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Houttuynia cordata water extract suppresses anaphylactic reaction and IgE-mediated allergic response by inhibiting multiple steps of FcεRI signaling in mast cells

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ABSTRACT

Houttuynia cordata has been used as a traditional medicine in Korea and is known to have antioxidant, anti-cancer and anti-allergic activities. The precise effect of *H. cordata*, however, remains unknown. In this study, we investigated the effects of *H. cordata* water extract (HCWE) on passive cutaneous anaphylaxis (PCA) in mice and on IgE-mediated allergic response in rat mast RBL-2H3 cells. Oral administration of HCWE inhibited IgE-mediated systemic PCA in mice. HCWE also reduced antigen (DNP-BSA)-induced release of β-hexosaminidase, histamine, and reactive oxygen species in IgE-sensitized RBL-2H3 cells. In addition, HCWE inhibited antigen-induced IL-4 and TNF-α production and expression in IgE-sensitized RBL-2H3 cells. HCWE inhibited antigen-induced activation of NF-κB and degradation of IκB-α. To investigate the inhibitory mechanism of HCWE on degranulation and cytokine production, we examined the activation of intracellular FcεRI signaling molecules. HCWE suppressed antigen-induced phosphorylation of Syk, Lyn, LAT, Gab2, and PLC γ2. Further downstream, antigen-induced phosphorylation of Akt and MAP kinases (ERK1/2 and JNK1/2 but not p38 MAP kinase) were inhibited by HCWE. Taken together, the *in vivo*/*in vitro* anti-allergic effect of HCWE suggests possible therapeutic applications of this agent in inflammatory allergic diseases through inhibition of cytokines and multiple events of FcεRI-dependent signaling cascades in mast cells.

1. Introduction

Type I allergy is an immune disorder that involves the production of immunoglobulin E (IgE) in response to allergens and antigens. Antigen-induced release of inflammatory mediators from mast cells causes the immediate symptoms of IgE-mediated allergic diseases, including allergic rhinitis, asthma, atopic dermatitis, and atopic eczema (Wedemeyer et al., 2000). Passive cutaneous anaphylaxis (PCA), which is an animal model of the IgE-mediated immediate allergic reaction, is also induced by mediators such as histamine secreted from mast cells (Kemp and Lockey, 2002; Kim et al., 1999). Thus, mast cells play a significant role in the allergic reaction. Indeed, mast cells are the primary effector cells involved in the allergic or immediate hypersensitivity response (Shin et al., 2004). After stimulation with antigen, cells release β-hexosaminidase, which is a marker of mast cell degranulation, and 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).

Mast cells also play an important role in initiating and perpetuating the inflammatory response in allergic reactions by secreting abundant amounts of cytokines such as IL-4, IL-5, IL-6, and tumor necrosis factor (TNF)-α (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). IL-4 also acts as a mast cell growth factor *in vitro* and down-modulates high affinity IgE receptor (FcεRI) on mouse bone marrow-derived mast cells (Ryan et al., 1998; Bischoff et al., 1999). Nuclear factor κB (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). 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). Reactive oxygen species (ROS) are also involved in allergic inflammation (Springer et al., 2007; Dharajiya et al., 2007). In addition, activation of signaling pathways in antigen-stimulated mast cells initially depends on the interaction of FcεRI with the Src kinases, Lyn, and on the subsequent downstream activation of Syk and other tyrosine kinases (Gilfillan and Tkaczyk, 2006; Parravicini et al., 2002). Degranulation of mast cells stimulated with IgE is markedly impaired, as is the activity of downstream signaling molecules phos-

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phatidylinositol 3-kinase (PI3-K) and Akt (Fukao et al., 2003). Furthermore, mitogen-activated protein kinase (MAPK) signaling cascades are important in the differentiation, activation, proliferation, degranulation and migration of 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), p38 MAPK, and c-Jun NH_2-terminal kinase (JNK).

Houttuynia cordata (*H. cordata*), known as 'E-Sung-Cho' in Korea, has been used to treat Herpes simplex, chronic sinusitis and nasal polyps (Chiang et al., 2003; Lee et al., 2008; Li et al., 2005). Chemical analysis of this extract identified of methyl nonyl ketone, caryophyllene, bornyl acetate, α-pinene, β-pinene, limonene, and other components. These components have antibacterial properties against the Gram-positive bacteria *Staphylococcus aureus* and *Sarcina ureae* (Lu et al., 2006a). Recently, the adjuvanticity and anticancer, antioxidant, and anti-allergy activities of *H. cordata* water extract (HCWE) were reported (Kim et al., 2007; Lee et al., 2008; Li et al., 2005). However, the precise anti-allergic effect and signaling pathway of HCWE remain unknown. In the present study, we investigated the effects of HCWE on the anaphylactic reaction in mice and on the IgE-mediated allergic response in multiple steps of the FcεRI signaling pathway.

