Piceatannol inhibits mast cell-mediated allergic inflammation

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Abstract. Piceatannol is a phenolic stilbenoid and a metabolite of resveratrol which is found in red wine. Piceatannol (PIC) commonly exhibits anti-inflammatory, antiplatelet and antiproliferative activity. In the present study, the anti-allergic and anti-inflammatory mechanisms of PIC were investigated by examining the effects of PIC on pro-inflammatory cytokine release and phosphorylation of mitogen-activated protein (MAP) kinases (ERK, JNK and p38) in a human mast cell line. PIC dose-dependently inhibited compound 48/80-induced systemic anaphylaxis and immunoglobulin E-mediated local allergic reactions. PIC reduced the immunoglobulin E (IgE)-mediated local allergic reaction and attenuated histamine release from rat peritoneal mast cells. Histamine and β-hexosaminidase release was markedly decreased dose-dependently by PIC treatment in RBL-2H3 cells. PIC treatments of HMC-1 cells definitely reduced mRNA expression and the release of the pro-inflammatory cytokines, tumor necrosis factor-α and interleukin-8. MAP kinase phosphorylation was also strongly decreased dose-dependently following PIC treatment. PIC regulated the production of cytokines and histamine in phorbol 12-myristate 13-acetate plus A23187-stimulated mast cells. Thus, PIC may alleviate allergic inflammation and may be a useful therapeutic agent for allergic diseases.

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Introduction

Resveratrol, *trans*-3,4',5-trihydoxystilbene, which is primarily derived from red grapes, exhibits anti-inflammatory, antiplatelet and antiproliferative activity, in addition to having anti-oxidative properties (1-4). Resveratrol has been reported to suppress the expression of pro-inflammatory markers, including cyclooxygenase (COX)-2, and reduce lipid peroxidation, and tumor initiation, promotion and metastasis (5). Naturally occurring hydroxystilbenes consist of 2 benzene rings connected by an olefin. Piceatannol (PIC) is a *trans*-3,4,3',5-tetra hydroxystilbene first isolated from the seeds of *Euphoribia lagascae* (6,7). This stilbene has been shown to be a potent inducer of apoptosis through activation of caspase-3, in lymphoma cells and in primary leukemic lymphoblasts derived from childhood acute lymphoblastic leukemia patients (8). Other studies have suggested that PIC is a potent inhibitor of the protein tyrosine kinases p56^{lck} and Syk (8-11), and a potent inhibitor of the following serine/threonine protein kinases: the catalytic subunit of cAMP-dependent protein kinase, phospholipid-dependent protein kinase C and Ca^2-calmodulin-dependent myosin light-chain kinase (12). PIC has also been shown to block FcεR I-mediated signaling in mast cells through the suppression of Syk activity (13). Resveratrol was found to exert a beneficial effect in the treatment of ischemia, and neurodegenerative and allergic diseases (14,15). However, the functional effects of PIC, a metabolite of resveratrol, are not known.

Mast cells are one of the major effector cells in the immune response system. Activated mast cells release pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, IL-13 and inflammatory mediators, including histamine, luekotrienes, serotonin, prostaglandin (PG)E2, as well as PGD2 (16-18). Cytokines, such as TNF-α, IL-6 and IL-8, are released in a coordinated fashion and play important roles in chronic inflammation. TNF-α is either preformed and stored in granules of mast cells, or is newly synthesized following mast cell activation; it is a multifunctional cytokine and an important mediator of the immune and inflammatory response (19). In contrast, IL-8 from mast cells

acts on surrounding cells such as neutrophils, T-lymphocytes and eosinophils, and plays a role in the activation of inflammatory effector cells (20).

Calcium (Ca^{2+}) acts as a second messenger during cell activation, and an increase in intracellular Ca^{2+} concentration has been proposed as an essential trigger for mast cell activation (21,22). Moreover, it has been reported that the release of intracellular Ca^{2+} from internal stores is required for mitogen-activated protein kinase (MAPK) activation (23).

The aim of the present study was to investigate the mechanisms by which PIC affects the production of inflammatory cytokines. This study was conducted using the human mast HMC-1 cell line and the rat basophilic RBL-2H3 leukemia cell line. The effects of PIC were evaluated on phorbol 12-myristate 13-acetate (PMA) plus A23187-induced expression of pro-inflammatory mediators, histamine, β-hexosaminidase and cytokines, and the inhibition of mitogen-activated protein kinase (MAPK) signaling pathways was analyzed.

