

Early Antiallergic Inflammatory Effects of *Rhus verniciflua* Stokes on Human Mast Cells

Myung Hee Hong,¹ Jeong-Hyun Kim,² Sea Yun Lee,¹ Ho Yeon Go,³ Ji Hye Kim,¹ Yong-Cheol Shin,¹ Sung-Hoon Kim^{2,4} and Seong-Gyu Ko^{1,2*}

¹Department of Preventive Medicine, College of Oriental Medicine, Kyunghee University, Republic of Korea

²Cancer Preventive Material Development Research Center (CPMDRC), College of Oriental Medicine, Kyunghee University, Republic of Korea

³Department of Oriental Internal Medicine, Semyung University, Chungbuk, Republic of Korea

⁴Graduate School of East-West Medical Science, Kyunghee University, Republic of Korea

Rhus verniciflua Stokes (RVS) is a traditional medicine used in Korea, Japan and China to treat various diseases including catharsis, diaphoretic gastritis and stomach cancer. However, the effects of RVS on allergic inflammatory diseases are unknown to date. This study showed the antiallergic inflammatory effects of RVS on human mast cells (HMC-1) which were stimulated by phorbol myristate acetate (PMA) and calcium ionophore A23187. RVS inhibited the expressions of TNF- α , IL-6 and IL-8 that were stimulated by treatment with both PMA and A23187. Among the mitogen-activated protein kinases (MAPKs), extracts of RVS suppressed the phosphorylation of ERK and p38, whereas RVS increased the phosphorylation of JNK in HMC-1. Consistent with the regulation of MAPKs, it was found that RVS inhibited the nuclear translocation of nuclear factor (NF)- κ B via inhibition of the phosphorylation of I κ B- α , which are important processes in controlling inflammatory responses. Taken together, these results suggest that RVS modulates the expressions of signal molecules related to allergic inflammatory responses mainly through the ERK signaling pathway, suggesting that RVS could be used as a treatment for mast cell-derived allergic inflammatory diseases. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: *Rhus verniciflua* Stokes (RVS); allergic inflammation; human mast cells (HMC-1); MAPK; cytokine.

INTRODUCTION

RVS is used as a traditional herbal medicine in Eastern Asia for the treatment of gastritis, stomach cancer and atherosclerosis (Kim, 1996). Several studies have shown that ethanol extracts of RVS have antioxidant effects, anticancer effects, antiproliferative activity and anti-inflammatory effects (Kim *et al.*, 2006; Jung *et al.*, 2006, 2007). In addition, the antiinflammatory effect of an ethanol extract of RVS in lipopolysaccharide (LPS)-stimulated Raw 264.7 macrophage cells has been reported recently (Jung *et al.*, 2007). However, the effect of RVS on mast cell-mediated allergic inflammatory responses has not been defined to date.

Mast cells are located in various tissues and in the mucosal membrane of most organs (Galli *et al.*, 2005a). As a result, mast cells play a central role in inflammatory and immediate allergic reactions. Mast cells are able to release potent inflammatory mediators including histamine, proteases, chemotactic factor and cytokines. Several recent studies have suggested that an important mechanism by which mast cells influence such biological responses is through the production of multifunctional cytokines such as tumor necrosis factor- α (TNF- α),

interleukin (IL)-6 and IL-8 (Galli *et al.*, 2005b, 2008; Bischoff, 2007; Thomas, 2001). Also, PMA/A23187 has been used widely for mast cell activation (Jiang *et al.*, 2007; Kim *et al.*, 2004; Strik *et al.*, 2007).

