

Inhibitory Effects of BiRyuChe-Bang on Mast Cell-Mediated Allergic Reactions and Inflammatory Cytokines Production

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Abstract: BiRyuChe-bang (BRC) is a Korean prescription medicine, which has been used to treat allergic rhinitis at Kyung Hee Medical Center. In this work, we investigated the effects of BRC on mast cell-mediated allergic reactions and inflammatory cytokines production, and identified the active component of BRC. Histamine release was measured from rat peritoneal mast cells (RPMCs). Ear swelling and passive cutaneous anaphylaxis (PCA) were examined in mouse models. Phorbol 12-myristate 13-acetate (PMA) plus A23187-induced inflammatory cytokines production was measured using enzyme-linked immunosorbent

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assay. Reverse transcriptase-polymerase chain reaction was used for the expressions of tumor necrosis factor (TNF)- α , interleukin (IL)-6, and IL-8. Activation of nuclear factor (NF)- κ B was analyzed by Western blotting. BRC significantly inhibited the compound 48/80-induced ear swelling response, histamine release from RPMCs, PCA activated by anti-dinitrophenyl IgE, and PMA plus A23187-induced inflammatory cytokines production ($p < 0.05$). In addition, BRC dose-dependently inhibited the mRNA expressions of TNF- α , IL-6, and IL-8 as well as the activation of NF- κ B in a human mast cell line, HMC-1 cells. BRC inhibited the levels of TNF- α and IL-6 in mice induced with PCA. Several components of BRC, such as 1,8-Cineole, Linalool, Linalyl acetate, α -Pinene, and α -Terpineol, significantly inhibited the release of histamine from RPMCs ($p < 0.05$). Among these components, Linalyl acetate was the most effective for inhibiting histamine release. These results indicate that BRC has a potential regulatory effect on allergic and inflammatory reactions mediated by mast cells.

Keywords: BRC; Mast Cells; Histamine; Inflammatory Cytokines; Nuclear Factor- κ B; Linalyl Acetate.

Introduction

Mast cells are regarded as critical effector cells in the allergic and inflammatory reactions that underlie immediate-type hypersensitivity reaction-mediated clinical conditions such as urticaria, allergic rhinitis, bronchial asthma, and atopic dermatitis (Miescher and Vogel, 2002; Emi and Maeyama, 2004). Activated mast cells can produce histamine as well as a wide variety of other inflammatory mediators and several pro-inflammatory and chemotactic cytokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-8, and IL-13 (Royer *et al.*, 2001; Stassen *et al.*, 2001; Song *et al.*, 2012). Histamine is one of the well characterized and potent vasoactive mediators implicated in the acute phase of immediate-type hypersensitivity reactions among the substances released on degranulation of mast cells (Moon *et al.*, 2007c). The cytokine TNF- α is an autocrine stimulator as well as a potent inducer of other inflammatory cytokines such as IL-1 β , IL-6, IL-8, and the granulocytemonocyte colony-stimulating factor (Arend and Dayer, 1995; Butler *et al.*, 1995). IL-6 is a pleiotropic inflammatory cytokine produced by T cells, monocytes, macrophages, and synovial fibroblasts, whereas IL-8, the first human chemokine, is often upregulated at inflammatory sites such as rheumatoid lesions (Baggiolini, 2001), and it acts on inflammatory effector cells such as neutrophils, T lymphocytes, B lymphocytes, and eosinophils (Mukaida, 2000).

The activation of NF- κ B induces the expression of various inflammatory cytokines. The expression of inflammatory cytokines, such as IL-6, IL-8, and TNF- α , depends on the activation of NF- κ B (Kim *et al.*, 2007; Huang *et al.*, 2012; Moon and Kim, 2012a). NF- κ B normally resides in the cytoplasm (Baeuerle and Henkel, 1994). However, when activated, NF- κ B translocates to the nucleus, binds the DNA, and activates genes (Moon *et al.*, 2007c).

BiRyuChe-bang (BRC) has been used to treat allergic rhinitis at Kyung Hee Medical Center in Seoul, Korea. BRC is used as an aerosol and bath oil. Many allergic patients have

been treated with BRC; however, its pharmacological effects have not been fully understood in experimental models. Thus, this study used a murine model to investigate how BRC affects histamine release, ear swelling response, and passive cutaneous anaphylaxis (PCA). Moreover, we used cells from the human mast cell line (HMC-1) to examine the effect of BRC on the production of inflammatory cytokines through NF- κ B activation.

