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Inhibitory mechanism of saponins derived from roots of *Platycodon grandiflorum* on anaphylactic reaction and IgE-mediated allergic response in mast cells

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ABSTRACT

The purpose of this study was to investigate the protective effects of saponins isolated from the root of *Platycodi Radix* (Changkil saponins: CKS) anti-allergic effects in mice and mast cells. Oral administration of CKS inhibited the dinitrophenyl (DNP)–IgE antibody-induced systemic PCA reaction in mice. CKS reduced the β-hexosaminidase and histamine release from anti-DNP–IgE-sensitized RBL–2H3 cells. In addition, CKS inhibited the IgE antibody-induced increases in IL-4 and TNF-α production and expression in RBL-2H3 cells. In order to explore the inhibitory mechanism of CKS in PCA and mast cell degranulation, we examined the activation of intracellular signaling molecules. CKS suppressed DNP–IgE antibody-induced Syk phosphorylation. Further downstream, CKS also inhibited the phosphorylation of Akt and MAP kinases. Taken together, the *in vivo*/*in vitro* anti-allergic effects of CKS suggest possible therapeutic applications for this agent in allergic diseases through the inhibition of inflammatory cytokines and Syk-dependent signaling cascades.

1. Introduction

In an allergic response, binding of antigen to the high-affinity IgE receptor (FcεRI) on the surface of mast cells and basophils induces the release of pre-formed intragranular mediators such as histamine, arachidonic acid metabolites, proteases, serotonin, and heparin (Beaven and Metzger, 1993; Turner and Kinet, 1999; Stassen et al., 2002). Mast cells participate in many biological responses such as allergic diseases and inflammatory disorders (Beaven and Metzger, 1993; Bochner and Schleimer, 2001). The FcεRI is abundant on the surface of these cells (Barsumian et al., 1981), and aggregation of IgE–FcεRI complexes by multivalent antigen generates a complex cascade of intracellular events leading to degranulation and subsequent release of chemical mediators of allergic response including histamine (Barsumian et al., 1981), serotonin (Taurog et al., 1979) and β-hexosaminidase (Schwartz et al., 1979). The β-hexosaminidase assay has been widely used to monitor RBL mast cell degranulation (Ortega Soto and Pecht, 1988; Pierini et al., 1997; Aketani et al., 2001). Many investigators have concentrated on finding effective therapeutics for allergic inflammation, using well established mast cell-dependent experimental model systems. For instance, passive cutaneous anaphylaxis (PCA), which is an animal model of the IgE-mediated immediate allergic reaction, is induced by mediators secreted from mast cells, such as histamine (Kemp and Lockey, 2002; Kim et al., 1999). Mast cells also play an important role in initiating and perpetuating the inflammatory response in allergic reactions by secreting abundant amounts of proinflammatory mediators such as histamine, interleukin-4 (IL-4), IL-5, IL-6, tumor necrosis factor (TNF)-α, and leukotrienes (Bradding et al., 1994). IL-4 is essential for IgE production (Kuhn et al., 1991) and promotes the switch from naive T cells to the allergic type Th2 cells (Hines, 2002; Huels et al., 1995). Nuclear factor κB (NF-κB) is a key transcription factor that regulates the expression of genes involved in immune and inflammatory responses that require inflammatory cytokine production (Marquardt and Walker, 2000). NF-κB is thought to play an important role in the regulation of proinflammatory molecules, especially TNF-α, IL-6, and IL-8 (Salamon et al., 2005). The activation of signaling pathways in antigen-stimulated mast cells depends initially on the interaction of FcεRI with the Src kinases, Lyn, and Fyn, in response to activation of Syk and other tyrosine kinases (Gilfillan and Tkaczyk, 2006). Mast cells then rapidly release various allergic mediators, including histamine, cytokines, and arachidonic acid derivatives (Gilfillan and Tkaczyk, 2006) that mediate various acute and chronic allergic reactions (Church and Levi-Schaffer, 1997; Metcalfe et al., 1981). Degranulation of mast cells stimulated with IgE is markedly impaired, as is the activity of the downstream signaling molecules phosphatidylinositol 3-kinase (PI3-K) and Akt (Fukao et al., 2003). Mitogen-activated protein kinase (MAPK) signaling cascades are also important in the differentiation, activation, proliferation, degranulation and migration of

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various immune cells such as mast cells (Duan and Wong, 2006). MAPK signaling modules are divided into at least 3 groups: extracellular signal-regulated kinase (ERK) 1/2, p38 MAPK, and c-Jun NH2-terminal kinase (JNK) 1/2.

Changkil (CK), the aqueous extract of the root from *Platycodon grandiflorum*, prevents hypercholesterolemia, hyperlipidemia and hepatic fibrosis (Kim et al., 1995; Lee et al., 2004). Our previous data showed that the CK saponin fraction (CKS) derived from CK has significant anti-inflammatory effects (Kim et al., 2005) and potent antioxidative effects such as superoxide radical scavenging activity (Kim et al., 2005). In addition, our previous data showed that CKS suppresses 12-O-tetradecanoylphorbol-13-acetate (PMA)-enhanced matrix metalloproteinase activation, which is related to tumor invasion and migration (Lee et al., 2008). In the present study, we investigated the anti-allergic effects of CKS on RBL–2H3 rat mast cells and on anti-DNP–IgE-mediated PCA in mice.

