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ORIGINAL ARTICLE

Peimine Inhibits the Production of Proinflammatory Cytokines Through Regulation of the Phosphorylation of NF-κB and MAPKs in HMC-1 Cells

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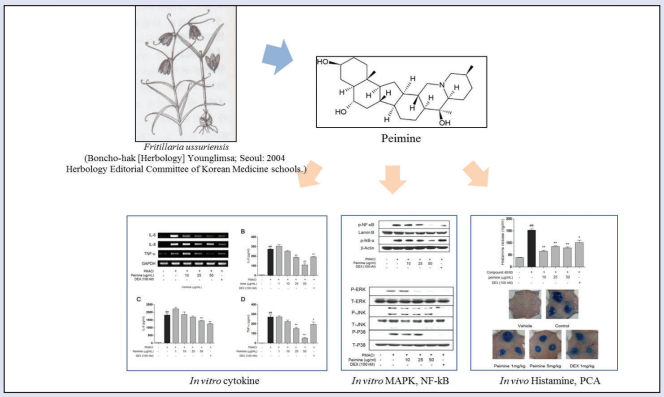
ABSTRACT

Background: Peimine is a major biologically active component of *Fritillaria ussuriensis*. Peimine was investigated in chronic inflammation response, but it has not been studied in mast cell-related immediate allergic reaction. The present study aimed to evaluate anti-allergic effect of peimine in human mast cell (HMC-1). **Materials and Methods:** The effect of peimine on cell viability was measured by MTS assay in HMC-1. Histamine release was investigated in rat peritoneal mast cells (RPMCs). Interleukin (IL)-6, IL-8, and tumor necrosis factor-α (TNF-α) expressions were measured by ELISA assay and reverse transcription-polymerase chain reaction. Mitogen-activated protein kinases (MAPKs) and nuclear factor-kappaB (NF-κB) were examined by Western blot. Passive cutaneous anaphylaxis (PCA) reactions were evaluated using Sprague-Dawley (SD) rats. **Results:** Peimine inhibited the production of pro-inflammatory cytokines, such as IL-6, IL-8, and TNF-α. Moreover, peimine reduced MAPKs phosphorylation and the nuclear NF-κB expression in PMACI-induced HMC-1. Peimine decreased PCA reactions in rats as well. **Conclusion:** Our study proved that peimine might be suitable for the treatment of mast cell-derived allergic inflammatory reactions.

Keywords: cytokine, human mast cell (HMC-1), inflammation, MAP kinases, nuclear factor-kappaB (NF-κB), peimine

SUMMARY

- Peimine inhibited the production of pro-inflammatory cytokines, such as IL-6, IL-8, and TNF-α
- Peimine reduced MAPKs phosphorylation and the nuclear NF-κB expression in PMACI-induced HMC-1
- Peimine decreased PCA reactions in rats
- Peimine has anti-allergic effect through regulation of pro-inflammatory mechanism on mast cell.



Abbreviations used: HMC-1: Human mast cell, MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, RPMCs: Rat peritoneal mast cells. IL-6: Interleukin 6, IL-8: Interleukin 8, TNF-α: Tumor necrosis factor-α, MAPKs: Mitogen-activated protein kinases; NF-κB: Nuclear factor-kappaB, PCA: Passive cutaneous anaphylaxis reactions, SD: Sprague-Dawley.

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INTRODUCTION

Peimine is the main compound of *Fritillaria ussuriensis* (FU, derived from the bulbs of various species of the genus *Fritillaria*, including *Fritillaria thunbergii* Miq.). FU is used as a reliving coughs, reducing heart rates, and lowering blood pressure.[1] In our previous study, we showed that FU had an affect on anti-allergy in human mast cell (HMC-1) that linked with allergy responses.[1] A recent study has found that peimine has potentials as pain reliever[2] and agent of inflammatory pulmonary diseases.[3] However, the anti-inflammatory reactions of peimine are not yet studied. In this study, we aimed to evaluate the mechanism of anti-allergic effects of peimine as effective compound of FU in HMC-1.

