Anti-allergic and anti-inflammatory effects of aqueous extract of *Pogostemon cablin*

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Abstract. Allergic disease is caused by exposure to normally innocuous substances that activate mast cells. Mast cell-mediated allergic inflammation is closely related to a number of allergic disorders, such as anaphylaxis, allergic rhinitis, asthma and atopic dermatitis. The discovery of drugs for treating allergic disease is an interesting subject and important to human health. The aim of the present study was to investigate the anti-allergic and anti-inflammatory effects of the aqueous extract of *Pogostemon cablin* (Blanco) Benth (AEPC) (a member of the Labiatae family) using mast cells, and also to determine its possible mechanisms of action. An intraperitoneal injection of compound 48/80 or a serial injection of immunoglobulin E and antigen was used to induce anaphylaxis in mice. We found that AEPC inhibited compound 48/80-induced systemic and immunoglobulin E-mediated cutaneous anaphylaxis in a dose-dependent manner. The release of histamine from mast cells was reduced by AEPC, and this suppressive effect was associated with the regulation of calcium influx. In addition, AEPC attenuated the phorbol 12-myristate 13-acetate plus calcium ionophore A23187 (PMACI)-stimulated expression of pro-inflammatory cytokines in mast cells. The inhibitory effects of AEPC on pro-inflammatory cytokines were dependent on the activation of nuclear factor (NF)-κB and p38 mitogen-activated protein kinase (MAPK). AEPC blocked the PMACI-induced translocation of NF-κB into the nucleus by hindering the degradation of IκBα and the phosphorylation of p38 MAPK. Our results thus indicate that AEPC inhibits mast cell-mediated allergic inflammation by suppressing mast cell degranulation and the expression of pro-inflammatory cytokines caused by reduced intracellular calcium levels and the activation of NF-κB and p38 MAPK.

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Introduction

Allergic diseases, such as allergic asthma, rhinitis and atopic dermatitis are a major health concern in the modern world, and are often caused by environmental pollution (1). Allergies develop from complex interactions between genes and the environment. In developed countries, the prevalence and risk of allergic disorders have both steadily increased for decades (2). Mast cells are known to be closely associated with immediate-type hypersensitivity through the release of allergic mediators and cytokines following activation by FcεRI. Allergen cross-linking with specific immunoglobulin E (IgE) bound to FcεRI triggers mast cell activation, inducing the rapid secretion of preformed allergic mediators and *de novo* synthesized mediators, such as histamine, cytokines, proteases and derivatives of arachidonic acid (3). Histamine, one of the major allergic mediators, plays a key role in various physiological and pathological responses, particularly allergic reactions (4).

Calcium, which acts as a secondary messenger in mast cells, is associated with the increasing degranulation of mast cells (3). Signal transducing enzymes, including phospholipase C and phosphoinositide 3-kinase, stimulate calcium influx following the activation of FcεRI (5). Activated mast cells can produce histamine, as well as a wide variety of inflammatory mediators, such as proteoglycans, eicosanoids, proteases, transforming growth factor-β, chemokines and cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, IL-4 and IL-13 (3,6). The activation of mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)-κB is also accompanied by the binding of allergens with IgE. MAPKs and NF-κB are important mediators of cellular responses from extracellular signals and are thought to regulate the expression of pro-inflammatory molecules, particularly TNF-α, IL-6 and IL-8 (7-9).

Pogostemon cablin (Blanco) Benth (*P. cablin*) is a widely used traditional medicine in Korea. *P. cablin* contains various biologi-

cally active compounds, such as patchouli alcohol, pogostone, eugenol, α-bulnesene, patchoulene and rosmarinic acid (10,11). The pharmacological activity of *P. cablin* is due to essential oils, which constitute approximately 1.5% of *P. cablin* and patchouli alcohol, which constitutes >50% of *P. cablin*. The anti-fungal, anticancer and anti-emetic properties of *P. cablin* have been demonstrated in a number of studies over the years (12-16). In Korea, the aqueous extract of *P. cablin* (AEPC) has long been used in the treatment of gastrointestinal disorders, such as indigestion, vomiting and diarrhea (17). Although various studies have been published on the biological effects of *P. cablin* (12-16), to the best of our knowledge, there is no study available to date on the anti-allergic and inflammatory effects of *P. cablin*. Thus, the aim of the present study was to examine the inhibitory effects of AEPC on allergic inflammation and to define the underlying mechanisms of these effects using animal models and mast cells.

Materials and methods

Animals. Imprinting control region (ICR) mice (n=150, male, aged 6 weeks) and Sprague-Dawley (SD) rats (n=10, male, aged 10 weeks) were purchased from Dae Han Biolink (Daejeon, Korea). The animals were housed 5 per cage in a laminar air flow room maintained at a temperature of 22±2˚C and a relative humidity of 55±5% throughout the study. The care and treatment of the mice and rats 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 of Kyungpook National University (Daegu, Korea).

