



Ethyl acetate extract of *Psidium guajava* inhibits IgE-mediated allergic responses by blocking FcεRI signaling

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ABSTRACT

Psidium guajava (*P. guajava*) is an important food crop and medicinal plant with antioxidant, anti-inflammatory, and anti-allergic activities, supporting its traditional uses. However, its precise effects remain unknown. We investigated the effects of *P. guajava* ethyl acetate extract (PGEA) on IgE-mediated allergic responses in rat mast RBL-2H3 cells. PGEA reduced antigen (DNP-BSA)-induced release of β-hexosaminidase and histamine in IgE-sensitized RBL-2H3 cells. In addition, it inhibited antigen-induced IL-4 and TNF-α mRNA expression and protein production in IgE-sensitized RBL-2H3 cells. PGEA also suppressed antigen-induced COX-2 mRNA and protein expression in these cells, as well as antigen-induced activation of NFAT and reactive oxygen species. Moreover, it inhibited antigen-induced activation of NF-κB and degradation of IκB-α. To identify the mechanisms underpinning the inhibition of degranulation and cytokine production by PGEA, we examined the activation of intracellular FcεRI signaling molecules. PGEA suppressed antigen-induced phosphorylation of Syk, LAT, Gab2, and PLCγ2 but not Lyn, and inhibited antigen-induced phosphorylation of downstream signaling intermediates including MAP kinases and Akt. Collectively, the anti-allergic effects of PGEA *in vitro* suggest its possible therapeutic application to inflammatory allergic diseases, in which its inhibition of inflammatory cytokine production and FcεRI-dependent signaling events in mast cells may be hugely beneficial.

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1. Introduction

Mast cells are secretory cells that are central to specific and innate immunity, allergic responses, and inflammation (McDermott et al., 2003; McLachlan et al., 2003). They also play an important role in initiating and perpetuating inflammatory responses in allergic reactions by secreting large amounts of cytokines such as interleukin-4 (IL-4) and tumor necrosis factor (TNF)-α (Bradding et al., 1994). In response to antigen stimulation, naive T helper cells differentiate into at least two types of effector cells, classified according to their distinct patterns of cytokine expression, as well as their different effects on ongoing immune responses (Kiani et al., 1997). The hallmark of T helper 1 (Th1) cells is the secretion of interferon-γ (IFNγ), which primarily promotes cell-mediated immunity, while Th2 cells secrete IL-4, IL-5, IL-10, and IL-13 and

activate immunoglobulin E (IgE) and mediate eosinophilic responses. IL-4 is essential for IgE production (Kuhn et al., 1991) and promotes the differentiation of naive T cells into Th2 cells (Hines, 2002). It also acts as a mast cell growth factor *in vitro* and down-regulates high-affinity IgE receptor (FcεRI) expression on mouse bone marrow-derived mast cells (Bischoff et al., 1999). The activation of mast cells by antigen is partially realized through the induction of nuclear factor κB (NF-κB) and nuclear factor of activated T-cells (NFAT) (Saito et al., 2009). NF-κB is thought to play an important role in the regulation of proinflammatory cytokines, notably TNF-α, IL-6, and IL-8 (Salamon et al., 2005). It is a transcription factor that regulates the expression of genes involved in immune and inflammatory responses that involve inflammatory cytokine production (Marquardt and Walker, 2000). NFAT is a transcription complex believed to mediate the final step in the signal transduction pathway linking T-cell receptor engagement with the expression of IL-2 (Hutchinson and McCloskey, 1995).

In cultured mast cells, including the well-studied RBL-2H3 cell line, free arachidonic acid is rapidly metabolized to prostaglandin by cyclooxygenase (COX) during allergic reactions (Hundley

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et al., 2001; Igarashi et al., 1993; Westcott et al., 1996; Wong et al., 1992). This initial prostaglandin synthesis is mediated by constitutively expressed COX-1, while a subsequent phase of prostaglandin production follows the induction of COX-2 mRNA and protein expression (Ashraf et al., 1996; Reddy and Herschman, 1997). While COX-1 is expressed constitutively in many cell types, in which it is believed to perform “housekeeping” activities central to normal cellular function, expression of COX-2 is induced in certain cells by various inflammatory cues (Mitchell et al., 1995; Herschman 1996). In general, the inflammatory responses in allergic reactions are associated with the induction of COX-2, but not COX-1. Several studies have reported that rat RBL-2H3 mast cells (Matsui et al., 2000; Yoshimaru et al., 2002) release reactive oxygen species (ROS) into the extracellular milieu in response to a range of stimuli and that mast cell-derived ROS are involved in allergic inflammation (Springer et al., 2007).

The activation of signaling pathways in antigen-stimulated mast cells initially depends on the interaction of FcεRI with the Src kinase Lyn, and on the subsequent 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). The MAP kinases are classified into at least three groups: extracellular signal-regulated kinase (ERK), p38 MAP kinase, and c-Jun N-terminal kinase (JNK).

P. guajava, an important food in tropical and subtropical countries, is widely used in folk medicines. It is commonly used to treat gastrointestinal and respiratory disturbances and as an anti-inflammatory medicine (Gutiérrez et al., 2008). Ethyl acetate extract of *P. guajava* leaves contains polyphenol and it is typical free radical scavengers (Hsieh et al., 2005, 2007). Polyphenols, as a class, have antioxidant properties, and depending on the specific type, often have other medicinal effects like anti-cancer, anti-inflammatory and anti-allergic activity (Hsieh et al., 2007). In mice, *P. guajava* extracts were shown to shift the Th1/Th2 balance to a Th1-dominant status by directly attenuating T cell activity (Seo et al., 2005). However, the precise anti-allergic effects of *P. guajava* and the signaling pathways involved remain unknown. We investigated the effects of *P. guajava* on IgE-mediated allergic responses and on multiple components of FcεRI signaling pathways.

