



Inhibition of the antigen-induced activation of RBL-2H3 cells by sinomenine

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Abstract

The present study was designed to investigate the effect of sinomenine (SIN), an alkaloid extracted from *sinomenium acutum*, on the antigen-induced activation of RBL-2H3. For this investigation, the RBL-2H3 cells were sensitized with dinitrophenyl (DNP)-specific IgE overnight in 1.0 ml of Eagle's MEM (EMEM), and varying doses of SIN were added to the culture medium for 30 min and challenged with dinitrophenyl-human serum albumin (DNP-HSA) to induce mast cell degranulation before supernatants were collected. The effects of SIN on antigen-induced release of β -hexosaminidase were measured by enzymatic assay, calcium influx by FACS, cytokines by ELISA, and signaling events by immunoblotting. The results showed that treatment with SIN was followed by a decrease in Fc ϵ RI-mediated mast cell release of β -hexosaminidase, production of IL-4 and TNF- α , phosphorylation of Gab2 (Scaffolding adapter Grb2-associated binder 2), Akt and p38 mitogen-activated protein kinase (MAPK). In addition, SIN had no effect on the phosphorylation of LAT and no significant difference on calcium mobilization was observed between control and SIN treated group. These results suggested that SIN might suppress the antigen-induced activation of RBL-2H3 cells via a Ca²⁺ independent pathway.

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1. Introduction

Sinomenine (7,8-didehydro-4-hydroxy-3,7-dimethoxy-17-methylmorphinan-6-one) (SIN) is an alkaloid extracted from the Chinese medicinal plant *sinomenium acutum*. SIN has been successfully used for the treatment of various autoimmune diseases [1]. Previous studies have demonstrated

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that SIN has wide range of actions including arthritis amelioration, anti-inflammation and immunosuppression. For example, the alkaloid reduces the production of prostaglandin E₂ (PGE₂), leukotriene C₄ (LTC₄) and nitric oxide by macrophages [2,3]. Our previous study showed that SIN had suppressive effect on both Th1 and Th2 immune responses [4]. However, little is known about its effect on mast cells functions induced by antigen.

Studies have shown that mast cells played important roles in both immediate allergic reactions and late-phase reactions. It is believed that mast cell activation is regulated by aggregation of the high-affinity IgE receptors (FcεRI), which cause degranulation and release of cytokines that ultimately contribute to allergic responses. The rat basophilic leukemia cell line RBL-2H3, a tumor analog of mast cells, indicates phenotypic characteristics of mucosal mast cells. After stimulation with antigen, cells release β-hexosaminidase which is a marker of mast cell degranulation. In addition, it is relatively difficult to obtain higher and purified mast cells, so RBL-2H3 cells are considered to be a good model for studying comprehensive events on mast cells induced by multivalent allergen [5,6]. In order to demonstrate the effect of SIN on antigen-induced activation of mast cells, we selected the antigen DNP-specific IgE to stimulate RBL-2H3 cells, which is a classic way to study the effect of unknown compounds on the antigen-induced activation of mast cells. Our finding showed that SIN had suppressive effects on the release of β-hexosaminidase, IL-4, and TNF-α, and phosphorylation of Gab2, Akt and p38 MAPK, whereas it had no effect on phosphorylation of LAT, ERK and JNK. In addition, SIN had no obvious effect on calcium mobilization. Overall, our study revealed a new mechanism for the immunoregulatory function of SIN.