Allergic diseases are the most common illnesses in developed countries.[4] Allergic diseases have increased in occurrence worldwide due to the consequence of the changing environment from urbanization.[5] It includes anaphylaxis, drug and food allergies, and certain forms of asthma, rhinitis, and eosinophilic disorders.[4] The occurrence of allergic diseases in children of developing countries is regarded as a serious concern.[6] In particular, anaphylaxis or immediate hypersensitivity is one allergy reaction caused by immunoglobulin E (IgE) antibodies against antigens that have the potential to develop into a fatal reaction.[5] Thus, the continuous study of the allergy mechanism occurrence is important.

Immediate allergy responses are initiated when mast cell activation results in antigen exposure and the activated mast cell release of

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histamines and other cytokines.[7,8] Cytokines, such as interleukin (IL)-6, IL-8, and tumor necrosis factor-α (TNF-α), are major cytokines in the inflammatory response and they play important roles in allergic diseases. These cytokines are released by mast cells and regulated by the activation of transcription factors, such as nuclear factor-kappaB (NF-κB).[9,10]

The most common treatment of allergic diseases is a corticosteroid widely called a "steroid," but long-term use may lead to severe or partially nonreversible side effects, such as deterioration of the immune system, bone loss, or growth suppression and Cushing's syndrome.[11] Recently, interest in alternative drugs or compounds has been increasing to find a better treatment that can reduce side effect.[12,13]

The HMC-1 model is useful for an immediate-allergy experiment because it secretes histamine and inflammatory cytokines only when it is stimulated by phorbol esters and calcium ionophore A23187.[14] The phosphorylation of tyrosine kinase and the mobilization of internal Ca^{2+} are led by the activation of mast cells, followed by the activation of protein kinase C, mitogen-activated protein kinase (MAPKs), and NF-κB, and the release of inflammatory cytokines follows.[15] In this study, we evaluated the effect of peimine on allergic responses in HMC-1, including the identification of the mechanism of action of peimine in cytokines, MAPKs, and NF-κB signaling pathways. Moreover, rat passive cutaneous anaphylaxis (PCA) is a useful experimental model for acute hypersensitivity.[14,15] It is an animal model related to the IgE-mediated immediate allergic reaction.[14,15] Therefore, we investigated the inhibitory effect of peimine in the rat PCA reaction.

MATERIALS AND METHODS

Preparation of drugs

Peimine was dissolved in dimethyl sulfoxide (DMSO). Peimine [Figure 1] was obtained from Ab cam, with a molecular weight of 431.65 g/mol (Cambridge, UK). Dexamethasone (DEX), general steroid, was dissolved in water and used as positive control.

Reagents

HMC-1 cell was provided from Prof. H.M. Kim (Department of Pharmacology, Kyung Hee University, Seoul, Korea). Iscove's modified Dulbecco's medium (IMDM) and fetal bovine serum (FBS) were gained from Gibco BRL (Grand Island, NY, USA) and streptomycin was purchased from Invitrogen (Carlsbad, CA, USA). Aqueous nonradioactive cell proliferation assay (MTS) was purchased from Promega (Madison, WI, USA). Compound 48/80, PMA and calcium ionophore A23187 were purchased from Sigma (St. Louis, MO, USA). Recombinant IL-6, IL-8, and TNF-α, biotinylated IL-6, IL-8, and TNF-α, and antihuman IL-6, IL-8, and TNF-α were obtained from BD Biosciences (San Jose, CA, USA). PCR primers were from Genotech (Daejeon, Korea). DEX-water soluble, anti-dinitrophenol (DNP) immunoglobulin (Ig)E, DNP-human serum albumin (HSA), and Evans blue were purchased from Sigma (St. Louis, MO, USA).

Antibodies

Antibodies (Abs) to phosphorylation-NF-κB (p-NF-κB) and phosphorylation-IκB-α (p-IκB-α) were purchased from Cell Signaling (Cell Signaling Technology, Danvers, MA, USA), phosphorylation-ERK (P-ERK), ERK, P-P38, P38, P-c-Jun-NH2-terminal kinase (JNK), JNK, Lamin B, and β-actin were obtained from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Peroxidase IgG was from purchased Jackson ImmunoResearch (Jackson ImmunoResearch, West Grove, PA, USA).

