

Clinopodium gracile inhibits mast cell-mediated allergic inflammation: involvement of calcium and nuclear factor- κ B

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Abstract

Mast cell-mediated allergic disease is involved in many diseases such as anaphylaxis, rhinitis, asthma and atopic dermatitis. The discovery of drugs for the treatment of allergic disease is an important subject in human health. In this study, we investigated the effect of the water extract of *Clinopodium gracile* Matsum var. multicaule (WECG) on the mast cell-mediated allergic inflammation and studied the possible mechanism of action. WECG inhibited compound 48/80-induced systemic anaphylaxis and immunoglobulin E-mediated cutaneous anaphylaxis in a dose-dependent manner. WECG dose-dependently reduced histamine release from rat peritoneal mast cells and human mast cells. The inhibitory effect of WECG on histamine release was mediated by the modulation of intracellular calcium. In addition, WECG attenuated the phorbol 12-myristate 13-acetate and calcium ionophore A23187-stimulated gene expression and secretion of proinflammatory cytokines such as tumor necrosis factor- α and interleukin-6 in human mast cells. The inhibitory effect of WECG on these proinflammatory cytokines was nuclear factor- κ B (NF- κ B) dependent. Our findings provide evidence that WECG inhibits mast cell-derived allergic inflammation and involvement of calcium and NF- κ B in these effects.

Keywords: *Clinopodium gracile*, allergic inflammation, mast cells, histamine, proinflammatory cytokines

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Introduction

Clinopodium gracile Matsum var. multicaule is a perennial herb that is widely distributed throughout Korea, China and Japan. The water extract of *C. gracile* (WECG) is used as a traditional medicinal herb for the treatment of infectious diseases. This crude drug contains saponins, mainly clinosaponin and saikosaponin.¹

Mast cells are broadly distributed throughout mammalian tissues and play a critical role in a wide variety of biological responses. Typically, mast cells have been considered not only in the association of immediate type hypersensitivity, but also in late reactions like inflammatory responses.^{2,3} Immediate-type hypersensitivity is mediated by histamine released in response to the antigen cross-linking of immunoglobulin E (IgE) bound to Fc ϵ RI on the mast cells.⁴ After activation via the Fc ϵ RI, the mast cells start the process of degranulation, which results in the releasing of mediators, such as products of arachidonic acid metabolism and an array of inflammatory cytokines.^{5,6} Among the inflammatory substances released from the mast cells, histamine is one of the

best characterized and most potent vasoactive mediators implicated in the acute phase of immediate hypersensitivity.^{7,8}

The activation of mast cells leads to the phosphorylation of tyrosine kinase and the mobilization of internal calcium. This is followed by the activation of protein kinase C, an increase of mitogen-activated protein kinases, nuclear factor- κ B (NF- κ B) and the release of inflammatory cytokines. Activated mast cells can produce histamine, as well as a wide variety of other inflammatory mediators such as eicosanoids, proteoglycans, proteases and several proinflammatory and chemotactic cytokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-8, IL-4, IL-13 and the transforming growth factor- β .^{4,5} The transcription factor NF- κ B has important activities as mediators of cellular responses to extracellular signals and is thought to play an important role in the regulation of proinflammatory molecules on cellular responses, especially TNF- α , IL-6 and IL-8.^{9,10}

In this study, we evaluated the effect of WECG on the systemic and local anaphylaxis, and histamine release from mast cells. The intracellular calcium content was

investigated to clarify the mechanism by which WECG inhibited histamine release from mast cells. In addition, the effect of WECG on phorbol 12-myristate 13-acetate and calcium ionophore A23187 (PMACI)-induced gene expression and secretion of proinflammatory cytokines and the role of NF- κ B in this effect were investigated using human mast cells (HMC-1).

Materials and methods

Animals

The original stock of male ICR mice (20–30 g) and male Sprague-Dawley rats (200–300 g) were purchased from the Dae-Han Biolink Co. Ltd (Daejeon, Korea). The animals were maintained in the College of Pharmacy, Woosuk University. The animals were housed 5–10 per cage in a laminar air flow room (conventional condition), maintained at a temperature of $22 \pm 2^\circ\text{C}$, with a relative humidity of $55 \pm 5\%$ 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, antidinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA) and PMACI were purchased from the Sigma Chemical Co. (St Louis, MO, USA). rTNF- α and rIL-6 and anti-TNF- α and -IL-6 antibodies were purchased from R&D Systems Inc. (Minneapolis, MN, 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 2 mmol/L glutamine at 37°C in 5% CO_2 . Passages 4–8 of cultures were used in all experiments.

