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Ripe fruit of *Rubus coreanus* inhibits mast cell-mediated allergic inflammation

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Abstract. In this study, we investigated the effect of a water extract of the ripe fruits of *Rubus coreanus* Miq. (Rosaceae) (RFRC) on mast cell-mediated allergic inflammation and studied the possible mechanism of action. Mast cell-mediated allergic disease is involved in many diseases such as anaphylaxis, rhinitis, asthma and atopic dermatitis. RFRC dose-dependently inhibited compound 48/80-induced systemic anaphylaxis and serum histamine release in mice. RFRC reduced the immunoglobulin E (IgE)-mediated local allergic reaction, passive cutaneous anaphylaxis. RFRC attenuated histamine release from rat peritoneal mast cells and human mast cells by the reduction of intracellular calcium. RFRC decreased the phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore A23187 (PMACI)-stimulated expression and secretion of pro-inflammatory cytokines in human mast cells. The inhibitory effect of RFRC on cytokine production was nuclear factor (NF)-κB- and mitogen-activated protein kinase (MAPK)-dependent. In addition, RFRC suppressed the activation of caspase-1. Our findings provide evidence that RFRC inhibits mast cell-derived allergic inflammatory reactions, and for the involvement of calcium, NF-κB, MAPKs and caspase-1 in these effects. Furthermore, *in vivo* and *in vitro* anti-allergic inflammatory effects of RFRC provide affirmative proof of a possible therapeutic application of this agent in allergic inflammatory diseases.

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Introduction

Mast cells are effector cells displaying various functions during immune responses and regulators of allergic inflammation such as asthma, atopic dermatitis, and sinusitis. Allergen provocation, cross linkage of immunoglobulin E (IgE) bound on mast cells, via the high-affinity receptors triggers the release of allergic inflammatory mediators including histamine, eicosanoid, and pro-inflammatory cytokines (1-4). Using these products mast cells regulate not only immediate type hypersensitivity but also late reaction, like inflammatory responses.

The signaling pathway leading to degranulation of mast cells has been extensively characterized. Cross-linking of FcεRI leads to phosphorylation of Src family kinases (Lyn, Syk and Fyn) (5). Calcium mobilization occurs after phosphorylation of Lyn and phospholipase C-γ induces mast cell degranulation through the granule membrane fusion (6). This is followed by activation of protein kinase C, mitogen-activated protein kinase (MAPKs), nuclear factor (NF)-κB, and the release of inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 (3,7). By the release of pro-inflammatory mediators, mast cells also affect on late phase responses of an allergic inflammation (5). Caspase-1 plays a crucial role in the regulation of cytokines. Caspase-1, a member of cysteine protease also known as an IL-1β converting enzyme, is found predominantly in the cytoplasm of cells, where it proteolytically converts pro-IL-1β into the mature form involved in inflammation (8).

Anal therapy is a drug delivery system through the anus and is utilized in patients who have difficulty with oral administration. Absorbing a drug in the rectum avoids the first-pass effect in the liver and allows it to circulate directly in the whole body (9,10). Thus, anal therapy is expected to have a better effect than oral therapy due to the increased absorption rate and potent medical action.

Rubus coreanus Miq. (Rosaceae) is a family of black raspberry. The dried unripe fruits of *R. coreanus*, more popularly known as Bokbunja in South Korea, are widely used as traditional medicines for spermatorrhea, enuresis and have stomachic and tonic actions (11,12). We previously reported the anti-allergic effect of unripe fruits of *R. coreanus* (13).

However, the anti-allergic inflammatory effect of ripe fruits of *R. coreanus* (RFRC) has not been studied. In this study, we evaluated the effects of RFRC on the systemic/local allergic reaction, and histamine release. The intracellular calcium was investigated to clarify the mechanism by which RFRC inhibited histamine release from mast cells. In addition, the effect of RFRC on the expression of pro-inflammatory cytokines and underlying mechanisms such as NF-κB, MAPKs, and caspase-1 were investigated in mast cells.

Materials and methods

Animals. The original stock of male Imprinting Control Region (ICR) mice (6 weeks) and male Sprague-Dawley rats (8 weeks) were purchased from the Dae-Han Experimental Animal Center (Daejeon, Korea). The animals were housed 5 per cage in a laminar air flow room maintained under a temperature of 22±2˚C and relative humidity of 55±5% throughout the study. The care and treatment of the mice were in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Reagents and cell culture. Compound 48/80, anti-dinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA), phorbol 12-myristate 13-acetate (PMA), and calcium ionophore A23187 were purchased from Sigma (St. Louis, MO, USA). The human mast cell line (HMC-1) was grown in Iscove's media (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and glutamine 2 mM at 37 ˚C in 5% CO_2. HMC-1 cells at passages ranging from 4 to 8 were used throughout the study.

Preparation of RFRC. The ripe fruits of *R. coreanus* were provided from the Black Raspberry Experiment Station, Gochang Agricultural Extension Center (Jeonbuk, Korea). The ripe fruits of *R. coreanus* were ground (400 x g, 30 sec) at room temperature using a Micro Hammer-Cutter mill (Culatti Co., Zurich, Switzerland). The particle size was 0.5-2 mm after grinding. The plant sample (60 g) was extracted twice with purified water (500 ml) at 70˚C for 5 h in a water bath. The extract was filtered through Whatman no. 1 filter paper and the filtrate was lyophilized using a 0.45 μm syringe filter. The yield of dried extract from crude materials was about 11.5%. The dried extract was dissolved in saline or Tyrode buffer A (HEPES 10 mM, NaCl 130 mM, KCl 5 mM, $CaCl_2$ 1.4 mM, $MgCl_2$ 1 mM, glucose 1.4 mM, 0.1% bovine serum albumin) before use.