Reagents and cell culture. Compound 48/80, anti-dinitrophenol (DNP) IgE, DNP-human serum albumin (HSA), pyrrolidine dithiocarbamate (PDTC), phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187, and *o*-phthaldialdehyde (OPA) were all purchased from Sigma Aldrich (St. Louis, MO, USA). Forward and reverse primers for human TNF-α, IL-6 and IL-8 were purchased from Genotech (Daejeon, Korea) and ELISA kits were purchased from BD Biosciences (San Diego, CA, USA). Human mast cells (HMC-1; kind gift from Professor D.K. Kim, Department of Immunology, School of Medicine, Chonbuk National University, Jeonju, Korea) and rat peritoneal mast cells (RPMCs) were grown in Iscove's modified Dulbecco's medium (IMDM) and α-minimum essential medium (Gibco, Grand Island, NY, USA), respectively, supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA), 100 U/ml penicillin G (HyClone), 100 μg/ml streptomycin (HyClone) and 250 ng/ml amphotericin B (HyClone) at 37˚C in 5% CO_2. HMC-1 cells at passages 4-8 were used throughout the study.

Preparation of RPMCs. The peritoneal cells were isolated from SD rats as previously described (18). In brief, the rats were anesthetized with CO_2 and injected with 20 ml of Tyrode's buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose and 0.1% bovine serum albumin) into the peritoneal cavity, and the abdomen was gently massaged for approximately 90 sec. The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells was aspirated using a Pasteur pipette. The peritoneal cells were sedimented at 150 g for 10 min at room temperature and resuspended in Tyrode's buffer A. Mast cells were separated from the major components of rat peritoneal cells, i.e., macrophages and small lymphocytes. Peritoneal cells were suspended in 1 ml of Tyrode buffer A, layered on 2 ml of Histodenz (Sigma-Aldrich) solution, and centrifuged at 400 x g for 10 min at 4˚C. The cells remaining at the buffer-Histodenz interface were aspirated and discarded, and the cells in the pellet were washed and resuspended. Mast cell preparations were approximately 95% pure based on toluidine blue staining. More than 95% of the cells were viable based on trypan blue exclusion (data not shown).

Preparation of AEPC. *P. cablin* used in the present study was purchased from the oriental drug store, Bohwa Dang (Jeonju, Korea) and identified by Dr D.K. Kim at the College of Pharmacy, Woosuk University (Jeonju, 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 (2 times) in a water bath. The extract was then filtered, lyophilized and stored at 4˚C. The yield of dried extract from the starting crude materials was approximately 15.3%. The dried extract of AEPC was dissolved in saline or Tyrode's buffer A prior to use.

Compound 48/80-induced systemic anaphylaxis. The mice (n=10/group) were administered an intraperitoneal injection of 8 mg/kg body weight (BW) of the mast cell degranulation compound 48/80, as previously described (18). The mice were divided into the following groups: group A, compound 48/80 + saline; group B, compound 48/80 + AEPC 10 mg/kg; group C, compound 48/80 + AEPC 50 mg/kg; group D, compound 48/80 + AEPC 100 mg/kg; group E, compound 48/80 + AEPC 500 mg/kg; group F, compound 48/80 + AEPC 1,000 mg/kg; group G, AEPC 1,000 mg/kg only. AEPC was intraperitoneally administered at doses of 10-1,000 mg/kg BW 1 h prior to the injection of compound 48/80. For the time-dependent experiments, AEPC (1,000 mg/kg) was intraperitoneally administered at 5, 10, 20 and 30 min after the injection of compound 48/80 (n=10/group). Mortality was monitored for 1 h after the induction of anaphylactic shock.

IgE-mediated passive cutaneous anaphylaxis (PCA). IgE-mediated PCA was induced as previously described (19). To induce the PCA reaction, the skin on the ears of the mice (n=5/group) was sensitized with an intradermal injection of anti-DNP IgE (0.5 μg/site) for 48 h. The mice were divided into the following groups: group A, saline only; group B, IgE/DNP-HSA; group C, IgE/DNP-HSA + AEPC 1 mg/kg, group D: IgE/DNP-HSA + AEPC 10 mg/kg, group E: IgE/DNP-HSA + AEPC 100 mg/kg, group F: IgE/DNP-HSA + AEPC 1,000 mg/kg AEPC was intraperitoneally administered at doses of 1-1,000 mg/kg BW 1 h prior to the intravenous injection of DNP-HSA (1 mg/mouse) and 4% Evans blue (1:1) mixture. Thirty minutes after the challenge, the mice were euthanized using carbon dioxide, and the skin of both ears was collected for measurements with pigment dye. The amount of dye was determined colorimetrically following extraction with a mixture of 1 ml of 1 M KOH and 9 ml of acetone and phosphoric acid (5:13). The absorbance intensity of the extract

was detected using a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 620 nm.