2. Materials and methods

2.1. Materials

Anti-dinitrophenyl (DNP)-IgE and 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide were obtained from Sigma Aldrich Co. (St. Louis, MO, USA); PGE₂ immunoassay reagents and anti-COX-2 antibody were purchased from Cayman Co. (Ann Arbor, MI, USA); Minimum essential medium with Eagle's salt (EMEM) and fetal bovine serum (FBS) were obtained from Life Technologies, Inc. (Carlsbad, CA, USA); DNP-bovine serum albumin (BSA), 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA), a WST-1 (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfonylphenyl]-2H-tetrazolium, monosodium salt) assay kit, a luciferase assay system, pCMV-β-gal, LipofectAMINE 2000, enzyme immunoassay reagents for cytokine assays, and a protein assay kit were purchased from Calbiochem (La Jolla, CA, USA), Molecular Probes (Eugene, OR, USA), Roche Co. (London, UK), Promega (Sunnyvale, CA, USA), Clontech (Palo Alto, CA, USA), Invitrogen Inc. (Carlsbad, CA, USA), R&D Systems (Minneapolis, MN, USA), and Bio-Rad Laboratories Inc. (Hercules, CA, USA), respectively. Primary antibodies (anti-IκB-α/phospho-IκB-α [Ser32/36], anti-MAPK [ERK1/2]/phospho-MAPK [ERK1/2] [Tyr202/204], anti-p38 MAPK/phospho-p38 MAPK [Tyr180/182], anti-SAPK/JNK/phospho-SAPK/JNK1/2 [Tyr183/185], anti-Syk/phospho-Syk [Tyr525/526], anti-Lyn/phospho-Lyn [Tyr507], anti-Syk/phospho-Syk, anti-PLCγ2/phospho-PLCγ2 [Tyr1217], anti-Gab2/phospho-Gab2 [Tyr452], and anti-Akt/phospho-Akt [Ser473]) and secondary antibody (HRP-linked anti-rabbit and anti-mouse IgG) were obtained from Cell Signaling Technology (Danvers, MA, USA). β-Actin was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA,

USA). ECL reagent and polyvinylidene difluoride (PVDF) membrane were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Oligonucleotide PCR primers were custom synthesized by Bioneer Co. (Korea). All chemicals were of the highest commercially available grade.

2.2. Plant material

P. guajava ethyl acetate extracts (PGEA) was prepared as described by Seo et al (2005). The air-dried leaves of *P. guajava* (130 g) were extracted with ethyl acetate (EtOAc) and the yield of PGEA corresponded to 0.15% and the extract powder was dissolved in dimethyl sulfoxide (DMSO).

2.3. Cell culture

The rat mast cell line RBL-2H3 was obtained from the American Type Culture Collection (Bethesda, MD) and grown in 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. Stock solutions of PGEA dissolved in DMSO were directly applied to the culture medium. Control cells were treated with DMSO alone. Final solvent concentrations did not exceed 0.1%. Moreover, DMSO was shown (at the concentrations used) not to affect the assay systems.

2.4. Cytotoxicity assay

Cell cytotoxicity was assessed using a WST-1 assay kit according to the manufacturer's instructions. Briefly, IgE-sensitized RBL-2H3 cells were seeded to 96-well plates. After growth in 10% FBS-EMEM at 37 °C for 24 h, various concentrations of PGEA (0.1–100 μg/mL) were applied to the culture medium. After incubation at 37 °C, cells were analyzed using the assay kit. Relative cytotoxicity was quantified by measuring spectrophotometer (Varioskan, Thermo Electron Co.) absorbances at 550 nm, a wavelength at which PGEA does not produce an interfering signal.

2.5. β-Hexosaminidase release assay

IgE-sensitized RBL-2H3 cells were treated with PGEA (10–50 μg/mL) and DNP-BSA in Tyrode's buffer (Han et al., 2009). Degranulation was terminated by placing cells on ice. To determine the quantities of β-hexosaminidase released by cells, supernatants and 1.2 mM β-hexosaminidase substrate (4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide) were mixed in sodium acetate buffer (pH 4.4) and incubated for 30 min at 37 °C. Cells were then lysed using 0.1% Triton-X 100 and the supernatants were isolated. Supernatant β-hexosaminidase activity was quantified by measuring levels of a fluorescent product using a spectrofluorometer (excitation, 360 nm; emission, 450 nm).

2.6. Histamine release assay

IgE-sensitized RBL-2H3 cells were pre-incubated with PGEA (10–50 μg/mL) for 30 min and then incubated with DNP-BSA for 15 min. Histamine content was measured by using the o-phthalaldehyde spectrofluorometric procedure. The fluorescence intensities at 440/360 nm were measured.

2.7. Measurement of ROS generation

Intracellular ROS levels were measured using H₂DCF-DA, which is deacetylated by intracellular esterases, yielding non-fluorescent 2',7'-dichlorodihydrofluorescein (DCFH). DCFH is in turn oxidized to the fluorescent compound 2',7'-dichlorodihydrofluorescein (DCF) by ROS. IgE-sensitized RBL-2H3 cells were pre-incubated with H₂DCF-DA for 30 min at 37 °C and then washed to remove excess H₂DCF-DA. IgE-sensitized RBL-2H3 cells were treated with PGEA (10–50 μg/mL) and/or DNP-BSA for 30 min. Finally, fluorescence intensity was measured using a spectrofluorometer (excitation, 485 nm; emission, 530 nm). DCF fluorescence intensities were calculated relative to control levels.

2.8. Transient transfection and luciferase activity assay

Cells were transfected with target luciferase vectors and pCMV-β-gal plasmid using a serum- and antibiotic-free LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA). The IL-4 and NFAT-Luc vectors were gifts from Dr. Kenneth M. Murphy (Washington University, USA). The COX-2 promoter-luciferase construct was a generous gift from Dr. Hiroyasu Inoue (Nara Women's University, Nara, Japan). The NF-κB luciferase reporter construct was purchased from Stratagene (Grand Island, NY). Four hours later, the growth medium was replaced with basal medium. IgE-sensitized RBL-2H3 cells were pretreated with PGEA (10–50 μg/mL) for 30 min and then treated with DNP-BSA for 18 h. Cells were lysed and cellular extract luciferase and β-galactosidase activities were measured. For each sample, the luciferase signal was normalized to β-galactosidase activity and expressed relative to control values.

2.9. Measurement of cytokine production

IgE-sensitized RBL-2H3 cells were treated with PGEA (10–50 µg/mL) and/or DNP-BSA for 3 or 24 h. Supernatants were harvested, and then IL-4 and TNF-α production were quantified at 24 and 3 h, respectively, by sandwich immunoassay, performed according to the protocols provided by R&D Systems.

