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Original Research

Elsholtzia ciliata inhibits mast cell-mediated allergic inflammation: role of calcium, p38 mitogen-activated protein kinase and nuclear factor-κB

Hui-Hun Kim[1], Jin-Su Yoo[2], Hyun-Shik Lee[3], Taeg Kyu Kwon[4], Tae-Yong Shin[2] and Sang-Hyun Kim[1]

[1]CMRI, Department of Pharmacology, School of Medicine, Kyungpook National University, Daegu 700-422; [2]College of Pharmacy, Woosuk University, Jeonju 565-701; [3]School of Life Sciences and Biotechnology, Kyungpook National University, Daegu 702-701; [4]Department of Immunology, School of Medicine, Keimyung University, Daegu 704-701, Republic of Korea
Corresponding authors: Sang-Hyun Kim and Tae-Yong Shin. Emails: shkim72@knu.ac.kr and tyshin@woosuk.ac.kr

Abstract

Mast cell-mediated allergic reaction is involved in many diseases such as asthma and allergic rhinitis. Therefore, discovery of drugs for the prevention or treatment of allergic disease is an important topic in human health. In this study, we evaluated the effects of water extract of *Elsholtzia ciliata* (Thunb.) Hyland (Labiatae) (WEEC) on mast cell-mediated allergic inflammation and studied the possible mechanisms of action. WEEC inhibited compound 48/80-induced systemic and immunoglobulin E-mediated local anaphylaxis, and serum histamine release in mice. WEEC reduced intracellular calcium levels and downstream histamine release from human mast cells (HMC-1) activated with phorbol 12-myristate 13-acetate and calcium ionophore A23187. In addition, WEEC decreased gene expression and secretion of proinflammatory cytokines such as tumor necrosis factor-α, interleukin (IL)-1β and IL-6 in HMC-1. The inhibitory effect of WEEC on cytokine expression was nuclear factor (NF)-κB and p38 mitogen-activated protein kinase (MAPK) dependent. Our results indicate that WEEC inhibits mast cell-mediated allergic inflammatory reactions by suppressing histamine release and proinflammatory cytokine expression, and involvement of calcium, NF-κB and p38 MAPK in these effects.

Keywords: *Elsholtzia ciliata*, allergic inflammation, histamine, nuclear factor-κB, mast cells

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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.[1] Immediate-type hypersensitivity (anaphylaxis) is mediated by a release of histamine in response to the antigen cross-linking of immunoglobulin E (IgE) bound to FcεRI on the mast cells. After stimulation of the FcεRI, mast cells rapidly secrete preformed and *de novo* synthesized allergic mediators such as histamine, cytokines, proteases and arachidonic derivatives. One of the allergic mediators, histamine, plays an important role both in normal physiology and in pathophysiology, and regulates several essential events in the allergic inflammation response.[2–4]

Activation of mast cells leads to phosphorylation of tyrosine kinase and mobilization of internal calcium. These are followed by the activation of protein kinase C, mitogen-activated protein kinases (MAPKs), nuclear factor (NF)-κB and the release of inflammatory cytokines.[1] Activated mast cells can release histamine and also other inflammatory mediators such as eicosanoids, proteoglycans, proteases and many proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-4, IL-6 and IL-13.[5,6] Although these inflammatory cytokines have helpful effects on the host defense mechanism, they can cause pathological conditions when overexpressed. Therefore, the reduction of these cytokines from mast cells is one of the key indicators of reduced allergic inflammatory symptoms.

Elsholtzia ciliata (Thunb.) Hyland (Labiatae) is an annual herb that is widely distributed throughout Korea, China and Europe. The seeds of *E. ciliate* are powdered and used for flavoring food. The herb has been used as a traditional oriental medication for the treatment of fever, headache, diarrhea and edema.[7–9] The chemical components of the crude drug are essential oil, elsholtzia ketone, flavonoids,

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steroids and triterpenes.[9] In this study, we evaluated the effect of water extract of *E. ciliata* (WEEC) on the systemic and local allergic reaction, and histamine release from mast cells. The investigation of intracellular calcium contents clarified the mechanism by which WEEC inhibited histamine release from mast cells. In addition, the effect of WEEC on expression and secretion of proinflammatory cytokines, and the role of p38 MAPK and NF-κB in this effect were investigated using human mast cells (HMC-1).

