JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Antiallergic Activity of Novel Isoflavone Methyl-glycosides from *Cordyceps militaris* Grown on Germinated Soybeans in Antigen-Stimulated Mast Cells

Dong Ki Park,[†,‡] Wahn Soo Choi,[§] and Hye-Jin Park*[,†,§]

[†]Department of Bioscience and Biotechnology, Konkuk University, Seoul 143-701, Republic of Korea

[§]Department of Immunology and Physiology, College of Medicine, Konkuk University, 322 Danwol-dong, Chungju, Republic of Korea

[‡]Cell Activation Research Institute, Seoul 143-701, Republic of Korea

ABSTRACT: Isoflavones are known to possess immunomodulating and antiallergic activities. Previously we identified novel isoflavone methyl-glycosides (daidzein 7-*O*-*β*-D-glucoside 4″-*O*-methylate (CDGM), glycitein 7-*O*-*β*-D-glucoside 4″-*O*-methylate (CGLM), genistein 7-*O*-*β*-D-glucoside 4″-*O*-methylate (CGNMI) and genistein 4′-*O*-*β*-D-glucoside 4″-*O*-methylate (CGNMII)) from *Cordyceps militaris* grown on germinated soybeans (GSC). The biological activity of novel isoflavone methyl-glycosides, however, remains unknown. In this study, CGNMII showed the strongest inhibition of degranulation. Additionally, the release of interleukin (IL)-4 and tumor necrosis factor (TNF)-α was decreased by CGNMII in antigen-stimulated RBL-2H3 cells. To elucidate the antiallergic mechanism of CGNMII, we examined whether it affected levels of signaling molecules responsible for degranulation. The levels of activated Lyn, Syk, PLCγ1 and LAT proteins were reduced in CGNMII treated RBL-2H3 cells. CGNMII also inhibited the activation of AKT and ERK1/2 proteins. These results suggest that CGNMII might be used as a therapeutic agent for allergic diseases.

KEYWORDS: *IgE-mediated allergic diseases, mast cell, isoflavone, Syk kinase, degranulation*

INTRODUCTION

The prevalence of type I hypersensitivity allergic diseases has been increased worldwide for several decades.[1] Mast cells and basophils are the key effector cells in IgE-mediated allergic diseases, including atopic dermatitis, allergic rhinitis, asthma and atopic eczema.[2] Binding of antigen to the high affinity IgE receptor (FcεRI) on the surface of mast cells or basophils induces the release of inflammatory mediators such as histamine, leukotriene C4, *β*-hexosaminidase, chemokines and cytokines that are responsible for allergic diseases.[3] When granules in mast cells are degranulated after antigen stimulation, *β*-hexosaminidase is released along with histamine. Histamine is one of the major chemical mediators, responsible the amplification of allergic symptoms and maintaining chronic inflammatory conditions.[4] Thus, the activity of *β*-hexosaminidase is used as a marker for degranulation. Antigen binding to IgE-sensitized mast cells or basophils promotes the activation of transcription factors for the production of cytokines, including tumor necrosis factor (TNF-α) and interleukin (IL-4).[5] IL-4 is necessary for Th2 cellular response development and promotes B cells to produce IgE.[6] TNF-α is responsible for allergic inflammation.[7] These cytokines are also major target molecules for antiallergic drugs.

Current approaches to the treatment of allergic diseases are largely based on allergen-specific immunotherapy, DNA vaccination, antihistamine drugs and steroid treatments.[8] Antagonists to leukotrienes (LTs) or histamine receptors are commonly used to treat allergic diseases, but they produce undesirable side effects, including drowsiness, dry mouth, chest congestion, and upset stomach.[9] Recently, many practitioners and researchers are paying attention to natural products as antiallergic drug candidates that are known to modulate immune responses with no adverse effects.[10]

Previously our group developed the method of growing *Cordyceps militaris* on germinated soybeans and identified several active compounds from it.[11,12] We reported the antiallergic activities of ethyl acetate extract of *Cordyceps militaris* grown on germinated soybeans (GSC) in vivo and in vitro.[13] Recently we identified novel isoflavone methyl-glycosides (daidzein 7-*O*-*β*-D-glucoside 4″-*O*-methylate (CDGM), glycitein 7-*O*-*β*-D-glucoside 4″-*O*-methylate (CGLM), genistein 7-*O*-*β*-D-glucoside 4″-*O*-methylate (CGNMI), and genistein 4′-*O*-*β*-D-glucoside 4″-*O*-methylate (CGNMII) from *Cordyceps militaris* grown on germinated soybeans (GSC).[12] Isoflavones are known to have immunomodulating and antioxidant activities[14] and to inhibit allergic inflammatory responses.[15]

In this study, we investigated the antiallergic activity of novel isoflavone methyl-glycosides in antigen-stimulated RBL-2H3 cells. First, the effect of novel isoflavone methyl-glycosides on degranulation in antigen-stimulated RBL-2H3 cells was examined. A further study was focused on whether novel isoflavone methyl-glycosides affected on the production and expression of inflammatory cytokines such as TNF-α and IL-4 and the FcεRI signaling events in antigen-stimulated RBL-2H3 cells.

