



Saikosaponin-d inhibits β -conglycinin induced activation of rat basophilic leukemia-2H3 cells

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ABSTRACT

β -Conglycinin is one of the major storage proteins in soybean and has been identified as a potential diagnostic marker for severe allergic reactions to soybean. Unfortunately, there is a lack of information on the signal transduction pathways of β -conglycinin induced mast cell activation and how to alleviate these allergic reactions. *Bupleurum falcatum*, a traditional oriental medicine, has been widely utilized in the treatment of influenza, fever, malaria and menstrual disorders. Furthermore, it has been reported that saikosaponins, the important principle of *B. falcatum*, possesses anti-allergic activities. Therefore, the present study investigated whether or not saikosaponin-d, an extract of *B. falcatum*, was effective in the treatment of allergic reactions caused by β -conglycinin, using a rat basophilic leukemia-2H3 cell line. There were multiple signaling pathways contributing to the development of β -conglycinin-mediated rat basophilic leukemia-2H3 cell activation. The intracellular calcium mobilization and tyrosine phosphorylation were early events, which in turn elicited reactive oxygen species production, gene activation of Cdc42 and c-Fos, and ultimately led to β -hexosaminidase release. Saikosaponin-d inhibited rat basophilic leukemia-2H3 cell degranulation by suppressing these critical incidents in the signal transduction pathway. These results suggest that saikosaponin-d exhibited anti-allergic activity and could become an effective herbal therapy for alleviating soybean allergy.

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1. Introduction

β -Conglycinin is one of the major storage proteins in soybean and has been demonstrated to bind immunoglobulin E (IgE) in subjects with soybean allergy [1,2]. Our previous studies showed that purified β -conglycinin induced IgE-mediated allergic reactions [3–5] and mast cells played an important role in these allergic reactions by secreting abundant amounts of pro-inflammatory mediators such as histamine and serotonin.

Mast cell activation by IgE-mediated stimuli is a central event in the pathogenesis of allergic disorders [6]. Tissue mast cells bind IgE on their surface by expressing the high-affinity Fc receptor for IgE (Fc ϵ RI) and then exert their biological effects by releasing mediators [7]. Stimulation with antigens triggers a cascade of events which leads to the rapid activation of several non-receptor-type protein tyrosine kinases and other signaling proteins, an increase in intracellular calcium ion

concentrations as well as intracellular production of reactive oxygen species and degranulation [8–10].

The granule-associated enzyme β -hexosaminidase, which is an exoglycosidase located in mast cells, is released in parallel to histamine upon mast cell activation. β -hexosaminidase release is in proportion to the degree of degranulation and is commonly used as a clinical marker for degranulation in mast cells [11]. The degranulation of mast cells and basophils elicited by antigen stimulation is dependent on the mobilization of intracellular calcium [12,13]. The Rho family of small GTPases, such as cell division control protein 42 (Cdc42), is also implicated in the regulation of calcium mobilization and degranulation in mast cells [14,15]. Cdc42 has been suggested to promote degranulation by enhancing the production of inositol trisphosphate and calcium mobilization in antigen-stimulated mast cells [16]. c-Fos, a member of the early gene family, is critical for the activation of mast cells [17,18]. The most important role of c-Fos in mast cells appears to be the regulation of the degranulation process of the secretory granules [19].

Currently, the most common intervention in the case of soybean allergy is strict avoidance of soybean products [20]. However, this is difficult to be achieved in practice due to the complexity of food composition. Traditional Chinese medicines are attractive to researchers as a therapy for food allergies. There have been many reports on the anti-allergic effects of traditional Chinese medicines [21–23].

Abbreviations: BSA, bovine serum albumin; DCFH-DA, 2',7'-dichlorofluorescein diacetate; FBS, fetal bovine serum; Fc ϵ RI, high-affinity receptor for IgE; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; IgE, immunoglobulin E; MEM, minimal essential medium; PTK, protein tyrosine kinase; RBL, rat basophilic leukemia; ROS, reactive oxygen species; TBST, tris buffered saline with Tween 20; TMB, tetramethylbenzidine.

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Rat basophilic leukemia-2H3 cells are mucosal mast cell types and are a useful model for the study of the FcεRI-mediated cell degranulation and signal transduction pathways [24,25]. Several studies have demonstrated that the herbal extract mixture containing *Bupleurum falcatum* (Radix Bupleuri) inhibited IgE-mediated leukotriene synthesis in rat basophilic leukemia-2H3 cells [26,27]. Park et al. [28] reported that saikosaponin-a isolated from *B. falcatum* has an inhibitory effect on animal allergic asthma. Saikosaponin-d, another saponin constituent extracted from *B. falcatum*, also has potent anti-inflammatory [29] and immunomodulatory properties [30]. Therefore, the following study was conducted to investigate the cellular modulating effects of saikosaponin-d on signal transduction pathways during rat basophilic leukemia-2H3 cell activation stimulated by β-conglycinin.