Materials and methods

Animals

The original stock of male Imprinting Control Region (ICR) mice (six weeks) and male Sprague-Dawley rats (eight weeks) were purchased from Dae-Han Biolink Co Ltd (Chungbuk, Korea). The animals were housed five per cage in a laminar air flow room maintained under a temperature of $22 \pm 2^{\circ}C$ and relative humidity of $55 \pm 5^{\circ}C$ 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), calcium ionophore A23187, and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma Chemical Co (St Louis, MO, USA). SB 203580 was purchased from Calbiochem (La Jolla, CA, 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 mmol/L at 37°C in 5% CO_2. The passage ranging 4–8 of HMC-1 was used throughout the study.

Preparation of WEEC

The *E. ciliata* was collected in Sunchang, Jeonbuk, South Korea, on the 25th of September, 2009. A voucher specimen (number WSP-09-11) was deposited in the Herbarium of the College of Pharmacy, Woosuk University. The *E. ciliata* was ground (400*g*, 30 s) 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 five hours. 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 7.5%. The dried extract was dissolved in saline or Tyrode buffer A (HEPES 10 mmol/L, NaCl 130 mmol/L, KCl 5 mmol/L, $CaCl_2$ 1.4 mmol/L, $MgCl_2$ 1 mmol/L, glucose 1.4 mmol/L, 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. WEEC was dissolved in saline and administered intraperitoneally at a dose of 10–1000 mg/kg BW one hour before the injection of compound 48/80 ($n = 10$/group). Mortality (%) within one hour following compound 48/80 injection is represented as the number of dead mice $\times$ 100/total number of experimental mice. In the time-dependent experiment, WEEC (1000 mg/kg BW) was injected at five, 10 and 15 min after compound 48/80 injection ($n = 10$/group). Mortality was monitored for one hour 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

An IgE-dependent cutaneous reaction was carried out as described previously.[10] The passive cutaneous anaphylaxis (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 killed 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 mmol/L 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 (Ultrospec 2100 pro; Amersham Biosciences, Cambridge, UK).

Preparation of serum and histamine assay

Preparation of serum for measurement of histamine contents was previously described.[11] Briefly, the blood was centrifuged at 400*g* for 10 min. The serum was withdrawn and the histamine contents were measured using the enzyme immunoassay kit (Oxford Biomedical Research, Oxford, MI, USA) according to the manufacturer's manual. For the histamine content from mast cells, cells were preincubated with WEEC for 30 min, and then incubated for eight hours with PMA (20 nmol/L) and calcium ionophore A23187 (1 μmol/L) (PMACI).

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 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 WEEC for 10 min before adding PMACI. The fluorescent intensity was recorded using a flow cytometer (BD Biosciences Pharmingen, San Diego, CA, USA) and visualized on a fluorescence microscope (Olympus BX51, Center Valley, PA, USA).

RNA extraction and mRNA detection

The total cellular RNA was isolated from the cells after stimulation with PMACI with or without WEEC for two hours using TRI reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's protocol. The first strand complementary DNA (cDNA) was synthesized using 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.[12] 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.

Enzyme-linked immunosorbent assay

The secretion of TNF-α and IL-6 was measured by enzyme-linked immunosorbent assay (ELISA) as described previously.[13] HMC-1 were cultured with media and re-suspended in Tyrode buffer A. The cells were sensitized with PMACI for eight hours in the absence or presence of WEEC. 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