Preparation of WECG

The plants of *C. gracile* Matsum var. multicaule were purchased from the oriental drug store, Bohwa Dang (Jeonbuk, Korea). A voucher specimen (number WSP-08-78) was deposited at the Herbarium of the College of Pharmacy, Woosuk University. *C. gracile* was ground (400 g, 30 s) at room temperature using Micro Hammer-Cutter Mill (Culatti Co., Zurich, Swiss). The particle size was 0.5–2 mm after grinding. The sample (50 g) was extracted with purified water (500 mL) at 70°C for five hours in water bath. The extract was filtered through Whatman No. 1 filter paper and the filtrate was lyophilized using 0.45 μm syringe filter. The yield of dried extract from starting crude materials was about 13.7%. The dried extract was dissolved in saline or Tyrode buffer A (10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 130 mmol/L NaCl, 5 mmol/L KCl, 1.4 mmol/L CaCl_2 , 1 mmol/L MgCl_2 , 5.6 mmol/L glucose, 0.1% bovine serum albumin) before use.

Compound 48/80-induced systemic anaphylaxis

Compound 48/80-induced systemic reaction was carried out as described previously.¹¹ Briefly, the mice ($n = 10/\text{group}$) were given an intraperitoneal injection of 8 mg/kg body weight (BW) of the mast cell degranulator, compound 48/80. WECG was dissolved in saline and administered intraperitoneally at doses of 1–100 mg/kg BW one hour before the compound 48/80 injection. In the time-dependent experiment, WECG (100 mg/kg) was administered 5, 10, 15 and 20 min after compound 48/80 injection ($n = 10/\text{group}$). Mortality was monitored for one hour after induction of anaphylactic shock.

Passive cutaneous anaphylaxis

Passive cutaneous anaphylaxis (PCA) reaction was carried out as described previously.¹² Briefly, mice were injected intradermally with 0.5 μg of anti-DNP IgE. After 48 h, each mouse ($n = 10/\text{group}$) received an injection of 1 μg of DNP-HSA in phosphate-buffered saline (PBS) containing 4% Evans blue (1:4) via the tail vein. WECG (1–100 mg/kg BW) was intraperitoneally administered one hour before the challenge. Thirty minutes after the challenge, the mice were killed and the dorsal skin (diameter, 1 cm) was removed in order to measure the pigment area. The amount of dye was determined colorimetrically after extraction with 1 mL of 1 mmol/L KOH and 9 mL of a mixture of acetone and phosphoric acid (5:13). The intensity of the absorbance was measured at 620 nm in a spectrophotometer (Shimadzu, UV-1201, Kyoto, Japan).

Preparation of rat peritoneal mast cells

Mast cells were separated from the rat peritoneal cavity cells as described previously.¹³ In brief, the peritoneal cells were suspended in Tyrode buffer A, layered on 2 mL of metrizamide (22.5 w/v%) and centrifuged at 400 g for 15 min at 4°C . The cells that remained at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 mL of Tyrode buffer A. Mast cell preparations were about 95% pure as assessed by toluidine blue staining. More than 95% of the cells were viable as judged by trypan blue exclusion.

Histamine assay

Histamine content was measured by the enzyme immunoassay kit (Oxford Biomedical Research, Oxford, MI, USA) according to the manufacturer's manual. Rat peritoneal mast cells (RPMC) and HMC-1 were incubated with WECG (1–100 $\mu\text{g}/\text{mL}$) for 10 min at 37°C before the addition of compound 48/80 (5 $\mu\text{g}/\text{mL}$) or PMA (20 nmol/L) and A23187 (1 $\mu\text{mol}/\text{L}$) and incubated for additional times. The cells were separated from the released histamine by centrifugation at 400 g for five minutes at 4°C .

Measurement of calcium

The intracellular calcium was measured with the use of the fluorescence indicator Fluo-3/AM (Molecular Probes,

Eugene, OR, USA). HMC-1 cells were preincubated with Fluo-3/AM for 30 min at 37°C. After washing the dye from the cell surface, the cells were treated with WECG for 10 min before adding PMACI. It was excited at 488 nm, and the emission was filtered with a 515 nm by flow cytometer (BD Biosciences Pharmingen, San Diego, CA, USA) and visualized in a fluorescence microscope (Olympus BX51, Center Valley, PA, USA).

RNA extraction and mRNA detection

The total cellular RNA was isolated using a TRI reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's protocol. The 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 and β -actin (internal control). The conditions for the reverse transcription and PCR steps were similar to those described previously.¹⁴ 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, USA). The band intensity was normalized to that of β -actin in the same sample.