2. Materials and methods

2.1. Materials

Chemicals and cell culture materials were obtained from the following sources: anti-dinitrophenyl (DNP)-IgE and 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide from Sigma–Aldrich Co.; DNP-bovine serum albumin (BSA) from Calbiochem; dichlorodihydrofluorescein diacetate (DCFHDA) from Molecular Probes (Eugene, OR, USA); 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; protein assay kit from Bio-Rad Laboratories, Inc.; primary antibodies [anti-IκB-αphospho-IκB-α (Ser32/36), anti-MAPK (Erkl/2)/phospho-MAPK (Erkl/2) (Tyr202/204), anti-p38 MAPK/phospho-p38 MAPK (Tyr180/182), anti-SAPK/JNK/phospho-SAPK/JNK1/2 (Tyr183/185), anti-Syk/phospho-Syk (Tyr525/526), anti-Lyn/phospho-Lyn (Tyr507), anti-Syk/phospho-Syk, anti-PLCγ2/phospho-PLCγ2 (Tyr1217), anti-Gab2/phospho-Gab2 (Tyr452), and anti-Akt/phospho-Akt (Ser473)] and secondary antibodies (HRP-linked anti-rabbit and anti-mouse IgG) from Cell Signaling Technology; β-actin from Santa Cruz Biotechnology, Inc.; 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. Plant material

HCWE, the aqueous extract of *H. cordata*, was supplied by sancheong-gun, gyeongsangnam-do, South Korea. HCWE was prepared as follows. Distilled water at 70 °C was added to dry *H. cordata* and the temperature was maintained for 5 h. The mixture was allowed to cool to room temperature, filtered, and lyophilized. The yield of lyophilized residue corresponded to 33.5% of the original dry *H. cordata* weight. The extract powder was dissolved in distilled water.

2.3. Animals

Specific pathogen-free ICR mice (female, 8 to 10 weeks old) were purchased from Dae Han Laboratory Animal Research 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

Mice were injected with anti-DNP IgE diluted in PBS intradermally in both ears with a 0.3-ml insulin syringe. One day later, mice were injected i.v. with DNP-BSA in 200 μl of PBS with 0.5% Evans blue. HCWE (0.5–3 g/kg BW) was administered orally 1 h before the challenge. Thirty minutes after the challenge, both ears were harvested and incubated at 80 °C in 1 ml of formamide for 2 h. The mixture was homogenized and centrifuged at 20,800*g* for 10 min. 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 (EMEM) supplemented with 10% fetal bovine serum (FBS), 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. HCWE 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, IgE-sensitized RBL-2H3 cells (5×10^5 cell/well) in 10% FBS-EMEM were seeded into 96-well plates. After 24 h, various concentrations of HCWE (1–120 μg/ml) were added to the wells and the plates were incubated at 37 °C. After cells were treat WST-1 assay kit. Relative cytotoxicity was quantified by measuring the absorbance at 550 nm using a spectrofluorometer (Varioskan, Thermo Electron Co.). HCWE did not interfere at this wavelength.

2.7. β-hexosaminidase release assay

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 with HCWE (1–20 μg/ml) and DNP-BSA in Tyrode's buffer (Pierini et al., 1997). 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. The cells were lysed with 0.1% Triton-X 100 prior to removing the supernatant for measurement of total 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 the substrate in buffer alone (no cell supernatant) was subtracted from all readings.

2.8. Histamine release assay

IgE-sensitized RBL-2H3 cells were pre-incubated with HCWE (1–20 μg/ml) 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. Reactive oxygen species measurement

The amount of intracellular reactive oxygen species (ROS) was measured using non-fluorescent 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA). As a fluorogenic, permeable tracer, H_2DCF-DA is specific for ROS assessment. It is deacetylated by intracellular esterases to non-fluorescent 2′,7′-dichlorodihydrofluorescein (DCFH), which is oxidized to the fluorescent compound 2′,7′-dichlorodihydrofluorescein (DCF) by ROS. IgE-sensitized RBL-2H3 cells were pre-incubated with 5 μM H_2DCF-DA for 30 min at 37 °C and then washed to remove excess H_2DCF-DA. IgE-sensitized RBL-2H3 cells were treated with HCWE (1–20 μg/ml) and/or DNP-BSA for 30 min. Finally, fluorescence intensity was measured at an excitation of 485 nm and an emission of 530 nm using a spectrofluorometer (Varioskan, Thermo Electron Co.). The values were calculated as relative intensity of DCF fluorescence compared to control.

2.10. Transient transfection and luciferase activity assay

For transient transfections, cells were seeded at 1×10^6 cell/well in a 48-well plate. The cells were then transfected with expression vector containing the NF-κB luciferase reporter construct (pNF-κB-LUC plasmid containing NF-κB binding site; Stratagene, Grand Island, NY) or with empty vector using 0.5 μl of serum- and antibiotic-free LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA). After 4 h, the medium was replaced with basal medium. IgE-sensitized RBL-2H3 cells were pretreated with HCWE (1–20 μg/ml) for 30 min, then treated with DNP-BSA 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.

2.11. Measurement of cytokine production

For cytokine immunoassays, IgE-sensitized RBL-2H3 cells (1×10^6 cell/well in 48-well plates) were treated with HCWE (1–20 μg/ml) and/or DNP-BSA for 3 h and 24 h. Supernatants were harvested 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.

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

IgE-sensitized RBL-2H3 cells were pretreated with HCWE (1–20 μg/ml) for 30 min and then treated with DNP-BSA for 3 h. Total RNA from the treated cells was prepared with RNAiso Reagent (Takara) according to the manufacturer's protocol and stored at −80 °C until use. For detection of cytokines, including TNF-α and IL-4, total RNA 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.

2.13. Subcellular fractionation

Cytosolic and nuclear fractions were prepared as described previously (Kabouridis et al., 1997). In brief, cells were pretreated with HCWE (1–20 μg/ml) for 30 min and then treated with DNP-BSA 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 480*g* for 5 min at 4 °C. Soluble and particulate fractions were separated by centrifugation at 100,000*g* for 30 min.