2. Materials and methods

2.1. Materials

Chemicals and cell culture materials were obtained from the following sources: anti-dinitrophenyl (DNP)–IgE, Evans blue and 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide from Sigma Aldrich Co.; DNP-bovine serum albumin (BSA) from Calbiochem; minimum essential medium with Eagle's salt and fetal bovine serum (FBS) from Life Technologies, Inc.; luciferase assay system from Promega; pCMV-β-gal from Clontech; LipofectAMINE 2000 from Invitrogen, Inc.; enzyme immunoassay reagents for cytokine assays from R&D Systems; β-actin from Santa Cruz Biotechnology, Inc.; protein assay kit from Bio-Rad Laboratories, Inc.; primary antibodies [anti-IκB-αphospho-IκB-α, anti-MAPK (Erkl/2)/phospho-MAPK (Erkl/2), anti-p38 MAPK/phospho-p38 MAPK, and anti- SAPK/JNK/phospho-SAPK/JNK1/2, anti-Syk/phospho-Syk, anti-Akt/phospho-Akt] and secondary antibody (HRP-linked anti-rabbit and anti-mouse IgG) from Cell Signaling Technology; ECL chemiluminescence system and polyvinylidene difluoride (PVDF) membrane from Amersham Pharmacia Biotech. Polymerase chain reaction (PCR) oligonucleotide primers were custom synthesized by Bioneer Co. (Korea). All chemicals were of the highest grade commercially available.

2.2. Preparation of CKS

CK refers to the aqueous extract from the 20-year-old roots of *P. grandiflorum*, which was supplied by Jang Saeng Doraji Co., Jinju, South Korea. The CKS were prepared as described elsewhere and their compositions were previously published (Kim et al., 1995; Tada et al., 1975). The composition of the root of CKS were deapioplatycoside E, platycoside E, deapioplatycodin D3, platycodin D3, polygalacin D2, platycodin D2, deapioplatycodin D and platycodin D (Kim et al., 2005). Briefly, CK was subjected to column chromatography over amberlite XAD-2, Diaion MCl Gel HP20 or Kogel BG4600. After removing the saccharides and amino acids with water, the column was eluted with methanol to obtain the CKS, which is the saponin fraction of CK (Tada et al., 1975).

2.3. Animals

Specific pathogen-free ICR mice (female, 8–10 weeks old) were purchased from Dae Han Laboratory Animal Research and Co. (Daejeon, Korea). The mice were housed under normal laboratory conditions, i.e., at 21–24 °C and 40–60% relative humidity, with a 12-h light/dark cycle and free access to standard rodent food and water.

2.4. Passive cutaneous anaphylaxis reactions

Anti-DNP IgE diluted in PBS alone was injected intradermally in both ears of mice with a 0.3-ml insulin syringe. One day later, DNP-BSA in 200 μl of PBS with 0.5% Evans blue was injected i.v. Thirty minutes after challenge, both ears were removed and incubated at 80 °C in 1 ml of formamide for 2 h. The mixture was homogenized and centrifuged at 20,800g for 10 min. The intensity of the absorbance was measured at 620 nm in a spectrofluorometer (Varioskan, Thermo Electron Co.).

2.5. Cell culture

The rat mast cell line, RBL-2H3, was obtained from the American Type Culture Collection (Bethesda, MD) and grown in minimum essential medium with Eagle's salt supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified incubator with a 5% CO_2/95% air atmosphere. CKS was dissolved in distilled water, and stock solutions were added directly to the culture media.

2.6. Assay for proliferative activity

Cell cytotoxicity was examined using a WST-1 assay kit according to the manufacturer's instructions. Briefly, RBL-2H3 cells (5×10^5 cell/well) in 10% FBS–EMEM were seeded into 96-well plates. After incubation for 48 h, various concentrations of CKS were added to the wells, and the plates were incubated at 37 °C. After the supernatant was removed, the cells were used in the WST-1 assay. Relative cytotoxicity was quantified by measuring the absorbance at 550 nm using a spectrofluorometer (Varioskan, Thermo Electron Co.). CKS did not interfere at this wavelength.

2.7. β-hexosaminidase release assay

IgE-sensitized RBL-2H3 cells attached to microtiter wells were washed twice in Tyrode's buffer (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.6 mM glucose, 20 mM HEPES, and 1 mg/ml BSA at pH 7.4) and stimulated by the addition of 250 μl of serially diluted antigen in Tyrode's buffer (Pierini et al., 1997). The cells were then incubated for 1 h at 37 °C, and degranulation was terminated by placing the cells on ice. To determine the amount of β-hexosaminidase activity released by the cells, 25 μl of supernatant and 100 μl of 1.2 mM β-hexosaminidase substrate (4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide) in 0.05 M sodium acetate buffer (pH 4.4) were mixed in 96-well plates and incubated for 30 min at 37 °C. Total β-hexosaminidase release was obtained by lysing the cells with 0.1% Triton-X 100 prior to removing the supernatant for measurement of β-hexosaminidase activity. β-Hexosaminidase activity in the supernatant was quantified by measuring the fluorescence intensity of the hydrolyzed substrate in a spectrofluorometer (Varioskan, Thermo Electron Co.) using 360 nm excitation and 450 nm emission filters. Background fluorescence of substrate in buffer alone (no cell supernatant) was subtracted from all readings.

