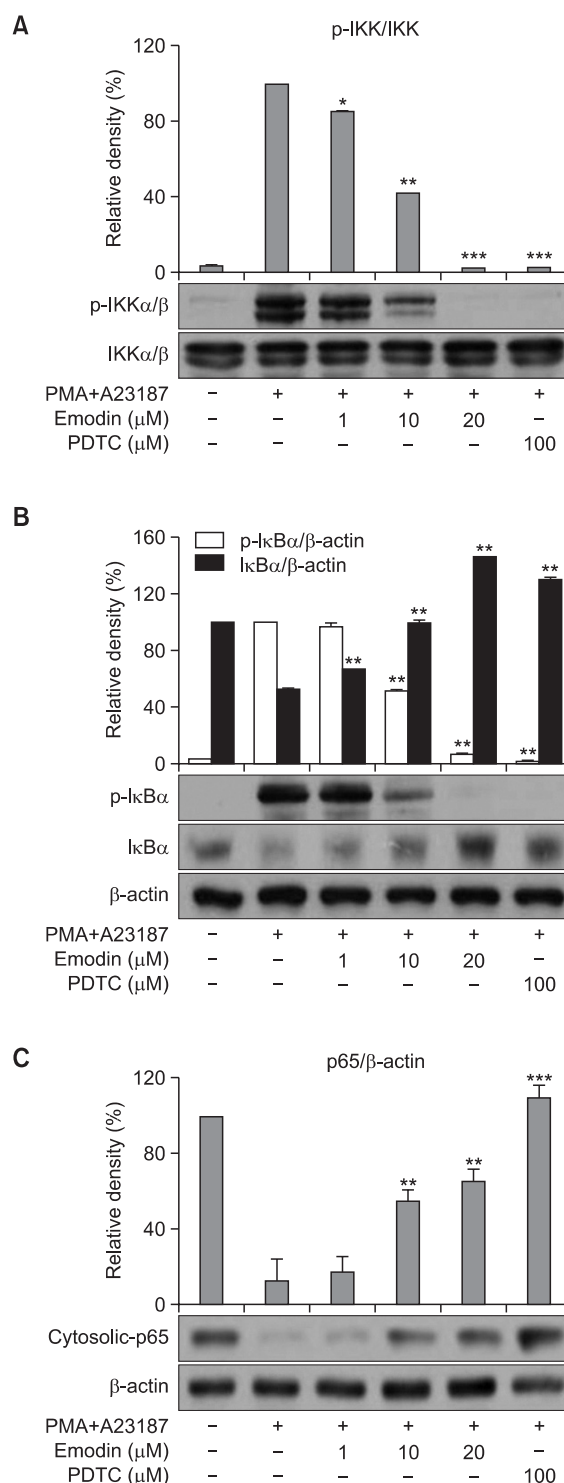


Fig. 2. Inhibitory Effects of emodin on PMA plus the A23187-stimulated IKK pathway in BMMCs. BMMCs were pre-incubated with emodin for 1 h and then incubated with PMA (50 nM) plus A23187 (1 μ M) for 15 min. Cells were then immunoblotted for IKK, I κ B α , NF- κ B-p65 and β -actin. p-IKK/IKK (A), p-I κ B α / β -actin (B) and p65/ β -actin (C) protein level ratios were determined by quantifying the intensities of immunoblots with scanning densitometry (**p*<0.05, ***p*<0.01 and ****p*<0.001 versus PMA plus A23187-stimulated BMMCs). Results were obtained from three independent experiments, and are represented as relative ratios (%).

RESULTS

Effect of emodin on the productions of pro-inflammatory cytokines

Pro-inflammatory cytokines, such as, IL-6 and TNF- α , are produced by macrophages and mast cells, and play important roles in various inflammatory responses (Guha and Mackman, 2001). Initially, we examined the cytotoxicity of emodin on BMMCs using an MTT assay, but found that it did not affect cell viability at 50 μ M (data not shown). Therefore, emodin was used at concentration below 20 μ M in all experiments. To evaluate the effect of emodin on the productions of IL-6 and TNF- α , BMMCs were pretreated with the indicated concentrations of emodin for 1 h and then stimulated with PMA (50 nM) plus A23187 (1 μ M) for 6h. As shown in Fig. 1A, concentrations of IL-6 and TNF- α in media were considerably increased after stimulation with PMA plus A23187, and the release of both was dose-dependently inhibited by emodin. Next, we examined whether emodin suppressed the expressions of IL-6 and TNF- α at the transcription level. As shown in Fig. 1B, real-time RT-PCR showed that emodin significantly inhibited the inductions of both mRNAs.