Materials and methods

Animals. The original stock of male ICR mice (20-30 g) was purchased from the Dae-Han Biolink Co. Ltd. (Daejeon, Korea). The animals were maintained in the Laboratory Animal Research Center (LARC) of Gyeongsang National University. The care and treatment of the mice were in accordance with the guidelines established by the Public Health Service Policy on the Human Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Reagents. Piceatannol (*trans*-3,4,3',5'-tetrahydoxystilbene) (PIC) (Fig. 1) was purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO), so that the final PIC concentrations in culture media were 1, 10, 50 and 100 µM. Compound 48/80, anti-dinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA), pyrrolidine dithiocarbamate (PDTC), phorbol 12-myristate 13-acetate (PMA), the calcium ionophore A23187 (calcimycin) and 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The easy-BLUE™ Total RNA Extraction kit was purchased from Intron Biotechnology. Iscove's modified Dulbecco's medium (IMDM) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco BRL (Grand Island, NY, USA). Anti-human TNF-α and IL-8 antibodies, and biotinylated anti-human TNF-α and IL-8 antibodies were obtained from BD Pharmingen (San Diego, CA, USA); antibodies to ERK, p-ERK, JNK, p-JNK, p38 and p-p38 were obtained from Cell Signaling (Beverly, MA, USA).

Compound 48/80-induced systemic anaphylaxis. As an *in vivo* model for the immediate-type allergic reaction, compound 48/80-induced systemic reaction was carried out as previously described (24). Briefly, the mice (n=10/group) received an intraperitoneal injection of 8 mg/kg body weight (BW) of the mast cell degranulator, compound 48/80. PIC was dissolved in saline and administered intraperitoneally at doses of 10-1000 mg/kg BW 1 h before the compound 48/80 injection. In the time-dependent experiment, PIC (1000 mg/kg)
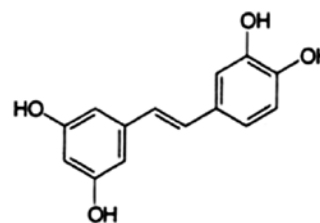

Figure 1. Chemical structure of piceatannol. Molecular weight, 244.2.

was administered 5, 10, 15 and 20 min after compound 48/80 injection (n=10/group). Mortality was monitored for 1 h after induction of anaphylactic shock.

Passive cutaneous anaphylaxis (PCA). PCA reaction was carried out as previously described (25). Briefly, mice were injected intradermally with 0.5 µg of anti-DNP IgE. After 48 h, each mouse (n=10/group) received an injection of 1 mg of DNP-HSA in PBS containing 4% Evans blue (1:1) via the tail vein. PIC (10-1000 mg/kg BW) was intraperitoneally administered 1 h before the challenge. Thirty minutes after the challenge, the mice were sacrificed 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 M KOH and 9 ml of a mixture of acetone and phosphoric acid (5:13). The intensity of the absorbent was measured at 620 nm in a spectrophotometer (Biochrom Libra S22 UV; Biochrom, Holliston, MA, USA).

Cell culture. The human mast cell line HMC-1 (a kind gift from Dr H.M. Kim of Kyeonghee University, Seoul, Korea) was grown in IMDM, supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated fetal bovine serum (FBS), in a humidified incubator at 37˚C in 5% CO_2. The adherent rat basophilic leukemia RBL-2H3 1 cell line (Korean Cell Line Bank no. 22256) was grown as a monolayer culture in complete DMEM (containing 10% heat-inactivated fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin). For experiments, the cells were detached by trypsinization, washed once with complete DMEM, once with serum-free DMEM, and then were resuspended in serum-free DMEM.

Treatment of cells with PIC. HMC-1 and RBL-2H3 cells were treated with PIC (1, 10, 50 and 100 µM) for 30 min. The cells were then stimulated by 50 nM of PMA plus 1 µM of A23187 and incubated at 37˚C for the indicated time periods.

MTT assay. Cell viability was determined by an MTT assay. Briefly, cells were pretreated with various concentrations of PIC (1, 10, 50 and 100 µM) for 30 min and then incubated for 12 h in the absence or presence of PMA plus A23187. After a 12-h incubation, 50 µl of MTT solution was added to the culture (total volume, 500 µl). After an 8-h incubation, the absorbance was measured at 540 nm with a microplate reader (PRS Scientific LLC, Frederick, MD, USA). The absorbance correlates with cell viability, and the number of cells (% of control) was calculated using the following formula: Cell number (% of control) = (absorbance of treated

Table I. Primers and PCR conditions for analysis of gene expression in the HMC-1 cells.

	Primer sequence (5'-3')	Annealing temperature (°C)	Number of cycles	Amplified fragment sizes (bp)
TNF-α	5'-CGGGACGTGGAGCTGGCCGAGGAG-3' 5'-CACCAGCTGGTTATCTCTCAGCTC-3'	57	35	355
IL-8	5'-TGAATTCTCAGCCCTCTTCAAAAA-3' 5'-CGATGTCAGTGCATAAAGACA-3'	59	35	443
GADPH	5'-CCTGCTTCACCACCTTCTTG-3' 5'-CAAAAGGGTCATCATCTCTG-3'	60	30	446

cells - absorbance of blank well)/(absorbance of control cells - absorbance of blank well) x 100. The control was not treated, and the blank control consisted of PMA- plus A23187-stimulated HMC-1 cells without PIC treatment.