2.8. Histamine release assay

Cells were preincubated with CKS for 30 min and then incubated with DNP-BSA for 15 min. Histamine content was measured by the *o*-phthaldialdehyde spectrofluorometric procedure. The fluorescence intensity was measured at 440/360 nm using a spectrofluorometer (Varioskan, Thermo Electron Co.).

2.9. Transient transfection and luciferase activity assay

For transient transfections, cells were seeded at 1×10^6 cell/well in a 48-well plate. Expression vector containing the NF-κB luciferase reporter construct (pNF-κB-LUC plasmid containing NF-κB binding site; Stratagene, Grand Island, NY) or empty vector was transfected with 0.5 μl of serum- and antibiotic-free LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA). After 4 h, the medium was replaced with basal medium. The cells were then treated with CKS for 18 h and lysed. The luciferase and β-galactosidase activities were measured in the cellular extract. The luciferase activity was normalized to the β-galactosidase activity and expressed relative to the activity of the control group.

2.10. Measurement of cytokine production

For cytokine immunoassays, cells were cultured for 3 h and 24 h at a density of 1×10^6 cell/well in 48-well plates. Supernatants were removed at the indicated times, and IL-4 (24 h) and TNF-α (3 h) production were quantified by sandwich immunoassays using the protocol supplied by R&D systems.

2.11. RNA preparation and mRNA analysis by real-time quantitative PCR

Cells were cultured with CKS for 30 min. Total RNA from the treated cells was prepared with RNAiso Reagent (Takara) according to manufacturer's protocol and stored at −80 °C until use. Total RNA for detection of macrophage-related cytokines, including TNF-α and IL-4, was extracted after stimulation and treatment. PCR product formation was continuously monitored during the PCR reaction using Sequence Detection System software, version 1.7 (Applied Biosystems, Foster City, CA). Accumulated PCR products were detected directly by monitoring the increase of the reporter dye (SYBR). The expression levels of cytokines in the exposed cells were compared to the expression levels in control cells at each collection time point using the comparative cycle threshold (Ct)-method (Johnson et al., 2000). The sequences of the primers used in this study were: TNF-α forward: 5′-CAA GGA GGA GAA GTT CCC AA-3′; TNF-α reverse: 5′-CGG ACT CCG TGA TGT CTA AG-3′; IL-4 forward: 5′-ACC TTG CTG TCA CCC TGT TC-3′; IL-4 reverse: 5′-TTG TGA GCG TGG ACT CAT TC-3′; β-actin forward: 5′-TCA TCA CCA TCG GCA ACG-3′, β-actin reverse: 5′-TTC CT GAT GTC CAC GTC GC-3′. The quantity of each transcript was calculated as described in the instrument manual and normalized to the amount of β-actin, a housekeeping gene.

2.12. Subcellular fractionation

Membrane, cytosolic, and nuclear fractions were prepared as described previously (Kabouridis et al., 1997). In brief, cells were incubated in CKS for 30 min. The cells were then resuspended in 0.5 ml hypotonic solution (25 mM Tris pH 7.5, 5 mM EGTA, 250 mM sucrose, 25 μg/ml aprotinin, 1 mM PMSF, 25 μg/ml leupeptin, 5 mM NaF, and 1 mM Na_3VO_4) and subjected to two successive freeze-thaw cycles. The cell suspension was homogenized on ice, and the salt concentration was adjusted to 150 mM NaCl. Nuclei were removed by two successive centrifugations at 480g for 5 min at 4 °C. Soluble and particulate fractions were separated by centrifugation at 100,000g for 30 min.

2.13. Western blotting

IgE-sensitized RBL–2H3 cells were cultured with CKS for 5 min (Syk, Akt and MAPK) and 30 min (IκB-α); equal amounts of cellular protein (50 μg) were resolved by 10% SDS–PAGE and transferred onto polyvinylidene difluoride membranes. After blocking, the membranes were incubated with the targeted antibody. Horseradish peroxidase-conjugated secondary antibody to IgG was used. Immunoreactive proteins were visualized using the ECL Western blot detection system. The protein level was compared to a loading control such as β-actin or non-phosphorylated protein.

2.14. Statistical analysis

All experiments were repeated at least three times. One-way analysis of variance (ANOVA) was used to determine the significance of differences between treatment groups. The Newman–Keuls test was used for multi-group comparisons. Statistical significance was accepted for p values of <0.01.

