



Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtoxInhibitory effect of *Pleurotus eryngii* extracts on the activities of allergic mediators in antigen-stimulated mast cellsEun Hee Han^a, Yong Pil Hwang^a, Hyung Gyun Kim^a, Jae Ho Choi^a, Ji Hye Im^a, Ji Hye Yang^a, Hyun-Uk Lee^b, Sung-Sik Chun^c, Young Chul Chung^c, Hye Gwang Jeong^{a,*}^a Department of Toxicology, College of Pharmacy, Chungnam National University, Daejeon 305-764, South Korea^b Mushroom Science Research Institute, Mushtopia Co. Ltd. Jinju, South Korea^c Division of Food Science, Korea International University, Jinju, South Korea

ARTICLE INFO

Article history:

Received 7 February 2011

Accepted 19 March 2011

Available online xxxx

Keywords:

Pleurotus eryngii

Anti-allergic activity

Mast cells

IL-4

NF-κB

COX-2

ABSTRACT

Pleurotus eryngii is an edible mushroom native to Europe, the Middle East, and North Africa, and is also grown in parts of Asia. The present study investigated the anti-allergy potential of *P. eryngii* extract (PEE) in antigen-stimulated RBL-2H3 mast cells. PEE inhibited allergy markers, including release of hexosaminidase and histamine, in antigen-sensitized RBL-2H3 cells. PEE also suppressed the expression and production of interleukin-4 and reduced antigen-induced NFAT and NF-κB transcriptional activity in antigen-sensitized mast cells. Moreover, PEE decreased the levels of proinflammatory cytokines and COX-2 and iNOS expression in antigen-sensitized mast cells. Finally, PEE suppressed antigen-induced signal protein phosphorylation of Lyn, PLCγ2, PKC, Akt, and MAP kinases. Taken together, these results suggest that *P. eryngii* extract may provide insight into the prevention and treatment of allergic and inflammatory diseases.

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1. Introduction

Mast cells are a major source of inflammatory mediators, including preformed mediators stored in secretory granules, cytokines, and lipid-derived eicosanoids (Thomas, 2001). After stimulation with antigen, mast cells release β-hexosaminidase, a marker of mast cell degranulation, and various allergic mediators such as histamine, cytokines, and arachidonic acid derivatives (Gilfillan and Tkaczyk, 2006). IgE-induced release of inflammatory mediators from mast cells causes the immediate symptoms of allergic diseases, including asthma, atopic dermatitis, and atopic eczema (Wedemeyer et al., 2000). Interleukin (IL)-4 is essential for IgE production (Kuhn et al., 1991) and promotes the switch from naïve T cells to Th2 cells (Hines, 2002).

The activation of mast cells is induced partially through the induction of nuclear factor κB (NF-κB) and nuclear factor of activated T-cells (NFAT) by antigen (Saito et al., 2009). NF-κB is a key transcription factor and regulates the expression of genes involved in immune and inflammatory responses (Marquardt and Walker, 2000). NFAT is a transcription complex and is required for the transcriptional regulation of immune responses and cytokines (Palanki, 2002).

The release of allergic mediators from mast cells can be stimulated through the antigen-induced aggregation of receptors with high affinity for IgE (FcεRI). This leads to the activation of signaling pathways that initially depend on an interaction of FcεRI with the Src kinase Lyn and subsequent downstream activation of Syk and other tyrosine kinases (Gilfillan and Tkaczyk, 2006). Degranulation of mast cells stimulated with IgE is markedly impaired, as is the activity of the downstream signaling molecules phosphatidylinositol 3-kinase (PI3-kinase) and Akt (Fukao et al., 2003). Furthermore, mitogen-activated protein kinase (MAP kinase) signaling cascades are important in the differentiation, activation, proliferation, degranulation, and migration of various immune cells, including mast cells (Duan and Wong, 2006). MAP kinase signaling modules are divided into at least three groups: extracellular signal-regulated kinase (ERK), p38 MAP kinase, and c-Jun NH₂-terminal kinase (JNK).

Several studies have reported that mast cells (Matsui et al., 2000) release reactive oxygen species (ROS) into the extracellular milieu in response to a range of stimuli, and ROS from mast cells are involved in allergic inflammation (Springer et al., 2007). Cyclooxygenase (COX) is the key enzyme responsible for the conversion of arachidonic acid, via oxygenation, to prostaglandins (PGs), which are pleiotropic mediators of immune responses (Hinz and Brune, 2002). COX-2 is inducible and plays a role in inflammatory cells such as macrophages, fibroblasts, and endothelial cells (Lim, 2010). Like COX-2, inducible nitric oxide synthase (iNOS) is also

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strongly induced by bacterial endotoxins and inflammatory cytokines (Lim, 2010). It has been reported that the expression of COX-2 and iNOS under inflammatory circumstances is elevated through the activation of transcriptional factors in response to pro-inflammatory cytokines and ROS (Hansen and Jorgensen, 2007; Han et al., 2001).

Pleurotus eryngii has been used for centuries in Korea as both a food and a traditional medicine. It contains little fat and digestible carbohydrates, but has higher protein content than most vegetables, and has been reported to have antimicrobial and antioxidant activities (Kalyoncu et al., 2010). It is also rich in vitamins B, D, and K, and sometimes vitamins A and C, making it suitable for low-calorie diets (Kurtzman, 1997; Mattila et al., 2001). However, the components of *P. eryngii* have not been completely identified. In the present study, we investigated the effects of *P. eryngii* on the IgE-mediated allergic response and the multiple steps of the FcεRI-mediated signaling pathway.

2. Materials and methods

2.1. Materials

Chemicals and cell culture materials were obtained from the following sources: anti-dinitrophenyl (DNP)-IgE and 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide from Sigma-Aldrich Co. (St. Louis, MO, USA); PGE₂ immunoassay reagents and antibody against COX-2 from Cayman Chemical Co. (Ann Arbor, MI, USA); DNP-bovine serum albumin (BSA) from Calbiochem; dichloro-dihydrofluorescein diacetate (DCFHDA) from Molecular Probes (Eugene, OR, USA); minimum essential medium with Eagle's salts and fetal bovine serum (FBS) from Life Technologies Inc.;

luciferase assay system from Promega (Madison, WI, USA); pCMV-gal from Clontech; Lipofectamine 2000 from Invitrogen Inc. (Carlsbad, CA, USA); enzyme immunoassay reagents for cytokine assays from R&D Systems (Minneapolis, MN, USA); protein assay kit from Bio-Rad Laboratories Inc. (Hercules, CA, USA); primary antibodies against IκB-α/phospho-IκB-α (Ser32/36), phospho-Erk1/2 (Tyr202/204), phospho-p38 (Tyr180/182), phospho-SAPK/JNK1/2 (Tyr183/185), phospho-Syk (Tyr525/526), phospho-Lyn (Tyr507), phospho-PLCγ2 (Tyr1217), phospho-Gab2 (Tyr452), and phospho-Akt (Ser473), and HRP-conjugated anti-rabbit and anti-mouse IgG as secondary antibodies from Cell Signaling Technology; anti-actin and anti-iNOS antibodies from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); and enhanced chemiluminescence system (ECL) and polyvinylidene difluoride membranes from Amersham Pharmacia Biotech. Polymerase chain reaction (PCR) oligonucleotide primers were custom-synthesized by Bioneer Co. (Korea). All chemicals were of the highest grade commercially available.

