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The ethyl acetate extract of *Cordyceps militaris* inhibits IgE-mediated allergic responses in mast cells and passive cutaneous anaphylaxis reaction in mice

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ABSTRACT

Ethnopharmacological relevance: Cordyceps militaris has been used as a traditional herbal medicine for treating allergy in East Asia.
Aim of the study: We investigated the anti-allergic efficacy of *Cordyceps militaris* and its mechanism of action.
Materials and methods: β-Hexosaminidase release of mast cells, a key parameter of degranulation, was evaluated. Anti-allergic potential of *Cordyceps militaris* was studied using passive cutaneous anaphylaxis (PCA) *in vivo*. The anti-allergic mechanism of *Cordyceps militaris* was investigated by immunoblotting analysis, RT-PCR and other biological approaches in mast cells.
Results: GSCM EtOAc extract (GSCME) inhibited antigen-induced degranulation with a IC50 value (28.5 μg/ml) in RBL-2H3 cells and antigen-induced passive cutaneous anaphylaxis (PCA) response with a ED50 value (665 mg/kg) *in vivo*. The release of interleukin (IL-4) and tumor necrosis factor (TNF-α were decreased by GSCME in RBL-2H3 cells. In order to elucidate the anti-allergic mechanisms of GSCME in mast cells, we examined the activated levels of signaling molecules. GSCME inhibited the phosphorylation Syk, ERK, p38 and JNK expression. Identified genistein, daidzein, genistein 7-*O*-β-D-glucoside 4″-*O*-methylate, genistein 4′-*O*-β-D-glucoside 4″-*O*-methylate, glycitein 7-*O*-β-D-glucoside 4″-*O*-methylate, daidzein 7-*O*-β-D-glucoside 4″-O-methylate and adenosine in GSCME, inhibited antigen-induced degranulation in RBL-2H3 cells.
Conclusions: Our study suggests that GSCME might be used as a therapeutic agent for allergic diseases.

1. Introduction

The prevalence of type I allergic disease (IgE-mediated allergic disease) has been increased worldwide during the past two decades (Asher et al., 2006). Mast cells are the major effector cells in IgE-mediated allergic diseases, including atopic dermatitis, allergic rhinitis, asthma and eczema. The allergic cascade is initiated after allergens bound to IgE (FcεRI) receptors expressed on the surface of either mast cells or basophils. Upon antigen stimulation, cells release inflammatory mediators from granules such as histamine, leukotriene C4, β-hexosaminidase, proteases and prostaglandin D2, chemokines and cytokines (Brightling et al., 2003). Interleukin (IL-4) is necessary for IgE production, and promotes the switch from naïve T-cells to the allergic type Th2 cells (Hines, 2002). Tumor necrosis factor (TNF-α) is a major initiator of inflammation, and induces other cytokine production (Kumar et al., 2004). Activation of signaling pathways in antigen-stimulated mast cells depends on activated Src kinases that interact with FcεRI receptor and subsequent activation of Syk and other downstream tyrosine kinases (Gilfillan and Tkaczyk, 2006). Mitogen-activated protein kinases (MAPKs) signaling cascades are involved in the degranulation and the regulation of gene expressions, including pro-inflammatory cytokines and chemokines (Gilfillan and Tkaczyk, 2006). Therefore, these kinases are major target molecules for screening anti-allergic drugs.

Current therapies for allergic diseases are largely based on allergen-specific immunotherapy, DNA vaccination, anti-histamine drugs and steroids treatments (Kaliner, 2009). Common small-molecule inhibitors to treat allergic diseases are mainly antagonists to leukotriens (LTs) or histamine receptors, but they produce undesirable side-effects, including drowsiness, dry mouth, chest congestion, and upset stomach (Oppenheimer and Casale, 2002). Recently, many practitioners and researchers are paying attention to traditional medicinal herbs and mushrooms as anti-allergic drugs that are known to modulate immune responses and neutralize allergic reactions without any adverse effects (Chan et al., 2008; Ellertsen and Hetland, 2009).

Cordyceps militaris (Family: Clavicipitaceae), a traditional medicine, has been widely used to treat inflammation, anemia, asthma, and cancer in East Asia (Kim et al., 2006; Shin et al., 2009).

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Recent studies have shown that the extracts of *Cordyceps militaris* has strong anti-inflammatory activity on the croton-oil induced ear edema in mice and are effective in treating asthma patient (Won and Park, 2005; Gao et al., 2009). Many nutritional compounds from soybeans have been reported to be effective in preventing various inflammatory diseases and cancers (Li et al., 2008). In this regard, we cultivated *Cordyceps militaris* on the germinated soybeans that might provide plentiful novel nutraceutical compounds that derive from the biologically effective components of the two materials (Choi et al., 2010). Recently, our group has reported that the methanol extracts of *Cordyceps militaris* grown on germinated soybeans (GSC) stimulated IL-8 production playing an important role in the activation of innate and adaptive immunity in A549 human pulmonary epithelial cells (Han et al., 2010). However, the evidence-based research into its anti-allergic activity of GSC is still in its infancy.

