

Chrysin suppresses mast cell-mediated allergic inflammation: Involvement of calcium, caspase-1 and nuclear factor- κ B

Yunju Bae, Soyoung Lee, Sang-Hyun Kim *

Department of Pharmacology, School of Medicine, Kyungpook National University, Daegu 700-422, Republic of Korea

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ABSTRACT

A great number of people are suffering from allergic inflammatory diseases such as asthma, atopic dermatitis, and sinusitis. Therefore discovery of drugs for the treatment of these diseases is an important subject in human health. Chrysin (5,7-dihydroxyflavone) is a natural flavonoid contained in propolis, blue passion flower, and fruits. Several studies reported that chrysin has beneficial effects including anti-tumor and anti-oxidant activities. The aim of the present study was to elucidate whether chrysin modulates the allergic inflammatory reaction and to study its possible mechanisms of action using mast cell-based in vitro and in vivo models. Chrysin inhibited immediate-type systemic hypersensitivity and serum histamine release. Chrysin attenuated immunoglobulin E-mediated local anaphylaxis. These inhibitory effects of chrysin on the systemic and local allergic reaction were more potent than cromolyn, a known anti-allergic drug. Chrysin reduced histamine release from mast cells. The inhibitory effect of chrysin on the histamine release was mediated by the modulation of intracellular calcium. In addition, chrysin decreased gene expression of pro-inflammatory cytokines such as, tumor necrosis factor- α , IL (interleukin)-1 β , IL-4, and IL-6 in mast cells. The inhibitory effect of chrysin on the pro-inflammatory cytokine was nuclear factor- κ B and caspase-1 dependent. Our findings provide evidence that chrysin inhibits mast cell-derived allergic inflammatory reactions by blocking histamine release and pro-inflammatory cytokine expression, and suggest the mechanisms of action. Furthermore, in vivo and in vitro anti-allergic inflammatory effect of chrysin suggests a possible therapeutic application of this agent in allergic inflammatory diseases.

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Introduction

Mast cells are effector cells displaying various functions during immune responses and regulators of allergic inflammation such as asthma, atopic dermatitis, and sinusitis. Allergen provocation, cross linkage of immunoglobulin E (IgE) bound on mast cell, via the high-affinity receptors triggers the release of allergic inflammatory mediators including histamine, eicosanoid, and several pro-inflammatory cytokines/chemokines (Galli et al., 2005a). Using these products mast cell regulates not only immediate type hypersensitivity but also late reaction, like inflammatory responses.

In the immediate phase of an allergic response such as anaphylaxis and asthma, histamine released from degranulation of mast cell acts as a major mediator (Galli et al., 2008). Mast cell degranulation can be elicited by nonimmunologic stimulators such as compound 48/80

(Ennis et al., 1980). Thus an appropriate amount of compound 48/80 has been used as a direct and convenient reagent to study the mechanism of anaphylactic allergic reactions. Anti-dinitrophenyl (DNP) IgE antibody and antigen have been established to induce a passive cutaneous anaphylaxis (PCA) reaction as typical in vivo model for immediate hypersensitivity.

The signaling pathway leading to degranulation of mast cells has been extensively characterized. Crosslinking of Fc ϵ RI leads phosphorylation of Src family kinases (Lyn, Syk and Fyn) (Galli et al., 2008). Calcium mobilization occurs after phosphorylation of Lyn and phospholipase C- γ induces mast cell degranulation through the granule membrane fusion (Castle et al., 2002). This is followed by activation of protein kinase C, mitogen-activated protein kinase (MAPKs), nuclear factor (NF)- κ B, and the release of inflammatory cytokines and chemokine (Kim et al., 2006; Gwack et al., 2007). By the release of pro-inflammatory mediators such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-4 and IL-6, mast cells also affect the late phase responses of an allergic inflammation (Galli et al., 2008). These cytokines released from mast cells change terminal microenvironment and attract neutrophils and basophils. Therefore reduction of these pro-inflammatory cytokines is one of key indicators of relieved allergic inflammatory symptoms. Caspase-1 and NF- κ B play a crucial role in the regulation of cytokines. Caspase-1, a member of cysteine

Abbreviations: HMC, human mast cell; RBL, rat basophilic leukemia; PCA, passive cutaneous anaphylaxis; DNP, dinitrophenyl; PMA, phorbol 12-myristate 13-acetate; TNF, tumor necrosis factor.

* Corresponding author at: Department of Pharmacology, School of Medicine, Kyungpook National University, 101 Dongin-dong, Jung-gu, Daegu 700-422, Republic of Korea. Fax: +82 53 423 4838.

E-mail address: shkim72@knu.ac.kr (S.-H. Kim).

protease also known as IL-1 β converting enzyme, is found predominantly in the cytoplasm of cells, where it proteolytically convert pro-IL-1 β into mature form, that is involved in inflammation (Thornberry et al., 1992). NF- κ B is an important transcriptional factor which regulates expression of pro-inflammatory cytokines. For this reason, NF- κ B is an obvious target of anti-inflammatory treatment (Kim and Shin, 2005; Kim et al., 2006).

Chrysin (5,7-dihydroxyflavone) is a natural flavonoid plentifully contained in propolis, blue passion flower (*Passiflora caerulea*), vegetables, and fruits (Sobocanec et al., 2006). It was reported that chrysin has an anti-cancer effect by enhancing TNF- α -mediated apoptosis in tumor cell, and inhibits pathological angiogenesis, like cancer metastasis process (Weng et al., 2005; Li et al., 2010). Some study reported that chrysin has anti-inflammatory effects by inhibiting several cytokines, nitric oxide, prostaglandin E, and cyclooxygenase-2 (Ha et al., 2010; Harasstani et al., 2010). Chrysin inhibits IgE and Th2 cytokine in ovalbumin-immunized mouse, and degranulation of mast cells (Yano et al., 2007; Nakamura et al., 2010). Chrysin has antioxidant, anti-viral, anti-anxiolytic, and aromatase inhibition effects as well (Dhawan et al., 2002; Lapidot et al., 2002; Schnitzler et al., 2010). Although chrysin has a variety of biological effects, its anti-allergic effect and mechanism are poorly understood. The objective of this study is to elucidate the effect of chrysin on the allergic inflammation and to define the underlying mechanisms of these effects. We also investigated the effect of chrysin on the systemic and local allergic reaction to assess its anti-allergic effect in vivo. In the present study, we clearly demonstrated that chrysin has an anti-allergic inflammatory effect on in vivo and in vitro allergy models.