Compound 48/80-induced systemic anaphylaxis. Mice were given an intraperitoneal injection of 8 mg/kg body weight (BW) of the mast cell degranulator, compound 48/80. RFRC was dissolved in saline and administered anally at a dose of 10-1000 mg/kg BW 1 h before the injection of compound 48/80 (n=10/group). In the time-dependent experiment, RFRC (1000 mg/kg, BW) was injected at 5, 10 and 20 min after compound 48/80 injection (n=10/group). Mortality was monitored for 1 h after induction of anaphylactic shock. After the mortality test, blood was obtained from the heart of each mouse to measure serum histamine contents.

Passive cutaneous anaphylaxis (PCA). An IgE-dependent cutaneous reaction was carried out as described previously (14). The PCA reaction was generated by sensitizing skin with an intradermal injection of anti-DNP IgE followed 48 h later with an injection of DNP-HSA into the mouse tail vein. The mice were injected intradermally with 0.5 μg of anti-DNP IgE. After 48 h, each mouse received an injection of 1 μg of DNP-HSA containing 4% Evans blue (1:4) via the tail vein. Thirty minutes after the challenge, the mice were sacrificed and the dorsal skin (diameter, 1 cm) was removed for measurement of the pigmented area. The amount of dye was determined colorimetrically after extraction with 1 ml of 1 mM KOH and 9 ml of mixture of acetone and phosphoric acid (5:13). The intensity of absorbance was measured at 620 nm in a spectrophotometer (Shimadzu, UV-1201, Kyoto, Japan).

Preparation of peritoneal mast cells (RPMC). RPMC were isolated as previously described (15). In brief, rats were anesthetized with carbon dioxide and injected with 20 ml of Tyrode buffer B (137 mM NaCl, 5.6 mM glucose, 12 mM $NaHCO_3$, 2.7 mM KCl, 0.3 mM NaH_2PO_4 and 1% gelatin), into the peritoneal cavity and the abdomen was massaged for about 90 sec. The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells were aspirated by a Pasteur pipette. The peritoneal cells were sedimented at 150 x g for 10 min at room temperature and resuspended in Tyrode buffer B. Peritoneal cells were suspended in 1 ml Tyrode buffer B, layered on 2 ml metrizamide (22.5% w/v), and centrifuged at 400 x g for 15 min at 4˚C. The cells remaining at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 ml Tyrode buffer A. Mast cell preparations were ~95% pure as assessed by toluidine blue staining. More than 97% of the cells were viable as judged by trypan blue exclusion.

Preparation of serum and histamine determination. Preparation of serum and determination of histamine contents were examined as previously described (9). Briefly, serum was withdrawn and the histamine contents were measured by the o-phthaldialdehyde spectrofluorometric procedure. The fluorescence intensity was measured at an emission wavelength of 438 nm and an excitation wavelength of 353 nm using a spectrofluorometer (Shimadzu).

Determination of intracellular Ca^{2+}. The intracellular calcium was measured with the use of the fluorescence indicator Fluo-3/AM (Molecular Probes, Eugene, OR, USA). HMC-1 cells were pre-incubated with Fluo-3/AM for 30 min at 37˚C. After washing the dye from the cell surface, RFRC was pretreated 10 min prior to treatment with PMA and the calcium ionophore A23187 (PMACI). The fluorescence intensity was recorded using a fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA) at an excitation of 488 nm and an emission of 515 nm and visualized with a fluorescence microscope (Olympus BX51, Center Valley, PA, USA).

RNA extraction and mRNA detection. The total cellular RNA was isolated from the cells ($1x10^6$/well in a 24-well plate) after stimulation with PMA (20 nM) and A23187 (1 μM) with or without RFRC for 2 h using TRI reagent (Molecular Research

Table I. Effects of RFRC on compound 48/80-induced systemic anaphylaxis.

RFRC treatment (mg/kg, BW)	Compound 48/80 (8 mg/kg, BW)	Mortality (%)
None (saline)	+	100
10	+	100
50	+	70
100	+	10
500	+	10
1000	+	0
1000	-	0

Groups of mice (n=10/group) were anally pretreated with 200 μl of saline or RFRC at various doses 1 h before intraperitoneal injection of compound 48/80. Mortality (%) within 1 h following compound 48/80 injection is represented as the number of dead mice x 100/total number of experimental mice.

Table II. Time-dependent effects of RFRC on compound 48/80-induced systemic anaphylaxis.

RFRC treatment (mg/kg, BW)	Compound 48/80 (8 mg/kg, BW)	Time (min)	Mortality (%)
None (saline)	+	-	100
1000	+	0	0
1000	+	5	40
1000	+	10	60
1000	+	20	100

Groups of mice (n=10/group) were anally pretreated with 200 μl of saline or RFRC. RFRC (1000 mg/kg) was given 5, 10 and 20 min after the intraperitoneal injection of compound 48/80. Mortality (%) within 1 h following compound 48/80 injection is represented as the number of dead mice x 100/total number of experimental mice.

Center, Cincinnati, OH, USA) according to the manufacturer's protocol. The first strand complementary DNA (cDNA) was synthesized using the SuperScript II reverse-transcriptase (Invitrogen, Carlsbad, CA, USA). A reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for TNF-α, IL-6, IL-1β, and β-actin (internal control). The conditions for the reverse transcription and PCR steps were similar to those described previously (16). The amplified products were separated by electrophoresis on 2% agarose gel containing ethidium bromide, documented using a Kodak DC 290 digital camera and digitized with the UN-SCAN-IT software (Silk Scientific, Orem, UT, USA). The band intensity was normalized to that of β-actin in the same sample.

Enzyme-linked immunosorbent assay (ELISA). The secretion of TNF-α and IL-6 was measured by the modification of an enzyme-linked immunosorbent assay (ELISA) as described previously (17). The cells were sensitized with PMACI for 8 h in the absence or presence of RFRC. The ELISA was performed by coating 96-well plates with 6.25 ng/well of monoclonal antibody with specificity for TNF-α and IL-6 respectively.