Histamine and β-hexosaminidase assay. To assess mast cell degranulation, the release of histamine from mast cells was detected using the *o*-phthaldialdehyde spectrofluorometric procedure and a fluorescent plate reader (Molecular Devices) at an excitation wavelength of 360 nm and an emission wavelength of 440 nm, as previously described, and the level of β-hexosaminidase was read using the spectrophotometer at 405 nm, as previously described (20,21). The RPMCs ($2x10^4$ cells/well in 24-well plates) were pre-treated with or without AEPC for 30 min and then stimulated with compound 48/80 (5 μg/ml) for 10 min. The cells were separated from the released histamine by centrifugation at 400 x g for 5 min at 4˚C. β-hexosaminidase substrate buffer (100 mM sodium citrate, 1 mM 4-nitrophenyl N-acetyl-β-D-galactosaminide, pH 4.5) was added following by incubation for 1 h at 37˚C. The reaction was terminated using stop solution (0.1 M Na_2CO_3 and $NaHCO_3$; Sigma Aldrich).

Measurement of intracellular calcium levels. Intracellular calcium levels were measured with the use of the fluorescence indicator, Fluo-3/AM (Invitrogen, Carlsbad, CA, USA), as previously described (20). The RPMCs ($1x10^4$ cells/well in 96-well plates) were pre-incubated with Fluo-3/AM for 1 h at 37˚C. After washing the dye from the cell surface with Tyrode's buffer B (137 mM NaCl, 5.5 mM glucose, 12 mM $NaHCO_3$, 2.7 mM KCl, 0.2 mM NaH_2PO_4, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$), the cells were pre-treated with or without AEPC for 30 min and then stimulated with compound 48/80 (5 μg/ml). The fluorescence intensity was detected using a fluorescent plate reader at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The intracellular calcium level was calculated using relative absorbance as the control value of 1.

Cell viability. Cell viability was assayed using an XTT assay kit (Welgene Inc., Seoul, Korea) according to the manufacturer's instructions and as previously described (22). The HMC-1 cells ($1x10^5$ cells/well in 96-well plates) were pre-treated with various concentrations of AEPC (1-1,000 μg/ml) for 24 h and incubated with XTT plus phenazine methosulfate reagent for 2 h at 37˚C. The absorbance intensity was detected using a spectrophotometer at 450 nm. Cell viability was calculated using relative absorbance as the control value of 100%.

RNA extraction and reverse-transcription-quantitative polymerase chain reaction (RT-qPCR). Prior to the isolation of total cellular RNA, the HMC-1 cells ($1x10^6$ cells/well in 24-well plates) were pre-treated with or without AEPC for 30 min and stimulated with PMA (40 nM) plus calcium ionophore A23187 (1 μM) (PMACI) for 2 h. RNAiso Plus reagent (Takara Bio Inc., Shiga, Japan) was used to extract the RNA, according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 2 μg of total RNA using the Maxime RT PreMix kit (iNtRON Biotechnology, Daejeon, Korea). To measure the expression levels of TNF-α, IL-6 and IL-8, RT-qPCR was carried out using the Maxime PCR PreMix kit (iNtRON Biotechnology) and the Thermal Cycler Dice TP850 (Takara Bio) according to the manufacturer's instructions. For RT-PCR, the total reaction mixture (20 μl) was composed of the following: 1 μl of cDNA (100 ng), 1 μl of each of forward and reverse primers (0.4 μM) and 17 μl of dH_2O. For quantitative (real-time) PCR (qPCR), the total reaction mixture (25 μl) was composed of the following: 1.5 μl of cDNA (200 ng), 1 μl of each of forward and reverse primers (0.4 μM), 12.5 μl of SYBR Premix Ex Taq (Takara Bio) and 9 μl of dH_2O. The primer sequences used were as follows: TNF-α forward, 5'-CCT ACC AGA CCA AGG TCA AC-3' and reverse, 5'-AGG GGG TAA TAA AGG GAT TG-3'; IL-6 forward, 5'-AAA GAG GCA CTG GCA GAA AA-3' and reverse, 5'-ATC TGA GGT GCC CAT GCT AC-3'; IL-8 forward, 5'-ACA GCA GAG CAC ACA AGC TT-3' and reverse, 5'-CTG GCA ACC CTA CAA CAG AC-3'; β-actin forward, 5'-GGA CTT CGA GCA AGA GAT GG-3' and reverse, 5'-AGC ACT GTG TTG GCG TAC AG-3'. The conditions for the qPCR steps were similar to those described in a previous study (22).

