



Anti-allergic effects of scoparone on mast cell-mediated allergy model

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ABSTRACT

Scoparone is known to have a wide range of pharmacological properties *in vitro*. However, the roles of scoparone in immediate-type allergic reactions have not yet been investigated. In this study, we demonstrated that scoparone attenuated IgE-mediated allergic response in mast cells. Oral administration of scoparone inhibited passive cutaneous anaphylaxis in rats. Presence of scoparone dose-dependently decreased histamine release from rat peritoneal mast cells (RPMC) stimulated by anti-dinitrophenyl IgE. Moreover, scoparone reduced the expression and secretion of pro-inflammatory cytokines, such as tumor necrosis factor- α and interleukin-6 in RPMC. Pretreatment with scoparone inhibited the calcium uptake and p38 mitogen-activated protein kinase (MAPK) activity. Furthermore, scoparone blocked translocation of nuclear factor-kappa B (NF-κB) p65 subunit by suppressing IκB α phosphorylation in RPMC. Reduced calcium uptake as well as the suppressed activity of p38 MAPK and NF-κB might be involved in the inhibitory effect of scoparone on the secretory response. Our findings suggest that scoparone may serve as an effective therapeutic agent for allergic diseases.

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Introduction

Allergy, especially immediate hypersensitivity such as asthma, allergic rhinoconjunctivitis, atopic dermatitis and eczema, is a principal health problem, and the prevalence of allergy has increased during the past two decades. Mast cells play a key role in immediate-type allergic response through the release of a number of mediators and cytokines. The secretory response of mast cells can be induced by aggregation of their cell surface-specific receptors (FcεRI) for IgE by the corresponding antigen (Kim et al. 2004). Activated mast cells can produce histamine, as well as a wide variety of other inflammatory mediators such as leukotrienes, prostaglandins, proteases and several pro-inflammatory and chemotactic cytokines such as tumor necrosis factor (TNF)- α , and interleukins (IL)-6, IL-4, IL-13, IL-8 (Kalesnikoff and Galli 2008). Among them, histamine remains the best-characterized and most potent vasoactive mediator implicated in the acute phase of immediate hypersensitivity (Petersen et al. 1996).

The signaling pathway leading to degranulation of mast cells has been extensively characterized. Activation of mast cells leads to phosphorylation of tyrosine kinase, mobilization of internal Ca²⁺ and external Ca²⁺ influx. This is followed by activation of protein kinase C, mitogen-activated protein kinases (MAPKs) and transcription factor nuclear factor-kappa B (NF-κB) and releasing of inflammatory cytokines (Kalesnikoff and Galli 2008). MAPKs

and NF-κB have important activities as mediators of cellular responses to extracellular signals. Some of the MAPKs important to mammalian cells include extracellular signal regulated kinase (ERK), c-jun N-terminal kinase (JNK) and p38. p38 MAPK and NF-κB are thought to play an important role in the regulation of pro-inflammatory cytokines on cellular responses, especially TNF- α , IL-1b and IL-6 (Azzolina et al. 2003; Lorentz et al. 2003).

Coumarins comprises of a group of phenolic compounds widely distributed in natural plants. They have recently attracted much attention because of their broad pharmacological activities. Scoparone (6,7-dimethoxycoumarin) (Fig. 1), a derivative of coumarin, has been shown to have biological properties including immunosuppressive effects, vasodilation, lipid lowering activity, antioxidant and anti-inflammatory effects (Huang et al. 1991, 1993; Jang et al. 2006). It has also been documented to exert strong anti-asthmatic action in the guinea pig model (Fang et al. 2003). However, the direct effect of scoparone on IgE-mediated mast cell activation has not yet been reported. Thus, we investigated whether scoparone can inhibit IgE-mediated allergic response in mast cells and its mechanism of action. Our results will give an insight into the prevention or treatment of mast cell-dependent allergic diseases.

Materials and methods

Materials

Scoparone (purity 98%, HPLC), azelastine, anti-dinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA) and HEPES were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

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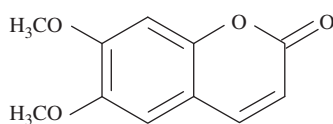


Fig. 1. Chemical structure of scoparone.

Percoll solution was purchased from Pharmacia (Uppsala, Sweden). Scoparone was dissolved in dimethyl sulfoxide (DMSO) and freshly diluted in HEPES-Tyrode buffer for all *in vitro* experiments.