MAPKs constitute three major subfamilies, including extracellular signal-regulated kinase (ERK), p38 MAPK and c-jun N-terminal kinase (JNK). MAPKs are a widely conserved family of serine/threonine protein kinases that are involved in the mediation of intracellular signal transduction and regulating cytokine production in response to a variety of extracellular stimuli (Kyriakis and Avruch, 1996; Shapiro and Dinarello, 1997; Zu *et al.*, 1998). For this reason, inhibition of the activation of NF- κ B and MAPKs is an important target of antiinflammation treatment (Yamamoto *et al.*, 1999). NF- κ B is a transcription factor that is involved in the cellular response to stimuli such as stress, cytokines and free radicals (Collins and Cybulsky, 2001). Activation of NF- κ B requires phosphorylation and degradation of I κ B- α through the ubiquitin pathway, followed by translocation of NF- κ B to the nucleus. This, in turn, activates the transcription of specific target genes such as TNF- α , IL-6 and IL-8 (Jobin *et al.*, 1999; Karin and Ben-Neriah, 2000; Baldwin, 1996). Because NF- κ B controls many genes involved in inflammation, it is chronically active in many inflammatory diseases such as arthritis, asthma and sinusitis (Simmonds and Foxwell, 2008; D'Acquisto and Ianaro, 2006). Therefore, inhibition of NF- κ B activation is an important target of antiinflammation treatment.

* Correspondence to: Seong-Gyu Ko, Cancer Preventive Material Development Research Center (CPMDRC), College of Oriental Medicine, Kyunghee University, Seoul 130-701, Republic of Korea.
E-mail: epiko@khu.ac.kr

This study showed the effects of RVS on allergic inflammatory responses in PMA and A23187-stimulated human mast cells through the regulation of MAP kinases and inactivation of NF- κ B, which might be involved in the subsequent production of inflammatory cytokines.

MATERIALS AND METHODS

Materials. Iscove's modified Dulbecco's media for cell culture media was obtained from Gibco BRL. The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) was purchased from Promega (Madison, WI). NF- κ B, I κ B- α , phospho-I κ B- α and laminB primary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187 and α -tubulin primary antibody were obtained from Sigma Aldrich (St Louis, MO). Anti-human IL-6/IL-8 antibody, biotinylated anti-human IL-6/IL-8 antibody and recombinant human IL-6/IL-8 protein for an ELISA assay were purchased from BD Biosciences (San Diego, CA). In addition, anti-human TNF- α antibody, biotinylated anti-human TNF- α antibody and recombinant human TNF- α protein were obtained from R&D Systems (Minneapolis, MN).

Preparation of *Rhus verniciflua* Stokes extract. The RVS used in this study was purchased from Omniherb (Gyeong Buk, Korea). The roots of RVS (100 g) were immersed in 1 L of 80% ethanol and then sonicated (Kodo, Korea) for 30 min. The resulting extract was then filtered through a 0.22 μ m filter and concentrated to approximately 100 mL under reducing pressure. The ethanol extracts were dried in a 42 °C water bath with the use of a vacuum pump evaporator (Eyela, Japan). The concentrated extract was then lyophilized, resulting in 6.3 g powder that was subsequently dissolved in DMSO to make a stock solution of 200 mg/mL. The stock solution was stored at -80 °C until used.

Cell culture. The HMC-1 cells were maintained as monolayer cultures in IMEM (Gibco BRL Co) supplemented with 10% fetal bovine serum (Welgene, Korea), 100 unit/mL of penicillin and 100 μ g/mL of streptomycin (Gibco BRL) at 37 °C in a humidified incubator under 5% CO₂ gas.

MTS assay. Cell proliferation was determined by an MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay. Briefly, HMC-1 cells were pretreated with various concentrations of RVS extract (0.05, 0.1 and 0.2 mg/mL) for 1 h and then incubated for 24 h in the absence or presence of PMA plus A23187. After 24 h of incubation, MTS reagents were added to the culture. After 2 h of incubation, the absorbance at 490 nm was measured with a microplate reader (Molecular Device). The absorbance is correlated with the viability of cells, therefore the number of cells (% of control) was calculated using the following formula: cell number (% of control) = (absorbance of cells treated with herb extract - absorbance of blank well)/

(absorbance of control cells - absorbance of blank well) $\times$ 100. The control was the PMA plus A23187 stimulated mast cells without herb extract and the blank was an empty well.