Enzyme-linked immunosorbent assay (ELISA). The secretion of pro-inflammatory cytokines was measured by ELISA, as previously described (8). The HMC-1 cells ($1x10^6$ cells/well in 24-well plates) were pre-treated with or without AEPC for 30 min and stimulated with PMACI for 8 h. ELISA was performed using an ELISA kit (BD Biosciences) on a 96-well Nunc-Immuno plate (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. After terminating the reaction with a substrate, the absorbance intensity was detected using a spectrophotometer at 450 nm.

Protein extraction and western blot analysis. Nuclear and cytosolic proteins were both extracted as previously described (23). Prior to protein extraction, the HMC-1 cells ($2x10^6$ cells/well in 6-well plates) were pre-treated with or without AEPC for 30 min and stimulated with PMACI for 2 h. Followiknng suspension in 100 μl of cell lysis buffer A (0.5% Triton X-100, 150 mM NaCl, 10 mM HEPES, 1 mM EDTA /Na_3VO_4, 0.5 mM PMSF/DTT and 5 μg/ml leupeptin /aprotinin), the cells were vortexed, incubated for 5 min on ice and centrifuged at 400 x g for 5 min at 4˚C, and the supernatant was then gathered as the cytosolic protein extract. The pellets were washed 3 times with 1 ml of PBS and suspended in 25 μl of cell lysis buffer B (25% glycerol, 420 mM NaCl, 20 mM HEPES, 1.2 mM $MgCl_2$, 0.2 mM EDTA, 1 mM Na_3VO_4, 0.5 mM PMSF/DTT and 5 μg/ml leupeptin/aprotinin), vortexed, sonicated for 30 sec, incubated for 20 min on ice and centrifuged at 15,000 x g for 15 min at 4˚C, and the supernatant was then gathered as the nuclear protein extract. Samples of protein were electrophoresed using 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. NF-κB, IκBα, actin and p38 MAPK were assayed using the following antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-NF-κB (sc-109), anti-IκBα (sc-371), anti-actin (sc-8432; mouse monoclonal; 1:1,000), and Cell Signaling Technology Inc. (Danvers, MA, USA); anti-phospho-p38 (#9211) and anti-p38 (#9212). Immunodetection was carried out using a chemiluminescent substrate (Thermo Fisher Scientific).

Transient transfection and luciferase activity assay. The HMC-1 cells ($2x10^6$ cells/well in 6-well plates) were seeded in serum/antibiotics-free IMDM 1 day prior to transient transfection. The expression vectors containing the NF-κB luciferase

Table I. Dose-dependent effects of AEPC on compound 48/80-induced systemic anaphylaxis.

AEPC treatment (mg/kg BW)	Compound 48/80 (8 mg/kg BW)	Mortality rate (%)
None (saline)	+	100
10	+	100
50	+	80
100	+	60
500	+	20
1,000	+	0
1,000	–	0

Groups of mice (n=10/group) were intraperitoneally injected with 200 μl of saline or AEPC prior to the induction of anaphylaxis. Various doses of aqueous extract of *Pogostemon cablin* (AEPC) were administered 1 h prior to the intraperitoneal injection of compound 48/80. The mortality rate (%) within 1 h following compound 48/80 injection was calculated as the number of dead mice/total number of experimental mice x100. BW, body weight.

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

AEPC treatment (mg/kg)	Time (min)	Compound 48/80 (8 mg/kg BW)	Mortality rate (%)
None (saline)	0	+	100
1,000	5	+	0
1,000	10	+	40
1,000	20	+	80
1,000	30	+	100

Groups of mice (n=10/group) were intraperitoneally injected with 200 μl of saline or aqueous extract of *Pogostemon cablin* (AEPC). AEPC (1,000 mg/kg) was administered at 5, 10, 20 and 30 min after the intraperitoneal injection of compound 48/80. The mortality rate (%) within 1 h following compound 48/80 injection was calculated as the number of dead mice/total number of experimental mice x100. BW, body weight.

reporter construct (pNF-κB-LUC, plasmid containing the NF-κB binding site; Stantagen, Grand Island, NY, USA) or empty vectors were transfected using 8 μl of Lipofectamine 2000 reagent (Invitrogen). Following incubation for 5 h, the medium was replaced with IMDM containing 10% FBS and antibiotics. The cells were allowed to recover at 37˚C for 20 h and subsequently were stimulated as indicated. The cell lysate was prepared and assayed for luciferase activity using a Luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Statistical analysis. Statistical analyses were performed using Prism5 (GraphPad Software, San Diego, CA, USA), and the

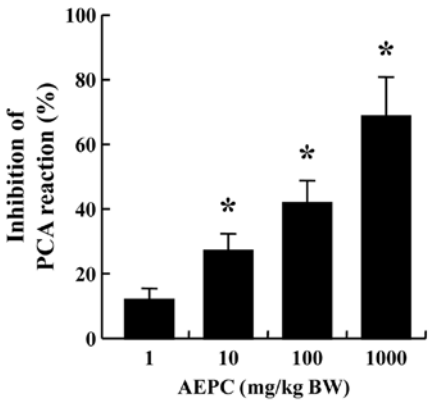

Figure 1. Effect of aqueous extract of *Pogostemon cablin* (AEPC) on passive cutaneous anaphylaxis (PCA). AEPC was intraperitoneally administered 1 h prior to the challenge with antigen. Each amount of dye was extracted as described in Materials and methods and measured using a spectrophotometer. Data are presented as the means ± SE (n=5/group). *P<0.05 indicates a statistically significant difference compared to DNP-HSA-challenged group.