Caspase-1 activity assay. The enzymatic activity of caspase-1 was measured according to the manufacturer's specification using a caspase assay kit (R&D Systems). The cell lysate was centrifuged at 14,000 rpm for 5 min. The protein supernatant was incubated with 50 μl reaction buffer and 5 μl substrates (WEHD-pNA) at 37˚C for 2 h. The absorbance was measured using a plate reader at a wavelength of 405 nm. Equal amounts of the total protein from each lysate were quantified using a bicinchoninic acid protein quantification kit (Sigma).

Western blot analysis. HMC-1 cells were washed 3 times with PBS and resuspended in lysis buffer. Samples were electrophoresed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described elsewhere (18), and then transferred to a nitrocellulose membrane. Immunodetection was performed using an enhanced chemiluminescence detection kit (Amersham Pharmacia, Piscataway, NJ, USA).

Transient transfection and luciferase activity assay. For transient transfection, HMC-1 cells were seeded at $2x10^6$ in a 6-well plate 1 day before transient transfection. The expression vectors containing the NF-κB luciferase reporter construct (pNF-κB-LUC, plasmid containing NF-κB binding site; Stratagen, Grand Island, NY, USA) were transfected with serum- and antibiotics-free Iscove's medium containing 8 μl Lipofectamine 2000 reagent (Invitrogen). After 5 h of incubation, the medium was replaced with Iscove's medium containing 10% FBS and antibiotics. Cells were allowed to recover at 37˚C for 30 h and subsequently were stimulated as indicated. Cell lysates were prepared and assayed for luciferase activity using the Luciferase Assay system (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Statistical analysis. Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC, USA). Treatment effects were analyzed using analysis of variance, followed by the Duncan's multiple range tests. $P<0.05$ was considered to indicate significant differences.

Results

RFRC inhibits compound 48/80-induced systemic allergic reaction. To determine the effect of RFRC on allergic reactions, an *in vivo* model of a systemic anaphylaxis was used. Compound 48/80 (8 mg/ml) was used as a model of induction of a systemic allergic reaction. After the intraperitoneal injection of compound 48/80, the mice were monitored for 1 h, after which the mortality rate was determined. As shown in Table I, injection of compound 48/80 into mice induced fatal shock in 100% of the animals. When mice were anally pretreated with RFRC at doses ranging from 10 to 1000 mg/kg for 1 h, the mortality with compound 48/80 was dose-dependently reduced. RFRC completely inhibited compound 48/80-induced fatal shock at 1000 mg/kg. In addition, the mortality of mice administered

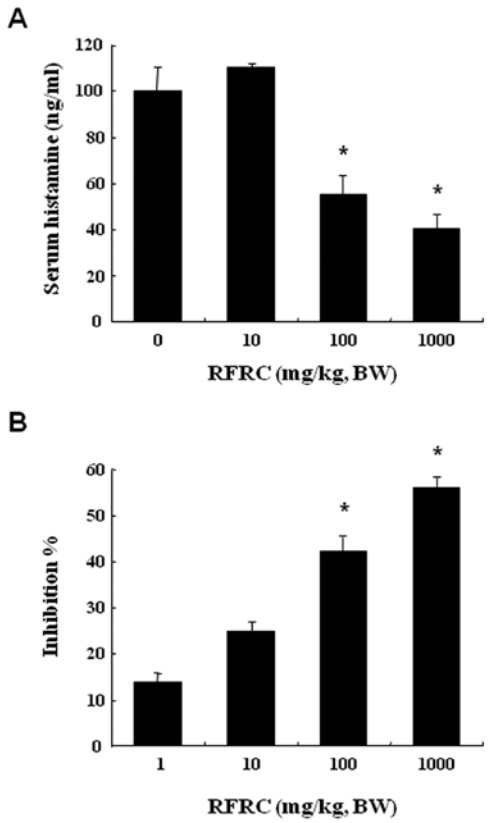

Figure 1. Effects of RFRC on serum histamine and PCA reactions. (A) Groups of mice (n=10/group) were anally pretreated with 200 μl of saline or RFRC. RFRC was given at various doses 1 h before the injection of compound 48/80. The compound 48/80 was given intraperitoneally to the group of mice. The blood was obtained from the heart of each mouse and histamine content was measured by a spectrofluorometer. (B) RFRC was anally administered 1 h prior to the challenge with antigen. Each amount of dye was extracted as described in Materials and methods and measured by spectrophotometry. Each bar represents the mean ± SEM of three independent experiments. *Significant difference at p<0.05.

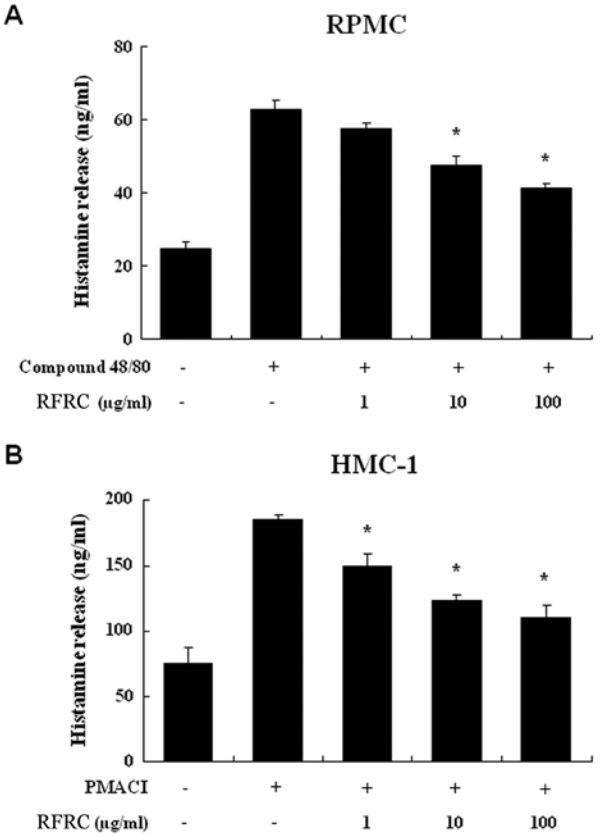

Figure 2. Effects of RFRC on histamine release from mast cells. (A) RPMC cells ($2x10^5$ cells/ml) were pre-incubated with RFRC for 10 min prior to incubation with compound 48/80 (5 μg/ml). (B) HMC-1 ($1x10^6$ cells/ml) were pre-incubated with RFRC 30 min prior to incubation with PMA (20 nM) and the calcium ionophore A23187 (1 μM) (PMACI). Each bar represents the mean ± SEM of three independent experiments. *Significant difference at p<0.05.