Received: July 26, 2011
Revised: January 31, 2012
Accepted: February 1, 2012
Published: February 1, 2012

 © 2012 American Chemical Society

■ MATERIALS AND METHODS

Reagents and Materials. *Cordyceps militaris* grown on germinated soybeans (Kucari 0903) (The Cell Activation Research Institute, Seoul, Korea), daidzein 7-*O*-*β*-D-glucoside 4″-*O*-methylate, glycitein 7-*O*-*β*-D-glucoside 4″-*O*-methylate, genistein 7-*O*-*β*-D-glucoside 4″-*O*-methylate and genistein 4′-*O*-*β*-D-glucoside 4″-*O*-methylate were purified as described in our previous study.[12] Fetal bovine serum (Invitrogen, Carlsbad, CA), penicillin, Minimum essential medium, Eagle (Invitrogen, Carlsbad, CA), Fluo-4 direct calcium kit (Invitrogen, Carlsbad, CA), DNP-specific IgE (Sigma-Aldrich, St. Louis, MO), DNP-BSA (Sigma-Aldrich, St. Louis, MO), PIPES (Sigma-Aldrich, St. Louis, MO), PP2 (Calbiochem, La Jolla, CA), phospho-Lyn antibody (Cell Signaling Technology Inc., Danvers, MA), phospho-Syk antibody (Cell Signaling Technology Inc., Danvers, MA), anti-phospho-PLC*γ* antibody (Santa Cruz, CA), antiphosphorylated linker for activation of T cells (LAT) antibody (Cell Signaling Technology Inc., Danvers, MA), anti-LAT antibody (Upstate, New York, USA), phospho-AKT antibody (Cell Signaling Technology Inc., Danvers, MA), phospho-p44/42 MAPK antibody (Erk1/2) (Cell Signaling Technology Inc., Danvers, MA), p44/42 MAPK (Erk1/2) antibody (Cell Signaling Technology Inc., Danvers, MA) and *β*-actin antibody (Santa Cruz, CA). Phosphorylated-Lyn and AKT were kind gifts of Dr. Choi W. S. (Konkuk University, Seoul, Korea).

Isolation of Novel Isoflavone Methyl-glycosides from *Cordyceps militaris* Grown on Germinated Soybeans. Isolation of novel isoflavone methyl-glycosides from *Cordyceps militaris* grown on germinated soybeans was done as previously described.[12]

Isolation and Culture of Bone Marrow-Derived Mast Cells (BMMCs) and Rat Basophilic Leukemia (RBL)-2H3 Cells. BMMCs were isolated according to a previous report with a minor modification. Briefly, BMMCs isolated from male BALB/c mice were cultured in a medium (RPMI 1640, containing 2 mM L-glutamine, 0.1 mM nonessential amino acids, antibiotics, and 10% fetal bovine serum (FBS)) containing 10 ng/mL of IL-3. The BMMCs were used 3−4 weeks after their isolation. RBL-2H3 cells were grown as monolayers in minimum essential medium (MEM) with Earle's salts, supplemented with 15% FBS and 1% penicillin. The cells were grown in 75 cm^2 culture flasks at 37 °C with 5% CO_2 at humidified atmospheric pressure.

Cell Viability Assay. Cytotoxicity was measured using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan), as described previously.[16] Cells (1×10^4/mL) were treated in the presence or absence of CGNMII, genistein and genistin (1, 3, 10, and 30 *μ*M) for 24 h. The cultures in 96-well plates were placed in 10 *μ*L of medium that contained CCK-8 and incubated for 2 h at 37 °C. The absorbance was measured with a microplate reader at 450 nm (Tecan, Mannedorf, Switzerland).

***β*-Hexosaminidase Assay.** RBL-2H3 cells (1×10^5 cells/well) were sensitized with 25 ng/mL of DNP-specific IgE overnight. After washing with PIPES buffer (25 mM PIPES (Sigma-Aldrich, St. Louis, MO), pH 7.2, 158 mM NaCl, 5 mM KCl (Daejung), 0.4 mM $MgCl_2$ (Origental chemical industry), 1 mM $CaCl_2$, 5.6 mM glucose, and 0.1% fatty acid free fraction V from a bovine serum (ACROS, New Jersey), cells were treated in the presence or absence of CGNMII, genistein and genistin (1, 3, 10, and 30 *μ*M) for 30 min at 37 °C. After incubation, cells were challenged with 25 ng/mL of DNP-BSA for 10 min at 37 °C. The 30 *μ*L of the supernatant was mixed with an equal volume of 2 mM substrate solution (*p*-nitrophenyl-*N*-acetyl-*β*-D-glucosaminide in 0.1 M citrate, pH 4.5 (Sigma-Aldrich, St. Louis, MO)) and then incubated for 1 h at 37 °C. The reaction was terminated by adding 250 *μ*L of stopping buffer (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0 (Sigma-Aldrich, St. Louis, MO)). The absorbance was measured with a microplate reader at 405 nm (Tecan, Männedorf, Switzerland).

Measurement of Intracellular [Ca^{2+}]i Concentration. The intracellular [Ca^{2+}]i level was determined with a Fluo-4 direct calcium kit (Invitrogen, Kumamoto, Japan). RBL-2H3 cells (5×10^4 cells/well) were seeded into poly-D-lysine coated plate 96-well microplates and incubated for 12 h. After incubation, cells were stimulated with 20 ng/mL mouse monoclonal anti-DNP IgE, and then the cells were incubated for 12 h. The IgE-sensitized cells were washed twice with PIPES buffer and incubated with 100 *μ*L of loading buffer containing Fluo-4 (Fluo-4 Direct Calcium Kit) for 30 min. Cells were incubated in the presence or absence of CGNMII (1, 3, 10, and 30 *μ*M) for 30 min. Then, cells were stimulated by DNP-BSA (200 ng/mL) or nonstimulated. Intracellular [Ca^{2+}]i was visualized by brightfield and fluorescence microscopy on an inverted microscope (Carl Zeiss, Chicago, IL, USA) using a filter (excitation = 494 nm, emission = 516 nm). To evaluate the concentration of intracellular calcium quantitatively, we assayed intracellular calcium levels by ELISA analysis. The fluorescent intensity was recorded using a fluorescent plate reader (excitation = 494 nm, emission = 516 nm) (Molecular Devices, Sunnyvale, CA).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from the RBL-2H3 cells using RNA-Bee Reagent (Tel test, Friendwood, TX, USA) and reverse transcribed using Revertra Ace qPCR RT kit (Toyobo Biologics Inc., Osaka, Japan) as described previously (Lee et al., 2008). The polymerase chain reaction was performed at 94 °C for 2 min, at 94 °C for 30 s, at 55 °C for 30 s and at 68 °C for 1 min for 30 cycles. The following primers were used: rat TNF-*α* forward 5-CACCACGCTCTTCTGTCTACTGAAC-3; rat TNF-*α* reverse 5-CCGGACTCCGTGATG-TCTAAGTACT-3′; rat IL-4 forward 5′-ACCTTGCTGTCACCCTGTTC-3′; rat IL-4 reverse 5′-TTGTGAGCGTGGACTCATTC-3′; rat glyceraldehydes-3-phosphate dehydrogenase forward 5′-CTTCACCACCATGGAGAAGGCTG-3′; rat glyceraldehydes-3-phosphate dehydrogenase reverse 5′-GACCACAGTCCATGCCATCACTG-3′.