effects of treatment were analyzed using one-way ANOVA followed by Dunnett's test. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Effects of AEPC on systemic and cutaneous anaphylaxis. To determine the effectS of AEPC on allergic reaction *in vivo*, compound 48/80-induced systemic anaphylaxis and IgE-mediated PCA were induced in mice for immediate-type hypersensitivity. The intraperitoneal injection of compound 48/80 (8 mg/kg BW), a mast cell degranulator, induces lethal systemic anaphylaxis (18). The mice (n=10/group) were intraperitoneally administered 200 μl saline or various doses of AEPC (10-1,000 mg/kg BW) 1 h prior to the intraperitoneal injection of compound 48/80. Following the administration of compound 48/80, we monitored the mice for 1 h and the mortality rate was recorded. All mice injected with compound 48/80 and saline suffered fatal anaphylactic shock, whereas the mortality rate was reduced in the mice administered AEPC in a dose-dependent manner (Table I). In addition, the mortality rate of the mice administered AEPC (1,000 mg/kg BW) at 5, 10, 20 and 30 min after the injection of compound 48/80 was increased in a time-dependent manner (Table II).

Another animal model, of IgE-mediated PCA, was used to confirm the anti-allergic effects of AEPC (19). Both ears of each mouse were sensitized by an intradermal injection of anti-DNP IgE (0.5 μg/site) prior to the intravenous injection of DNP-HSA (1 mg/mouse) and a 4% Evans blue (1:1) mixture to induce the PCA reaction. After the antigen challenge, a blue spot was visible at the sensitized site due to the increased vascular permeability caused by the release of histamine from mast cells. AEPC was administered intraperitoneally 1 h prior to the challenge with antigen. The administration of AEPC reduced the size and blue color of the spot, thus inhibiting the PCA reaction in a dose-dependent manner (Fig. 1).

Effect of AEPC on compound 48/80-induced degranulation and calcium influx. Histamine and β-hexosaminidase released from mast cells are important allergic mediators, and

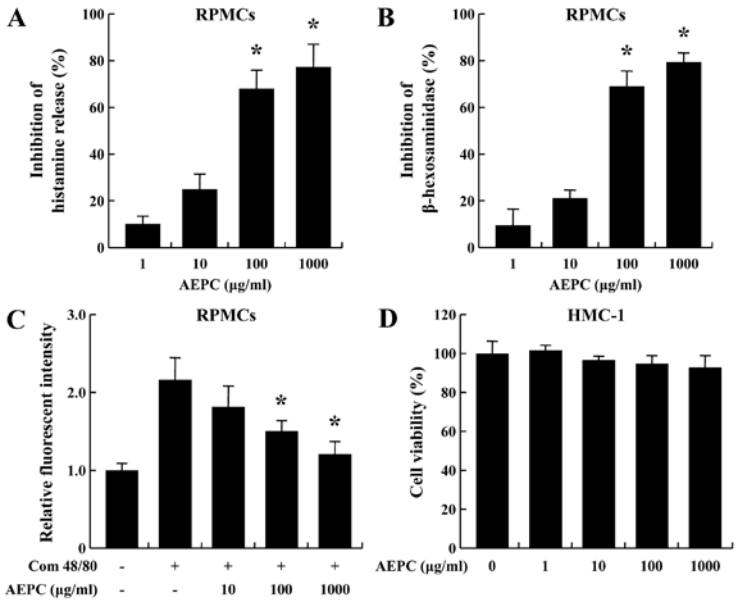

Figure 2. Effect of aqueous extract of *Pogostemon cablin* (AEPC) on compound 48/80-induced degranulation and calcium influx. Rat peritoneal mast cells (RPMCs) ($2x10^4$ cells/well in 24-well plates) were treated with or without AEPC for 30 min and then stimulated with compound 48/80 (5 μg/ml) for 10 min. (A) Histamine content was detected using *o*-phthaldialdehyde spectrofluorometric procedure with a fluorescent plate reader. (B) β-hexosaminidase assay was performed as described in Materials and methods. (C) The level of relative fluorescent intensity was recorded using Fluo-3/AM by fluorescent plate reader. (D) Cell viability was assayed using an XTT assay kit. Human mast cells (HMC-1; $1x10^5$ cells/well in 96-well plates) were pre-treated with various concentrations of AEPC for 24 h and incubated with XTT plus phenazine methosulfate reagent for 2 h at 37˚C. The absorbance intensity was detected using a spectrophotometer. Cell viability was calculated using relative absorbance as the control value of 100%. Data are presented as the means ± SE of 3 independent experiments. *P<0.05 indicates a statistically significant difference compared to compound 48/80-stimulated group.