After activation of HMC-1 (3×10^6 in a six-well plate) for two hours, cells were washed in 1 mL of ice-cold phosphate-buffered saline (PBS), centrifuged at 1200*g* for five minutes, re-suspended in 400 μL of ice-cold hypotonic buffer (10 mmol/L HEPES/KOH, 2 mmol/L $MgCl_2$, 0.1 mmol/L EDTA, 10 mmol/L KCl, 1 mmol/L DTT, 0.5 mmol/L PMSF, pH 7.9), left on ice for 10 min, vortexed and centrifuged at 15,000*g* for 30 s. After several times of washing, pelleted nuclei were re-suspended in 50 μL 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 analysis

HMC-1 were washed three times with PBS and re-suspended in lysis buffer. Samples were electrophoresed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described elsewhere,[14] and then transferred onto a nitrocellulose membrane. The NF-κB, IκBα and p38 MAPK were assayed using anti-NF-κB (p65), anti-IκBα, anti-p38 and anti-phospho-p38 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively. Immunodetection was done by using an enhanced chemiluminescence detection kit (Amersham Pharmacia, Piscataway, NJ, USA).

Transient transfection and luciferase activity assay

For transient transfections, HMC-1 were seeded at 2×10^6 in a six-well plate one day before transient transfection. The expression vectors containing the NF-κB luciferase reporter construct (pNF-κB-LUC, plasmid containing NF-κB binding site; Stantagen, Grand Island, NY, USA) were transfected with serum- and antibiotics-free Iscove's medium containing 8 μL of Lipofectamine 2000 reagent (Invitrogen). After five hours of incubation, the 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 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 Duncan's multiple range tests. $P < 0.05$ indicated significance.

Results

WEEC inhibits systemic and local allergic reaction

To determine the effect of WEEC 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 allergic reaction. As shown in Table 1, injection of compound 48/80 into mice induced fatal shock in 100% of animals. When WEEC (10–1000 mg/kg BW) was intraperitoneally pretreated one hour before the compound 48/80 injection, the mortality with compound 48/80 was dose-dependently reduced. WEEC completely inhibited compound 48/80-induced fatal shock at 500 mg/kg. In addition, the mortality of mice administered with WEEC (1000 mg/kg) five, 10 and 15 min after compound 48/80 injection increased time-dependently (Table 2). The effect of WEEC on compound 48/80-induced serum histamine release was also investigated. The increase of serum histamine caused by compound 48/80 was inhibited by WEEC in a

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

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

Groups of mice ($n = 10$/group) were intraperitoneally pretreated with 200 μL of saline or WEEC 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 × 100/total number of experimental mice. WEEC, water extract of *Elsholtzia ciliata*; BW, body weight

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

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

Mice ($n = 10$/group) were intraperitoneally pretreated with 200 μL of saline or WEEC (1000 mg/kg) 0, 5, 10 and 15 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. WEEC, water extract of *Elsholtzia ciliate*; BW, body weight

dose-dependent manner (Figure 1a). To confirm the antiallergic effects of WEEC, we used an IgE-mediated PCA model. The PCA model is one of the most important *in vivo* models of local allergic reaction.[15] Local extravasation was induced by a local injection of anti-DNP IgE followed by an intravenous antigenic challenge. To compare the amount of dye with control, the left dorsal skin of these mice was injected with saline alone. WEEC was intraperitoneally administered one hour prior to the challenge with antigen. WEEC dose-dependently inhibited the PCA reaction (Figure 1b).