Histamine assay. Preparation of serum and determination of histamine contents were performed as previously described (26). Trypsinized RBL-2H3 cells were resupended in serum-free DMEM ($1x10^6$ cells/ml) in a 24-well cell culture plate and stabilized for 1 h in an incubator. The cells were pretreated with various concentrations of PIC (1, 10, 50 and 100 μM) for 30 min and then incubated for 8 h with PMA plus A23187. After treatments, the cell suspensions were centrifuged at 13,000 x g for 5 min and the pellets were discarded. The concentrations of released histamine were determined using a histamine enzyme immunoassay kit (Oxford Biomedical Research, Oxford, MI, USA), and 50-μl samples of supernatants were tested following the manufacturer's instructions. Fifty microliter samples or the standard, plus the enzyme conjugate were added to the 96-well plate of the kit, which was coated with monoclonal antibody, followed by incubation for 45 min at room temperature. The plate was then washed 3 times, and after the addition of 150 μl of tetramethylbenzidine (TMB) substrate, it was incubated for 15-20 min. The reaction was stopped using 50 μl of 1 N HCl, and the absorbance was read at 450 nm. All subsequent steps took place at room temperature, and all standards and samples were assayed in triplicate.

β-hexosaminidase release assay. Mast cell degranulation was determined by measuring the release of a granule marker, β-hexosaminidase. RBL-2H3 cells were seeded into 24-well plates ($1x10^6$ cells/well) and incubated overnight. Cells were washed 3 times with Siraganian buffer (125 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 20 mM HEPES, pH 7.3). The cells were then stimulated with 400 ng/ml dinitrophenol-bovine serum albumin (DNP-BSA) (Sigma-Aldrich) suspended in 500 μl Siraganian buffer containing 0.1% BSA, and incubated at 37°C for 1 h. Following incubation, 50 μl samples of the cell culture supernatant were each incubated with 200 μl of 1 mM p-nitrophenyl N-acetyl-β-D-glucosamine (Sigma) in 0.05 M citrate buffer (pH 4.5) for 3 h at 37°C. The enzyme reaction was terminated by the addition of 500 μl of 0.05 M sodium carbonate buffer (pH 10.0), and the absorbance of each reaction was determined at 405 nm.

The effects of various treatments on β-hexosaminidase release were described as the percentage of the IgE-only control absorbance value: % of inhibition = (Treated - Blank - Spontaneous)/(Control - Blank - Spontaneous); Control, normal allergen-IgE response evoked with test material not added; Treated, normal allergen-IgE response evoked with test material added; Blank, only test material and substrate added to ELISA well; Spontaneous, allergen-IgE response was not evoked with test material not added.

Cytokine assay. The secretion of TNF-α and IL-8 was measured by an enzyme-linked immunosorbent assay (ELISA). HMC-1 cells were stimulated with PMA (50 nM) and A23187 (1 μM) for 8 h with or without PIC. The ELISA was performed by coating 96-well plates with 6.25 ng/well of monoclonal antibodies with specificity for TNF-α or IL-8. Before use and between subsequent steps in the assay, the coated plates were washed twice with washing buffer (PBS containing 0.05% Tween-20). For the standard curve, recombinant TNF-α and IL-8 were added to serum which was previously determined to be negative for endogenous TNF-α and IL-8. After exposure to the cell supernatant from the experiment, the assay plates were exposed sequentially to biotinylated 2,2' azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) substrate. The absorbances at 405 nm were determined within 10 min after addition of the substrate. All subsequent steps took place at room temperature, and all standards and samples were assayed in triplicate.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. HMC-1 cells ($1x10^6$/ml) were pretreated with PIC for 30 min before PMA (50 nM) plus A23187 (1 μM) stimulation for 2 h. Total RNA was isolated according to the manufacturer's specifications using an easy-BLUE RNA extraction kit. The concentration of total RNA was determined by spectrophotometry. Each sample was reverse transcribed to cDNA for 60 min at 45°C using a cDNA synthesis kit (Amersham Biosciences, Piscataway, NJ, USA). The primers and PCR conditions are shown in Table I. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide. The gels were photographed using a Kodak DC 290 digital camera (Eastman Kodak, Rochester, NY, USA) and digitized using UN-SCAN-IT software (Silk Scientific, Orem, UT, USA). Relative band intensity was analyzed by using ImageJ image analyzing software.

Table II. Effect of PIC on compound 48/80-induced systemic anaphylaxis.