the inhibition of mast cell degranulation is the proper therapeutic target for allergic disorders (24). Thus, in this study, we evaluated the effects of AEPC on the release of histamine and β-hexosaminidase induced by compound 48/80. Treatment with compound 48/80 resulted in the increased release of histamine and β-hexsosaminidase from the RPMCs (data not shown). By contrast, AEPC suppressed the release of histamine and β-hexsosaminidase induced by compound 48/80 in a dose-dependent manner (Fig. 2A and B). These results support the hypothesis that AEPC inhibits compound 48/80-induced anaphylaxis by blocking degranulation of mast cells.

To investigate the mechanisms responsible for the inhibitory effects of AEPC on the release of histamine, we measured the intracellular calcium levels. Calcium influx in mast cells is critical to the release of histamine (25). Antigen cross-linking of IgE bound to FcεRI causes the calcium influx, which stimulates granule fusion-to-cell membranes consequentially through the binding of synaptotagmin to the soluble NSF attachment protein receptor (SNARE) complex (26). Compound 48/80 induced an increase in the intracellular calcium levels in the RPMCs; however, AEPC counteracted this increase (Fig. 2C). Therefore, our findings suggest that AEPC inhibits the release of histamine by blocking calcium movement into mast cells. The concentration of AEPC used in this study was not cytotoxic to the HMC-1 cells (Fig. 2D).

Effect of AEPC on the expression of pro-inflammatory cytokines. Pro-inflammatory cytokines are important to the progression of chronic allergic inflammation (27). Thus, we examined the effects of AEPC on the gene expression of pro-inflammatory cytokines in HMC-1 cells, such as TNF-α, IL-6 and IL-8. AEPC attenuated the PMACI-induced increase in the mRNA expression of TNF-α, IL-6 and IL-8 in a dose-dependent manner (Fig. 3A and B). To confirm the effects of AEPC on the mRNA expression of pro-inflammatory cytokines, cultured media were used in the ELISA for assaying the secretion of TNF-α, IL-6 and IL-8. Stimulation of the cells with PMACI for 8 h induced the secretion of cytokines. On the contrary, AEPC inhibited the secretion of TNF-α, IL-6 and IL-8 from the PMACI-stimulated HMC-1 cells (Fig. 3C).

Effects of AEPC on the activation of NF-κB and p38 MAPK. To elucidate the mechanisms responsible for the inhibitory effects of AEPC on cytokine expression, we investigated the activation of the transcription factors, NF-κB and p38 MAPK, which have previously been reported to play important roles in immune and inflammatory responses (23). Stimulation of the HMC-1 cells with PMACI caused the degradation of IκBα and the translocation of p65 NF-κB into the nucleus. AEPC reduced the PMACI-induced activation of NF-κB by blocking the degradation of IκBα (Fig. 4A). To confirm the suppressive effects of AEPC on the activation of NF-κB, we performed an NF-κB-dependent gene reporter assay. PDTC, an inhibitor of NF-κB, was used as a positive control. The HMC-1 cells were transiently transfected with an NF-κB-luciferase reporter construct or an empty vector. Stimulation of the cells with PMACI increased the luciferase activity in the cells transfected with the NF-κB-luciferase reporter construct. AEPC significantly diminished the increased luciferase activity induced by PMACI (Fig. 4B). The MAPK pathway is also known to play a major role in the regulation of pro-inflammatory mediators (28,29). Thus, we evaluated the effects of AEPC on the activation of MAPKs. It is clear that AEPC attenuated PMACI-induced p38 MAPK phosphorylation (Fig. 4C).