2. Materials and methods

2.1. Preparation of β-conglycinin

The purified β-conglycinin was prepared previously in our laboratory following the preparation procedure described by You et al. [31]. The purity of the β-conglycinin was determined as >90%.

2.2. Animals, diets and experimental protocol

Sixteen female Brown Norway rats, 3 weeks of age, were individually housed in polycarbonate cages with a soft wood granulate floor. The cages were located in a room with controlled lighting (12 h light:dark), temperature (23 °C) and relative humidity (65%). Drinking water and feed were available ad libitum. Rats were fed soybean-free, laboratory-produced diets with casein as their only source of dietary protein [32]. All procedures were approved by the China Agricultural University Animal Care and Use Committee (Beijing, China).

Immunization and anti-β-conglycinin serum preparation were performed according to a modified method of Lee et al. [33]. Rats were divided into two groups (n=8) according to body weight. Rats were either sensitized by means of intragastric gavage with 10 mg protein/mL purified β-conglycinin in PBS (pH 7.4) or with PBS only on d 0, 1, 2, 7 and 21 (1 mL/animal). Rats were challenged by intragastric gavage with 20 mg of β-conglycinin on d 35 provided in two doses at 30 to 40 min intervals. Serum samples were collected from the tail vein on 1 day before β-conglycinin challenge and stored at −80 °C for analysis of β-conglycinin-specific IgE.

2.3. Cell culture

Rat basophilic leukemia-2H3 mast cells were a generous gift from Mr. Lishi Zhang from the Institute of Basic Theory, China Academy of Traditional Chinese Medicine (Beijing, China), and were cultured in Eagles' Minimal Essential Medium (MEM) containing 10% (v/v) heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), 2 mmol/L L-glutamine (Invitrogen, Paisley, Scotland), 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen, Paisley, Scotland). Cultures were maintained at 5% CO₂ and 37 °C in tissue culture flasks. Cells were detached with trypsin–EDTA solution (Invitrogen, Paisley, Scotland). After the cells were washed, they were re-suspended in medium and used for subsequent experiments.

2.4. Measurement of serum β-conglycinin-specific IgE

The levels of β-conglycinin-specific IgE were measured by indirect ELISA following the procedures described by Gizzarelli et al. [34] with slight modifications. Immulon II 96-well microplates (Dynatech Laboratories, Chantilly, VA) were coated with 20 μg/mL purified β-conglycinin in coating buffer (Sigma, St Louis, MO) and incubated for 3 h at 37 °C. After washing with PBS/0.05% Tween-20, the plates

were blocked with 1% bovine serum albumin (BSA)–PBS overnight at 4 °C. Serum samples were diluted 1:10 in 1% BSA–PBS and incubated for 2 h at 37 °C. Bound antibodies were detected by adding 100 μL horseradish peroxidase-labeled sheep anti-rat IgE (Bethyl Laboratories, Montgomery, TX) for 1 h at 37 °C. After three washings, the peroxidase substrate tetramethylbenzidine (Sigma, St Louis, MO) was added and left for 20 min at room temperature. The reaction was stopped by the addition of 1 M H₂SO₄ and the optical density was determined with an ELISA reader (Tecan, Groedig, Austria) at 450 nm.

2.5. Assay of β-hexosaminidase release

Rat basophilic leukemia-2H3 cells were grown in 48-well plates (1 × 10⁵ cells/400 μL/well) and were sensitized overnight with 20 μL anti-β-conglycinin serum (1:2 dilution), and incubated for 10 h in MEM supplemented with 0.5% fetal bovine serum. The cells were then washed twice with tyrode buffer (135 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L CaCl₂, 1 mmol/L MgCl₂, 5.6 mmol/L glucose, 1 mg/mL BSA, and 20 mmol/L Hepes). The IgE-sensitized rat basophilic leukemia-2H3 cells were pre-incubated for 15 min, and then exposed to 50 ng/mL of saikosaponin-d (Winherb Medical Science Company, Shanghai, China; purity >98%) for 1 h. After incubation, the cells were stimulated at 37 °C with 10 μg/mL β-conglycinin protein in tyrode buffer for 5, 15, 30, 45 or 60 min, respectively. The assay procedure for β-hexosaminidase was performed as previously described with slight modifications [10]. In brief, the culture supernatant (50 μL) was incubated for 1.5 h at 37 °C with 100 μL of p-nitrophenyl-N-acetyl-β-D-glucosaminide (5 mmol/L, AppliChem, Darmstadt, Germany) in 0.1 mol/L sodium citrate buffer (pH 4.5), after which the reaction was terminated by the addition of 200 μL of 0.2 mol/L sodium carbonate buffer (pH 10.5). The generation of p-nitrophenol was monitored by measurement of absorbance at 405 nm. To determine the total amount of β-hexosaminidase released, the remaining cells were lysed by treatment with assay buffer containing 0.1% (v/v) triton X-100 (Sigma, St Louis, MO) prior to incubation with substrate using the same procedure as for the determination of activity in the supernatant. The percentage of β-hexosaminidase released (% R) was calculated as follows: % R = (A₁ − A₀) / (A₂ − A₀) × 100. A₀ is the absorbance of the un-stimulated cells in the supernatant, A₁ is the absorbance of the stimulated cells in the supernatant and A₂ is the absorbance of the total cell lysate.