PIC treatment (mg/kg BW)	Compound 48/80 (8 mg/kg BW)	Mortality (%)
None (saline)	+	100
10	+	100
50	+	100
100	+	70
500	+	40
1000	+	0
1000	-	0

Groups of mice (n=10/group) were intraperitoneally pretreated with 200 μl of saline or PIC at various doses 1 h before the intraperitoneal injection of compound 48/80. Mortality (%) within 1 h following compound 48/80 injection is represented as the number of dead mice x100/total number of experimental mice.

Table III. Time-dependent effect of PIC on compound 48/80-induced systemic anaphylaxis.

PIC treatment (mg/kg BW)	Time (min)	Compound 48/80 (8 mg/kg BW)	Mortality (%)
None (saline)	-	+	100
1000	5	+	30
	10	+	70
	15	+	100

Mice (n=10/group) were intraperitoneally pretreated with 200 μl of saline or PIC (1000 mg/kg) 0, 5, 10 and 15 min after the intraperitoneal injection of compound 48/80. Mortality (%) within 1 h following compound 48/80 injection is represented as the number of dead mice x100/total number of experimental mice.

Protein extraction and western blot analysis. HMC-1 cells were washed twice with PBS and resuspended for protein extraction with reagents from an Intron Pro-Prep Protein Extraction kit. Processed samples were subjected to 8% SDS-PAGE and transferred to a PVDF membrane. The membrane was then washed with 5% skim milk in PBST (0.05% Tween-20 in PBS) for 1 h at room temperature and then incubated overnight with anti-MAPK antibodies. After the blot was washed 3 times with PBST, it was incubated with the secondary antibody for 1 h. The antibody-specific proteins were then visualized using an enhanced chemiluminescence detection kit (Amersham Biosciences), and the blot was exposed to X-ray film.

Statistical analysis. Statistical significances were compared between each treated group and analyzed by the Student's t-test. Data from the experiments are presented as means ± SEM. The numbers of independent experiments assessed are provided in the figure legends.

Results

Effect of PIC on systemic and local anaphylaxis. To determine the effect of PIC on allergic reaction, an *in vivo* model of a systemic reaction 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 1 h, after which the mortality rate was determined. As shown in Table II, injection of compound 48/80 into mice induced fatal shock in 100% of the animals. When PIC was intraperitoneally administered at concentrations ranging from 10 to 1000 mg/kg BW 1 h prior to exposure to compound 48/80, the mortality rate was dose-dependently reduced. In addition, the mortality of mice administered PIC (1000 mg/kg) following compound 48/80 injection increased in a time-dependent manner (Table III). The effect of PIC on the compound 48/80-induced serum histamine release was investigated. PIC was administered at concentrations ranging from 10 to 1000 mg/kg 1 h prior to the compound
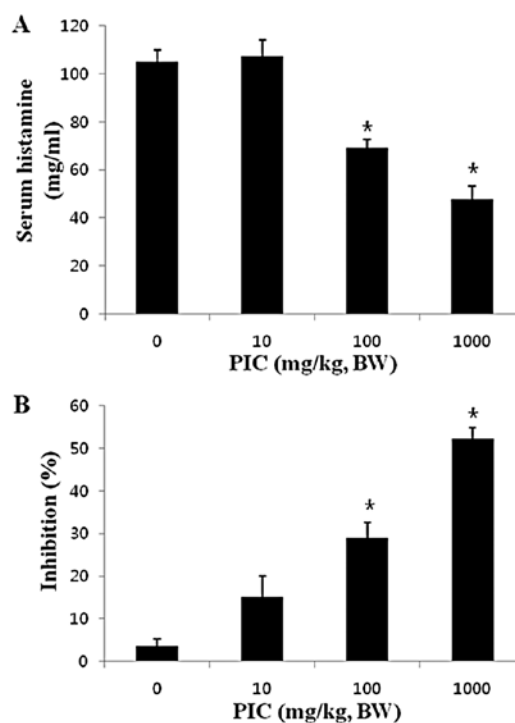
Figure 2. Effect of piceatannol on serum histamine release and PCA reactions. (A) Groups of mice (n=10/group) were intraperitoneally pretreated with 200 μl of saline or PIC. PIC was administered at various doses 1 h prior to the injection of compound 48/80. The compound 48/80 solution was administered intraperitoneally to the group of mice. The blood was obtained from the heart of each mouse, and the histamine content was measured by histamine assay kit. (B) PIC 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 by spectrophotometry. Each bar represents the mean ± SEM of three independent experiments. Significant difference at *p<0.05.