Effect of emodin on the activation of the IKK/I κ B α /NF- κ B pathway

To evaluate the mechanism by which emodin inhibits the productions of IL-6 and TNF- α , we examined its effects on NF- κ B activation. The expressions of these pro-inflammatory cytokines are regulated by the transcription factor NF- κ B as mediated by IKK (Azzolina *et al.*, 2003; Hayden and Ghosh, 2004). IKK complex includes regulatory scaffold protein NF- κ B essential modulator (NEMO or IKK γ) and IKK α and IKK β kinases. Once activated, IKK phosphorylates I κ B α , and subsequent ubiquitination of phosphorylated I κ B α by 26S proteasome releases NF- κ B dimers from cytoplasmic I κ B α /NF- κ B (Peng *et al.*, 2005). To determine whether emodin inhibits the phosphorylation of IKK α / β , the phosphorylation and degradation of I κ B α , the nuclear translocation of NF- κ B p65, and cytosolic NF- κ B p65 activation, we pretreated BMMCs with indicated concentrations of emodin or 100 μ M PDTC (a NF- κ B pathway inhibitor and positive control). As shown in Fig. 2, both PDTC and emodin inhibited the phosphorylations of IKK α / β and I κ B α and prevented I κ B α degradation and disappearance of cytosolic p65. Furthermore, after pretreating BMMCs with emodin or PDTC for 1 h and then stimulating them with PMA plus A23187 for 30 min, the nuclear localization of the p65 subunit was dose-dependently blocked by emodin and PDTC (Fig. 3). Lamin B was used as an internal control in the nuclear fraction. Next, we examined the effect of emodin and of PDTC on the DNA binding activity of NF- κ B using a DNA binding assay kit. As shown in Fig. 3B, emodin obviously suppressed the DNA binding activity of NF- κ B in a dose dependent manner. PDTC was also found to inhibit NF- κ B DNA binding activity strongly.

Effect of emodin on activation of MAP kinases

The MAP kinase pathway has been reported to play an major role in the regulation of proinflammatory mediator production (Beyaert *et al.*, 1996; Baldassare *et al.*, 1999). Thus, we examined the roles played by the MAPKs JNK, ERK1/2, and p38 on the productions of IL-6 and TNF- α in PMA plus A23187 stimulated BMMCs. As illustrated in Fig. 4, PMA plus A23187

strongly induced the expressions of phosphorylated ERK1/2, p38, and JNK without altering total protein levels. However, under the same experimental conditions, emodin significantly and dose-dependently suppressed the phosphorylations of these three MAPKs. Furthermore, the application of U0126 (an ERK1/2 inhibitor, 25 μ M), SB203580 (a p38 inhibitor, 30 μ M), or SP600125 (a JNK inhibitor, 25 μ M) almost completely inhibited MAPK phosphorylation. These observations suggest that inhibition of the productions of IL-6 and TNF- α by emodin is mediated by blocking the phosphorylations of these three MAPKs in PMA plus A23187 stimulated BMMCs.

DISCUSSION

Emodin is an anthraquinone and is the major bioactive compound found in several herbs. Recently, several studies have shown emodin ameliorates metabolic disorder, has anti-arthritis activity, anti-allergic activity and that on molecular level, it activated AMPK, has PPAR γ -agonist activity and acts