Materials and methods

Reagents and cell culture. Compound 48/80, anti-DNP IgE, DNP-human serum albumin (HSA), *o*-phthalaldehyde, phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187, cromolyn, and chrysin were purchased from Sigma (St. Louis, MO). Human mast cell (HMC)-1 and rat basophilic leukemia (RBL)-2H3 cells were grown in IMDM and DMEM (GIBCO, Grand Island, NY), respectively and supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin, and heat-inactivated 10% fetal bovine serum (GIBCO) in 5% CO₂ at 37 °C.

Animals. The male ICR mice (6 weeks) and male Sprague–Dawley rats (8 weeks) were purchased from Dae-Han Experimental Animal Center (Daejeon, Korea). The animals were housed in a laminar air flow room maintained under a temperature of 22 $\pm$ 2 °C and relative humidity of 55 $\pm$ 5% throughout the study. The care and treatment of the animals were in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Compound 48/80-induced systemic anaphylaxis. Mice were given an intraperitoneal injection of 8 mg/kg body weight (BW) of the mast cell degranulator, compound 48/80 (n = 10/group). Chrysin and cromolyn were dissolved in DMSO and saline, respectively. Chrysin and cromolyn were intraperitoneally administered at doses of 1–100 mg/kg BW 1 h before the compound 48/80 injection. Mortality was monitored for 1 h after induction of anaphylactic shock. After the mortality test, blood was obtained from the heart of each mouse to measure serum histamine contents.

Passive cutaneous anaphylaxis (PCA). An IgE-dependent cutaneous reaction was carried out as described previously (Shin et al., 2005). The rats were injected intradermally with 0.5 μ g of anti-DNP IgE. After 48 h, each rat received an injection of 1 μ g of DNP-HSA containing 4% Evans blue (1:4) via the tail vein. Chrysin was dissolved in olive oil

and cromolyn was dissolved in saline. Chrysin and cromolyn were intraperitoneally administered at doses 1–100 mg/kg BW 1 h before the challenge. Thirty minutes after the challenge, the rats were killed and the dorsal skin was removed for measurement of the pigment area. The amount of dye was determined colorimetrically after extraction with 1 ml of 1 M KOH and 9 ml of a mixture of acetone and phosphoric acid (5:13). The intensity of absorbance was measured at 620 nm in a spectrophotometer (UV-1201; Shimadzu, Kyoto, Japan).

Histamine content. Histamine contents in serum and cells were measured by the *o*-phthalaldehyde spectrofluorometric procedure as previously described (Kim et al., 2007a). The blood from the mice was centrifuged at 400 g for 10 min and the serum was withdrawn to measure histamine content. For IgE-mediated histamine release, cells were sensitized with anti-DNP IgE (1 μ g/ml) for 16 h. The cells were preincubated with chrysin at 37 °C for 30 min prior to the challenge with DNP-HSA (500 ng/ml). For PMA plus A23187-induced histamine release, cells were stimulated with PMA (40 nM) plus A23187 (1 μ M) for 2 h. The cells were separated from the released histamine by centrifugation at 10,000 g for 5 min at 4 °C.

Intracellular calcium. To determine intracellular calcium, Fluo-3/AM (Molecular Probes Inc. Eugene, OR) was used according to the manufacturer's protocol, a brief description of which follows. IgE-sensitized RBL-2H3 and HMC-1 were preincubated with Fluo-3/AM for 30 min at 37 °C. After washing the dye from the cell surface, chrysin (10 μ g/ml) was pretreated 10 min prior to the DNP-HSA or PMA plus A23187 stimulation. The fluorescent intensity was measured using flow cytometer (BD Biosciences, San Diego, CA).

Quantitative real-time PCR. IgE-sensitized RBL-2H3 cells (2×10^5 /well in 24-well plate) were preincubated with chrysin at 37 °C for 30 min prior to the challenge with DNP-HSA. HMC-1 cells (1×10^6 /well in 24-well plate) were stimulated with PMA (40 nM) plus A23187 (1 μ M). The total cellular RNA was isolated using an RNAiso Plus (Takara Bio Inc., Shiga, Japan). The first strand complementary DNA (cDNA) was synthesized using Maxime RT Premix (iNtRON Biotech, Sungnam, Korea). The conditions for the reverse transcription steps were similar to those previously described (Kim et al., 2007b). Quantitative real-time PCR was carried out to analyze the expression of mRNA for TNF- α , IL-1 β , IL-4, IL-6 and β -actin (internal control) using the Thermal Cycler Dice TP850 (Takara Bio Inc.) according to the manufacturer's protocol. Briefly, 2 μ l of cDNA (100 ng), 1 μ l of sense and antisense primer solution (0.4 μ M), 12.5 μ l of SYBR Premix Ex Taq (Takara Bio Inc.), and 9.5 μ l of dH₂O were mixed together to obtain a final 25 μ l reaction mixture in each reaction tube. The primer sets for cytokines are summarized in Table 1. The amplification conditions were 10 s at 95 °C, 40 cycles of 5 s at 95 °C and 30 s at 60 °C, 15 s at 95 °C, 30 s at 60 °C, and 15 s at 95 °C. Relative quantification of mRNA expression was performed using the TP850 software.

Nuclear protein extraction. After RBL-2H3 (2×10^6 in a 6-well plate) and HMC-1 (3×10^6 in a 6-well plate) were stimulated with DNP-HSA or PMA plus A23187, cells were washed in 1 ml of ice-cold PBS, centrifuged at 1200 g for 5 min, resuspended in 400 μ l of ice-cold hypotonic buffer (10 mM HEPES/KOH, 2 mM MgCl₂, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 10 min, vortexed, and centrifuged at 15,000 g for 30 s. After washing, pelleted nuclei were resuspended in 50 μ l of ice-cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 20 min, vortexed, centrifuged at 15,000 g for 5 min at 4 °C, and supernatant gathered.

Western blotting. Cell extracts were prepared by detergent lysis procedure as described elsewhere (Kim and Sharma, 2004). Samples

Table 1
Primer sets for real-time PCR.

