

Sopoongsan Inhibits Mast Cell-Mediated Anaphylactic Reactions and Inflammatory Cytokine Secretion

Ho-Jeong Na^a Hyun-Ja Jeong^{b,c} Hye-Young Shin^c Nam-Song Kim^d
Jae-Young Um^b Seung-Heon Hong^c Hyung-Min Kim^b

^aCollege of Pharmacy, Woosuk University, Jeonbuk, ^bCollege of Oriental Medicine, Kyung Hee University, Seoul, and ^cCollege of Pharmacy, Wonkwang University and ^dWonkwang University School of Medicine, Iksan City, South Korea

Key Words

Sopoongsan · Atopic dermatitis · Mast cells · Histamine · Passive cutaneous anaphylaxis · Cytokines

Abstract

Background: Mast cells are key effector cells in the early-phase allergic inflammation and in diverse immunological and pathological processes. Sopoongsan (SPS), a traditional Korean medicine, has been used as therapeutics for allergic diseases such as atopic dermatitis (AD). The precise effect in experimental models of SPS, however, remains unknown. In this report, we investigated the effect of SPS on mast cell-mediated anaphylactic reactions and cytokine production in in vivo and in vitro murine models. **Methods:** Compound 48/80-induced histamine and ear swelling were measured with the various concentrations of SPS. The amount of dye was determined colorimetrically after antinitrophenyl IgE antibody-induced passive cutaneous anaphylaxis reaction. Secretion of tumor necrosis factor- α (TNF- α), interleukin-8 (IL-8) and IL-6 in supernatants from HMC-1 cells was measured by a sandwich enzyme-linked immunosorbent assay. The expression level of nuclear factor (NF)- κ B/Rel A in the nucleus and the activation of mitogen-activated protein kinases (MAPKs) were exam-

ined by Western blot analysis. **Results:** SPS inhibited the degranulation and histamine release from the rat peritoneal mast cells activated by compound 48/80. Compound 48/80-induced ear swelling was significantly reduced. SPS also showed an inhibitory effect of passive cutaneous anaphylaxis reaction. Significantly reduced levels ($p < 0.05$) of TNF- α , IL-8 and IL-6 were observed in the human mast cell line with SPS and SPS components. In addition, SPS inhibited an increase of NF- κ B and extracellular signal-regulated kinase 1/2 activity. **Conclusions:** These findings suggest that SPS has an inhibitory effect on atopic allergic reaction and this might be useful for the clinical application to treat allergic diseases such as AD.

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Introduction

Sopoongsan (SPS) is a genuine Korean prescription drug, which has been used for the treatment of allergic diseases, such as atopic dermatitis (AD) in Korea. It is claimed to have medicinal value in allergic disorders in folk medicine, but its mechanism of action is poorly understood.

Mast cells participate in the pathogenesis of several inflammatory skin disorders such as AD. They are mono-

nuclear, granule-containing secretory cells that reside mostly in the skin, and which are increased in number in chronic AD lesions [1–3]. Histamine, the main constituent of granules in mast cells, has many effects related to the immediate phase of allergic inflammation, including vasodilation, increased vascular permeability, and tissue edema [3–5]. One of the well-used approaches in the examination of the immunopathological mechanisms of anaphylactic and inflammatory disorders is to elicit the formation of edema. The ear swelling test is traditionally predictive of dermal sensitization in humans using mice [6]. In recent years, it has been established that activated mast cells synthesize and release various cytokines and chemokines, of which the mast cell-derived tumor necrosis factor- α (TNF- α) is probably of particular importance in causing allergic inflammation [7]. In addition, mast cells have also been shown to produce interleukin-8 (IL-8) and IL-6. The release of these cytokines may be of major importance in the development of many inflammatory skin disorders [8].

Nuclear factor (NF)- κ B is a key transcription factor required for the expression of many inflammatory genes, including TNF- α , IL-8 and IL-6. The three major families of mitogen-activated protein kinases (MAPKs), c-JUN NH₂-terminal kinases (JNK), extracellular signal-regulated kinases (ERK), and p38 MAPK are regulated by distinct but cross-talking signaling cascades [9]. Such signals culminate in the activation of transcription factors such as NF- κ B [10].

In this study, the results suggest that SPS has an inhibitory effect on atopic allergic reaction and this might be useful for clinical application to treat allergic diseases such as AD through regulating cytokine production from downmodulating mast cell activation.