Experimental animals

Male Sprague-Dawley rats (8 weeks old, 230–280 g each) were purchased from Damool Science (Daejeon, Korea). Animals were housed 3–5 per cage in laminar air-flow cabinet maintained at $22 \pm 1^\circ\text{C}$ and relative humidity of $55 \pm 10\%$ throughout the study. All animal experiments were performed in compliance with the NIH guidelines for the care and use of laboratory animals (NIH Publication No. 85–23; revised 1985) and were approved by the Institutional Animal Care and Use Committee of Yanbian University School of Medical Sciences.

Anti-DNP IgE-mediated passive cutaneous anaphylaxis (PCA)

Anti-DNP IgE-mediated PCA was examined as reported previously (Choi et al. 2006). Rats were sensitized in the right dorsal skin by the intradermal injection of 500 ng anti-DNP IgE in 50 μl PBS and were given a sham PBS injection in the left dorsal skin. Twenty-four hours later, the rats received into the penile vein an injection of 200 μl of PBS containing 100 μg DNP-HSA with 1% Evans blue. Scoparone [10, 25, 50 mg/kg body weight (BW)] was administered orally at 1 h before the antigen challenge. As a control, a mast cell-stabilizing agent, azelastine, was orally given at 1 h prior to the challenge with antigen. Thirty minutes after the challenge, the rats were killed by terminal anaesthesia, tissue sections around the intradermal injection site were excised and weighed, followed by extraction of extravasated Evans blue dye by incubation of biopsies in 1 ml formamide at 55°C for 24 h and measurement of absorbance at 620 nm using a spectrophotometer (Spectra MAX PLUS, Molecular Devices, CA, USA). Tissue Evans blue concentrations were quantified by interpolation on a standard curve of dye concentrations in the range of 0.01 to 30 $\mu\text{g}/\text{ml}$.

Preparation of rat peritoneal mast cells (RPMC) suspension

RPMC were isolated as described previously (Choi et al. 2006). Isolated RPMC were purified by using a Percoll density gradient as described in detail elsewhere (Hachisuka et al. 1988). RPMC preparations were at least 95% pure as assessed by toluidine blue staining and at least 98% of these cells were viable as judged by trypan blue exclusion. Purified mast cells (1×10^6 cells/ml) were resuspended in HEPES-Tyrode buffer.

Assay of histamine release

Mast cell suspensions (2×10^5 cells in 200 μl) were sensitized with 10 $\mu\text{g}/\text{ml}$ anti-DNP IgE for 6 h and preincubated with scoparone (25, 50 and 100 μM), the vehicle (0.1% DMSO, used as a control) or azelastine at 37°C for 30 min. The cells were stimulated with DNP-HSA (100 ng/ml) for 30 min. Following centrifugation at $150 \times g$ for 10 min, the amount of histamine in the supernatant was determined by the radioenzymatic method (Harvima et al. 1988). The inhibition percentage of histamine release was calculated using the following formula: Inhibition (%) = $(A-B) \times 100/A$

(A): histamine release without scoparone; (B): histamine release with scoparone

Assay of TNF- α and IL-6 secretion

RPMC were sensitized with 10 $\mu\text{g}/\text{ml}$ anti-DNP IgE for 6 h and then stimulated with 100 ng/ml DNP-HSA for 30 min with or without scoparone. TNF- α and IL-6 concentrations in the supernatant were determined by using commercial ELISA kits, according to the manufacturer's instructions (Invitrogen-Biosource Cytokine & Signaling, Camarillo, CA, USA).

Western blot analysis

RPMC extracts were prepared by lysis procedure described previously (Nunomura et al. 2006). Samples (30 μg of protein per lane) were loaded on a 12% SDS-PAGE gel. After electrophoresis at 120 V for 90 min, separated proteins were transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA) by the wet transfer method (250 mA, 90 min). The amount of TNF- α and IL-6 was determined using anti-TNF- α and -IL-6 antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The p38 MAPK, ERK and JNK activation was evaluated using anti-phospho-p38, -ERK and -JNK antibodies (Cell Signaling, Beverly, MA, USA). Immunodetection was done using enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Measurement of ^{45}Ca uptake