2. Materials and methods

2.1. Materials

The following materials were purchased from the indicated commercial sources: SIN (Tiancheng Drug and Bio-engineering Co., XIAN, China, >99% HPLC); Fetal bovine serum (FBS) and Eagle's minimal essential medium/EMEM (Gibco Life Technologies, Paisley, UK); G-Sepharose (Amersham Biosciences, Piscataway, NJ, USA); DNP-specific-IgE, DNP-HSA, Azelastine (Aze) and Methylthiazolotetrazolium (MTT) (Sigma, St. Louis, MO, USA); Rat IL-4 ELISA (BD Biosciences, San Jose, CA, USA); Rat TNF-α ELISA kit (BioSource, Camarillo, CA, USA); PD98059, anti-Phospho-Gab2 (Tyr452), peroxidase-conjugated anti-phosphotyrosine, Anti-Akt and anti-phospho-Akt (Ser473), anti-ERK and anti-phospho-ERK (Thr202/Tyr204), anti-phospho-p38 MAPK (Thr180/Tyr182) and anti-p38 MAPK, anti-phospho-JNK (Thr183/Tyr185) and anti-JNK (Cell Signaling Technology, Beverly, MA, USA); anti-Gab2 (Upstate Biotechnology, Charlottesville, VA, USA); anti-LAT (BD Biosciences, Franklin Lakes, NJ, USA); LY294002, SB203580, SP600125 (Calbiochem-Novabiochem, San Diego, CA, USA); Fluo-3/AM (Molecular Probes, Oregon, WA, USA); ECL chemiluminescence kit (Pierce, Rockford, IL, USA); RBL-2H3 cell line (American Type Culture Collection, Rockville, MD, USA).

2.2. Cell culture

RBL-2H3 cells were incubated overnight in 1.0 ml of EMEM containing 10% of (v/v) FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin. Cultures were maintained at 37 °C in a 5% CO₂ incubator. Cells were detached with trypsin–EDTA solution. After washing, the cells were resuspended in fresh medium and used for subsequent experiments.

2.3. MTT assay [7]

RBL-2H3 cells (0.3×10^5 /ml) were cultured with 200 ng/ml of DNP-specific IgE overnight. After 3 times of washing with PIPES buffer (119 mM NaCl, 5 mM KCl, 25 mM PIPES, 5.6 mM glucose, 1 mM CaCl₂, 0.4 mM MgCl₂, and 0.1% BSA, pH 7.2), they were pretreated with varying doses of SIN (0.5 mM, 1 mM, 2 mM, 4 mM) for 30 min. Cells were washed and then stimulated with 20 ng/ml of DNP-HSA for 2 h at 37 °C. Subsequently, the culture medium was changed to EMEM containing 10% FBS and cells were incubated for 24 h. The medium was removed and the cells were washed 3 times with EMEM. 200 μl EMEM supplemented with 10% FBS and 10% MTT (2.5 mg/ml) was added. After incubation for another 4 h, the formazan formed was dissolved in 150 μl of DMSO. Cell viability was determined by a microplate reader at 570 nm (BioRad 680 microplate reader, Hercules, CA, USA). The density of formazan formed in control (medium alone) cells was taken as 100% of viability.

2.4. β-Hexosaminidase secretion assay [8]

RBL-2H3 cells (1.5×10^5 /ml) were sensitized with 200 ng/ml of DNP-specific IgE overnight. After 3 times of washing with PIPES buffer, they were exposed to varying doses of SIN (0.5 mM, 1 mM, 2 mM) for 30 min at 37 °C. After incubation, the cells were challenged with 20 ng/ml of DNP-HSA for 30 min at 37 °C. At the end of the experiment, 20 μl of the supernatant was mixed with an equal volume of substrate solution (*p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide 2 mM in citrate 0.1 M, pH 4.5) for 1 h at 37 °C. The reaction was terminated by adding 200 μl of stopping buffer (0.1 M Na₂CO₃/NaHCO₃, pH 10.0). The absorbance was measured with a microplate reader at 405 nm (BioRad 680 microplate reader).

2.5. Calcium measurement [9]

RBL-2H3 cells were sensitized with 200 ng/ml of DNP-specific IgE overnight, and they (1.0×10^6 /ml) were resuspended and incubated with or without SIN (2 mM) in PIPES buffer for 30 min. Subsequently, cells were washed 3 times and loaded with 5 μM Fluo-3/AM for 1 h at 37 °C. After 3 times of washing, cells were stimulated with 20 ng/ml of DNP-HSA and Fluo-3 fluorescence was analyzed by a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Mean fluorescence intensity was calculated using CellQuest software.