Materials and Methods

Chemical Reagents

The chemicals α -Pinene, α -Terpineol, Linalool, Linalyl acetate, compound 48/80, anti-dinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA), metrizamide, phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187 (calcymycin; C₂₉H₃₇N₃O₆), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), *o*-phthaldialdehyde (OPA), avidin-peroxidase, 2,2-azio-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and Evans Blue were purchased from Sigma Chemical Co. (St. Louis, MO, USA). In addition, 1,8-Cineole was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Antibodies

For antibodies, anti-human TNF- α antibody, biotinylated anti-human TNF- α antibody, and recombinant human TNF- α were purchased from R&D Systems (Minneapolis, MN, USA). Anti-human IL-6 and IL-8 antibodies, biotinylated anti-human IL-6 and IL-8 antibodies, and recombinant human IL-6 and IL-8 were purchased from BD PharMingen (San Diego, CA, USA). NF- κ B and β -actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Preparation of BRC

BRC was obtained from the Kyung Hee Medical Center (Seoul, Republic of Korea) and consisted of lavender oil, pine oil, and eucalyptus oil (1:2:2). Dilutions were made in distilled water then filtered through a 0.22- μ m syringe filter.

Animals

The original stock of male ICR mice (four weeks old) and male Wistar rats (seven weeks old) were purchased from the Dae-Han Experimental Animal Center (Eumsung, Republic of Korea), and the animals were maintained at the College of Korean Medicine, Kyung Hee University. Animal care and experimental procedures were performed under the approval of the animal care committee at Kyung Hee University [Approval No. KHUASP (SE)-10-019]. The rats were housed five to ten per cage in a laminar air-flow room maintained at a temperature of 22 $\pm$ 1°C and a relative humidity of 55 $\pm$ 1% throughout the study. No animal was used more than once. The research was conducted in accordance with

internationally accepted principles for laboratory animal use and care as found in US guidelines (NIH publication #85-23, revised in 1985).

Cell Culture

For cell culture, α -minimal essential medium was purchased from Flow Laboratories (Irvine, UK). Fetal bovine serum was purchased from Life Sciences (Grand Island, NY, USA). Iscove's modified Dulbecco's medium (IMDM) was purchased from Gibco BRL (Grand Island, NY, USA). Human mast cell line, HMC-1 cells were grown in IMDM and supplemented with 100 U/mL of penicillin, 100 μ g/mL of streptomycin, and 10% fetal bovine serum at 37°C in 5% CO₂ with 95% humidity. Rat peritoneal mast cells (RPMCs) were isolated as previously described (Jippo-Kanemoto *et al.*, 1993). Mast cells were separated from the major components of rat peritoneal cells (i.e. macrophages and small lymphocytes) according to the method described by Yurt *et al.* (1997).

MTT Assay

For the MTT colorimetric assay of cell survival, the method of Ben Trivedi *et al.* (1990) was used with minor modifications. First, cell aliquots (3×10^5) were seeded in microplate wells and incubated with 20 μ L of an MTT solution (5 mg/mL) for 4 h at 37°C under 5% CO₂ and 95% air. Next, 250 μ L of dimethyl sulfoxide was added to extract the MTT formazan, and an automatic microplate reader was used to read the absorbance of each well at 540 nm.

Histamine Assay

Purified RPMCs were resuspended in Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 0.1% bovine serum albumin) containing calcium for the treatment with compound 48/80. RPMC suspensions (2×10^5 cells/mL) were preincubated for 10 min at 37°C before the addition of compound 48/80 for stabilization. The cells were preincubated with BRC or various components of BRC for 20 min, and then incubated for 15 min with compound 48/80 (6 μ g/mL). The reaction was stopped by cooling the tubes in ice. The cells were separated from the released histamine by centrifugation at $400 \times g$ for 5 min at 4°C. Residual histamine in the cells was released by disrupting the cells with perchloric acid and centrifugation at $400 \times g$ for 5 min at 4°C. The histamine content was measured by the OPA spectrofluorometric procedure, as previously described (Moon *et al.*, 2007a). The fluorescent intensity was measured at 440 nm (excitation at 360 nm) in a spectrofluorometer. The inhibition percentage of histamine release was calculated using the following equation: % inhibition = $(A - B) \times 100/A$; where A is the histamine release without BRC and B is the histamine release with BRC.

Ear Swelling Response

Compound 48/80 was freshly dissolved in saline and injected intradermally into the dorsal aspect of a mouse ($n = 5$) ear using a microsyringe with a 28-gauge hypodermic needle.

Ear thickness was measured with a digimatic micrometer (Mitutoyo, Japan) under mild anesthesia. BRC (0.01 and 0.1%) was applied to both sides of the ears 1 h before the compound 48/80-injection (100 μ g/site). The ear swelling response evoked by physiologic saline returned to almost baseline thickness within 40 min.

