



Inhibitory Effects of *Paeonia suffruticosa* on Allergic Reactions by Inhibiting the NF-kappaB/IkappaB-alpha Signaling Pathway and Phosphorylation of ERK in an Animal Model and Human Mast Cells

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The root cortex of *Paeonia suffruticosa* Andrews (PSA), also known as Moutan Cortex, is known to have anti-allergic and anti-inflammatory properties. This study investigates the effect and mechanism of PSA by *in vivo* and *in vitro* methods. Treatments the root cortex from PSA with up to 0.4 mg/ml of an ethanol extract showed no cytotoxicity in human mast cells. The ethanol extract of PSA (200 mg/kg) significantly inhibited the passive cutaneous anaphylaxis reaction *in vivo*, and suppressed the release of histamine from rat peritoneal mast cells induced by compound 48/80. It was also found that PSA decreased the expressions of TNF-alpha and IL-6 in PMA- and A23187-stimulated HMC-1 cells. The results show the inactivation of IkappaB-alpha and NF-kappaB, as well as suppression of the phosphorylation of extracellular signal-regulated kinase (ERK). Our findings therefore suggest that PSA could be promising for anti-allergic inflammation by inhibiting the NF-kappaB/IkappaB-alpha signaling pathway and the phosphorylation of ERK.

Key words: *Paeonia suffruticosa* Andrews; allergic reaction; mast cells; ERK; NF-kappaB

The root cortex of *Paeonia suffruticosa* Andrews has been a widely used and important herbal medicine in Asia for a long time. PSA has been used to treat such diseases as atherosclerosis, infection, and inflammation as well as cutaneous disease.^{1,2)} Recent studies have mainly shown its effects on inflammation, neurotoxicity and oxidation,^{3,4)} whereas there are few reports on the anti-allergic effects of PSA.⁵⁾

Mast cells, as the key effector cell for immediate allergic reactions mediated by immunoglobulin E (IgE), express the high-affinity Fc receptor for IgE (FcepsilonRI) on their surface and release histamine and proinflammatory cytokines after crosslinking FcepsilonRI-bound IgE to the allergen.^{6,7)} Histamine, which has a key role in allergic inflammation, is mostly

released by mast cells and basophils and reaches a high extracellular level in generalized allergic events.⁸⁾ TNF-alpha, as an important mediator in many inflammatory events, is well known to be released from mast cells in IgE-mediated allergic reactions.⁷⁾ Interleukin (IL)-6 is also known to be produced by allergic inflammation-mediated activation in mast cells.^{9,10)}

FcepsilonRI-mediated signaling in mast cells has been revealed to activate mitogen-activated protein kinases (MAPKs), degranulation, and the release of proinflammatory cytokines *via* the LAT (linker of activated T cells) molecule.¹¹⁾ MAPKs of extracellular signal-regulated kinase (ERK), p38 and c-jun N-terminal kinase (JNK) are similarly activated in a LAT-dependent manner in mast cells, but the mechanism that regulates these responses is not fully understood.¹²⁾ Nuclear factor-kappaB is a transcription factor that is involved in regulating inflammatory responses, leading to enhanced the transcription of many genes important in inflammation.^{13,14)} Under the basal condition, NF-kappaB generally exists in the cytoplasm bound to inhibitory IkappaB protein, whereas, upon stimulation by which IkappaB is phosphorylated by MAPKs and degraded, NF-kappaB is translocated into the nucleus to bind specific elements in the upstream promoter region of such target genes as cytokines.¹⁵⁾ Inhibiting NF-kappaB and MAPKs activation are therefore important targets for an anti-inflammation treatment.¹⁶⁾

Materials and Methods

Preparation of PSA. PSA was purchased from Omniherb (Korea). The roots of PSA (100 g) were immersed in 1 liter of 80% ethanol and then sonicated for 30 min. The resulting extract was passed through a 0.22-μm filter and concentrated to approximately 100 ml under reducing pressure. The concentrated extract was then lyophilized, resulting in about 20.5 g of powder that was subsequently dissolved in DMSO to make a stock solution of 200 mg/ml. This stock solution was stored at -80 °C until needed.