Gene		Forward primer	Reverse primer
Rat	TNF- α	5'-TCCCAATGGGCTCCCTCTC-3'	5'-AAATGGCAAACCGCTGACG-3'
	IL-1 β	5'-GCTGTGGCAGCTACCTATGTCTTG-3'	5'-AGGTCGTCATCATCCACAG-3'
	IL-4	5'-TGCACCGAGATCTTTGTACCAGA-3'	5'-TTGCGAAGCACCTTGAAG-3'
	IL-6	5'-TGTGCAATGGCAATTCTGAT-3'	5'-GAGCATTGGAAGTTGGGGTA-3'
	β -actin	5'-GAAGCGGTGCTATGTGCCCTAGA-3'	5'-GTACTCCTGCTTGCTGATCCACAT-3'
Human	TNF- α	5'-CCTACCAGACCAAGGTCAAC-3'	5'-AGGGGGTAATAAAGGGATTG-3'
	IL-1 β	5'-GGATATGAGCAACAAGTGG-3'	5'-ATGTACCAGTTGGGAACTG-3'
	IL-4	5'-ATGGGTCTCACCTCCCACTGCT-3'	5'-CAGCTCGAAGACTTTGAATATTTCTCTC-3'
	IL-6	5'-AAAGAGGCACTGCCAGAAA-3'	5'-ATCTGAGGTGCCCATGCTAC-3'
	β -actin	5'-GGACTTCGAGCAAGAGATGG-3'	5'-AGCACTGTGTGGCGTACAG-3'

of protein (50 μ g) were electrophoresed using 10% SDS-PAGE, and then transferred to nitrocellulose membrane. The nucleus and cytosolic p65 NF- κ B, I κ B α , caspase-1 was assayed using anti-NF- κ B (p65), anti-I κ B α , and anti-caspase-1 antibodies (Santa Cruz Biotech., Santa Cruz, CA), respectively. Immunodetection was done using chemiluminescent substrate (Thermo scientific, Waltham, MA).

Transient transfection and luciferase activity. NF- κ B luciferase reporter gene constructs (pNF- κ B-LUC, plasmid containing NF- κ B binding site; STANTAGEN, Grand Island, NY) were transiently transfected into cells with FBS and antibiotics free medium containing 8 μ l of Lipofectamin 2000 reagent (Invitrogen, Carlsbad, CA) as previously described (Lee et al., 2008). After 6 h of incubation, medium was replaced with 10% FBS and antibiotic containing medium. Cells were allowed to recover for 18 h and stimulated with DNP-HSA or PMA plus A23187 at 37 °C for 2 h. Chrysin was pretreated to cells for 30 min prior to stimulation. Cells were harvested and washed in ice-cold PBS before lysis in 100 μ l of cell lysis buffer (Luciferase assay kit; Promega, Madison, WI), according to the manufacturer's protocol. Luciferase activity was determined using the luminometer.

Secretion of TNF- α . The secretion of TNF- α was measured by modification of an enzyme-linked immunosorbent assay (ELISA). Cells were stimulated with DNP-HSA or PMA plus A23187. The ELISA was performed by coating 96-well plates with 6.25 ng/well of monoclonal antibody with specificity for TNF- α . Before use and between subsequent steps in the assay, the coated plates were washed twice with PBS containing 0.05% Tween-20 and twice with PBS alone. After exposure to the medium, the assay plates were exposed sequentially to biotinylated anti-human or anti-rat TNF- α and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) tablet substrates. Optical density was read within 10 min of the addition of the substrate with a 405 nm filter.

Table 2
Effect of chrysin on compound 48/80-induced systemic anaphylaxis.

Dose (mg/kg BW)	Compound 48/80 (8 mg/kg BW)	Mortality (%)
None (saline)	+	100
None (DMSO)	+	100
Chrysin 1	+	30
10	+	10
100	+	0
100	—	0
Cromolyn 1	+	100
10	+	80
100	+	40
100	—	0

Groups of mice ($n = 10$ /group) were intraperitoneally pretreated with 200 μ l of saline or drugs at various doses 1 h before the intraperitoneal injection of compound 48/80. Mortality (%) within 1 h following compound 48/80 injection is represented as the number of dead mice $\times$ 100/total number of experimental mice.

Statistical analysis. Statistical analyses were performed using Prism 5 (Graph-pad software, San Diego, CA). Treatment effects were analyzed using one way analysis of variance followed by Dunnett's test. A value of $P < 0.05$ was used to indicate significant differences.

Results

Effect of chrysin on systemic and local anaphylaxis

To examine the effects of chrysin on systemic immediate-type allergic reaction, systemic anaphylaxis was used. Compound 48/80 was used as a model of induction for a systemic fatal allergic reaction. After the intraperitoneal injection of compound 48/80, the mice were monitored for 1 h and the mortality rate was determined. As shown in Table 2, compound 48/80 induced 100% fatal shock in DMSO injected mice. Administration of chrysin (1–100 mg/kg BW) dose-dependently blocked compound 48/80-induced fatal shock. We compared the anti-anaphylactic effect of chrysin with cromolyn, a known anti-allergic drug. At the 10 mg/kg BW, the mortality of chrysin treated group was 10%, while that of cromolyn treated group was 80%. The effect of chrysin on the compound 48/80-induced serum histamine release was investigated as well. Injection of compound 48/80 caused high increase of serum histamine release which was markedly suppressed by chrysin administration in a dose-dependent manner, while cromolyn barely reduced serum histamine release (Fig. 1).