PCA Reaction

The IgE-dependent cutaneous reaction was generated by sensitizing the skin with an intradermal injection of anti-DNP IgE followed 48 h later with an injection of DNP-HSA into the mouse tail vein. BRC was applied to both sides of dorsal skin. The mice ($n = 5$) were sacrificed 40 min after the intravenous challenge. The amount of dye was determined colorimetrically based on the method of Katayama *et al.* (1978). The absorbent intensity of the extraction was measured at 620 nm in a spectrofluorometer, and the amount of dye was calculated with the Evans Blue measuring-line.

Isolation of Tissue Protein

After the induction of PCA, the mice were sacrificed and their dorsal skin rapidly removed, and then frozen in liquid nitrogen. Samples were dissected and homogenized in ice-cold buffer (20 mM HEPES, pH 7.5, 1.5 mM MgCl_2 , 0.2 mM EDTA, 0.1 M NaCl) supplemented with 0.2 mM DTT, 0.5 mM sodium vanadate, and protease inhibitors (0.4 mM PMSF and 2 mg/mL each of leupeptin, pepstatin, and aprotinin). Subsequently, NaCl was added to a final concentration of 0.45 M, and the homogenate was centrifuged at $15,000 \times g$ for 30 min. Supernatants were collected and mixed with an equal volume of homogenization buffer containing 40% (vol/vol) glycerol before being stored at -70°C . Samples were assayed for the TNF- α and IL-6. Protein was determined using a bicinchoninic acid (Sigma, St. Louis, MO, USA).

Cytokine Assays

HMC-1 cells were pretreated with BRC (0.01 and 0.1%) for 1 h before PMA plus A23187 stimulation. The supernatants of homogenized tissue proteins from mice induced with PCA were used to investigate the effects of BRC in an *in vivo* model. The enzyme-linked immunosorbent assay (ELISA) method was used to assay the culture supernatants for TNF- α , IL-6, and IL-8 protein levels. To measure the levels of these cytokines, a modified ELISA method was used (Moon and Kim, 2012a).

Reverse Transcriptase-polymerase Chain Reaction (RT-PCR) Analysis

Using an easy-BLUETM RNA extraction kit (iNtRON Biotech, Republic of Korea), the total RNA was isolated from HMC-1 cells according to the manufacturer's specifications, as previously described (Moon and Kim, 2011, 2012b). The concentration of total RNA in the final eluate was determined by spectrophotometry. PCR was performed with the

following primers: TNF- α (5'CAC CAG CTG GTT ATC TCT CAG CTC 3'; 5'CGG GAC GTG GAG CTG GCC GAG GAG 3'); IL-6 (5'GAT GGA TGC TTC CAA TCT GGA T 3'; 5'AGT TCT CCA TAG AGA ACA ACA TA 3'); and IL-8 (5'CGA TGT CAG TGC ATA AAG ACA 3'; 5'TGA ATT CTC AGC CCT CTT CAA AAA 3'). To verify if equal amounts of RNA were used for the reverse transcription and PCR amplification from different experimental conditions, GAPDH (5'CAA AAG GGT CAT CAT CTC TG 3'; 5'CCT GCT TCA CCA CCT TCT TG 3') was used. The annealing temperature was 56°C for IL6 and 60°C for TNF- α , IL8, and GAPDH. The amplified fragment sizes were 355 bp for TNF- α , 443 bp for IL-6, 200 bp for IL-8, and 446 bp for GAPDH. Finally, the products were electrophoresed on a 2.0% agarose gel and visualized by staining with ethidium bromide.

Nuclear Protein Extraction

Nuclear extracts were prepared as previously described (Moon *et al.*, 2012). Briefly, after cell activation, 5×10^6 cells were washed in 1 mL of ice-cold PBS, centrifuged at $15,000 \times g$ for 5 min, resuspended in 400 μ L of ice-cold hypotonic buffer (10 mM HEPES/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 10 min, vortexed, and centrifuged at $15,000 \times g$ for 30 s. The pellets of nuclei were gently resuspended in 40 μ L of cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, and 0.5 mM PMSF, pH 7.9) and left for 20 min on ice. After centrifugation ($15,000 \times g$ for 15 min at 4°C), we froze the aliquots of supernatant containing the nuclear proteins in liquid nitrogen and stored them at -70°C until ready for analysis. The bicinchoninic acid protein assay (Sigma, St. Louis, MO, USA) was used to measure protein concentrations.

Western Blot Analysis

The HMC-1 cells (5×10^6 cells/well) were stimulated with PMA plus A23187. Cell lysates were then prepared in a sample buffer containing sodium dodecyl sulfate (SDS). The results were visualized using antibody-specific proteins with an enhanced chemiluminescence detection kit through recommended procedures (Amersham Corp., Newark, NJ, USA).