2.6. Measurement of IL-4 and TNF-α [10]

RBL-2H3 cells were sensitized with 200 ng/ml of DNP-specific IgE overnight, and after 3 washes the cells (5.0×10^6 /ml) were incubated in 1.0 ml of PIPES buffer in the presence or absence of varying doses of SIN (0.5 mM, 1 mM, 2 mM) for 30 min. RBL-2H3 cells were then stimulated with 20 ng/ml of DNP-HSA for 2 h and the supernatant was used for ELISA.

2.7. Immunoblotting and immunoprecipitation [11]

RBL-2H3 cells were sensitized with 200 ng/ml of DNP-specific IgE overnight. After the antigen stimulation, 10^7 cells were washed and lysed in 100 μl of lysis buffer (20 mM HEPES, pH 7.3, 1% Triton X-100, 10% glycerol, 50 mM NaF, 0.1 mM EDTA, 10 μg/ml PMSF, 1 mM Na₃VO₄, 5 μg/ml leupeptin) on ice for 30 min. Lysates were centrifuged at $10,000 \times g$ and 4 °C for 10 min. About 20 μl (80 μg of total protein) of the supernatant was subjected to SDS-PAGE and immunoblotting with anti-Akt and anti-phospho-Akt, anti-ERK and anti-phospho-ERK, anti-phospho-p38 MAPK and anti-p38 MAPK, anti-phospho-JNK and anti-JNK. The second part of the supernatant was subjected to immunoprecipitation assay using anti-LAT, anti-Gab2 and G-Sepharose according to standard procedures. Immunoprecipitated proteins

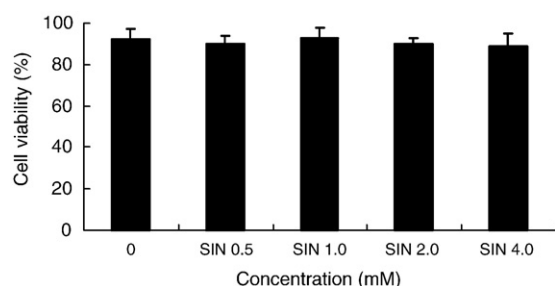


Figure 1 Cytotoxic effect of SIN on RBL-2H3. RBL-2H3 cells (0.3×10^5 /ml) were cultured with 200 ng/ml of DNP-specific IgE overnight. After treatment with varying doses of SIN (0.5 mM, 1 mM, 2 mM, 4 mM) for 30 min and stimulating with 20 ng/ml of DNP-HSA for 2 h, cells were cultured for 24 h. The cytotoxicity of SIN on RBL-2H3 cells was determined by MTT assay. Results are expressed as mean $\pm$ SD, Bonferroni's test.

were separated by SDS-PAGE and detected by western blot with anti-phosphotyrosine.

2.8. Statistical analysis

The data obtained were statistically evaluated using an one-way analysis of variance followed by Bonferroni's test; $P < 0.05$ was considered to indicate significance.

3. Results

3.1. Cytotoxicity effect of SIN on RBL-2H3

To investigate the effect of SIN on cell viability, the cells were exposed to SIN for 30 min. As shown in Fig. 1, the concentrations (0.5–4 mM) of SIN had no effect on the viability of RBL-2H3 cells compared with the control.