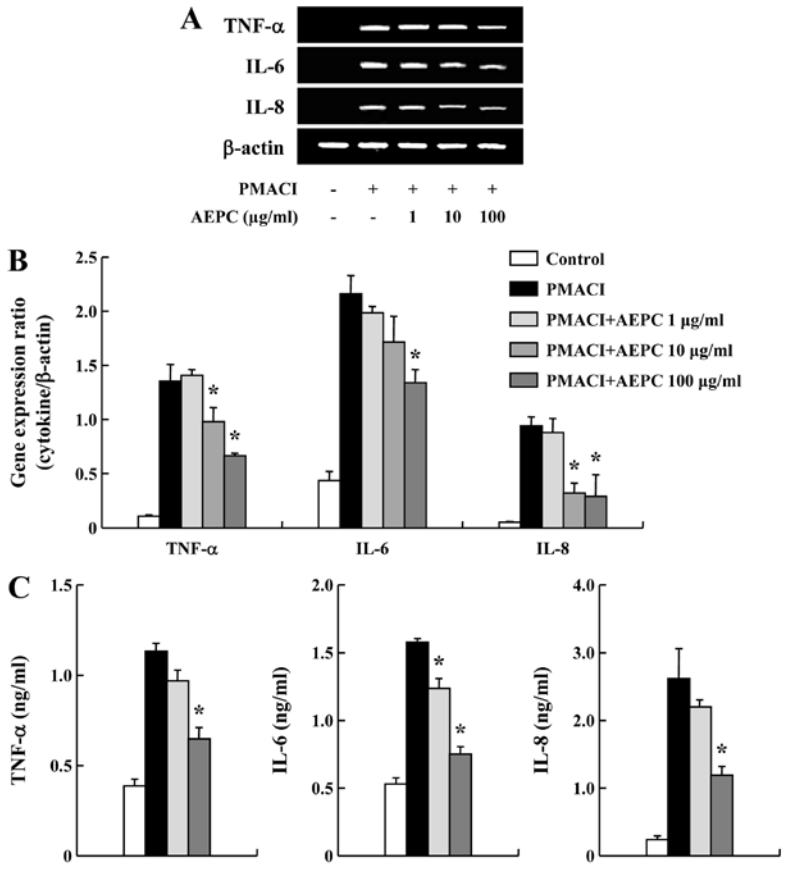

Figure 3. Effect of aqueous extract of *Pogostemon cablin* (AEPC) on the expression of pro-inflammatory cytokines. Human mast cells (HMC-1; $1x10^6$ cells/well in 24-well plates) were pre-treated with or without AEPC for 30 min and stimulated with phorbol 12-myristate 13-acetate plus calcium ionophore A23187 (PMACI). (A and B) mRNA levels of tumor necrosis factor (TNF)-α, interleukin (IL)-16 and IL-8 were determined by RT-qPCR. (C) The secretion of TNF-α, IL-6 and IL-8 was measured by ELISA. The band is representative of 3 independent experiments. The band of actin is used as a loading control. Data are presented as the means ± SE of 3 independent experiments. *P<0.05 indicates a statistically significant difference compared to PMACI-stimulated group.

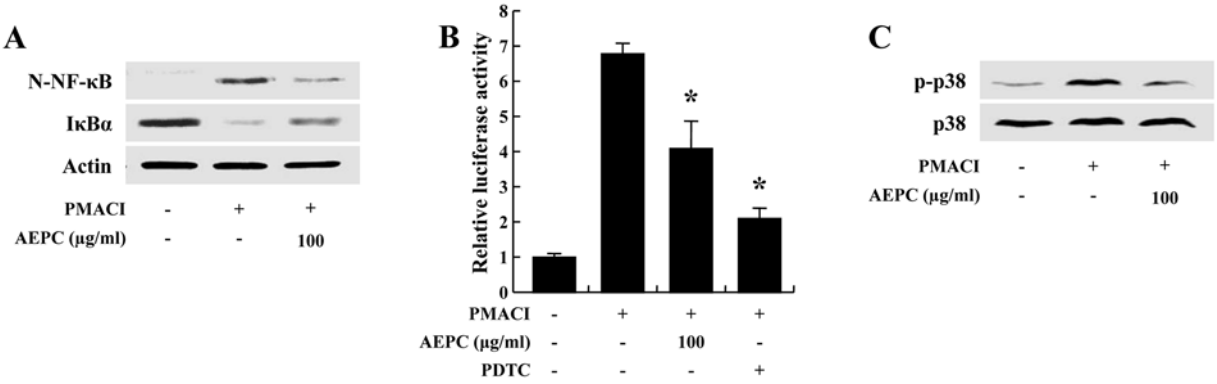

Figure 4. Effect of aqueous extract of *Pogostemon cablin* (AEPC) on the activation of nuclear factor (NF)-κB and p38 mitogen-activated protein kinase (MAPK). Human mast cells (HMC-1; $2x10^6$ cells/well in 6-well plates) were treated with or without AEPC for 30 min and then stimulated with phorbol 12-myristate 13-acetate plus calcium ionophore A23187 (PMACI) for 2 h. (A) The nuclear translocation of NF-κB and the degradation of IκBα were assayed by western blot analysis. (B) Cells were transiently transfected with the NF-κB-luciferase reporter construct or empty vectors as described in the Materials and methods. NF-κB-dependent transcriptional activity was determined by luciferase activity assay. (C) Phosphorylation of p38 MAPK was analyzed by western blot analysis (p-, phosphorylated). The band is representative of 3 independent experiments. The bands of actin and total p38 are used as loading controls. Data are presented as the means ± SE of 3 independent experiments. *P<0.05 indicates a statistically significant difference compared to PMACI-stimulated group.