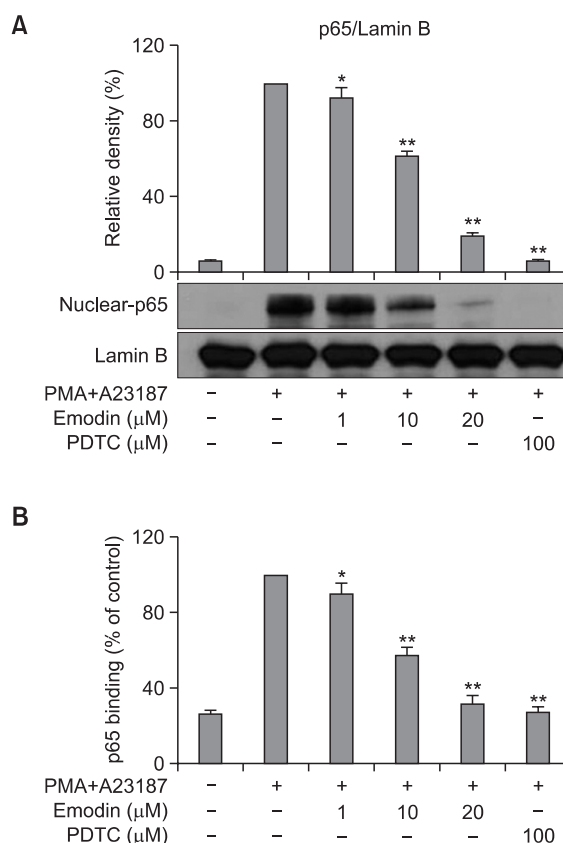


Fig. 3. Effects of emodin on the nuclear translocation of NF- κ B and on its DNA binding activity in PMA plus A23187-stimulated BMMCs. BMMCs were pretreated for 1 h with emodin and then treated for 30 min with PMA (50 nM) plus A23187 (1 μ M). Nuclear extracts were then isolated and used for the immunoblot of NF- κ B (p65) (A) and tested for DNA binding activity (B). Ratio of the protein levels of p-65 to lamin B were determined by measuring immunoblot band intensities by scanning densitometry (* p <0.05 and ** p <0.01 versus PMA plus A23187 stimulated BMMCs). Results are the averages of three independent experiments.

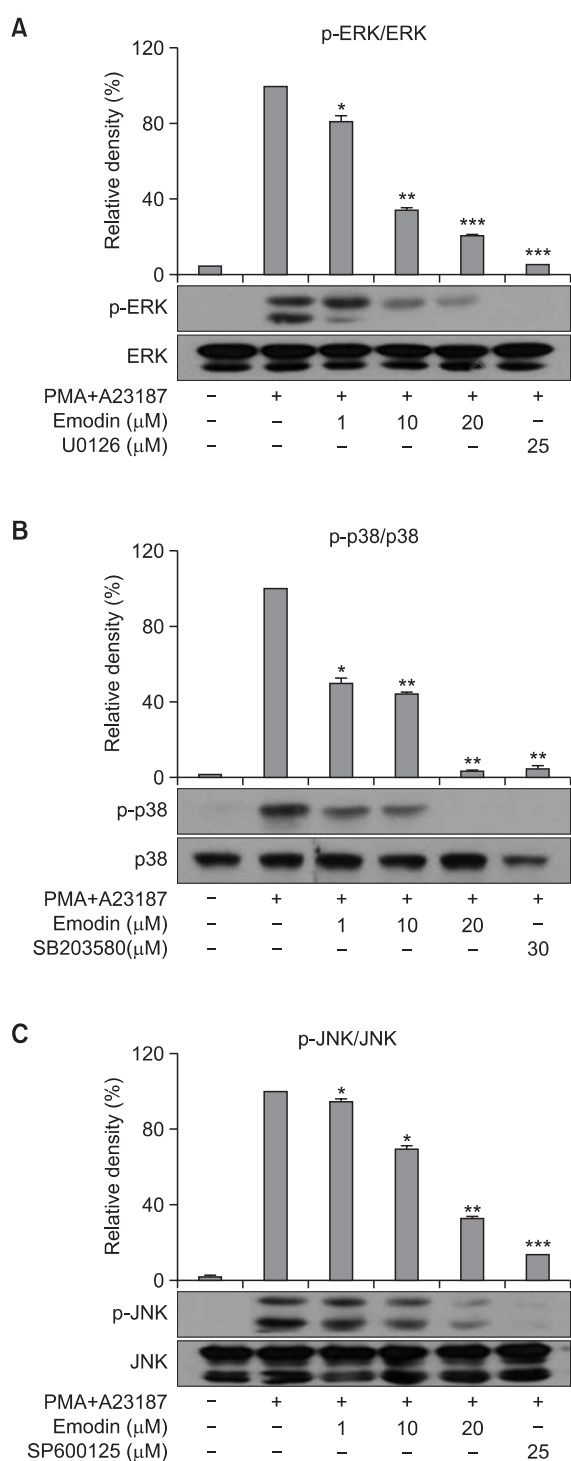


Fig. 4. Inhibitory effects of emodin on PMA plus A23187-Stimulated MAPK Phosphorylation in BMMCs. BMMCs were pre-incubated with emodin for 1 h and then incubated with PMA (50 nM) plus A23187 (1 μ M) for 15 min. The phosphorylations of MAPKs were evaluated by immunoblot analysis. The results of three independent experiments are shown. p-ERK/ERK (A), p-p38/p38 (B) and p-JNK/JNK (C) protein level ratios were determined by measuring immunoblot band intensities by scanning densitometry (* p <0.05, ** p <0.01 and *** p <0.001 versus PMA plus A23187-stimulated BMMCs). The results shown are representative of three independent experiments.

as a potent phosphatase inhibitor (Feng *et al.*, 2010; Chen *et al.*, 2012; Han *et al.*, 2012; Wang *et al.*, 2012; Hwang *et al.*, 2013). In this study, we investigated the effects of emodin on the productions of proinflammatory cytokine in phorbol myristate acetate (PMA) plus A23187-stimulated BMMCs and the mechanism responsible for its effects.