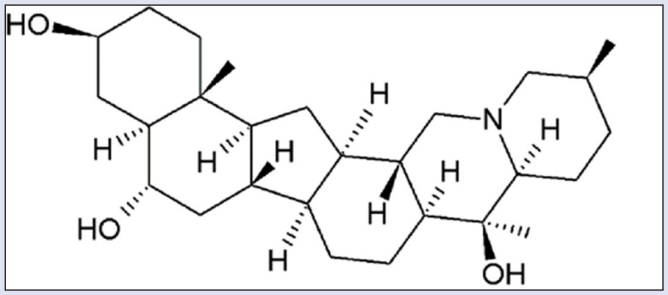

Figure 1: Structure of peimine

Culture of HMC-1 cells

HMC-1 cells were maintained at 37°C, 5% CO_2, and 95% humidity in IMDM that supplemented with 100 Units/mL penicillin, 100 mg/mL streptomycin, and 10% heat-inactivated FBS.

MTS assay for cell viability

HMC-1 cells were seeded density of 1×10^5 cells/well, pretreated with 1, 10, 25, 50, and 100 μg/mL concentrations of peimine. Cells were also treated with 1, 10, and 100 μg/mL concentrations of DEX as a positive control. The cells were incubated in a 37°C incubator for 24 h. Cell viability was measured using the MTS colorimetric assay. MTS solution was treated to each well. After 2 h, the optical density (OD) value of the 96-well culture plates was read at 490 nm with a spectrophotometer (Versa Max ELISA Microplate Reader; Molecular Devices, CA, USA). The formazan OD value of the untreated control cells was taken to represent 100% viability.

Measurement of cytokines

HMC-1 cells were pretreated with 10, 25, and 50 μg/mL concentrations of peimine and stimulated with PMACI (PMA 25 nM plus A23187 1 μM) for 8 h. The levels of IL-6, IL-8, and TNF-α were measured by enzyme-linked immunosorbent assay (ELISA) using a commercial cytokine/chemokine ELISA kit following the manufacturer's recommendations (BD Biosciences, San Jose, CA, USA). The cells were measured with a Spectrophotometer (Molecular Devices, CA, USA). The effect of peimine on allergic responses was calculated from the equation obtained with a standard curve plot for each cytokine standard solution in the kit.

Reverse transcriptase-polymerase chain reaction analysis

HMC-1 cells were pretreated with various peimine concentrations (10, 25, and 50 μg/mL) for 1 h and stimulated with PMACI for 6 h. Total RNA was prepared using Trizol (TAKARA BIO, Otsu, Japan) and cDNAs were synthesized from 2 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). RT-generated cDNAs encoding IL-6, IL-8, and TNF-α were produced by PCR using selective primers as shown in Table 1. The cDNA samples were separated on a 1-1.2% agarose gel after the reaction, stained with SYBR staining solution (Invitrogen, Carlsbad, CA, USA) and identified using a NαBI (Neo science, Suwon, Korea).

Preparation of Western proteins

HMC-1 cells (5×10^6 cells/well) were pretreated with various peimine concentrations (10, 25, and 50 μg/mL) for 1 h and stimulated with PMACI. The cells were incubated 2 h for nuclear protein and 30 min for total protein. To obtain the cell extract, harvested cells were lysed using lysis buffer (HEPES, KCl, $MgCl_2$, DTT, PMSF, NaCl glycerol, EDTA) to

Table 1: Primer sets for RT-PCR

Name	Forward primer	Reverse primer
IL-6	5' GAT GGC TGA AAA AGA TGG ATG C 3'	5' GTT TTG GGT CAG GGG TGG TT 3'
IL-8	5' CGA TGT CAG TGC ATA AAG ACA 3'	5' TGA ATT CTC AGC CCT CTT CAA AAA 3'
TNF-α	5' CCT ACC AGA CCA AGG TCA AC 3'	5' AGG GGG TAA TAA AGG GAT TG 3'
GAPDH	5' CGT CTA GAA AAA CCT GCC AA 3'	5' TGA AGT CAA AGG AGA CCA CC 3'