3.2. Effect of SIN on β -hexosaminidase release from RBL-2H3

As an indicator of the degranulation of RBL-2H3 cells, release of β -hexosaminidase into the supernatant was measured. SIN dose-

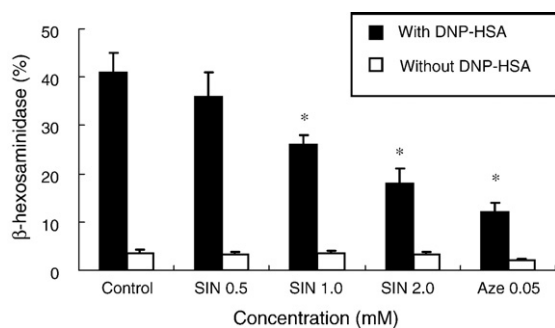


Figure 2 Effect of SIN on the β -hexosaminidase release from RBL-2H3 cells. RBL-2H3 cells (1.5×10^5 /ml) were sensitized with 200 ng/ml of DNP-specific IgE overnight and pretreated with varying doses of SIN (0.5 mM, 1 mM, 2 mM) for 30 min. Cells were stimulated with 20 ng/ml of DNP-HSA for 30 min. The absorbance was measured with a microplate reader at 405 nm. The indicated dose of Aze was given as a positive control. Results are expressed as mean $\pm$ SD, $*P < 0.05$ vs control group. Bonferroni's test.

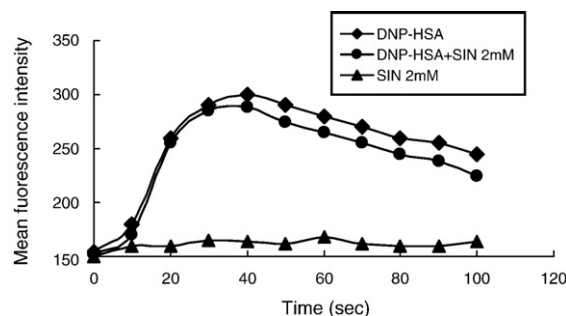


Figure 3 Effect of SIN on calcium mobilization. RBL-2H3 cells were sensitized with 200 ng/ml of DNP-specific IgE overnight. Cells (1.5×10^5 /ml) were treated with or without SIN (2 mM) in PIPES buffer for 30 min and loaded with 5 μ M Fluo-3/AM. After stimulating with 20 ng/ml of DNP-HSA, cells were analyzed by a FACScan flow cytometer. Mean channel fluorescence intensities were calculated using CellQuest software.

dependently reduced the release of β -hexosaminidase from RBL-2H3 stimulated with DNP-HSA (Fig. 2). Aze was selected as the positive control.

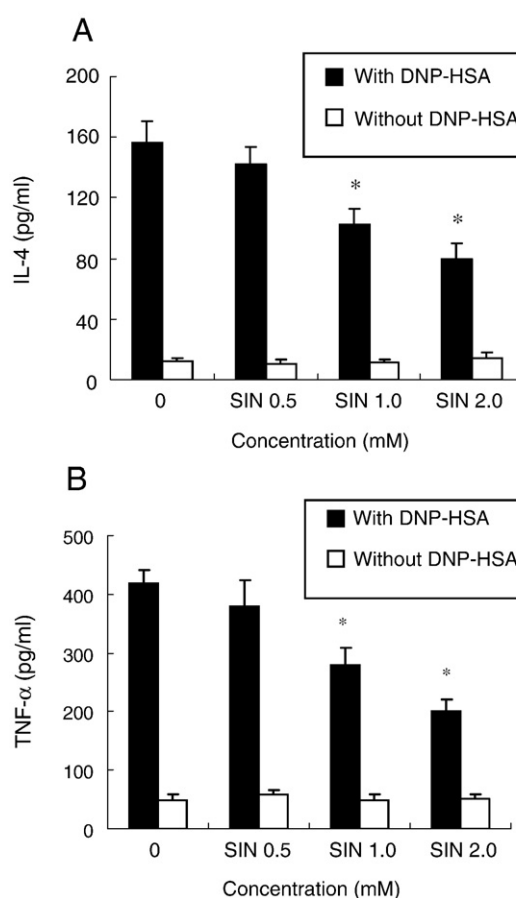


Figure 4 Effect of SIN on production of IL-4 and TNF- α . RBL-2H3 cells were sensitized with 200 ng/ml of DNP-specific IgE overnight. After washing, cells (5.0×10^6 /ml) were pretreated with varying doses of SIN (0.5 mM, 1 mM, 2 mM) in PIPES buffer for 30 min, and were then stimulated with 20 ng/ml of DNP-HSA for 2 h and the supernatant was used for ELISA. (A) IL-4; (B) TNF- α . Results are expressed as mean $\pm$ SD, $*P < 0.05$ vs control group. Bonferroni's test.