Assay of TNF- α and IL-6 secretion

The secretion of TNF- α and IL-6 was measured by the modification of an enzyme-linked immunosorbent assay (ELISA) as described previously.¹⁵ HMC-1 cells were cultured with media and resuspended in Tyrode buffer A. The cells were sensitized with PMACI for 16 h in the absence or presence of WECG. 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.

Nuclear protein extraction

The preparation of nuclear extract was basically as described elsewhere.¹⁶ Briefly, after cell activation for the times indicated, cells were washed in 1 mL of ice-cold PBS, centrifuged at 400 g for five minutes, resuspended in 400 μ L of ice-cold hypotonic buffer (10 mmol/L HEPES/KOH, 2 mmol/L MgCl₂, 0.1 mmol/L EDTA, 10 mmol/L KCl, 1 mmol/L dithiothreitol [DTT], 0.5 mmol/L phenylmethanesulfonylfluorid [PMSF], pH 7.9), left on ice for 10 min, vortexed and centrifuged at 15,000 g for 30 s. Pelleted nuclei were gently resuspended in 50 μ L of ice-cold saline buffer (50 mmol/L HEPES/KOH, 50 mmol/L KCl, 300 mmol/L NaCl, 0.1 mmol/L EDTA, 10% glycerol, 1 mmol/L DTT, 0.5 mmol/L PMSF, pH 7.9), left on ice for 20 min, vortexed and centrifuged at 15,000 g for five minutes at 4°C. Aliquots of the supernatant that contained nuclear proteins were frozen in liquid nitrogen and stored at -70°C.

Western blot analyses

HMC-1 cells were washed with PBS and resuspended in lysis buffer. Samples were electrophoresed using 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described elsewhere,¹⁷ and then transferred to a nitrocellulose membrane. The NF- κ B was assayed using anti-NF- κ B (p65) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunodetection was carried out 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 2×10^6 in a six-well plate one day before transient transfection. The expression vectors containing the NF- κ B luciferase reporter construct (pNF- κ B-LUC, plasmid containing NF- κ B binding site; STANTAGEN, Grand Island, NY, USA) were transfected with serum- and antibiotics-free Iscove's medium containing 8 μ L of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). After five hours of incubation, medium was replaced with Iscove's medium containing 10% FBS and antibiotics. Cells were allowed to recover at 37°C for 20 h and subsequently were stimulated as indicated. Cell lysates were prepared and assayed for luciferase activity using 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 Duncan's multiple range tests. $P < 0.05$ was used to indicate significance.

Results

Effect of WECG on systemic and local anaphylaxis

To determine the effect of WECG on allergic reaction, an *in vivo* model of a systemic anaphylaxis was used. Compound 48/80 (8 mg/kg) was used as a model of induction for a systemic fatal allergic reaction. After the intraperitoneal injection of compound 48/80, the mice were monitored for one hour, after which the mortality rate was determined. As shown in Table 1, injection of compound 48/80 into mice induced fatal shock in 100% of animals. When WECG was intraperitoneally administered at concentrations ranging from 1 to 100 mg/kg BW for one hour, the mortality with compound 48/80 was dose-dependently reduced. In addition, the mortality of mice administered with WECG (100 mg/kg) 5, 10, 15 and 20 min after compound 48/80 injection increased time dependently (Table 2).

Another way to test the anaphylaxis is to induce PCA. A local extravasation was induced by a local injection of IgE followed by an antigenic challenge. Intraperitoneal injection of WECG dose-dependently inhibited PCA reaction (Figure 1).

Table 1 Effect of WECG on compound 48/80-induced systemic anaphylaxis

WECG treatment (mg/kg BW)	Compound 48/80 (8 mg/kg BW)	Mortality (%)
None (saline)	+	100
1	+	100
5	+	70
10	+	30
50	+	0
100	+	0
100	—	0

WECG, water extract of *C. gracile*; BW, body weight

Groups of mice ($n = 10$ /group) were intraperitoneally pretreated with 200 μ L of saline or WECG at various doses one hour before the intraperitoneal injection of compound 48/80. Mortality (%) within one hour following compound 48/80 injection is represented as the number of dead mice $\times$ 100/total number of experimental mice

Effect of WECG on histamine release from mast cells

We next evaluated the ability of WECG to inhibit compound 48/80 and PMACI-induced histamine release from primary mast cells (RPMC) and human mast cell line (HMC-1). Treatment with WECG dose-dependently inhibited compound 48/80- and PMACI-induced histamine release at concentrations of 1–100 μ g/mL (Figure 2). Up to 10 mg/mL of WECG did not show cytotoxicity (data not shown).