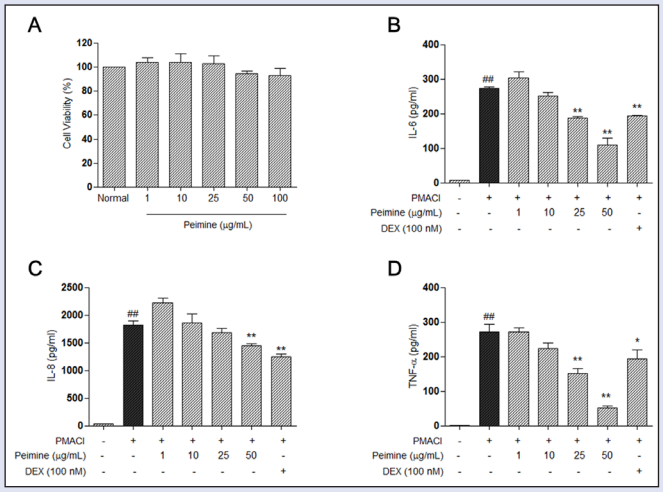

Figure 2: Cell viability of peimine in HMC-1 cells (A). The optical density of the culture plates at 490 nm was measured with a spectrophotometer. The value represents the mean ± SEM from three independent experiments. The effect of peimine on the production of (B) IL-6, (C) IL-8, and (D) TNF-α cytokine levels in PMACI-stimulated HMC-1 cells. The value represents the mean ± SEM of three independent experiments. $^{\#\#}P < 0.01$, significantly different from the normal group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the PMACI only group

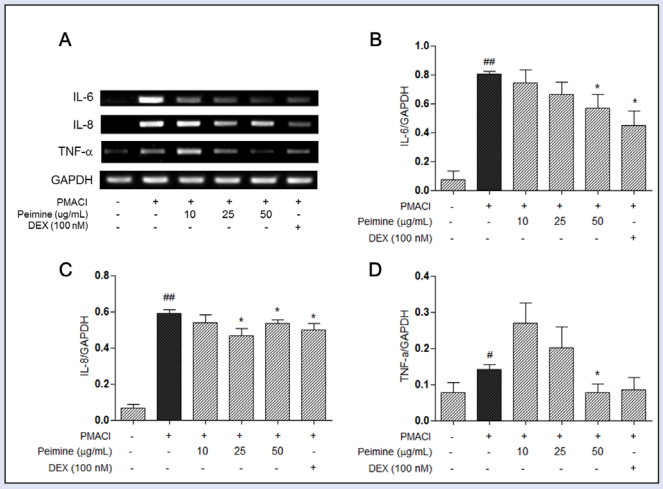

Figure 3: Effects of peimine on PMACI-induced expression of inflammatory cytokines mRNA in HMC-1 cells (A). Cells were pretreated with peimine (10, 25, and 50 μg/mL) and DEX at 100 nM for 1 h prior to PMACI stimulation for 6 h. The levels of (B) IL-6, (C) IL-8, (D) TNF-α mRNA expression were measured using Image J. Columns and error bars represent the mean ± SEM for three independent experiments. $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ significantly different from the control group. $^{*}P < 0.05$ significantly different from PMACI alone

obtain total protein lysis. The cells were lysed with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA) for nuclear and cytosolic fractions. The proteins were centrifuged and heated at 95°C for 5 min.