2.10. RNA preparation and quantitative real-time PCR

IgE-sensitized RBL-2H3 cells were pretreated with PGEA (10–50 µg/mL) for 30 min and then with DNP-BSA for 3 h (TNF-α), 20 h (COX-2), or 24 h (IL-4). Total RNA was extracted from treated cells using RNeasy Reagent (Takara) according to the manufacturer's protocol. Product formation was continuously monitored during PCR reactions using Sequence Detection System software, version 1.7 (Applied Biosystems, Foster City, CA). PCR product accumulation was detected by monitoring reporter dye (SYBR) fluorescence. Cytokine expression in treated cells was compared to that in controls using the comparative cycle threshold-method (Johnson et al., 2000). The following primers were used: TNF-α forward, 5'-CAA GGA GGA GAA GTT CCC AA-3'; TNF-α reverse, 5'-CGG ACT CCG TGA TGT CTA AG-3'; IL-4 forward, 5'-ACC TTG CTG TCA CCC TGT TC-3'; IL-4 reverse, 5'-TTG TGA GCG TGG ACT CAT TC-3'; COX-2 forward, 5'-AAG AAA TGG CTG CAG AGT TGA AA-3'; COX-2 reverse, 5'-AGG GCG GGA TAC AAG TCC A-3'; β-actin forward, 5'-TGG CAC CCA GCA CAA TGA A-3'; β-actin reverse, 5'-CTA AGT CAT AGT CCT AGA AGC A-3'. Levels of each target transcript were calculated as described in the instrument manual and normalized to those of the housekeeping gene β-actin.

2.11. Subcellular fractionation

Cytosolic and nuclear fractions were prepared as described previously (Kabouridis et al., 1997). Briefly, cells were pretreated with PGEA (10–50 µg/mL) for 30 min and then treated with DNP-BSA for 30 min. They were resuspended in hypotonic solution (25 mM Tris, pH 7.5, 5 mM EGTA, 250 mM sucrose, 25 µg/mL aprotinin, 1 mM PMSF, 25 µg/mL leupeptin, 5 mM NaF, 1 mM Na₃VO₄) and subjected to two successive freeze-thaw cycles. Cell suspensions were then homogenized on ice and their NaCl concentrations were adjusted to 150 mM. Cell nuclei were removed by centrifugation twice at 480g for 5 min at 4 °C. Soluble and particulate fractions were separated by centrifugation at 100,000g for 30 min.

2.12. Western blotting

IgE-sensitized RBL-2H3 cells were pretreated with PGEA (10–50 µg/mL) for 30 min and then treated with DNP-BSA for 5 min (Syk, Lyn, LAT, Gab2, PLCγ2, Akt, and MAP kinase), 30 min (IκB-α) and 24 h (COX-2). Equal amounts of cellular protein (50 µg) were resolved by 10% SDS-PAGE and transferred to PVDF. After blocking, membranes were successively incubated with primary antibody and horseradish peroxidase-conjugated anti-IgG secondary antibody. Immunoreactive proteins were visualized using the ECL reagent. Target protein levels were compared to those of a loading control (β-actin or non-phosphorylated protein).

2.13. PGE₂ production

IgE-sensitized RBL-2H3 cells were incubated with PGEA (10–50 µg/mL) or DNP-BSA for 24 h, after which culture media were collected. Amounts of PGE₂ released into the culture medium were measured using a specific enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol. PGE₂ concentrations were determined by measuring absorbances at 415 nm.

2.14. Statistical analysis

All experiments were repeated at least three times. One-way analysis of variance (ANOVA) was used to identify statistically significant differences between treatment groups. The Newman-Keuls test was used for multi-group comparisons. A *p* value of <0.01 was considered to be statistically significant.

3. Results

3.1. PGEA inhibits antigen-induced degranulation and histamine release in IgE-sensitized mast cells

We first measured the cytotoxicity of PGEA to RBL-2H3 cells by WST-1 assay. Treatment for 24 h with PGEA at concentrations ranging from 0.1 to 50 µg/mL did not produce any significant cytotoxic effects (Fig. 1A). We thus treated DNP-IgE-sensitized RBL-2H3 mast cells with PGEA at concentrations ranging from 10 to 50 µg/mL in subsequent experiments. The rat basophilic leuke-

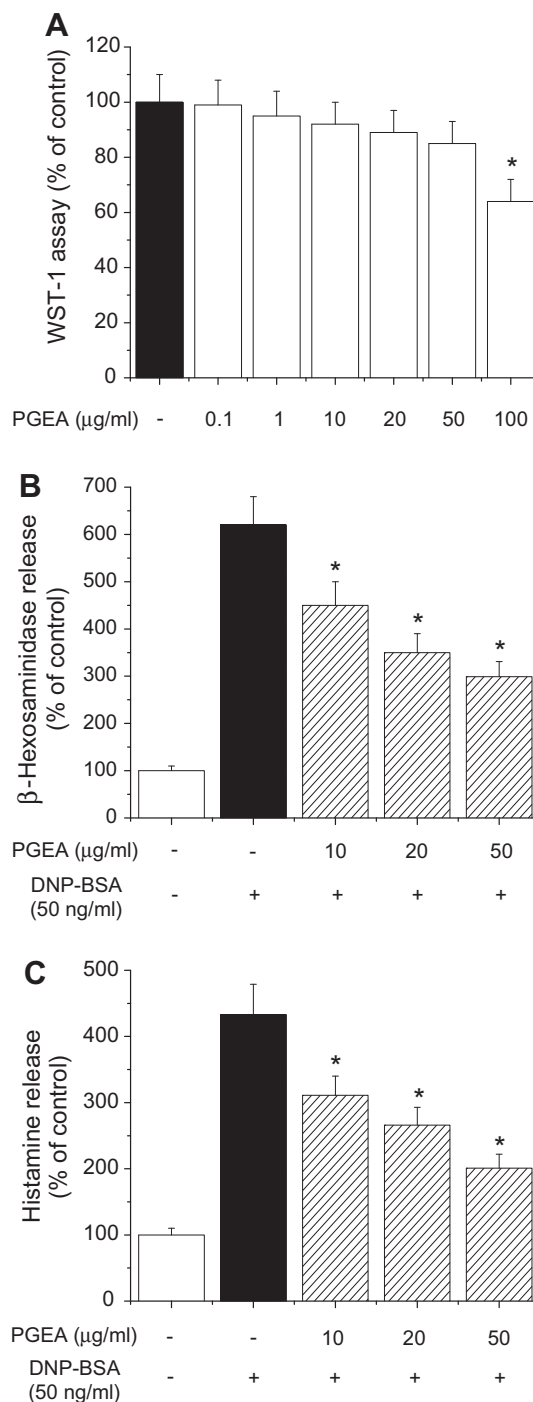


Fig. 1. Effects of PGEA on antigen-induced RBL-2H3 cell degranulation and histamine release. (A) Effects of PGEA on cytotoxicity. Cells were seeded to a 96-well plate and treated with various concentrations of PGEA for 24 h. Cell viability was assessed by WST-1 assay. (B and C) Effects of PGEA on antigen-induced degranulation and histamine release. Cells were incubated overnight in 48-well plates in medium containing DNP-specific IgE (100 ng/mL). Growth medium was replaced with Tyrode's buffer containing PGEA (10–50 µg/mL) prior to stimulation with DNP-BSA (50 ng/mL) for 10 min. Each bar represents the mean of three independent experiments. **P* < 0.01 vs. positive control (DNP-BSA).