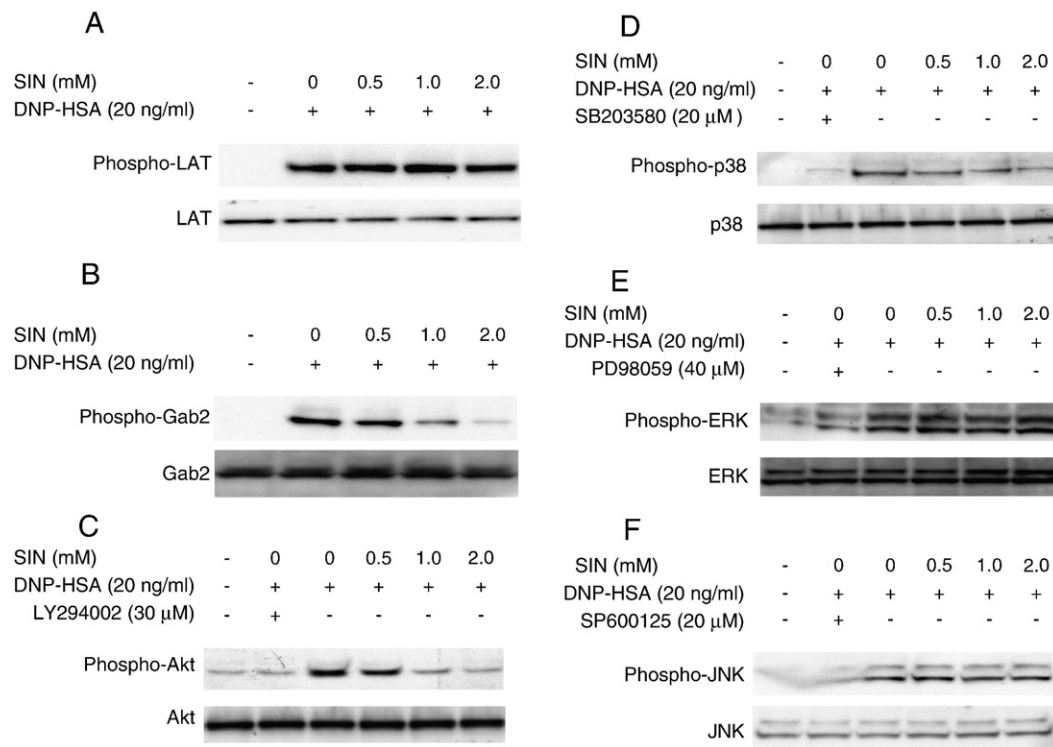


Figure 5 Effect of SIN on signal events in RBL-2H3 cells induced with DNP-specific IgE. RBL-2H3 cells were sensitized with 200 ng/ml of DNP-specific IgE overnight. After washing, cells were pretreated with varying doses of SIN (0.5 mM, 1 mM, 2 mM) in PIPES buffer for 30 min, and were then stimulated with 20 ng/ml of DNP-HSA. After adding lysis buffer, one part of the lysates was used for immunoprecipitation with (A) anti-LAT and (B) Gab2, and subjected to western blot with anti-phosphotyrosine. Another part of the lysate was directly subjected to SDS-PAGE and immunoblotting with the indicated antibodies. (C) Akt; (D) p38; (E) ERK; (F) JNK. LY294002, SB203580, PD98059 and SP600125 were selected as the positive controls for anti-Phospho-Akt, Phospho-p38, Phospho-ERK and Phospho-JNK, respectively.