Western blot analysis

Proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis. Proteins were electrotransferred to nitrocellulose membranes (Whatman, Maidstone, Kent, UK). The membranes were blocked for 1 h with 5% skim milk and incubated with primary antibodies (1:1000). The membranes were reacted with peroxidase-conjugated secondary antibodies at room temperature and the antibody-specific proteins were visualized using a chemiluminescence (ECL) detection system according to the manufacturer's protocol (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Animals

Sprague-Dawley (SD) rats were purchased from Nara biotech (Gangnam-gu, Seoul, Korea). All animal experiments were in accordance with the guidelines of the Kyung Hee University Institutional Animal Care and Use Committee (KHUASP(SE)-14-032). The rats were housed under controlled temperature (23 ± 3°C) with a relative humidity of 40-60% and 12-h light/dark cycles. Food and water were provided *ad libitum*.

Histamine assay

The SD rat (350 g) peritoneal cavity was washed with 20 mL HEPES Tyrode's buffer containing 10 mM HEPES, 136 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2.75 mM $MgCl_2$, 5.6 mM glucose, 11 mM $NaHCO_3$, 0.6 mM NaH_2PO_4, and 1% bovine serum albumin (BSA), pH 7.4. After the rats' abdominal area was massaged for 2 min, peritoneal exudate cells (PECs) containing mast cells were obtained. The collected PECs were washed with HEPES Tyrode's solution and mast cells were purified to more than 95% with Percoll (Sigma-Aldrich). The collected mast cells were pretreated with peimine (final concentrations: 10, 25, and 50 μg/mL) for 10 min before incubation with compound 48/80 (5 μg/mL) for 20 min. The cells were centrifuged at 3000 rpm for 15 min at 4°C; then histamine was measured using Histamine EIA Kit (Oxford, MI, USA).

PCA reaction

SD rats of 6 weeks were injected anti-DNP IgE into three outlined dorsal skin sites, followed 48 h later by an injection of DNP-HAS, diluted in phosphate-buffered saline (PBS), containing 4% Evans blue into the dorsal vein of the rat's penis. One hour before the injection, peimine was administered by peroral administration (p.o.) at two doses of 1 and 5 mg/kg. DEX was administered by p.o. at a dose of 1 mg/kg also. Rats were sacrificed 30 min after the intravenous challenge. The dorsal skin of the rats was removed and dissolved with formamide in an incubator for 72 h. The amount of dye was then measured at 620 nm using a spectrophotometer (Molecular Devices, USA).

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using the Graph Pad PRISM software (Graph Pad Software Inc., CA, USA). One-way ANOVA was used to

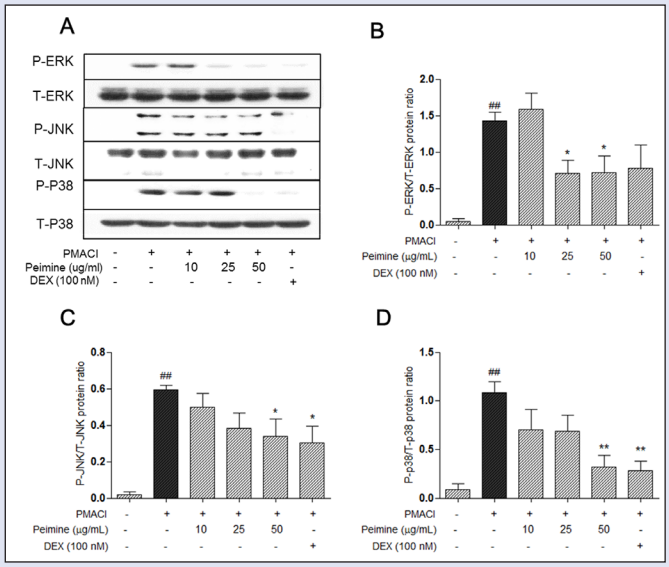

Figure 4: The effects of peimine on PMACI induced the MAPKs expressions in HMC-1 cells. (A) A typical band image. (B-D) A relative level of MAPK expression band by Image J. The value represents the mean ± SEM of three independent experiments. $^{\#\#}P < 0.01$, significantly different from the normal group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the PMACI-only group