Enzyme-linked immunosorbent assay. TNF- α , IL-6 and IL-8 production was measured by an indirect enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates were coated with 100 μ L/well of anti-human TNF- α , IL-6 and IL-8 monoclonal antibody in 0.1 M sodium carbonate buffer at pH 9.5 and then incubated overnight at 4 °C. After washing the wells, the plates were blocked with 10% FBS in PBS and then incubated at room temperature for 1 h. After additional washes, 100 μ L/well of cell medium or TNF- α , IL-6 and IL-8 standards were incubated at room temperature for 2 h. Next, the wells were washed with PBS containing 0.05% Tween 20 (PBST), and then 0.2 μ g/mL biotinylated anti-human TNF- α , IL-6 and IL-8 was added, after which the plates were incubated at room temperature for 1 h. The wells were then washed again with PBST, and then streptavidin-horseradish peroxidase was added, after which the plates were incubated for 30 min at room temperature. The wells were then washed with PBST, and tetramethylbenzidine and hydrogen peroxide substrate solution was added to each well. The plates were then incubated for 20-30 min in the dark, at which time color development was stopped using 2NH₂SO₄. Color development was measured using a microplate reader set to 450 nm. A standard curve was created using data generated by evaluating the absorbance of serial dilutions of recombinant TNF- α , IL-6 and IL-8. The inhibition percentage of cytokines production was calculated using the following equation: % inhibition = $(A - B) \times 100/A$, where A is the cytokine production without herb extract and B is the cytokine production with herb extract.

RNA extraction and reverse transcription-polymerase chain reaction. The cells were harvested by centrifugation at 4 °C and the pellet was then washed twice with ice-cold PBS. Next, the total cellular RNA was isolated using an easy-BLUE™ RNA extraction kit (iNtRON Biotech) according to the manufacturer's instructions. The concentration of the isolated total RNA was determined by spectrophotometry. For reverse transcriptase (RT) reactions, 2 μ g of total RNA was used as a template to synthesize cDNA. To accomplish this, total RNA was added to 2.5 μ g of random hexamers (Amersham Biosciences), incubated at 65 °C for 10 min and then cooled on ice for 2 min. The RT reaction was conducted in a 30 μ L solution that contained 2 units of M-MLV reverse transcriptase (Invitrogen) at 42 °C for 1 h, followed by heating at 95 °C for 5 min to stop the reaction. Polymerase chain reaction (PCR) was conducted in a 20 μ L reaction mixture consisting of DNA template, 10 pmol of each gene-specific primer, 10X Taq buffer, 2.5 mM dNTP mixture and 1 unit of Taq DNA polymerase (Takara). PCR was performed using the following primers for TNF- α (5'-TGAGCACT GAAAGCATGATCC-3', 5'-ATCACTCCAAAGTG CAGCAG-3'), IL-6 (5'-AACCTTTCCAAAGATG GCTGAA-3', 5'-CAGGAAGTGGATCAGGACTTT-3'), IL-8 (5'-TCAGTGCATAAAGACATACTCC-3', 5'-TGGCATCTTCACTGATTCTTG-3') and GAPDH (5'-CGTCTTACCACCATGGAGA-3', 5'-CGGCCA

TCACGCCACAGTTT-3'). The annealing temperature was 55 °C for TNF- α , IL-6, IL-8 and GAPDH. The resultant PCR products were resolved on a 2.0% agarose gel containing ethidium bromide.

Preparation of nuclear and cytosolic protein. The cells were washed with ice-cold PBS and then resuspended in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol (DTT) and 0.2 mM phenyl-methylsulfonyl fluoride (PMSF). The cells were then allowed to swell on ice for 5 min, after which they were centrifuged at 5000 rpm for 4 min. The pellet was then lysed with buffer B (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.1% NP-40, 0.5 mM DTT and 0.2 mM PMSF) and centrifuged at 5000 rpm for 5 min at 4 °C. The cytoplasmic proteins were then extracted from the supernatant, after which the pellet was resuspended in buffer C (20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF), incubated on ice for 30 min, and then centrifuged at 5000 rpm for 10 min at 4 °C. The supernatant that contained nuclear proteins was stored at -70 °C until used.