Materials and Methods

Reagents

Compound 48/80, *o*-phthalaldehyde, pulegone, 18 β -glycyrrhetic acid, glycyrrhizic acid, ergosterol, limonene, pinene, PMA and A23187 were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). IMDM was purchased from Gibco BRL, USA (Grand Island, N.Y., USA). Fetal bovine serum (FBS) was purchased from Life Sciences (Grand Island, N.Y., USA). Recombinant IL-6 and IL-8, biotinylated IL-6 and IL-8, and anti-human IL-6 and IL-8 were purchased from Pharmingen (San Diego, Calif., USA). Recombinant TNF- α , biotinylated TNF- α , and anti-human TNF- α were purchased from R&D Systems, USA. NF- κ B, JNK, ERK, p38 and phosphorylated antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., USA).

Preparation of SPS

Extract of SPS was prepared by decocting the dried prescription of herbs with boiling distilled water. The extraction decocted for approximately 3 h was filtered, lyophilized and stored at 4°C. The plant materials of SPS were obtained from the Oriental Drugstore (College Oriental Pharmacy, Iksan, South Korea). 25.2-gram of SPS ingredients included the following: *Schizonepetae herba* 3.75 g, *Glycyrrhizae radix* 3.75 g, *Ginseng radix alba* 1.87 g, *hoelen* 1.87 g, *Bombycis corpus* 1.87 g, *Cnidii rhizoma* 1.87 g, *Ledebouriellae radix* 1.87 g, *Agastache herba* 1.87 g, *Cicadidae periostracum* 1.87 g, *Rhizoma seu radix notopterygii* 1.87 g, *Aurantii nobilis pericarpium* 1.12 g, *Magnoliae cortex* 1.12 g, and *Folium camelae* 0.5 g.

Cell Cultures

The human leukemic mast cell line-1 (HMC-1) was maintained in IMDM with 10% FBS 37°C in 5% CO₂.

Animals

The original stock of ICR mice and Sprague-Dawley rats was purchased from the Damul Experimental Animal Center (Daejeon, South Korea). They were housed five to ten per cage in a laminar air-flow room maintained at a temperature of 22 $\pm$ 1°C and relative humidity of 55 $\pm$ 10% throughout the study.

Ear Swelling Response

Compound 48/80 was freshly dissolved in saline and injected intradermally into the dorsal aspect of a mouse ear using a microsyringe with a 28-gauge hypodermic needle. Ear thickness was measured with a digital micrometer (Mitutoyo, Japan) under mild anesthesia. Ear swelling response represented an increment in thickness above baseline control values, and was determined 40 min after compound 48/80 or vehicle injection (100 μ g/site). SPS was administered orally for 1 h before the compound 48/80 injection. The values obtained would appear to represent the effect of compound 48/80 rather than the effect of the vehicle injection (physical swelling), since the ear swelling response evoked by physiological saline returned to almost baseline thickness within 40 min.

Rat Peritoneal Mast Cell Preparation

Rat peritoneal mast cells (RPMCs) were isolated as previously described [11]. Mast cells were separated from the major components of rat peritoneal cells (i.e. macrophages and small lymphocytes) according to the method described by Yurt et al. [12]. RPMC preparations were about 95% pure as assessed by toluidine blue staining. More than 97% of the cells were viable as judged by trypan blue uptake.

Histamine Assay

RPMC suspensions (2 $\times$ 10⁵ cells/ml) were preincubated for 30 min at 37°C before the addition of compound 48/80 for stabilization. The cells were preincubated with the SPS for 30 min, and then incubated for 15 min with compound 48/80 (6 μ g/ml). The cells were separated from the released histamine by centrifugation at 400 g for 5 min at 4°C. The histamine content was measured by the *o*-phthalaldehyde spectrofluorometric procedure of Shore et al. [13]. The fluorescent intensity was measured at 440 nm (excitation at 360 nm) in a spectrofluorometer.

Passive Cutaneous Anaphylaxis Reaction

ICR mice were passively sensitized according to a method previously described [11]. They were injected intradermally into their dorsal skin with 0.5 µg of anti-DNP IgE, followed 48 h later by injection of 1 µg of DNP-HSA in PBS containing 4% Evans blue via the tail vein. After 40 min, the mice were sacrificed and reaction sites were excised for determination of extravasated dye. The amount of dye was then determined colorimetrically after extraction with 1.0 *N* KOH and a mixture of acetone and phosphoric acid based on the method Katayama et al. [14]. SPS was suspended in sterile saline at appropriate concentrations and administered orally for 1 h, before challenging with DNP-HSA.