RFRC (1000 mg/kg) 5, 10 and 20 min after compound 48/80 injection time-dependently increased (Table II). The effect of RFRC on the compound 48/80-induced serum histamine release was investigated. The increase of serum histamine caused by compound 48/80 was inhibited by RFRC in a dose-dependent manner (Fig. 1A). To confirm the anti-allergic effects of RFRC, we used an IgE-mediated passive cutaneous anaphylaxis (PCA) model. PCA is one of the most important *in vivo* models of local allergic reaction. A local extravasation was induced by a local injection of IgE following an antigenic challenge. To compare the amount of dye with control, the left dorsal skin of these mice was injected with saline alone. RFRC was anally administered 1 h prior to the challenge with antigen. Anal injection of RFRC dose-dependently inhibited the PCA reaction (Fig. 1B).

RFRC reduces histamine release and intracellular calcium levels on mast cells. We evaluated the ability of RFRC to induce histamine release in compound 48/80-induced RPMC cells and PMACI-induced HMC-1 cells. Mast cells released a high level of histamine when stimulated with compound 48/80 or PMACI (Fig. 2). RFRC dose-dependently inhibited compound 48/80-induced histamine release from RPMC. In

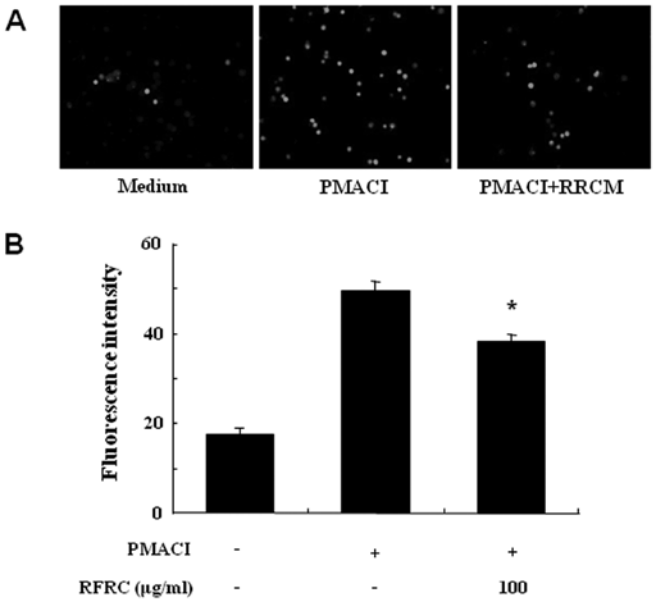

Figure 3. Effects of RFRC on intracellular calcium levels in HMC-1 cells. Cells ($1x10^6$ cells/ml) were pre-incubated with RFRC at 37˚C for 30 min prior to incubation with PMA (20 nM) and calcium ionophore A23187 (1 μM) (PMACI). Intracellular calcium was detected by fluorescence microscope (A) and fluorescence plate reader. (B) Each bar represents the mean ± SEM of three independent experiments. *Significant difference from PMACI value at p<0.05.

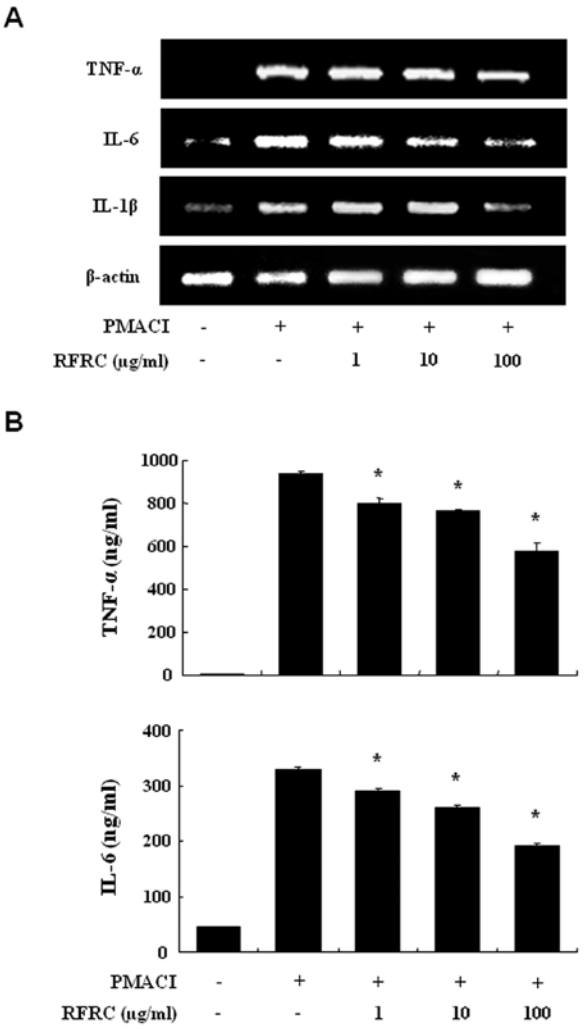

Figure 4. Effects of RFRC on the gene expression and secretion of pro-inflammatory cytokines in HMC-1 cells. Cells were treated with RFRC for 30 min before being stimulated with PMA (20 nM) and ionophore A23187 (1 μM) (PMACI). (A). Extraction and analysis of mRNA was performed as described in Materials and methods. The gene expression of TNF-α, IL-6 and IL-1β were determined by RT-PCR. (B) The level of TNF-α and IL-6 in supernatant was measured using ELISA and represented as the mean ± SEM of three independent experiments. *Significant difference from PMACI value at $p<0.05$.