Western Blot Analysis. Immunoblotting analysis was performed as described previously.[13] 20 *μ*g aliquots of protein were subjected to electrophoresis on 4−15% gradient polyacrylamide gels and electrophoretically transferred onto polyvinylidene fluoride membranes (Bio-Rad Laboratories, Berkeley, CA, USA). The membranes were incubated in 5% skim milk solution and then with antibodies against phospho-Lyn, phospho-Syk, phospho-linker for activation of T cells (LAT), phospho-AKT, phospho-PLC*γ*, phospho-p44/42 (ERK1/2) antibodies and anti-*β*-actin antibody. The membranes were washed and incubated horseradish peroxidase-conjugated secondary antibody for 1 h. The protein bands were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, U.K.).

Statistical Analysis. Results are expressed as means ± standard error (SE). Student's *t*-test or one-way ANOVA/Dunnett's *t*-test was used to assess significance between controls and treatments. Statistical analysis was performed using SPSS, version 12 (SPSS Inc., Chicago, IL, USA).

■ RESULTS

Identification Novel Isoflavone Methyl-glycosides from *Cordyceps militaris* Grown on Germinated Soybeans. Using LC−ESI-IT-MS/MS, we identified the four major peaks in the ethyl acetate or the butanol fraction of GSC: novel isoflavone methyl-glycosides such as genistein 4′-*O*-*β*-D-glucoside 4″-*O*-methylate (CGNMII), genistein 7-*O*- *β*-D-glucoside 4″-*O*-methylate (CGNMI), daidzein 7-*O*-*β*-D-glucoside 4″-*O*-methylate (CDGM) and glycitein 7-*O*-*β*-D-glucoside 4″-*O*-methylate (CGLM) (Figure 1B). The extraction procedure and the chemical structures of novel isoflavones methyl-glycosides from *Cordyceps militaris* grown on germinated soybeans (GSC) are shown in Figure 1A,C.

Effect of Novel Isoflavones from *Cordyceps militaris* on Degranulation from Antigen-Stimulated RBL-2H3 Cells. Previously our group identified and purified novel isoflavones from GSC.[12] To investigate the antiallergic activity of these compounds, we checked their inhibitory effects on the degranulation of antigen-stimulated RBL-2H3 cells. *β*-Hexosaminidase release was determined as a marker of degranulation.[17] We observed that the inhibitory effect of CGNMII on the degranulation of antigen-stimulated RBL-2H3 cells was stronger than those of CDGM, CGLM and CGNMI (Figure 2A). Therefore, we

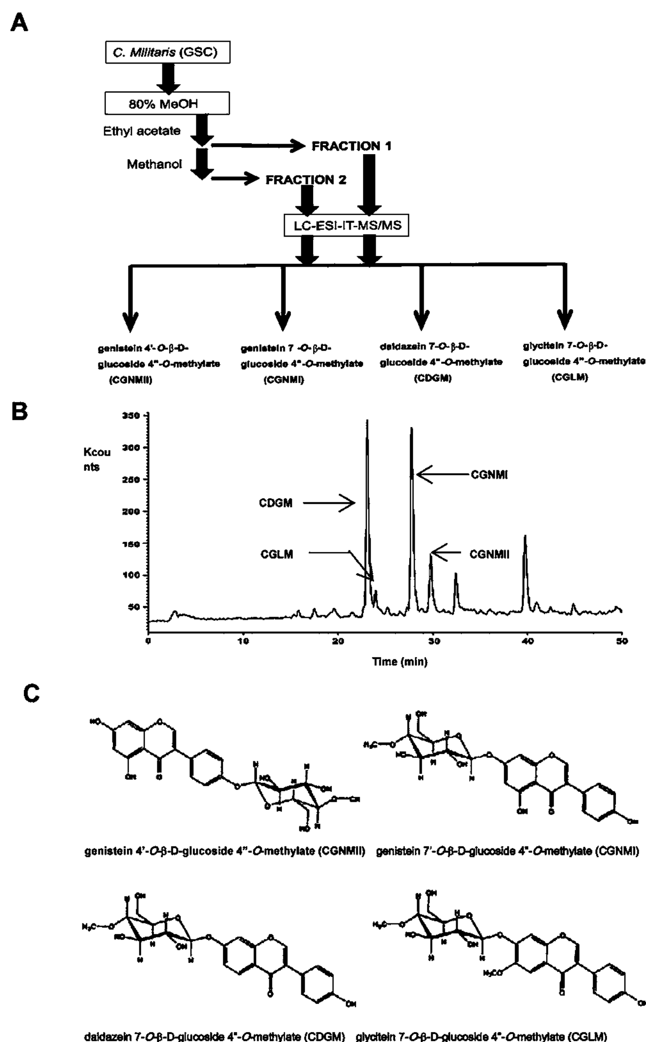

Figure 1. Isolation of novel isoflavone methyl-glycosides from *Cordyceps militaris* grown on germinated soybeans. (A) The extraction procedure of novel isoflavone methyl-glycosides from *Cordyceps militaris* grown on germinated soybeans (GSC). (B) The compounds from GSC were analyzed using LC−ESI-IT-MS/MS as described in Materials and Methods. (C) The chemical structures of novel isoflavone methyl-glycosides from *Cordyceps militaris* grown on germinated soybeans (GSC).