3. Results

3.1. CKS suppresses the mast cell-mediated passive cutaneous anaphylaxis reaction in mice

PCA reaction is one of the most frequently used models for evaluating anti-allergic drugs (Kabu et al., 2006). We examined the anti-allergic activity of CKS in PCA in the mouse ear model. PCA was induced through local injection of DNP–IgE in the mouse ear and then systemic injection of antigen in mice. As shown in Fig. 1, when mice were administered CKS orally for 1 h, the ear-swelling responses derived from DNP-BSA were significantly reduced in a dose-dependent manner.

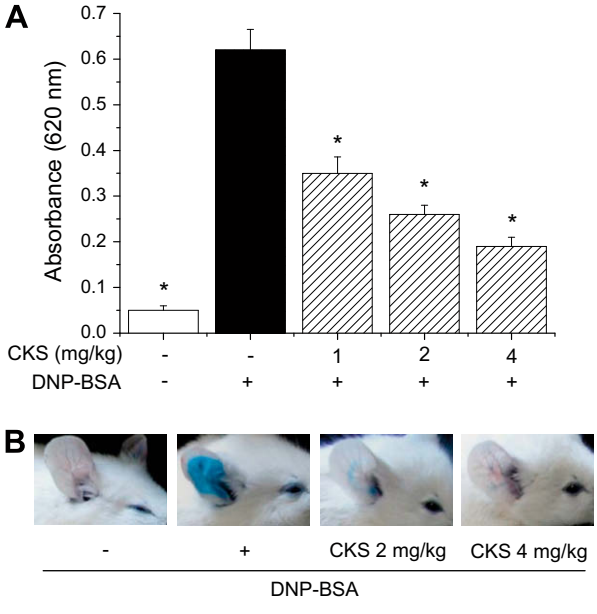

Fig. 1. Effects of CKS on mast cell-mediated allergic reaction in mice. Anti-DNP-specific IgE (0.5 μg) was intradermally injected into the ear of a mouse. One day later, DNP-BSA in 200 μl of PBS containing 0.5% Evans blue was injected i.v. CKS was administered 1 h before administration of the antigen (DNP–BSA). The mice were then euthanized after 1 h, and the right ear was excised in order to measure the extravasated dye. (A) The dye was extracted overnight in 1 ml of formamide at 80 °C, and the intensity was measured at 620 nm. Each bar shows the mean ± S.D. of three independent experiments. $^{*}P < 0.01$, significantly different from DNP-BSA alone. (B) Representative pictures of the ears are shown.

3.2. CKS inhibits DNP–IgE-induced degranulation and histamine release of RBL–2H3 cells

The cytotoxic effect of CKS in RBL–2H3 cells was measured by WST-1 assay. CKS at the tested concentrations did not significantly affect the cytotoxicity (Fig. 2A). Thus, we treated cells with CKS ranging from 1–4 μg/ml during subsequent experiments. We examined whether CKS affected allergic inflammation *in vitro*. The rat basophilic leukemia cell line RBL–2H3, a tumor analog of mast cells, exhibits phenotypic characteristics of mucosal mast cells. After stimulation with antigen, cells release β-hexosaminidase, which is a marker of mast cell degranulation; thus RBL–2H3 cells are considered a good model for studying comprehensive events in mast cells induced by multivalent allergens (McDermott et al., 2007; Marchand et al., 2003). CKS significantly suppressed antigen-induced degranulation in a dose-dependent manner in RBL–2H3 cells (Fig. 2A). The release of chemical mediators such as histamine plays an important role in allergic inflammation (Marchand et al., 2003). Therefore, we examined the effects of CKS on histamine release. Fig. 2B shows the inhibitory effect of CKS on the DNP–IgE-induced release of histamine from RBL–2H3