2.6. Measurement of intracellular calcium

The calcium responses were measured with the use of a Fluorescent indicator Fluo-3/AM following the procedures described by Matsubara et al. [35] with some modifications. Rat basophilic leukemia-2H3 cells were incubated and treated with saikosaponin-d as described above. After washing twice, the cells were incubated in the dark for 50 min at 37 °C in tyrode buffer containing 5 μmol/L Fluo-3 acetoxymethyl ester (Dojindo, Tokyo, Japan). The Fluo-3-loaded rat basophilic leukemia-2H3 cells were washed two times with assay buffer and then stimulated with 10 μg/mL β-conglycinin in tyrode buffer for 1, 2, 5 or 15 min. The change in fluorescence of fluo-3 was monitored at an excitation wavelength of 488 nm and an emission wavelength of 515 nm.

2.7. Measurement of reactive oxygen species

Intracellular H₂O₂ was measured by means of flow cytometric analysis of 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Fluka, Buchs, Switzerland) fluorescence. Rat basophilic leukemia-2H3 cells were incubated and treated with saikosaponin-d as described above. After washing twice, the cells were incubated in the dark for 30 min at 37 °C in tyrode buffer containing 10 μmol/L DCFH-DA. The rat basophilic leukemia-2H3 cells were washed two more times with assay buffer and

then stimulated at 37 °C with 10 µg/mL β -conglycinin in tyrode buffer for 5, 15, 30 or 45 min. DCFH-DA fluorescence was excited at 488 nm and the evoked emission was filtered with a 515 nm long-pass filter.

2.8. Assessment of tyrosine phosphorylation by flow cytometry

After saikosaponin-d treatment, sensitized rat basophilic leukemia-2H3 cells were stimulated at 37 °C with 10 µg/mL β -conglycinin in tyrode buffer for 2, 5, 15, 30 or 45 min. The cells were detached with trypsin, washed with PBS and fixed in 4% paraformaldehyde for 20 min at room temperature followed by permeation with 0.1% triton X-100 for 10 min. The permeation was blocked with 5% BSA in tris buffered saline with Tween 20 for 30 min at room temperature. The cells were then incubated with 10 µg/mL of phospho-tyrosine mouse mAb (Abcam, Cambridge, United Kingdom) at 4 °C for 45 min. After washing, the cells were incubated with fluorescein isothiocyanate labeled sheep anti-mouse IgG (1:50, KPL, Gaithersburg, Maryland) at 4 °C in the dark for 45 min. Cells incubated only with secondary antibody were used as a negative control. The cells were then washed and flow cytometric analysis was performed using the FACS Calibur System (BD Biosciences, Heidelberg, Germany).

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

After saikosaponin-d treatment, sensitized rat basophilic leukemia-2H3 cells were stimulated at 37 °C with 10 µg/mL of β -conglycinin in tyrode buffer for 45 min. Total RNA from the treated cells was prepared with RNeasy Plus Mini Kit (Macherey-Nagel, Germany) according to the manufacturer's instructions and stored at –80 °C until use. First-strand cDNA synthesis was performed as described by Lai et al. [36]. Real-Time PCR was performed using DNA Engine Opticon-2 (MJ Research; Waltham, MA) and DyNAmo SYBR Green qPCR commercial kits (Finnzymes, Espoo, Finland), in which SYBR Green I was a double-stranded DNA-specific fluorescent dye. β -Actin was used as the reference gene. The specific sense and anti-sense primers used in this study were: β -actin (product size, 291 bp; annealed temperature, 63 °C) 5'-GCCAACCGTAAAAGATG-ACC-3', 5'-ACGCACGATTCCCTCTCAG-3'; c-Fos (298 bp; 63 °C) 5'-GCAGAAAGGGCAAAGTAGAG-3', 5'-AAGTCCAGGGAGGTCACAGA-3'; Cdc42 (171 bp; 61 °C) 5'-GCAGGGCAAGAGGATTATGA-3', 5'-GGTC-CCGACAAGCAAGAAA-3'. To quantify the mRNA expression, the real-time PCR reaction system contained 5.25 µL of SYBR Green qPCR mix, 1.0 µL of cDNA template (sample or standard), 3.45 µL of double distilled H₂O and 0.15 µL of primer pairs (25 µmol/L forward and 25 µmol/L reverse) in a total volume of 10 µL. Cycling conditions were 50 °C for 5 min, followed by 95 °C for 5 min. The temperature was then set at 95 °C for 30 s with the corresponding annealing temperatures for 30 s and 72 °C for 45 s. The melting curve program was 65 to 95 °C with a heating rate of 0.5 °C/s and a continuous fluorescence measurement. Reaction mixture without the cDNA was used as negative control in each run. All samples were measured in triplicate. Specificity of the reaction was controlled by separation of the PCR products on 1% agarose gel. To calculate the relative transcription level of the genes of interest, the copy number for the mRNA of the gene of interest was calculated and divided by that of β -actin mRNA [37].