Mast cells are key effector cells and abundant in the airways of asthmatic patients, where they contribute to allergic inflammation by secreting an array of inflammatory mediators that exacerbate vasodilation and vascular permeability, airway smooth muscle contraction, mucus secretion, and immune cell recruitment (Galli *et al.*, 2008; Price *et al.*, 2013). Under inflammatory conditions, proinflammatory cytokines recruit activated immune and inflammatory cells to lesion sites, and thus, exacerbate inflammation (Church and Levi-Schaffer, 1997; Metcalfe *et al.*, 1997). Mast cells release several cytokines, including TNF- α , IL-1 β , IL-4, IL-5, IL-6, IL-8, and IL-13 (Galli *et al.*, 2005). TNF- α is either preformed and stored in the granules of mast cells or newly synthesized following mast cell activation, and is a multifunctional cytokine that mediates a variety of immune and inflammatory responses. IL-6, another pleiotropic inflammatory cytokine produced by mast cells, also supports the biological roles of mast cells during allergy-induced inflammation (Nakae *et al.*, 2005). In addition, MAPK signaling pathways have been implicated in the regulation of cytokine production after mast cell stimulation (Ishizuka *et al.*, 1997; Turner and Cantrell, 1997; Boudreau *et al.*, 2004). It is also well known that cytokine gene expression is regulated by a combination of three different mechanisms, namely, the derepression of gene promoter, gene transcriptional activation by NF- κ B, JNK, and ERK, and by the stabilization of mRNA via the p38 MAPK pathway. Furthermore, it has been reported the inductions of the gene expressions of IL-6 and TNF- α and the productions of IL-6 and TNF- α are depressed when cells are pretreated with either an inhibitor of the p38 MAPK pathway (SB203580) or an inhibitor of the ERK pathway (PD98059) (Guo *et al.*, 2003; Glushkova *et al.*, 2013). In the present study, emodin dose-dependently inhibited the PMA plus A23187-induced phosphorylations of ERK1/2, JNK, and p38, and inhibited the IKK-mediated NF- κ B pathway, thus, decreasing the productions of IL-6 and TNF- α .

The expressions of various inflammatory cytokines are regulated by the NF- κ B, which is strongly linked to inflammatory and immune responses. Thus, we postulate that emodin mediates the effects of NF- κ B, at least partly, by suppressing its activation. The activation of NF- κ B is dependent on the phosphorylation and degradation of I κ B, an endogenous inhibitor that binds to NF- κ B in the cytoplasm (Li *et al.*, 2006; Moon *et al.*, 2007). In the present study, stimulation by PMA plus A23187 led to activation of the key enzyme IKK, a complex composed of the regulatory IKK γ (NEMO) subunit and two enzymatically active subunits, IKK α and IKK β , and IKK activation resulted in the phosphorylation of I κ B and its subsequent dissociation from NF- κ B (Fig. 2). IKK β is considered primarily responsible for I κ B phosphorylation, whereas IKK α is required for the processing of NF- κ B (Senftleben *et al.*, 2001). Furthermore, when NF- κ B binding subunits are released and translocated to the nucleus, they bind to specific elements in the upstream promoter regions of target genes, such as, TNF- α , IL-6, and IL-1 β . (Peng *et al.*, 2005). Since activation of the IKK-I κ B-NF- κ B axis leads to the productions of TNF- α and IL-6, we examined the effect of emodin or PDTC on the IKK/

NF- κ B pathway. As illustrated in Fig. 2, 3, emodin and PDTC inhibited activation of the IKK-I κ B α -NF- κ B and NF- κ B DNA binding activity, which suggests that the NF- κ B pathway plays an essential role in the productions of TNF- α and IL-6 in PMA plus A23187-induced BMMCs. MAPKs can also regulate the expressions of many genes via their actions on transcription factors, such as, NF- κ B. In a previous study, ERK and p38 inhibitors were found to inhibit NF- κ B activation and cytokine production (Sigala *et al.*, 2011), whereas in the present study, MAPKs inhibitors significantly suppressed the productions of IL-6 and TNF- α . These findings suggest that crosstalk between MAPKs and NF- κ B could mediate cytokine production.

Summarizing, this study shows that emodin; 1) regulates the productions of IL-6 and TNF- α , 2) inhibits MAPK phosphorylation, 3) suppresses the nuclear translocation of NF- κ B, 4) suppresses the degradation of cytoplasmic I κ B α , and 5) inhibits the phosphorylations of I κ B α and IKK in PMA plus A23187-induced BMMCs. These findings and those of our previous studies (Lu *et al.*, 2011; Alisi *et al.*, 2012; Chu *et al.*, 2012) suggest that emodin should be regarded as a potential means of treating mast cell-mediated allergic inflammatory diseases.

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