Effect of WECG on intracellular calcium in HMC-1 cells

Calcium movements across membranes of mast cells are critical to histamine release.¹⁸ To investigate the mechanism of WECG on the reduction of histamine release, we assayed intracellular calcium levels. Figure 3 shows the stimulation of intracellular calcium when mast cells are treated with PMACI. Preincubation of WECG with cells decreased the intracellular calcium level induced by PMACI.

Effect of WECG on the gene expression and secretion of proinflammatory cytokines in HMC-1 cells

TNF- α and IL-6 are the most important proinflammatory cytokines. Therefore, we tested the effect of WECG on the gene expression of TNF- α and IL-6 induced by PMACI in HMC-1 cells. HMC-1 cell line is a useful cell for studying

Table 2 Time-dependent effect of WECG on compound 48/80-induced systemic anaphylaxis

WECG treatment (mg/kg BW)	Compound 48/80 (8 mg/kg BW)	Time (min)	Mortality (%)
None (saline)	+		100
100	+	0	0
	+	5	10
	+	10	30
	+	15	50
	+	20	100

WECG, water extract of *C. gracile*; BW, body weight

Mice ($n = 10$ /group) were intraperitoneally pretreated with 200 μ L of saline or WECG. WECG (100 mg/kg) was given 5, 10, 15 and 20 min after the intraperitoneal injection of compound 48/80. Mortality (%) within one hour following compound 48/80 injection is represented as the number of dead mice $\times$ 100/total number of experimental mice

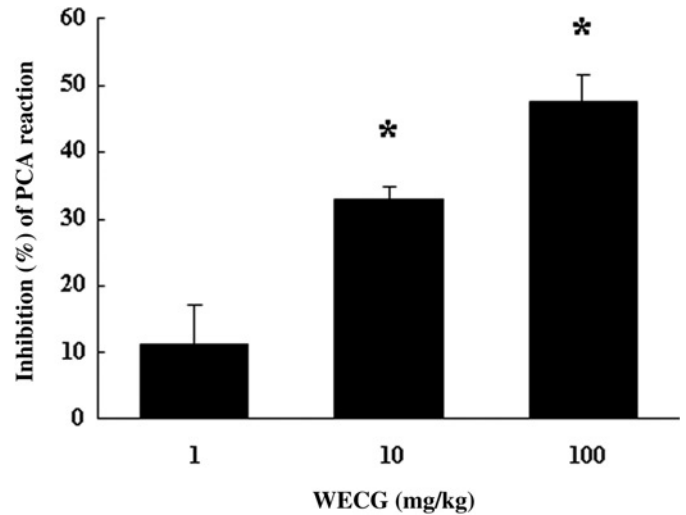


Figure 1 Effect of WECG on PCA reactions. WECG was intraperitoneally administered one hour prior to the challenge with antigen. Each amount of dye was extracted as described in Materials and methods and measured by spectrophotometry. Each bar represents the mean $\pm$ standard error of mean of three independent experiments. * $P < 0.05$, statistically significant. WECG, water extract of *C. gracile*; PCA, passive cutaneous anaphylaxis

the cytokine activation pathway.^{6,19} Previously, we reported that gene expression of TNF- α and IL-6 peaked at four hours after treatment of PMACI.¹⁵ Therefore, stimulation of HMC-1 with PMACI was induced for four hours, and the cells were pretreated with WECG for 30 min. WECG dose-dependently inhibited PMACI-induced expression of TNF- α and IL-6 (Figure 4a). To confirm the effect of WECG on the gene expression of proinflammatory cytokines, culture supernatants were assayed for TNF- α and IL-6 levels by ELISA. The stimulation of cells with PMACI for 16 h induced the secretion of cytokines. WECG