The calcium uptake of mast cells was measured according to the method described by Choi et al. (2006). Purified mast cells were resuspended in HEPES-Tyrode buffer containing ^{45}Ca (1.5 mCi/ml; $1 \text{ Ci} = 3.7 \times 10^{10}$ becquerels; Perkin-Elmer Life Sciences, MA, USA) and incubated at 4°C for 10 min. Mast cell suspensions were sensitized with 10 $\mu\text{g}/\text{ml}$ anti-DNP IgE for 6 h and preincubated with scoparone at 37°C for 30 min prior to the challenge with DNP-HSA (100 ng/ml). The reaction was stopped by the addition of 1 mM lanthanum chloride. The samples were centrifuged 3 times at $150 \times g$ for 10 min at 4°C , and then the mast cells were lysed with 10% Triton X-100 and vigorous shaking. Radioactivity of the solution was measured in a scintillation β -counter (Liquid Scintillation Analyzer, A Canberra Company, Australia).

Cytosolic and nuclear protein extractions for analysis of NF- κB

RPMC were sensitized with 10 $\mu\text{g}/\text{ml}$ anti-DNP IgE for 6 h and preincubated with scoparone or the vehicle at 37°C for 30 min prior to the challenge with DNP-HSA (100 ng/ml). The cells were harvested and washed twice with ice-cold PBS. The washed cell pellets were resuspended in 2 volumes of buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 0.5 mM DTT, 5 mM MgCl_2 , 1 mM PMSF and protease inhibitor cocktails). This suspension was centrifuged at $1,000 \times g$ for 15 min at 4°C . The supernatant fraction was incubated on ice for 10 min and centrifuged at $100,000 \times g$ for 1 h at 4°C to obtain cytosolic protein extracts. The pellets were washed twice in buffer A and resuspended in buffer B (1.3 M sucrose, 1.0 mM MgCl_2 and 10 mM potassium phosphate buffer, pH 6.8) and pelleted at $1,000 \times g$ for 15 min. The pellets were suspended in buffer B with a final sucrose concentration of 2.2 M and centrifuged at $100,000 \times g$ for 1 h. The resulting nuclear pellets were washed once with a solution containing 0.25 M sucrose, 0.5 mM MgCl_2 and 20 mM Tris-HCl, pH 7.2, and centrifuged at $1,000 \times g$ for 10 min. The pellets were solubilized

with a solution containing 50 mM Tris-HCl (pH 7.2), 0.3 M sucrose, 150 mM NaCl, 2 mM EDTA, 20% glycerol, 2% Triton X-100, 2 mM PMSF and protease inhibitor cocktails. The mixture was kept on ice for 1 h with gentle stirring and centrifuged at $12,000 \times g$ for 30 min. The resulting supernatant was used as soluble nuclear proteins. For Western blotting analysis, samples (30 μ g of protein per lane) were loaded on a 12% SDS-PAGE gel. After electrophoresis at 120 V for 90 min, separated proteins were transferred to polyvinylidene difluoride membranes by the wet transfer method (250 mA, 90 min). The NF- κ B and I κ B α activation was assayed using anti-NF- κ B p65 and anti-I κ B α antibodies (Upstate Biotech, Lake Placid, NY, USA). Immunodetection was done using enhanced chemiluminescence detection kit.

Statistical analysis

The results obtained were expressed as mean $\pm$ S.E.M. for the number of experiments. Statistical evaluation of the results was performed using one-way ANOVA, followed by Duncan's multiple range tests. Results with $p < 0.05$ were considered statistically significant.

Results and discussion

The PCA animal model used here is a well-established model for evaluating localized mast cell-mediated allergic reactions *in vivo*. PCA is reported to be induced by the rapid local release of inflammatory mediators such as histamine, serotonin and various cytokines from mast cells (Kabu et al. 2006). Our result has revealed that scoparone effectively suppressed the local allergic reaction in this model in a dose-dependent fashion (Table 1). In view of the inhibitory effect of scoparone on PCA *in vivo*, we examined its effect on release of preformed mediators from mast cells. In the scoparone-treated RPMC, histamine release induced by Fc ϵ RI stimulation was inhibited in a dose-dependent manner (Fig. 2). Accordingly, it is postulated that scoparone inhibits the immediate-type allergic reaction through interference with the degranulation system.