2.10. Statistical analysis

All statistical analysis was performed using SAS Statistical Version 8.2 (Cary, NC). The results were reported as means $\pm$ SE. Data for serum β -conglycinin-specific IgE were analyzed statistically with an unpaired t-test. Other data were analyzed using a one-way ANOVA followed by the Duncan's Multiple Range Test where appropriate. Differences between means were considered statistically significant for values of $P < 0.05$.

3. Results

3.1. Induced production of β -conglycinin-specific IgE

To investigate β -conglycinin induced mast cell degranulation in vitro, we first measured allergen-specific IgE responses in the rat sera obtained from animals treated with β -conglycinin or PBS. High levels of specific IgE antibodies were obtained (Fig. 1) indicating that a β -conglycinin induced immediate hypersensitivity model was successfully initiated and therefore the serum was used in the following study.

3.2. Saikosaponin-d inhibited rat basophilic leukemia-2H3 cell degranulation

β -Hexosaminidase is a granule marker for mast cell degranulation. In a stationary state, rat basophilic leukemia-2H3 cells spontaneously released small amounts of β -hexosaminidase, but after being sensitized by anti- β -conglycinin IgE and stimulated by β -conglycinin, the β -hexosaminidase release rate significantly increased compared with the control group ($P < 0.001$), which reached a maximum at 45 min of stimulation (Fig. 2). Furthermore, saikosaponin-d significantly inhibited β -hexosaminidase release ($P < 0.001$). The percentage declined from 41.15% down to 27.78% at 45 min of stimulation.

3.3. Saikosaponin-d suppressed calcium release from intracellular stores

There was almost no change of intracellular calcium signal in the mast cells of the control group. A transient increase in calcium was observed after β -conglycinin stimulation of sensitized rat basophilic leukemia-2H3 cells for 1 min (Fig. 3). Fluorescence intensity reached its maximum after being stimulated for 2 min, which was approximately 5-fold greater compared with cells in the control group. The intensity gradually decreased after 5 min of stimulation and returned to the basal level at 15 min after stimulation. Pretreatment with saikosaponin-d suppressed calcium influx. The extent of inhibition for calcium influx is positively related to its effects on β -hexosaminidase release.

3.4. Saikosaponin-d alleviated the production of reactive oxygen species

Fig. 4 demonstrates the generation of reactive oxygen species after rat basophilic leukemia-2H3 cell activation induced by β -conglycinin. When stimulated with β -conglycinin for 5 min, the intracellular reactive oxygen species were produced in the activation group in which

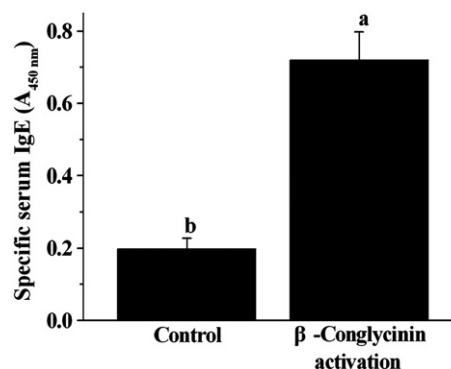


Fig. 1. Levels of β -conglycinin-specific IgE one day before β -conglycinin challenge. Rats were sensitized by means of intragastric gavage with 10 mg protein/mL purified β -conglycinin in PBS (pH 7.4) or with PBS only on d 0, 1, 2, 7 and 21 (1 mL/animal). Rats were challenged by intragastric gavage with 20 mg of β -conglycinin on d 35 in two divided doses at 30 to 40 min intervals. The group of rats that were sensitized and challenged with β -conglycinin is described as the activation group. The group of rats treated only with PBS is described as the control group. Values are expressed as means $\pm$ SE, $n = 8$ for each treatment group. Values with different letters are significantly different $P < 0.05$.