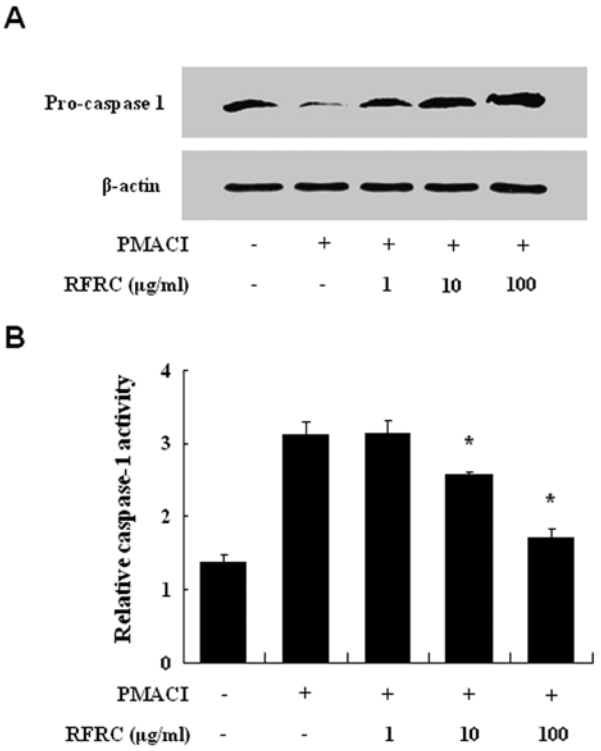

Figure 5. Effects of RFRC on PMACI-induced capase-1 activation in HMC-1 cells. Cells were pretreated with RFRC for 30 min prior to PMA (20 nM) and A23187 (1 μM) (PMACI) stimulation for 2 h. (A) The level of pro-caspase-1 was assayed by Western blotting. (B) The enzymatic activity of caspase-1 was confirmed by colorimetric assay. Each bar represents the mean ± SEM of three independent experiments. *Significant difference from PMACI value at $p<0.05$.

addition, RFRC inhibited PMACI-induced histamine release from HMC-1 in a dose-dependent manner. The concentration and duration of RFRC treatment used in these studies had no significant effect on the viability of RPMC and HMC-1 (data not shown). To further investigate the mechanisms of RFRC on the inhibition of histamine release, we assayed the intracellular calcium levels. Calcium movements across membranes of mast cells are critical to histamine release (19). Fig. 3 shows the elevation of intracellular calcium when HMC-1 cells were stimulated with PMACI. Pre-treatment with RFRC (100 μg/ml) decreased the intracellular calcium levels induced by PMACI.

RFRC inhibits gene expression and secretion of pro-inflammatory cytokines. We assessed the effect of RFRC on the expression of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 in HMC-1. The HMC-1 cell line is a useful cell for studying cytokine activation pathway (10,20). We previously reported that gene expression of TNF-α, IL-1β and IL-6 peaked at 4 h after treatment of PMACI (21). Therefore, stimulation of HMC-1 with PMACI was induced during 4 h, and the cells were pretreated with RFRC for 30 min. RFRC inhibited the gene expression of all cytokines (Fig. 4A). To confirm the correlation of mRNA expression with protein production, we measured the secretion of TNF-α and IL-6. Culture supernatants were assayed for TNF-α and IL-6 levels by ELISA. The stimulation of cells with PMACI for 8 h induced the secretion of cytokines. RFRC inhibited the secretion of TNF-α and IL-6 in PMACI-stimulated HMC-1 (Fig. 4B).

RFRC reduces activation of caspase-1. Caspase-1 activates the inactive precursors of IL-1β, IL-6, and TNF-α cytokines involved in immune responses (22,23). We investigated the effect of RFRC on caspase-1 activation. HMC-1 cells were pretreated with RFRC for 30 min and then treated with PMACI for 2 h. The level of pro-caspase-1 was decreased, after stimulation with PMACI (Fig. 5A). However, pro-caspase-1 was significantly increased by the treatment of RFRC. To confirm the attenuation of caspase-1, we measured the caspase-1 activity. The enhanced caspase-1 activity by PMACI was markedly decreased by RFRC (Fig. 5B).

RFRC inhibits the activation of NF-κB and of MAPKs. In order to evaluate the mechanism of the RFRC effect on cytokine expression, we examined the effect of RFRC on the activation of the transcription factors, NF-κB and MAPKs. NF-κB is an