Chromatography Conditions

For the DB-WAX column, the injector temperature was 240°C, detector temperature was set at 250°C, the injection volume was 1 μ L, and the column flow rate was 1 mL/min. The oven temperature program was as follows: held for 3 min at 40°C, then 3°C/min to 110°C, 10°C/min to 180°C, then a final ramp of 15°C/min to 240°C where this temperature was held for 5 min. For the HP5-MS column, the conditions were the same, except the oven program was held for 1 min at 40°C, then ramped at 7.5°C/min to 250°C and held.

Statistical Analysis

Statistical analysis was performed using SPSS software (version 14.0, SPSS Inc, Chicago, IL, USA). Our results are expressed as the mean $\pm$ standard error of the mean (S.E.M.). The statistical evaluation of the results was performed by an independent *t*-test and an analysis of variance with a Tukey post hoc test. The results were deemed significant at $p < 0.05$.

Results

Effect of BRCon Cell Survival and Histamine Release from RPMCs

First of all, we pretreated RPMCs with BRC at concentrations ranging from 0.01 to 1%. The cytotoxicity of BRC was observed at a dose of 1% (Fig. 1A, $p < 0.05$); thus, we evaluated the effects of BRC at concentrations of 0.01 and 0.1% in the next set of experiments. The inhibitory effect of BRC on compound 48/80-induced histamine release from RPMCs is shown in Fig. 1B. BRC significantly inhibited compound 48/80-induced histamine release in a dose-dependent manner (Fig. 1B, $p < 0.05$). The maximal inhibition rate from 0.1% of BRC was $47.86 \pm 1.98\%$. The histamine intensities at doses of 0.01 and 0.1% were 28.67 ± 2.78 and 22.49 ± 2.29 , respectively. Control and spontaneous values were 37.61 ± 4.56 and 1.54 ± 0.40 , respectively.

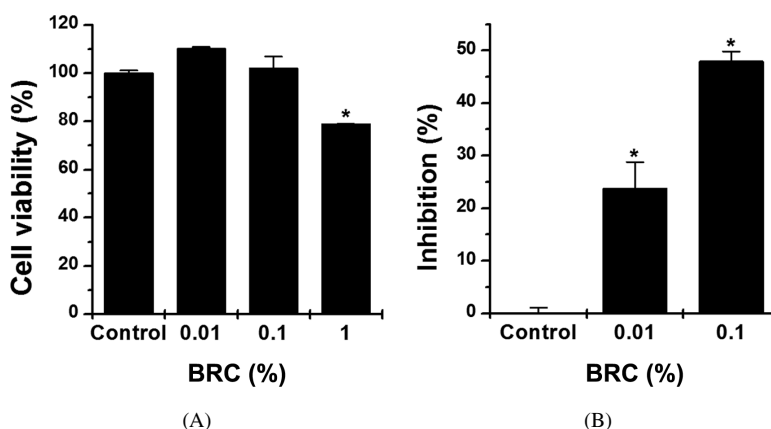


Figure 1. Effect of BRC on cell survival and histamine release from RPMCs. (A) RPMCs (2×10^5) were treated with various concentrations (0.01 to 1%) of BRC for 30 min, and then stimulated with compound 48/80 for 20 min. Cell viability was determined with the MTT assay. The results represent three independent experiments conducted in duplicate. The values from each treatment were expressed as a percentage relative to the control (100%). (B) RPMCs (2×10^5 cells) were preincubated with various concentrations of BRC at 37°C for 30 min prior to incubation with compound 48/80. Each datum represents the mean $\pm$ S.E.M. of three independent experiments. Control, PBS-treated, and then compound 48/80-stimulated cells. * $p < 0.05$; significantly different from the control values.

Table 1. Effect of BRC on the Compound 48/80-induced Ear Swelling Response in Mice

Treatment	Dose (%)	Pre-Thickness	Post-Thickness	Inhibition (%)
Control	—	0.35 ± 0.01	0.61 ± 0.02	0.00 ± 5.35
BRC	0.01	0.34 ± 0.00	0.52 ± 0.02	32.24 ± 9.14*
	0.1	0.37 ± 0.02	0.54 ± 0.03	37.99 ± 6.71*

Note: BRC was applied to both sides of a mouse ear for 1 h prior to compound 48/80 injection. Injections were then performed intradermally with 20 μ L of compound 48/80 (100 μ g/site). Control, vehicle-applied, and then compound 48/80-injected group. Each datum represents the mean $\pm$ S.E.M. of three independent experiments.

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

Effect of BRC on the Ear Swelling Response

The previous experiment (Moon *et al.*, 2007a) showed that the intradermal application of compound 48/80 at a concentration of 100 μ g/site induces an optimal ear swelling response in normal mice. As shown in Table 1, when mice were pretreated with BRC for 1 h, the ear swelling responses derived from compound 48/80 were significantly inhibited ($p < 0.05$).