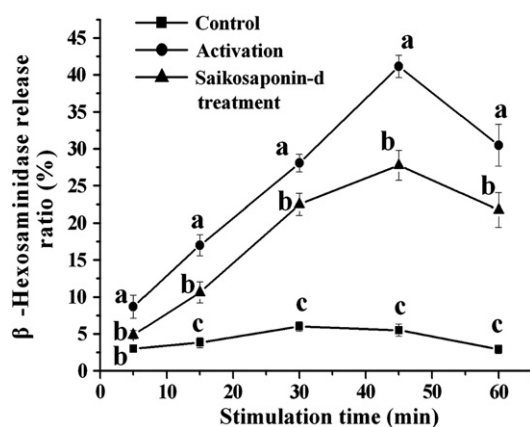


Fig. 2. Effects of saikosaponin-d on rat basophilic leukemia-2H3 cell degranulation induced by β -conglycinin. Rat basophilic leukemia-2H3 cells were sensitized overnight with 20 μ L anti- β -conglycinin serum (1:2 dilution), and incubated for 10 h in EMEM supplemented with 0.5% FBS. The IgE-sensitized rat basophilic leukemia-2H3 cells were then exposed to 50 ng/mL of saikosaponin-d for 1 h. After incubation, the cells were stimulated at 37 °C with 10 μ g/mL β -conglycinin protein for 5, 15, 30, 45 and 60 min, respectively. Cells were divided into three groups, including a control group, activation group and saikosaponin-d treatment group. Cells cultured with serum from rats which were treated with PBS served as control. In the activation group, the IgE-sensitized cells were stimulated by β -conglycinin without saikosaponin-d treatment. The group in which sensitized cells were cultured with saikosaponin-d and β -conglycinin are described as saikosaponin-d treatment group. Values are expressed as means $\pm$ SE, $n = 9$ for each treatment group. Values with different letters are significantly different $P < 0.05$.

fluorescence intensity was about twice as high as the control group. Reactive oxygen species production reached its maximum at 15 min, at which point, it was more than 10-fold greater compared with the control. After 15 min, the generation of reactive oxygen species decreased and thereafter maintained a constant level in which fluorescence intensity was about 4-fold greater than the control. Saikosaponin-d showed anti-oxidation activity, which significantly inhibited the production of reactive oxygen species in activated rat basophilic leukemia-2H3 cells from 15 min of stimulation.

3.5. Saikosaponin-d inhibited protein tyrosine dependent signal pathway of rat basophilic leukemia-2H3 cells

Flow cytometry was used to measure the level of phosphorylated tyrosine after stimulation by β -conglycinin, and the results showed that protein tyrosine kinase was involved in signal transduction of mast cell activation induced by β -conglycinin (Fig. 5). IgE-sensitized cells were rapidly activated after β -conglycinin stimulation, in which the FITC-label fluorescence intensities were significantly higher than those of the control group ($P < 0.05$). After 15 min of stimulation, the phosphorylation of tyrosine reached its peak value (23.46 ± 0.38).

There were no significant effects of saikosaponin-d on the inhibition of tyrosine phosphorylation following 2 or 5 min of stimulation. However, saikosaponin-d significantly suppressed tyrosine phosphorylation at 15 min of stimulation.

3.6. Saikosaponin-d suppressed the corresponding gene expression with rat basophilic leukemia-2H3 cells activation

Fig. 6 reflects expression levels for the genes Cdc42 (A) and c-Fos (B) during the rat basophilic leukemia-2H3 cell activation. The expression of these genes was significantly increased following stimulation for 45 min in sensitized rat basophilic leukemia-2H3 cells ($P < 0.001$). Moreover, saikosaponin-d significantly inhibited the mRNA expression of Cdc42 and c-Fos, which were reduced 8-fold and 150-fold compared with the activation group, respectively. These results indicate that saikosaponin-d might affect pivotal signal gene expression in the mast cell activation process.

4. Discussion

Soybean allergy represents a significant health problem. With the popularized application of soybean in food, the incidence of soybean allergy is rising in children and adults [38]. β -Conglycinin has been demonstrated as one of the major triggers for severe allergic reactions to soybean [1,2,39].

In this study, we investigated β -conglycinin induced allergic reaction in rat basophilic leukemia-2H3 cells. The data indicates that β -conglycinin triggered mast cell activation, which included a series of signal transductions such as calcium influx, protein tyrosine phosphorylation and intracellular production of reactive oxygen species with a concomitant increase in corresponding gene expression. In addition, we examined the anti-allergic activity of saikosaponin-d an extract of *B. falcatum* (Radix Bupleuri). It inhibited mast cell activation and degranulation most likely via the regulation of calcium mobilization, protein tyrosine phosphorylation, reactive oxygen species generation and the mRNA expression of Cdc42 and c-Fos during certain stimulation times.