2.2. Plant material

Air-dried *P. eryngii* (2.5 kg) was extracted with ethanol; the yield of *P. eryngii* extract (PEE) corresponded to 0.23%. The *P. eryngii* ethanol extract was analyzed to contain 86.1% water, 1.72% crude protein, 0.31% crude lipid, 0.63% ashes, 6.14% crude sugar, and 5.3% crude fiber. The contents of moisture, crude protein, crude lipid, ashes, crude sugar, and crude fiber content were determined by the air oven method, Micro-Kjeldahl determination, soxhlet method, drying ash method, Phenol-H₂SO₄ method, and H₂SO₄ and NaOH method, respectively. The minerals analysis was performed by the use of a PerkinElmer[®] ICP model Optima™ 4300 DV. The amount of minerals in *P. eryngii* ethanol extract is 98.9 mg/kg K, 4.5 mg/kg Ca, 15.8 mg/kg Mg, 23.9 mg/kg Na, 1.8 mg/kg Mn, 2.9 mg/kg Fe, 0.6 mg/kg Zn, 0.5 mg/kg Cu, 0.4 mg/kg Al, and 75.5 mg/kg P. The fungal sterol, ergosterol and ergosterol peroxide, were also determined by high-pressure liquid chromatography (HPLC) system and analyzed to contain 5.5% and 15.4%, respectively. The extract powder was dissolved in dimethyl sulfoxide (DMSO), and stock solutions were added directly to the culture media.

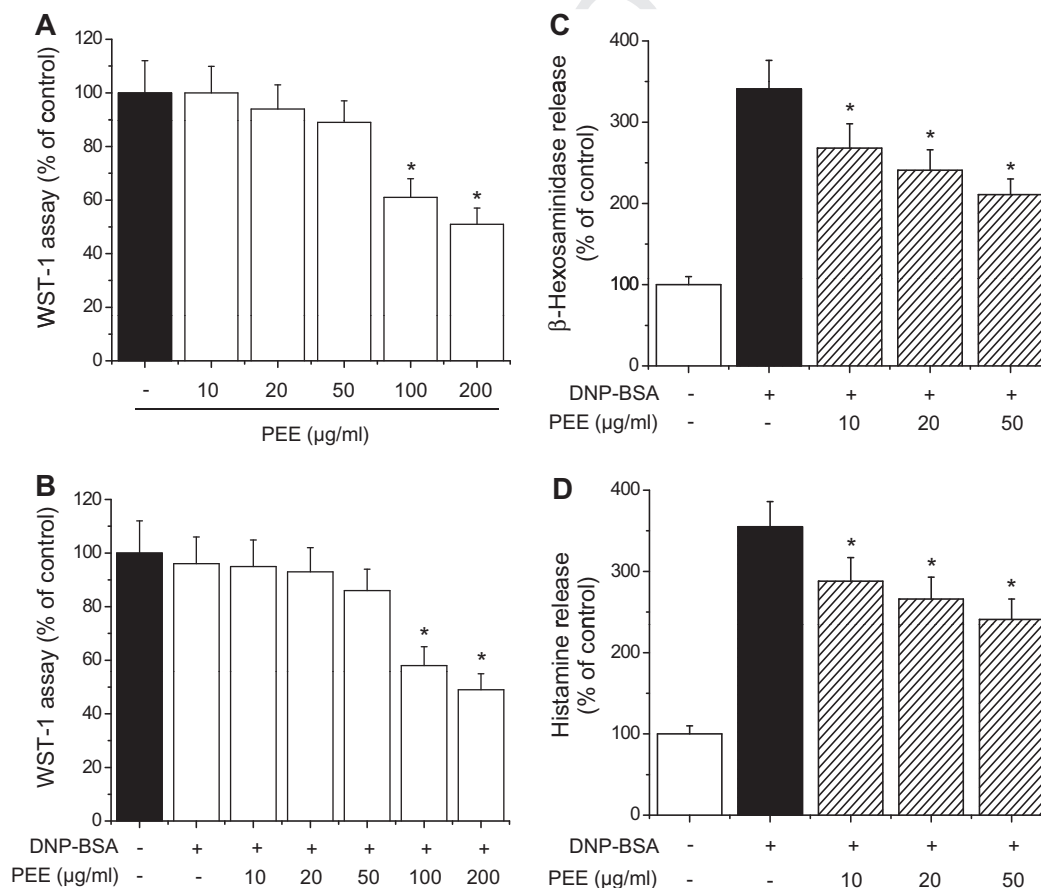


Fig. 1. Effects of PEE on degranulation and histamine release in antigen-stimulated mast cells. (A) Effects of PEE on cytotoxicity. The cells were seeded in a 96-well plate and treated with the indicated concentrations of PEE for 24 h. (B) Cells were treated with the indicated concentrations of PEE and/or DNP-BSA (50 ng/mL) for 24 h. Cell viability was assessed using the WST-1 assay. **P* < 0.01, vs. control. (C) Effects of PEE on degranulation. The cells were incubated overnight in 48-well plates with 100 ng/mL of DNP-specific IgE in the medium. The medium was replaced with Tyrode's buffer containing the indicated concentrations of PEE (10–50 µg/mL) followed by stimulation with 50 ng/mL DNP-BSA for 10 min, and the release of -hexosaminidase was measured. (D) Effects of PEE on histamine release. Same as (B) except that histamine release was measured. Each bar shows three independent experiments. **P* < 0.01, significantly different from DNP-BSA alone (Newman-Keuls test).

2.3. Cell culture

The rat mast cell line RBL-2H3 was obtained from the American Type Culture Collection (Manassas, VA) and grown in minimum essential medium with Eagle's salts (EMEM), supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a humidified incubator with a 5% CO₂/95% air atmosphere. Control cells were treated with solvent alone. The final concentration of DMSO did not exceed 0.1% and did not affect the assay systems.

2.4. Assay for proliferative activity

RBL-2H3 cells in 10% FBS-EMEM were seeded in 96-well plates. Briefly, PEE (0.1–100 µg/mL) and/or DNP-BSA (50 ng/mL) were added to the wells, and the plates were incubated for 24 h. After, the cells were treated with 10 µL of WST-1 solution. Cell viability was examined using a WST-1 assay kit according to the manufacturer's instructions (Roche, Mannheim). Relative cytotoxicity was quantified by measuring the absorbance at 550 nm; PEE did not interfere with the measurement at this wavelength.

2.5. -Hexosaminidase release assay

IgE-sensitized RBL-2H3 cells were treated with PEE (10–50 µg/mL) and DNP-BSA for 16 h in Tyrode's buffer (Han et al., 2011). Degranulation was terminated by placing the cells on ice. To determine the amount of β-hexosaminidase activity released from the cells, 25 µL of culture supernatant and 100 µL of 1.2 mM β-hexosaminidase substrate (4-methylumbelliferyl-N-acetyl-β-D-glucosaminide) in sodium acetate buffer (pH 4.4) were mixed in 96-well plates and incubated for 30 min at 37 °C. For measurement of total β-hexosaminidase activity, the cells were lysed with 0.1% Triton-X 100 prior to removing the supernatant. β-Hexosaminidase activity was quantified by measuring the fluorescence intensity of the hydrolyzed substrate in a fluorometer (Varioskan, Thermo Electron Co.) using 360 nm excitation and 450 nm emission filters. Background fluorescence of the substrate in buffer alone (no cell supernatant) was subtracted from all readings.

2.6. Histamine release assay

IgE-sensitized RBL-2H3 cells were preincubated with PEE (10–50 µg/mL) for 30 min and then incubated with DNP-BSA for 15 min. To examine the amount of histamine released from the cells, 100 µL of supernatant and 500 µL of o-phthalaldehyde substrate were mixed in 48-well plates and incubated for 10 min at 37 °C. Histamine content was measured at 440/360 nm using a fluorometer (Varioskan).

2.7. Reactive oxygen species (ROS) measurement

The amount of intracellular ROS was measured using 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA), which is deacetylated by intracellular esterases to non-fluorescent 2',7'-dichlorodihydrofluorescein (DCFH), which in turn is oxidized to the fluorescent compound 2',7'-dichlorodihydrofluorescein (DCF) by ROS. IgE-sensitized RBL-2H3 cells were preincubated with 5 µM of H₂DCF-DA for 30 min at 37 °C in 48-well plates, washed to remove excess H₂DCF-DA, and treated with PEE (10–50 µg/mL) and/or DNP-BSA for 30 min. The fluorescence intensity was measured with excitation at 485 nm and emission at 530 nm using a fluorometer (Varioskan). The values were calculated as DCF fluorescence intensity relative to the control. Cellular fluorescence images were obtained using a confocal laser scanning microscope (Zeiss, Jena, Germany).