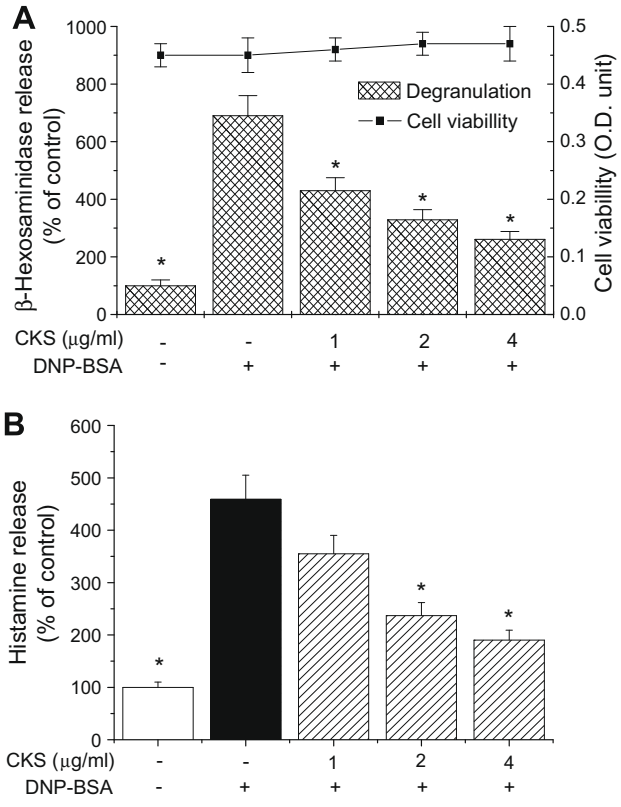

Fig. 2. Effects of CKS on antigen-induced degranulation and histamine release in RBL–2H3 cells. (A) The cells were incubated overnight in 48-well plates with 100 ng/ml of DNP-specific IgE in medium. The medium was replaced with Tyrode buffer that contained the indicated concentrations of CKS before stimulation with 50 ng/ml of DNP–BSA for 10 min in order to measure the release of β-hexosaminidase. Cell viability was assessed using WST-1 assays. Each bar shows the mean ± S.D. of three independent experiments. $^{*}P < 0.01$, significantly different from DNP–BSA alone. (B) Same as (A) except that histamine release was measured. Each bar shows the mean ± S.D. of three independent experiments. $^{*}P < 0.01$, significantly different from DNP–BSA alone.

cells. These results suggest a potential use for CKS as an allergy therapeutic.

3.3. CKS inhibits DNP–IgE-induced production and expression of TNF-α and IL-4 in RBL-2H3 cells

Various cytokines, including IL-4 and TNF-α, are critical for allergic inflammation (Theoharides and Kalogeromitros, 2006). IL-4 is essential for IgE production (Kuhn et al., 1991) and promotes the switch from naive T cells to the allergic type Th2 cells (Hines, 2002; Huels et al., 1995). TNF-α is a potent inflammatory mediator in the cytokine family. TNF-α is mainly produced by activated macrophages and T cells in response to infection, although it is also formed and secreted by mast cells as a result of IgE challenge (Gordon and Galli, 1990). Accordingly, we examined whether CKS suppressed the expression and secretion of IL-4 and TNF-α in antigen-stimulated RBL–2H3 cells. CKS significantly inhibited the antigen-stimulated secretion of IL-4 and TNF-α production in a dose-dependent manner (Fig. 3A). We further tested whether CKS suppressed expression of IL-4 and TNF-α mRNA in the cells. In agreement with the ELISA results, CKS inhibited the mRNA expression of both cytokines in a dose-dependent manner (Fig. 3B).

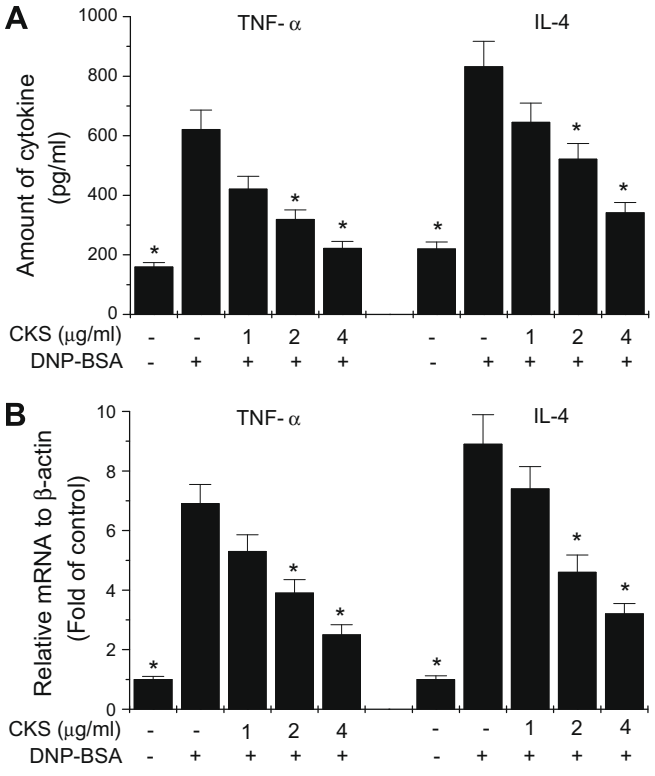

Fig. 3. Effects of CKS on antigen-induced expression and secretion of TNF-α and IL-4 in RBL–2H3 cells. (A) The IgE-primed cells were stimulated with 50 ng/ml of DNP–BSA for 4 h or were left unstimulated with or without CKS. The concentrations of TNF-α and IL-4 released into the culture media were assessed using commercial ELISA kits. Each bar shows the mean ± S.D. of three independent experiments. $^{*}P < 0.01$, significantly different from DNP–BSA alone. (B) The cells were sensitized with 100 ng/ml of DNP-specific IgE overnight and pretreated with CKS for 30 min. Cells were stimulated with 50 ng/ml of DNP–BSA for 30 min. The cells were lysed and total RNA was prepared for analysis of TNF-α and IL-4 gene expression. PCR amplification of the housekeeping gene, β-actin, was performed for each sample. TNF-α and IL-4 mRNA expression in treated cells was compared to the expression in untreated cells at each time point by real-time PCR. Each bar shows the mean ± S.D. of three independent experiments. $^{*}P < 0.01$, significantly different from DNP–BSA alone.