Activation of mast cells also stimulates cytokine release. Thus, we first determined whether scoparone could modify the protein expression of cytokines. Scoparone dose-dependently inhibited the antigen-stimulated protein expression for TNF- α and IL-6 in RPMC (Fig. 3). To confirm the above results, we next tested whether scoparone suppressed the secretion of both cytokines in the cells. In consistent with the Western blotting results, in the presence of scoparone, a dose-dependent suppression of the secretion of both cytokines was shown (Fig. 3). Pro-inflammatory cytokines, particularly TNF- α and IL-6, are known to play a central

biological role in triggering and sustaining allergic inflammation in mast cells (Nigo et al. 2006). TNF- α is a key mediator in many cytokine-dependent inflammatory events. It induces chemotaxis of neutrophils and T cells, enhances the cytotoxicity of macrophages and stimulates the expression of adhesion molecules from endothelial cells (Thomas 2001). IL-6 is one of the most important mediators of fever and acute phase response, and is secreted mainly by T cells, macrophages and mast cells (Hu et al. 2007). Moreover, its local accumulation is associated with a PCA reaction (Mican et al. 1992). From this, reduction of pro-inflammatory cytokines from mast cells is considered to be one of the key indicators of attenuated allergic symptom. Therefore, our findings propose that scoparone could suppress acute allergic inflammation through the regulation of cytokines production.

To gain insight into the mechanism of mast cell inhibition by scoparone, we investigated its effect on calcium uptake, MAPKs and NF- κ B activation in mast cells. Intracellular calcium response after Fc ϵ RI cross-linking on mast cells is supposed to occur in (at least) two steps: first one is calcium release from the intracellular store (mainly endoplasmic reticulum), and second one is calcium influx from the external environment, and the latter one is considered to be essential step for Fc ϵ RI-induced degranulation

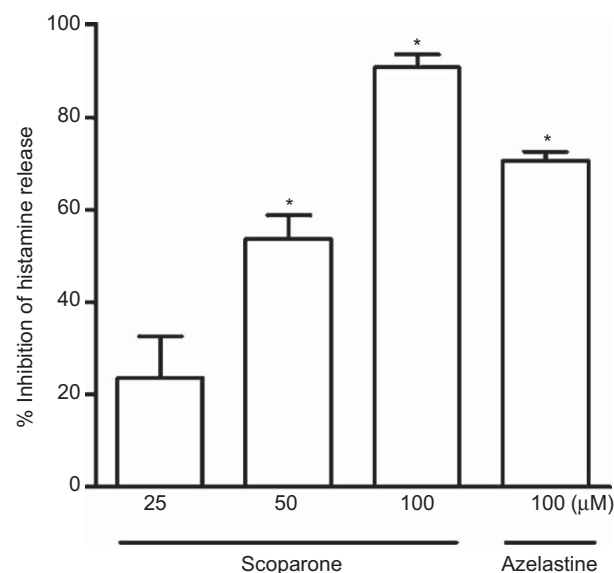


Fig. 2. Inhibitory effect of scoparone on anti-DNP IgE-mediated histamine release from rat peritoneal mast cells (RPMC). RPMC were preincubated with scoparone at 37 °C for 30 min prior to the incubation with DNP-HSA. Each data value represents the mean $\pm$ S.E.M. of five independent experiments. * $p < 0.05$, significantly different from the control value.

Table 1

Inhibitory effect of scoparone on passive cutaneous anaphylaxis in rats.

Treatment (mg/kg BW)	Anti-DNP IgE	Amount of Evans blue (μ g/g)	Inhibition (%) ^a
Scoparone			
0	–	46.71 $\pm$ 5.22	–
0	+	317.65 $\pm$ 28.43	–
10	+	299.26 $\pm$ 20.31	5.79
25	+	214.38 $\pm$ 15.80	32.51*
50	+	162.19 $\pm$ 12.57	48.94*
Azelastine			
50	+	189.52 $\pm$ 11.15	40.33*

Scoparone or azelastine was orally administrated at 1 h prior to the challenge with antigen (DNP-HSA). Each amount of Evans blue represents the mean $\pm$ S.E.M. of five independent experiments. ^a) Inhibition (%) = [(amount of Evans blue without scoparone – amount of Evans blue with scoparone) / amount of Evans blue without scoparone] $\times$ 100.

* $p < 0.05$, significantly different from the control value.