3.3. Effects of SIN on intracellular Ca^{2+} level

The increasing level of intracellular Ca^{2+} is an important factor for degranulation in Ca^{2+} -dependent pathway. As shown in Fig. 3, the highest dose of SIN (2 mM) had no obvious effect on calcium mobilization induced by antigen.

3.4. Effect of SIN on secretion of IL-4 and TNF- α

As IL-4 and TNF- α play important roles in allergic diseases, the effect of SIN on the production of IL-4 and TNF- α was examined. Treatment with SIN resulted in significant suppression of secretion of IL-4 and TNF- α from RBL-2H3 cells (Fig. 4).

3.5. Effect of SIN on signaling events in RBL-2H3

LAT is essential for activation of $\text{PLC}\gamma$ activation which is important for increasing the intracellular Ca^{2+} level in the Ca^{2+} -dependent pathway. SIN could not change the level of LAT (Fig. 5). Gab2 and Akt are the key factors for degranulation in the Ca^{2+} -independent pathway and MAPK is related to the production of cytokines. SIN suppressed phosphorylated Gab2, Akt and p38 MAPK in a dose-dependent manner, whereas it had no effect on the phosphorylation of ERK and JNK.

4. Discussion

Reports have indicated that Fc ϵ RI-mediated signaling events in RBL-2H3 cells stimulated with antigen can be classified

into two independent but complementary pathways. The first way is Ca^{2+} -dependence, which is activated by Lyn/Syk/LAT axis and results in the activation of phospholipase $\text{C}\gamma$ ($\text{PLC}\gamma$), critically increasing the intracellular Ca^{2+} level. Action of Fyn/Gab2/PI3K (phosphoinositide 3-kinase) axis, the Ca^{2+} -independent pathway, also contributes an important role in the signaling pathway proximal to Fc ϵ RI [12].

In RBL-2H3 cells, antigen stimulation causes the activation of IP_3 , resulting in the release of Ca^{2+} from intracellular Ca^{2+} stores. Depletion of the calcium stores induces Ca^{2+} influx via CRAC channels to replenish the depleted Ca^{2+} stores. The Ca^{2+} influx is an important event for degranulation and cytokine production. The increase in Ca^{2+} influx is followed by the degranulation of mast cells and production of inflammatory mediators such as PGE_2 , LTC_4 and arachidonic acid. The linker molecule LAT is a substrate of the tyrosine kinases activated upon TCR engagement. It plays important roles in the Ca^{2+} -dependent pathway of Fc ϵ RI-mediated mast cell activation. Tyrosine phosphorylation of $\text{PLC}\gamma$ 1 and $\text{PLC}\gamma$ 2, calcium mobilization and degranulation were obviously reduced in LAT-deficient bone marrow-derived mast cells (BMMC) [13]. The data indicated that SIN markedly decreased the β -hexosaminidase release (Fig. 2). To investigate the mechanism of SIN on degranulation, we firstly investigated whether SIN could suppress the release of β -hexosaminidase through the Ca^{2+} -dependent pathway, but our results suggested that SIN could not influence the degranulation level via this pathway since it could not change the phosphorylation of LAT (Fig. 5A) and had no

significant effect on the calcium level (Fig. 3) stimulated with DNP-HSA.