48/80 injection. Injection of compound 48/80 caused a marked increase in serum histamine release, and this induction was inhibited by PIC treatment in a dose-dependent manner (Fig. 2A). To confirm the anti-allergic effects of PIC, we used an IgE-mediated passive cutaneous anaphylaxis (PCA) model. PCA is one of the most important *in vivo* models of
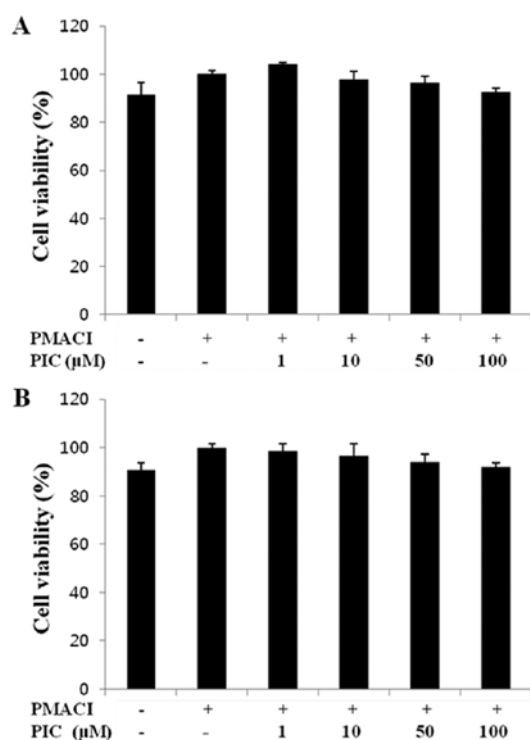
Figure 3. Effect of piceatannol on cell viability in PMA- plus A23187-induced HMC-1 (A) and RBL-2H3 (B) cells. Cells were pretreated with PIC (1, 10, 50, and 100 μM) for 30 min prior to PMA (50 nM) + A23187 (1 μM) (PMACI) stimulation. Each bar represents the mean ± SEM of three independent experiments. *Significant difference at p<0.05.
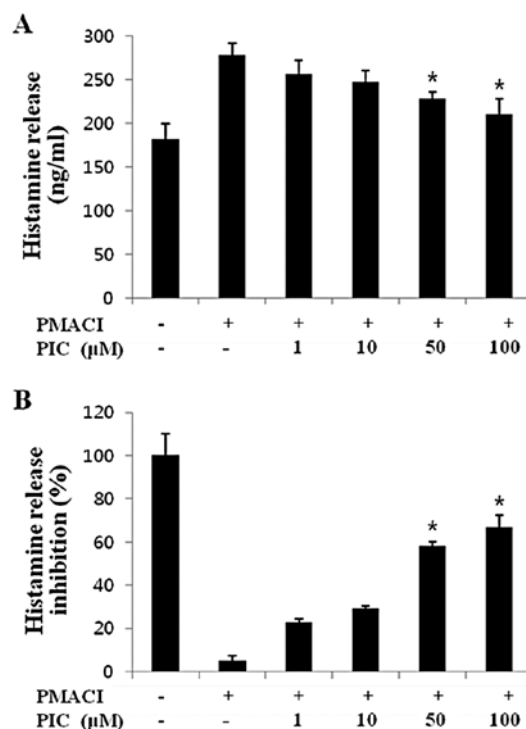

Figure 4. (A) Effect of piceatannol on histamine release in stimulated RBL-2H3 cells. (B) Effect of piceatannol on histamine release inhibition in stimulated RBL-2H3 cells. Cells were pretreated with PIC (1, 10, 50, and 100 μM) for 30 min prior to PMA (50 nM) + A23187 (1 μM) (PMACI) stimulation. Each bar represents the mean ± SEM of three independent experiments. *Significant difference at p<0.05.

local allergic reaction. A local extravasation was induced by a local injection of IgE followed by an antigenic challenge. Intraperitoneal injection of PIC dose-dependently inhibited the PCA reaction (Fig. 2B).

Effect of PIC on cell viability. To evaluate the cytotoxic effects of PIC, an MTT viability assay in HMC-1 and RBL-2H3 cells was performed. Fig. 3 shows the viability of cells incubated 24 h after stimulation in the absence or presence of PIC (1, 10, 50 and 100 μM). Stimulation and treatment with PIC did not appreciably affect cell viability. Kang *et al* (27). reported on the cytotoxic effect of resveratrol, the precursor of PIC, on HMC-1 cells using the MTT assay. Resveratrol was not cytotoxic against HMC-1 and RBL-2H3 cells up to 100 μM.