Effect of BRC on PCA

When BRC was applied to the dorsal skin of mice, the PCA reaction was inhibited in a dose-dependent manner. The highest inhibition was obtained at a dose of 0.1% (Fig. 2, $p < 0.05$). The amount of dye at doses of 0.01 and 0.1% were 0.58 ± 0.04 μ g/mL and 0.33 ± 0.03 μ g/mL, respectively. The amount of dye in control mice was 0.68 ± 0.03 μ g/mL.

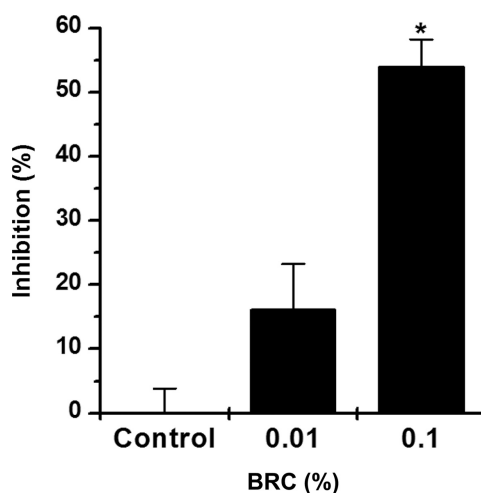


Figure 2. Effect of BRC on PCA in mice. BRC was applied to both sides of dorsal skin for 1 h prior to the challenge with antigen (DNP-HSA). Each datum represents the mean $\pm$ S.E.M. of three independent experiments. Control, vehicle-applied, and then antigen-challenged group. * $p < 0.05$; significantly different from the control value.

Effect of BRC on PMA Plus A23187-Induced TNF- α , IL-6, and IL-8 Production in HMC-1 Cells

Treatment with PMA (0.05 μ M) plus A23187 (1 μ M) increased the production of cytokines from the HMC-1 cells. We tested the effect of BRC on the PMA plus A23187-induced TNF- α , IL-6, and IL-8 production. The increases in TNF- α , IL-6 and IL-8 due to PMA plus A23187 were significantly decreased by BRC ($p < 0.05$; Figs. 3A–3C). To determine