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MTS assay. Cell proliferation was analyzed by the MTS assay as previously reported. In brief, HMC-1 cells were pretreated with different concentrations of the PSA extract (0.1, 0.2, and 0.4 mg/ml) for 1 h and then incubated for 24 h in the absence or presence of phorbol 12-myristate 13-acetate (PMA) plus calcium ionophore A23187. MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, was measured at 490 nm.

Animals. Male Spargue-Dawley rats were purchased from Orient Bio Experimental Animal Center (Korea). The animals were examined in compliance with Guide for Animal Experiments edited by the Korean Academy of Medical Sciences. The animals were housed in a laminar air flow room maintained at a temperature of $22 \pm 1^\circ\text{C}$ and relative humidity of $55 \pm 10\%$ throughout the study.

Preparation of rat peritoneal mast cells (RPMC). PSA of 200 mg/kg was orally administered 1 h prior to challenging with the antigen. The rats were anesthetized, and 10 ml of Tyrode buffer A (10 mM HEPES, 136 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 2.75 mM MgCl_2 , 5.6 mM glucose, 11 mM NaHCO_3 , 0.56 mM NaH_2PO_4 , and 0.1% bovine serum) containing 0.1% gelatin was injected into the peritoneal cavity. The cavity was carefully opened, and peritoneal cells were obtained and centrifuged at $150 \times g$ for 10 min at room temperature, before being resuspended in Tyrode buffer A. Mast cells were then isolated from the peritoneal cells according to the previous method,¹⁷⁾ and then assessed by toluidine blue staining.

Passive cutaneous anaphylaxis (PCA) reaction. Three dorsal skin sites of each rat that had been shaved earlier were intradermally injected with 0.5 μg (50 μl) of anti-DNP IgE. After 48 h, each rat was injected with 1 μg (50 μl) of DNP-human serum albumin (DNP-HSA) in PBS containing 4% Evans blue (1:4) via the dorsal vein of the penis. PSA (200 mg/kg of body weight) was orally administered 1 h before the reaction, and 30 min later, the rats were sacrificed and the dorsal skin was obtained to measure the pigment area. The amount of dye was colorimetrically determined after extracting with formamide. The intensity of absorbance of the extract was measured at 620 nm with a spectrophotometer (Beckman).

Histamine assay. The isolated mast cells of rats were resuspended in Tyrode buffer A for a treatment with compound 48/80. RPMC suspensions (4×10^5 cells/ml) were pre-incubated with PSA (0.2 mg/ml) and then induced with compound 48/80 (5 μg /ml). Each activated RPMCs was centrifuged at $150 \times g$ for 10 min at 4°C , and its supernatant was obtained. The amount of histamine released was measured by a histamine enzyme-linked immunosorbent assay (ELISA) test kit at 450 nm of optical density.

Productions of TNF-alpha and IL-6 by ELISA. 96-well plates were coated with 100 μl /well of anti-human TNF-alpha and IL-6 monoclonal antibodies in a 0.1 M sodium carbonate buffer at pH 9.5, and then incubated overnight at 4°C . After blocking and washing the wells, 0.2 μg /ml each of biotinylated anti-human TNF-alpha and IL-6 was added, and the mixture incubated for 1 h. Streptavidin-horseradish peroxidase, tetramethylbenzidine and a hydrogen peroxide substrate solution were added, and the mixture further incubated. The plates were incubated for 20–30 min in the dark, and the reaction was stopped by adding $2\text{NH}_2\text{SO}_4$, and the result measured at 450 nm.

Luciferase assay. NF-kappaB luciferase reporter genes (Stratagene) were transiently transfected into HMC-1 cells by using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. After 24 h of incubation, the cells were pretreated with PSA for 1 h and then stimulated with PMA plus A23187 for 24 h. After lysing with a luciferase assay kit (Promega), the luciferase activity was measured with a luminometer (Perkin Elmer) in accordance with the manufacturer's protocol.