In this study, we investigated whether *Cordyceps militaris* grown on germinated soybean (GSC) could inhibit IgE-mediated allergic response and regulate the mechanisms involved in allergic events.

2. Material and methods

2.1. 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, genistein 4′-*O*-β-D-glucoside 4″-*O*-methylate and adenosine used in this study that were purified as described in our previous study (Choi et al., 2010). Fetal bovine serum (Invitrogen, Carlsbad, CA), Penicillin (Invitrogen, Carlsbad, CA), Minimum essential medium, Eagle (Invitrogen, Carlsbad, CA), DNP-specific IgE (Sigma–Aldrich, St. Louis, MO), DNP-BSA (Sigma–Aldrich, St. Louis, MO), Piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) (Sigma–Aldrich, St. Louis, MO), Evans blue (Sigma–Aldrich, St. Louis, MO), Cetrazine (CZ, Sigma–Aldrich, St. Louis, MO), Src tysoine kinase inhibitor (PP2) (Calbiochem, La Jolla, CA), anti-phospho-Syk (Cell Signaling Technology Inc., Danvers, MA) antibody, anti-phosphorylated linker for activation of T cells ((LAT) antibody (Cell Signaling Technology Inc., Danvers, MA)), anti-phospho-PKC α/β II (Cell Signaling Technology Inc., Danvers, MA) antibody, anti-phospho-p44/42 MAPK (Erk1/2) antibody (Cell Signaling Technology Inc., Danvers, MA), anti-phospho-SAPK/JNK antibody (Cell Signaling Technology Inc., Danvers, MA), anti-phospho-p38 antibody (Santa Cruz, CA) and anti-β-actin antibody (Santa Cruz, CA).

2.2. Preparation of extract of Cordyceps militaris grown on germinated soybean

Cordyceps militaris was grown on germinated soybeans (GSCs) as previously described (Han et al., 2010). An authenticated voucher specimen of *Cordyceps militaris* (CM, Kucari 0906) is deposited in the Herbarium at the College of Bioscience and Biotechnology, Konkuk University (Seoul, Korea). Briefly, mycelium of *Cordyceps militaris* (CM, Kucari 0906) was inoculated on germinated soybeans (*Glycine max* (L.) Merr), and was cultured at 20–25 °C for 4 weeks. The cultured material (1 kg) was ground and extracted with 80% MeOH (methanol extract of *Cordyceps militaris* grown on germinated soybeans (GSCM)) for 48 h under reflux. The total extract (178 g, yield 17.8%) were dissolved with water, after remove the insoluble solid by filtration, the liquid phase was extracted sequentially from non-polar to polar solvents by hexane, EtOAc, BuOH (1:10 (w/v) for all solvents), yielding four fractions. The liquid–liquid phase extraction was performed in Erlenmeyer flasks shaking and concentrated by a rotary evaporator to dryness, i.e. hexane fraction (16 g, yield (w/w) 1.6%), EtOAc fraction (4.5 g, yield (w/w) 0.45%), BuOH fraction (8.25 g, yield (w/w) 0.825%) and water fraction (10.86 g, yield (w/w) 1.086%).

2.3. Cell culture

RBL-2H3 cells were obtained from ATCC (A Biological Resource Center, Manassas, VA). The RBL-2H3 were cultured in MEM supplemented with 15% FBS and 1% Penicillin. The cells were grown in 75 cm^2 culture flasks at 37 °C with 5% CO_2 in humidified atmospheric pressure.

2.4. Experimental animals

Male Balb/c mice (aged 4 weeks) were supplied from the Dae Han Experimental Animal Center (Eumsung, Korea). They were kept in cages with a temperature of (23 ± 2 °C) and a relative humidity of 55%. The animal study was performed in accordance with the institutional guidelines (The Institutional Animal Care and Use Committee (IACUC) at Konkuk University (Seoul, Korea)).

2.5. Cell viability assay

Cell cytotoxicity was measured using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan), as described previously (Park et al., 2010a). Cells (1×10^4/ml) were treated with GSCME extract (10, 30, 100 and 300 μg/ml) for 24 h. The cultures in 96-well plates were placed in 10 μl 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, Männedorf, Switzerland).

2.6. β-Hexosaminidase secretion assay

As a marker of degranulation, the release of β-hexosaminidase was measured as previously described (Lee et al., 2008).

2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the RBL-2H3 cells using RNA-Bee Reagent (Tel test, Friendwood, Texas) 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′-CCGGACTCCGTGATGTCTAAGTACT-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′

2.8. Immunoblotting

RBL-2H3 cells (1×10^6/ml) were stimulated with 25 ng/ml DNP-BSA for 10 min. Cells were lysed in 100 μl cell lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM PMSF) on ice for 10 min. Lysates were centrifuged at 14,000 × *g* for 10 min at 4 °C. The protein concentrations were determined using a BCA Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) as previously described (Park et al., 2010b). Then, 20 μg aliquots of protein were subjected to electrophoresis on 4–15% gradient polyacrylamide gels and electrophoretically transferred onto polyvinylidene fluoride