3.4. CKS inhibits DNP–IgE-induced activation of NF-κB and Syk-dependent signal pathway of RBL–2H3 cells

NF-κB is thought to play an important role in the regulation of proinflammatory molecules of cellular responses, especially TNF-α, IL-6, and IL-8 (Salamon et al., 2005). To evaluate the mechanisms of the effects of CKS on cytokine secretion, we examined the effect of CKS on NF-κB activation using a NF-κB-luciferase reporter vector. CKS markedly suppressed DNP–BSA-induced NF-κB-luciferase activity in antigen-stimulated RBL–2H3 cells (Fig. 4A). Unstimulated NF-κB is found in the cytoplasm bound to IκB-α. IκB-α was rapidly degraded when antigen-stimulated RBL–2H3 cells were treated with CKS (Fig. 4B). Anti-β-actin antibodies were used as quantitative controls (Fig. 4B). We also examined the effects of CKS on phosphatidylinositol 3-kinase and the MAP kinases because of their role in the production of TNF-α and IL-4 (Fukao et al., 2003; Duan and Wong, 2006). To gain insight into how CKS suppresses mast cell activation, we examined its effects on early FcεRI-mediated signaling events, namely the phosphorylation of Syk. As shown in Fig. 5A, the tyrosine phosphorylation of Syk was inhibited by CKS in antigen-stimulated RBL–2H3 cells. The phosphorylation of Akt, an indicator of the activation of phosphatidylinositol 3-kinase (PI3-K), and the phosphorylation of the MAP kinases p38, Erk1/2, and JNK1/2 were also significantly suppressed by CKS in a dose-dependent manner (Fig. 5B).

4. Discussion

Platycodi Radix, the root of *Platycodon grandiflorum* (*P. grandiflorum*), commonly known as Doraji in Korea (Chinese drug, 'Jiegeng', and Japanese name, 'Kikyo'), has been used as a traditional oriental medicine (Lee, 1973). In Korea, the root of *P. grandiflorum* (4-year-old) has been used as a food, as a folk remedy for diseases of adulthood, such as bronchitis, asthma, pulmonary tuberculosis, hyperlipidemia, diabetes, inflammatory diseases, and as a sedative and analgesic (Lee, 1973; Kim et al., 1995). Changkil (CK) is an aqueous extract of the root from 20-year-old *Platycodon grandiflorum* plants in Korea, which prevented hypercholesterolemia, hyperlipidemia and hepatic fibrosis (Kim et al., 1995; Lee et al., 2004). Our previous data showed that the CK saponin fraction (CKS) derived from CK had significant anti-inflammatory effects (Kim et al., 2005) and potent antioxidative effects, exhibiting superoxide radical scavenging activity and inhibiting ROS production (Kim et al., 2005). In addition, our previous data showed that CKS suppressed 12-O-tetradecanoylphorbol-13-acetate (PMA)-enhanced matrix metalloproteinase (MMP)-9 and MMP-2 activation, which is important in tumor invasion and migration (Lee et al., 2008).

In this study, we investigated the effects of CKS on IgE-mediated passive cutaneous anaphylaxis (PCA) in mice and the anti-allergic effect in RBL–2H3 cells. We examined the anti-allergic activity of CKS in PCA in the mouse ear model. Because the number of allergic patients is increasing worldwide, many investigators have concentrated their efforts on finding effective therapeutics for allergic inflammation, using well established mast cell-dependent experimental model systems. For instance, PCA, which is an animal model of the IgE-mediated immediate allergic reaction, is also induced by mediators, such as histamine, which are secreted from mast cells (Kemp and Lockey, 2002; Kim et al., 1999). As shown in Fig. 1, when mice were administrated oral CKS for 1 h, the ear-swelling responses derived from DNP-BSA were significantly reduced in a dose-dependent manner. This result suggests that CKS might be useful in the treatment of allergic disease.

Mast cells participate in many biological responses such as allergic diseases and inflammatory disorders (Beaven and Metzger, 1993; Bochner and Schleimer, 2001). The β-hexosaminidase assay