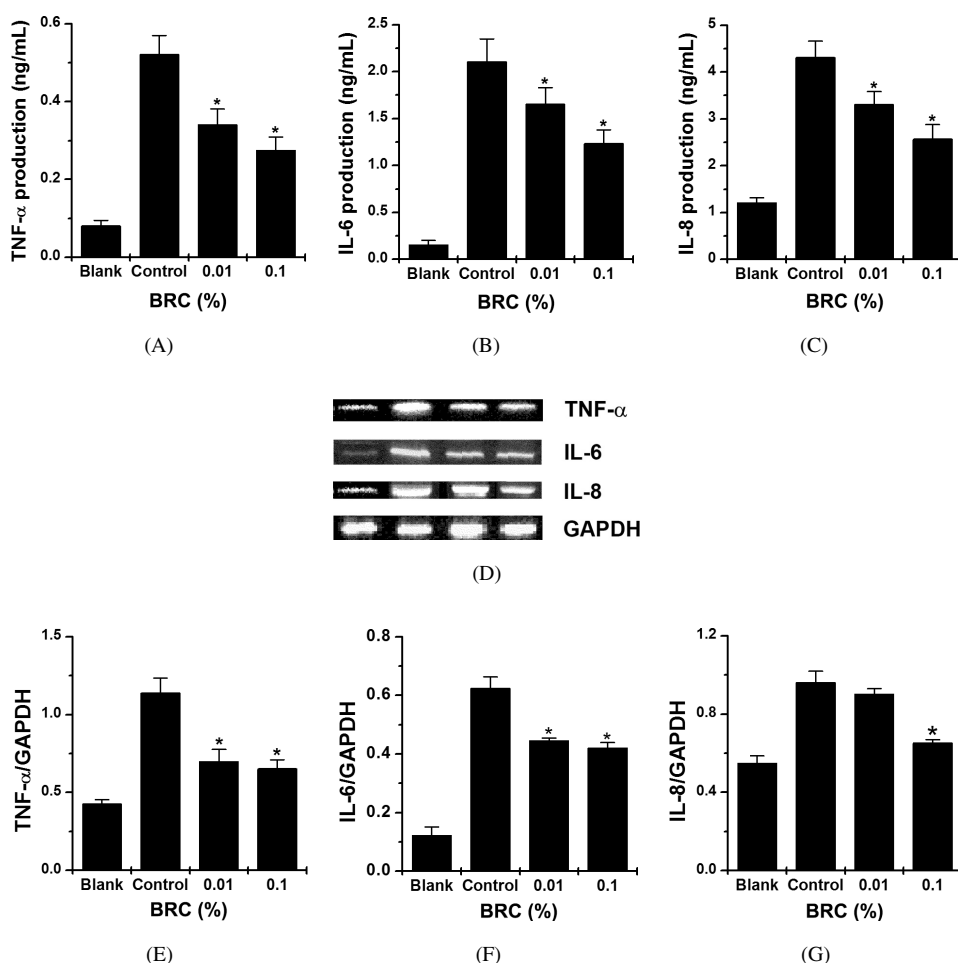


Figure 3. Effect of BRC on cytokine production and mRNA expression after PMA plus A23187 stimulation. (A–C) HMC-1 cells (3×10^5) were treated with BRC (0.01 and 0.1%) for 1 h, then stimulated with PMA plus A23187 for 8 h. Cytokine levels in the supernatant were measured with the ELISA method. (D) HMC-1 cells (5×10^6) were treated with BRC (0.01 and 0.1%) for 1 h, and then stimulated with PMA plus A23187 for 6 h. The mRNA was measured with the RT-PCR method. (E)–(G) The mRNA expression levels of each cytokine were quantified by densitometry. Blank, PBS-treated cells; Control, PBS-treated, and then PMA plus A23187-stimulated cells. Each datum represents the mean $\pm$ S.E.M. of three independent experiments. * $p < 0.05$; significantly different from the control value.

whether BRC can modulate the PMA plus A23187-induced mRNA expression of each cytokine, RT-PCR analysis was performed. The mRNA expression of TNF- α , IL-6 and IL-8 was up-regulated by PMA plus A23187, but decreased by treatment with BRC (Figs. 3E–3G).

Effect of BRC on NF- κ B Activation in HMC-1 Cells

The production of TNF- α , IL-6, and IL-8 is regulated by the transcription factor NF- κ B/Rel A, a subunit of NF- κ B. To determine the effect of BRC on the activation of NF- κ B, we performed Western blot analysis for NF- κ B/Rel A in cells stimulated by PMA plus A23187. In the control group stimulated by PMA plus A23187, the protein level of NF- κ B/Rel A increased in the nucleus. However, the protein level of NF- κ B/Rel A in the nucleus decreased from treatment with BRC (Fig. 4). The protein level of β -actin in nuclear extract was unchanged by any treatment.

Effect of BRC on Inflammatory Responses in Mice

BRC inhibited the inflammatory responses in mast cells *in vitro*. To determine whether BRC can inhibit inflammatory responses in an *in vivo* model, we assessed the levels of inflammatory cytokines in mice induced with PCA. When BRC was applied to the dorsal skin of mice, the levels of TNF- α and IL-6 were inhibited in a dose-dependent manner. The highest inhibition was obtained at a dose of 0.1% (Fig. 5, $p < 0.05$).

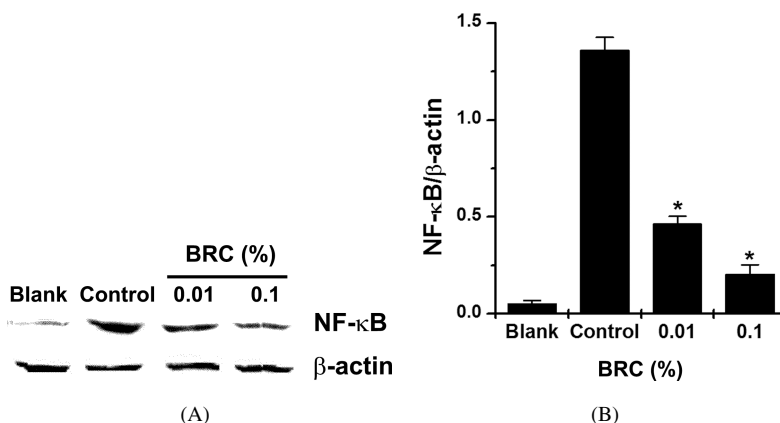


Figure 4. Effect of BRC on PMA plus A23187-induced NF- κ B activation. (A) HMC-1 cells (5×10^6) were treated with BRC (0.01 and 0.1%) for 1 h, then stimulated with PMA plus A23187 for 2 h. Nuclear proteins were prepared and analyzed for NF- κ B by Western blotting as described in the experimental procedures. (B) NF- κ B levels were quantified with densitometry. Blank, PBS-treated cells; Control, PBS-treated, and then PMA plus A23187-stimulated cells. Each datum represents the mean $\pm$ S.E.M. of three independent experiments. * $p < 0.05$; significantly different from the control value.