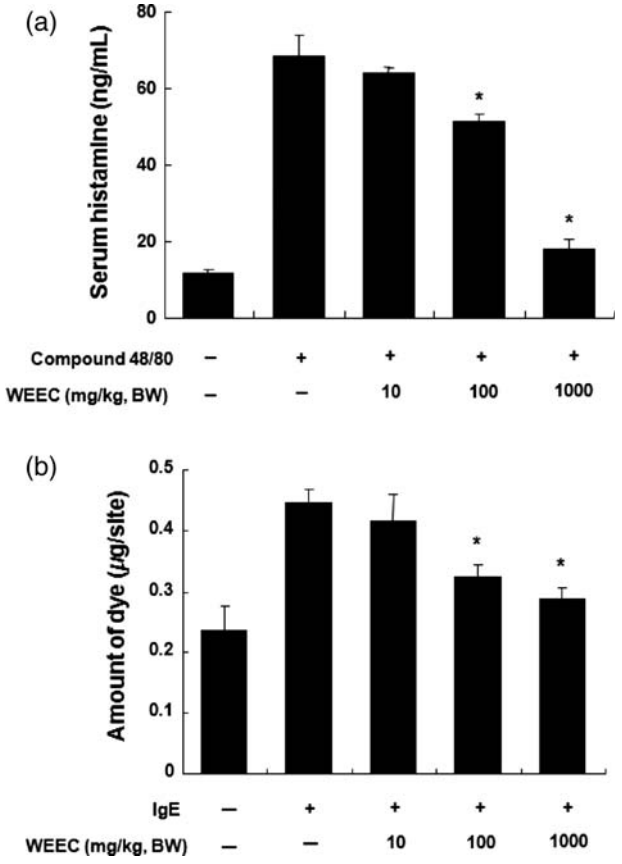

Figure 1 Effects of water extract of *Elsholtzia ciliata* (WEEC) on compound 48/80-induced serum histamine and passive cutaneous anaphylaxis reaction. (a) Groups of mice ($n = 10$/group) were intraperitoneally pretreated with 200 μL of saline or WEEC at various doses one hour before the intraperitoneal injection of compound 48/80. After one hour, the blood was obtained from the heart of each mouse and histamine contents were measured using the enzyme immunoassay kit. (b) WEEC was intraperitoneally administered one hour prior to the challenge with antigen. Each amount of dye was determined colorimetrically. The intensity of absorbance was measured at 620 nm using a spectrophotometer. Each bar represents the mean $\pm$ SEM of three independent experiments. *Significant difference from compound 48/80 or IgE value at $P < 0.05$

WEEC reduces histamine release and intracellular calcium levels

Next, we evaluated the effects of WEEC on PMACI-induced histamine release from HMC-1. WEEC dose-dependently inhibited PMACI-induced histamine release at concentrations of 10 and 100 μg/mL (Figure 2a). Up to 10 mg/mL of WEEC did not show cytotoxicity (data not shown). Calcium