Statistical analysis. Student's *t*-test was used for single variable comparisons, a *p*-value < 0.05 being considered as statistically significant. All data are presented as the mean $\pm$ SEM.

Results

Effect of PSA on the cytotoxicity and production of TNF-alpha and IL-6

An HPLC analysis was used to determine the standard quantity of paeoniflorin, as a standard compound, in the ethanol extract from PSA. The average content of paeoniflorin in PSA was calculated to be about 16.263 ± 0.994 mg/g (data not shown). In order to determine the influence of a PSA extract on the viability of human mast cells, HMC-1 cells were pretreated with PSA under conditions of induction with both PMA and A23187. Treatment with each PSA extract up to 0.4 mg/ml had no cytotoxic effect on cell viability (Fig. 1A). The next study evaluated the effect of PSA on the production of TNF-alpha and IL-6 in human mast cells. The protein level of TNF-alpha was dose-dependently decreased by the PSA pre-treatment (Fig. 1B). The release of TNF-alpha and IL-6 from the cells was also significantly reduced in response to PSA (Fig. 1C and D), suggesting that PSA inhibited the production of TNF-alpha and IL-6 in human mast cells without any significant cytotoxic effect.

Inhibitory effects of PSA on the histamine release and anti-DNP IgE-mediated PCA reaction

To confirm the effects of the PSA extract on the degranulation and histamine release, peritoneal mast cells from rats were induced by synthetic compound 48/80 and the histamine release assessed depending on the treatment with PSA. When stimulated with compound 48/80, the histamine release from the mast cells was approximately three times higher than the controls without induction. However, the PSA treatment decreased the histamine release by 12.3% (Fig. 2A, $p < 0.05$). Since an allergic reaction is characterized by the crosslinking of FcepsilonRI-bound IgE to the allergen in mast cells, an *in vivo* model of the anti-DNP IgE-mediated PCA reaction was investigated. Anti-DNP IgE was injected into the dorsal skin sites of rats to determine the effect of PSA on the allergic reaction, before injecting specific antigen of DNP-HSA containing Evans blue as an indicator. The PSA extract (200 mg/kg of body weight) was orally administered before injecting the antigen. Interestingly, about 51.5% of the IgE-mediated PCA reaction was significantly reduced after treating with PSA when compared to the control which was shown by a visual inspection and spectrophotometric quantification when extracted from the reaction site (Fig. 2B, $p < 0.05$).

Inhibitory effect of PSA by the inhibition of NF-kappaB/IkappaB-alpha and the phosphorylation of ERK in HMC-1 cells

The expression of ERK, p38 and JNK were analyzed to determine which MAPKs were modulated by PSA in an allergic reaction. A preliminary experiment to determine the experimental conditions showed that the phosphorylation of ERK was markedly increased 30 min after induction with both PMA and A23187 in HMC-1 cells, whereas the phosphorylation of p38 and JNK was not significantly increased (data not shown). Pretreatment with 0.2 mg/ml of the PSA extract then decreased the phosphorylation level of ERK 30 min after PMA and