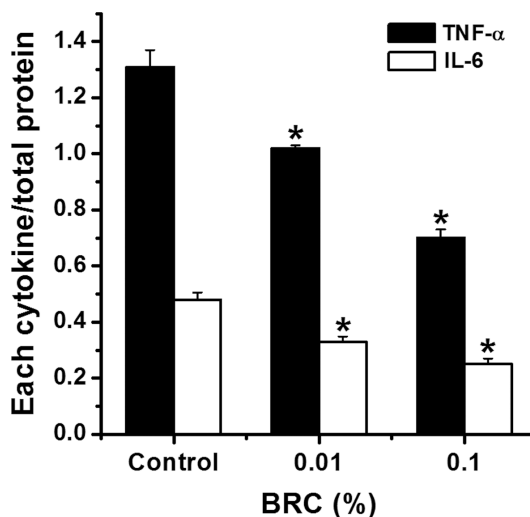


Figure 5. Effect of BRC on inflammatory responses in mice. BRC was applied to both sides of dorsal skin for 1 h prior to antigen challenge (DNP-HSA). Cytokine levels in the supernatants of homogenized tissue proteins from mice induced with PCA were measured with the ELISA method. Each datum represents the mean $\pm$ S.E.M. of three independent experiments. Control, vehicle-applied, and then antigen-challenged group. * $p < 0.05$; significantly different from the control value.

Effect of Components of BRC on Histamine Release from RPMCs

BRC is a mixture of three essential oils. Thus, we investigated which is an active component in BRC. First of all, we selected the major components (1,8-Cineole, α -Pinene, α -Terpineol, Linalool, Linalyl acetate) from each essential oil (Lee *et al.*, 2000; Manninen *et al.*, 2002;

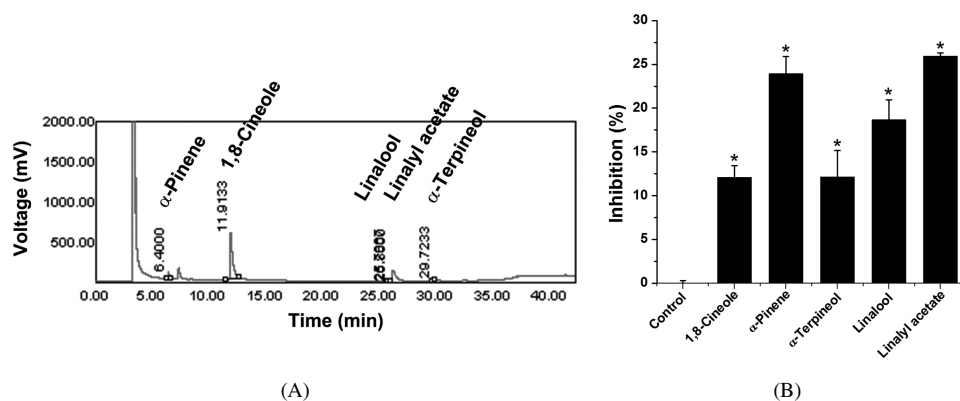


Figure 6. Effect of components of BRC on histamine release from RPMCs. (A) Gas chromatogram of BRC. (B) RPMCs (2×10^5 cells) were preincubated with each component of BRC at 37°C for 30 min prior to incubation with compound 48/80. Each datum represents the mean $\pm$ S.E.M. of three independent experiments. Control, PBS-treated, and then compound 48/80-stimulated cells. * $p < 0.05$; significantly different from the control values.

Ballabeni *et al.*, 2004; Prashar *et al.*, 2004; Juan *et al.*, 2011; Mann *et al.*, 2011). Gas chromatography was used to determine the ratio of each component in the BRC mixture. The ratios of each component are as follows: 1,8-Cineole (91.44%), α -Pinene (6.75%), α -Terpineol (1.65%), Linalool (0.10%), Linalyl acetate (0.06%) (Fig. 6A). To compare the efficiency of each component, we pretreated PRMCs for 30 min with each component at the same dose (0.4 mg/mL) considering the report of Santos *et al.* (2004). The inhibition rates of each component were $25.92 \pm 0.37\%$ (Linalyl acetate), $23.90 \pm 2.00\%$ (α -Pinene), $18.64 \pm 2.32\%$ (Linalool), $12.14 \pm 3.00\%$ (α -Terpineol), and $12.06 \pm 1.35\%$ (1,8-Cineole), respectively (Fig. 6B).

Discussion

The stimulation of mast cells with compound 48/80 is believed to initiate the activation of a signal transduction pathway, which leads to histamine release. There have been some reports that compound 48/80 is able to activate G proteins (Mousli *et al.*, 1990a,b). (Chadi *et al.*, 2000) reported that compound 48/80 activates mast cell phospholipase D (PLD) via heterotrimeric GTP-binding proteins. They identified recombinant $G_{\beta 2\gamma 2}$ subunit markedly synergizes PLD activation by compound 48/80 in permeabilized RBL-2H3 cells.