Effect of PIC on histamine release. The release of chemical mediators such as histamine plays an important role in allergic inflammation (27). Therefore, we examined the effects of PIC on histamine release. We evaluated the ability of PIC to inhibit PMA and A23187-induced histamine release from RBL-2H3 cells (Fig. 4A). Histamine (~188 ng/ml) was detectable in unstimulated cells; however, RBL-2H3 cells released ~275 ng/ml of histamine when stimulated with PMA plus A23187. PIC inhibited PMA and A23187-induced histamine release in a dose-dependent manner as follows: 22.14% inhibition at 1 μM, 30.18% inhibition at 10 μM, 51.92% inhibition at 50 μM and 61.61% inhibition at 100 μM (Fig. 4B).
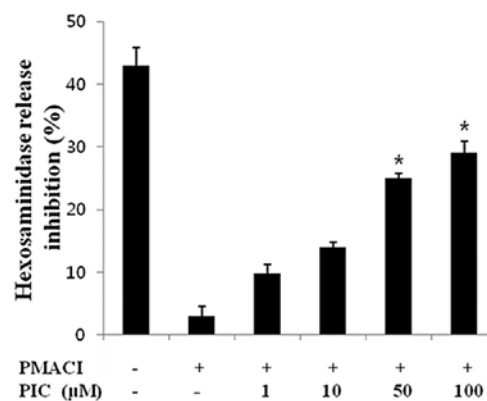

Figure 5. Effect of piceatannol on β-hexosaminidase release inhibition in stimulated RBL-2H3 cells. Cells were pretreated with PIC (1, 10, 50, and 100 μM) for 30 min prior to PMA (50 nM) + A23187 (1 μM) (PMACI) stimulation. Each bar represents the mean ± SEM of three independent experiments. *Significant difference at p<0.05.

Effect of PIC on β-hexosaminidase release. The rat basophilic leukemia RBL-2H3 cell line, a tumor analog of mast cells, exhibits phenotypic characteristics of mucosal mast cells. After stimulation with antigens, cells release β-hexosaminidase, which is a marker of mast cell degranulation. Thus, RBL-2H3 cells are considered a good model for the comprehensive study of events induced by multivalent allergens in mast cells (27-29). In our study, PIC strongly suppressed PMA plus A23187-induced degranulation in a dose-dependent manner in the RBL-2H3 cells (Fig. 5).
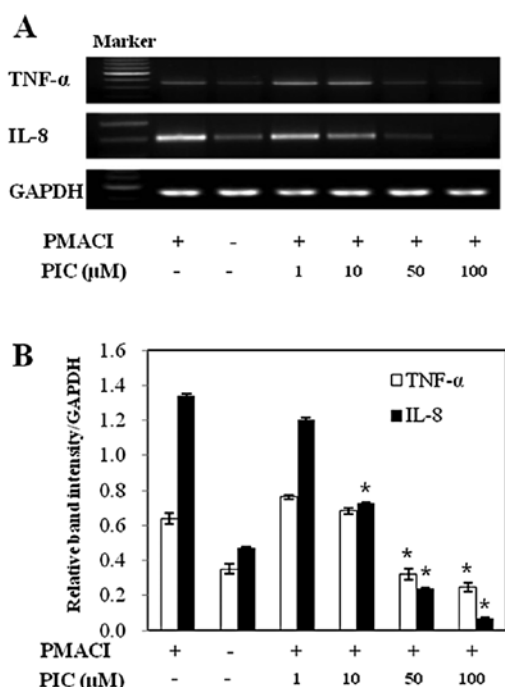
Figure 6. Effect of piceatannol on pro-inflammatory cytokine (TNF-α and IL-8) mRNA expression in PMA- plus A23187-induced HMC-1 cells (A) and its relative intensity (B). Cells were pretreated with PIC (1, 10, 50 and 100 μM) for 30 min prior to PMA (50 nM) + A23187 (1 μM) (PMACI) stimulation. Expression of cytokine mRNAs was analyzed by RT-PCR. Band intensity for each gene expressions was normalized against GAPDH.
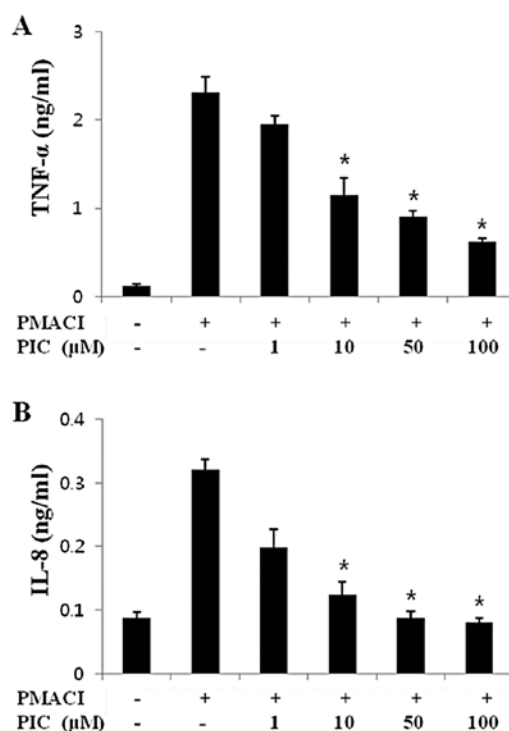

Figure 7. Effect of piceatannol on pro-inflammatory cytokine secretion (TNF-α and IL-8) by PMA- plus A23187-induced HMC-1 cells. Cells were pretreated with PIC (1, 10, 50 and 100 μM) for 30 min prior to PMA (50 nM) + A23187 (1 μM) (PMACI) stimulation. Each bar represents the mean ± SEM of three independent experiments. *Significant difference at $p < 0.05$.