2.14. Western blotting

IgE-sensitized RBL-2H3 cells were pretreated with HCWE (1–20 μg/ml) for 30 min and then treated with DNP-BSA for 5 min (Syk, Lyn, LAT, Gab2, PLCγ2, Akt and MAPK) or 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 and then with horseradish peroxidase-conjugated secondary antibody to IgG. 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.

2.15. 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. HCWE suppresses IgE-mediated passive cutaneous anaphylaxis in mice

PCA is one of the most frequently used models for evaluating anti-allergic drugs (Kabu et al., 2006). To investigate the anti-allergic activity of HCWE *in vivo*, we evaluated the ability of HCWE to inhibit the passive cutaneous anaphylaxis (PCA) reaction in mice. PCA was induced through local injection of DNP-IgE in the mouse ear and then systemic injection of antigen (DNP-BSA). When mice were administered HCWE at concentrations ranging from 0.5 to 3 g/kg orally for 1 h, the ear-swelling response derived from DNP-BSA was significantly reduced in a dose-dependent manner (Fig. 1). The results suggest that HCWE has potential as an allergy therapeutic.

3.2. HCWE inhibits antigen-induced degranulation and histamine release in IgE-sensitized mast cells

To investigate the inhibitory mechanism of HCWE on PCA, we observed the IgE-mediated mast cell activation. Initially, we measured the cytotoxicity of HCWE in RBL-2H3 cells using the WST-1 assay. Dose-dependent cytotoxic effects of HCWE against RBL-2H3 cells are shown in Fig. 2A. HCWE at concentrations ranging from 1–20 μg/ml did not significantly affect the cytotoxicity for 24 h (Fig. 2A). Thus, we treated DNP-IgE-sensitized RBL-2H3 mast cells with HCWE ranging from 1–20 μg/ml in subsequent experiments. 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). The secretion of β-hexosaminidase, known as a degranulation marker, is the hallmark of an allergic reaction resulting from allergen exposure. We therefore examined the effects of HCWE on β-hexosaminidase. IgE-sensitized RBL-2H3 cells were pre-incubated with HCWE (1–20 μg/ml) for 30 min and then incubated with antigen (DNP-BSA; 50 ng/ml) for 15 min. DNP-BSA induced degranulation in IgE-sensitized RBL-2H3 cells (Fig. 2B), and HCWE significantly suppressed antigen-induced degranulation in a dose-dependent manner (Fig. 2B).

The release of chemical mediators such as histamine plays an important role in allergic reaction (Marchand et al., 2003). We further examined the effects of HCWE on histamine release. DNP-IgE sensitized RBL-2H3 cells were pre-incubated with HCWE (1–20 μg/ml) for 30 min and then incubated with DNP-BSA for 15 min. Fig. 2C shows the inhibitory effect of HCWE on DNP-BSA-induced

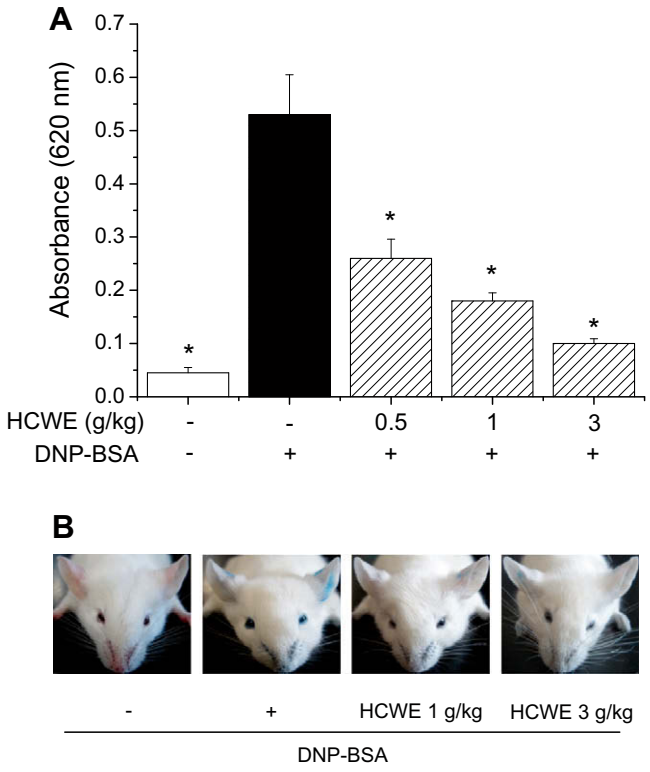

Fig. 1. Effects of HCWE on IgE-mediated PCA in mice. Anti-DNP-specific IgE (0.5 μg) was intradermally injected into the mouse ear. One day later, DNP-BSA in 200 μl of PBS containing 0.5% Evans blue was injected i.v. HCWE was administered orally 1 h before antigen (DNP-BSA) administration. The mice were 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.

