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Effect of Ripe Fruits of *Rubus coreanus* on Anaphylactic Allergic Reaction and Production of Cytokines

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Abstract – The anaphylactic allergic reaction is involved in many allergic diseases such as asthma and allergic rhinitis. In this report, I investigated the effect of ripe fruits of *Rubus coreanus* Miq. (Rosaceae) (RFRC) on the anaphylactic allergic reaction and studied its possible mechanisms of action. RFRC inhibited compound 48/80-induced systemic anaphylactic reaction in mice. RFRC dose-dependently decreased the IgE-mediated passive cutaneous anaphylaxis. In addition, RFRC decreased the gene expression and production of tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 in phorbol 12-myristate 13-acetate (PMA) plus calcium ionophore A23187 (A23187)-stimulated human mast cells. These findings provide evidence that RFRC could be a candidate as an anti-allergic agent.

Keywords – *Rubus coreanus* ripe fruit, Anaphylactic allergic reaction, Tumor necrosis factor-α, Interleukin-6

Introduction

Mast cells play a pivotal role in a wide variety of biological responses. They are important in the development of many physiologic changes during allergic and anaphylactic responses (Kemp and Lockey, 2002; Galli *et al*., 2005a). Anaphylactic reaction is a life threatening syndrome induced by the sudden systemic release of inflammatory mediators, such as histamine, heparin, lipid-derived mediators and various cytokines from mast cells. Among the inflammatory substances released from mast cells, histamine remains the best-characterized and most potent vasoactive mediator implicated in the acute phase of immediate hypersensitivity (Galli *et al*., 2005b; Kim and Shin, 2005). Activated mast cells also produce a wide variety of other inflammatory mediators such as eicosanoids, proteoglycans, proteases and several proinflammatory and chemotactic cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-4, IL-8, IL-13 and transforming growth factor (TGF)-β (Galli *et al*., 1991; Bradding *et al*., 1993). Therefore, modulation of production of these cytokines and histamine from mast cells can provide a useful therapeutic strategy for allergic disease.

Mast cell activation by IgE, bring about the process of degranulation that results in the fusion of the cytoplasmic membranes with the plasma membrane. This is accompanied by both the fast external release of granule-associated stored mediators as well as by the generation and release of newly generated mediators (Metcalfe *et al*., 1981; Church and Levi-Schaffer, 1997). Mast cell degranulation can be elicited by a number of positively charged substances, collectively known as the basic secretagogues of mast cells (Lagunoff *et al*., 1983; Shin *et al*., 2005). The synthetic compound 48/80 is known to be one of the most potent secretagogues. An appropriate amount of compound 48/80 has been used as a direct and convenient reagent to study the mechanism of anaphylaxis (Kim *et al*., 2005; Kim and Shin, 2005).

The dried unripe fruits of *Rubus coreanus* Miq. (Rosaceae) have been used for the management of impotence, spermatorrhea, enuresis, asthma and it also has been used as a stomachic and tonic in Korea (Perry, 1980; But *et al.*, 1997). Shin (Shin *et al*., 2002) reported that *Rubus coreanus* unripe fruits inhibited systemic and local anaphylaxis. However, the effect of *Rubus coreanus* ripe fruits on anaphylactic reaction has not been studied.

The aim of this study is to evaluate the anti-allergic effects of ripe fruits of *Rubus coreanus* Miq. (Rosaceae) (RFRC).

Experimental

Reagents and cell culture – Compound 48/80, anti-dinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA), phorbol 12-myristate 13-acetate and calcium

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ionophore A23187 were purchased from Sigma (St. Louis, MO). The human mast cell line (HMC-1) was grown in Iscove's media (Life Technologies, Grand Island, NY) supplemented with 10% FBS and 2 mM glutamine at 37 °C in 5% CO_2. The passage ranging 4 - 8 of HMC-1 cells was used throughout the study.

Preparation of RFRC – The ripe fruits of *Rubus coreanus* were purchased from Black Raspberry Experiment Station, Gochang Agricultural Extension Center (Jeonbuk, Korea). A voucher specimen was deposited at the Herbarium of the College of Pharmacy, Woosuk University. The sample was extracted with purified water at 70 °C for 5 h in water bath. The extract was filtered and lyophilized. The dried extract was dissolved in PBS before use.