Effect of PIC on pro-inflammatory cytokine expression. TNF-α and IL-8 are the most important pro-inflammatory cytokines. Therefore, we tested the effect of PIC on the gene expression of TNF-α and IL-8 induced by PMA plus A23187 in HMC-1 cells. Gene expression of pro-inflammatory cytokines was then analyzed using RT-PCR. HMC-1 cells were treated with PMA plus A23187 in the presence or absence of PIC (1, 10, 50 and 100 μM) for 2 h and then harvested for transcriptional analysis via RT-PCR. Enhanced TNF-α and IL-8 mRNA expression induced by PMA plus A23187 was inhibited by pretreatment of HMC-1 cells with PIC. Pretreatment with PIC at concentrations of 1, 10, 50 and 100 μM inhibited PMA and A23187-induced gene expression of TNF-α and IL-8. Gene expression of TNF-α and IL-8 was decreased in a dose-dependent manner by pretreatment with PIC (Fig. 6).

Effect of PIC on pro-inflammatory cytokine production. To confirm the effect of PIC on the gene expression of pro-inflammatory cytokines, culture supernatants were assayed for TNF-α and IL-8 levels by ELISA methods. HMC-1 cells were pretreated with PIC (1, 10, 50 and 100 μM) before stimulation with PMA (50 nM) and A23187 (1 μM) for 8 h, and culture supernatants were analyzed using ELISA. As shown in Fig. 7A and B, the concentrations of TNF-α and IL-8 were considerably increased after HMC-1 cells were stimulated with PMA plus A23187. PIC pretreatment strongly inhibited these increases in a dose-dependent manner.

Effect of PIC on activation of mitogen-activated protein kinases (MAPKs). MAPK pathways play a crucial role in the regulation
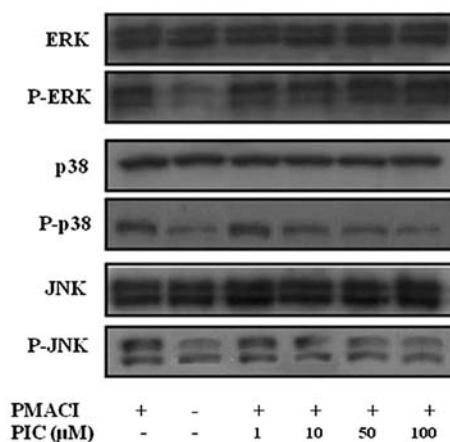

Figure 8. Effect of piceatannol on activation of MAPKs. Cells were pretreated with PIC (1, 10, 50 and 100 μM) for 30 min prior to PMA (50 nM) + A23187 (1 μM) (PMACI) stimulation. Phosphorylation of MAPKs was analyzed by western blotting.

of pro-inflammatory molecules affecting cellular responses. In order to elucidate the mechanisms underlying the effects of PIC, we examined the possible effects of PIC on the activation of MAPKs (Fig. 8). The stimulation of HMC-1 cells with PMA plus A23187 resulted in increased phosphorylation of all 3 types of MAPKs (p38, JNK, and ERK) 30 min post-treatment. Pretreatment with PIC (1, 10, 50 and 100 μM) attenuated PMA and A23187-induced p38 and JNK MAPK activation but did not significantly affect the phosphorylation of ERK.

Discussion

The present study revealed that piceatannol (PIC) demonstrates anti-allergic inflammatory properties via regulation of MAPK signaling. Anaphylaxis is a life-threatening syndrome induced by the sudden systemic release of inflammatory mediators, such as histamine, heparin and various cytokines from mast cells (30). PIC inhibited compound 48/80-induced systemic anaphylaxis and histamine release from mast cells. Mice administered PIC were protected from IgE-mediated local allergic reaction. PCA is one of the most important *in vivo* models of anaphylaxis. These results indicate that PIC inhibited mast cell-mediated allergic reactions. In addition, PIC decreased phorbol-12-myristate 13-acetate and calcium ionophore A23187 (PMACI)-stimulated expression of pro-inflammatory cytokines. These results suggest that PIC may be useful in the treatment of allergic inflammatory diseases.