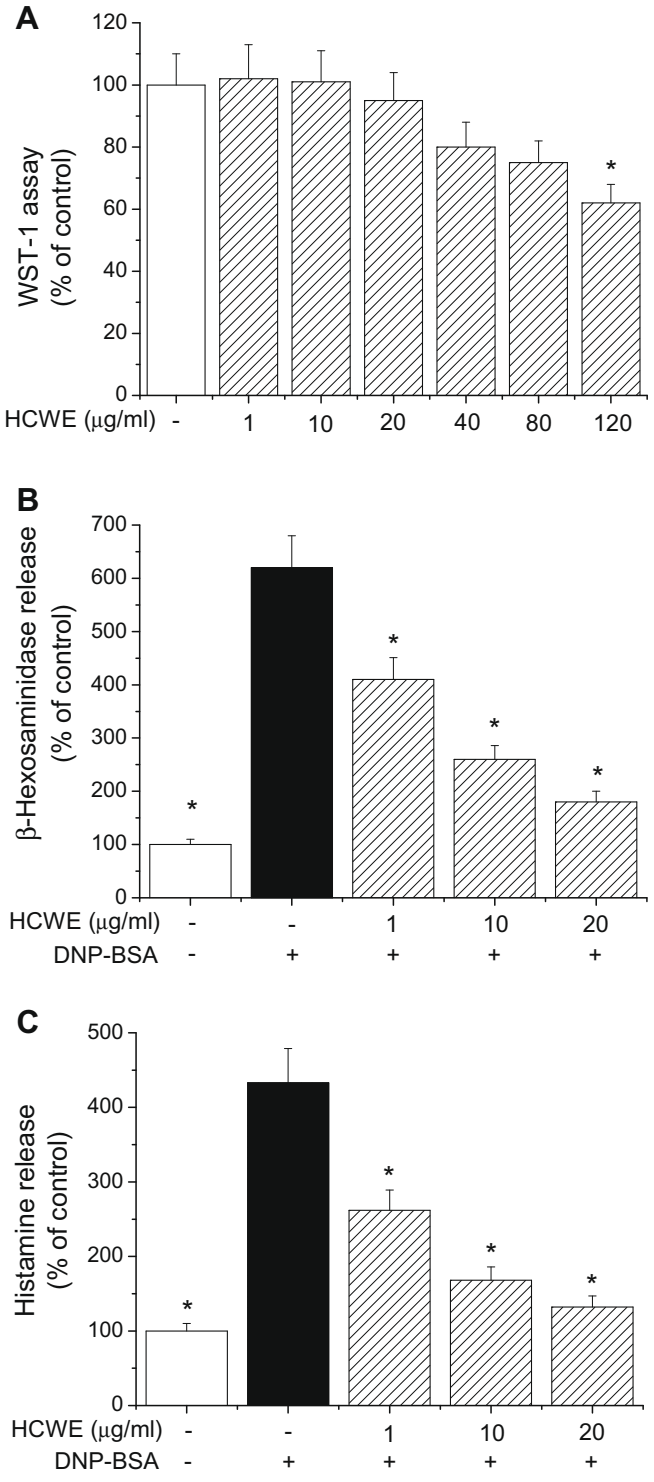

Fig. 2. Effects of HCWE on antigen-induced degranulation and histamine release in RBL-2H3 cells. (A) Effects of HCWE on cytotoxicity. The cells were seeded in a 96-well plate and treated with various concentrations of HCWE for 24 h. Cell viability was assessed using WST-1 assays. Each bar shows the mean ± S.D. of three independent experiments. $^{*}P < 0.01$, significantly different from control. (B) 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's buffer that contained the indicated concentrations of HCWE before stimulation with 50 ng/ml of DNP-BSA for 10 min in order to measure the release of β-hexosaminidase. Each bar shows the mean ± S.D. of three independent experiments. $^{*}P < 0.01$, significantly different from DNP-BSA alone. (C) Same as (B) 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.

release of histamine in IgE-sensitized RBL-2H3 cells. The results suggest that HCWE significantly inhibits antigen-induced mast cell degranulation and histamine release.

3.3. HCWE inhibits antigen-induced production and expression of TNF-α and IL-4 in IgE-sensitized mast 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). Mast cell-derived TNF-α is probably of particular importance in causing allergic inflammation. 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 HCWE suppressed the expression and secretion of IL-4 and TNF-α in IgE-sensitized RBL-2H3 cells. Cells were treated with HCWE (1–20 μg/ml) and/or DNP-BSA for 3 h (TNF-α) and 24 h (IL-4). HCWE significantly inhibited the antigen-induced IL-4 secretion and TNF-α production in a dose-dependent manner (Fig. 3A). We further tested whether HCWE suppressed expression of IL-4 and TNF-α mRNA in the cells. IgE-sensitized RBL-2H3 cells were treated with HCWE (1–20 μg/ml) and/or DNP-BSA for 3 h. In agreement with the ELISA results, HCWE inhibited antigen-induced mRNA expression of both cytokines in a dose-dependent manner (Fig. 3B). The results suggest that HCWE significantly inhibits the antigen-induced gene expression and production of cytokines related to the allergic reaction.

3.4. HCWE inhibits antigen-induced activation of NF-B and production of ROS in IgE-sensitized mast cells

To evaluate the mechanisms of the effects of HCWE on cytokine secretion, we examined the effect of HCWE on NF-κB activation using a NF-κB-luciferase reporter vector. NF-κB is thought to play an important role in the regulation of proinflammatory molecules in cellular responses, especially TNF-α, IL-6, and IL-8 (Salamon et al., 2005). HCWE markedly suppressed antigen induced NF-B-luciferase activity in IgE-sensitized RBL-2H3 cells (Fig. 4A). Unstimulated NF-κB is bound to IκB-α in the cytoplasm. Phosphorylation of IκB-α, which regulates NF-κB translocation, was somewhat decreased by HCWE in IgE-sensitized RBL-2H3 cells (Fig. 4B). IκB-α was rapidly degraded when IgE-sensitized RBL-2H3 cells were treated with HCWE (Fig. 4B). Anti-β-actin antibodies were used as quantitative controls (Fig. 4B).