Animals – The original stock of male ICR mice were purchased from Dae-Han Biolink Co. Ltd. (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.

Compound 48/80-induced systemic reaction – Mice were given an intraperitoneal injection of 8 mg/kg, body weight (BW), of the mast cell degranulator, compound 48/80. RFRC was intraperitoneally administered 1 h before the injection of compound 48/80 (n = 10/group). In the time dependent experiment, RFRC (1000 mg/kg) was administered intraperitoneally at 5, 10, 15, and 20 min after injection of compound 48/80 (n = 10/group). Mortality was monitored for 1 h after induction of anaphylactic shock.

Passive cutaneous anaphylaxis (PCA) reaction – An IgE-dependent cutaneous reaction was examined as previously described (Shin *et al.*, 2005). 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 was received an injection of 1 μg of DNP-HSA containing Evans blue via the tail vein. RFRC (1 - 1000 mg/kg, BW) was intraperitoneally administered 1 h before the challenge. Then 30 min after the challenge, the mice were killed and the dorsal skin was removed for measurement of the pigment area. The amount of dye was determined colorimetrically after extraction with 1 ml of 1 M KOH and 9 ml of a mixture of acetone and phosphoric acid (5 : 13) based on the previous report (Shin *et al.*, 2006). The intensity of absorbance was measured at 620 nm in a spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences).

Western blot analysis – HMC-1 cells (3×10^6/well in a 6-well plate) were washed with PBS and resuspended in lysis buffer. Samples of protein were electrophoresed using 12% SDS-PAGE, as described elsewhere (Kim and Sharma, 2004) and then transferred to nitrocellulose membrane. The TNF-α and IL-6 were assayed using anti-TNF-α and IL-6 antibody (R&D Systems Inc. Minneapolis, MN). Immunodetection was done using enhanced chemiluminescence detection kit (Amersham, Piscataway, NJ).

Reverse-transcriptase polymerase chain reaction (RT-PCR) – The total cellular RNA was isolated from the cells (1×10^6 /well in a 24-well plate) after stimulation with PMA (20 nM) and A23187 (1 μM) with or without RFRC for 2 h using a TRI reagent (Molecular Research Center, Cincinnati, OH, U.S.A.) according to the manufacturer's protocol. The first-strand complementary DNA (cDNA) was synthesized using the Superscript II reverse transcriptase (Life Technologies). RT-PCR was used to analyze the expression of mRNA for TNF-α, IL-6 and β-actin (internal control). The conditions for the reverse transcription and PCR steps were similar to those previously described (Kim and Shin, 2006). The primer sets were chosen with the Primer 3 program (Whithead Institute, Cambridge, MA, U.S.A.). The cycle number was optimized to ensure product accumulation in the exponential range. 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 UN-SCAN-IT software (Silk Scientific, Orem, UT, U.S.A.). The band intensity was normalized to that of β-actin in the same sample.

Statistical Analysis – Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC). Treatment effects were analyzed using one way analysis of variance followed by Duncan's Multiple Range test. A value of $p < 0.05$ was used to indicate significant differences.

Results

RFRC inhibits compound 48/80-induced systemic reaction – To determine the effect of RFRC on allergic reaction, an *in vivo* model of systemic reaction was used. Compound 48/80 (8 mg/kg BW) was used to induce a systemic fatal allergic reaction. After the intraperitoneal

Table 1. Effect of RFRC on compound 48/80-induced systemic allergic reaction

RFRC treatment (mg/kg BW)	Compound 48/80 (8 mg/kg BW)	Mortality (%)
None (saline)	+	100
50	+	100
100	+	60
500	+	10
1000	+	0
1000	–	0

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

Table 2. Time-dependent effect of RFRC on compound 48/80-induced systemic allergic reaction

RFRC treatment (mg/kg BW)	Time (min)	Compound 48/80 (8 mg/kg BW)	Mortality (%)
None (saline)	0	+	100
1000	5	+	30
	10	+	60
	15	+	80
	20	+	100

Groups of mice (n = 10/group) were intraperitoneally pretreated with 200 μl of saline or RFRC. RFRC (1000 mg/kg) was given at 5, 10, 15 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 × 100/total number of experimental mice.

injection of compound 48/80, the mice were monitored for 1 h, after which the mortality rate was determined. As shown in Table 1, injection of compound 48/80 to mice induced fatal shock in 100% of animals. When RFRC was intraperitoneally administered at concentrations ranging from 50 to 1000 mg/kg BW for 1 h, the mortality with compound 48/80 was dose-dependently reduced. In addition, the mortality of mice administered with RFRC (1000 mg/kg) 5, 10, 15, and 20 min after compound 48/80 injection increased time-dependently (Table 2).