To confirm the anti-allergic effect of chrysin in vivo, we induced PCA in rats. PCA is one of the most appropriate in vivo models of local allergic reaction (Kim et al., 2006). Local injection of anti-DNP IgE

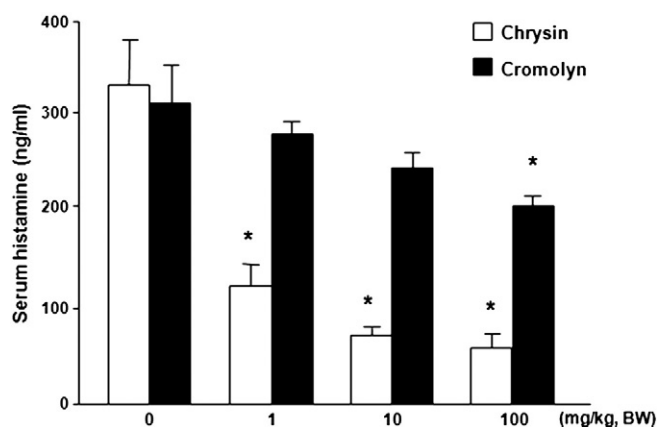


Fig. 1. Effect of chrysin on compound 48/80-induced serum histamine release. Groups of mice were intraperitoneally pretreated with 200 μ l of DMSO or drugs at various doses 1 h before the intraperitoneal injection of compound 48/80. The blood was obtained from the heart of each mouse and histamine content was measured. Each bar represents the mean $\pm$ SEM of three independent experiments. *Significant difference at $P < 0.05$. BW, body weight.

followed by an intravenous antigenic challenge induced local extravasation. After injection of 4% Evans blue mixed with antigen, the PCA reaction site showed blue color. Administration of chrysin (1–100 mg/kg BW) visibly reduced PCA reaction in a dose-dependent manner. In comparison, cromolyn showed relatively low reduction effects (Fig. 2).

Effect of chrysin on histamine release and intracellular calcium

We assessed the ability of chrysin on the histamine release from RBL-2H3 and HMC-1 cells, which are the most widely used mast cell lines for histamine assay. Anti-DNP IgE sensitized RBL-2H3 cells released a high level of histamine when challenged with DNP-HSA. PMA plus A23187-stimulated RBL-2H3 cells released large amounts of

histamine as well. Chrysin reduced DNP-HSA and PMA plus A23187-induced histamine release in RBL-2H3 cells in a concentration-dependent manner (Fig. 3A). The PMA plus A23187-induced histamine release in HMC-1 cells was also inhibited by chrysin (Fig. 3B). To check the effect of chrysin on the cell viability of RBL-2H3 and HMC-1, we performed MTT and XTT assay during 24 h, respectively. Up to 100 μ g/ml of chrysin did not affect cell viability in both RBL-2H3 and HMC-1 (data not shown).

To investigate the mechanism of chrysin on the reduction of histamine release, we assayed the intracellular calcium. Calcium movements across membranes of mast cells are critical to histamine release (Beaven et al., 1984; Kim et al., 2006). We used Fluo-3/AM for intracellular calcium assay. IgE-sensitized RBL-2H3 cells were pretreated with chrysin (10 μ g/ml) for 10 min prior to DNP-HSA (Fig. 4A). HMC-1

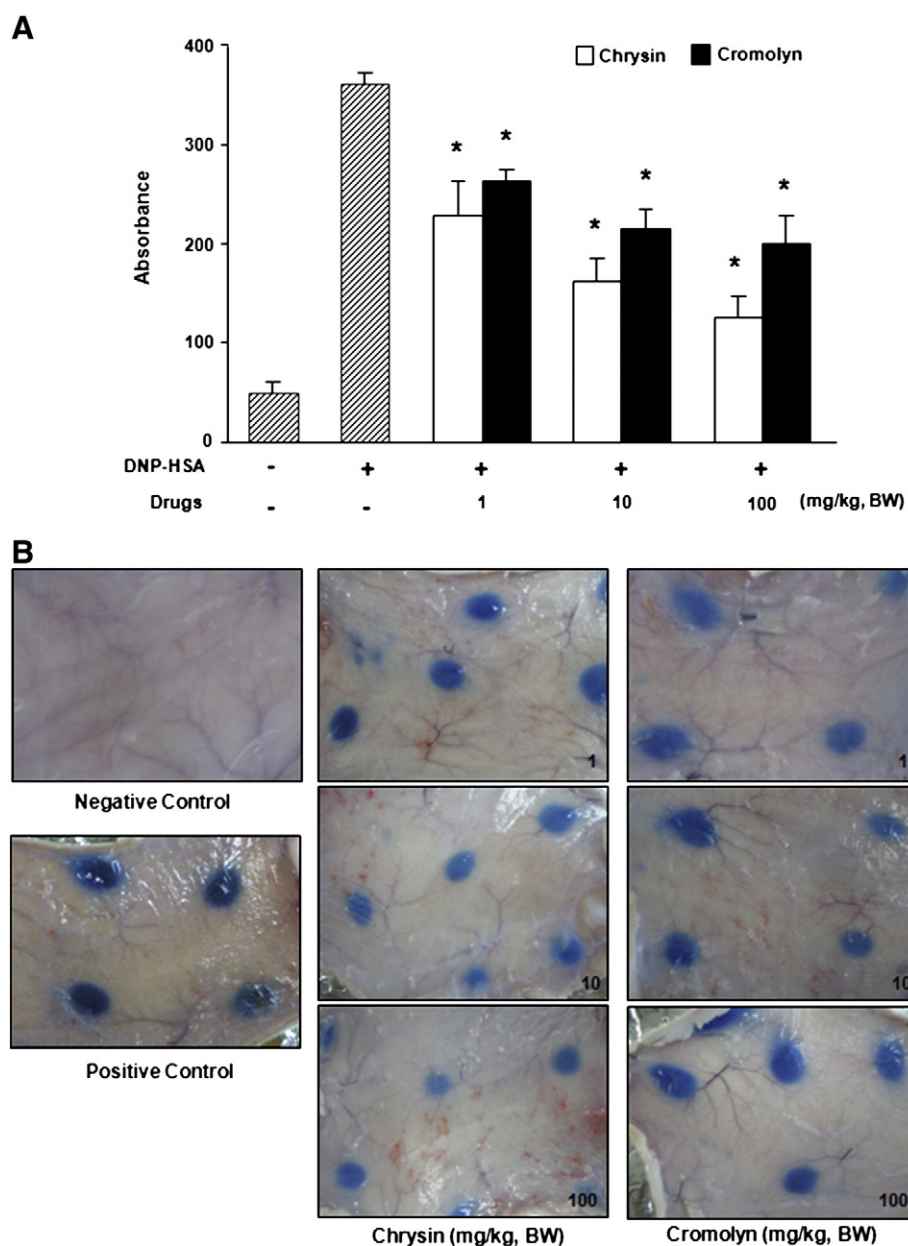


Fig. 2. Effect of chrysin on PCA reaction. The rats were injected intradermally with 0.5 μ g of anti-DNP IgE. After 48 h, each rat received an injection of 1 μ g of DNP-HSA containing 4% Evans blue (1:4) via the tail vein. Chrysin or cromolyn were administered 1 h prior to the challenge with antigen. (A) Each amount of dye was determined colorimetrically. The intensity of absorbance was measured at 620 nm using a spectrophotometer. (B) Dorsal skins were removed and photographed. Blue spots indicate PCA reaction site. Each bar represents the mean $\pm$ SEM of three independent experiments. *Significant difference at $P < 0.05$. BW, body weight.

cells were pretreated with chrysin prior to PMA plus A23187 stimulation (Fig. 4B). Calcium influx was analyzed by flow cytometry. Chrysin inhibited calcium mobilization in both RBL-2H3 and HMC-1 cells.