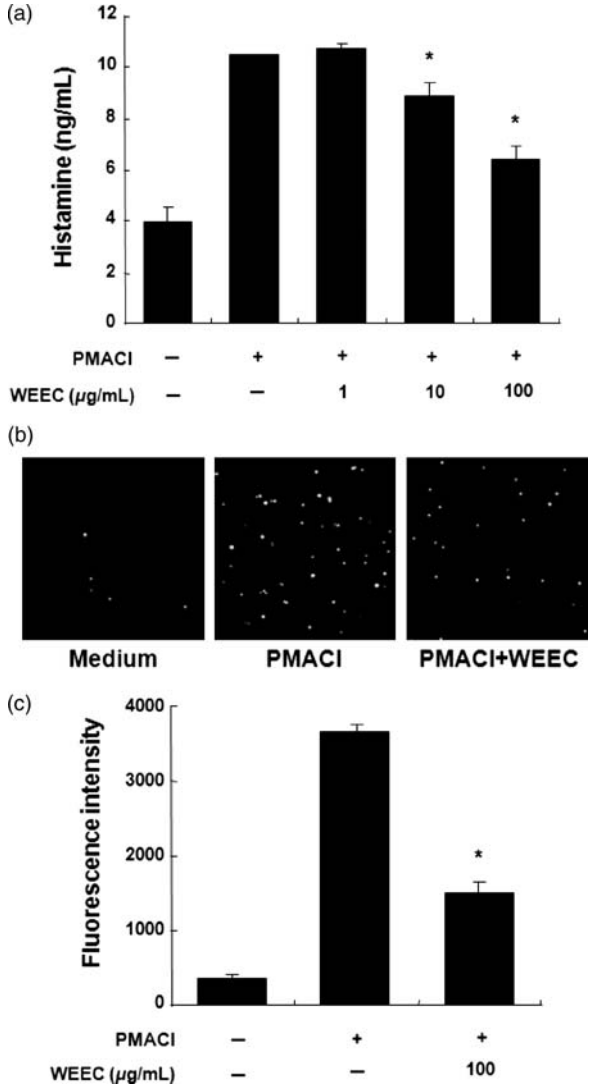

Figure 2 Effects of water extract of *Elsholtzia ciliata* (WEEC) on phorbol 12-myristate 13-acetate and calcium ionophore A23187 (PMACI)-induced histamine release and intracellular calcium in human mast cell-1 (HMC-1). Cells (1×10^6 cells/mL) were preincubated with WEEC 30 min prior to incubation with PMA (20 nmol/L) and calcium ionophore A23187 (1 μmol/L). (a) The histamine contents were measured using the enzyme immunoassay kit. Intracellular calcium was detected by a fluorescence microscope (b) and flow cytometer (c). Each bar represents the mean $\pm$ SEM of three independent experiments. *Significant difference from PMACI (without WEEC) value at $P < 0.05$

movements across membranes of mast cells are critical to histamine release.[16] To investigate the possible mechanism of WEEC on reduction of histamine release, we assayed the level of intracellular calcium. Preincubation of WEEC (100 μg/mL) with HMC-1 decreased the PMACI-induced intracellular calcium levels (Figures 2b and c).

WEEC inhibits gene expression and secretion of proinflammatory cytokines from mast cells

We evaluated the effect of WEEC on the gene expression and secretion of proinflammatory cytokines such as TNF-α, IL-6 and IL-1β in PMACI-stimulated HMC-1. Stimulation of HMC-1 with PMACI was induced for two hours, and the cells were preincubated with WEEC for 30 min. As shown in Figures 3a and b, WEEC dose-dependently inhibited PMACI-induced gene expression of TNF-α, IL-6 and IL-1β. To confirm the effects of WEEC on the gene expression of proinflammatory cytokines, culture supernatants were collected and assayed for TNF-α and IL-6 levels by ELISA. The stimulation of cells with PMACI for eight hours induced the secretion of cytokines. WEEC inhibited the secretion of TNF-α and IL-6 in PMACI-stimulated HMC-1 (Figure 3c).

WEEC inhibits the activation of NF-κB and p38 MAPK

To evaluate the mechanism of effect on cytokine expression, we examined the effect of WEEC on the activation of transcription factors, NF-κB and MAPKs. NF-κB is an important transcriptional regulator of inflammatory cytokines and plays a crucial role in immune and inflammatory responses. The stimulation of HMC-1 with PMACI induced the degradation of IκBα and nuclear translocation of p65 NF-κB after two hours of incubation. WEEC inhibited the PMACI-induced degradation of IκBα and nuclear translocation of p65 NF-κB (Figure 4a). To confirm the inhibitory effect of WEEC on the NF-κB activation, we examined the effect of WEEC on the 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 the NF-κB-luciferase reporter construct (Figure 4b). WEEC significantly reduced the PMACI-induced luciferase activity.

MAPK pathways also play a crucial role in the regulation of proinflammatory molecules. To evaluate the mechanisms of the effect of WEEC on the expression of proinflammatory cytokines, we examined the effect of WEEC 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.[10] WEEC reduced the PMACI-induced p38 MAPK activation (Figure 4c) but did not affect the phosphorylation of ERK and JNK (data not shown). Our previous report using the dominant-negative form of p38 MAPK also showed that p38 MAPK is an important factor for the expression of TNF-α and IL-6.[4] To strengthen the role of p38 MAPK and NF-κB on