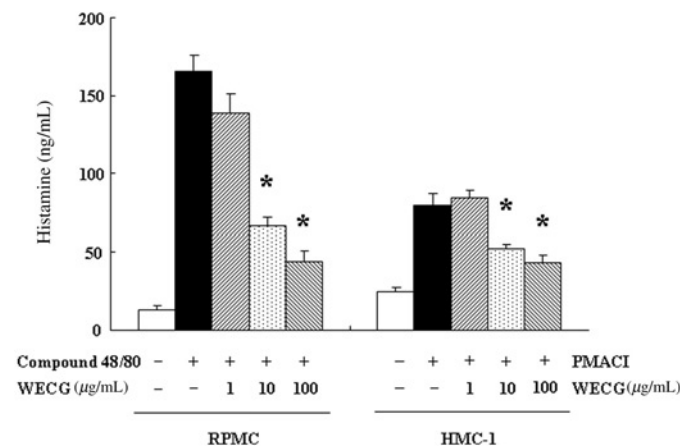


Figure 2 Effect of WECG on compound 48/80 or PMACI-induced histamine release in mast cells. RPMC (2×10^5 cells/ml) and HMC-1 cells (2×10^6 cells/ml) were preincubated with WECG at 37°C for 10 min prior to incubation with compound 48/80 (5 μ g/mL) or PMA (20 nmol/L) and A23187 (1 μ mol/L). Each value represents the mean $\pm$ standard error of mean of three independent experiments. * $P < 0.05$ (significantly different from the compound 48/80 or PMACI value). WECG, water extract of *C. gracile*; PMACI, phorbol 12-myristate 13-acetate and calcium ionophore A23187; RPMC, rat peritoneal mast cells; HMC, human mast cells

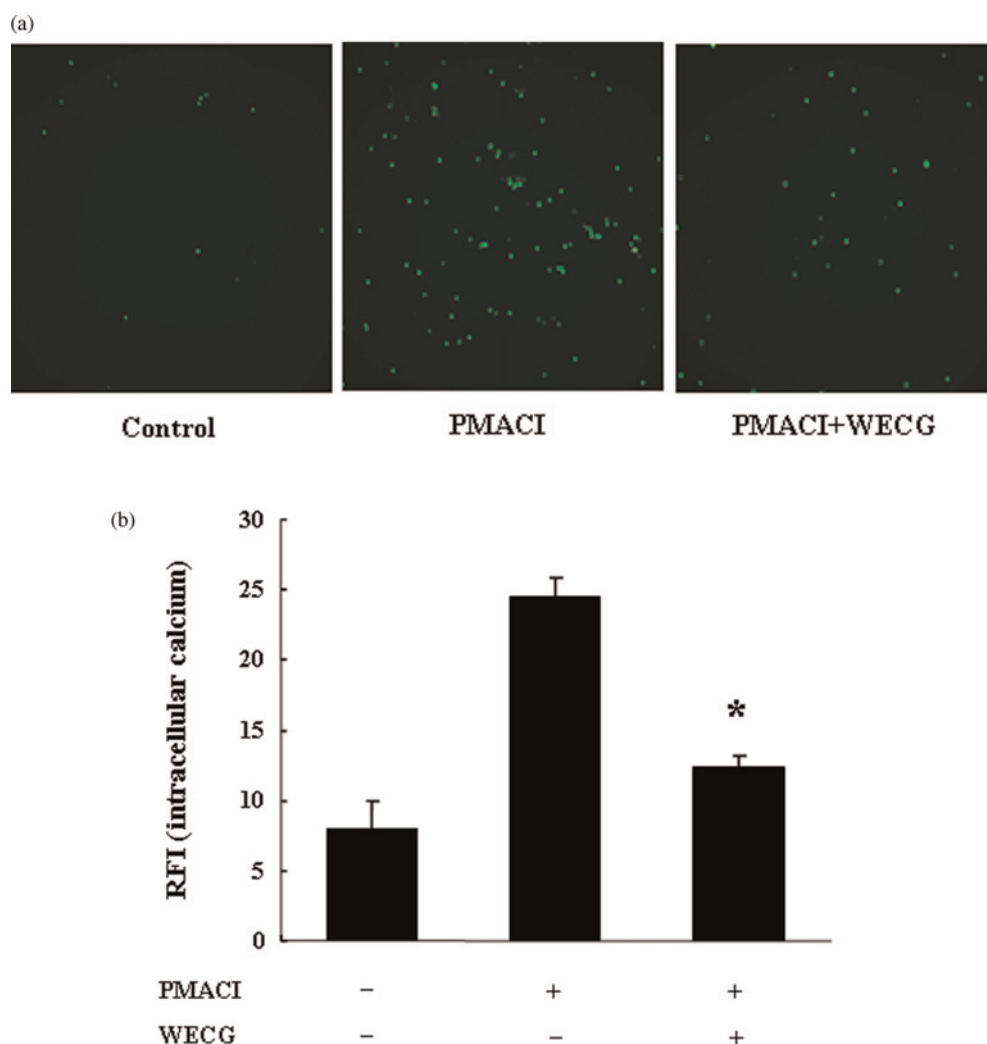


Figure 3 Effect of WECG on intracellular calcium in mast cells. HMC-1 cells were stained with Fluo-3/AM and then cells were preincubated for 10 min with WECG before adding PMA (20 nmol/L) and A23187 (1 μ mol/L), and then for another 10 min with PMACI. Intracellular calcium was detected by fluorescence microscope (a) and flow cytometer (b). Each value represents the mean $\pm$ standard error of mean of three independent experiments. *Significant difference from PMACI value at $P < 0.05$. WECG, water extract of *C. gracile*; PMACI, phorbol 12-myristate 13-acetate and calcium ionophore A23187; HMC, human mast cells (A color version of this figure is available in the online journal)

dose-dependently inhibited the secretion of TNF- α and IL-6 in PMACI-stimulated HMC-1 cells (Figure 4b).