2.8. Transient transfection and luciferase activity assay

For transient transfections, cells were seeded at 1×10^6 cells/well in a 24-well plates and transfected with 0.5 µg of luciferase vector or 0.1 µg of pCMV-gal plasmid using 0.5 µL of serum- and antibiotic-free Lipofectamine 2000 reagent (Invitrogen). The IL-4- and NFAT-Luc vectors were gifts from Dr. Kenneth M. Murphy (Washington University, St. Louis, MO, USA). The COX-2 promoter-luciferase construct was a gift from Dr. Hiroyasu Inoue (Nara Women's University, Nara, Japan). The NF-κB luciferase reporter construct was purchased from Stratagene (Grand Island, NY). After 4 h, the medium was replaced with basal medium. IgE-sensitized RBL-2H3 cells were pretreated with PEE (10–50 µg/mL) for 30 min, then treated with DNP-BSA for 18 h, and lysed. Luciferase and -galactosidase activities were measured in the cellular extracts. The luciferase activity was normalized to the -galactosidase activity and is expressed relative to the activity in the control group.

2.9. Measurement of cytokine production

For cytokine immunoassays, IgE-sensitized RBL-2H3 cells were treated with PEE (10–50 µg/mL) and/or DNP-BSA. Supernatants were harvested at the indicated times, and IL-4 (24 h), IL-1 (24 h), IL-6 (6 h), and TNF-α (3 h) production was quantified by sandwich immunoassays using the protocol supplied by R&D Systems.

2.10. RNA preparation and mRNA analysis by real-time quantitative PCR

IgE-sensitized RBL-2H3 cells were pretreated with PEE (10–50 µg/mL) for 30 min and then treated with DNP-BSA for 6, 20, and 24 h. Total RNA from the treated cells was prepared using RNAiso reagent (Takara) according to the manufacturer's protocol and was stored at –80 °C until use. For detection of cytokines,

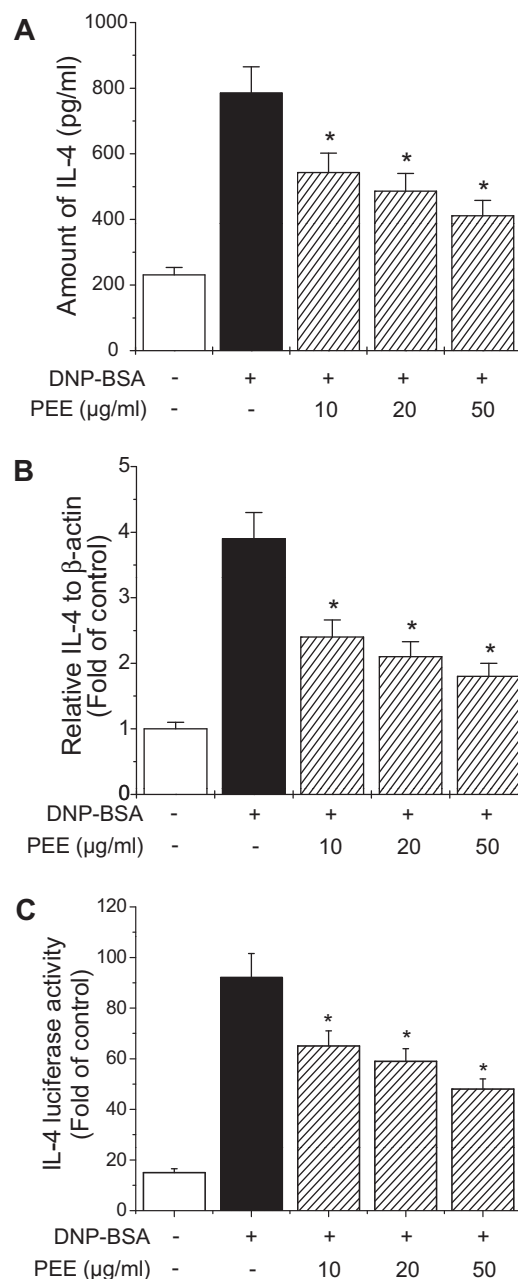


Fig. 2. Effects of PEE on the level of IL-4 in antigen-stimulated mast cells. (A) Effects of PEE on the production of IL-4. The IgE-primed cells were stimulated with DNP-BSA (50 ng/mL) for 4 h or were left unstimulated, with or without PEE. The concentrations of IL-4 released into the culture media were assessed using commercial ELISA kits. (B) Effects of PEE on mRNA expression of IL-4. The cells were sensitized with 100 ng/mL DNP-specific IgE overnight, pretreated with PEE for 30 min, and stimulated with DNP-BSA (50 ng/mL) for 24 h. The cells were lysed, and total RNA was prepared for analysis of IL-4 gene expression. PCR amplification of a housekeeping gene, actin, was performed for each sample. IL-4 mRNA expression in treated cells was compared with expression in untreated cells at each time point, by real-time PCR. (C) Effect of PEE on IL-4 promoter activity. Cells were transfected with an IL-4-Luc construct and then treated with PEE (10–50 µg/mL) and/or DNP-BSA for 20 h. The cells were then harvested and assayed for luciferase activity. Each bar shows three independent experiments. **P* < 0.01, significantly different from DNP-BSA alone (Newman-Keuls test).

including TNF- α (6 h), IL-6 (6 h), IL-1 (24 h), and IL-4 (24 h), and COX-2 (20 h) and iNOS (20 h), total RNA was extracted after stimulation and treatment. PCR product formation was continuously monitored during the PCR reaction using Sequence Detection System software (ver. 1.7; Applied Biosystems, Foster City, CA). Accumulated PCR products were detected directly by monitoring the increase of the reporter dye (SYBR). Expression levels of cytokines in the exposed cells were compared with the expression levels in control cells at each collection time point using the comparative cycle threshold (Ct) method. The sequences of the primers used were (5' $\rightarrow$ 3'): IL-4 forward, ACC TTG CTG TCA CCC TGT TC and reverse, TTG TGA GCG TGG ACT CAT TC; TNF- α forward, CAA GGA GGA GAA GTT CCC AA and reverse, CGG ACT CCG TGA TGT CTA AG; IL-6 forward, TGG AGT TCC GTT TCT ACC TG and reverse, TTC ATA TTG CCA GTT CTT CG; IL-1 forward, CAC CTT CTT TTC CTT CAT CTT TG and reverse, GTC GTT GCT TGT CTC TCC TTG TA; iNOS forward, CAG CCC TCA GAG TAC AAC GAT and reverse, CAG CAG GCA CAC GCA ATG AT; COX-2 forward, AAG AAA TGG CTG CAG AGT TGA AA and reverse, AGG GCG GGA TAC AGT TCC A; and actin forward, TGG CAC CCA GCA CAA TGA A and reverse, CTA AGT CAT AGT CCG CCT AGA AGC A. The quantity of each transcript was calculated as described in the instrument manual (Applied Biosystems, Foster City, CA) and normalized to the amount of actin, a housekeeping gene.

2.11. Subcellular fractionation

Cytosolic and nuclear fractions were prepared as described previously (Kabouridis et al., 1997). Briefly, cells were pretreated with PEE (10–50 μ g/mL) for 30 min, treated with DNP-BSA for 30 min, and resuspended in hypotonic solution (25 mM Tris-HCl, pH 7.5, 5 mM EGTA, 250 mM sucrose, 25 μ g/mL aprotinin, 1 mM PMSF, 25 μ g/mL leupeptin, 5 mM NaF, and 1 mM Na₂VO₄). The cell suspension was homogenized on ice, and the salt concentration was adjusted to

150 mM NaCl. Nuclei were removed by two successive centrifugations (480g, 5 min, 4 $^{\circ}$ C). The soluble and particulate fractions were separated by centrifugation (100,000g, 30 min).