TNF-α, IL-8 and IL-6 Assay

TNF-α, IL-8 and IL-6 in supernatants from HMC-1 cells (3×10^5 cells/ml, culture medium IMDM with 10% FBS) were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol. Absorption of the avidin-horseradish peroxidase color reaction was measured at 405 nm and compared with serial dilutions of human TNF-α, IL-8 and IL-6 recombinant as a standard.

Nuclear Protein Extraction

Preparation of crude nuclear extract was basically the same as described previously [15]. Briefly, after cell activation for the times indicated, 1×10^7 cells were washed in 1 ml of ice-cold PBS, centrifuged at 1,000 *g* for 5 min, resuspended in 400 µl of ice-cold hypotonic buffer (10 mM Hepes/KOH, 2 mM MgCl₂, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, and 0.5 mM PMSF, pH 7.9), left on ice for 10 min, vortexed, and centrifuged at 15,000 *g* for 30 s. Pelleted nuclei were gently resuspended in 50 µl of ice-cold saline buffer (50 mM Hepes/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, and 0.5 mM PMSF, pH 7.9), left on ice for 20 min, vortexed, and centrifuged at 15,000 *g* for 5 min at 4°C. Aliquots of the supernatant that contain nuclear proteins were frozen in liquid nitrogen and stored at -70°C. Protein was determined using a bicinchoninic acid protein assay method (Sigma, St. Louis, Mo., USA).

Western Blot Analysis

Cell extracts were prepared by detergent lysis procedure. Cells (5×10^6 cells) were scraped, washed once with PBS, and resuspended in lysis buffer. Samples were vortexed for lysis for a few seconds in every 15 min at 4°C for 1 h and centrifuged at 15,000 *g* for 5 min at 4°C. Supernatants were assayed. Samples were heated at 95°C for 5 min and briefly cooled on ice. Following the centrifugation at 15,000 *g* for 5 min, 50-µg aliquots were resolved by 10% SDS-PAGE. Resolved proteins were electrotransferred overnight to nitrocellulose membranes in 25 mM Tris, pH 8.5, 200 mM glycerol, and 20% methanol at 25 V. Blots were blocked for at least 2 h with $1 \times$ TBST containing 5% nonfat dried milk. Protein levels were analyzed essentially according to the manufacturer's instructions.

Statistical Analysis

The results were expressed as means $\pm$ SEM. The statistical significance was compared between each treated group and control with the independent *t* test. Results with *p* < 0.05 were considered statistically significant.

Results and Discussion

The inhibitory effect of SPS on degranulation of RPMCs was investigated after stimulation with compound 48/80 for 15 min. As shown in table 1, pretreatment of SPS inhibited degranulation of RPMCs by 35.5% compared with RPMCs not pretreated with SPS.

Among the preformed and newly synthesized inflammatory substances released on the degranulation of mast cells, histamine remains the best-characterized and most potent vasoactive mediator implicated in the acute phase of anaphylaxis. Histamine release from HMC-1 was found to be smaller than that from RPMC. Thus RPMC was chosen for the better investigation of the effect of SPS on histamine release.

RPMCs were preincubated with SPS at 37°C for 30 min prior to the treatment with compound 48/80. SPS significantly inhibited compound 48/80-induced histamine release from RPMCs, which showed 32.89 and 65.84% inhibition at the doses of 0.1 and 1.0 mg/ml, respectively (table 1).

Ear swelling response and passive cutaneous anaphylaxis reaction were performed to investigate the effect of SPS *in vivo*. In IgE-independent allergic disorders, intracellular calcium, cAMP and histamine release in murine mast cells were effectively stimulated by compound 48/80 [16]. Intradermal application of compound 48/80 at the dose of 100 µg/site can induce an ear swelling response in normal mice [17]. The same dose was chosen for compound 48/80-induced optimal ear swelling response in this experiment. As shown in table 1, when mice were pretreated with SPS for 1 h, the ear swelling responses derived from compound 48/80 were significantly reduced in a dose-dependent manner (*p* < 0.05).

In IgE-dependent allergic disorders, including anaphylaxis, allergic asthma, rhinitis and AD [18, 19], mast cell activation induces many of the acute changes observed. When orally administered for 1 h, SPS showed a marked inhibitory effect (35.4 and 41.0% at the doses of 0.1 and 1.0 g/kg) on passive cutaneous anaphylaxis reaction induced by anti-DNP IgE and DNP-HSA (table 1).