chose CGNMII as the test sample. Genistein and genistin, a major isoflavone in most soy products, are known to modulate immune responses.[11] Since CGNMII shares genistein backbone structure, we compared the inhibitory activity of CGNMII, genistein and genistin on the degranulation of antigen-stimulated RBL-2H3 cells. We observed that β-hexosaminidase release was inhibited in the order CGNMII > genistein > genistin (Figure 2B). We used the CCK-8 assay to access the cytotoxicity of compounds (1, 3, 10, and 30 μM) on RBL-2H3 cells. As shown in Figure 2B, CGNMII did not affect cell viability. But genistin and genistein inhibited cell viability (Figure 2C). We tested whether CGNMII inhibited antigen-induced degranulation using bone marrow-derived mast cells (BMMCs), which express the IgE high-affinity receptor, FcεRI, at equivalent levels to RBL-2H3 cells. CGNMII inhibited degranulation in antigen-stimulated BMMCs in a dose-dependent manner ($^{*}p < 0.01$; $^{**}p < 0.005$) (Figure 2D).

Effect of CGNMII on Inhibition [Ca^{2+}]i in Antigen-Stimulated RBL-2H3 Cells. The levels of intracellular calcium

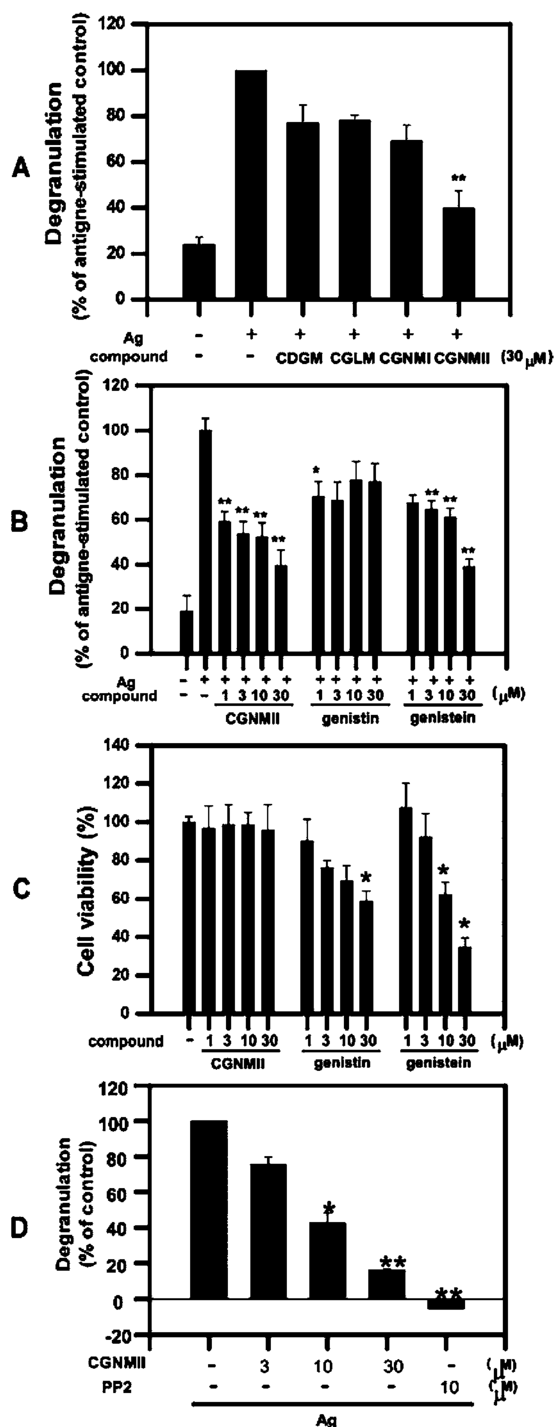

Figure 2. Effect of novel isoflavones from *Cordyceps militaris* on degranulation and cell viability of antigen-stimulated mast cells. (A) The degranulation was determined by measuring β-hexosaminidase release in antigen-stimulated RBL-2H3 cells. The RBL-2H3 cells were treated in the presence or absence of CDGM, CGLM, CGNMI and CGNMII (30 μM) for 30 min and then stimulated for 10 min with antigen. (B) The RBL-2H3 cells were treated in the presence or absence of CGNMII, genistin or genistein (1, 3, 10, and 30 μM) for 30 min and then stimulated for 10 min with DNP-BSA. (C) RBL-2H3 cell viability was measured using the Cell Counting Kit-8 (CCK-8) assay. RBL-2H3 cells (1×10^4 cells/mL) were treated in the presence or absence of CGNMII, genistin or genistein (1, 3, 10, and 30 μM) for 24 h. (D) BMMCs were treated in the presence or absence of CGNMII, genistin or genistein (1, 3, 10, and 30 μM) for 30 min and then stimulated for 10 min with DNP-BSA. Each value represents the mean ± SE of three experiments ($^{*}p < 0.01$; $^{**}p < 0.005$).

dx.doi.org/10.1021/jf205199j | J. Agric. Food Chem. 2012, 60, 2309−2315

in mast cells are critical in degranulation.[22] We checked intracellular [Ca^{2+}]i levels in RBL-2H3 cells after CGNMII treatment. Upon antigen stimulation, the [Ca^{2+}]i level was rapidly increased, while preincubation of CGNMII in IgE sensitized RBL-2H3 cells decreased the antigen-induced [Ca^{2+}]i level (Figure 3A). To evaluate the concentration of intracellular calcium quantitatively, we assayed intracellular calcium levels by ELISA analysis. CGNMII inhibited the release of intracellular calcium in a dose-dependent manner (*p < 0.05) (Figure 3B).

Effects of CGNMII on the Expression of Inflammatory Cytokines in Antigen-Stimulated RBL-2H3 Cells. Proinflammatory cytokines, IL-4 and TNF-α, are responsible for allergic responses.[7] We investigated whether CGNMII could regulate gene expression of IL-4 and TNF-α in antigen-stimulated RBL-2H3 cells. CGNMII significantly inhibited the IL-4 and TNF-α gene expression of antigen-stimulated RBL-2H3 cells in a dose-dependent manner (Figure 4).