Mast cells secrete chemical mediators such as histamine, prostaglandin, reactive oxygen species (ROS) and cytokines during specific immune responses (Yoshimaru et al., 2002; Matsui et al., 2000). RBL-2H3 cells release ROS in response to various stimuli (Matsui et al., 2000). Thus, we tested whether HCWE suppressed production of ROS in IgE-sensitized RBL-2H3 cells. Antigen stimulation resulted in ROS production (Fig. 4C) and, as expected, HCWE inhibited ROS production from IgE-sensitized RBL-2H3 cells (Fig. 4C). These results indicate that NF-κB and ROS were responsible for allergic reaction and that HCWE has potential as an allergy therapeutic.

3.5. HCWE inhibits antigen-induced FcεRI-mediated signaling events in IgE-sensitized mast cells

To gain insight as to how HCWE suppresses mast cell activation, we examined its effects on FcεRI-mediated signaling events, namely the activating phosphorylation of Syk by Lyn and the phosphorylation of LAT and Gab2 by Syk. As shown in Fig. 5A, antigen-induced tyrosine phosphorylation of Syk was inhibited by HCWE in

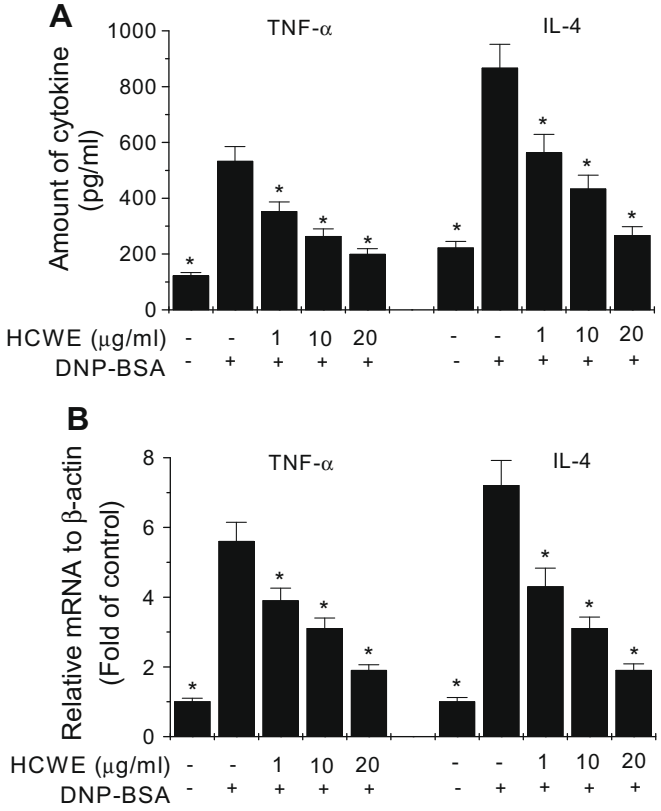

Fig. 3. Effects of HCWE on antigen-induced expression and secretion of TNF-α and IL-4 in RBL-2H3 cells. (A) The IgE-primed cells were stimulated with 50 ng/mL of DNP-BSA for 4 h or were left unstimulated with or without HCWE. The concentrations of TNF-α and IL-4 released into the culture media were assessed 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 HCWE 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.

IgE-sensitized RBL-2H3 cells. The Syk-dependent downstream activating phosphorylations of LAT and Gab2 were also significantly inhibited by HCWE in a dose-dependent manner (Fig. 5A). The phosphorylation and activation of phospholipase C (PLC) γ, as well as calcium mobilization, are dependent on the phosphorylation of Syk and LAT (Rivera and Gilfillan, 2006). Indeed, antigen-induced phosphorylation of PLCγ2 was inhibited by HCWE in a concentration-dependent manner (Fig. 5A). We also examined the effects of HCWE 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 antigen-induced phosphorylation of Akt, an indicator of PI3-K activation, and the phosphorylation of the MAP kinases Erk1/2 and JNK1/2 were also significantly suppressed by HCWE in a dose-dependent manner (Fig. 5B). However, DNP-BSA-induced p38 MAP kinase activation was not affected by HCWE. These results indicate that HCWE inhibits antigen-induced FcεRI-mediated signaling events in IgE-sensitized mast cells.