Western blot analysis. The harvested cells were lysed with buffer containing 40 mM Tris-HCl, pH 7.4, 10 mM EDTA, 120 mM NaCl, 1 mM dithiothreitol, 0.1% Nonidet P-40, 1 mM NaF, 1 mM Na₃VO₄, P1 cocktail and 1 mM PMSF on ice for 30 min, followed by centrifugation at 13000 rpm for 20 min at 4 °C. The supernatant

was then used to conduct a Bradford protein assay (Bio-rad). Next, the total proteins (20–40 μ g) were electrophoresed using 10–15% reducing polyacrylamide gels and immunoblot analysis was conducted using anti-phospho-ERK, anti-phospho-p38, anti-phospho-JNK, anti-ERK, anti-p38, anti-JNK, anti-NF- κ B, anti-I κ B- α , anti-phospho-I κ B- α anti-TNF- α and α -tubulin antibody with emission chemiluminescence-based detection.

Statistical analysis. All data are expressed as the mean $\pm$ standard error (SE). A Student's *t*-test was used for single variable comparisons and a value of *p* < 0.05 was considered to be statistically significant.

RESULTS

Decreased TNF- α in treatment of RVS in human master cell

In order to determine whether RVS prevents allergic effects in HMC-1 cells without inducing cytotoxicity, the viability of the HMC-1 cells following treatment with various concentrations of RVS was evaluated (Fig. 1A). HMC-1 cells were pretreated with RVS prior to treatments with PMA and A23187. As shown in Fig. 1A, treatments with up to 0.2 mg/mL RVS showed no effect on cell viability. Since TNF- α is a major cytokine