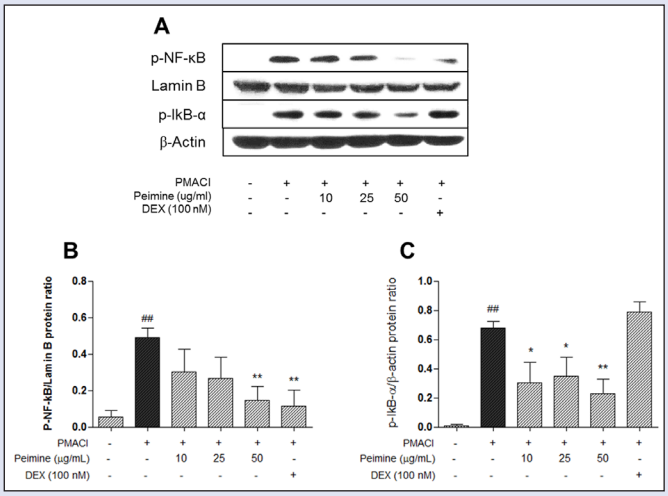

Figure 5: The effect of peimine on the degradation of p-IκB-α in the cytosol and p-NF-κB activation in the nuclei of PMACI-induced HMC-1 cells. The relative expression levels of p-IκB-α and p-NF-κB were measured using an Image J (B and C). The value represents the mean ± SEM of three independent experiments. $^{\#\#}P < 0.01$, significantly different from the normal group; $^{*}P < 0.05$ and $^{**}P < 0.01$ significantly different from the PMACI-only group

evaluate the treatment effect, followed by Dunnett's multiple comparison test. Values of *P* less than 0.05 were considered to be significant.

RESULTS

Effect of peimine on HMC-1 cell viability and PMACI-induced cytokine expressions

We examined the viability of HMC-1 cells on peimine treatment using the MTS assay [Figure 2]. There was no significant cytotoxicity in HMC-1 cells with peimine at concentrations of 1, 10, 25, 50, and 100 μg/mL. Based on these results, 50 μg/mL of peimine was used as the optimal concentration based on viability results.

The levels of IL-6, IL-8, and TNF-α were increased after stimulation with PMACI in HMC-1 cells. To evaluate the effect of peimine pro-inflammatory cytokines, we pretreated cells with peimine (1, 10, 25, and 50 μg/mL) before stimulation with PMACI for 8 h. The inhibition of the PMACI-induced expressions of IL-6, IL-8, and TNF-α in mast cells was reduced following dose-dependent peimine treatment [Figure 2]. DEX used a positive control, as well as significantly decreased those cytokines.

Effects of peimine on cytokine mRNA expression in HMC-1 cells

To measure the effect of peimine on the production of pro-inflammatory cytokines mRNA, we pretreated the cells with peimine before stimulation with PMACI for 6 h and performed RT-PCR. The enhanced expressions of IL-6, IL-8, and TNF-α mRNAs induced by PMACI were inhibited by pretreatment of the cells with peimine [Figure 3]. Peimine at 50 μg/mL ($P < 0.05$) and DEX ($P < 0.05$) significantly decreased the expressions of IL-6. Pretreatment with peimine at concentrations of 25, 50 μg/mL ($P < 0.05$), and DEX ($P < 0.05$) significantly inhibited the PMACI-induced gene expressions of IL-8. Peimine at 50 μg/mL significantly decreased the expressions of TNF-α ($P < 0.05$) expression.

Effects of peimine on the activation of MAPKs in PMACI-stimulated HMC-1 cells

To elucidate the mechanisms underlying the effects of peimine, we examined the activation of MAPKs using Western blot analysis. The stimulation of HMC-1 cells with PMACI resulted in an increased phosphorylation of all three types of MAPKs, such as ERK, JNK, and p38. The cells were pretreated for 1 h with peimine and then treated for 30 min with PMACI. As shown in figure, peimine attenuated the PMACI-induced phosphorylation of ERK concentration of 25 and 50 μg/mL, JNK, and p38 MAPK at a concentration of 50 μg/mL significantly [Figure 4]. In addition, 100 nM DEX reduced only the P-P38 expression significantly.

