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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.