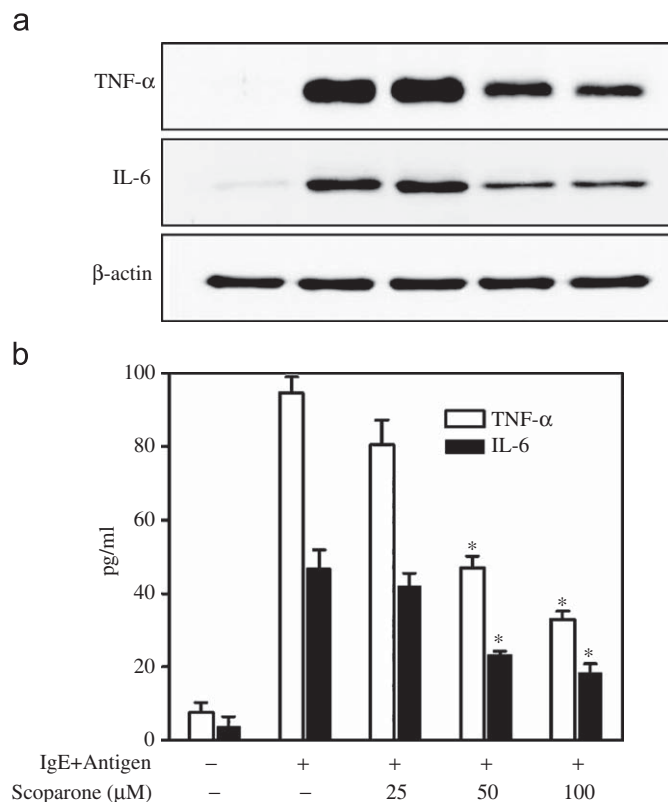


Fig. 3. Inhibitory effect of scoparone on the anti-DNP IgE-mediated protein expression and production of cytokines (TNF- α and IL-6) in rat peritoneal mast cells (RPMC). RPMC were sensitized with anti-DNP IgE and preincubated at 37 °C for 30 min in the absence or presence of scoparone before the challenge with DNP-HSA. The protein expression of these cytokines was measured by Western blotting. A representative result was presented among at least five separate experiments with similar results. The level of cytokine in the supernatant was measured by using ELISA. Each data value represents the mean $\pm$ S.E.M. of five independent experiments. * $p < 0.05$, significantly different from the control value.

(Shumilina et al. 2008). Thus, we measured calcium uptake using radiotracer assay. Activation of RPMC by IgE increased the levels of calcium uptake (Fig. 4). However, as expected from inhibition of degranulation and cytokine production by scoparone, scoparone markedly inhibited antigen-evoked calcium influx into RPMC. Based on this finding, it is inferred that scoparone inhibits mediator release through suppressing calcium uptake into mast cells.

The MAPK cascade is one of the important signaling pathways in immune response (Arbabi and Maier 2002). The exact signaling pathways among three types of MAPKs such as p38, ERK and JNK are still unclear; however, p38 are thought to play an important role in the regulation of inflammatory responses. Phosphorylation of p38 is essential for the expression of the pro-inflammatory cytokines (Ashwell 2006; Davis et al. 2004). According to the present results, antigenic stimulation activated all three types of MAPKs in RPMC (Fig. 5). Among the MAPKs, scoparone inhibited the activation of p38 but not ERK and JNK. Furthermore, the specific p38 inhibitor, SB203580 (10 μ M), decreased TNF- α and IL-6 secretion (Table 2). These data suggest that scoparone has the inhibitory activity on p38 activation and downstream TNF- α and IL-6 secretion.

Previous reports have demonstrated that NF- κ B plays a critical role in immune and inflammatory responses, including asthma and anaphylaxis (Siebenlist et al. 1994). Activation of NF- κ B has also been observed after Fc ϵ RI-mediated allergic response in mast cells. The NF- κ B is present in the cytoplasm and it is associated