4. Discussion

H. cordata, known as 'E-Sung-Cho' in Korea, has been used to treat Herpes simplex, chronic sinusitis and nasal polyps (Chiang

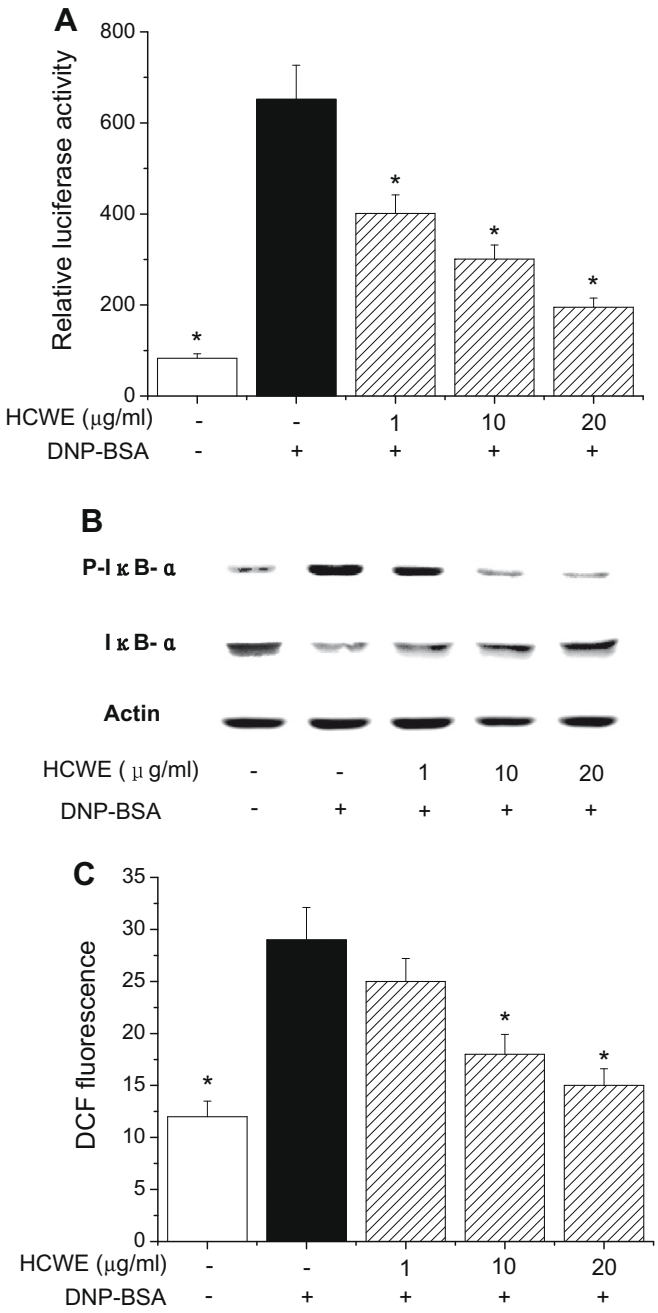

Fig. 4. Effects of HCWE on antigen-induced activation of NF-κB and production of ROS in RBL-2H3 cells. (A) Cells were transiently co-transfected with pGL3-NF-κB-Luc and pCMV-β-gal. After 4 h, the cells were sensitized with DNP-specific IgE (100 ng/ml) for 16 h. The IgE-sensitized cells were treated with HCWE 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 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) HCWE inhibits the antigen-induced phosphorylation of IκB-α. IgE-sensitized cells pretreated with HCWE 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. (C) HCWE inhibits antigen-induced ROS production in the cells. The amount of intracellular reactive oxygen species (ROS) was measured using non-fluorescent 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA). IgE-sensitized RBL-2H3 cells were pre-incubated with 5 μM H_2DCF-DA for 30 min at 37 °C and then washed. Cells were treated with HCWE (1–20 μg/ml) and/or DNP-BSA for 30 min. Finally, fluorescence intensity was measured at an excitation of 485 nm and an emission of 530 nm using a spectrofluorometer (Varioskan, Thermo Electron Co.). Each bar shows the mean ± S.D. of three independent experiments. $^{*}P < 0.01$, significantly different from DNP-BSA alone.