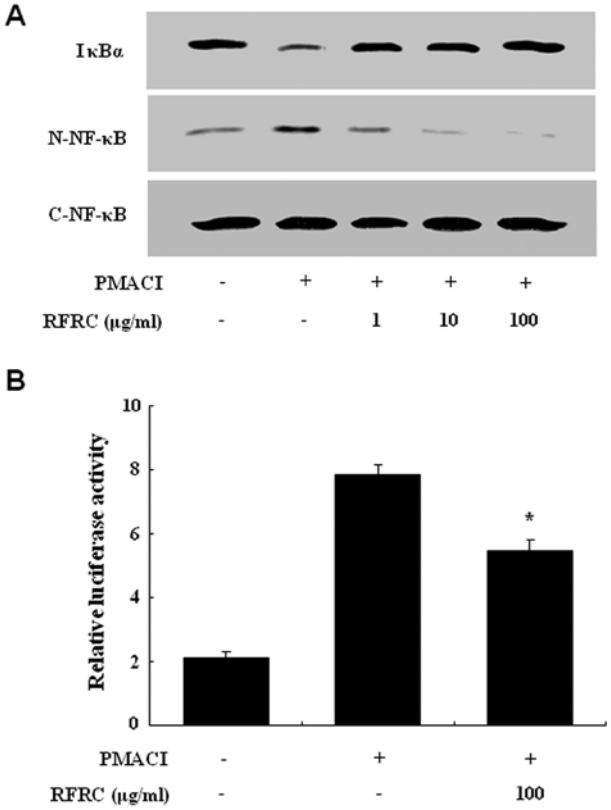

Figure 6. Effects of RFRC on the activation of NF-κB in HMC-1. Cells were pretreated with RFRC for 30 min prior to PMA (20 nM) and A23187 (1 μM) (PMACI) stimulation for 2 h. (A) Nuclear translocation of NF-κB and IκBα degradation were assayed by Western blotting (N-NF-κB, nuclear NF-κB; C-NF-κB, cytoplasmic NF-κB). (B) Cells were transiently transfected with the NF-κB-luciferase reporter construct or empty vector. Then, the cells were incubated with PMA and A23187 with or without RFRC. NF-κB-dependent transcriptional activity was determined by luciferase activity assay. Each bar represents the mean ± SEM of three independent experiments. *Significant difference from PMACI value at $p<0.05$.

important transcriptional regulator of inflammatory cytokines and plays a crucial role in immune and inflammatory responses. Stimulation of HMC-1 cells with PMACI induced the nuclear translocation of p65 NF-κB and degradation of IκBα after 2 h of incubation (Fig 6A). RFRC inhibited the PMACI-induced nuclear translocation of NF-κB and degradation of IκBα. To confirm the inhibitory effect of RFRC on NF-κB activation, we examined the effect of RFRC on NF-κB-dependent gene reporter assay. HMC-1 were transiently transfected with a NF-κB-luciferase reporter construct or an empty vector. Exposure of cells to PMACI increased the luciferase activity in the cells transfected with NF-κB-luciferase reporter construct (Fig. 6B). RFRC significantly reduced PMACI-induced luciferase activity.

MAPKs also play a crucial role in the regulation of pro-inflammatory molecules on cellular responses (24). To evaluate the mechanisms of the effect of RFRC on pro-inflammatory cytokine expression, we examined the effect of RFRC on the phosphorylation of MAPKs. Previously we documented that PMACI activates all three types of MAPKs such as p38, JNK, and ERK at 15-30 min in HMC-1 cells (25). Stimulation of cells with PMACI induced the phosphorylation of MAPKs (Fig. 7). RFRC markedly attenuated the PMACI-induced activation of all three types of MAPKs.

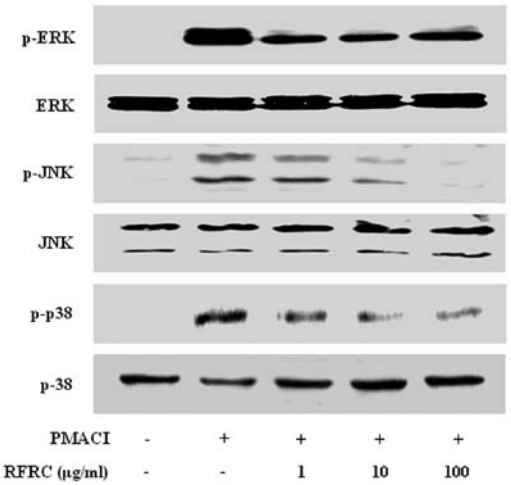

Figure 7. Effect of RFRC on phosphorylation of MAPKs in HMC-1. Cells were pretreated with RFRC for 30 min prior to PMA (20 nM) and A23187 (1 μM) (PMACI) stimulation for 30 min, and phosphorylation of p38, JNK,and ERK was assayed by Western blotting.

Discussion

Anaphylaxis is a life-threatening syndrome induced by the sudden systemic release of inflammatory mediators such as histamine, various cytokines and lipid-derived mediators (26). Using *in vitro* and *in vivo* models, we showed that RFRC reduces mast cell-derived allergic inflammatory responses. RFRC inhibited compound 48/80-induced systemic anaphylaxis and histamine release from mast cells. RFRC administered mice are protected from IgE-mediated local allergic reaction, PCA, which is one of the most important *in vivo* models of anaphylaxis. These results indicate that RFRC inhibited mast cell-mediated allergic reactions. In addition, RFRC decreased PMACI-stimulated expression of pro-inflammatory cytokines. These results suggest that RFRC might be useful in the treatment of allergic inflammatory diseases.

Histamine was originally considered as a mediator of acute inflammatory and immediate hypersensitivity responses. Recently, it has been reported that histamine affects chronic inflammation and regulates several essential events of immune response such as immune cell maturation, polarization, and lymphocyte responsiveness (27). Histamine also induces actin polymerization and chemotaxis (28). Compound 48/80, the synthetic mast cell stimulator, increases the permeability of the lipid bilayer membrane by perturbation in the membrane. These reports indicate that the increase of membrane permeability may be an essential element for the release of the mediator from mast cells. In our study, RFRC dose-dependently reduced compound 48/80-induced histamine release from mast cells. Therefore, we speculate that RFRC might stabilize the actin polymerization of the membrane, thus preventing the compound 48/80-induced membrane perturbation.

Mobilization of calcium plays a critical role in the degranulation and release of histamine in mast cells (29). Calcium movements across membranes of mast cells represent a major target for effective anti-allergic drugs, as these are essential events linking stimulation to secretion. Our results showing an attenuation of intracellular calcium in mast cells with RFRC

treatment suggest the decreased intracellular calcium might be involved in the inhibitory effect of RFRC on histamine release.

HMC-1 stimulated by PMACI produce wide range of cytokines and supports the well-recognized role of mast cells in immediate-type hypersensitivity (3). Pro-inflammatory cytokines such as TNF-α and IL-6 are major mediators in triggering and sustaining the allergic inflammatory response in mast cells. TNF-α promotes inflammation, granuloma formation and tissue fibrosis and is an initiator of cytokine related inflammatory responses by stimulating cytokine production (30). IL-6 is also produced from mast cells and its accumulation is associated with the PCA reaction (31). These reports show that reduction of TNF-α and IL-6 from mast cells is one of the major indicators of relief from allergic symptoms. In this study, RFRC dose-dependently reduced the expression of TNF-α and IL-6. These results suggest that RFRC has anti-allergic inflammatory effect by controlling pro-inflammatory cytokines in mast cells.