mia cell line RBL-2H3, a tumor analog of mast cells, exhibits phenotypic characteristics of mucosal mast cells. After stimulation with antigen, RBL-2H3 cells release β-hexosaminidase, a marker of mast cell degranulation. They are therefore considered a good model for studying the responses of mast cells to multivalent allergens (McDermott et al., 2007; Marchand et al., 2003). The secretion

of β -hexosaminidase is a hallmark of allergic reactions resulting from exposure to allergen. We therefore examined the effects of PGEA on β -hexosaminidase release. IgE-sensitized RBL-2H3 cells were pre-incubated with PGEA (10–50 $\mu\text{g}/\text{mL}$) for 30 min and then incubated with antigen (DNP-BSA, 50 ng/mL) for 15 min. PGEA significantly and dose-dependently suppressed DNP-BSA-induced degranulation of IgE-sensitized RBL-2H3 cells (Fig. 1B).

Chemical mediators including histamine released from mast cells play important roles in allergic reactions (Marchand et al., 2003). We next examined the effects of PGEA on histamine release. DNP-IgE-sensitized RBL-2H3 cells were pre-incubated with PGEA (10–50 $\mu\text{g}/\text{mL}$) for 30 min and then incubated with DNP-BSA for 15 min. PGEA inhibited the DNP-BSA-induced release of histamine from IgE-sensitized RBL-2H3 cells (Fig. 1C). Collectively, these results suggest that PGEA significantly inhibits antigen-induced mast cell degranulation and histamine release.

3.2. PGEA inhibits antigen-induced TNF- α and IL-4 mRNA expression and protein production in IgE-sensitized mast cells

We next examined whether PGEA influences the expression and secretion of IL-4 and TNF- α in IgE-sensitized RBL-2H3 cells. Cells were treated with PGEA (10–50 $\mu\text{g}/\text{mL}$) and/or DNP-BSA for 3 h (TNF- α) or 24 h (IL-4). PGEA significantly and dose-dependently inhibited antigen-induced IL-4 and TNF- α production (Fig. 2A). We further tested whether PGEA similarly suppressed the expression of IL-4 and TNF- α mRNA. IgE-sensitized RBL-2H3 cells were treated with PGEA (10–50 $\mu\text{g}/\text{mL}$) and/or DNP-BSA for 3 h or 24 h, respectively. PGEA dose-dependently inhibited antigen-induced expression of mRNA encoding both cytokines (Fig. 2B). It also significantly reduced antigen-induced IL-4 luciferase activity in IgE-sensitized RBL-2H3 cells (Fig. 2C). These results suggest that PGEA markedly inhibits antigen-induced gene expression and the production of cytokines associated with allergic reactions.

3.3. PGEA inhibits antigen-induced COX-2 mRNA expression and protein production in IgE-sensitized mast cells

Several studies have reported that induction of allergic reactions in RBL-2H3 cells is regulated by COX-2 (Hundley et al., 2001; Igarashi et al., 1993; Westcott et al., 1996; Wong et al., 1992). We investigated whether or not PGEA influences COX-2 expression and/or activity in IgE-sensitized RBL-2H3 cells. Cells were treated with PGEA (10–50 $\mu\text{g}/\text{mL}$) and/or DNP-BSA for 20 h. PGEA dose-dependently inhibited antigen-induced COX-2 mRNA expression (Fig. 3A). To clarify the nature of this response, we transfected RBL-2H3 cells with an hCOX-2-Luc reporter construct. Treatment with PGEA and/or DNP-BSA inhibited antigen-induced COX-2 luciferase activity (Fig. 3B), suggesting that PGEA may regulate the transcriptional activation of COX-2 in RBL-2H3 cells. We next used a COX-2-specific antibody to investigate the effect of PGEA on antigen-induced COX-2 protein expression. PGEA significantly reduced COX-2 protein levels in antigen-exposed cells (Fig. 3C). COX-2 catalyzes the conversion of arachidonic acid to prostanooids and thus is central to the generation of PGE₂ (Hla et al., 1999; Simpson, 2004). PGEA inhibited antigen-induced production of PGE₂ (Fig. 3D). These results indicate that the inhibition of allergic reactions in PGEA-treated RBL-2H3 cells correlates with reduced COX-2 activity.

3.4. PGEA inhibits antigen-induced activation of NFAT and NF- κB and production of ROS in IgE-sensitized mast cells

To evaluate the mechanisms by which PGEA inhibits IL-4 mRNA expression and protein production, we measured NFAT transcriptional activity. NFAT transcriptional activity is tightly regulated