Effect of chrysin on the expression of pro-inflammatory cytokines

We tested the effect of chrysin on expression of pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-4 and IL-6 in mast cells. We previously reported that gene expression of TNF- α , IL-1 β , IL-4 and IL-6 was peaked at 4 h after treatment of PMA plus A23187 in HMC-1 (Park et al., 2007). Therefore, stimulation of HMC-1 with PMA plus A23187 was induced during 4 h, and the cells were pretreated with chrysin for 30 min. IgE-sensitized RBL-2H3 cells were preincubated with chrysin for 30 min and challenged with DNP-HSA for 2 h. The results of quantitative real-time PCR showed that chrysin (0.1, 10 μ g/ml) inhibited gene expression of all cytokines in RBL-2H3 (Fig. 5A) and HMC-1 (Fig. 5B).

Effect of chrysin on the activation of NF- κ B and caspase-1

To find out the mechanism responsible for the inhibitory effect of chrysin on the cytokine expression, we investigated the effect of chrysin on NF- κ B activation. Challenge of IgE-sensitized RBL-2H3 with DNP-HSA and stimulation of HMC-1 with PMA plus A23187 induced translocation of p65 NF- κ B to the nucleus following degradation of I κ B α . Chrysin hindered IgE-mediated and PMA plus A23187-induced degradation of I κ B α and translocation of p65 NF- κ B (Fig. 6A). To confirm the inhibitory effect of chrysin on NF- κ B translocation, we examined the effect of chrysin on the NF- κ B-dependent gene reporter assay. We used PDTC (50 μ M), a potent inhibitor for NF- κ B as a positive control. Stimulation of cells with DNP-HSA or PMA plus A23187 increased the luciferase activity in the cells transfected with the NF- κ B-luciferase reporter construct (Fig. 6B). Chrysin significantly reduced IgE-mediated and PMA plus A23187-induced luciferase activity. To further strengthen the relation of NF- κ B and expression of pro-inflammatory cytokines in our system, we next investigated the effects of pharmacological agents that modulate NF- κ B activity. Treatment of cells with the selective NF- κ B inhibitor, PDTC (50 μ M), blocked the IgE-mediated and PMA plus A23187-induced TNF- α secretion (Fig. 6C). These data indicate that NF- κ B activation contributes to the regulation of TNF- α . The pharmacologic agents (PDTC) used in this experiment did not show cytotoxicity (data not shown).

Caspase-1 plays a key role in inflammatory responses by cleaving pro-IL-1 β into secreted pro-inflammatory cytokines. For the additional underlying mechanism responsible for the inhibitory effect of chrysin on the cytokine expression, we assayed caspase-1 in mast cells. Caspase-1 cleaves precursor of IL-1 β to biologically active form. Active caspase-1 is derived from the cleavage of the 45 kDa pro-caspase-1. DNP-HSA challenge and PMA plus A23187 induced decrease of pro-caspase-1, that is, caspase-1 activation is increased and chrysin reduced caspase-1 activation (Fig. 7).

Discussion

In the present study, we showed that chrysin relieved mast cell-mediated allergic inflammation using *in vivo* and *in vitro* models. Anaphylaxis is a severe hypersensitivity induced by the mast cell degranulation and following the release of inflammatory mediators such as histamine, proteolytic enzymes and cytokines. Numerous reports established that stimulation of mast cells with compound 48/80 or IgE initiates the activation of the signal transduction pathway, which leads to degranulation (Galli et al., 2005a; Galli et al., 2005b). We compared the effect of chrysin with cromolyn on the compound 48/80-induced systemic and local allergic reaction. Cromolyn is a clinically used medication for treating asthma and allergy and it works by stabilizing mast cells. We observed that chrysin inhibited systemic