Discussion

Currently, there are many allergic diseases for which no cure has been found. In particular, anaphylaxis, which can be lethal, is induced by the systemic release of allergic mediators, such as histamine, chemokines and cytokines from mast cells in only a few minutes (30). The first aim of the present study was to examine whether AEPC has anti-allergic properties.

As shown by our results, AEPC inhibited compound 48/80-induced systemic anaphylaxis and IgE-mediated PCA. Numerous reports have previously mentioned that stimulation with compound 48/80 or antigen-IgE initiates the signaling pathway, which results in histamine being released from mast cells (3,9,24). Histamine is considered a crucial mediator in acute inflammation and immediate-type hypersensitivity. It has previously been demonstrated that histamine affects the development of the antigen-specific immune response, the maturation of dendritic cells, alters T cell-polarizing capacity, and leads to chronic allergies by selectively recruiting major effector cells into the allergic region and via the maturation, activation and polarization of immune cells (4,31). We observed in the present study that the rapidly elevated degranulation of histamine and β-hexosaminidase in RPMCs stimulated with compound 48/80 was reduced by AEPC. Therefore, we posit that AEPC regulates anaphylaxis by inhibiting the degranulation of mast cells. Intracellular calcium levels are critical to the release of allergic mediators from mast cells (8). In addition, blocking calcium movement across the membranes of mast cells is a useful strategy for anti-allergic drugs to hinder the secretion of mast cells (25). In the present study, we noted that AEPC decreased calcium influx in a dose-dependent manner. Thus, we propose that AEPC exerts a suppressive effect on immediate-type hypersensitivity by reducing the degranulation of mast cells caused and blocking calcium influx.

HMC-1 cells are appropriate tools for the *in vitro* examination of the expression of pro-inflammatory cytokines (32). Various cytokines, including TNF-α, IL-6 and IL-8, which are produced in HMC-1 cells by stimulation with PMACI, are well-recognized to trigger and sustain allergic inflammatory reactions. Mast cells are major donors of cytokines in the human dermis. TNF-α, a well-known pro-inflammatory factor from mast cells, is important for the development of mast cells despite the fact that it is not a growth factor and that it promotes the interaction of endothelial leukocytes by inducing the expression of adhesion molecules (33-35). Local accumulation of IL-6 mediates the PCA reaction as well as promoting type 2 T helper (Th2) modulation by assisting T cell survival (36,37). The IL-8-dependent recruitment of neutrophils enhances inflammation in chronic allergic diseases (38). These reports suggest that the inhibition of pro-inflammatory cytokines is a most important aspect of reducing allergic inflammation. In the present study, we noted that AEPC inhibited the gene expression and secretion of TNF-α, IL-6 and IL-8 in HMC-1 cells stimulated with PMACI. As a result, we suggest that the anti-allergic and anti-inflammatory effects of AEPC are caused by the inhibition of TNF-α, IL-6 and IL-8 in mast cells.

In order to elucidate the mechanisms responsible for the anti-allergic and anti-inflammatory effects of AEPC on mast cells, we examined the activation of NF-κB. The activation of NF-κB, a transcription factor which is of significance for inflammatory mediators, plays a critical role in chronic inflammatory diseases as it regulates the expression of various inflammatory and immune genes, including TNF-α and IL-1β (39). The phosphorylation and proteolytic degradation of IκBα are required for the tranlocation of NF-κB into the nucleus (7). In our study, PMACI stimulated the nuclear translocation of NF-κB, which was regulated by AEPC,which then blocked the degradation of IκBα in mast cells. Moreover, the transcription caused by NF-κB was also obstructed. Thus, it is possible that AEPC attenuates the expression of downstream cytokines by inhibiting the activation of NF-κB. It is well known that the MAPK signaling cascade is also involved in inflammation (28). There are three types of MAPKs, namely p38, ERK and JNK. We examined the effects of AEPC on the activation of p38 MAPK, as previous studies noted that the phosphorylation of p38 MAPK is essential for the expression of the pro-inflammatory cytokines (40,41). In the present study, we demonstrated that AEPC inhibited the activation of p38 MAPK, as was expected. According to,our results, AEPC reduced the expression of allergic inflammatory mediators by suppressing the activation of transcription factors, particularly NF-κB and p38.

In the present study, we provide evidence that AEPC inhibits the allergic inflammatory reaction. We suggest that AEPC inhibits the degranulation of mast cells and the expression of pro-inflammatory cytokines via the reduction of calcium influx, and the activation of NF-κB and p38 MAPK. As we used whole aqueous extract of *P. cablin (*AEPC), not a purified single compound, the biological effects of the individual active components are not clear at this time. Efforts to identify the active components from AEPC in allergic inflammatory symptoms are ongoing in our laboratory. However, the results presented herein provide insight into the mechanisms responsible for the anti-allergic and anti-inflammatory effects of AEPC, as well as evidence that AEPC contributes to the prevention or treatment of mast cell-mediated allergic inflammatory diseases.

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