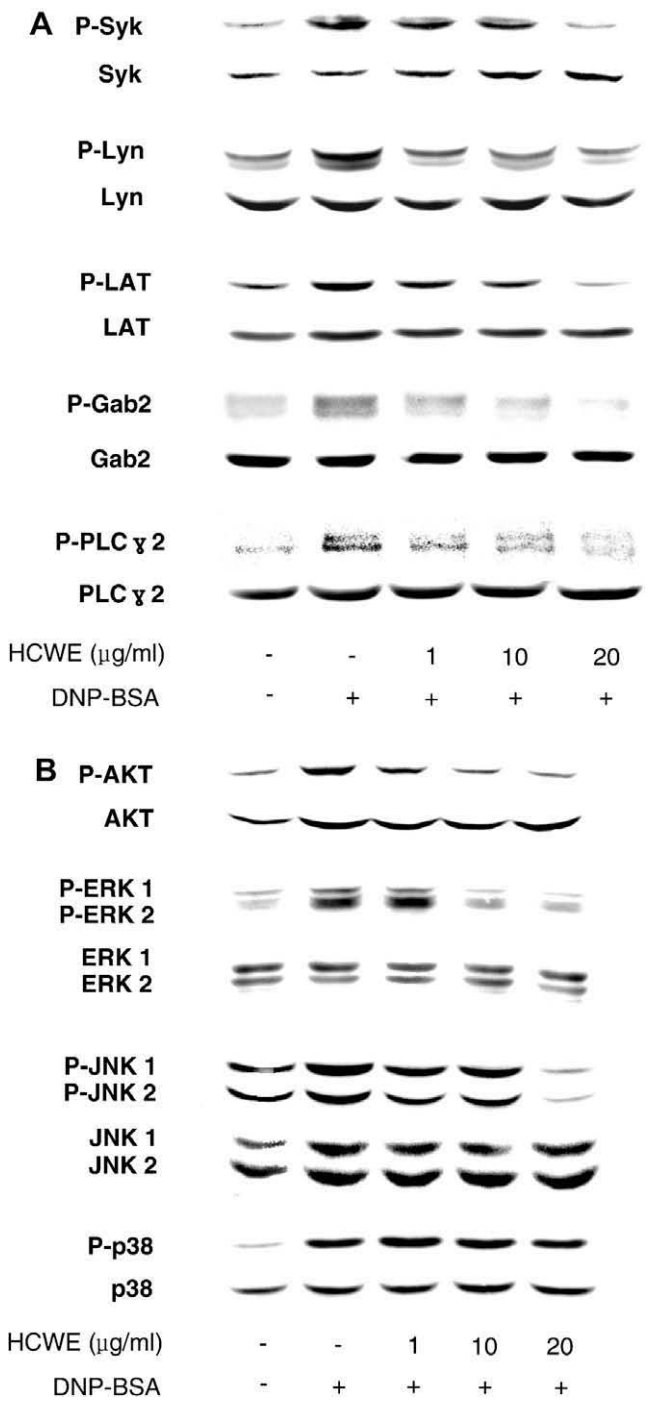

Fig. 5. Effects of HCWE on antigen-induced FcεRI-mediated signaling events and phosphorylation of Akt and MAPK in RBL-2H3 cells. (A) IgE-sensitized cells were treated with HCWE and stimulated with DNP-BSA (50 ng/ml) for 5 min. The extracts were analyzed for Syk, Lyn, LAT, Gab2, and PLC γ2 activation by western blot analysis using antibodies against phosphorylated Lyn, LAT, Gab2, and PLC γ2. (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.

et al., 2003; Lee et al., 2008; Li et al., 2005). Recently, several studies also provided scientific data to support its anti-inflammatory (Park et al., 2005; Lu et al., 2006b), anti-allergic (Li et al., 2005; Kim et al., 2007), virucidal (Hayashi et al., 1995; Chiang et al., 2003), anti-oxidative (Chen et al., 2003; Cho et al., 2003; McDermott et al., 2007) and anti-cancer (Chang et al., 2001; Kim et al., 2001) activities. In this study, we investigated the effects of *H. cordata* water extract (HCWE) on IgE-mediated passive cutaneous anaphylaxis (PCA) in mice and its anti-allergic effects in rat basophilic leukemia RBL-2H3 cells.

We first examined the anti-allergic activity of HCWE in PCA in mouse ear. Because the number of allergic patients is increasing worldwide, many investigators have concentrated on finding effective therapeutics for allergic inflammation, using well-established mast cell-dependent experimental model systems. For instance, PCA, animal model of the IgE-mediated immediate allergic reaction, is induced by mediators such as histamine that are secreted from mast cells (Kemp and Lockey, 2002; Kim et al., 1999). As shown in Fig. 1, when mice were administered oral HCWE, the ear-swelling response derived from antigen (DNP-BSA) was significantly reduced in a dose-dependent manner. This result suggests that HCWE might be useful in treating 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 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). The release of chemical mediators such as histamine plays an important role in allergic inflammation (Marchand et al., 2003). HCWE significantly suppressed antigen-induced degranulation and histamine release in IgE-sensitized RBL-2H3 cells (Fig. 2B and C). These results also suggest potential value for HCWE 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 HCWE suppressed antigen-induced gene expression and secretion of IL-4 and TNF-α in IgE-sensitized RBL-2H3 cells. HCWE significantly inhibited the antigen-stimulated secretion and gene expression of IL-4 and the TNF-α production (Fig. 3A and B). This result suggests that the anti-allergic effect of HCWE is a result of its reduction of IL-4 and TNF-α production in mast cells. To evaluate the mechanism of the effect of HCWE on cytokine secretion, we examined the effect of HCWE on NF-κB activation. HCWE markedly suppressed antigen-induced NF-κB-luciferase activity (Fig. 4A). Moreover, phosphorylation of IκB-κ, which regulates NF-κB translocation, was somewhat decreased by HCWE in IgE-sensitized RBL-2H3 cells (Fig. 4B). These data demonstrate that HCWE attenuates NF-κB activation and downstream TNF-α and IL-4 production. Stimulation of IgE-sensitized mast cells with specific antigen triggers a cascade of events leading to degranulation, mediator release, MAPK activation, tyrosine kinase and phospholipase C activation, increased reactive oxygen species (ROS), calcium influx, and cytokine production (Reth, 1989; Cambier, 1995). ROS are known to be involved in allergic inflammation (Springer et al., 2007; Dharajiya et al., 2007). Thus, we tested whether HCWE suppressed production of ROS in IgE-sensitized RBL-2H3 cells. Antigen stimulation resulted in ROS production