Mast cells play a central role in allergic reactions by releasing various mediators, such as histamine, serotonin, leukotrienes and cytokines [7,40]. Stimulation of rat basophilic leukemia-2H3 cells has been used as a model for the investigation of degranulation and signal transduction pathways initiated by cross-linkage of the high affinity IgE receptor [25]. In this study, we obtained a high level of β -conglycinin-specific IgE in rat serum and used the serum and β -conglycinin to establish a rat basophilic leukemia-2H3 activated model in vitro. We observed that IgE-sensitized mast cells were activated after β -conglycinin stimulation, in which β -hexosaminidase release was significantly increased. β -hexosaminidase is usually released along with histamine during mast cell degranulation, thus it was used as a biomarker for antigen induced degranulation in rat basophilic leukemia-2H3 cell line [41].

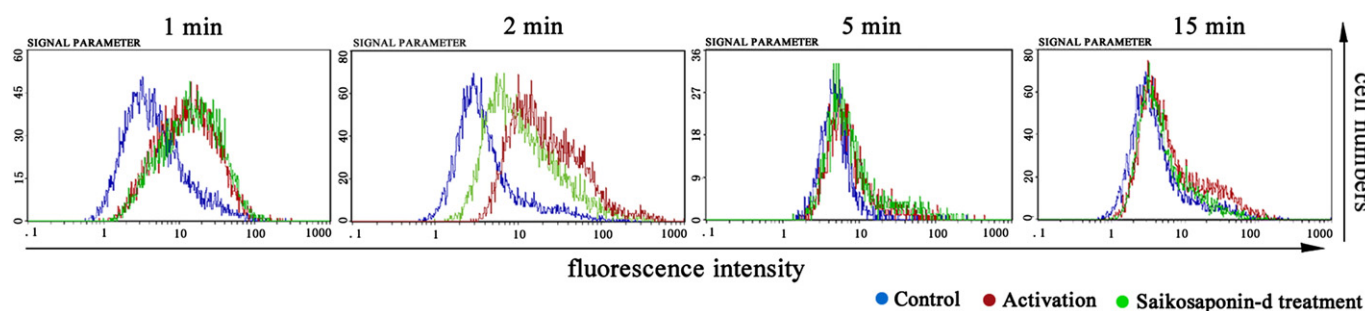


Fig. 3. Effects of saikosaponin-d on calcium mobilization in rat basophilic leukemia-2H3 cells after β -conglycinin activation. Rat basophilic leukemia-2H3 cells were sensitized and treated with saikosaponin-d. After washing twice, the cells were incubated in the dark for 50 min at 37 °C in tyrode buffer containing 5 μ mol/L Fluo-3 acetoxymethyl ester. The fluo-3-loaded rat basophilic leukemia-2H3 cells were then stimulated with 10 μ g/mL β -conglycinin for 1, 2, 5 and 15 min. The cell groups were the same as those described in Fig. 2. Each histogram was obtained from 1×10^4 cells. Data shown in line plots are the means of three independent experiments.

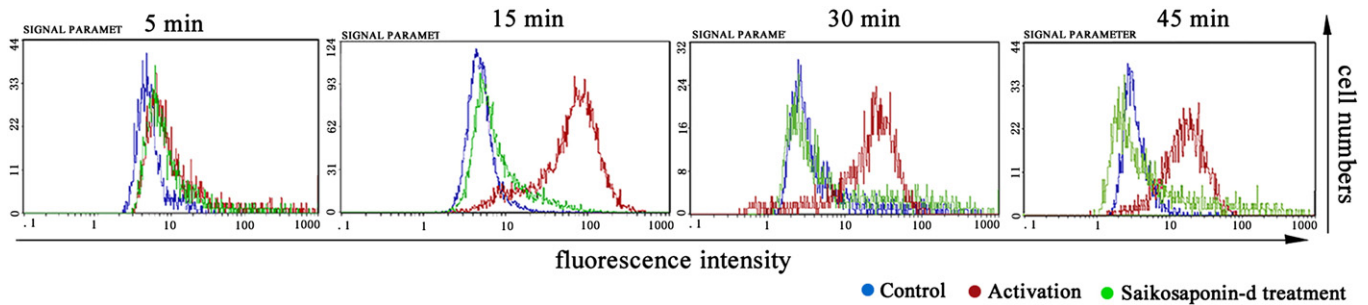


Fig. 4. Effects of saikosaponin-d on intracellular reactive oxygen species production in rat basophilic leukemia-2H3 cells after β -conglycinin activation. Rat basophilic leukemia-2H3 cells were sensitized and treated with saikosaponin-d. After washing twice, the cells were incubated in the dark for 30 min at 37 °C in tyrode buffer containing 10 μ mol/L DCFH-DA. The rat basophilic leukemia-2H3 cells were then stimulated at 37 °C with 10 μ g/mL β -conglycinin for 5, 15, 30 and 45 min. The cell groups are the same as those described in Fig. 2. Each histogram was obtained from 1×10^4 cells. Data shown in line plots are the means of three independent experiments.