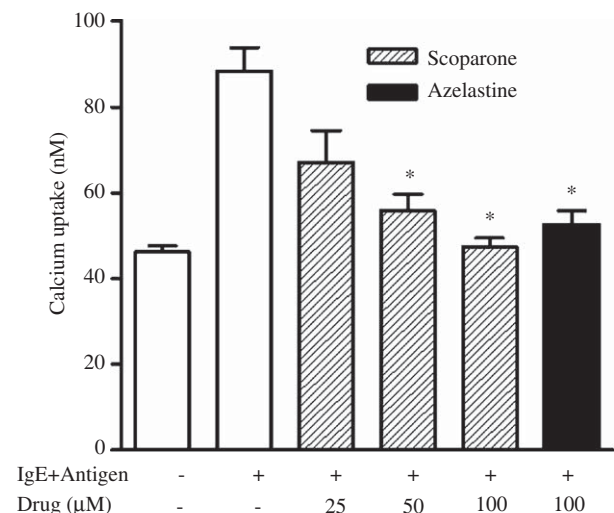


Fig. 4. Inhibitory effect of scoparone on anti-DNP IgE-mediated calcium uptake in rat peritoneal mast cells (RPMC). RPMC were sensitized with anti-DNP IgE and preincubated at 37 °C for 30 min in the absence or presence of scoparone before the challenge with DNP-HSA. Each data value represents the mean $\pm$ S.E.M. of five independent experiments. * $p < 0.05$, significantly different from the control value.

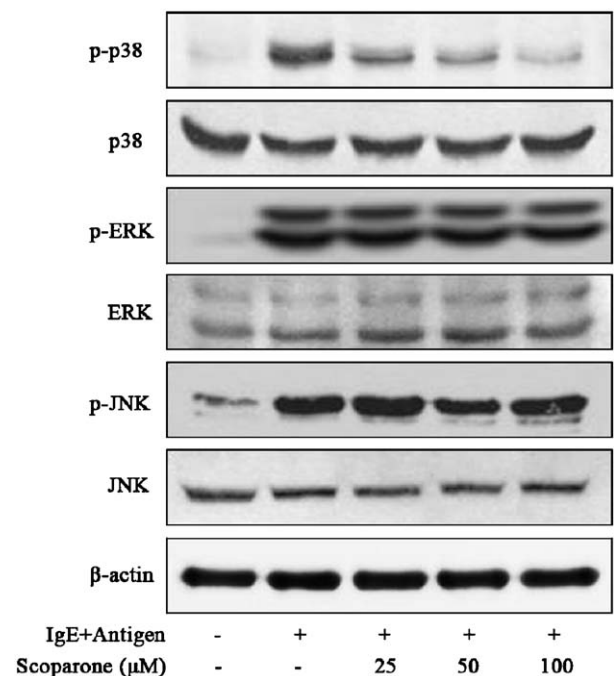


Fig. 5. Effect of scoparone on mitogen-activated protein kinases (MAPKs) activation in rat peritoneal mast cells (RPMC). RPMC were sensitized with anti-DNP IgE and preincubated at 37 °C for 30 min in the absence or presence of scoparone before the challenge with DNP-HSA. Phosphorylation of MAPKs was analyzed by Western blotting assay. A representative result was presented among at least five separate experiments with similar results.

with I κ B α , which is a NF- κ B inhibitory protein. Upon stimulation, I κ B α phosphorylates and undergoes subsequent proteolytic degradation, which ultimately results in the release of NF- κ B subunits from I κ B α . The released NF- κ B subunits translocate into the nucleus and then promote gene transcription, which is related with pro-inflammatory cytokines (Klemm and Ruland 2006). In the present study, scoparone inhibited the phosphorylation of I κ B α , which may result in the reduction of degradation of I κ B α

Table 2

Role of p38 and NF- κ B on anti-DNP IgE-mediated pro-inflammatory cytokine secretion from rat peritoneal mast cells (RPMC).

Treatment	Anti-DNP IgE	TNF- α (pg/ml)	IL-6 (pg/ml)
None	–	7.51 $\pm$ 2.86	3.88 $\pm$ 1.73
None	+	94.55 $\pm$ 4.35	46.82 $\pm$ 3.60
SB203580	+	56.23 $\pm$ 6.72*	21.94 $\pm$ 2.57*
PDTC	+	48.14 $\pm$ 2.19*	24.03 $\pm$ 1.66*

RPMC were sensitized with anti-DNP IgE and then were treated with SB203580 (10 μ M) or PDTC (10 μ M) for 30 min prior to the challenge with antigen (DNP-HSA). The level of cytokine in the supernatant was measured by using ELISA. Each data value represents the mean $\pm$ S.E.M. of five independent experiments.

* $p < 0.05$, significantly different from the control value.