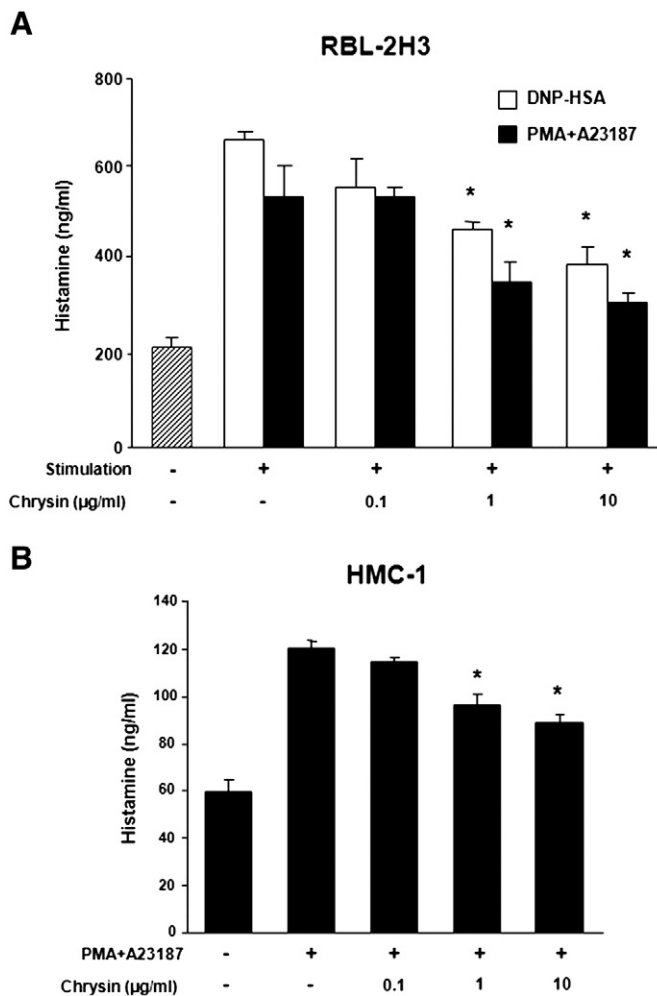


Fig. 3. Effect of chrysin on histamine releases from mast cells. (A) RBL-2H3 cells were sensitized overnight with 1 μ g/ml of DNP-specific IgE. The IgE-sensitized cells were pretreated chrysin for 30 min prior to 500 ng/ml of DNP-HSA stimulation for 2 h. In addition, cells were pretreated with chrysin for 30 min and stimulated with PMA (40 nM) plus A23187 (1 μ M) for 2 h. (B) HMC-1 cells were pretreated with chrysin for 30 min and stimulated with PMA plus A23187 for 2 h. Supernatant was used for analysis using the *o*-phthalaldehyde spectrofluorometric procedure. Each bar represents the mean $\pm$ SEM of three independent experiments. *Significant difference from DNP-HSA or PMA plus A23187 value at $P < 0.05$.

allergic reaction and serum histamine release, which is an index of mast cell degranulation. In addition, chrysin administered to rats results in protection from IgE-mediated PCA, one of the most important *in vivo* modes of acute local anaphylaxis. In both systemic and local anaphylaxis, the inhibitory effect of chrysin was superior to cromolyn. These findings suggest that chrysin would be a useful treatment of allergic reactions.

Mast cells function as a key mediator of allergic inflammation. After allergen exposure, mast cells are activated by crosslinking of adjacent IgE, aggregation of Fc ϵ RI triggers intracellular signaling process that results in the degranulation and secretion of histamine, protease, cytokine, and chemokine. These events cause vasodilation, vascular permeability and contraction of bronchial smooth muscle. After several hours from allergen exposure various inflammatory cells, such as T cells, eosinophils, macrophages and basophils, are activated and migrate to the allergic region. These responses are mediated by mast cell products (Galli et al., 2008). Mast cell degranulation is preceded by the calcium influx. Calcium leads the granule membrane fusion via the SNAP receptor protein and secretory carrier membrane protein finally granules are broken up (Castle et al., 2002). Calcium is a critical factor for the degranulation of mast cells. Calcium movements

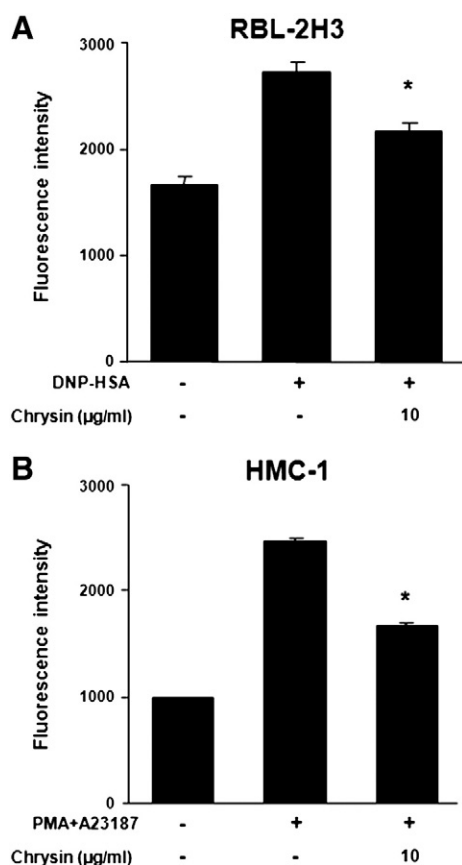


Fig. 4. Effect of chrysin on the intracellular calcium levels in mast cells. (A) IgE-sensitized RBL-2H3 cells preincubated for 10 min with chrysin (10 µg/ml) and then challenged with DNP-HSA. (B) HMC-1 cells were preincubated with chrysin (10 µg/ml) and then stimulated with PMA (40 nM) plus A23187 (1 µM) for 10 min. Intracellular calcium was measured using flow cytometer. Each bar represents the mean $\pm$ SEM of three independent experiments. *Significant difference from the DNP-HSA or PMA plus A23187 value at $P < 0.05$.

across membranes of mast cells represent a major target for effective anti-allergic drugs, as these are essential events linking stimulation to secretion (Beaven et al., 1984). Our results showed that chrysin decreased histamine and intracellular calcium in mast cells. These results suggest that chrysin inhibits the degranulation of mast cells by the decreasing of intracellular calcium level.

HMC-1 is a useful cell for studying cytokine activation pathways (Moller et al., 1998; Kim et al., 2005). The spectrum of cytokines produced by HMC-1 with PMA plus A23187 stimulation supports the well-recognized role of mast cell in immediate-type hypersensitivity. However, due to absence of Fc ϵ RI in HMC-1, normal physiological activation through IgE is not possible in this cell. Therefore, we confirmed the anti-allergic inflammatory effect of chrysin using antigen-stimulated RBL-2H3 cells. TNF- α is a major cytokine stored and released by mast cells. It promotes inflammation, leukocyte infiltration, chemotaxis of neutrophils and T cells (Thomas, 2001). TNF- α is also involved in eosinophil survival, and thereby contributes to chronic inflammation (Levi-Schaffer et al., 1998). IL-1 β induces expression of adhesion molecules on endothelium to enhance PMN transmigration (Lin et al., 2002). IL-4 is produced by Th2 cells and mast cells and play an important role in allergic inflammation, such as isotype switching of B cells to IgE synthesis and it can cause mast cell activation (Bischoff et al., 1999). IL-6 is also produced from mast cells and its local accumulation is associated with PCA reaction (Mican et al., 1992). These reports indicate that the reduction of pro-inflammatory cytokines from mast cells is one of the key indicators of reduced allergic symptoms. In the present study, chrysin inhibited the expression of TNF- α , IL-1 β , IL-4 and IL-6 from