In Asian countries, many traditional herbal medicines have been used to cure various diseases including allergic illnesses [42]. *B. falcatum* is a well-known Chinese traditional medicine. In an earlier study by Zhou et al. [43], the anti-allergic inflammatory activity of saikosaponin mixtures was demonstrated by observing that saikosaponins could block passive cutaneous anaphylaxis, the Arthus reaction, adjuvant arthritis and delayed hypersensitiveness. As one of its major bioactive compounds, saikosaponin-d extracted from *B. falcatum* showed favorable anti-allergic effects in this study. As shown in Fig. 2, when sensitized rat basophilic leukemia-2H3 cells were pretreated with saikosaponin-d for 1 h, the antigen-induced degranulation was significantly suppressed. Our results also indicate that at least some of the anti-allergic actions of saikosaponins can be derived from the saikosaponin-d component.

The elevation of calcium in rat basophilic leukemia-2H3 cells has been proposed as an essential trigger for mast cell activation and degranulation [34,44]. In agreement with the results of Ali et al. [12] and Tanifuji et al. [13], the degranulation of mast cells elicited by antigen stimulation is dependent on the mobilization of intracellular calcium (Fig. 3). Calcium movements in mast cells represent a major target for effective anti-allergic drugs as this is an essential event linking stimulation to secretion [45]. From our observations, it appears that the decreased calcium is involved in the inhibitory effect of saikosaponin-d on mast cell activation after 2 min of stimulation. However, it failed to reduce the increase in Ca^{2+} at 1 min. We speculate that this may depend

on the chemical structure and the concentration of saikosaponin-d. But the precise mechanism remains to be determined.

It has been reported that mast cells generate intracellular reactive oxygen species in response to stimulation with divergent physiologically relevant stimulants [46,47]. Cho et al. [10] pointed out that the increase in calcium induced by antigen stimulation triggers the generation of reactive oxygen species. Consistent with this, our results also suggest that reactive oxygen species were generated in response to an increase in calcium and that the generation of reactive oxygen species reached a maximum within 15 min and maintained that level for at least 30 min (Fig. 4). These results indicate that reactive oxygen species production played an important role in the activation of mast cells via Fc ϵ RI signaling. However, the precise mechanism by which calcium induces reactive oxygen species generation and the

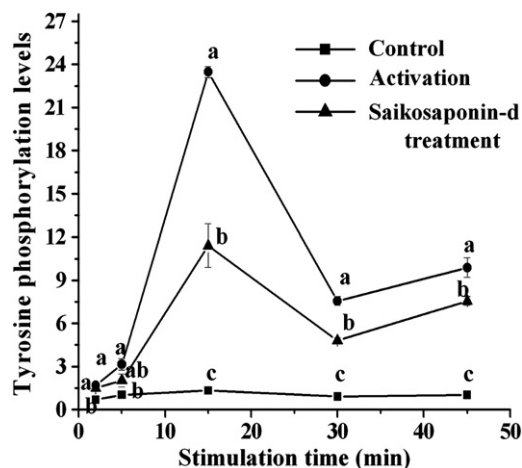


Fig. 5. Effects of saikosaponin-d on tyrosine phosphorylation after rat basophilic leukemia-2H3 cell activation. After saikosaponin-d treatment, sensitized rat basophilic leukemia-2H3 cells were stimulated at 37 °C with 10 μ g/mL β -conglycinin for 2, 5, 15, 30 and 45 min. The cell groups are the same as those described in Fig. 2. Flow cytometric analysis was performed. Each histogram was obtained from 1×10^4 cells. Values are expressed as means $\pm$ SE, $n = 3$ for each treatment group. Values with different letters are significantly different $P < 0.05$.