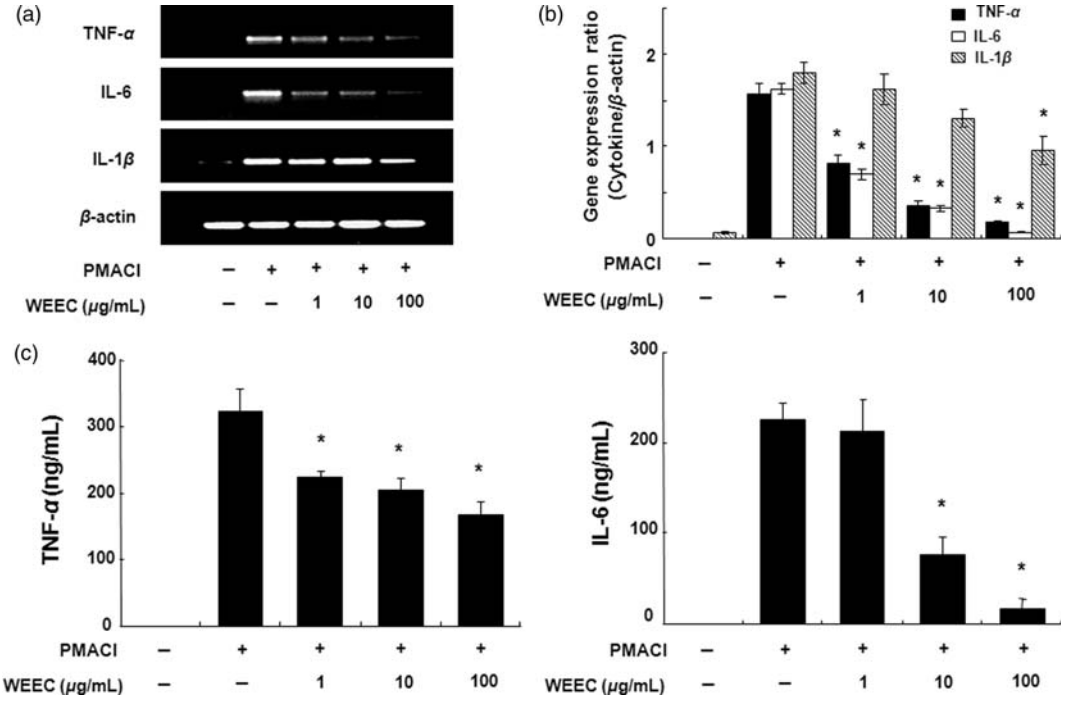

Figure 3 Effects of water extract of *Elsholtzia ciliata* (WEEC) on gene expression and secretion of proinflammatory cytokines in human mast cell-1 (HMC-1). Cells were pretreated with WEEC for 30 min before the stimulation of phorbol 12-myristate 13-acetate (PMA) (20 nmol/L) and ionophore A23187 (1 μmol/L). (a) Extraction and analysis of mRNA was performed as described in Materials and methods. The gene expression of tumor necrosis factor α (TNF-α), interleukin (IL)-6 and IL-1β were determined by reverse transcription polymerase chain reaction. (b) Gel pictures were taken using a Kodak DC 290 digital camera, and densitometric analysis was performed using UN-SCAN-IT software. (c) The level of TNF-α and IL-6 in supernatant was measured by enzyme-linked immunosorbent assay and represented as the mean $\pm$ SEM of three independent experiments. *Significant difference from PMA and calcium ionophore A23187 (without WEEC) value at $P < 0.05$