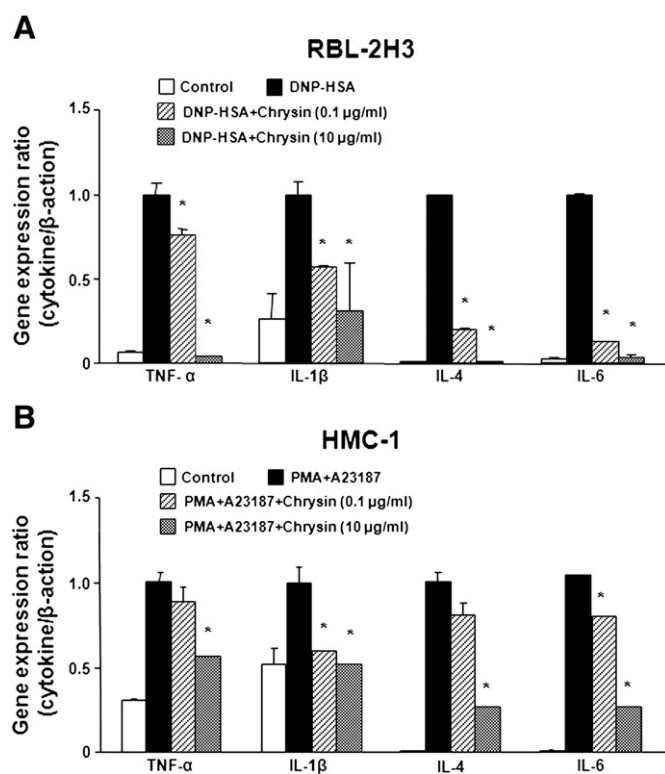


Fig. 5. Effect of chrysin on expression of pro-inflammatory cytokines in mast cells. (A) RBL-2H3 cells were sensitized overnight with anti-DNP IgE and were pretreated with chrysin for 30 min prior to DNP-HSA challenge for 2 h. (B) HMC-1 cells were pretreated with chrysin for 30 min prior to being stimulated with PMA (40 nM) plus A23187 (1 µM) for 4 h. The expression level of TNF- α , IL-1 β , IL-4 and IL-6 were determined by real-time PCR. Each bar represents the mean $\pm$ SEM of three independent experiments. *Significant difference from the DNP-HSA or PMA plus A23187 value at $P < 0.05$.

mast cells. These results indicate that chrysin has anti-allergic inflammatory effects by reducing pro-inflammatory cytokines in mast cells. Recently it was reported that the extract of propolis attenuates IL-4 and MCP-1 production from RBL-2H3 cells (Yano et al., 2007; Nakamura et al., 2010). Furthermore, chrysin inhibits pro-inflammatory cytokines and relieve inflammation responses in macrophages and monocytes. Chrysin inhibits TNF- α and IL-1 β from LPS-stimulated RAW 264.7 and J774.1 cells (Blonska et al., 2004; Harasastani et al., 2010). Chrysin and its structural analog, apigenin, inhibit the production of TNF- α , IL-1 β and IL-6 in LPS-stimulated human peripheral blood mononuclear cells (Hougee et al., 2005). These accumulated reports support our results showing the inhibition of pro-inflammatory cytokines in mast cells.

Intracellular calcium is crucially involved in gene transcription of inflammatory cytokines as well. Several reports have demonstrated associations between intracellular calcium and inflammatory cytokine production from mast cells (Jeong et al., 2002; Tanaka et al., 2005). Depletion of intracellular calcium with various calcium blockers inhibits IgE-induced TNF- α and IL-6 production in mast cells. Furthermore, NF- κ B pathway mediates these effects. Because of the reducing effect of chrysin on the intracellular calcium, we suggest that one possible pathway of the inhibitory effect of chrysin of the inflammatory cytokines is mediated by the reduction of intracellular calcium in mast cells.

Among pro-inflammatory cytokines, IL-1 β is synthesized in cytoplasm as a 31 kDa molecule and is processed and released from cells by a mechanism involving an enzyme called IL-1 β converting enzyme, known as caspase-1. Pro-IL-1 β is biologically inactive and must be converted to the mature 17 kDa IL-1 β to acquire the ability to bind to receptors and activate cells. Caspase-1 presents in a