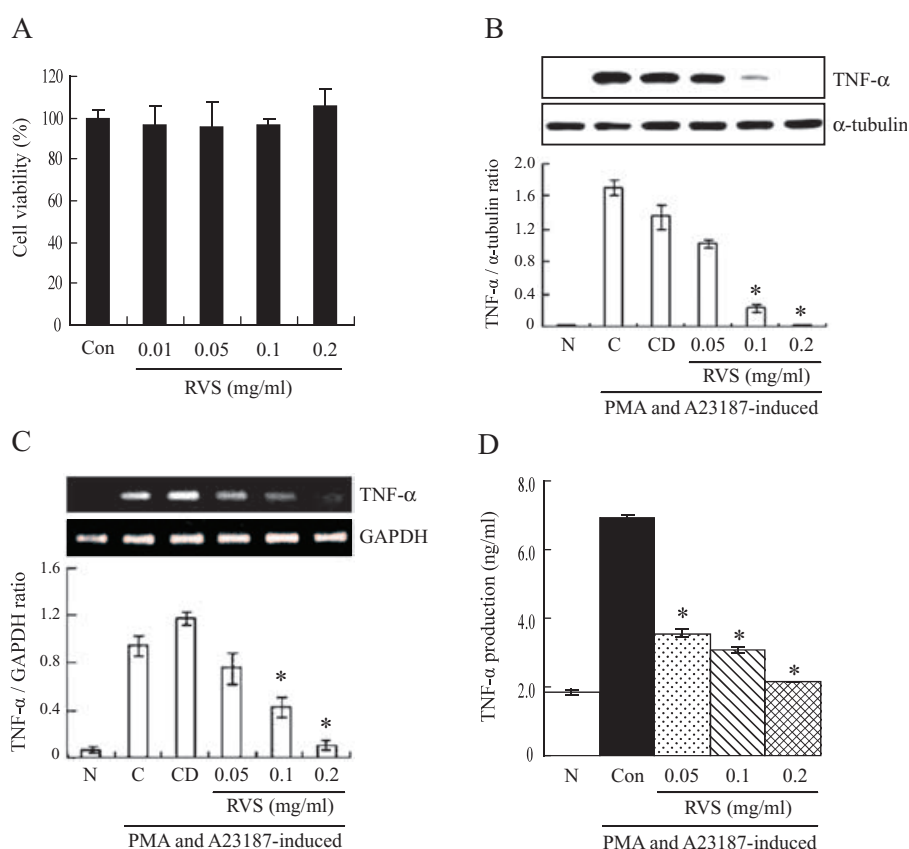


Figure 1. Effects of RVS on cell viability and TNF- α in human mast cells. Cell viability (A) in treatment with various RVS for 24 h was determined by MTS assay. HMC-1 cells were pretreated with RVS extract (0.01–0.2 mg/mL) and then incubated with PMA and A23187 for 4 h. The expressions of TNF- α were measured by immunoblot (B) and RT-PCR (C). The production of TNF- α (D) was determined by ELISA assay (D). N, not-induced normal; Con and C, induced control; CD, induced control with DMSO treatment. Each data point represents the mean $\pm$ SE of three independent experiments (**p* < 0.05).

for allergic responses, the study next evaluated whether the RVS extract has an effect on the production of TNF- α . Interestingly, RVS decreased significantly the expression of protein and mRNA in TNF- α in a dose-dependent manner, respectively (Fig. 1B and 1C). DMSO was used for cytotoxicity as a control. Consistently, the production of TNF- α from culture supernatants was decreased in response to RVS (Fig. 1D), indicating that RVS could suppress the production of TNF- α in human mast cells without cytotoxicity.

Inhibitory effects of RVS on IL-6 and IL-8

Since IL-6 and IL-8, as pro-inflammatory cytokines, are also induced during allergic responses, the effect of RVS on the expressions of IL-6 and IL-8 in HMC-1 was examined. When pre-treated with various concentrations of RVS, the release and mRNA expression of IL-6 from HMC-1 cells were significantly decreased in response to even 0.05 mg/mL of RVS (Fig. 2A and 2B). Although there was no dramatic change in mRNA expression of IL-8 (Fig. 2D), RVS still decreased significantly the secretion of IL-8 in PMA and A23187-stimulated HMC-1 cells (Fig. 2C). These results suggest

that RVS might exert its early anti-inflammatory effect through IL-6 rather than IL-8.

Effects of RVS on MAPKs in human mast cells

MAPKs are also important signals in allergic and inflammatory responses. To determine whether RVS regulates the molecules of MAPK and which pathways are involved, the phosphorylation of MAPKs was evaluated in RVS-pretreated and PMA and A23187-stimulated HMC-1 cells. In advance, the induction time of MAPK phosphorylation in the treatment of both PMA and A23187 was evaluated. Although all the MAPKs were induced within 30 min of treatment with PMA and A23187, the phosphorylation of ERK was characteristically increased (Fig. 3A). Furthermore, ERK and p38 were induced within the short time of 30 min, whereas the induction of JNK was prolonged up to 4 h. Based on the induction time, HMC-1 cells were then pre-treated with 0.05 mg/mL to 0.2 mg/mL of RVS and treated with PMA and A23187 for 30 min to evaluate the effects of RVS on MAPKs (Fig. 3B). Not only was the phosphorylation of ERK significantly reduced but also p38 MAPK was marginally decreased. However,