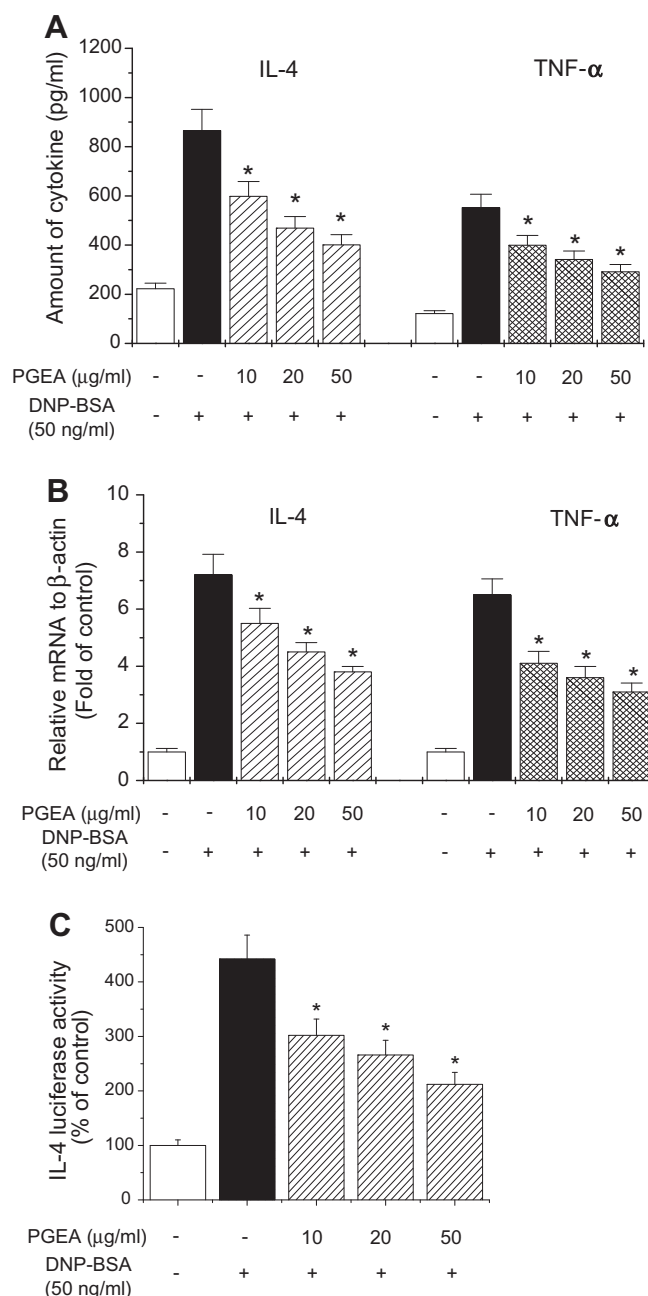


Fig. 2. Effects of PGEA on antigen-induced TNF- α and IL-4 mRNA expression and protein secretion in RBL-2H3 cells. (A) Effects of PGEA on antigen-induced production of TNF- α and IL-4. IgE-primed cells were stimulated with DNP-BSA (50 ng/mL) (or left unstimulated) and treated with PGEA for 3 h (TNF- α) and 24 h (IL-4). Quantities of TNF- α and IL-4 released into the culture medium were assessed using commercially available ELISA kits. (B) Effects of PGEA on antigen-induced TNF- α and IL-4 mRNA expression. Cells were sensitized overnight with DNP-specific IgE (100 ng/mL) and pretreated with PGEA for 30 min. Then they were stimulated with DNP-BSA (50 ng/mL) for 3 h (TNF- α) and 24 h (IL-4) and lysed. Total RNA was subsequently prepared and analyzed for the expression of TNF- α , IL-4, and the housekeeping gene β -actin by real-time PCR. Expression of TNF- α and IL-4 mRNA in treated cells was compared to that in untreated cells at each time point. (C) Effect of PGEA on IL-4 promoter activity. Cells were transfected with IL-4-Luc and then treated with PGEA (10–50 $\mu\text{g}/\text{mL}$) and/or DNP-BSA for 20 h. Then they were harvested and assayed for luciferase activity. Each bar represents the mean of three independent experiments. * $P < 0.01$ vs. positive control (DNP-BSA).

during Th1/Th2 development in NFAT reporter transgenic mice (Rincón and Flavell, 1997), and NFAT is an indispensable transactivator of several cytokine genes (Rao et al., 1997). Indeed, NFAT