Effects of peimine on the activation of NF-κB in PMACI-stimulated HMC-1 cells

We examined whether peimine affects the expression of NF-κB-signaling molecules or NF-κB transcriptional activity. We found that peimine suppressed the expression of nuclear NF-κB induced by PMACI dose dependently and the concentration of 50 μg/mL was significant [Figure 5]. We also found that peimine inhibited the phosphorylation of IκB-α in stimulated HMC-1 cells significantly in concentrations of 10, 25, and 50 μg/mL.

Effects of peimine on histamine release from HMC-1 cells

To investigate the effects of peimine on histamine release from rat peritoneal mast cells (RPMCs), we measured compound 48/80-induced mast cell activation. Peimine inhibited compound 48/80-induced histamine release significantly. DEX decreased histamine release significantly compared with the compound 48/80 group [Figure 6].

Effects of peimine on the IgE-mediated PCA reaction in rats

To determine the effect of peimine on allergic reactions *in vivo*, we established the PCA rat model. Peimine concentrations of 1 and 5 mg/

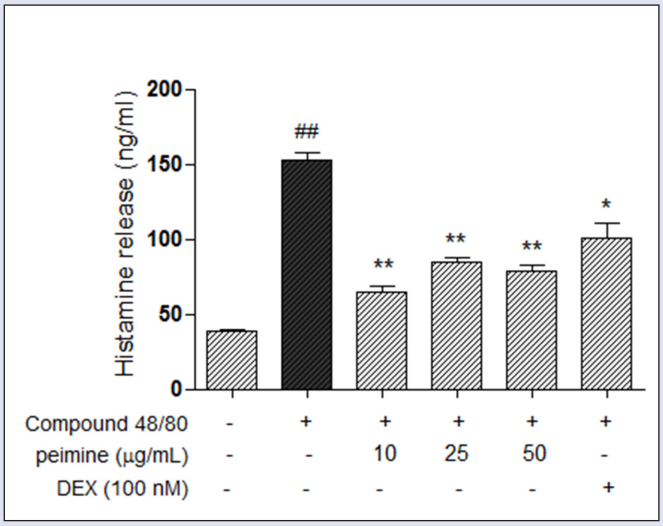

Figure 6: The effect of peimine on compound 48/80-induced histamine release from RPMCs. The value represents the mean ± SEM from three independent experiments. $^{\#\#}P < 0.01$ compared with the normal group. $^{*}P < 0.05$ compared with the compound 48/80-only group

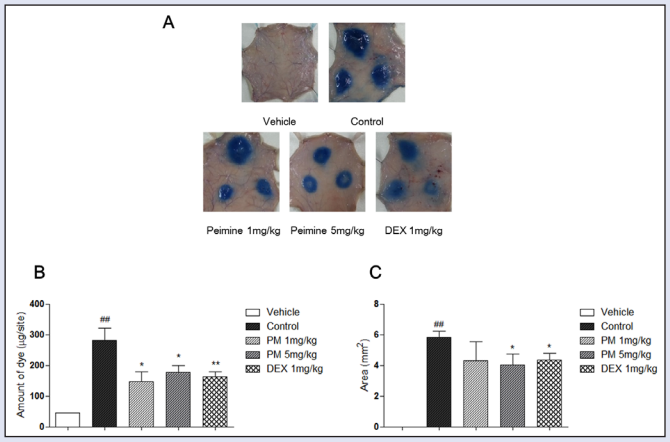

Figure 7: The inhibitory effect of peimine on 48 h PCA. (A) Photograph of internal surfaces of the rat skins. (B) Extravasation of Evans blue (μg/site) on internal surfaces of the rat. (C) Area of Evans blue on internal surface of dorsal skin. The value represents the mean ± SEM. $^{\#\#}P < 0.01$ compared with the vehicle group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group

mL significantly inhibited the PCA reaction in both areas and the amount of dye [Figure 7]. In addition, 1 mg/mL DEX significantly decreased the area of the PCA reaction and the amount of dye.