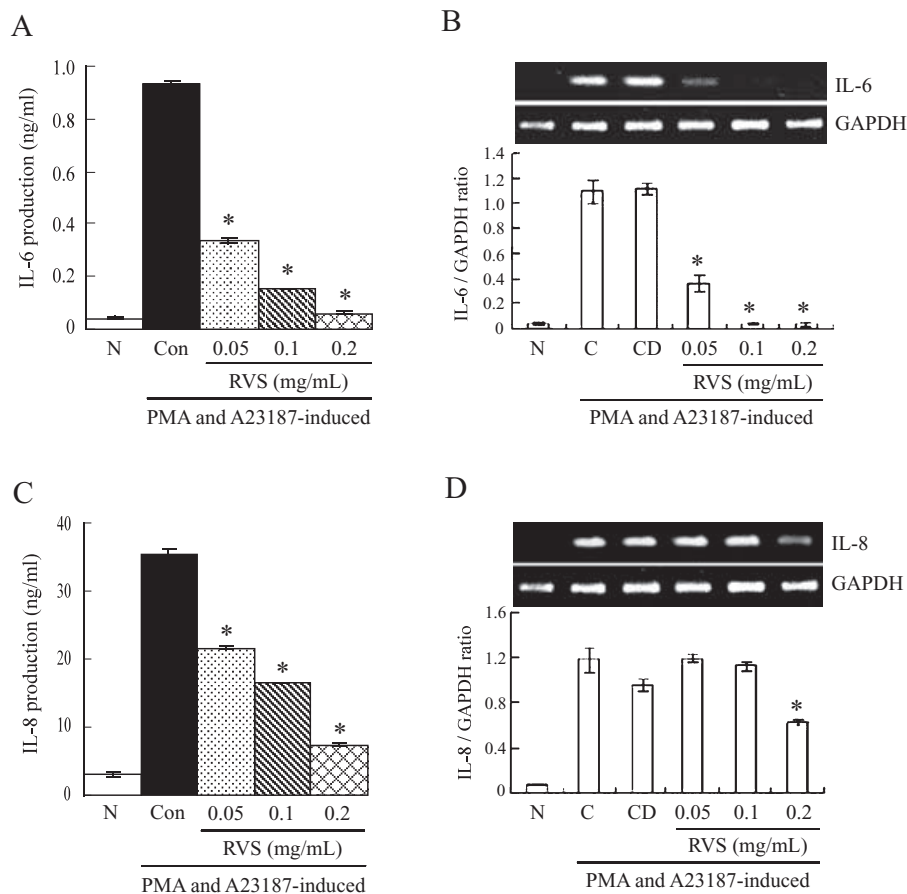


Figure 2. Reduced release and expression of IL-6 and IL-8 by inhibitory effect of RVS. Pretreatments of 0.05–0.2 mg/mL of RVS were followed by stimulation with PMA and A23187 in HMC-1 cells. The production and mRNA expression of IL-6 (A and B) and IL-8 (C and D) in treatment of RVS were measured by ELISA assay and RT-PCR analysis, respectively. N, not-induced normal; Con and C, induced control; CD, induced control with DMSO treatment. Each data point represents the mean $\pm$ SE of three independent experiments (* p < 0.05).