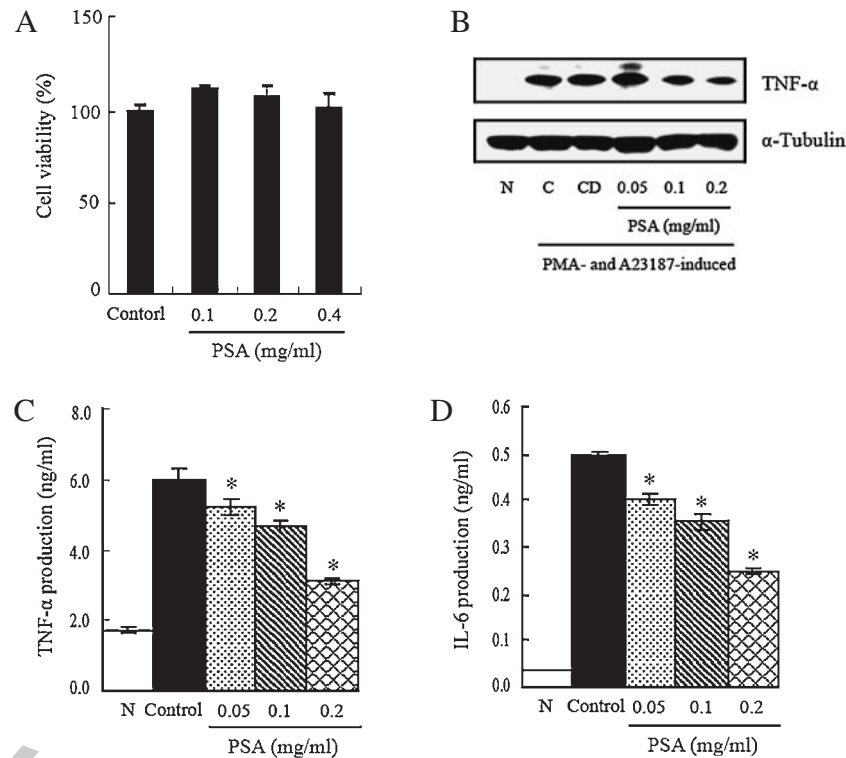


Fig. 1. Effects of PSA on the Cell Viability and Expression of TNF-α and IL-6.

A, HMC-1 cells for assessing cytotoxicity were pretreated with 0.1 to 0.4 mg/ml of PSA for 1 h and then incubated for 24 h. B–D, HMC-1 cells were pretreated with 0.05 to 0.2 mg/ml of PSA for 1 h and then incubated for 4 h with both PMA and A23187. The TNF-α protein levels in the cell lysate and supernatant were then respectively measured by a western blot analysis (B) and ELISA (C). The production of IL-6 was also determined by ELISA (D). Each value is presented as the mean ± SEM of three independent experiments (* $p < 0.05$). N, not-induced normal; Control and C, induced control; CD, induced control with the DMSO treatment.

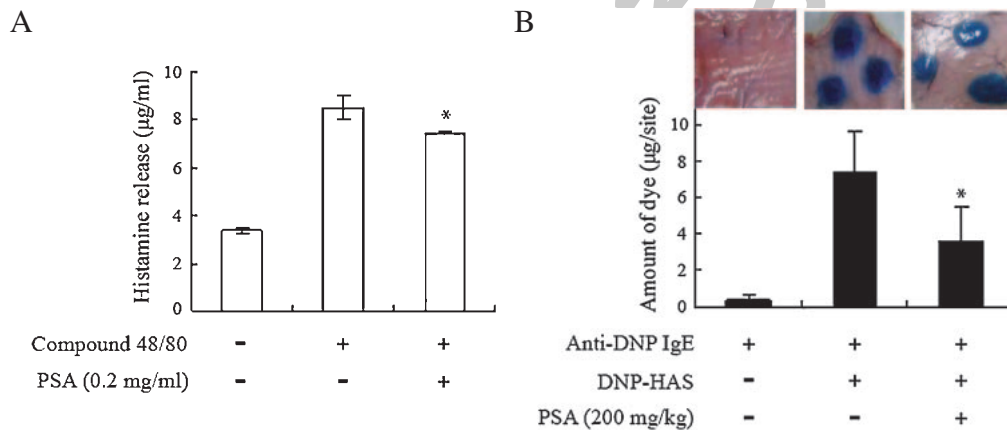


Fig. 2. Effects of PSA on the Histamine Release and Anti-DNP IgE-Mediated PCA Reaction.

A, RPMCs were pre-incubated with PSA at 37°C for 10 min prior to incubating with compound 48/80. The amount of histamine release was measured by ELISA. B, PSA was administered 1 h prior to challenging with the antigen. The amount of dye was extracted and measured by a spectrophotometer. Each value is presented as the mean ± SEM ($n = 3$) of three independent experiments (* $p < 0.05$).