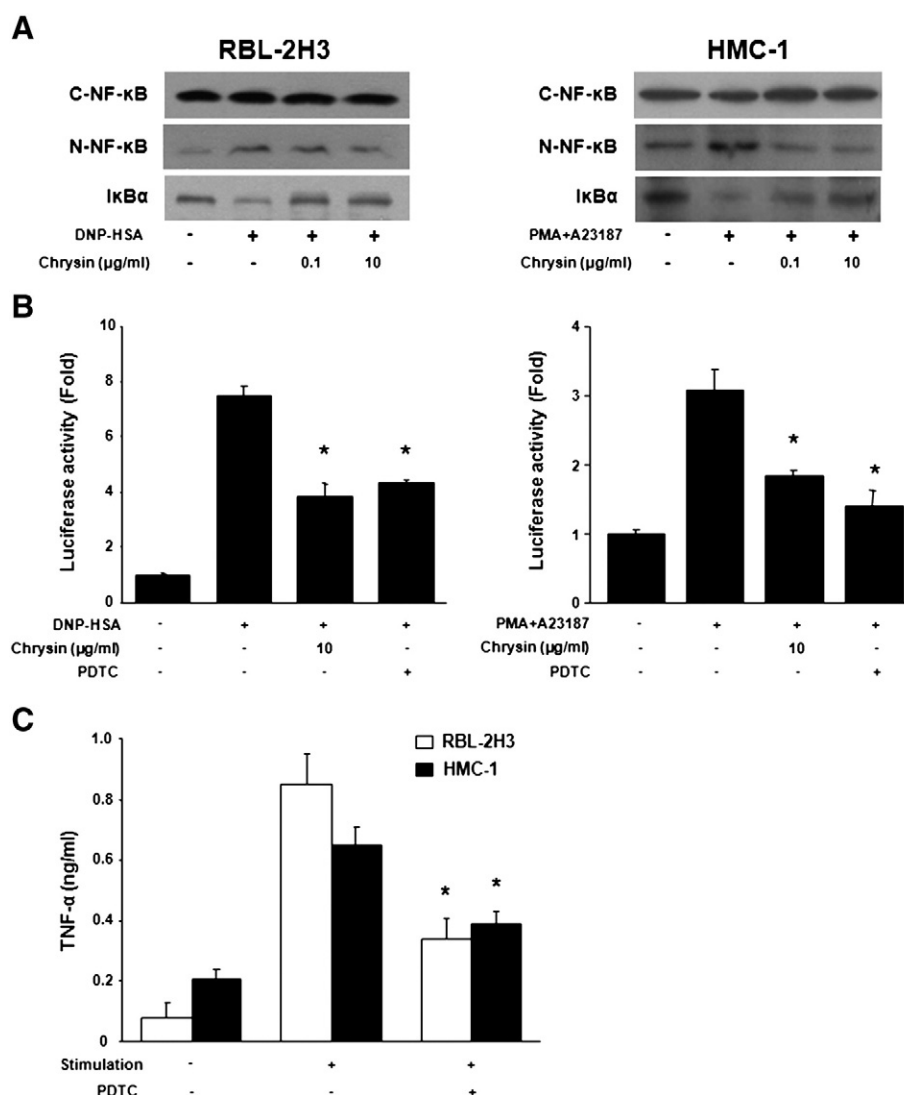


Fig. 6. Effect of chrysin on the activation of NF- κ B in mast cells. Cells were pretreated with chrysin for 30 min prior to DNP-HSA or PMA plus A23187 stimulation. (A) NF- κ B translocation and I κ B α degradation were assayed by Western blot (C-NF- κ B, cytoplasmic NF- κ B; N-NF- κ B, nucleus NF- κ B). (B) Cells were transiently transfected with the NF- κ B-luciferase reporter construct gene. Then, the cells were incubated with or without chrysin and stimulated with DNP-HSA or PMA plus A23187. NF- κ B dependent transcriptional activity was determined by luciferase activity assay. (C) Cells were pretreated with pharmacologic inhibitor of NF- κ B (PDTc, 50 μ M) for 30 min prior to DNP-HSA or PMA plus A23187 stimulation, and secretion of TNF- α was measured by ELISA. These data are representative of three independent experiments. *Significant difference from the DNP-HSA or PMA plus A23187 value at $P < 0.05$.

catalytically inactive 45 kDa proenzyme and is activated by cleavage into two non-identical subunits, p20 and p10. Active caspase-1 can also cleave other members of the IL-1 family, IL-18 and IL-33 (Thornberry et al., 1992). The administration of caspase-1 inhibitors attenuates the course of collagen-induced arthritis (Ku et al., 1996) and inhibits the pro-inflammatory responses of monocytes isolated from patients with familial cold auto-inflammatory syndrome (Stack et al., 2005). In our study, we observed that DNP-HSA or PMA plus A23187-stimulated caspase-1 activation was inhibited by chrysin in mast cells. From that result we postulate that chrysin inhibits IL-1 β via the regulation of caspase-1 activation.

NF- κ B is a transcription factor that induces the transcription of a variety of genes. Many of these genes encode molecules important in inflammatory processes, such as cytokines and adhesion molecules. The role of NF- κ B and its regulation of cytokine production in allergic inflammation has been characterized (Blackwell et al., 1997). In our study, chrysin decreased the nuclear translocation of NF- κ B and NF- κ B-dependent gene expression. These data demonstrate that chrysin attenuates allergic inflammation via the inhibition of NF- κ B and downstream pro-inflammatory cytokines. This finding is in agree-

ment with recent reports showing that chrysin restrains LPS-stimulated pro-inflammatory mediators by regulating NF- κ B translocation in microglia cells (Ha et al., 2010) and TNF- α -induced inflammation by inhibiting NF- κ B activation in HEK 293 cells (Lee et al., 2009). The MAPK cascade is also an important signaling pathway in immune responses and cross talks with NF- κ B to regulate inflammation signaling (Arbabi and Maier, 2002). The expression of pro-inflammatory cytokines is known to be regulated by MAPKs in mast cells (Kim et al., 2006). However, interestingly, chrysin did not affect the activation of all three types of MAPKs, such as p38, ERK, and JNK (data not shown). These data suggest that the inhibitory effect of chrysin on the pro-inflammatory cytokines is mediated by NF- κ B but not MAPKs.

Chrysin has been isolated from propolis, blue passion flower, vegetables and fruits. In practice, chrysin is an over the counter supplement for increase of testosterone level. Although chrysin has many beneficial properties in vitro, absorption of chrysin following oral administration in animals and humans is low due to rapid metabolism in the liver and small intestine (Walle et al., 1999). However, it has been suggested that black pepper derivative, piperine,

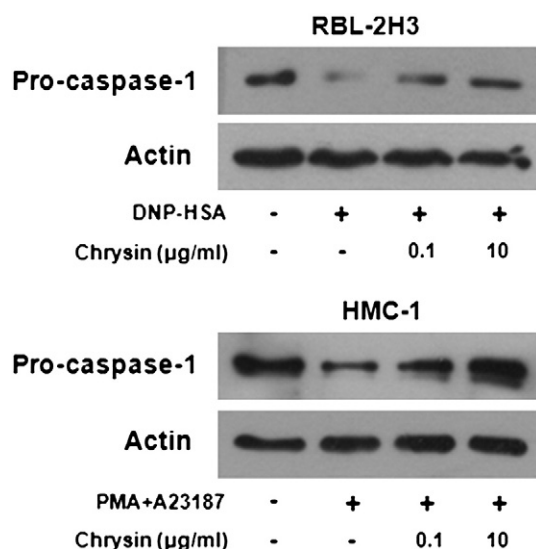


Fig. 7. Effect of chrysin on the caspase-1 activation in mast cells. Cells were pretreated with chrysin for 30 min being stimulated with DNP-HSA or PMA plus A23187 for 2 h. Activation of pro-caspase-1 was assayed by Western blot. Actin was used as a loading control.

a known inhibitor of hepatic and intestinal glucuronidation, increases flavonoid uptake in the intestine (Shoba et al., 1998; Lambert et al., 2004). Several methylated flavones, including 5,7-dimethoxyflavone, 7-methoxyflavone and 7,4'-dimethoxyflavone, are much more resistant to metabolism and showed higher intestinal absorption than their unmethylated analogs (Ta and Walle, 2007). Further studies are needed to improve bioavailability by increasing the absorption into the bloodstream when chrysin is taken orally.

In conclusion, our results suggest the mechanisms responsible for anti-allergic inflammatory activity of chrysin. We suggest that chrysin reduces histamine release via regulating intracellular calcium and inhibits inflammatory cytokines through suppressing caspase-1 and NF- κ B. We provide evidence that chrysin could contribute to the prevention or treatment of mast cell-mediated allergic inflammatory disorders.

Conflict of interest statement

The authors have no conflicts to disclose.

Acknowledgments

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