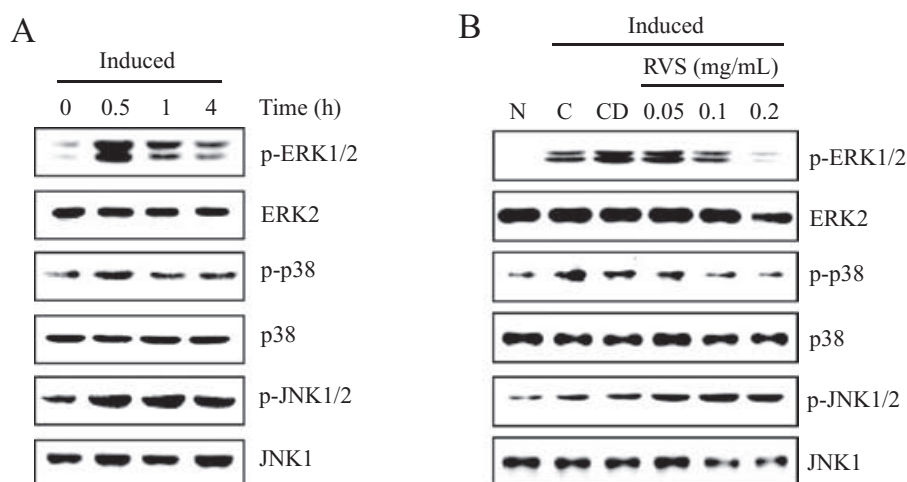


Figure 3. Inhibitory effects of RVS on MAPKs in HMC-1 cells. The time course of MAPKs in HMC-1 cells following treatment with PMA and A23187. After HMC-1 cells were stimulated for 0, 0.5, 1 and 4 h, the induction time of MAPKs was determined by immunoblot (A). HMC-1 cells were pretreated with RVS for 1 h and then stimulated with PMA and A23187 for 30 min. The phosphorylation of ERK, p38 and JNK was evaluated by immunoblot analysis (B). The total protein value of each MAPK was used as a control. N, not-induced normal; C, induced control; CD, induced control with DMSO treatment.

the phosphorylation of JNK was shown to be increased by RVS treatment.

RVS inhibited the translocation of NF- κ B

Since it is known that pro-inflammatory cytokines such as TNF- α , IL-6 and IL-8 were regulated by NF- κ B, the effects of RVS on the degradation of I κ B- α and following translocation of NF- κ B into the nucleus were examined. The level of NF- κ B in the nucleus was decreased in response to treatment with RVS. Consistently, the phosphorylation of I κ B- α in the cytosol extracts was also inhibited by RVS in HMC-1 cells (Fig. 4). LaminB and tubulin were used as controls of nuclear and cytosolic proteins, respectively. These results suggest that RVS inhibits the production of TNF- α , IL-6 and IL-8 through inactivation of NF- κ B.

DISCUSSION

RVS has been used traditionally to treat many diseases including stomach cancer, arteriosclerosis and gastritis. Additionally, it was reported that RVS acted as a scavenger of reactive oxygen species (ROS) (Lim *et al.*, 2001; Kim, 1996; Jung *et al.*, 2007; Lee *et al.*, 2002). These facts indicate that an ethanol extract of RVS has a wide range of effects including antioxidant, antiproliferative activity and antiinflammatory effects. In the present study, the effect of RVS on antiallergic inflammatory responses was identified. The findings demonstrated that RVS inhibited the expressions of TNF- α , IL-6 and IL-8 through the inactivation of NF- κ B in human mast cells that were stimulated by treatment with PMA and A2187. In addition, this study showed that RVS extracts exert its early antiinflammatory effect mainly through ERK and NF- κ B pathway in mast cells, leading to a regulation of target genes and the subsequent induction of pro-inflammatory cytokine. These findings suggest that RVS could be used as an additional antiallergic inflammatory medicine.

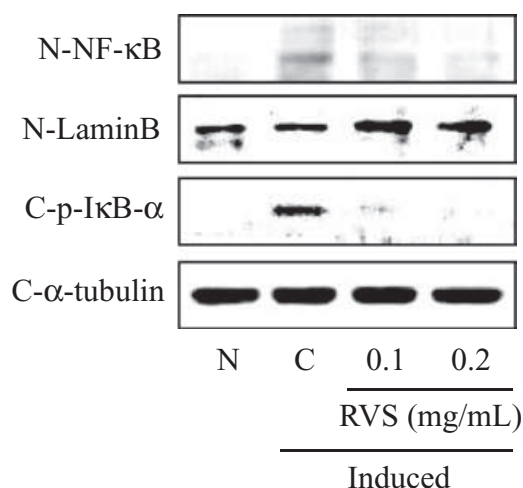


Figure 4. Inactivation of NF- κ B and I κ B- α by RVS. HMC-1 cells were pretreated with RVS (0.1–0.2 mg/mL) for 1 h and then stimulated by PMA and A23187. Nuclear and cytoplasmic proteins were separately isolated and then measured for the levels of NF- κ B and p-I κ B- α by immunoblot. LaminB and α -tubulin were used as controls for nuclear and cytoplasmic proteins, respectively. N-, nuclear extract; C-, cytosol extract. N, not-induced normal; C, induced control.