NF-κB regulates the expression of multiple inflammatory and immune genes and plays a critical role in chronic inflammatory diseases. The role of NF-κB activation and regulation of cytokine production in allergic inflammatory processes have been characterized (32). Activation of NF-κB required phosphorylation and proteolytic degradation of the inhibitory protein IκBα, an endogenous inhibitor that binds to NF-κB in the cytoplasm (33). In PMACI-stimulated mast cells, RFRC decreased the degradation of IκBα and the nuclear translocation of p65 NF-κB. These results demonstrate that RFRC attenuates the activation of NF-κB and downstream expression of TNF-α and IL-6.

The MAPK cascade is an important signaling pathway in immune responses. The MAPK signaling cascades regulate important cellular processes including gene expression, cell proliferation, cell survival and death, and cell mobility (24). The precise signaling pathways among the three types of MAPKs, i.e., ERK, JNK and p38 are still unclear. However, the induction of inflammatory cytokine genes requires activation of all three types of MAPKs. In this study, PMACI-induced activation of all three types of MAPKs was reduced by RFRC. The data suggest that RFRC has an inhibitory activity on the all three of MAPKs and downstream cytokine production.

Inflammasomes are cytoplasmic multi-molecular protein complexes that mediate the activation of inflammatory caspase-1 (34). The activation of caspase-1 regulates the inflammatory reactions via production of inflammatory cytokines and recruitment of neutrophils (35). A previous report showed that stimulation of caspase-1$^{-/-}$ mice with lipopolysaccharide inhibited the production of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β (36).

The protein kinase receptor-interacting protein (RIP)-2 is a member of the CARD protein family (caspase activation and recruitment domain, also known as CARD3, RIPK2, CARDIAK, RICK, and CCK) and has been shown to be an activator of NF-κB (37). RIP-2 promotes NF-κB activation as well as activation of the MAPKs, and transcription of inflammatory-related genes, such as IL-1β, IL-6, and TNF-α. RIP-2 knockout inhibited the secretion of pro-inflammatory cytokines such as TNF-α and IL-6 (22,23). RIP-2 is a specific adaptor molecule and regulates the activation of caspase-1 (38,39). These studies suggest that the activation of RIP-2/caspase-1 is an attractive target for the treatment of inflammatory diseases. Accordingly, we postulated that RFRC mediates its effects at least partly through the suppression of RIP-2/caspase-1. In this study, we confirmed that RFRC inhibited PMACI-induced caspase-1 activation in HMC-1 cells. This result suggested that the suppressive effects of RFRC on the production of cytokines may be derived through the regulation of PMACI-induced caspase-1 activation. However, further studies will be needed in order to clarify the function of RFRC on the RIP-2/caspase-1 pathway.

Recently changes of the metabolic profiles of fruits of *R. coreanus* during maturation were reported (40). Sucrose, most of the amino acids, and organic acids were decreased, whereas fructose, glucose, and cyanidins were increased according to maturation. This report suggests that the maturation stage of fruits of *R. coreanus* may have result in different biological effects. Because we used the whole water extract of ripe fruits of *R. coreanus* not a purified single compound, the specific active components that are responsible for the biological effect are not clear at this time. The effort to identify the active components of RFRC in allergic inflammation is ongoing in our laboratory. However, the results of this study show that the mechanism responsible for the anti-allergic inflammatory effects of RFRC and RFRC contribute to the prevention or treatment of mast cell-mediated allergic inflammation.

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References

1. Galli SJ: New concepts about the mast cell. N Engl J Med 328: 257-265, 1993.
2. Galli SJ, Kalesnikoff J, Grimbaldeston MA, Piliponsky AM, Williams CM and Tsai M: Mast cells as 'tunable' effector and immunoregulatory cells: recent advances. Annu Rev Immunol 23: 749-786, 2005.
3. Kim SH, Jun CD, Suk K, *et al*: Gallic acid inhibits histamine release and pro-inflammatory cytokine production in mast cells. Toxicol Sci 91: 123-131, 2006.
4. Kim SH, Park SB, Kang SM, *et al*: Anti-allergic effects of *Teucrium japonicum* on mast cell-mediated allergy model. Food Chem Toxicol 47: 398-403, 2009.
5. Galli SJ, Tsai M and Piliponsky AM: The development of allergic inflammation. Nature 454: 445-454, 2008.
6. Castle JD, Guo Z and Liu L: Function of the t-SNARE SNAP-23 and secretory carrier membrane proteins (SCAMPs) in exocytosis in mast cells. Mol Immunol 38: 1337-1340, 2002.
7. Gwack Y, Feske S, Srikanth S, Hogan PG and Rao A: Signalling to transcription: store-operated Ca^{2+} entry and NFAT activation in lymphocytes. Cell Calcium 42: 145-156, 2007.
8. Thornberry NA, Bull HG, Calaycay JR, *et al*: A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. Nature 356: 768-774, 1992.
9. Kim SH, Lee S, Kim IK, *et al*: Suppression of mast cell-mediated allergic reaction by *Amomum xanthiodes*. Food Chem Toxicol 45: 2138-2144, 2007.
10. Shin TY, Kim SH, Suk K, *et al*: Anti-allergic effects of *Lycopus lucidus* on mast cell-mediated allergy model. Toxicol Appl Pharmacol 209: 255-262, 2005.