Gab2 contains an N-terminal pleckstrin homology (PH) domain, several proline-rich motifs, and multiple tyrosyl phosphorylation sites. Studies have shown that Gab2 is a key component of FcεRI signaling in mast cells. Gu H et al. [14] indicated that the degranulation of mast cells stimulated with IgE was markedly impaired as well as the activity of downstream signaling molecules PI3K and Akt in Gab2-deficient BMMC. Perravicini V et al. [15] showed that degranulation was impaired which was related to the down-regulated phosphorylation of Gab2 in Fyn-deficient BMMC. Yu M. et al. [16] reported that Syk could activate Gab2 in a critical way, indicating an important role in the degranulation of RBL-2H3 cells. As a whole, Gab2 plays a critical and key role in the FcεRI-mediated degranulation response in the Ca²⁺-independent pathway. It transfers the signal from upstream to downstream molecules and leads to β-hexosaminidase release, chemical mediators and cytokines. In addition, a previous study proved that PI3K also played an important role in FcεRI-mediated activation of mast cells. PI3K is a downstream target of the Gab2. It is originally isolated as a heterodimeric enzyme which includes a regulatory subunit, p85α and a catalytic subunit, p110α. Akt is a downstream effector of PI3K and an index of PI3K activity. Phosphorylation of Akt is a significant event in mast cell proliferation and cytokine production. Gab2 can activate Akt by binding the p85 subunit of PI3K [17]. Activation of Akt was markedly impaired in Gab2-deficient BMMC [14]. Our results indicated that SIN might suppress the FcεRI-mediated mast cell degranulation via the Ca²⁺-independent pathway since treatment of SIN could lead to a decrease of phosphorylation of Gab2 and Akt.

It is well known that mast cells play important roles in Th2 response; they are critical responsive cells in Th2 and IgE-mediated immune response. The antigen-induced action of RBL-2H3 cells induces degranulation and production of Th2 cytokines including IL-4, IL-6 and IL-13, which are involved in immediate allergic response [17]. Studies have revealed that some factors are related to Th2 cytokine production in antigen-induced mast cell activation. Chan JH et al. [18] indicated that blocking of Gab2 with antisense oligonucleotide resulted in down-regulation of production of IL-4 in antigen-induced activation of RBL-2H3 cells. Hirasawa N et al. [19] found that the PI3-kinase inhibitors could partially decrease the expression of IL-4 mRNA and the production of IL-4 in RBL-2H3 cells induced by DNP-HSA. They also found that the specific p38 MAPK inhibitor SB203580 inhibited the antigen-induced expression of IL-4 mRNA. Frossi B et al. [20] demonstrated that Fyn/PI3K and p38 MAPK regulated the production of IL-4 in BMMC. These results implied that Gab2, PI3K and p38 MAPK might be involved in the production of IL-4 in antigen-induced activation of RBL-2H3 cells. Therefore, SIN may suppress the Th2 cytokine IL-4 (Fig. 4) by the down-regulation of phosphorylation of Gab2 (Fig. 5B), p38 MAPK (Fig. 5D) and Akt (Fig. 5C), an index of PI3K activity.

TNF-α is an important pro-inflammatory cytokine which plays a critical role in late-phase reactions of hypersensitivity. Production of TNF-α is decreased by blocking of Gab2 in RBL-2H3 cells [18]. Gu H et al. [14] proved that TNF-α was impaired in Gab2-deficient BMMCs stimulated with DNP-HSA. Kitaura J et al. [21] found that Akt regulated the FcεRI-

induced production of TNF-α in RBL-2H3 cells. According to the above data, the production of IL-4 and TNF-α were both down-regulated by Gab2 and Akt. In addition, the p38 MAPK may not be involved in the down-regulation of production of TNF-α in FcεRI-mediated cytokines production in RBL-2H3 cells [22]. Therefore, we could conclude that SIN suppressed the production of TNF-α via inhibition of phosphorylation of Gab2 (Fig. 5B) and Akt (Fig. 5C).

In summary, SIN might suppress degranulation and cytokines production in antigen-induced action of RBL-2H3 cells, perhaps through a Ca²⁺ independent pathway. SIN could not change the level of phosphorylation of LAT and had no significant effect on calcium mobilization, while it markedly suppressed the phosphorylation of Gab2 and Akt. In addition, SIN might inhibit the production of IL-4 by down-regulation of the level of phosphorylation of Gab2, Akt and p38, and TNF-α by decreasing the level of phosphorylation of Gab2 and Akt.

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