Mast cell-derived pro-inflammatory cytokines, such as TNF- α , IL-6 and IL-8, have been known to play an important role in allergic inflammation. In this study, RVS showed a specificity to changes in TNF- α and IL-6 rather than IL-8, indicating that RVS might participate in the early response of allergic inflammation. These results could be explained by the fact that TNF- α and IL-6 are involved in IgE-mediated acute reactions, whereas IL-8 is a mediator for neutrophil-mediated transition to late-phase reaction (Galli *et al.*, 2008; Bischoff, 2007; Kempuraj *et al.*, 2002; Lippert *et al.*, 2000). Furthermore, the productions of IL-6 and IL-8 were found to be mediated by IgE and adenosine A2B receptor, respectively (Feoktistov *et al.*, 1999; Boudreau *et al.*, 2004; Wong *et al.*, 2006). Therefore, our results that production and expression of TNF- α , IL-6 and IL-8 were significantly decreased in HMC cells following treatment with RVS gives strong evidence that RVS

exerts an inhibitory effect on the production of these cytokines.

MAPKs play a significant role in the signaling pathway that leads to cytokine expression (Kyriakis and Avruch, 1996; Adwanikar *et al.*, 2004; Kaminska, 2005; Ashwell, 2006). In this study, it was found that RVS decreased the phosphorylation of ERK and p38, whereas RVS increased the phosphorylation of JNK (Fig. 3B). According to other reports, the NF- κ B pathway also negatively regulates the activation of JNK in the mediation of TNF- α (Tang *et al.*, 2001; Javelaud and Besancon, 2001). In addition, it was shown that ERK and p38 are mediated, but not JNK, by the expression of cytokines using selective inhibitors in HMC-1 cells (Wong *et al.*, 2006), and that the inter-pathways repressed each other in inflammation (Hall and Davis, 2002). From these facts, our results suggest that RVS could differentially regulate MAPKs in the stimulated HMC-1 cells. Therefore, when it is considered that each MAPK signaling plays a discrete and complementary role in inflammation and that RVS has an effect on the allergic inflammatory response, further study is necessary to elucidate the more detailed signals and molecules by which RVS regulates an antiallergic and inflammatory effect.

Upon stimulation by various agents, I κ B- α is rapidly phosphorylated at the serine residues, which leads to ubiquitin-dependent degradation by the 26S proteasome (Baeuerle and Baltimore, 1988). The released NF- κ B heterodimer (p65/RelA and NF- κ B p50) then translocates to the nucleus and activates target genes by binding to their promoter/enhancer regions (Jobin *et al.*, 1999; Karin and Ben-Neriah, 2000; Baldwin, 1996). Here, it was found that RVS blocked the nuclear translocation of NF- κ B and phosphorylation of I κ B- α , sug-

gesting that RVS might inhibit the expression of TNF- α , IL-6 and IL-8 through modulation of MAPKs followed by NF- κ B inactivation.

In the present study, a combined treatment with PMA and A2187 for the stimulation of human mast cells was used because they are known to produce pro-inflammatory cytokines that cause allergic responses (Hosoda *et al.*, 2002; Strik *et al.*, 2007) and mimic these allergic and inflammatory conditions. On the other hand, since the lyophilized PSA extract was dissolved in DMSO, the expression levels of cytokines and MAPKs were compared with the treatment with DMSO only. However, the inhibitory effects of RVS in the production of cytokines and the induction of MAPKs were not affected by DMSO, indicating that RVS has obvious antiallergic activity in itself.

Our previous analysis showed that RVS suppressed the inflammatory response in LPS-induced macrophages (Jung *et al.*, 2007). In addition, resveratrol, a phenolic antioxidant, has also been demonstrated to act as an antiinflammatory agent (Schroecksnadel *et al.*, 2005). Therefore, it is suggested that phenolic-rich compounds of RVS might play a role in the antiinflammatory effects. Taken together, the results of this study demonstrate that RVS inhibits the expression of pro-inflammatory cytokines through the inhibition of ERK and p38 MAPK leading to inactivation of NF- κ B, indicating that RVS might contribute to the treatment of allergic inflammatory diseases.

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