11. But PPH, Kimura T, Guo JX and Sung CK (eds): International Collation of Traditional and Folk Medicine, Part II. World Scientific, River Edge, pp68-69, 1997.
12. Perry LM (ed): Medicinal Plants of East and Southeast Asia: Attributed Properties and Uses. MIT Press, Cambridge, p346, 1980.
13. Shin TY, Kim SH, Lee ES, Eom DO and Kim HM: Action of *Rubus coreanus* extract on systemic and local anaphylaxis. Phytother Res 16: 508-513, 2002.
14. Kim SH, Kwon TK and Shin TY: Antiallergic effects of *Vitis amurensis* on mast cell-mediated allergy model. Exp Biol Med (Maywood) 233: 192-199, 2008.
15. Kim SH and Shin TY: *Amomum xanthiodes* inhibits mast cell-mediated allergic reactions through the inhibition of histamine release and inflammatory cytokine production. Exp Biol Med (Maywood) 230: 681-687, 2005.
16. Kim SH and Sharma RP: Mercury-induced apoptosis and necrosis in murine macrophages: role of calcium-induced reactive oxygen species and p38 mitogen-activated protein kinase signaling. Toxicol Appl Pharmacol 196: 47-57, 2004.
17. Min YD, Choi CH, Bark H, *et al*: Quercetin inhibits expression of inflammatory cytokines through attenuation of NF-kappaB and p38 MAPK in HMC-1 human mast cell line. Inflamm Res 56: 210-215, 2007.
18. Kim SH, Lee S, Suk K, *et al*: Discoidin domain receptor 1 mediates collagen-induced nitric oxide production in J774A.1 murine macrophages. Free Radic Biol Med 42: 343-352, 2007.
19. Beaven MA, Rogers J, Moore JP, Hesketh TR, Smith GA and Metcalfe JC: The mechanism of the calcium signal and correlation with histamine release in 2H3 cells. J Biol Chem 259: 7129-7136, 1984.
20. Moller A, Henz BM, Grutzkau A, *et al*: Comparative cytokine gene expression: regulation and release by human mast cells. Immunology 93: 289-295, 1998.
21. Park HH, Lee S, Oh JM, *et al*: Anti-inflammatory activity of fisetin in human mast cells (HMC-1). Pharmacol Res 55: 31-37, 2007.
22. Chin AI, Dempsey PW, Bruhn K, Miller JF, Xu Y and Cheng G: Involvement of receptor-interacting protein 2 in innate and adaptive immune responses. Nature 416: 190-194, 2002.
23. Kobayashi K, Inohara N, Hernandez LD, *et al*: RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. Nature 416: 194-199, 2002.
24. Arbabi S and Maier RV: Mitogen-activated protein kinases. Crit Care Med 30: S74-S79, 2002.
25. Kim SH, Choi CH, Kim SY, Eun JS and Shin TY: Anti-allergic effects of *Artemisia iwayomogi* on mast cell-mediated allergy model. Exp Biol Med (Maywood) 230: 82-88, 2005.
26. Kemp SF and Lockey RF: Anaphylaxis: a review of causes and mechanisms. J Allergy Clin Immunol 110: 341-348, 2002.
27. Jutel M, Blaser K and Akdis CA: Histamine in allergic inflammation and immune modulation. Int Arch Allergy Immunol 137: 82-92, 2005.
28. Jutel M, Watanabe T, Akdis M, Blaser K and Akdis CA: Immune regulation by histamine. Curr Opin Immunol 14: 735-740, 2002.
29. Cho SH, Woo CH, Yoon SB and Kim JH: Protein kinase Cdelta functions downstream of Ca^{2+} mobilization in FcepsilonRI signaling to degranulation in mast cells. J Allergy Clin Immunol 114: 1085-1092, 2004.
30. Hide I, Toriu N, Nuibe T, *et al*: Suppression of TNF-alpha secretion by azelastine in a rat mast (RBL-2H3) cell line: evidence for differential regulation of TNF-alpha release, transcription, and degranulation. J Immunol 159: 2932-2940, 1997.
31. Walsh LJ, Trinchieri G, Waldorf HA, Whitaker D and Murphy GF: Human dermal mast cells contain and release tumor necrosis factor alpha, which induces endothelial leukocyte adhesion molecule 1. Proc Natl Acad Sci USA 88: 4220-4224, 1991.
32. Blackwell TS, Blackwell TR and Christman JW: Impaired activation of nuclear factor-kappaB in endotoxin-tolerant rats is associated with down-regulation of chemokine gene expression and inhibition of neutrophilic lung inflammation. J Immunol 158: 5934-5940, 1997.
33. Azzolina A, Bongiovanni A and Lampiasi N: Substance P induces TNF-alpha and IL-6 production through NF kappa B in peritoneal mast cells. Biochim Biophys Acta 1643: 75-83, 2003.
34. Stutz A, Golenbock DT and Latz E: Inflammasomes: too big to miss. J Clin Invest 119: 75-83, 2009.
35. Faubel S, Lewis EC, Reznikov L, *et al*: Cisplatin-induced acute renal failure is associated with an increase in the cytokines interleukin (IL)-1beta, IL-18, IL-6, and neutrophil infiltration in the kidney. J Pharmacol Exp Ther 322: 8-15, 2007.
36. Kuida K, Lippke JA, Ku G, *et al*: Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. Science 267: 2000-2003, 1995.
37. Yin X, Krikorian P, Logan T and Csizmadia V: Induction of RIP-2 kinase by proinflammatory cytokines is mediated via NF-kappaB signaling pathways and involves a novel feed-forward regulatory mechanism. Mol Cell Biochem 333: 251-259, 2010.
38. Lee SH, Stehlik C and Reed JC: Cop, a caspase recruitment domain-containing protein and inhibitor of caspase-1 activation processing. J Biol Chem 276: 34495-34500, 2001.
39. Druilhe A, Srinivasula SM, Razmara M, Ahmad M and Alnemri ES: Regulation of IL-1beta generation by Pseudo-ICE and ICEBERG, two dominant negative caspase recruitment domain proteins. Cell Death Differ 8: 649-657, 2001.
40. Kim HS, Park SJ, Hyun SH, *et al*: Biochemical monitoring of black raspberry (*Rubus coreanus* Miquel) fruits according to maturation stage by ^{1}H NMR using multiple solvent systems. Food Res Int 44: 1977-1987, 2011.