Effect of CGNMII on the Activation of Syk, PLCγ, LAT and ERK in Antigen-Stimulated RBL-2H3 Cells. It is well reported that FcεRI signaling events, including phosphorylation of Lyn, Syk and Syk downstream molecules such as LAT and PLCγ, regulate mast cell activation. PP2, a general Scr-family kinase inhibitor, potently inhibited phosphorylation of Syk.[18] To unravel the antiallergic mechanism of CGNMII, we checked whether CGNMII could affect the level of activated Lyn and Syk proteins. As shown in Figure 5, the levels of phosphorylated Lyn and Syk proteins were suppressed. Next, we examined whether CGNMII inhibited other downstream molecules. The phosphorylations of LAT and PLCγ were also significantly

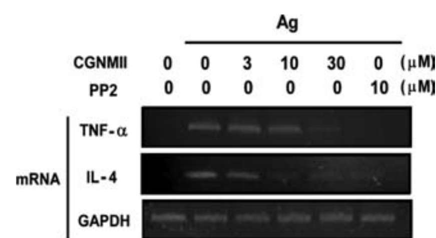

Figure 4. Effect of CGNMII on proinflammatory cytokines in antigen-stimulated RBL-2H3 cells. IgE-primed RBL-2H3 cells were stimulated with 25 ng/mL of DNP-BSA (Ag), or not stimulated. Total RNA was isolated and then reversely transcribed. IL-4 or TNF-α mRNA was determined by quantitative RT-PCR. The representative images from three independent experiments are shown.

inhibited by CGNMII in a dose-dependent manner (Figure 5). We also examined the effects of CGNMII on phosphatidylinositol 3-kinase (PI3-K) and the MAP kinases because of their role in the production of TNF-α and IL-4. The antigen-induced phosphorylation of Akt, an indicator of PI3-K activation, and that of the Erk1/2 were also inhibited by CGNMII in a dose-dependent manner (Figure 6). These results indicate that CGNMII inhibits antigen-induced FcεRI-mediated signaling events in IgE-sensitized RBL-2H3 cells.

DISCUSSION

The incidences of type I hypersensitivity allergic disorders, including allergic rhinitis, asthma, atopic dermatitis and atopic eczema, are constantly increasing in many developed countries.[2]

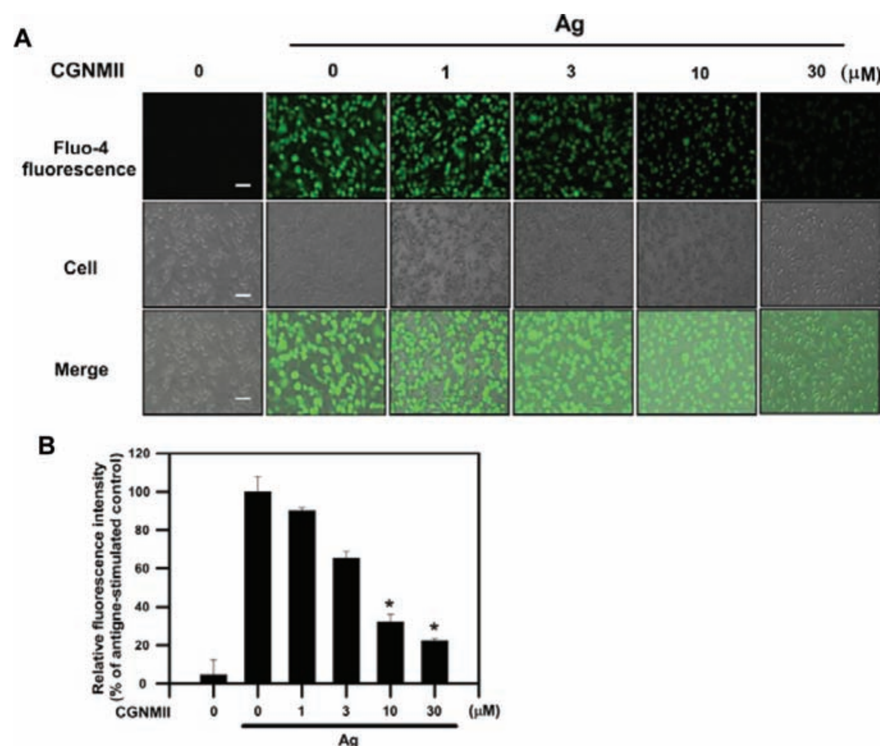

Figure 3. Effect of CGNMII on inhibition [Ca^{2+}]i in antigen-stimulated RBL-2H3 cells. Intracellular calcium levels were measured as described under Materials and Methods. RBL-2H3 cells were loaded with Fluo-4 for 30 min at 37 °C. The cells were treated in the presence or absence of CGNMII (1, 3, 10, and 30 μM) for 30 min and then stimulated with DNP-BSA (200 ng/mL) or not stimulated. (A) Fluorescence image of Fluo-4 (upper); phase contrast image of cells (middle); overlapped images of upper and middle panels (lower). The cells labeled with Fluo-4 fluorescence were detected under an inverted microscope with a filter (excitation = 494 nm, emission = 516 nm). Bar, 5 μm. Images are representative of 3 independent experiments. (B) Intracellular calcium was detected by ELISA with excitation at 494 nm, emission = 516 nm. Each value represents the mean ± SE of three experiments (antigen vs CGNMII, *p < 0.05).