A23187 induction, suggesting a possibility that PSA could modulate allergic reactions mainly through the ERK pathway. Additionally, the phosphorylation of p38 was found to be marginally reduced in response to 0.2 mg/ml of PSA, although there was no significant change in JNK (Fig. 3A). To elucidate the effect of PSA on NF-κappaB activation in human mast cells, the protein levels of cytosolic phosphorylated IκappaB-α and nuclear NF-κappaB after the PSA treatment were evaluated in HMC-1 cells. Both the degradation of IκappaB and subsequent translocation of NF-κappaB into the nucleus were inhibited by the PSA treatment

(Fig. 3B). In addition, the luciferase activity of NF-κappaB was significantly decreased with treatment by the PSA extract (Fig. 3C), suggesting that PSA exerted its inhibitory effect on allergic reactions by inhibiting the NF-κappaB/IκappaB-α signaling pathway and phosphorylation of ERK in the mast cells.

Discussion

Allergic inflammation is classified into early-phase (immediate) and late-phase reactions which subsequently result in chronic allergic inflammation. The immediate

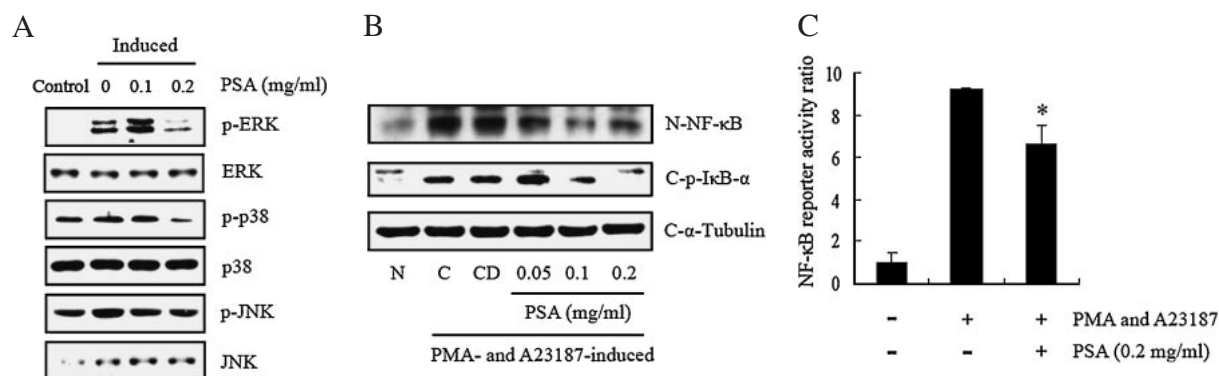


Fig. 3. Inactivation of ERK, NF-kappaB and IkappaB-alpha Phosphorylation by PSA.

A, HMC-1 cells were pretreated with PSA (0.1 and 0.2 mg/ml) before stimulating with PMA and A23187, and the expression of MAPKs was then determined after 30 min. Each total form of p38, JNK and ERK was used as a loading control. B, HMC-1 cells were pretreated with PSA for 1 h and then stimulated with PMA plus A23187. The levels of nuclear NF-kappaB and cytoplasmic p-IkappaB-alpha were determined by a western blot analysis. N-, nuclear extract; C-, cytosol extract. N, not-induced normal; C, induced control; CD, induced control with DMSO treatment. C, The NF-kappaB activity was examined by a luciferase assay in HMC-1 cells after pretreating with PSA for 1 h and then stimulating with PMA plus A23187. Each value is presented as the mean $\pm$ SEM of three independent experiments (* $p < 0.05$).

allergic reaction, which is characterized by mast cell activation through IgE bound FcepsilonRI aggregation, occurs within seconds to minutes after allergen exposure, leading to the release of mediators from mast cells, when compared to the late-phase reaction which is induced after several hours.^{6,7} The inhibitory effect of PSA on the immediate allergic response in rat peritoneal mast cells induced by compound 48/80, but not in human mast cells, has recently been studied.⁵ This present study further shows an inhibitory effect of PSA on early-phase response in human mast cells through the suppression of ERK (Fig. 3). The IgE-mediated PCA reaction *in vivo* is a method for studying the mechanism of the immediate allergy reaction and is also a sensitive method for detecting very small quantities of antibodies.¹⁸ This study is the first to show an inhibitory effect of PSA by the IgE-mediated PCA reaction, suggesting that PSA might be involved in the early-phase reaction of allergic inflammation.