2.12. Western blot analysis

IgE-sensitized RBL-2H3 cells were pretreated with PEE (10–50 μ g/mL) for 30 min and then treated with DNP-BSA for 5 min (Syk, Lyn, LAT, Gab2, PLC γ 2, Akt, MAP kinase), 30 min (I κ B- α), or 24 h (COX-2, iNOS). Equal amounts of cellular protein (50 μ g) were resolved by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. After blocking, the membranes were incubated with the appropriate primary antibody and then with horseradish peroxidase-conjugated secondary antibody to IgG. Immunoreactive proteins were visualized using an ECL Western blot detection system. The protein levels were compared with a loading control such as actin or non-phosphorylated protein.

2.13. PGE₂ production

IgE-sensitized RBL-2H3 cells were incubated with PEE (10–50 μ g/mL) or DNP-BSA for 24 h, and the culture medium was collected. The PGE₂ released into the culture medium was detected using a specific enzyme immunoassay (Cayman Chemical) according to the manufacturer's protocol. PGE₂ concentrations were determined by measuring the absorbance at 415 nm.

2.14. Statistical analyses

All experiments were repeated at least three times. One-way analysis of variance (ANOVA) was used to determine the significance of differences between treat-

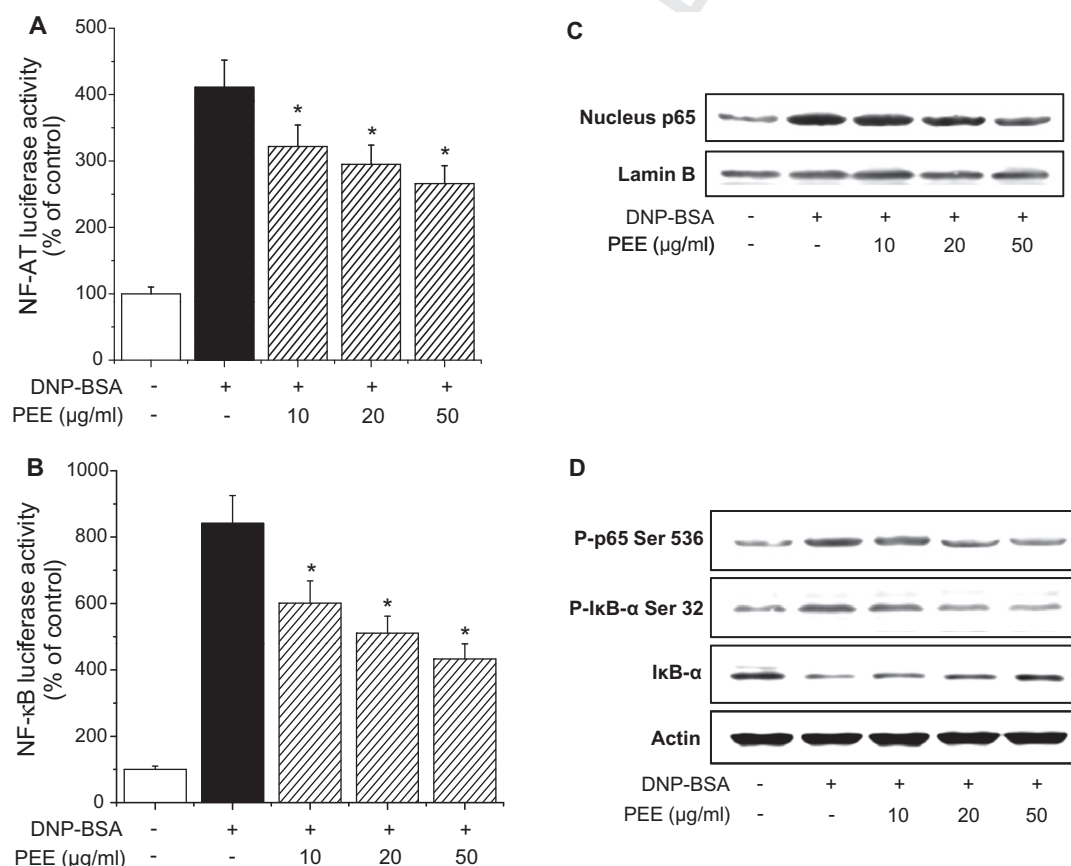


Fig. 3. Effects of PEE on the activation of NFAT and NF- κ B in antigen-stimulated mast cells. (A) Effects of PEE on NFAT transcriptional activity. Cells were transiently co-transfected with NFAT-Luc and pCMV-gal. After 4 h, the cells were sensitized with DNP-specific IgE (100 ng/mL) for 20 h. The IgE-sensitized cells were treated with PEE and stimulated with DNP-BSA (50 ng/mL). The cells were harvested and assayed for luciferase activity. (B) Effects of PEE on NF- κ B transcriptional activity. Cells were transiently co-transfected with pGL3-NF- κ B-Luc and pCMV-gal, and treated as in (A), except that NF- κ B transcriptional activity was measured. The cells were harvested, and luciferase and -galactosidase activities were determined. The luciferase activity was normalized to the -galactosidase activity and is expressed relative to the activity of the control. (C) Effects of PEE on the nuclear translocation of NF- κ B. Cells were incubated for 15 min in the presence or absence of DNP-BSA (50 ng/mL) and PEE, and nuclear proteins were extracted and subjected to Western blot analysis using antibodies against NF- κ B p65. To provide a loading control, anti-lamin B antibody was included. (D) Effects of PEE on the phosphorylation of NF- κ B and I κ B- α . Cells were incubated with or without DNP-BSA in the presence or absence of PEE, or were pretreated with PEE and then incubated with DNP-BSA. After 30 min, the total cell protein was analyzed by Western blotting with antibodies against I κ B- α , phospho-I κ B- α , and phospho-p65. Western blot analysis was performed as described in the Section 2. Each blot is representative of three independent experiments with similar results.

ment groups. The Newman–Keuls test was used for multi-group comparisons. Statistical significance was accepted for p values <0.01 .

3. Results

3.1. PEE inhibits degranulation and histamine release in antigen-stimulated mast cells

To determine the optimal concentrations of PEE to use in our studies, PEE was tested for potential cytotoxicity in RBL-2H3 cells. PEE did not affect cell viability at concentrations of 10, 20, or 50 $\mu\text{g}/\text{mL}$ for 24 h (Fig. 1A). We also assessed the cytotoxic effect of PEE and antigen (DNP–BSA) co-treatment. Treatments for 24 h at PEE concentrations from 10 to 50 $\mu\text{g}/\text{mL}$ produced no significant cytotoxic effect (Fig. 1B). Thus, we treated the RBL-2H3 mast cells with PEE at concentrations ranging from 10–50 $\mu\text{g}/\text{mL}$ in subsequent experiments.

To investigate the mechanism of the inhibitory effect of PEE on the allergic reaction, this study used DNP–IgE, which specifically binds Fc ϵ RI complexes in mast cells. Fc ϵ RI is abundant on the surface of mast cells, and the aggregation of IgE–Fc ϵ RI complexes by multivalent antigen initiates a complex cascade of intracellular events, leading to degranulation and subsequent release of chemical mediators of allergic responses, including histamine and β -hexosaminidase (Schwartz et al., 1979). The secretion of β -hexosaminidase, a known degranulation marker, is the hallmark of an allergic reaction resulting from allergen exposure. DNP–IgE-sensitized RBL-2H3 cells were pretreated with PEE (10–50 $\mu\text{g}/\text{mL}$) for 30 min and then treated with antigen (DNP–BSA; 50 ng/mL) for 15 min. DNP–BSA induced degranulation in IgE-sensitized RBL-2H3 cells (Fig. 1B), and PEE significantly suppressed the antigen-induced degranulation (Fig. 1B). We also examined the effects of PEE on histamine release in antigen-stimulated mast cells. DNP–IgE-sensitized cells were preincubated with PEE (10–50 $\mu\text{g}/\text{mL}$) for 30 min and then incubated with DNP–BSA for 15 min, as for the β -hexosaminidase assay. PEE inhibited antigen-induced histamine release in IgE-sensitized RBL-2H3 cells (Fig. 1C). The results suggest that PEE significantly inhibits antigen-induced activation of the allergic response, including mast cell degranulation and histamine release.