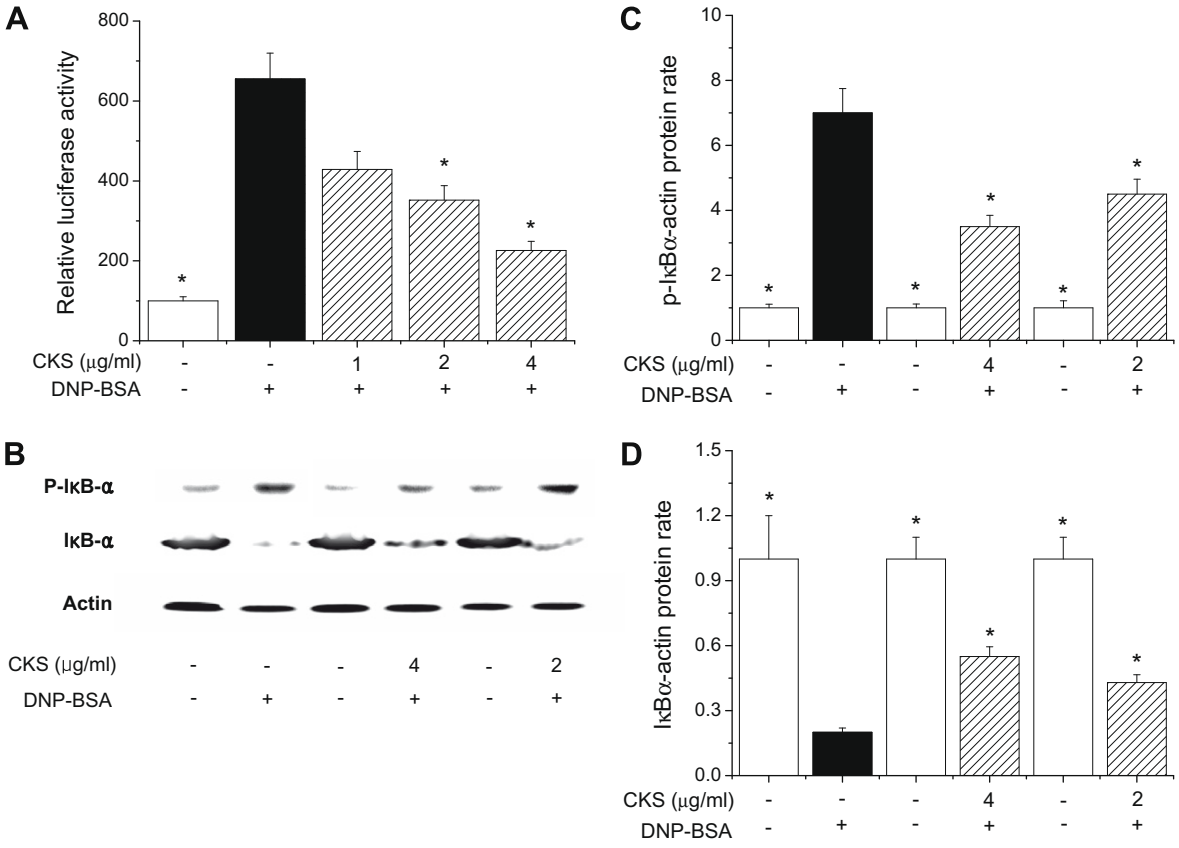

Fig. 4. Effects of CKS on antigen-induced activation of NF-κB in RBL–2H3 cells. (A) Cells were transiently co-transfected with pGL3-NF-κB-Luc and pCMV-β-gal. After 4 h, the cells were sensitized with DNP-specific IgE (100 ng/ml) for 16 h. The IgE-sensitized cells were treated with CKS and stimulated with DNP-BSA (50 ng/ml). The cells were harvested, and luciferase and β-galactosidase activities were determined. The luciferase activity was normalized to the β-galactosidase activity and is expressed relative to the activity of the control. Each bar shows the mean ± S.D. of three independent experiments. $^{*}P < 0.01$, significantly different from DNP–BSA alone. (B) CKS inhibits the antigen-induced phosphorylation and degradation of IκB-α in the cells. IgE-sensitized cells pretreated with CKS (1–4 μg/ml) were stimulated with 50 ng/ml of DNP–BSA for 30 min. Western blot analysis was performed as described in "Section 2." Each blot in this figure is representative of three independent experiments with similar results. Densitometric analyses of P-IκB-α C and IκB-α degradation (D). Each bar shows the mean ± S.D. of three independent experiments. $^{*}P < 0.01$, significantly different from DNP–BSA alone.

has been widely used to monitor RBL-2H3 mast cell degranulation (Ortega Soto and Pecht, 1988; Pierini et al., 1997; Aketani et al., 2001) and this assay is a convenient method for studying the signal transduction mechanisms that lead to exocytosis (Smith et al., 1997), as well as for monitoring the capacity of potential new drugs to block mast cell activation and degranulation (Granberg et al., 2001). CKS significantly suppressed antigen-induced degranulation in a dose-dependent manner in RBL–2H3 cells (Fig. 2A). The release of chemical mediators such as histamine plays an important role in allergic inflammation (Marchand et al., 2003). CKS inhibited the DNP–IgE-induced histamine release in a dose-dependent manner (Fig. 2B). These results also suggest a potential value for CKS as an allergy therapeutic.

Various cytokines, including IL-4 and TNF-α, are critical for allergic inflammation (Theoharides and Kalogeromitros, 2006). In particular, IL-4 is an important cytokine in allergic reactions. IL-4 is an eosinophil chemoattractant that induces endothelial cells to produce eosinophil chemotactic factor and eotaxin (Rothenberg et al., 1995) and that enhances endothelial cell surface adhesion molecules such as vascular cell adhesion molecule-1 (Schleimer et al., 1992). IL-4 is essential in IgE production (Kuhn et al., 1991) and promotes the switch from naive T cells to allergic type Th2 cells (Hines, 2002; Huels et al., 1995). TNF-α is a potent inflammatory mediator of the cytokine family. TNF-α is mainly produced by activated macrophages and T cells in response to infection, although it is also formed and secreted by mast cells in response to IgE challenge (Gordon and Galli, 1990). We therefore examined whether CKS suppressed the expression and secretion of IL-4 and TNF-α in antigen-stimulated RBL–2H3 cells. CKS significantly inhibited the antigen-stimulated secretion of IL-4 and the TNF-α production in a dose-dependent manner (Fig. 3A). We then further tested whether CKS suppressed expression of IL-4 and TNF-α mRNA in the cells. In agreement with the ELISA results, CKS inhibited expression of both cytokines in a dose-dependent manner (Fig. 3B). This result suggests that the anti-allergic effect of CKS is a result of its reduction of IL-4 and TNF-α production from mast cells.