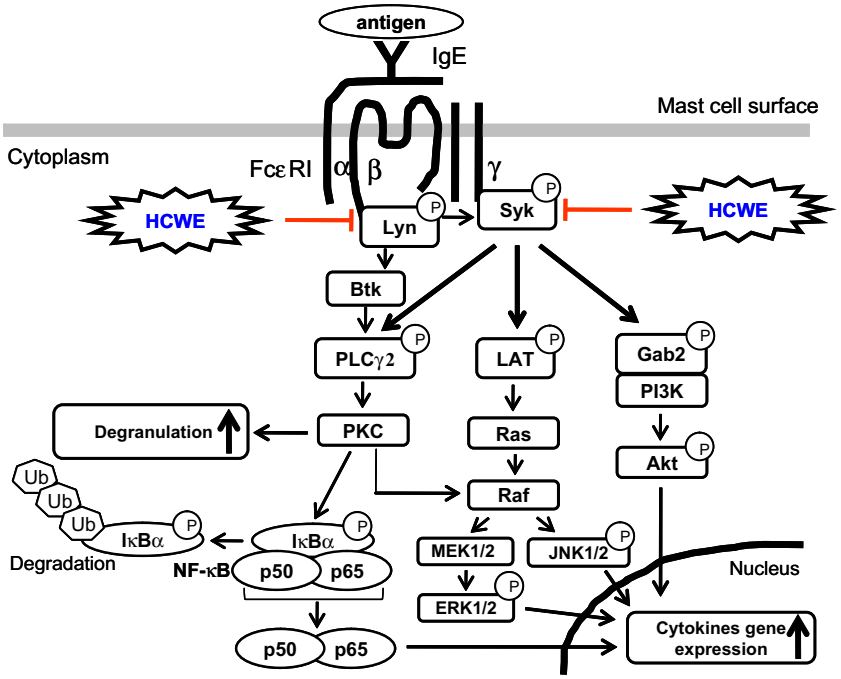

Fig. 6. A scheme showing the FcεRI signal transduction by HCWE in RBL-2H3 cells. Antigen-mediated aggregation of FcεRI on mast cells leads to transphosphorylation of ITAMs by Src family protein tyrosine kinase Lyn. The protein tyrosine kinase Syk was recruited to the phosphorylated ITAMs through its SH2 domains. Activated-Syk leads to tyrosine phosphorylation of other proteins such as LAT, PLCγ2, Gab2, and so on. These initial interactions finally lead to degranulation and cytokine gene transcription. Furthermore, it activates PKC, which selectively controls IKK activation and subsequent IκB-α degradation and NF-κB activation for cytokine gene transcription. HCWE significantly suppressed FcεRI-mediated signaling, such as Syk and Lyn and NF-κB activation, resulting in anti-allergic effect.

(Fig. 4C) and, as expected, HCWE inhibited ROS production from DNP-IgE sensitized RBL-2H3 cells (Fig. 4C). These results indicate that ROS were responsible for allergic reaction and that HCWE has potential allergy therapeutic effects.

The activation of signaling pathways in antigen-stimulated mast cells depends initially on the interaction of FcεRI with the Src kinases, Lyn and Fyn, and subsequently on the downstream activation of Syk and other tyrosine kinases (Gilfillan and Tkaczyk, 2006; Parravicini et al., 2002). When IgE-antigen binds to FcεRI, the FcεRI is activated, resulting in the release of biologically active mediators that cause several allergic reactions such as degranulation and expression and secretion of cytokines (Siraganian et al., 2002). Antigen-mediated aggregation of FcεRI on mast cells leads to transphosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) by Src family protein tyrosine kinase (PTK) Lyn. The protein tyrosine kinase Syk was recruited to the phosphorylated ITAMs through its tandem Src homology 2 region (SH2) domains. Activated-Syk leads to tyrosine phosphorylation of other proteins such as LAT, PLCγ2, Gab2, and so on. These initial interactions finally lead to degranulation and cytokine gene transcription. It was reported that PKC and calcium are related to the activation of ERK1/2, JNK, and p38 MAP kinase (Gilfillan and Tkaczyk, 2006; Church and Levi-Schaffer, 1997; Metcalfe et al., 1981). Moreover, the activated Syk also regulates small GTPases such as Rac, Ras, and Rho. These signaling also activate downstream factors such as ERK, JNK, and p38 MAP kinase, which were associated with cytokine gene transcription. From the above-mentioned, the Syk/PLCcs/PKC pathway coupled with Ca2 + influx plays an important role in degranulation. Furthermore, it activates PKC, which selectively controls IKK activation and subsequent IκB-α degradation and NF-κB activation for cytokine gene transcription. To gain insight as to how HCWE suppresses mast cell activation, we examined its effects on early FcεRI-mediated signaling events, namely the activating phosphorylation of Syk by Lyn and the phosphorylation of LAT and Gab2 by Syk. HCWE suppressed antigen-induced phosphorylation of Syk, Lyn, LAT, Gab2, and PLC γ2 (Fig. 5A). Further downstream, antigen-induced phosphorylation of Akt and MAP kinases (ERK1/2 and JNK1/2 but not p38 MAP kinase) were also inhibited by HCWE (Fig. 5B). As shown in Fig. 6, HCWE significantly suppressed FcεRI-mediated signaling, such as Syk and Lyn and NF-κB activation, resulting in anti-allergic effect.

There are many approaches for treating allergic diseases, such as allergen-specific immunotherapy, DNA vaccination, administration of humanized anti-IgE antibody, treatment with soluble IL-4 receptor, and treatment with leukotriene and histamine receptor antagonists. Novel approaches are being explored to develop new therapies, including blocking mast cell activation with tyrosine kinase inhibitors (Luskova and Draber, 2004). Also, complementary and alternative medicines provide a promising avenue for the development of new therapies. In conclusion, this study is the first report indicating that HCWE inhibits degranulation, cytokine secretion, and FcεRI-mediated signaling activation in antigen-stimulated RBL-2H3 mast cells and reduces mast cell-mediated PCA in mice. These results suggest that HCWE exhibits anti-allergic activity by inhibiting NF-κB- and FcεRI-mediated signaling in mast cells.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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