3.2. PEE inhibits transcriptional activation of IL-4 in antigen-stimulated mast cells

Mast cell degranulation stimulated by antigen-crosslinked IgE releases histamine as well as cytokines, especially IL-4 (Bruhns et al., 2005; Zhao et al., 2005). IL-4 has a critical role in the allergic reaction and promotes the switching of naïve T cells to allergic-type Th2 cells (Hines, 2002). ELISA results showed that PEE (10–50 $\mu\text{g}/\text{mL}$) significantly inhibited IL-4 secretion in antigen-stimulated DNP–IgE-sensitized mast cells (Fig. 2A). We further examined whether PEE suppressed the mRNA expression and transcriptional activity of IL-4 in IgE-sensitized mast cells. Consistent with the ELISA results, PEE reduced antigen-induced mRNA expression and luciferase reporter activity of IL-4, in a dose-dependent manner (Fig. 2B, C). These results suggest that PEE markedly inhibits the antigen-induced gene expression and production of IL-4 related to allergic reactions.

3.3. PEE inhibits the activation of NFAT and NF- κ B in antigen-stimulated mast cells

To evaluate the inhibitory mechanism of IL-4 production and gene expression by PEE, we observed the effect of PEE on the transcriptional activities of NFAT and NF- κ B in antigen-stimulated mast cells. PEE dose-dependently suppressed antigen-induced

NFAT luciferase activity in IgE-sensitized mast cells (Fig. 3A). Likewise, PEE markedly reduced antigen-induced NF- κ B transcriptional activity in IgE-sensitized mast cells (Fig. 3B).

NF- κ B normally binds to regulatory I κ B- α in the cytoplasm and is activated by the signal-induced degradation of I κ B proteins. NF- κ B stimuli act through distinct signaling pathways that converge on the activation of I κ B kinase (IKK), which initiates I κ B phosphorylation at specific amino-terminal serine residues (Baeuerle and Henkel, 1994), leading to the ubiquitination and degradation of I κ B. When I κ B is degraded, the NF- κ B complex is released and translocates to the nucleus, where it turns on the expression of target proteins, including IL-4 (Shen and Stavnezer, 1998).

To examine the inhibition mechanism of NF- κ B transcriptional activity by PEE, we assessed its effect on the nuclear translocation and phosphorylation of NF- κ B. PEE inhibited the nuclear translocation of NF- κ B p65 protein induced by antigen stimulation (Fig. 3C). PEE also reduced the antigen-inducible activation of serine 536-phosphorylated NF- κ B p65, but did not affect the total level of p65 protein in IgE-sensitized cells (Fig. 3D). The phosphorylation of I κ B- α , which regulates the nuclear translocation of NF- κ B, was decreased by PEE, and I κ B- α degradation also was inhibited by PEE in antigen-stimulated cells (Fig. 3D). Lamin B and actin antibodies were used as quantitative controls (Fig. 3C, D). Thus, PEE

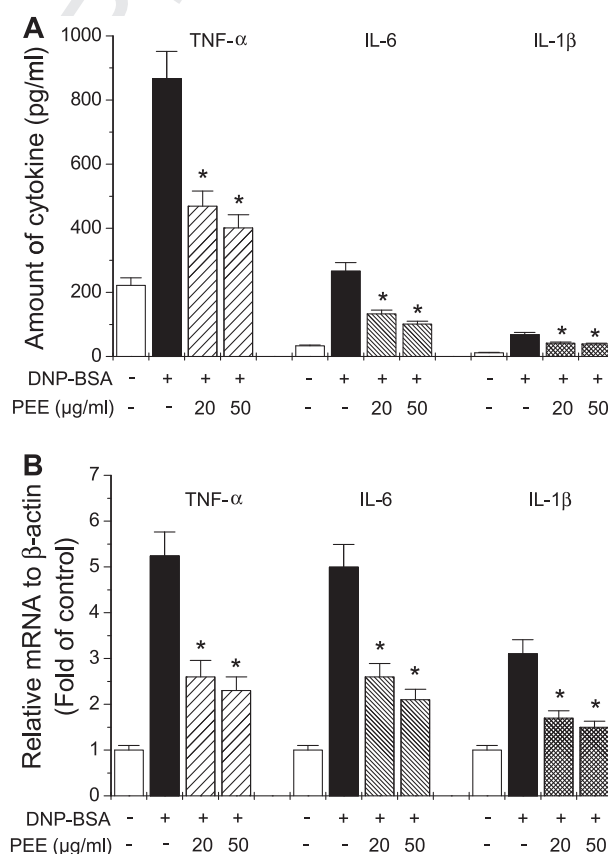


Fig. 4. Effects of PEE on the amount of inflammatory cytokines in antigen-stimulated mast cells. (A) Cells were cultured for 6 h (TNF- α and IL-6) or 24 h (IL-1) in medium alone or with PEE (20 or 50 $\mu\text{g}/\text{mL}$). The amounts of TNF- α , IL-1, and IL-6 released into the culture medium were measured by immunoassay. * $P < 0.01$, significantly different from control as determined by analysis of variance and Newman–Keuls test. (B) Cells were treated with PEE (20 or 50 $\mu\text{g}/\text{mL}$) for 6 h and then lysed. Total RNA was prepared for analysis of TNF- α , IL-6, and IL-1 gene expression. PCR amplification of a housekeeping gene, actin, was also performed for each sample. TNF- α , IL-6, and IL-1 mRNA expression was compared between PEE-treated and untreated cells at each time point, by real-time PCR. * $P < 0.01$, significantly different from control as determined by analysis of variance and Newman–Keuls test.

may inhibit the activities of allergic mediators via the inhibition of NFAT and NF- κ B transcriptional activation.

3.4. PEE inhibits proinflammatory cytokines in antigen-stimulated mast cells

Once activated, mast cells secrete numerous pro-inflammatory mediators (Galli et al., 2005). Some of these mediators such as histamine, serotonin, TNF, kinins, and proteases are preformed molecules stored in secretory granules. Leukotrienes (LT), prostaglandins, and platelet-activating factor are synthesized from arachidonic acid liberated by the action of phospholipases during mast cell activation. Additionally, several cytokines, including vascular endothelial growth factor (Grutzkau et al., 1998), TNF- α , and IL-1, -2, -5, -6, -8, -9, and -13, are synthesized *de novo* and released several hours after stimulation.

To investigate the inhibitory mechanism of PEE on the allergic reaction, we examined its effects on the production of the pro-inflammatory cytokines IL-6, IL-1, and TNF- α in antigen-stimulated mast cells. DNP-IgE-sensitized cells were treated with PEE (10–50 μ g/mL) and/or DNP-BSA, and the quantities of these cytokines secreted into the culture supernatants were monitored by ELISA. PEE dose-dependently inhibited the levels of TNF- α and IL-6 in the supernatant of antigen-stimulated cells after 6 h (Fig. 4A). As PEE reduced the secretion of cytokines, a real-time

quantitative PCR assay was performed to determine whether PEE regulated cytokine secretion at the mRNA level. PEE dose-dependently inhibited the expression of TNF- α , IL-6, and IL-1 mRNA (Fig. 4B). As a control, -actin was constitutively expressed and unaffected by PEE treatment. Thus, PEE may regulate the transcriptional activation of TNF- α , IL-1, and IL-6 in antigen-stimulated cells.