Histamine was originally considered as a mediator of acute inflammatory and immediate hypersensitivity responses. It has been reported that histamine affects chronic inflammation and regulates several essential events of the immune response such as immune cell maturation, polarization and lymphocyte responsiveness (31). Histamine also induces actin polymerization and chemotaxis (32). Compound 48/80, the synthetic mast cell stimulator, increases the permeability of the lipid bilayer membrane by perturbation in the membrane. These studies indicate that an increase in membrane permeability may be essential for the release of the mediator from mast cells. In our study, PIC dose-dependently reduced compound 48/80-induced histamine release from mast cells. Taken together, we speculate that PIC stabilizes the actin polymerization of the membrane, thus preventing the compound 48/80-induced membrane perturbation. Seow *et al* (28) reported that, at 30 and 100 μM, PIC significantly suppressed ovalbumin-induced histamine release from lung fragments by 30 and 74%, respectively.

Another study on flavonoids reported that at 30 μM, fisetin, kaempferol, myricetin, quercetin and rutin inhibited the release of histamine from antigen-stimulated RBL-2H3 cells (29). Those results were similar to an investigation using PMACI-stimulated RBL-2H3 cells, in which fisetin, kaempferol, myricetin, quercetin and rutin efficiently inhibited histamine release. While fisetin, kaempferol, quercetin and rutin significantly reduced antigen- and PMACI-induced histamine release from mast cells, astragalin was not effective (29). Matsuda *et al* (33) studied the inhibitory effects of 15 stilbenes and 4 dihydrostilbenes on the release of β-hexosaminidase in RBL-2H3 cells. 3,5,40-Trimethylpiceatannol (IC$_{50}$, 2.1 μM) exhibited the most potent activity, followed by trimethylresveratrol (5.1 μM), rhapontigenin (11 μM), isorhapontigen (12 μM), tetramethylpiceatannol (13 μM), 3,30,40-trimethylpiceatannol (16 μM), resveratrol (17 μM), desoxyrhapontigenin (18 μM) and PIC (24 μM). Their activities were stronger than those of 2 anti-allergic compounds, tranilast (0.28 mM) and ketotifen fumarate (0.22 mM). 12-*trans*-stilbene (>100 μM), which lacks oxygen functions on the ring, was inactive. Thus, the effects of substitution on aromatic rings of stilbenes indicated that stilbenes substituted with methoxyl groups at the 3-, 5- and 40-positions exhibited higher activity than those with hydroxyl groups.

Mast cells play a critical role in inflammation and protective immune responses by releasing preformed vasoactive amines, prostaglandins, cytokines and chemokines (34). Various inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-8, which act as triggers or regulators in allergic inflammatory response, are crucial for mast cell development (35). TNF-α is a well-known inflammatory factor produced mainly by macrophages, and plays a critical role in systemic inflammation (36). It is also a potent inducer of other inflammatory cytokines, including IL-1β, IL-6, IL-8 and GM-CSF. IL-6 is one of the most important mediators of a multifunctional pro-inflammatory cytokines, and the major cytokine produced by activated mast cells (37). These studies indicate that the inhibition of pro-inflammatory cytokines is one of the critical points of reduced allergic inflammatory symptoms. In our study, PIC inhibited gene expression and secretion of cytokines. These results imply that PIC has anti-allergic inflammatory effects by controlling pro-inflammatory cytokines in mast cells.

The MAPK cascade is an important signaling pathway in immune responses. The MAPK signaling cascade regulates important cellular processes including gene expression, cell proliferation, cell survival and death and cell mobility (38). The precise signaling pathways among the three types of MAPKs, i.e., ERK, JNK and p38 are still unclear. However, the induction of inflammatory cytokine genes requires activation of all three types of MAPKs. In this study, PMACI-induced activation of p38 and JNK MAPKs was reduced by PIC. The data suggest that PIC has an inhibitory activity on all three MAPKs and downstream cytokine production. Kang *et al* (27) examined the possible effects of resveratrol on activation of MAPKs. Stimulation of HMC-1 cells with PMA plus A23187 resulted in increased phosphorylation of all 3 types of MAPKs, p38, JNK, ERK, and activation of protein kinase C (PKC) 15-30 min post-treatment. Resveratrol attenuated PMA plus A23187-induced ERK 1/2, but did not affect the phosphorylation of JNK 1/2 and p38 MAPK. In addition, resveratrol had no effect on PMA plus A23187-induced PKC activation.

PIC is an anti-inflammatory, immunomodulatory and antiproliferative stilbene that has been shown to interfere with the cytokine signaling pathway (39). In the present study, we provided evidence that PIC inhibits mast cell-mediated allergic inflammatory reactions and their possible mechanisms such as histamine release and MAPK for pro-inflammatory cytokines. The results obtained in the present study reveals that PIC contributes to the prevention or treatment of mast cell-mediated allergic inflammatory diseases.

Acknowledgements

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