DISCUSSION

Immediate hypersensitivity can develop into a fatal reaction, and the occurrence of the effects of steroids is growing, so the necessity for alternative drug developments has become important.[5,6] A previous study proved the potential of peimine to have an inhibitory effect in LPS-induced inflammation in macrophages.[16] We estimated that peimine has an effect on immediate allergic responses. To verify the anti-allergy effect of peimine, in this study, we investigated pro-inflammatory cytokines, MAPK phosphorylation, and the nuclear NF-κB expression in PMACI-induced HMC-1. PCA reactions and histamine release in rats were also measured.

The reason for the increase in allergy diseases is observed to be societies becoming more affluent and urbanized, as well as because of increases in environmental risk factors, such as outdoor and indoor pollutions.[4] Allergy responses are initiated when mast cell activation results in allergen exposure. Mast cells are major immune cells involved in the release of histamine, and it induces cytokines, such as IL-6, IL-8, and TNF-α in mast cells.[17-20] In the present study, peimine reduced cytokines including IL-6, IL-8, and TNF-α expressions in PMACI-stimulated HMC-1 significantly. These cytokines play an important role in triggering the mast cell-mediated allergic inflammatory response.[21,22] Therefore, we suggest that peimine inhibits allergic responses through the regulation of the expression of cytokines.

Three major subfamilies of MAPKs, such as ERK1/2, JNK, and p38 MAPK, mediate cellular responses to cytokines to control cell growth and differentiation.[23] Especially, ERK is essential for cell proliferation and differentiation in these signaling pathways, while JNK and p38 are known to promote cell growth inhibition, inflammatory response, and apoptosis.[24-26] Moreover, MAPK acts upstream of NF-κB and regulates the NF-κB DNA binding activity.[27] In immune responses, therefore, the MAPK cascade is suggested to be an important pathway; therefore, it has been used as a target for the treatment of inflammatory disorders.[28] To identify whether the intracellular mechanisms of peimine inhibition affect PMACI-induced inflammation, we examined the effect of peimine on the MAPK cascade in the PMACI-induced HMC-1. In this study, 25 and 50 μg/mL of peimine reduced the p-ERK expression and 50 μg/mL of peimine inhibited the P-JNK and P-P38 expressions. This result indicates that peimine decreased the inflammatory reaction in HMC-1 associated with the production of inflammatory cytokines blocking the phosphorylation of MAPK.

In the cellular inflammation response, NF-κB plays a major role in controlling the expression of a network of genes and inflammatory cytokines, such as TNF-α, IL-6, and IL-8.[29] Moreover, the phosphorylation and proteolytic degradation of the inhibitory protein IκB-α are required for the NF-κB expression to regulate cytokine expressions.[30] The inhibition of NF-κB has been suggested as a main target for the treatment of inflammatory reactions.[29] Therefore, we investigated the effect of peimine in the phosphorylation of NF-κB and IκB-α. Peimine reduced both NF-κB and IκB-α phosphorylation. This result shows that peimine suppressed the nuclear translocation of NF-κB by inhibiting IκB-α degradation, which would inhibit the expressions of IL-6, IL-8, and TNF-α in HMC-1 cells.

An acute allergic reaction is initiated by the release of histamine from mast cells.[31] Histamine induces allergic reactions, including anaphylaxis and atopic dermatitis.[32] Histamine is known to control the expression and secretion of cytokines and chemokines.[32] PCA reaction is a most useful experimental animal model for acute hypersensitivity, which can be used to measure anti-anaphylaxis ability *in vivo*.[33,34] Therefore, we examined histamine and PCA reactions in animals. Peimine reduced histamine release slightly, but peimine showed a significant anti-allergic effect against IgE-mediated PCA reactions after oral administration in rats. These results mean that peimine is a potential complementary treatment of anti-allergy events.

CONCLUSIONS

Our data suggest that peimine inhibits the production of pro-inflammatory cytokines through the inhibition of the NF-κB signaling pathway in HMC-1 human mast cells. Moreover, peimine has an effect *in vivo* on allergic events, such as PCA reactions. It considered that peimine has anti-allergic effect through regulation of pro-inflammatory mechanism in mast cell.

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Conflicts of interest

There are no conflicts of interest

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