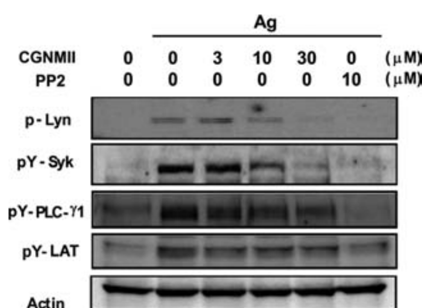

Figure 5. CGNMII inhibited the activation of signaling molecules in antigen-stimulated RBL-2H3 cells. IgE-sensitized RBL-2H3 cells (2×10^6 cells/well) were stimulated with 25 ng/mL antigen for 7 min in the presence or absence of CGNMII. The levels of phosphorylated forms of Lyn, Syk, PLCγ1 and LAT proteins were measured by immunoblotting analysis. PP2 is a general Src-family kinase inhibitor. The representative images from three independent experiments are shown.

These days active medicinal compounds or extracts from natural products come into the spotlight due to the unexpected side effects of current therapies.[20] Previously our group identified novel nutraceutical compounds from germinated soybeans cultivated with *Cordyceps militaris* (GSC) that may be second metabolites.[11,12] The structures of novel compounds from GSC are similar to the known isoflavones such as genistein and daidzein compounds that are rich in soybeans except additional glucoside and methyl group. The additional glucoside and methyl groups on these isolated compounds might be derived from components such as polysaccharide of *C. militaris.*[12] However, the antiallergic efficacies and mechanisms of novel isoflavones from GSC have not been reported yet. In this study, we investigated that CGNMII exerted antiallergic activities.

Mast cells are the primary effector cells involving IgE-mediated allergic responses by secreting inflammatory mediators and cytokines. Aggregation of the high affinity receptor for IgE (FcεRI) on mast cells initiates a biochemical cascade that results in the degranulation and the release of inflammatory mediators from them.[21] We observed that the inhibitory effect of CGNMII on the degranulation of antigen-stimulated RBL-2H3 cells was stronger than those of CDGM, CGLM and CGNMI (Figure 2A). Since CGNMII shares genistein backbone structure, we compared the inhibitory activity of CGNMII, genistein and genistin on the degranulation of antigen-stimulated RBL-2H3 cells. We showed that CGNMII inhibited the degranulation the most among genistin and genistein in antigen stimulated RBL-2H3 cells without affecting cell viability (Figures 2B and 2C).

It is well reported that FcεRI signaling events, including Lyn, Syk and PLCγ, regulate degranulation.[22] The FcεRI receptor is a tetrameric complex of subunits, binding IgE and antigen, and initiates intracellular allergic signaling. The FcεRI receptor signaling is dependent on Spleen tyrosine kinase (Syk). Syk becomes phosphorylated after binding to the immunoreceptor tyrosine-based activation motifs (ITAMs) of the FcεRI β and γ chains. Phosphorylated tyrosine residues on Syk then act as docking sites for adaptor molecules including LAT and PLCγ, which are then phosphorylated by Syk to propagate downstream signaling.[27] Activated Syk dependent signaling events increase intracellular calcium level, which leads to the degranulation. Therefore, Syk is one of the attractive targets in developing antiallergic drugs. The tyrosine residues on the adaptor proteins linker of the activated T cells (LAT) also provide docking sites for assembly of other signaling molecules to ensure further propagation of activating signals.[25] The phosphorylation of phospholipase C (PLC) γ is dependent on the activation of Syk and LAT.[26] We showed that CGNMII treated group RBL-2H3 cells decreased the antigen-induced $[Ca^{2+}]$i level (Figure 3). We showed that CGNMII inhibited the activation of Lyn, Syk, LAT and PLCγ (Figure 5). These results indicated CGNMII might possess antiallergic activity in mast cell-dependent allergic disease, most likely through its ability of inhibiting the activation of Lyn, Syk, LAT and PLCγ proteins that led to elevate degranulation in mast cells.

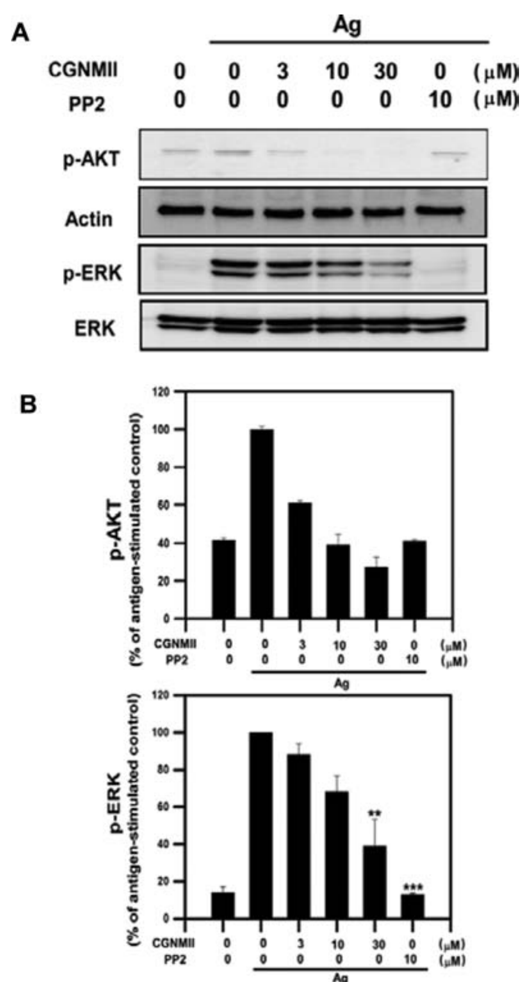

Figure 6. CGNMII inhibited the phosphorylation of AKT and ERK proteins in antigen-stimulated RBL-2H3 cell. IgE-sensitized RBL-2H3 cells (2×10^6 cells/well) were stimulated with 25 ng/mL antigen for 7 min in the presence or absence of CGNMII. (A) The levels of phosphorylated AKT and ERK proteins were measured by immunoblotting analysis. PP2 is a general Src-family kinase inhibitor. The representative images from three independent experiments are shown. (B) The densitometric data are shown as mean ± SEM of $n = 3$ independent observations expressed as a percentage of values for corresponding antigen-stimulated groups in the lower panel (antigen vs CGNMII, $*p < 0.05$; $**p < 0.01$; $***p < 0.005$). Densitometry data presented for pY-AKT and p-ERK are normalized to the intensity of actin bands and ERK, respectively.