Mast cells are capable of synthesizing various cytokines [20]. TNF-α, IL-8 and IL-6 play crucial roles in the regulation of many dermal and epidermal genes that participate in the immune and inflammatory responses as well as chronic inflammatory disease [21, 22]. To assess the effect of SPS on PMA and A23187-induced TNF-α, IL-8 and IL-6 secretion, HMC-1 cells (3×10^5 cells/ml) were pretreated with various concentrations (0.01, 0.1 and 1.0 mg/ml) of SPS for 30 min prior to the stimulators.

Table 1. Effects of SPS on allergic reaction in the murine model

Dose of SPS g/kg	RPMC degranulation ^a %	Histamine release ^b inhibition, %	Ear swelling response ^c		PCA ^d	
			thickness of ear, mm	inhibition %	amount of dye, µg/site	inhibition %
None (DW)	73.87 ± 1.455		0.312 ± 0.034		2.678 ± 0.275	
0.01		11.10	0.236 ± 0.023	24.35	2.347 ± 0.538	12.35
0.1		32.89*	0.163 ± 0.010	47.75*	1.730 ± 0.242	35.40*
1.0	35.49 ± 0.009*	65.84*	0.130 ± 0.046	58.33*	1.580 ± 0.354	41.00*

Effects of SPS on RPMC degranulation, on compound 48/80-induced histamine release, on compound 48/80-induced ear swelling response, and on PCA in mice are shown. DW = Distilled water.

^a Inhibitory effect of SPS on degranulation of RPMCs stimulated with compound 48/80. Each point represents the means ± SEM of three experiments. * $p < 0.05$, significantly different from compound 48/80-stimulated value.

^b Effect of SPS on compound 48/80-mediated histamine release from RPMCs. RPMCs (2×10^5 cells/ml) were preincubated with SPS (0.01–1 mg/ml) at 37°C for 30 min prior to addition of compound 48/80 (6 µg/ml). Each point represents the means ± SEM of three experiments. * $p < 0.05$, significantly different from the saline value.

^c 20 µl of compound 48/80 (100 µg/site) were applied intradermally. The mice were orally administered with the various concentrations (0.01, 0.1 and 1.0 g/kg) of SPS for 1 h prior to the compound 48/80 application. Each datum represents the means ± SEM of three independent experiments. * $p < 0.05$, significantly different from the saline value.

^d Saline or SPS was applied orally 1 h prior to the challenge with antigen. Each amount of dye represents the means ± SEM of three independent experiments. * $p < 0.05$, significantly different from the saline value.

The cells were stimulated with PMA (50 nM) and A23187 (1 µM) for 8 h. TNF-α, IL-8 and IL-6 levels in culture supernatant were measured using ELISA. As shown in figure 1a, the secretion of TNF-α was dose dependently and significantly inhibited ($p < 0.05$) by the treatment of SPS. The inhibition rates were 61.41 and 63.03% at the doses of 0.1 and 1 mg/ml. IL-8 secretion was decreased 32.44% by the treatment of SPS (1 mg/ml). The secretion of IL-6 was significantly inhibited at the dose of 1.0 mg/ml of SPS ($p < 0.05$). The inhibition rate reached up to 74.8%. In an effort to reveal the components of SPS that inhibit mediators released from mast cells, we investigated some major components contained in the herbs constituting SPS. Well-known major chemical constituents of individual herbs include pulegone (*S. herba*), 18β-glycyrrhetic acid, glycyrrhizic acid (*G. radix*), ergosterol (hoelen), limonene (*A. nobilis pericarpium*), and pinene (*M. cortex*). To evaluate the effect of SPS components on PMA and A23187-induced TNF-α, IL-8 and IL-6 secretion, HMC-1 cells (3×10^5 cells/ml) were pretreated with various concentrations for 1 h prior to stimulators. The cells were stimulated with PMA (50 nM) and A23187 (1 µM) for 8 h. As shown in figure 1c–h, the components of SPS inhibited the TNF-α, IL-8 and IL-6

secretion. These components did not significantly affect the HMC-1 cell toxicity (data not shown). Maximal inhibition rates of these cytokine were 80% for TNF-α (at limonene 1 mM), 52% for IL-8 (at glycyrrhizic acid 50 µM), and 94.8% for IL-6 (at pulegone 1 mM). Even though inhibition rate with SPS components was higher than that with SPS, these effects are not the same as with SPS. Each component reduced one or two cytokines, but SPS significantly inhibited the secretion of all three cytokines (TNF-α, IL-8 and IL-6). Thus, the six components are believed to play important roles for SPS to regulate those inflammatory cytokines. Further studies need to be done to find additional active components.