The anti-allergic and anti-inflammatory activities of PSA by modulating histamine, TNF-alpha and NF-kappaB have been elucidated,^{19,20} whereas a more specific pathway for the effect of PSA has not yet been studied. Although integrated complex signaling pathways, including LAT (the linker for activating T cells) and NTAL (the non-T-cell activation linker) adaptors are involved in activating mast cells, this study has investigated the expression of MAPKs in advance. The results show that PSA significantly inhibited the activation of ERK, and also marginally p38 (Fig. 3A). In addition, we found that PSA blocked both the nuclear translocation of NF-kappaB and the phosphorylation of IkappaB-alpha (Fig. 3B). Although 0.1 mg/ml of PSA transiently increased the phosphorylation of ERK, 0.2 mg/ml of PSA clearly decreased the phosphorylation of ERK from the results of our repeated experiments. These results suggest that PSA exerted its anti-allergic activity mainly through inhibition of the NF-kappaB/IkappaB-alpha signaling pathway and subsequent phosphorylation of ERK, leading to suppression of the target genes. On the other hand, since selective conditions are also involved in mast cell activation,^{21,22} it can be expected that there might be other pathways along

which p38 MAPK could be inactivated by PSA. Further studies on upstream signaling pathways are needed to understand details of the mechanism modulated by PSA.

The production of several cytokines by allergic inflammation-mediated activation in mast cells has been reported.^{9,10} PSA and its major compounds, such as penta-O-galloyl-beta-D-glucose (PGG) and paeonol, have inhibited the production of IL-8 in human monocytic U937 cells,^{4,20} and IL-4 in B cells from mice.¹⁹ IL-6 is important in the acute-phase response, and inflammation and is also known to be induced by allergic inflammation-mediated activation in mast cells.^{9,10,23} Our results show that PSA significantly suppressed the production of IL-6 in PMA and A23187-induced HMC-1 cells, indicating that PSA might modulate the gene expression of IL-6 *via* reduced activation of NF-kappaB.

Mast cells are well known for their marked role in allergic responses. The modulation of allergic events in mast cells is complex, whereas the most effective stimulation of mast cells is IgE-mediated crosslinking to the allergen. A variety of environmental and chemical agents can lead to the activation of mast cells. Since compound 48/80, anti-DNP IgE and PMA/A23187 have been widely used for mast cell activation,^{5,19,24} mast cells were activated by each of these inducers to evaluate the effects of PSA under a variety of inducible conditions in this study. In the case of the PCA reaction, a dose of 200 mg/kg was the most effective among several tests of amounts. Even if the lyophilized PSA extract was dissolved in DMSO, no significant cytotoxicity was found in human mast cells. Moreover, there was no significant difference in the expression of TNF-alpha between the induced controls and an additional treatment of DMSO (Fig. 1B).

Several reports have shown that PSA and such active components as paeonol and PGG had a clinical effect on diseases including anti-allergic inflammation and neuronal damage.^{4,5,25} In addition, the chemical constituents of PSA have recently been analyzed by high-performance liquid chromatography coupled to electrospray ionization mass spectrometry.²⁶ When considering the cell viability, experimental results and clinical treatment

as a traditional medicine over a long period, PSA could be useful to relieve such allergic disorders as anaphylaxis, atopic dermatitis and asthma. In conclusion, the results of this study show that PSA inhibited inflammatory responses by regulating the NF-kappaB/IkappaB-alpha signaling pathway and the phosphorylation of ERK.

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