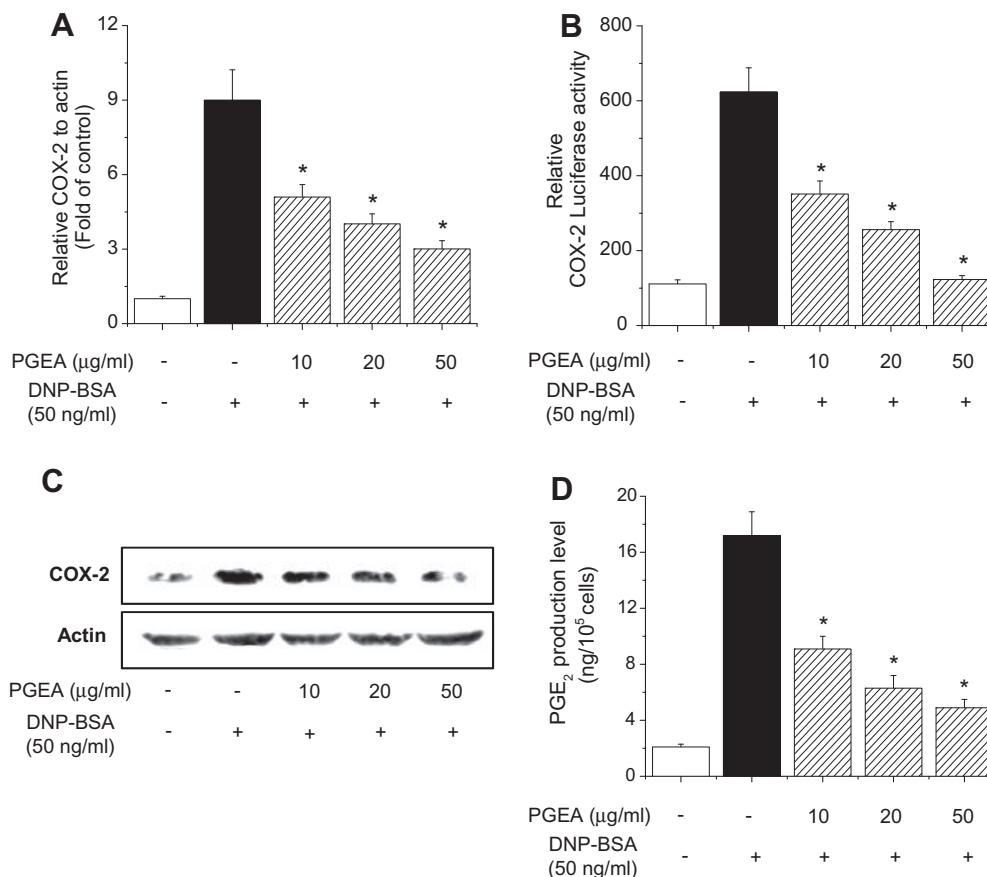


Fig. 3. Effects of PGEA on antigen-induced COX-2 expression and PGE₂ production in RBL-2H3 cells. (A) Effect of PGEA on COX-2 mRNA levels. Cells were treated with PGEA (10–50 μg/mL) and/or DNP-BSA (50 ng/mL) for 20 h. They were then lysed and total RNA was extracted and analyzed by PCR for the expression of COX-2 and the housekeeping gene β-actin. (B) Effect of PGEA on COX-2 gene transcription. Cells were transfected with COX-2-Luc and pCMV-β-gal and then treated with PGEA (10–50 μg/mL) and/or DNP-BSA (50 ng/mL) for 20 h. Then they were harvested and assayed for luciferase activity. (C) Effect of PGEA on COX-2 protein expression. Cells were treated with PGEA (10–50 μg/mL) and/or DNP-BSA (50 ng/mL) for 24 h. PVDF membranes were probed using a COX-2-specific antibody. Each blot shown is representative of three independent experiments, each yielding similar results. COX-2 protein levels were compared to those of β-actin. (D) Effect of PGEA on PGE₂ production. Cells were treated with PGEA (10–50 μg/mL) and/or DNP-BSA (50 ng/mL) for 24 h. Growth media were collected and assayed for PGE₂ levels by enzyme immunoassay. Each bar represents the mean of three independent experiments. **P* < 0.01 vs. positive control (DNP-BSA).

binds to and transactivates the IL-4 promoter (Li-Weber and Krammer, 2003). PGEA significantly reduced antigen-induced NFAT-luciferase activity in IgE-sensitized RBL-2H3 cells (Fig. 4A). To further investigate the mechanisms underpinning the effects of PGEA on mast cell cytokine secretion, we probed the effects of PGEA on NF-κB activation using an NF-κB-luciferase reporter vector. PGEA markedly suppressed antigen-induced NF-κB-luciferase activity in IgE-sensitized RBL-2H3 cells (Fig. 4B). In unstimulated cells, NF-κB binds to IκB-α in the cytoplasm. Phosphorylation of IκB-α, which regulates the nuclear translocation of NF-κB, was lower in IgE-sensitized RBL-2H3 cells treated with PGEA (Fig. 4C). IκB-α was rapidly degraded when IgE-sensitized RBL-2H3 cells were treated with PGEA (Fig. 4C). β-Actin was used as a loading control (Fig. 4C).

Mast cells secrete chemical mediators such as histamine, prostaglandin, ROS, and cytokines during specific immune responses (Yoshimaru et al., 2002; Matsui et al., 2000). RBL-2H3 cells release ROS in response to various stimuli (Matsui et al., 2000). We thus tested whether PGEA suppresses the production of ROS in IgE-sensitized RBL-2H3 cells. We found that antigen stimulated ROS production (Fig. 4D) and that, as anticipated, PGEA inhibited ROS production in IgE-sensitized RBL-2H3 cells (Fig. 4D). These results indicate that NFAT, NF-κB, and ROS are responsible for allergic reactions and that PGEA has potential as an anti-allergy therapeutic.

3.5. PGEA inhibits antigen-induced, FcεRI-mediated signaling events in IgE-sensitized mast cells

To gain insight into how PGEA suppresses mast cell activation, we examined its effects on FcεRI-mediated signaling events, specifically the phospho-activation of Syk by Lyn and phosphorylation of LAT and Gab2 by activated Syk. Antigen-induced tyrosine phosphorylation of Syk was inhibited by PGEA in IgE-sensitized RBL-2H3 cells (Fig. 5A). Moreover, PGEA significantly and dose-dependently inhibited Syk-mediated downstream phospho-activation of LAT and Gab2 (Fig. 5A).

Calcium mobilization and the phosphorylation and activation of phospholipase C (PLC)γ depend on the phosphorylation of Syk and LAT (Rivera and Gilfillan 2006). We found that PGEA dose-dependently inhibited antigen-induced phosphorylation of PLCγ2 (Fig. 5A). However, PGEA did not influence DNP-BSA-induced activation of Lyn.

Owing to their roles in the production of TNF-α and IL-4 (Fukao et al., 2003; Duan and Wong 2006), we examined the effects of PGEA on PI3-kinase and the MAP kinase signaling. PGEA significantly and dose-dependently inhibited antigen-induced phosphorylation of Akt (a marker of PI3-kinase activation) and the MAP kinases ERK1/2, JNK1/2, and p38 MAP kinase (Fig. 5B). These results indicate that PGEA inhibits antigen-induced, FcεRI-mediated signaling events in IgE-sensitized mast cells.