3.5. PEE inhibits inflammatory mediators in antigen-stimulated mast cells

Increasing evidence indicates that mast cells are critical for the pathogenesis of inflammatory diseases (Theoharides et al., in press). Previous studies in mast cells have shown that the induction of allergic reactions is regulated by the inflammatory protein COX-2 (Hundley et al., 2001). We investigated whether PEE had an inhibitory effect on COX-2 in antigen-stimulated cells. Cells were treated with PEE (10–50 μ g/mL) and/or DNP-BSA for 20 h. PEE dose-dependently inhibited COX-2 mRNA expression (Fig. 3A). To clarify the mechanism of COX-2 gene expression, we transiently transfected the cells with the COX-2-Luc reporter construct. Treatment with PEE inhibited the COX-2 luciferase activity in antigen-stimulated cells (Fig. 3B), suggesting that PEE may regulate the transcriptional activation of COX-2 in these cells. To investigate the effect of PEE on the COX-2 protein level, we used a COX-2-specific antibody. PEE significantly reduced the COX-2

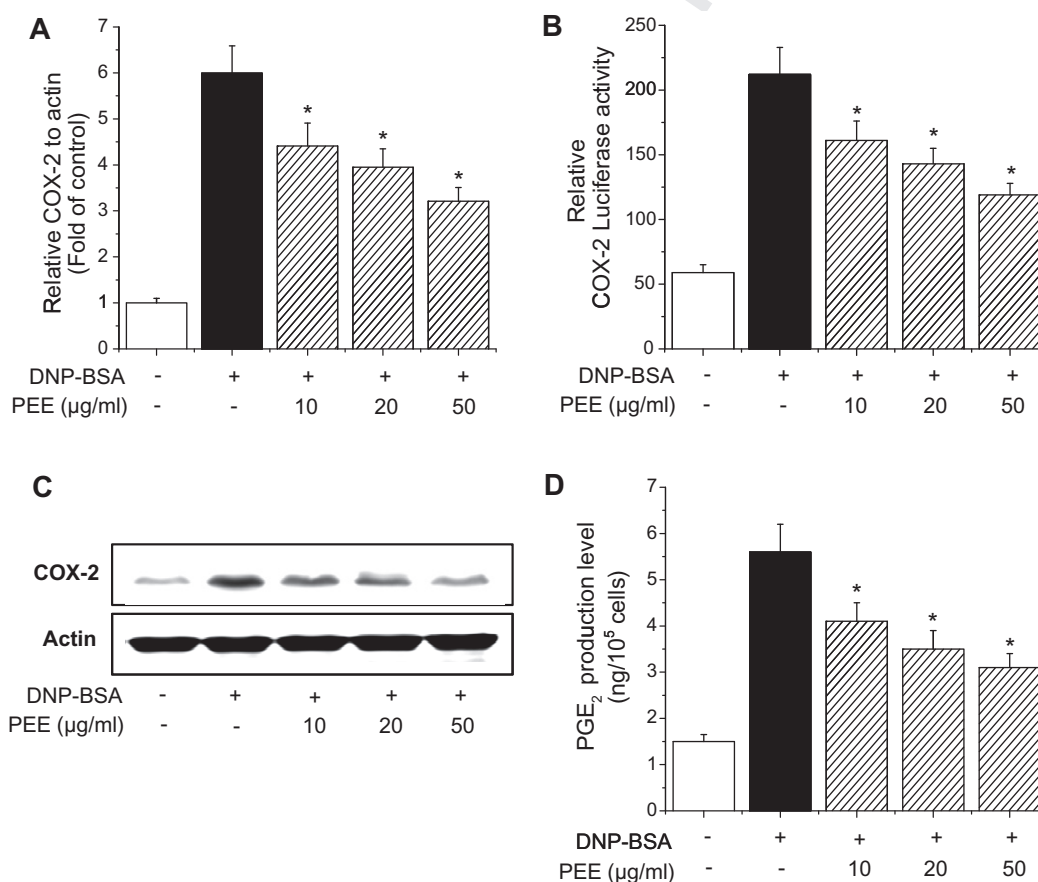


Fig. 5. Effects of PEE on COX-2 expression and PGE₂ production in antigen-stimulated mast cells. (A) Effect of PEE on COX-2 mRNA levels. Cells were treated with PEE (10–50 μ g/mL) and/or DNP-BSA (50 ng/mL) for 20 h. The cells were lysed, and total RNA was prepared for analysis of COX-2 gene expression. PCR amplification of a housekeeping gene, actin, was also performed for each sample. (B) Effect of PEE on COX-2 transcriptional activity. Cells were transfected with COX-2-Luc and pCMV-gal and then treated with PEE (10–50 μ g/mL) and/or DNP-BSA (50 ng/mL) for 20 h. The cells were harvested and assayed for luciferase activity. (C) Effect of PEE on COX-2 protein level. Cells were treated with PEE (10–50 μ g/mL) and/or DNP-BSA (50 ng/mL) for 24 h. Total cell protein was analyzed by Western blotting with antibody against COX-2. The COX-2 protein level was compared with that of actin. Each blot is representative of three independent experiments with similar results. (D) Effect of PEE on PGE₂ production. Cells were treated with PEE (10–50 μ g/mL) and/or DNP-BSA (50 ng/mL) for 24 h. The medium was collected and assayed for PGE₂ production by enzyme immunoassay. Each bar shows three independent experiments. *P < 0.01, significantly different from DNP-BSA alone (Newman-Keuls test).

protein level in antigen-stimulated cells (Fig. 3C). Prostaglandin E₂ (PGE₂) is generated through COX-2 activity, which catalyzes the conversion of arachidonic acid to prostanoids in the inflammation signaling pathway (Hla et al., 1999; Simpson, 2004). PEE inhibited the production of PGE₂, one of the major metabolites of COX-2, in these cells (Fig. 3D). Additionally, we examined whether PEE modulated the expression of a proinflammatory protein (iNOS) in antigen-stimulated RBL-2H3 cells. As expected, the relative levels of iNOS mRNA and protein were significantly reduced after PEE treatment of antigen-stimulated cells (Fig. 5A, B).

Mast cells release ROS in response to various stimuli (Matsui et al., 2000). Thus, we assessed whether PEE suppressed ROS production in antigen-stimulated cells. PEE inhibited ROS production in these cells (Fig. 5C). These results indicate that anti-inflammatory mediators are correlated with the inhibition of allergic reactions in PEE-treated antigen-stimulated RBL-2H3 mast cells.

3.6. PEE regulates FcεRI-mediated signaling events in antigen-stimulated mast cells

To identify potential signals for the inhibition of allergic mediators by PEE, we examined its effects on FcεRI-mediated signaling events in antigen-stimulated mast cells. Upon FcεRI aggregation, the Src-family protein tyrosine kinase (PTK) Lyn is activated and

phosphorylates the α and γ subunits of the FcεRI complex (Yamashita et al., 1994), which in turn recruits and activates Syk PTK (Costello et al., 1996). Activated PTKs subsequently catalyze the phosphorylation of phospholipase C (PLC) γ (Park et al., 1991) and linker of activated T cells (LAT) (Yamazaki et al., 2007). The Src-family PTK Fyn plays an essential role in initiating FcεRI signaling by phosphorylating Grb2-associated binder-2 (Gab2) (Parravicini et al., 2002), which recruits and activates PI3 kinase. PI3 kinase (Ali et al., 2004) also plays a critical role by contributing to the recruitment of Akt and PLCγ to the plasma membrane. DNP-IgE-sensitized cells were treated with PEE (10–50 μg/mL) and/or DNP-BSA for 5 min. PEE significantly inhibited the phosphorylation of Lyn, PLCγ2, and PKC in antigen-stimulated mast cells (Fig. 6A, Fig. 7). However, Syk and Syk-dependent downstream activating phosphorylation of LAT and Gab2 were not changed by PEE treatment in these cells (Fig. 6B). Actin, the control gene, was constitutively expressed and unaffected by the chemical treatment. The phosphorylation of Akt, an indicator of PI3-kinase activation, and the phosphorylation of the MAP kinases Erk1/2, JNK1/2, and p38 were also significantly suppressed by PEE in antigen-stimulated mast cells (Fig. 5B). These results indicate that PEE may have potential as an allergy therapeutic owing to its inhibition of FcεRI-mediated signaling events, including Lyn, PLCγ, PKC, Akt, and MAP kinases, in antigen-stimulated mast cells.