Various cytokines, including IL-4 and TNF-α, are produced by antigen stimulated mast cells.[7] In particular, IL-4 is essential for Th2 cellular response development and promotes B cell to produce IgE.[6] TNF-α is one of the potent inflammatory cytokines.[23] CGNMII significantly suppressed the TNF-α and IL-4 gene expression in antigen-stimulated RBL-2H3 cells

dx.doi.org/10.1021/jf205199j | J. Agric. Food Chem. 2012, 60, 2309–2315

(Figure 4). It is well documented that when LAT, Akt and ERK kinases are activated, they stimulate proinflammatory cytokine production in mast cells.[24] We observed that CGNMII attenuated activation of LAT, Akt and ERK kinases. These signaling molecules are responsible for the production of IL-4 and TNF-α in RBL-2H3 mast cells.[28] The results showed that CGNMII inhibited the activation of Akt and ERK kinases in a dose-dependent manner (Figure 6). These results indicated CGNMII might possess antiallergic activity through inhibiting the activation of Akt and ERK proteins that led to the production of IL-4 and TNF-α cytokines in antigen-stimulated mast cells. Further experiments need to be done to clarify the antiallergic mechanism of CGNMII in vivo.

In conclusion, CGNMII suppressed degranulation, the level of intracellular calcium and the release of proinflammatory cytokines in antigen-stimulated RBL-2H3 cells. CGNMII inhibited the activation of the FcεRI receptor signaling molecules such as Lyn, Syk, LAT, PLCγ, Akt and ERK that are responsible for allergic responses in antigen-stimulated RBL-2H3 cells (Figure 7). Our findings suggest that CGNMII might be applied as a therapeutic agent for preventing or treating IgE-mediated allergic diseases.

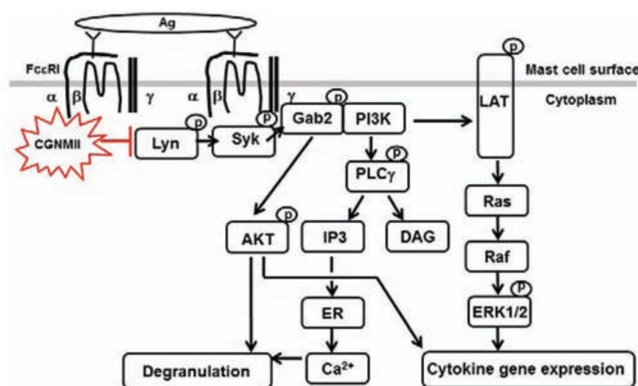

Figure 7. CGNMII suppressed signaling pathways that are initiated by the high-affinity receptor for IgE (FcεRI). CGNMII inhibits the activation of FcεRI signaling molecules. The level of phosphorylated Lyn and Syk proteins were reduced by CGNMII. CGNMII inhibited other downstream signaling molecules, including LAT, AKT and PLCγ involved in calcium signaling. The activation of AKT and ERK1/2 were also suppressed by CGNMII.

AUTHOR INFORMATION

Corresponding Author

*Department of Bioscience and Biotechnology, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, Republic of Korea. E-mail: hyejinpak@gmail.com. Tel: +82 2 455 5589. Fax: +82 2 455 5517.

Funding

This paper was supported by the SMART Research Professor Program of Konkuk University, Seoul, Korea.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. Wan Soo Choi, Konkuk University, for kindly presenting anti-phospho Lyn, Gab2, Akt antibodies and Dr. Choong Hwan Lee, Konkuk University (Korea) for providing novel isoflavone methyl-glycosides from *Cordyceps militaris* grown on germinated soybeans.

ABBREVIATIONS USED

LC–ESI-IT-MS/MS, liquid chromatography coupled with electrospray ionization and ion trap tandem mass spectrometry; PIPES, piperazine-*N*,*N*′-bis(1-ethanesulfonic acid); PLCγ, phospholipase Cγ; LAT, linker of activated T cells; ERK, extracellular receptor kinase

REFERENCES

(1) Asher, M. I.; Montefort, S.; Bjorksten, B.; Lai, C. K.; Strachan, D. P.; Weiland, S. K.; Williams, H. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. *Lancet* **2006**, *368*, 733–743.

(2) Wedemeyer, J.; Tsai, M.; Galli, S. J. Roles of mast cells and basophils in innate and acquired immunity. *Curr. Opin. Immunol.* **2000**, *12*, 624–631.

(3) Brightling, C. E.; Symon, F. A.; Holgate, S. T.; Wardlaw, A. J.; Pavord, I. D.; Bradding, P. Interleukin-4 and −13 expression is co-localized to mast cells within the airway smooth muscle in asthma. *Clin. Exp. Allergy* **2003**, *33*, 1711–1716.

(4) Walter, M.; Kottke, T.; Stark, H. The histamine H(4) receptor: Targeting inflammatory disorders. *Eur. J. Pharmacol.* **2011**, *668* (1–2), 1–5, DOI: 10.1016/j.ejphar.2011.06.029.

(5) Frossi, B.; Rivera, J.; Hirsch, E.; Pucillo, C. Selective activation of Fyn/PI3K and p38 MAPK regulates IL-4 production in BMMC under nontoxic stress condition. *J. Immunol.* **2007**, *178*, 2549–2555.

(6) Huels, C.; Germann, T.; Goedert, S.; Hoehn, P.; Koelsch, S.; Hultner, L.; Palm, N.; Rude, E.; Schmitt, E. Co-activation of naive CD4+ T cells and bone marrow-derived mast cells results in the development of Th2 cells. *Int. Immunol.* **1995**, *7*, 525–532.

(7) Theoharides, T. C.; Kalogeromitros, D. The critical role of mast cells in allergy and inflammation. *Ann. N.Y. Acad. Sci.* **2006**, *1088*, 78–99.