The expression of inflammatory cytokines is known to be regulated by the transcription factor, NF-κB/*Rel A*. The effect of SPS on NF-κB/*Rel A* expression was investigated. HMC-1 cells (5×10^6 cells/ml) were treated with SPS (1 mg/ml) for 30 min and then stimulated with PMA (50 nM) and A23187 (1 µM) for 1 h. The expression level of NF-κB/*Rel A* in the nucleus was examined by Western blot analysis. In PMA and A23187-stimulated cells, the expression level of NF-κB/*Rel A* was increased, but it was decreased by SPS treatment (fig. 1b).

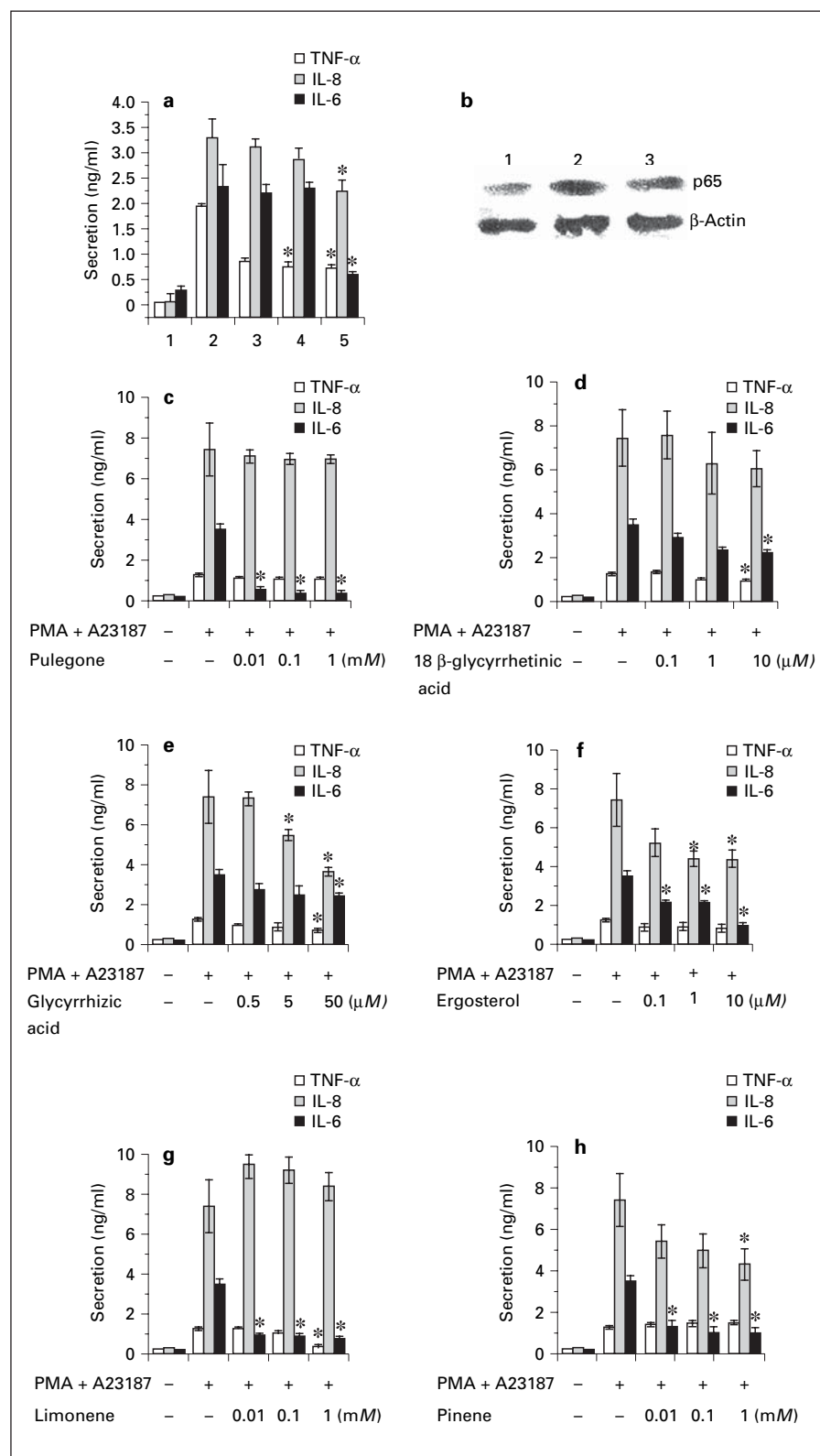
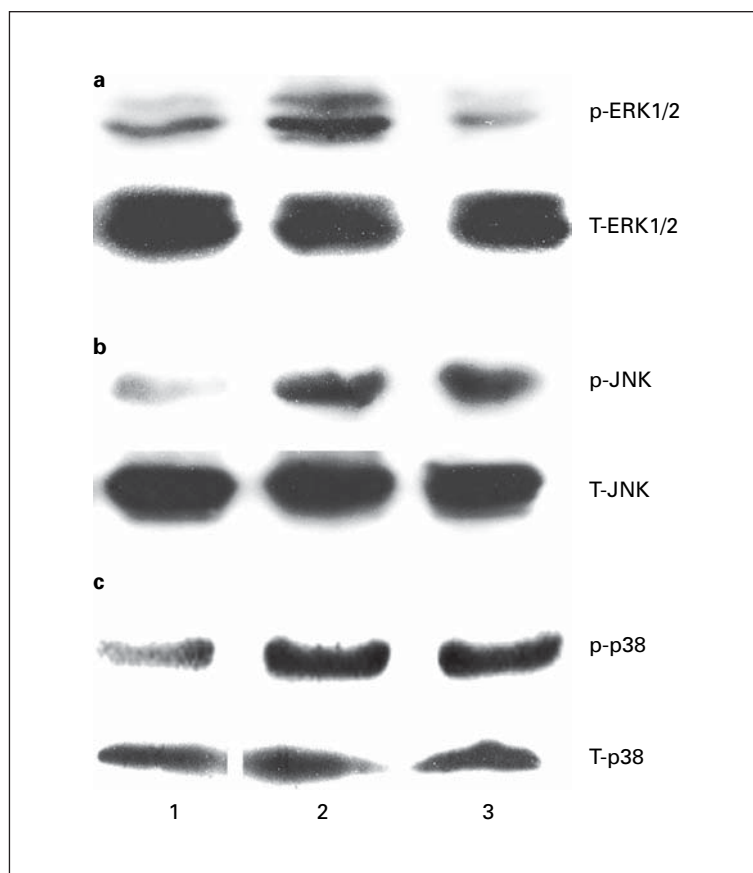