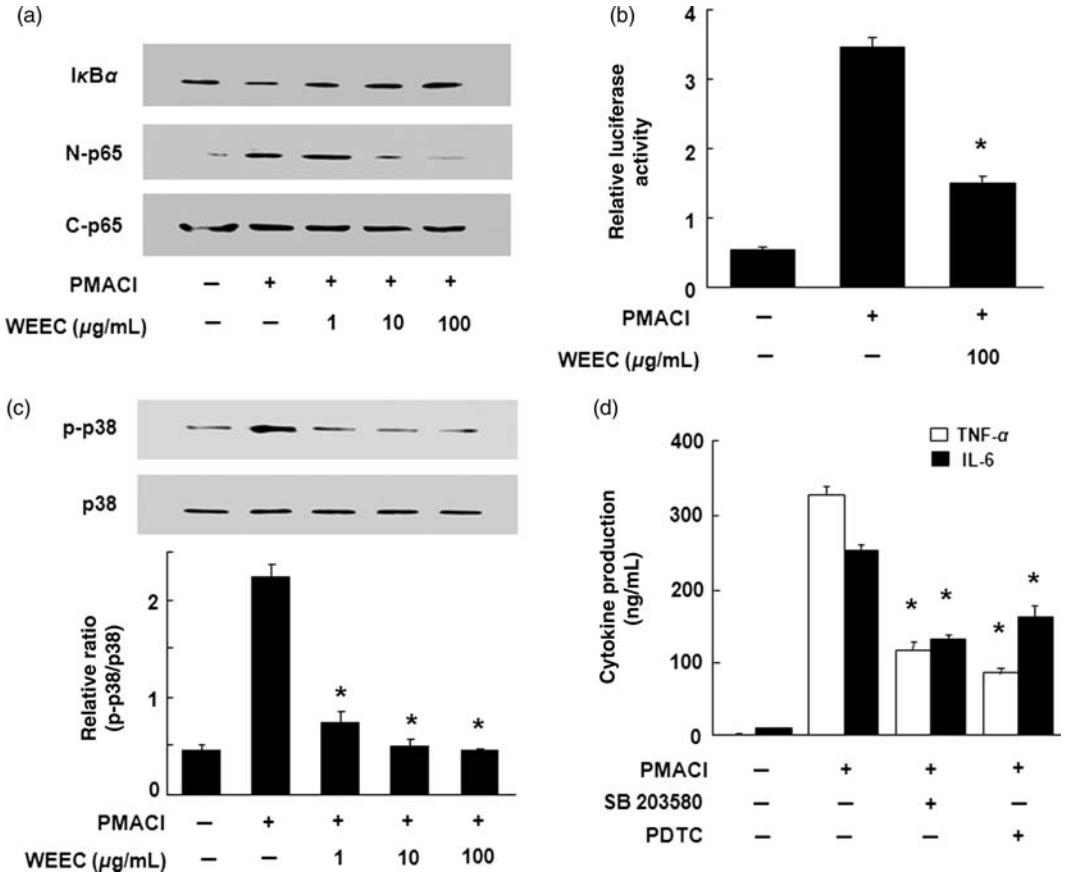

Figure 4 Effects of water extract of *Elsholtzia ciliata* (WEEC) on the activation of nuclear factor κB (NF-κB) and p38 mitogen-activated protein kinase (MAPK) in human mast cell-1 (HMC-1). Cells (3×10^6 cells) were pretreated with WEEC for 30 min prior to phorbol 12-myristate 13-acetate (PMA) (20 nmol/L) and A23187 (1 μmol/L) stimulation. (a) Nuclear translocation of p65 NF-κB and degradation of IκBα were assayed by Western blot (N-p65, nucleus p65 NF-κB; C-p65, cytoplasmic p65 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 calcium ionophore A23187 (PMACI) with or without WEEC. NF-κB-dependent transcriptional activity was determined by luciferase activity assay. (c) Phosphorylation of p38 MAPK was analyzed by Western blot. The pictures were taken using a Kodak DC 290 digital camera, and densitometric analysis was performed using a UN-SCAN-IT software. (d) Cells were pretreated with pharmacologic inhibitor of p38 MAPK (SB 203580, 5 μmol/L) and NF-κB (pyrrolidine dithiocarbamate, 50 μmol/L) for 30 min prior to PMACI stimulation. Secretion of tumor necrosis factor α (TNF-α) and interleukin (IL)-6 was measured by enzyme-linked immunosorbent assay. Each bar represents the mean ± SEM of three independent experiments. *Significant difference from PMACI (without WEEC) value at $P < 0.05$

the expression of proinflammatory cytokines in our system, we used the pharmacological agents modulating p38 MAPK and NF-κB. Treatment of cells with the selective p38 inhibitor, SB 203580 (5 μmol/L), and NF-κB inhibitor, PDTC (50 μmol/L), blocked the PMACI-induced TNF-α and IL-6 secretion (Figure 4d). These data indicate that both p38 MAPK and NF-κB contribute to the regulation of TNF-α and IL-6. All the pharmacological agents (SB 203580 and PDTC) used in this experiment did not show cytotoxicity (data not shown).