Murine mast cells are a good experimental model for the study of compound 48/80-induced histamine release (Alfonso *et al.*, 2000). The report that compound 48/80 increased the permeability of the lipid bilayer membrane by causing a perturbation of the membrane (Tasaka *et al.*, 1986) indicates that the increase in membrane permeability may be an essential trigger for the release of mediators from mast cells. Our results showed that BRC profoundly affected compound 48/80-induced histamine release and the ear swelling response (Fig. 1B and Table 1). Thus, we hypothesize that BRC might act on the lipid bilayer membrane affecting the prevention of the perturbation induced by compound 48/80 and regulate the degranulation of mast cells in mouse skin by stabilizing membrane fluidity.

The high-affinity IgE-receptor (Fc ϵ RI) is primarily expressed by mast cells (Daeron, 1997). Cross-linking of IgE-bound Fc ϵ RI by a multivalent Ag on mast cells induces the release of biologically active mediators: the preformed mediators stored in the cytoplasmic granules, such as histamine (Galli *et al.*, 2005). To reproduce these reactions in animals, a PCA model was used in passively sensitized mice. Our results showed that an application of BRC to the dorsal skin of mice inhibited PCA (Fig. 2). It is conceivable that an application of BRC inhibits the immediate type allergic reactions, probably by interfering with the degranulation system.

The PKC activator PMA is a substitute for diacylglycerol and A23187 is a widely used ionophore. Co-stimulation of mast cells with PMA plus A23187 produces TNF- α , IL-6, and IL-8 (Fig. 3). TNF- α promotes inflammation, leukocyte infiltration, granuloma formation, and tissue fibrosis and is thought to be an initiator of cytokine-related inflammatory states by stimulating cytokine production in other types of cells (Baumgartner *et al.*, 1994). The cytokine IL-6 is a pleiotropic inflammatory cytokine produced by T cells, monocytes, and

macrophages (Van Snick, 1990); and IL-8, which is a potent activator and chemoattractant of neutrophils, mediates the migration of neutrophils to local inflammation (Karakurum *et al.*, 1994). Elevated levels of circulating TNF- α , IL-1 β , IL-4, IL-6, and IL-8 have been reported in various pathological states, including sepsis, rheumatoid arthritis, osteoarthritis, asthma, and cerebral infarction (Lin *et al.*, 1994; Hammami *et al.*, 1997; Jeong *et al.*, 2002; Hougee *et al.*, 2005). We have shown here that BRC inhibited the production of TNF- α , IL-6, and IL-8 in HMC-1 cells. Furthermore, BRC inhibits the levels of TNF- α and IL-6 in mice induced with PCA. Thus, the inhibition of proinflammatory cytokine production by BRC may contribute to the treatment of inflammatory diseases.

The expression of inflammatory cytokines (TNF- α , IL-6, and IL-8) is regulated by the transcription factor, NF- κ B (Moon *et al.*, 2007b; Lee *et al.*, 2012; Oh *et al.*, 2012). This pathway is activated by the appropriate cellular stimulation, most often by signals related to pathogens or stress. A therapeutic strategy aimed at limiting the activity of NF- κ B, a critical transcription factor in the regulation of acute inflammation, may have a beneficial effect in these pathological states (Bouchama and Knochel, 2002). As BRC inhibited NF- κ B activation (Fig. 4), we can therefore deduce that BRC acts as a specific inhibitor of the NF- κ B transcription factor to inhibit acute inflammation.

BRC is a mixture of three essential oils. We selected five representative components from each essential oil based on several reports (Lee *et al.*, 2000; Manninen *et al.*, 2002; Ballabeni *et al.*, 2004; Prashar *et al.*, 2004; Juan *et al.*, 2011; Mann *et al.*, 2011). When we pretreated RPMCs with the same dose of each component; Linalyl acetate was the most effective component (Fig. 6B). Although Linalyl acetate is an active component of BRC, 1,8-Cineole is the most abundant component and may heavily contribute to effects of BRC as well.

In conclusion, we have shown that BRC regulates the allergic and inflammatory reactions that are mediated by mast cells. BRC affects the allergic reactions by regulating the degranulation of mast cells, and affects the expression of inflammatory cytokines by regulating the NF- κ B signal cascade. Overall, our results suggest that BRC is a potent inhibitor of histamine release and inflammatory cytokines production in mast cells, potentially explaining its beneficial effects for the treatment of allergic and inflammatory diseases.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0005591) and by a grant from the Kyung Hee University in 2013 (KHU-20130163).

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