RFRC inhibits IgE-mediated PCA reaction – PCA is one of the most important *in vivo* models of anaphylaxis in a local allergic reaction (Wershil *et al*., 1987). Local extravasation is induced by a local injection of anti-DNP IgE followed by an intravenous antigenic challenge. Anti-DNP IgE was injected into the right dorsal skin sites. As a control, the left dorsal skin site of these mice was injected with saline alone. After 48 h, all animals were injected

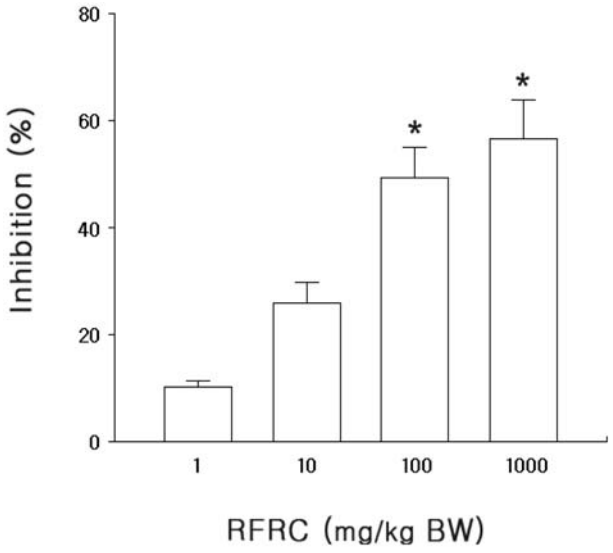

Fig. 1. Effect of RFRC on the 48 h PCA. RFRC was intraperitoneally administered 1 h prior to the challenge with antigen. Each data represents the mean ± SEM of three independent experiments. *Significantly different from the saline value at $p < 0.05$.

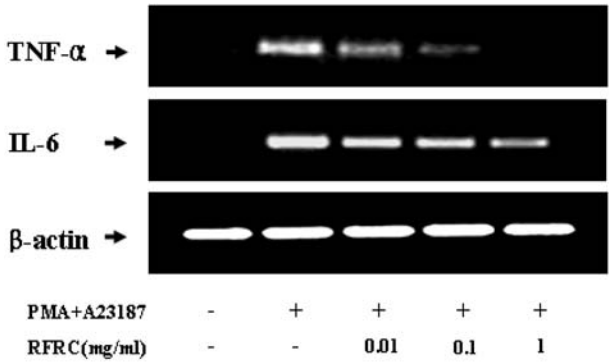

Fig. 2. Effect of RFRC on gene expression of TNF-α and IL-6 in HMC-1 cells. The gene expression of TNF-α and IL-6 was analyzed using RT-PCR.

intravenously with DNP-HSA and Evans blue dye. RFRC was administered intraperitoneally 1 h prior to the challenge with antigen. The administration of RFRC (1 - 1000 mg/kg, BW) showed a dose-dependent inhibition in the PCA reaction (Fig. 1).