Discussion

Anaphylaxis is a life-threatening syndrome induced by a sudden systemic release of inflammatory mediators such as histamine, various cytokines and lipid-derived mediators.[17] Using *in vitro* and *in vivo* models, we showed that WEEC reduces mast cell-derived allergic inflammatory responses. WEEC inhibited compound 48/80-induced systemic anaphylaxis and histamine release from mast cells. WEEC-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 WEEC inhibits mast cell-mediated allergic reactions. In addition, WEEC decreased PMACI-stimulated expression of proinflammatory cytokines. These results suggest that WEEC 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.[18] Histamine also induces actin polymerization and chemotaxis.[19] 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, WEEC dose-dependently reduced compound 48/80-induced

histamine release from mast cells. Therefore, we speculate that WEEC might stabilize the actin polymerization of 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.[20] Calcium movements across the membranes of mast cells represent a major target for effective antiallergic drugs, as these are essential events linking stimulation to secretion. Our results showing an attenuation of intracellular calcium in mast cells with WEEC treatment might suggest that the decreased intracellular calcium might be involved in the inhibitory effect of WEEC on histamine release.

HMC-1 stimulated by PMACI produce a wide range of cytokines and support the well-recognized role of mast cells in immediate-type hypersensitivity.[4] Proinflammatory cytokines such as TNF-α and IL-6 play a major role in triggering and sustaining the allergic inflammatory response in mast cells. TNF-α is a major cytokine stored and released by mast cells. It promotes inflammation, leukocyte infiltration and chemotaxis of neutrophils and T-cells.[21] IL-6 is also produced from mast cells and its accumulation is associated with PCA reaction.[22] These reports indicate that reduction of TNF-α and IL-6 from mast cells is one of the major indicators of reduced allergic symptoms. In this study, WEEC dose-dependently reduced the expression of TNF-α and IL-6. These results suggest that WEEC has an anti-allergic inflammatory effect by controlling proinflammatory 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.[23] 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.[24] In PMACI-stimulated mast cells, WEEC decreased the degradation of IκBα and nuclear translocation of p65 NF-κB. These results demonstrate that WEEC attenuates the activation of NF-κB and downstream TNF-α and IL-6 expression.

To confirm the effects of WEEC on activation of NF-κB and expression of cytokines, we evaluated the inhibitory effect of WEEC on the activation of p38 MAPK in PMACI-stimulated mast cells. The MAPK cascade is one of the important signaling pathways in immune responses.[25] The expression of TNF-α and IL-6 is regulated by MAPKs.[4] Although the signaling pathway of the three types of MAPKs such as p38, ERK and JNK is still not well understood, p38 MAPK is thought to be an important factor in regulation of allergic inflammatory responses.[26] The activation of p38 MAPK regulates NF-κB and NF-κB-dependent gene expression through the transcriptional active subunit (p65) phosphorylation in a MSK1-dependent fashion.[27] In this study, WEEC specifically inhibited the activation of p38 MAPK but not ERK or JNK on PMACI-induced HMC-1. These results suggest that the inhibitory effects of WEEC on NF-κB and proinflammatory cytokines were mediated by p38 MAPK.

Because we used the whole water extract of *E. ciliata* and not a purified single compound, the biological effects of the individual active components are not clear at this time. The effort to identify the active components from WEEC in allergic inflammatory symptoms is ongoing in our laboratory. However, the results presented in this report give an insight into the mechanism responsible for antiallergic inflammatory activity of WEEC and evidence that WEEC could contribute to the prevention or treatment of mast cell-mediated allergic inflammatory diseases.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. H-HK and J-SY performed the major experiments and wrote the manuscript. H-SL and TKK have made substantial contributions to the conception and design of the study. T-YS and S-HK supervised the research and co-wrote the manuscript.

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