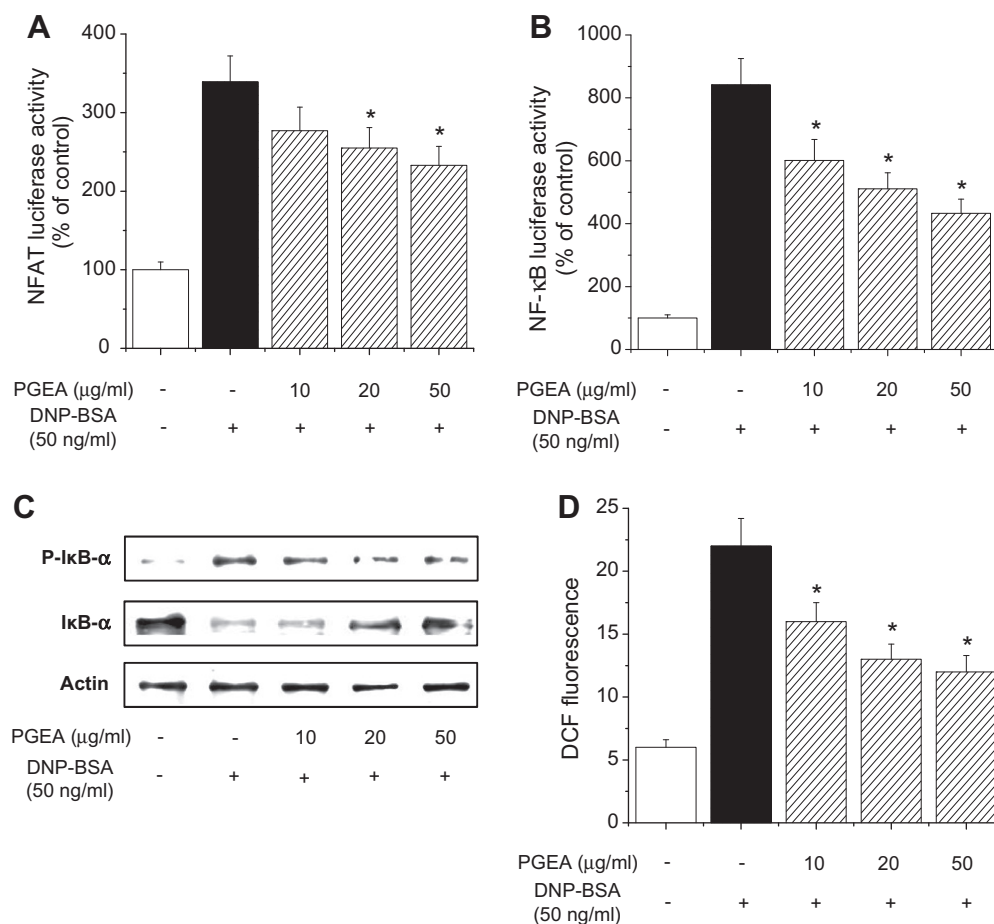


Fig. 4. Effects of PGEA on antigen-induced activation of NFAT and NF-κB, and production of ROS, in RBL-2H3 cells. (A and B) Effects of PGEA on antigen-induced NFAT and NF-κB transcriptional activity. Cells were transiently co-transfected with pCMV-β-gal and NFAT-Luc or pGL3-NF-κB-Luc. Four hours later, they were sensitized with DNP-specific IgE (100 ng/mL) for 16 h. IgE-sensitized cells were then treated with PGEA and stimulated with DNP-BSA (50 ng/mL). They were harvested, and luciferase and β-galactosidase activities were determined. Luciferase activity was normalized to β-galactosidase activity and expressed relative to that detected in the control. (C) PGEA inhibits antigen-induced phosphorylation of IκB-α. IgE-sensitized cells pretreated with PGEA were stimulated with DNP-BSA (50 ng/mL) for 30 min. Western blot analysis was performed as described in the Section 2. Each blot is representative of three independent experiments, each yielding similar results. (D) PGEA inhibits antigen-induced ROS production. Intracellular ROS levels were measured using non-fluorescent H₂DCF-DA. IgE-sensitized RBL-2H3 cells were pre-incubated with H₂DCF-DA (5 μM) for 30 min at 37 °C and then washed. Cells were then treated with PGEA (1–20 μg/mL) and/or DNP-BSA for 30 min. Finally, levels of fluorescence were measured spectrofluorometrically (excitation, 485 nm; emission, 530 nm). Each bar represents the mean of three independent experiments. **P* < 0.01 vs. positive control (DNP-BSA).

4. Discussion

P. guajava is widely consumed as a food and is used in folk medicine to treat gastrointestinal and respiratory disturbances and to combat inflammation (Gutiérrez et al., 2008). Several recent studies have provided evidence to support its putative anti-inflammatory effects (Gutiérrez et al., 2008; Seo et al., 2005). In the present study we investigated the effects of PGEA on IgE-mediated anti-allergic effects in rat basophilic leukemia RBL-2H3 cells.

The β-hexosaminidase assay is a convenient method for monitoring the capacity of novel drugs and other compounds to block mast cell activation and degranulation (Granberg et al., 2001). The release of chemical mediators such as histamine plays an important role in allergic inflammation (Marchand et al., 2003). PGEA significantly suppressed antigen-induced degranulation and histamine release in IgE-sensitized RBL-2H3 cells.

IL-4 is an important cytokine in allergic reactions. As an eosinophil chemoattractant, it stimulates endothelial cells to secrete eosinophil chemotactic factor and eotaxin (Rothenberg et al., 1995) and enhances endothelial cell surface expression of adhesion molecules including vascular cell adhesion molecule-1 (Schleimer et al., 1992). PGEA significantly inhibited IL-4 and TNF-α mRNA expression and protein secretion. These results suggest that the

anti-allergic effect of PGEA stems from its inhibition of mast cell IL-4 and TNF-α production.

COX-2 catalyzes the synthesis of inflammatory prostaglandins, notably PGE₂, which stimulates matrix deposition and suppresses immune responses (Martelletti, 1995). During allergic inflammation, its expression is induced by several cytokines. We found that PGEA suppressed COX-2 mRNA and protein expression and the production of PGE₂. To identify the mechanisms underpinning the effects of PGEA on cytokine secretion, we examined the effects of PGEA on the activation of NFAT and NF-κB. PGEA markedly suppressed the activation of both of these transcription factors. Moreover, it decreased the phosphorylation of IκB-α, which regulates NF-κB translocation, in IgE-sensitized RBL-2H3 cells. These data indicate that PGEA inhibits the production of TNF-α and IL-4 by attenuating NF-κB activation.

Antigen stimulation of IgE-sensitized mast cells triggers a cascade of events leading to degranulation, the release of mediators, MAP kinase activation, the activation of tyrosine kinases and PLC, increased ROS production, calcium influx, and cytokine production (Cambier, 1995). ROS are involved in allergic inflammation (Springer et al., 2007). The observed capacity of PGEA to inhibit ROS production in RBL-2H3 cells suggests that it may be effective in the treatment of allergies.