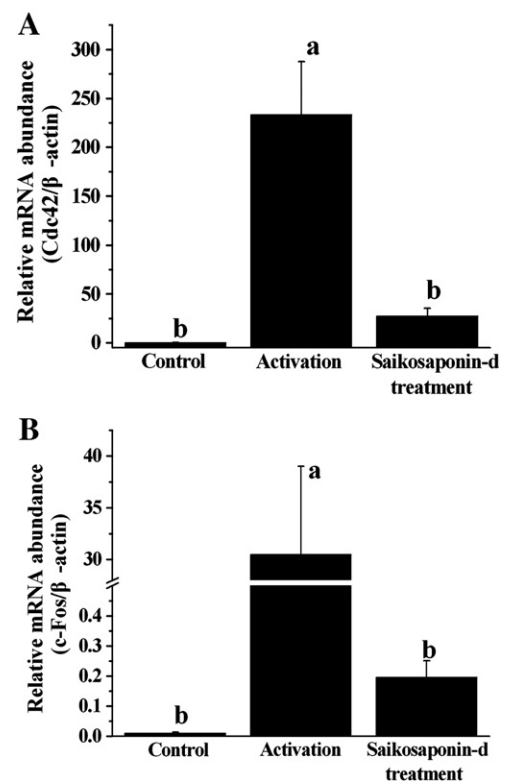


Fig. 6. Effects of saikosaponin-d on mRNA expression of signal genes Cdc42 (A) and c-Fos (B) after rat basophilic leukemia-2H3 cell activation. After saikosaponin-d treatment, sensitized rat basophilic leukemia-2H3 cells were stimulated at 37 °C with 10 μ g/mL β -conglycinin in tyrode buffer for 45 min. The cell groups are the same as those described in Fig. 2. Total RNA was prepared, and then real-time quantitative PCR was performed. Values are expressed as means $\pm$ SE, $n = 9$ for each treatment group. Values with different letters are significantly different $P < 0.05$.

source of the reactive oxygen species remains to be determined. We also observed that saikosaponin-d inhibited reactive oxygen species production during the process of mast cell activation. We therefore speculate that saikosaponin-d possesses anti-oxidative activity, meaning it could effectively remove free radicals which might in turn decrease β -hexosaminidase release.

Protein tyrosine phosphorylation is a key element in cellular signal transduction cascades and is therefore a highly regulated process. Cross-linking of Fc ϵ RI in rat basophilic leukemia-2H3 led to the phosphorylation of several proteins on tyrosine [48,49]. The results of this study also confirmed that rat basophilic leukemia-2H3 cells, which were activated through Fc ϵ RI, showed increased tyrosine phosphorylation which was the earlier signaling event. Tyrosine phosphorylation happened rapidly, in which the significant increase was detected 2 min after stimulation and had reached a maximum within 15 min (Fig. 5). Saikosaponin-d was found to suppress the tyrosine phosphorylation of proteins in activated rat basophilic leukemia-2H3 after 15 min of stimulation with β -conglycinin. We believe that saikosaponin-d might suppress the aggregation of Fc ϵ RI, the reversal of Fc ϵ RI, since cross-linking prevented continuation of the degranulation process and thus resulted in a rapid decrease of tyrosine phosphorylation.

Previous studies have indicated that Cdc42 is involved in Fc ϵ RI activated responses in the rat basophilic leukemia cells, including actin cytoskeleton re-distribution, calcium mobilization, degranulation and JNK activation [14,15]. No Cdc42 expression in rat basophilic leukemia cells inhibited Fc ϵ RI mediation of the degranulation response [50]. The expression of the proto-oncogene c-Fos plays an important role in the activation of Fc ϵ RI in rodent mast cells [17]. Upon activation of mast cells either by cross-linking the Fc ϵ RI or by the addition of calcium ionophore, the mRNA levels of c-Fos are elevated [51]. Our results also confirmed that Cdc42 and c-Fos were important downstream signaling genes for Fc ϵ RI-mediated mast cell activation and degranulation. Furthermore, we found that saikosaponin-d could significantly decrease the transcription levels of Cdc42 and c-Fos in activated rat basophilic leukemia-2H3 cells (Fig. 6). However, the regulation mechanism is unclear and remains to be resolved.

The results of the present study demonstrate that the inhibitory actions of saikosaponin-d on the production of ROS and the expression of cdc42 and c-Fos were strong. We speculate that saikosaponin-d may trigger the generation of ROS, which might in turn inhibit the expression of cdc42 and c-Fos. Previous studies support this hypothesis. The result of Li et al. [52] indicated that saikosaponin-d might enhance body antioxidant defense ability by inhibiting the production of free radicals. Wong et al. [53] pointed out that saikosaponin-d significantly inhibited c-Fos signaling pathways. Moreover, we think that the chemical structure of saikosaponin-d may play an important role in inhibiting the production of ROS. However, the precise mechanism through which it functions remains to be determined.

In conclusion, the present study demonstrated that there were multiple signaling pathways contributing to the development of β -conglycinin-mediated rat basophilic leukemia-2H3 cell activation. The intracellular calcium mobilization and tyrosine phosphorylation were early events, which in turn elicited reactive oxygen species production, gene activation of Cdc42 and c-Fos, and ultimately led to β -hexosaminidase release. Saikosaponin-d inhibited rat basophilic leukemia-2H3 cell degranulation by suppressing these critical incidents in the signal transduction pathway. These results suggest that saikosaponin-d exhibits anti-allergic activity, which could be used as a herbal therapy to treat soybean allergy.

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