Effect of WECG on the activation of NF- κ B

NF- κ B is an important transcriptional regulator of inflammatory cytokines and plays a crucial role in immune and inflammatory responses. To investigate the intracellular mechanism responsible for the inhibitory effect of WECG on the expression of proinflammatory cytokines, we examined the effect of WECG on NF- κ B. The stimulation of HMC-1 cells with PMACI induced degradation of I- κ B α and nuclear translocation of p65 NF- κ B after two hours of incubation (Figure 5a). WECG inhibited the PMACI-induced degradation of I- κ B α and nuclear translocation of p65 NF- κ B. To confirm the inhibitory effect of WECG on NF- κ B activation, we examined the effect of WECG on the NF- κ B-dependent gene reporter assay. HMC-1 cells were transiently transfected with an NF- κ B luciferase reporter construct or an empty vector. The

exposure of cells to PMACI increased the luciferase activity in the cells transfected with the NF- κ B luciferase reporter construct (Figure 5b). WECG significantly reduced the PMACI-induced luciferase activity.

Discussion

The results of this study demonstrated that WECG has antiallergic inflammatory properties. WECG inhibited compound 48/80-induced systemic allergic reaction (anaphylaxis) and histamine release from mast cells. These results indicate that mast cell-mediated immediate type allergic reactions are inhibited by WECG. In addition, the WECG-administered mice are protected from IgE-mediated PCA, which is one of the most important *in vivo* models of anaphylaxis in a local allergic reaction. This finding suggests that WECG might be useful in the treatment of allergic diseases, especially, skin reactions.

The intracellular calcium is the critical factor for the degranulation of mast cells. Calcium movements across