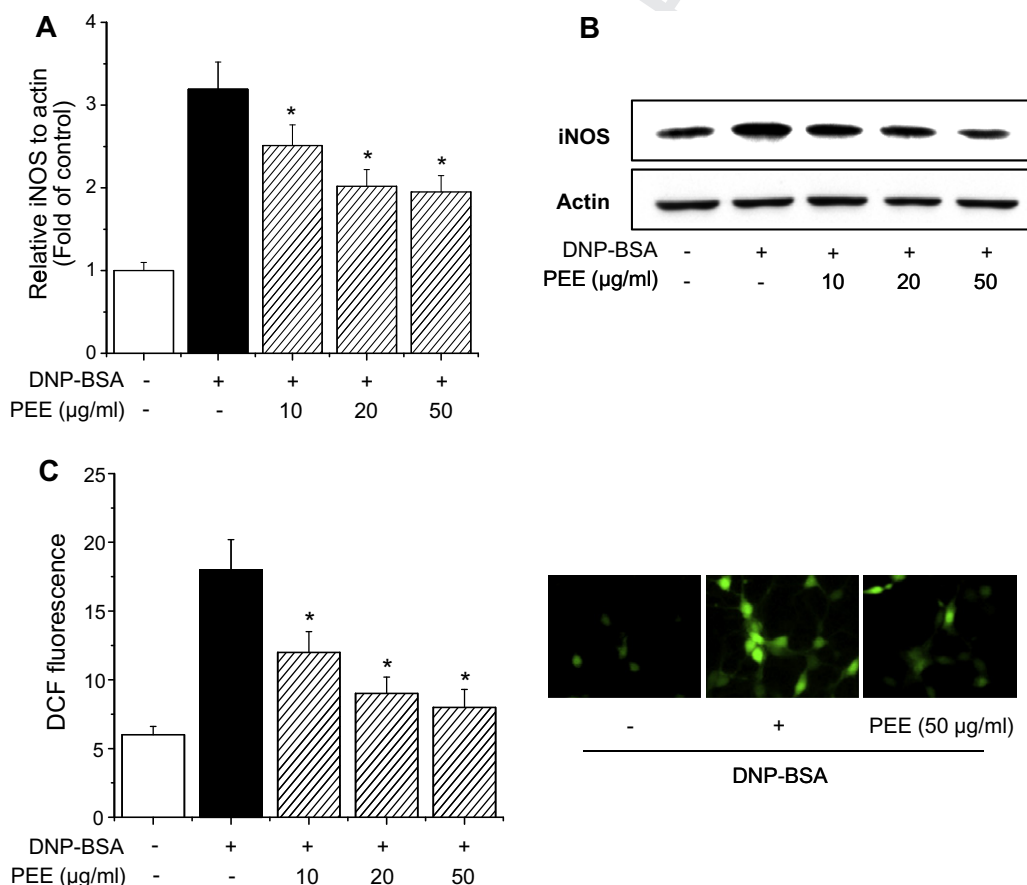


Fig. 6. Effects of PEE on iNOS expression and NO and ROS production in antigen-stimulated mast cells. (A) Effect of PEE on iNOS mRNA levels. Cells were treated with PEE (10–50 μg/mL) and/or DNP-BSA (50 ng/mL) for 20 h and then lysed. Total RNA was prepared for analysis of iNOS gene expression. PCR amplification of a housekeeping gene, actin, was also performed for each sample. (B) Effect of PEE on iNOS protein expression. Cells were treated with PEE (10–50 μg/mL) and/or DNP-BSA (50 ng/mL) for 24 h. Total cell protein was analyzed by Western blotting with antibody against iNOS. The iNOS protein level was compared with that of actin. Each blot is representative of three independent experiments with similar results. (C) Effect of PEE on antigen-induced ROS production. Intracellular ROS was measured using 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA). IgE-sensitized RBL-2H3 cells were pre-incubated with 5 μM H₂DCF-DA for 30 min at 37 °C and then washed. Cells were treated with PEE (1–20 μg/mL) and/or DNP-BSA for 30 min. Then, fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorometer (Varioskan). Each bar shows three independent experiments. **P* < 0.01, significantly different from DNP-BSA alone (Newman–Keuls test). Cellular fluorescence images were obtained using a confocal laser scanning microscope (original magnification, ×200).

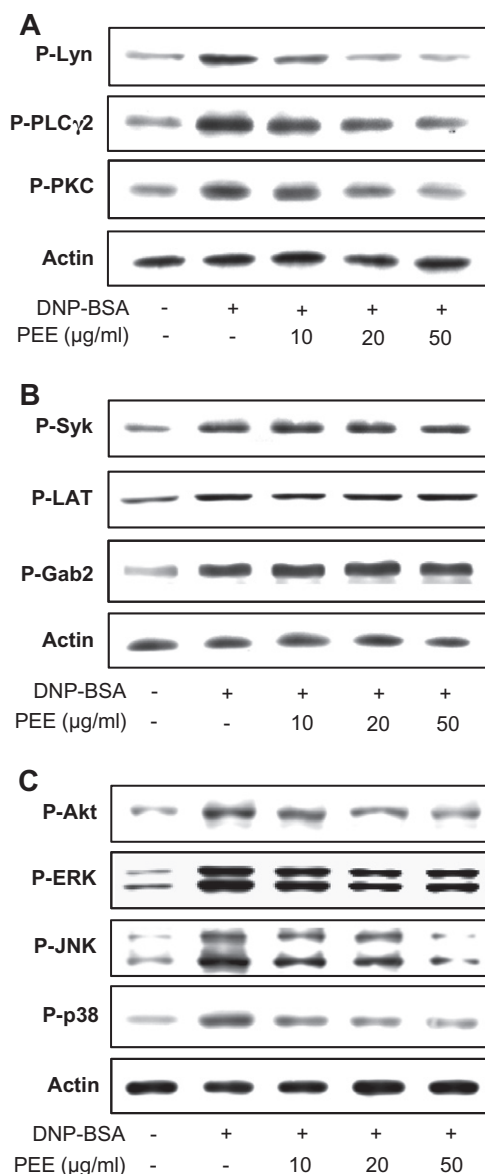


Fig. 7. Effects of PEE on Fc ϵ RI-mediated signaling events and the phosphorylation of Akt and MAP kinases in antigen-stimulated mast cells. IgE-sensitized cells were treated with PEE and stimulated with DNP-BSA (50 ng/mL) for 5 min. (A, B) Cell extracts were analyzed for levels of Lyn, PLC γ 2, PKC, Syk, LAT, and Gab2 activation by Western blot analysis. (C) Cell extracts were analyzed for Akt and MAP kinase (ERK, JNK, and p38) activation by Western blot analysis.