Nuclear factor κB (NF-κB) is a key transcription factor that regulates the expression of genes involved in immune and inflammatory responses that require cytokine production (Marquardt and Walker, 2000). NF-κB is thought to play an important role in the regulation of proinflammatory molecules of cellular responses, especially TNF-α, IL-6, and IL-8 (Salamon et al., 2005). To evaluate the mechanism of the effect of CKS on cytokine secretion, we examined the effect of CKS on NF-κB activation using a NF-κB-luciferase reporter vector. CKS markedly suppressed DNP–BSA-induced NF-κB-luciferase activity in antigen-stimulated RBL–2H3 cells (Fig. 4A). Unstimulated NF-κB is found in the cytoplasm bound to IκB-α. Upon stimulation, IκB-α is phosphorylated on one or more serine residues, ubiquitinated, and degraded, allowing the transport of NF-κB to the nucleus to bind recognition elements in the upstream promoter region of cytokine DNA (Alkalay et al., 1995). IκB-α was rapidly degraded when antigen-stimulated RBL–2H3 cells were treated with CKS (Fig. 4B). These data

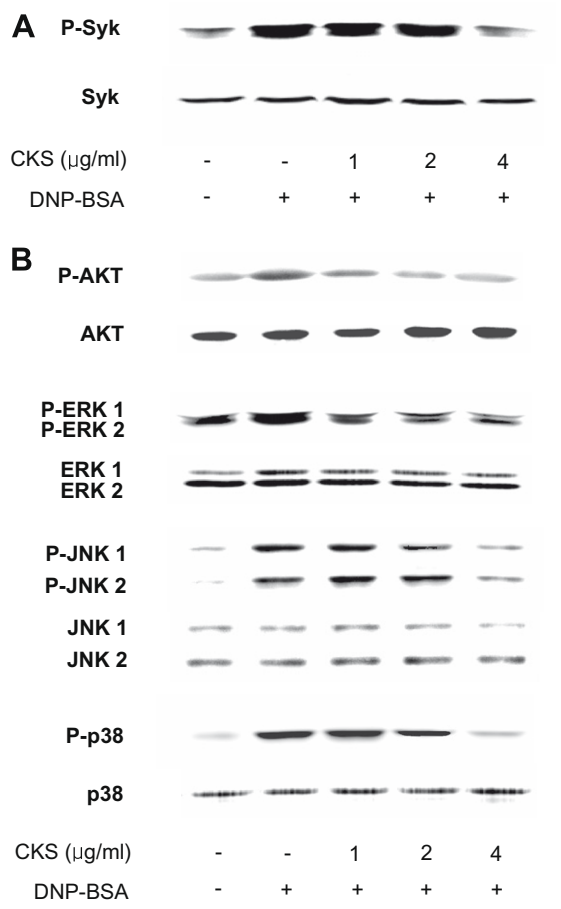

Fig. 5. Effects of CKS on antigen-induced phosphorylation of Syk, Akt and MAPK in RBL–2H3 cells. (A) IgE-sensitized cells were treated with CKS and stimulated with DNP–BSA (50 ng/ml) for 5 min. The extracts were analyzed for Syk activation by western blot analysis using antibodies against phosphorylated Syk. (B) Same as (A) except that Akt and MAP kinase activation was assessed. The extracts were analyzed for Akt and MAP kinase activation by western blot analysis using antibodies against phosphorylated Akt, ERK, p38 and JNK.

demonstrate that CKS attenuates activation of NF-κB and downstream TNF-α and IL-4 production.

We also examined the effects of CKS on phosphatidylinositol 3-kinase (PI3-K) and the MAP kinases because of their role in the production of TNF-α and IL-4 (Fukao et al., 2003; Duan and Wong, 2006). The phosphorylation of Akt, an indicator of the activation of PI3-K and the phosphorylation of the MAP kinases, p38, Erk1/2, and c-Jun N-terminal kinase, were significantly suppressed by CKS in a dose-dependent manner (Fig. 5B). To gain insight into how CKS suppresses mast cell activation, we examined its effects on early FcεRI-mediated signaling events, namely the phosphorylation of Syk. As shown in Fig. 5A, the tyrosine phosphorylation of Syk was inhibited by CKS in antigen-stimulated RBL–2H3 cells. Collectively, these results suggest that CKS acts initially at the level of Syk kinase to suppress downstream signaling events and mast cell activation.

In conclusion, this study is the first report indicating that CKS inhibits degranulation, cytokine secretion, and Syk phosphorylation in antigen-stimulated RBL–2H3 mast cells and reduces mast cell-mediated PCA in mice. These results suggest that CKS exhibits anti-allergic activity by inhibiting NF-κB and Syk activation in the cells.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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