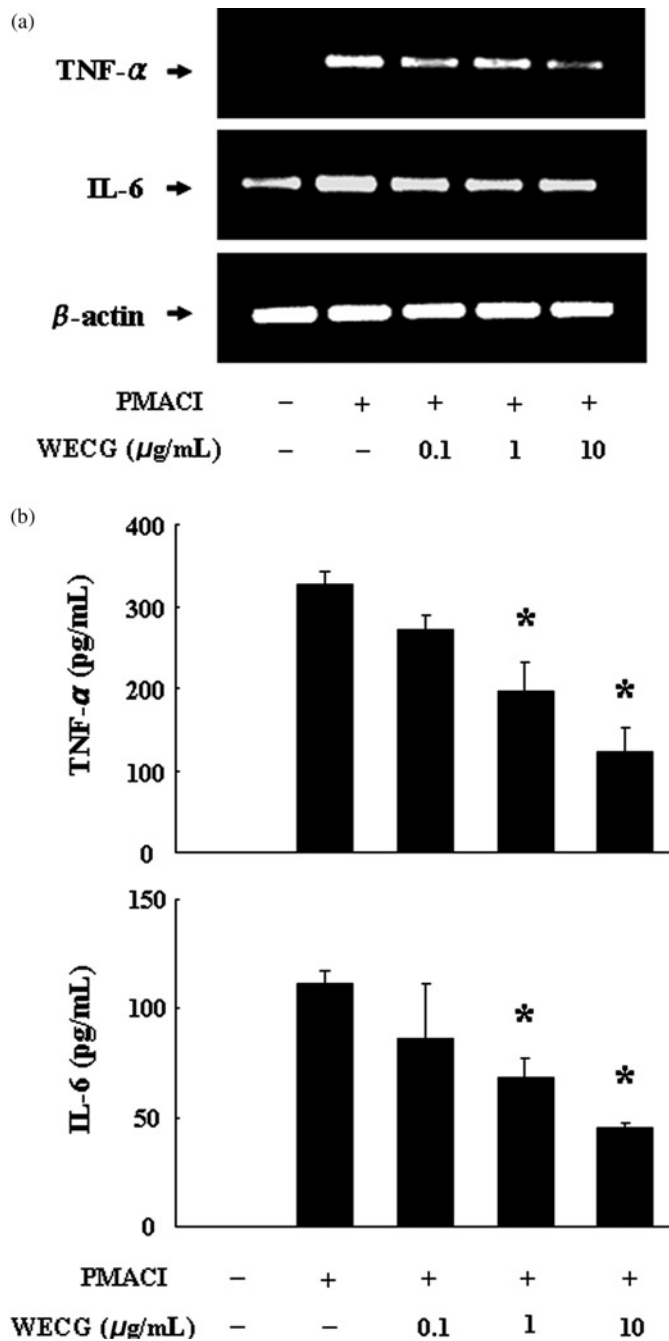


Figure 4 Effect of WECG on the gene expression and secretion of proinflammatory cytokines in mast cells. HMC-1 cells were pretreated with WECG for 30 min prior to PMA (20 nmol/L) and A23187 (1 μmol/L) stimulation. The levels of TNF-α and IL-6 were determined by RT-PCR (a). TNF-α and IL-6 level in supernatant was measured using ELISA and represented as the mean ± standard error of mean of three independent experiments (b). *Significant difference from PMACI value at $P < 0.05$. WECG, water extract of *C. gracile*; PMA, phorbol 12-myristate 13-acetate; HMC, human mast cells TNF, tumor necrosis factor; IL, interleukin; PMACI, phorbol 12-myristate 13-acetate and calcium ionophore A23187; RT-PCR, reverse-transcriptase polymerase chain reaction

the membranes of mast cells represent a major target for effective antiallergic drugs, as these are essential events that link stimulation to secretion.^{4,20} The transduction pathways modulating intracellular calcium are modified by ADP-ribosylates G-protein binding protein.²¹ Our results,

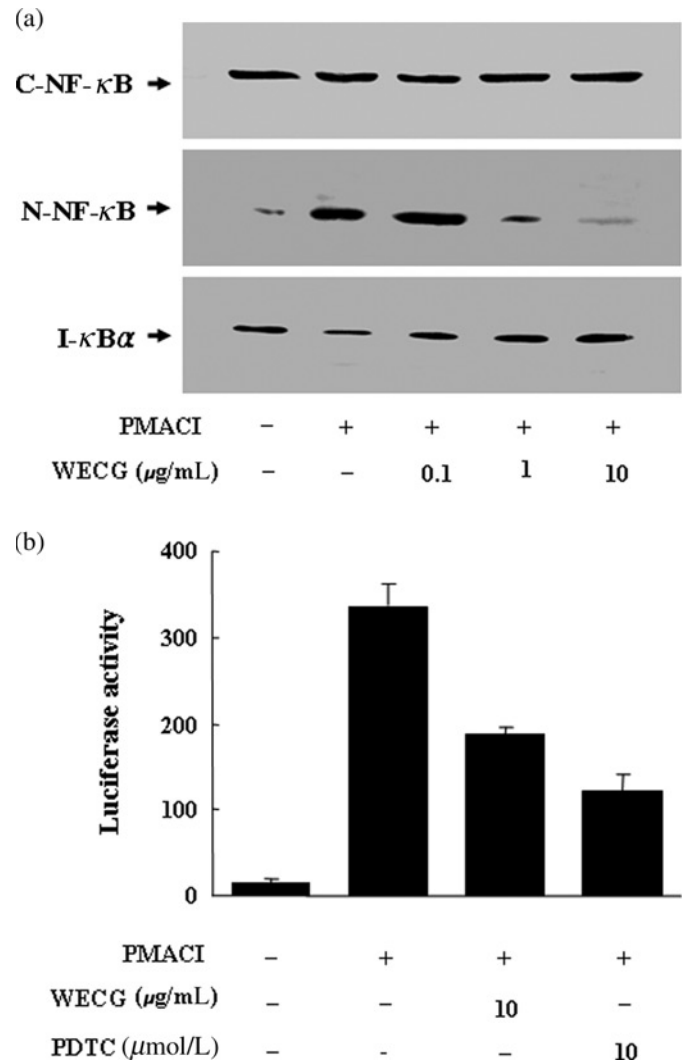


Figure 5 Effect of WECG on the activation of NF-κB in mast cells. HMC-1 cells were pretreated with WECG or PDTC (10 μmol/L) for 30 min prior to PMA (20 nmol/L) and A23187 (1 μmol/L) stimulation. (a) Nuclear translocation of NF-κB and degradation of I-κBα were assayed by Western blot (N-NF-κB, nucleus 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 PMACI with or without WECG. NF-κB-dependent transcriptional activity was determined by luciferase activity assay. PDTC was used as a positive control. *Significant difference from PMACI value at $P < 0.05$. WECG, water extract of *C. gracile*; NF-κB, nuclear factor-κB; HMC, human mast cell; PDTC, pyrrolidine dithiocarbamate; PMACI, phorbol 12-myristate 13-acetate and calcium ionophore A23187

which show an attenuation of intracellular calcium in mast cells with WECG treatment, is consistent with other reports. According to these observations, we strongly suggest that decreased intracellular calcium might be involved in the inhibitory effect of WECG on histamine release, and WECG might have membrane-stabilizing activity through G-protein.