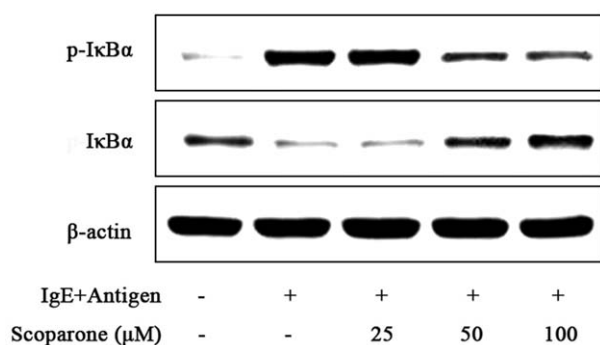


Fig. 6. Effect of scoparone on anti-DNP IgE-mediated NF- κ B activation in rat peritoneal mast cells (RPMC). RPMC were sensitized with anti-DNP IgE and preincubated at 37 °C for 30 min in the absence or presence of scoparone before the challenge with DNP-HSA. The levels of phosphorylated I κ B α (p-I κ B α) and I κ B α protein were determined by Western blotting assay. A representative result was presented among at least five separate experiments with similar results.

(Fig. 6). These observations encourage the view that scoparone may inhibit NF- κ B activity by preventing I κ B α degradation. Therefore, we next investigated the effect of scoparone on the activation of NF- κ B p65 subunit in IgE-stimulated RPMC. Determination of NF- κ B protein level in nuclear extracts revealed that this protein level was substantially increased in IgE-stimulated RPMC, suggesting that NF- κ B was activated (Fig. 7). However, the NF- κ B p65 levels in nucleus were decreased by the treatment of scoparone. In contrast, levels of NF- κ B p65 protein in cytosolic extracts from IgE-stimulated RPMC were decreased compared with the levels in the normal group. The NF- κ B p65 levels in cytosol preparations were increased by the treatment of scoparone. These results indicate that scoparone inhibits NF- κ B activity by preventing translocation of this transcription factor into nucleus. This suppression of NF- κ B translocation by scoparone may be due to inhibitory effect of scoparone on I κ B α phosphorylation. Although the different cell types were used, our results are in close agreement with those of Jang et al (Jang et al. 2006). Meanwhile, several evidences showed that expression of TNF- α and IL-6 is dependent on the activation of NF- κ B (Azzolina et al. 2003). In fact, our study has shown that PDTC, the potent inhibitor of NF- κ B, reduced anti-DNP IgE-mediated TNF- α and IL-6 secretion from RPMC (Table 2). Taken together, it is suggested that the inhibition of NF- κ B activity by scoparone modulates allergic inflammatory processes through suppression of the production of inflammatory cytokines.

In conclusion, our results provide evidence that scoparone can inhibit IgE-mediated allergic response in *in vivo* and *in vitro* murine

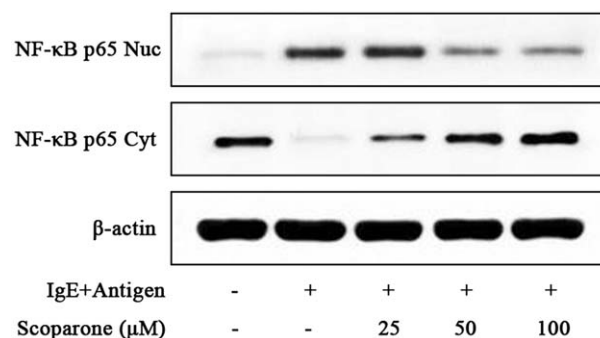


Fig. 7. Effect of scoparone on NF- κ B expression in nuclear and cytosolic protein extracts from anti-DNP IgE-stimulated rat peritoneal mast cells (RPMC). NF- κ B p65 expression in nuclear (Nuc) and cytosolic (Cyt) protein extracts from RPMC was shown. A representative result was presented among at least five separate experiments with similar results.

models. The inhibition of pro-inflammatory mediator release by scoparone appears to be involved in inhibition of p38 MAPK and NF- κ B activity as well as suppression of calcium uptake. Scoparone may therefore be suitable for the treatment of allergic and inflammatory diseases. Further studies are needed to elucidate the possibility that scoparone may also be effective in the human mast cells and in the treatment of human allergic disorders.

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