4. Discussion

Mushrooms have been used as traditional medicines since ancient times, and the results of recent studies have elucidated the anticancer actions of various mushroom components (Kobori et al., 2007). The best known of these is the immunomodulatory effect of polysaccharides belonging to the β -glucans. In clinical trials, the β -D-glucans were shown to have some beneficial effects against cancer (Kidd, 2000; Zaidman et al., 2005). The fungal sterol, ergosterol, is abundant in mushrooms and is known to be pro-vitamin D2. Ergosterol was shown to inhibit inflammatory ear oedema in mice (Yasukawa et al., 1994), and vitamin D2 has been shown to contribute to prevention of prostate and colon cancer (Guyton et al., 2003). The ergosterol peroxide, 5 α ,8 α -epidioxy-22E-ergosta-6, 22-dien-3-ol, is common in mushrooms (Yaoita et al., 2002; Takei et al., 2005), and shows antitumor, antioxidative and

immunosuppressive properties (Takei et al., 2005), but the molecular mechanism of its antitumor action has not been clarified. *P. eryngii* is an edible mushroom and has been shown to possess antimicrobial and antioxidant activities (Kalyoncu et al., 2010). *P. eryngii* extract (PEE) used in this study has an ergosterol and ergosterol peroxide and these contain respectively 5.5% and 15.4%. In the present study, we investigated the effects of *P. eryngii* PEE on the inflammatory allergic reaction and possible mechanisms by which PEE may inhibit the activities of allergic mediators in allergen-stimulated mast RBL-2H3 cells.

The mast cell is a key player in the early allergic response, which typically starts within minutes of exposure to an appropriate antigen, and in other biological responses, including inflammatory disorders (Bochner and Schleimer, 2001). These cells are best known for their role as the critical effector cells in IgE-dependent immediate-type hypersensitivity reactions (Galli, 1993). Mast cell degranulation can initiate an acute inflammatory response and contribute to the progression of chronic diseases (Nguyen et al., 2005). When IgE-antigen binds to Fc ϵ RI, the Fc ϵ RI is activated, resulting in the release of biologically active mediators that cause several allergic reactions such as degranulation and expression and secretion of cytokines (Siraganian et al., 2002). Antigen-mediated aggregation of Fc ϵ RI on mast cells leads to transphosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) by Src family protein tyrosine kinase (PTK) Lyn. The protein tyrosine kinase Syk was recruited to the phosphorylated ITAMs through its tandem Src homology 2 region (SH2) domains. Recent studies emphasize the key role of the protein tyrosine kinases Lyn and Fyn in mast cell signal transduction (Kopeck et al., 2006). In these cells, surface-bound IgE is cross-linked by antigen, leading to mast cell activation and release of potent mediators such as histamine (Marchand et al., 2003). PEE significantly suppresses degranulation and histamine release in antigen-sensitized mast cells. During the pathogenesis of allergic diseases, IL-4 plays crucial roles in processes such as the induction of IgE synthesis and the development of mast cells (Nabeshima et al., 2005). IL-4 also modulates the inflammatory response. This is due in part to its ability to affect adhesion molecule expression and cytokine production by endothelial cells and to influence the growth and/or activation states of neutrophils, mast cells, T cells, and eosinophils (Boulay and Paul, 1992). PEE markedly reduced IL-4 secretion, mRNA expression, and transcriptional activity in antigen-stimulated mast cells, suggesting that the anti-allergic effect of PEE is a result of IL-4 reduction in antigen-stimulated mast cells.

NFAT is a complex of transcription factors that was first described on the basis of its ability to regulate the expression of the IL-2 gene (Israel, 1994). Several recent observations implicate a component of NFAT in IL-4 gene transcription in T cells, and NFAT has been proposed as a transcriptional activator of cytokine genes (Cron et al., 1999). NF- κ B is a dimeric transcription factor formed by the hetero- or homodimerization of proteins in the Rel family, including p50 and p60 (Hanada and Yoshimura, 2002). It plays important roles in immune responses and in cell differentiation induced by cytokines, growth factors, and other cell activators (Shen and Stavnezer, 1998). Our data demonstrate that PEE inhibits the transcriptional activity of NFAT and NF- κ B in antigen-stimulated mast cells. These results suggest that PEE may block the induction of IL-4 in antigen-stimulated mast cells by inhibiting NFAT and NF- κ B activation.

Mast cells also participate in allergy and inflammation by secreting inflammatory mediators such as histamine and proinflammatory cytokines, including IL-1, IL-6, and TNF- α (Park et al., 2008). Activation of NF- κ B proteins also plays a central role in inflammation through the regulation of genes encoding proinflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes such as COX-2 and iNOS (Baldwin,

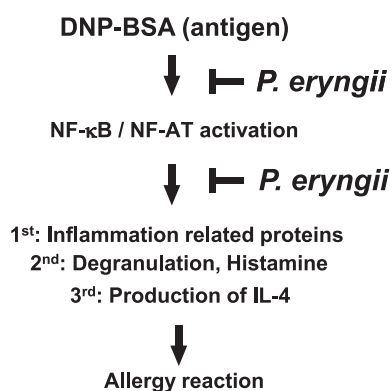


Fig. 8. Potential anti-allergic effects of PEE in antigen-stimulated mast cells. Antigen-mediated aggregation of FcεRI on mast cells induces transcriptional activation of NFAT and NF-κB, which ultimately leads to mast cell degranulation and the induction of IL-4 gene expression. PEE inhibits the activation of NFAT and NF-κB, and thereby inhibits their consequent effects in mast cells. PEE also reduces the levels of allergy-related signal proteins, including inflammation-related proteins. Thus, PEE has potential anti-allergic effects by inhibiting allergy-related signal proteins and NFAT-, NF-κB- and FcεRI-mediated signaling in antigen-stimulated mast cells.

1996). COX-2 expression is activated by several cytokines in allergic inflammation (Han et al., 2011), and it catalyzes the synthesis of inflammatory prostaglandins, notably PGE₂, which may cause matrix deposition and suppresses the immune response (Martelletti, 1995). The ability of PGE₂ to modulate mast cell function has been noted for many years, and PGE₂ has been reported to both enhance mast cell degranulation and inhibit the release of mast cell mediators (Stock et al., 2001). Nitric oxide (NO), which is produced by iNOS, is associated with mast cell-dependent inflammatory events (Lim, 2010). Therefore, allergy-related signaling systems (e.g., COX-2, PGE₂, NO, and iNOS) have attracted attention as potential therapeutic targets for the prevention or treatment of many inflammatory allergic diseases. Interestingly, treatment with PEE decreases the levels of these proteins in antigen-stimulated mast cells, and thus it may downregulate mast cell-dependent inflammatory events.

Antigen-mediated aggregation of FcεRI on mast cells triggers the transphosphorylation of immunoreceptor tyrosine-based activation motifs (Yamashita et al., 1994). PEE reduces antigen-induced activation of Lyn, but does not influence antigen-induced activation of Syk. The activation of Lyn results in the tyrosine phosphorylation of other proteins, including PLCγ2 and PKC. These initial interactions ultimately lead to mast cell degranulation and the induction of cytokine gene expression, resulting in the subsequent activation of transcriptional factors that may stimulate IL-4 and COX-2 gene expression. Thus, by suppressing FcεRI-mediated signaling, PEE produces potential anti-allergic effects.

In conclusion, *P. eryngii* extract downregulated the level of IL-4 and allergy-related signaling proteins by inhibiting NFAT-, NF-κB-, and FcεRI-mediated signaling in antigen-stimulated mast cells (Fig. 8). Its clinical effectiveness remains to be determined in an *in vivo* model, as does the identification of the active components in *P. eryngii* extract. We are still investigating that what is the active components in an ethanol extract of *P. eryngii* for preventive effects on antigen-stimulated allergic reactions. Previous study reported that ergosterol and its peroxides from edible mushroom *Hypsizipus marmoreus* have antioxidant and anti-inflammatory effect (Yoshino et al., 2008). Further study is needed to identify the anti-allergic components in the ethanol extract of *P. eryngii*. Taken together, these results suggest that *P. eryngii* extract may provide insight into the prevention or treatment of mast cell-dependent allergic diseases.

Conflict of Interest

The authors declare that there are no conflicts of interest.

5. Uncited reference

Kobori et al. (2006).

Acknowledgments

This research was financially supported by grants from the Ministry of Knowledge Economy (MKE) and Korea Institute for Advancement of Technology (KIAT) through the Research and Development for Regional Industry (2009-70006470) and the Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by Ministry of Education, Science and Technology (2009-0093815), Republic of Korea.

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