Numerous reports have established that stimulation of mast cells with compound 48/80 or IgE initiates the activation of signal transduction pathways, which lead to histamine release. Several recent studies have shown that compound 48/80 and other polybasic compounds are able, apparently directly, to activate G-proteins.²² Compound 48/80 increases the permeability of the lipid bilayer membrane by causing a

perturbation in 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, antiallergic agents having a membrane-stabilizing action may be desirable. WECG might stabilize the lipid bilayer membrane, thus preventing the perturbation being induced by compound 48/80.

HMC-1 cell line is a useful cell for studying cytokine activation pathways.²³ The spectrum of cytokines produced by HMC-1 cells with PMACI stimulation supports the well-recognized role of mast cells in immediate-type hypersensitivity. Proinflammatory cytokines, including TNF- α and IL-6, play a major role in triggering and sustaining the allergic inflammatory response in mast cells.^{24,25} Mast cells are a principal source of TNF- α in human dermis. TNF- α has an important amplifying effect in asthmatic inflammation and potently stimulates airway epithelial cells to produce cytokines.⁵ It is also a potent inducer of other inflammatory cytokines, including IL-1, IL-6, IL-8 and GM-CSF. IL-6 is produced from mast cells and its local accumulation is associated with PCA reaction.²⁵ These reports indicate that the reduction of proinflammatory cytokines from mast cells is one of the key indicators of reduced allergic inflammatory symptoms. In our present study, WECG inhibited the secretion of TNF- α and IL-6 in PMACI-stimulated HMC-1 cells. This result suggests that the antiallergic inflammatory effect of WECG results from its reduction of TNF- α and IL-6 from mast cells.

Intracellular calcium plays an important role in the expression of inflammatory cytokines. Depletion of intracellular calcium blocked the expression of IgE-induced TNF- α and IL-6 in RBL-2H3 mast cells.²⁶ In our experiments, WECG decreased the level of intracellular calcium in mast cells. We suggest that the inhibitory effect of WECG on the inflammatory cytokines results from the reduction of intracellular calcium.

The expression of TNF- α and IL-6 gene is dependent on the activation of transcription factor NF- κ B.²⁷ The activation of NF- κ B requires phosphorylation and proteolytic degradation of the inhibitory protein I- κ B α , an endogenous inhibitor that binds to NF- κ B in the cytoplasm.¹⁰ In PMACI-stimulated mast cells, WECG inhibited DNA binding of NF- κ B and NF- κ B-dependent gene transcription. We also reported previously that pyrrolidine dithiocarbamate (PDTC), the potent inhibitor of NF- κ B, reduced PMACI-induced production of TNF- α , IL-6 and IL-8 in HMC-1 cells.^{6,7} These data demonstrate that WECG attenuates the activation of NF- κ B and downstream TNF- α and IL-6 production.

Because we used whole water extract of *C. gracile*, not a purified component, the active components that are responsible for the biological effect are not clear at this time. The effort to identify active components from the fruits of *C. gracile* in the mast cell-mediated allergic inflammation is ongoing in our laboratory. In the present report, we provide evidence that WECG inhibits a model of mast cell-mediated allergic inflammation and the possible mechanisms such as intracellular calcium for histamine release and NF- κ B for proinflammatory cytokines. The results obtained in the present study show that WECG contributes

to the prevention or treatment of mast cell-mediated allergic inflammatory diseases.

Author contributions: S-BP: first author, primary work and manuscript writing; S-HK: co-corresponding author (*in vitro* experiment); KS: co-author, *in vitro* experiment and molecular work (PCR); H-SL: co-author, molecular work and manuscript editing; TTK: co-author, *in vitro* experiment and molecular work (calcium, Western);

M-GJ: co-author, animal experiment and histamine assay (PCA); HJ: co-author, animal experiment and histamine assay (anaphylaxis); D-KK: co-author, animal experiment and histamine assay (anaphylaxis); J-PL: co-author, animal experiment and histamine assay (PCA); T-YS: corresponding author (*in vivo* experiment).

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