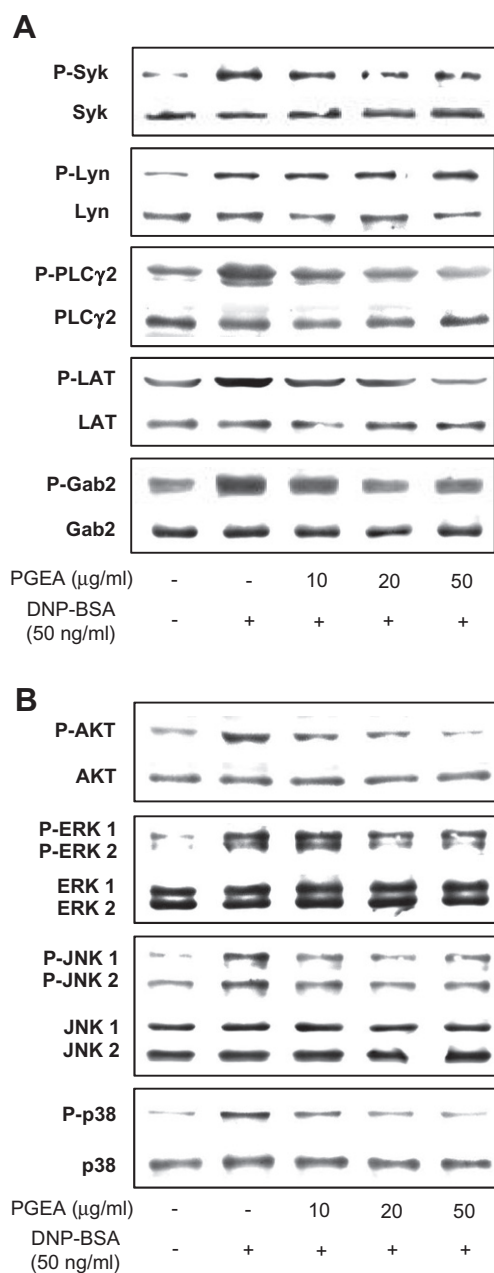


Fig. 5. Effects of PGEA on antigen-induced, FcεRI-mediated signaling events and the phosphorylation of Akt and MAP kinases in RBL-2H3 cells. IgE-sensitized cells were treated with PGEA and stimulated with DNP-BSA (50 ng/mL) for 5 min. (A) Cell extracts were analyzed for levels of Syk, Lyn, PLC γ2, LAT, and Gab2 activation by Western blot analysis using antibodies raised against phosphorylated forms of Syk, Lyn, PLC γ2, LAT, and Gab2. (B) Assessment of Akt and MAP kinase activation. Cell extracts were subjected to Western blot analysis using antibodies raised against phosphorylated forms of Akt, ERK, JNK, and p38.

The activation of signaling pathways in antigen-stimulated mast cells depends initially on the interaction of FcεRI with the Src kinases Lyn and Fyn, and subsequently on the activation of Syk and other downstream tyrosine kinases (Gilfillan and Tkaczyk, 2006). The binding of IgE-antigen to FcεRI activates the latter, triggering the release of biologically active mediators that themselves stimulate allergic processes including degranulation and the expression and secretion of cytokines (Siraganian et al., 2002). Antigen-mediated aggregation of FcεRI on mast cells leads to transphosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) by the Src family protein tyrosine kinase Lyn.

These initial interactions ultimately lead to degranulation and cytokine gene expression. It has been reported that PKC and calcium contribute to the activation of ERK1/2, JNK, and p38 MAP kinase (Gilfillan and Tkaczyk, 2006), while activated Syk regulates small GTPases including Rac, Ras, and Rho. They in turn activate other factors including ERK, JNK, and p38 MAP kinase, which have been implicated in IL-4 and COX-2 gene expression. It thus appears that Syk/PLC/PKC signaling, coupled with an influx of Ca²⁺ ions, may play an important role in degranulation. Moreover, PKC selectively controls IKK activation and subsequent IκB-α degradation. The consequent activation of NF-κB increases the expression of cytokine-encoding genes. NFAT, meanwhile, activates MAP kinase, PKC, and Akt.

To gain insights into the mechanism by which PGEA suppresses mast cell activation, we examined its effects on early FcεRI-mediated signaling events, specifically the phospho-activation of Syk by Lyn and the phosphorylation of LAT and Gab2 by Syk. PGEA suppressed antigen-induced phosphorylation of Syk, LAT, Gab2, and PLC γ2 but not Lyn. It also inhibited antigen-induced phosphorylation of the downstream signaling intermediates Akt, ERK1/2, JNK1/2, and p38 MAP kinase (Fig. 6).

There are many options for treating allergic diseases. They include allergen-specific immunotherapy, DNA vaccination, adminis-

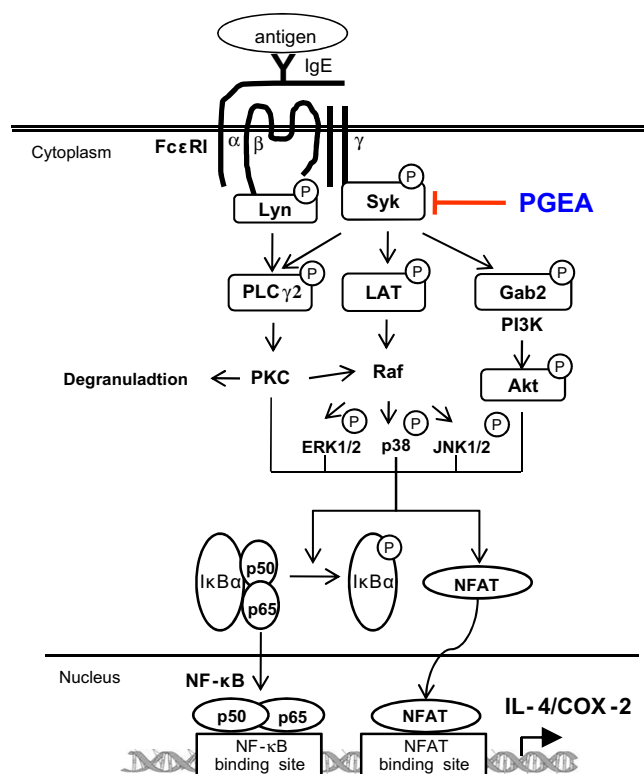


Fig. 6. The anti-allergic effects of PGEA in RBL-2H3 cells. Antigen-mediated aggregation of FcεRI on mast cells triggers the transphosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs). The protein tyrosine kinase Syk is recruited to phosphorylated ITAMs through its SH2 domains. PGEA reduces antigen-induced activation of Syk, but does not influence antigen-induced activation of Lyn, a Src family tyrosine kinase. Activation of Syk results in the tyrosine phosphorylation of other proteins, including LAT, PLCγ2, and Gab2. These initial interactions ultimately lead to mast cell degranulation and the induction of cytokine gene expression. PGEA inhibits antigen-induced activation of proteins that act downstream of Syk, including LAT, PLCγ2, and Gab2, which in turn activate PKC. This results in subsequent IκB-α degradation and consequent activation of NF-κB, which, together with the parallel activation of NFAT, stimulates IL-4 and COX-2 gene expression. PGEA suppresses antigen-induced activation of NF-κB and NFAT. By thus suppressing FcεRI-mediated signaling, PGEA produces strong anti-allergic effects.

tration of humanized anti-IgE antibody, treatment with soluble IL-4 receptor, and treatment with leukotriene and histamine receptor antagonists. New and improved therapeutic strategies, including blocking mast cell activation using tyrosine kinase inhibitors (Luskova and Draber, 2004) are constantly being developed. It should be noted that complementary and alternative medicines provide a promising avenue for the development of new therapies.

In conclusion, this study is the first to show that PGEA inhibits degranulation, cytokine secretion, COX-2 expression, and FcεRI-mediated signaling in antigen-stimulated RBL-2H3 mast cells. Our results suggest that its anti-allergic effects stem from its inhibition of NFAT and NF-κB- and FcεRI-signaling in mast cells.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgement

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