Fig. 1. Effect of SPS and SPS components on TNF-α, IL-8 and IL-6 secretion (**a, c–h**) and on NF-κB activation (**b**) from HMC-1. Cytokine levels in culture supernatant were measured using ELISA. **a** 1 = Unstimulated cells; 2 = PMA + A23187; 3 = SPS (0.01 mg/ml) + PMA + A23187; 4 = SPS (0.1 mg/ml) + PMA + A23187; 5 = SPS (1 mg/ml) + PMA + A23187. Each point represents the means ± SEM of three experiments. * $p < 0.05$, significantly different from PMA- and A23187-treated values. Nuclear protein was prepared and analyzed for NF-κB by Western blotting as described in the experimental procedures. **b** 1 = Unstimulated cell; 2 = PMA + A23187; 3 = PMA + A23187 + SPS.

Fig. 2. Effect of SPS on MAPK activation. HMC-1 cells (5×10^6 cells/ml) were treated with SPS (1 mg/ml) for 30 min and then stimulated with PMA (50 nM) and A23187 (1 μ M) for 15 min. Phospho-ERK1/2 (**a**), phospho-JNK (**b**), and phospho-p38 (**c**) were determined by Western blotting as described in the experimental procedures. 1 = Unstimulated cell; 2 = PMA + A23187; 3 = PMA + A23187 + SPS (1 mg/ml).



The MAPK signaling cascade plays an essential role in the initiation of inflammatory responses [23]. Suppression of MAPKs in mast cells may be a useful tool to reduce the mast cell number in the inflammatory response [24]. Investigation of the activation of JNK, ERK1/2, and p38 pathways, determined by assaying their phosphorylation, showed that ERK1/2 activation was inhibited by SPS, when stimulated with PMA and A23187 for 15 min (fig. 2a). However, phosphorylation of JNK and p38, induced by PMA and A23187, was not altered by SPS treatment (fig. 2b, c).

In conclusion, the results obtained in the present study provide evidence that SPS potently contributes to the treatment of atopic allergic reaction, and inhibits inflammatory cytokine production via NF- κ B and ERK1/2 pathway inhibition. Future studies on the isolation and characterization of the active chemical constituents are needed.

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