RFRC inhibits gene expression and production of TNF-α and IL-6 from HMC-1 cells – TNF-α and IL-6 are the most important pro-inflammatory cytokines. Therefore, I examined the effects of RFRC on the gene expression and production of TNF-α and IL-6 using RT-PCR and Western blotting, respectively. HMC-1 is useful for studying the synthesis of mediators and cytokine activation pathways (Kim *et al*., 2006). As shown in Fig. 2, the gene expression of TNF-α and IL-6 were increased

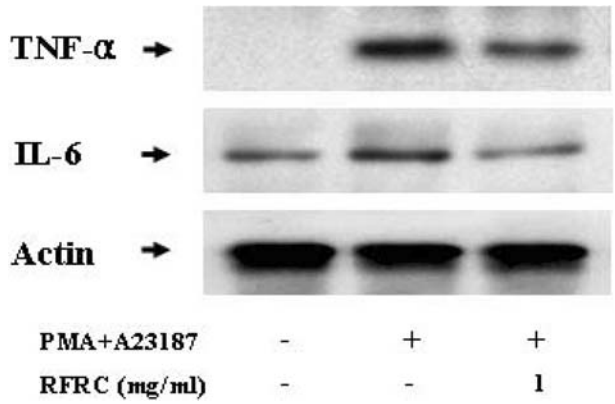

Fig. 3. Effect of RFRC on secretion of TNF-α and IL-6 in HMC-1 cells. The secretion of TNF-α and IL-6 was analyzed using Western blotting.

after stimulation with PMA plus A23187. Pretreatment with RFRC inhibited PMA plus A23187-induced production of TNF-α and IL-6 mRNA. Pretreatment with RFRC also decreased PMA plus A23187-induced production of TNF-α and IL-6 protein (Fig. 3). The concentration of RFRC used in the experiment did not interfere with the viability of cells as assessed in the trypan blue dye-exclusion test.

Discussion

Mast cell-induced anaphylactic allergic reaction is a life threatening syndrome induced by the sudden systemic release of inflammatory mediators, such as histamine, heparin, lipid-derived mediators and various cytokines from mast cells. Mast cells are located throughout the human body, and upon allergen exposure, they are stimulated via the IgE receptor (Kemp and Lockey, 2002).

The results of this study demonstrated that RFRC has anti-allergic properties. Pretreatment with RFRC profoundly inhibited compound 48/80-induced systemic reaction and IgE-mediated local allergic reaction. These results indicate that mast cell-mediated allergic reactions are inhibited by RFRC. It is known that stimulation of mast cells with compound 48/80 or IgE initiates the activation of a signal transduction pathway which leads to histamine release. Several studies have shown that compound 48/80 and other polybasic compounds are able, apparently directly, to activate G-proteins (Mousli *et al*., 1990). The evidence indicates that the protein is Gi-like and that the activation is inhibited by benzalkonium chloride (Bueb *et al.*, 1990). Tasaka (Tasaka *et al*., 1986) reported that compound 48/80 increases the permeability of the lipid bilayer membrane by causing a perturbation of the membrane. This result indicates that the increase in membrane permeability may be an essential trigger for the release of the mediator from mast cells. In this sense, anti-allergic agents having a membrane-stabilizing action may be desirable. RFRC may act on the lipid bilayer membrane, thus preventing the perturbation induced by compound 48/80.

PCA is one of the most important *in vivo* models of anaphylaxis in local allergic reactions. The mice administered with RFRC were protected from IgE-mediated PCA. This finding suggests that RFRC might be useful in the treatment of allergic skin reactions.

Mast cell-derived cytokines, especially TNF-α and IL-6 have a critical biological activity in the allergic reaction. It has been reported that mast cells are a principal source of TNF-α in human dermis, and degranulation of mast cells in the dermal endothelium is abrogated by the anti-TNF-α antibody (Walsh *et al*., 1991). IL-6 is also produced from mast cells and its local accumulation is associated with a PCA reaction (Mican *et al*., 1992). These reports indicate that reduction of pro-inflammatory cytokines from mast cell is a one of the key indicator of reduced allergic symptom. The HMC-1 cell line is useful for studying cytokine activation pathways (Sillaber *et al*., 1993). RFRC inhibited the production of TNF-α and IL-6 in PMA plus A23187-stimulated HMC-1 cells. This result may suggest that one possible pathway of the anti-allergic effect of RFRC results from the reduction of TNF-α and IL-6 from mast cells. The effect of RFRC on TNF-α and IL-6 production by mast cells *in vivo* and the relative importance of mast cells as a source of TNF-α and IL-6 during inflammatory and immune responses are important areas for future studies. In conclusion, the results obtained in the present study provide evidence that RFRC might contribute to the treatment of mast cell-mediated allergic diseases.

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