(8) Holgate, S. T.; Polosa, R. Treatment strategies for allergy and asthma. *Nat. Rev. Immunol.* **2008**, *8*, 218–230.

(9) Oppenheimer, J. J.; Casale, T. B. Next generation antihistamines: therapeutic rationale, accomplishments and advances. *Expert Opin. Invest. Drugs* **2002**, *11*, 807–817.

(10) Chan, B. C.; Hon, K. L.; Leung, P. C.; Sam, S. W.; Fung, K. P.; Lee, M. Y.; Lau, H. Y. Traditional Chinese medicine for atopic eczema: PentaHerbs formula suppresses inflammatory mediators release from mast cells. *J. Ethnopharmacol.* **2008**, *120*, 85–91.

(11) Ohta, Y.; Lee, J. B.; Hayashi, K.; Fujita, A.; Park, D. K.; Hayashi, T. In vivo anti-influenza virus activity of an immunomodulatory acidic polysaccharide isolated from Cordyceps militaris grown on germinated soybeans. *J. Agric. Food Chem.* **2007**, *55*, 10194–10199.

(12) Choi, J. N.; Kim, J.; Lee, M. Y.; Park, D. K.; Hong, Y. S.; Lee, C. H. Metabolomics revealed novel isoflavones and optimal cultivation time of Cordyceps militaris fermentation. *J. Agric. Food Chem.* **2010**, *58*, 4258–4267.

(13) Oh, J. Y.; Choi, W. S.; Lee, C. H.; Park, H. J. The ethyl acetate extract of Cordyceps militaris inhibits IgE-mediated allergic responses in mast cells and passive cutaneous anaphylaxis reaction in mice. *J. Ethnopharmacol.* **2011**, *135*, 422–429.

(14) Duffy, C.; Perez, K.; Partridge, A. Implications of phytoestrogen intake for breast cancer. *CA—Cancer J. Clin.* **2007**, *57*, 260–277.

(15) Sakai, T.; Kogiso, M. Soy isoflavones and immunity. *J. Med. Invest.* **2008**, *55*, 167–173.

(16) Park, H. J.; Han, E. S.; Park, D. K. The ethyl acetate extract of PGP (Phellinus linteus grown on Panax ginseng) suppresses B16F10 melanoma cell proliferation through inducing cellular differentiation and apoptosis. *J. Ethnopharmacol.* **2010**, *132*, 115–121.

(17) Lee, J. H.; Kim, J. W.; Ko, N. Y.; Mun, S. H.; Kim, D. K.; Kim, J. D.; Kim, H. S.; Lee, K. R.; Kim, Y. K.; Radinger, M.; Her, E.; Choi, W. S. Camellia japonica suppresses immunoglobulin E-mediated

allergic response by the inhibition of Syk kinase activation in mast cells. *Clin. Exp. Allergy* **2008**, *38*, 794−804.

(18) Lee, J. H.; Kim, J. W.; Ko, N. Y.; Mun, S. H.; Her, E.; Kim, B. K.; Han, J. W.; Lee, H. Y.; Beaven, M. A.; Kim, Y. M.; Choi, W. S. Curcumin, a constituent of curry, suppresses IgE-mediated allergic response and mast cell activation at the level of Syk. *J. Allergy Clin. Immunol.* **2008**, *121*, 1225−1231.

(19) Gilfillan, A. M.; Tkaczyk, C. Integrated signalling pathways for mast-cell activation. *Nat. Rev. Immunol.* **2006**, *6*, 218−230.

(20) Bradding, P. Novel approaches to the inhibition of mast cells in allergic disease. *Clin. Exp. Allergy* **2008**, *38*, 704−708.

(21) Beaven, M. A.; Metzger, H. Signal transduction by Fc receptors: the Fc epsilon RI case. *Immunol. Today* **1993**, *14*, 222−226.

(22) Yamamoto, T.; Yumioka, T.; Sekine, Y.; Sato, N.; Minoguchi, M.; Yoshimura, A.; Matsuda, T. Regulation of FcepsilonRI-mediated signaling by an adaptor protein STAP-2/BSK in rat basophilic leukemia RBL-2H3 cells. *Biochem. Biophys. Res. Commun.* **2003**, *306*, 767−773.

(23) Gordon, J. R.; Galli, S. J. Mast cells as a source of both preformed and immunologically inducible TNF-alpha/cachectin. *Nature* **1990**, *346*, 274−276.

(24) Lorentz, A.; Klopp, I.; Gebhardt, T.; Manns, M. P.; Bischoff, S. C. Role of activator protein 1, nuclear factor-kappaB, and nuclear factor of activated T cells in IgE receptor-mediated cytokine expression in mature human mast cells. *J. Allergy Clin. Immunol.* **2003**, *111*, 1062−1068.

(25) Rivera, J.; Gilfillan, A. M. Molecular regulation of mast cell activation. *J. Allergy Clin. Immunol.* **2006**, *117*, 1214−1225 quiz 1226.

(26) Saitoh, S.; Arudchandran, R.; Manetz, T. S.; Zhang, W.; Sommers, C. L.; Love, P. E.; Rivera, J.; Samelson, L. E. LAT is essential for Fc(epsilon)RI-mediated mast cell activation. *Immunity* **2000**, *12*, 525−535.

(27) Wong, B. R.; Grossbard, E. B.; Payan, D. G.; Masuda, E. S. Targeting Syk as a treatment for allergic and autoimmune disorders. *Expert Opin. Invest. Drugs* **2004**, *13*, 743−762.

(28) Zhang, C.; Baumgartner, R. A.; Yamada, K.; Beaven, M. A. Mitogen-activated protein (MAP) kinase regulates production of tumor necrosis factor-alpha and release of arachidonic acid in mast cells. Indications of communication between p38 and p42 MAP kinases. *J. Biol. Chem.* **1997**, *272*, 13397−13402.