

EUPATILIN BLOCKS MEDIATOR RELEASE VIA TYROSINE KINASE INHIBITION IN ACTIVATED GUINEA PIG LUNG MAST CELLS

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Eupatilin, an extract from Artemisia asiatica Nakai, is known to exert anti-gastric ulcer, anticancer, and anti-inflammatory effects. The aim of this study was to elucidate whether eupatilin has antiallergic reactions in activated guinea pig lung mast cells compared to apigenin and genistein. Mast cells were purified from guinea pig lung tissues by using enzyme digestion and rough and discontinuous density Percoll gradient. The purified mast cells were sensitized with immunoglobulin (Ig) G₁ (anti-OVA antibody) and challenged with ovalbumin (OVA). Histamine was assayed using an automated fluorometric analyzer, leukotrienes by radioimmunoassay, and tyrosine phosphorylation by immunoblotting. Intracellular Ca²⁺ was analyzed by confocal laser scanning microscopy, protein kinase C (PKC) activity using protein phosphorylated with [γ -³²P]ATP, and phospholipase D activity (PLD) and phosphatidic acid by using labeled phosphatidyl alcohol. Eupatilin, apigenin, or genistein reduced histamine release and leukotriene synthesis in a dose-dependent manner. Eupatilin inhibited mediators to a greater extent than apigenin or genistein. Eupatilin, apigenin, and genistein initially blocked phosphorylation of Syk tyrosine and Ca²⁺ influx, PLD activity, phosphatidic acid, and Ca²⁺-dependent PKC α/β activities during mast cell activation in a dose-dependent manner. Our data suggest that eupatilin initially inhibits Syk kinase, and then blocks downstream multisignal pathways and Ca²⁺ influx during mast cell activation triggered by a specific antigen-antibody reaction. Thus, eupatilin may have use clinically as a treatment for inflammatory disorders associated with allergic diseases including asthma.

Artemisia asiatica Nakai (Asteraceae) is widely used as a traditional Asian medical plant, and its pharmacologically active ingredient is a flavonoid named eupatilin (Seo & Surh, 2001). Eupatilin (5,7-dihydroxy-3',4',6-trimethoxyflavone) has inhibitory effects on gastric ulcer, ulcerative colitis of rats (Ahn et al., 2001), experimentally induced reflux esophagitis (Lee et al., 2001), and naproxen-induced and ethanol-induced gastric damage (Huh et al., 2003). It also has

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antioxidative effects and inhibits oxidant-mediated gastric disease (Hahm et al., 2001; Lee et al., 2001; Oh et al., 2001), and induces apoptosis in human promyelocytic leukemia cells (Seo & Surh, 2001). In mouse skin, eupatilin was found to inhibit the activation of nuclear transcription factor κ B (NF- κ B), which is a factor involved in the transcription of proinflammatory cytokines such as interleukin (IL)-6, IL-8, tumor necrosis factor α (TNF α), etc. (Seo et al., 2002).

Mast cells are multifunctional hematopoietic effector cells that produce and release an array of biologically active mediators (histamine, leukotrienes, TNF α , etc.) in the pathogenesis of allergic diseases like asthma. The aggregation of Fc receptors on mast cells activates these cells to release granular mediators (histamine, TNF α), the newly synthesized mediators (leukotrienes, prostaglandins, platelet-activating factor, etc.) (Plaut et al., 1989), and several cytokines (Kempuraj et al., 2003; Kettner et al., 2004). Mast cells are stimulated by the cross-linking of receptor-bound immunoglobulin (Ig) E antibody with a specific antigen, which leads to the activation of tyrosine kinases (Jouvin et al., 1994; Shichijo et al., 2003; Akin et al., 2004; Kettner et al., 2004) and phospholipase C γ_1 (PLC γ_1) (Tkaczyk et al., 2003), PLC γ_2 (Kettner et al., 2004), phospholipase D (PLD) (Gruchalla et al., 1990; Cockcroft, 2001; Choi et al., 2002; Chahdi et al., 2003), and phospholipase A $_2$ (PLA $_2$) (Fonteh et al., 2000; Ishiwarra et al., 2003), and to Ca $^{2+}$ influx (Weintraub et al., 1994; Kempuraj et al., 2003). The activation of PLC or PLD ultimately leads to the production of second messengers such as protein kinase C (PKC) (Altricher et al., 1995; Chahdi et al., 2003), which is known to be activated by 1,2-diacylglycerol and intracellular Ca $^{2+}$.

Many studies focused on the signal pathway that activates mast cell (RBL-2H3, rat peritoneal mast cells, human cord blood-derived cells, bone marrow-derived cells, human mast cell line-1, etc.), which are stimulated by antigen/antibody reactions (Gruchalla et al., 1990; Fujii et al., 2003; Kempuraj et al., 2003; Kyo et al., 2003; Shichijo et al., 2003; Kettner et al., 2004). However, few reports are available on the signaling pathway of guinea pig lung mast cell activation, although it is known that activated guinea pig lung mast cells activate PLD enzyme more than PLC in the release of mediators (Ro et al., 2000). Membrane-located PLD during mast cell activation hydrolyzes phospholipids, especially phosphatidylcholine, to yield phosphatidic acid, which is a known cellular second messenger. Phosphatidic acid is enzymatically converted to diacylglycerol, and triggers free arachidonic acid, which contributes to the arachidonic acid pool used to synthesize prostanoids and leukotrienes. The diacylglycerol also activates PKC in the presence of calcium, and then induces mast cell degranulation (Gould et al., 2001). The activation of guinea pig lung mast cells produces responses that resemble those of human lung mast cell activation. Therefore, these mast cells are used in basic studies on therapeutic agents for allergic disorders such as asthma (Wong et al., 1999; Campos et al., 2000; Arimura et al., 2001; Seow et al., 2002).

Recently, it was reported that the inhibition of non-transmembrane protein tyrosine kinases, such as Lyn and Syk, which are known to be the earliest

detectable signal response to Fc ϵ RI cross-linking on mast cells, attenuate mast cell degranulation (Wong et al., 1999; Seow et al., 2002; Akin et al., 2004; Shichijo et al., 2003; Yamamoto et al., 2003).

Eupatilin exerts an inhibitory effect on various forms of inflammation. It has also been reported that eupatilin isolated from the Chinese plant *Artemisia rubripes* Nakai has an inhibitory effect on 5-lipoxygenase activity in a cultured mastocytoma cell line (Koshihara et al., 1983). Eupatilin may be subject to pharmacological modulation by various inflammatory mediators, such as eicosanoids (Kuhn et al., 1995) and cytokines (Driscoll et al., 1995; Lemaire & Ouellet, 1996; Broeckaert et al., 1997; Huang et al., 2002; Zeidler et al., 2003), produced by environmental toxic substances. Furthermore, the anti-inflammatory effect of eupatilin may be effective in cases of allergic hypersensitivity and asthmatic disorders. Flavonoids inhibit mediator release and production of cytokines by human basophils or rat mast cells (Kim et al., 2001; Hirano et al., 2004). Genistein and apigenin are known as flavonoids and tyrosine kinase inhibitors, and inhibit signaling events and intracellular calcium upregulation induced by the cross-linking of Fc γ RI (Shichijo et al., 2003; Yamamoto et al., 2003). Therefore, it was proposed that eupatilin, a flavonoid, might act in the tyrosine kinase signaling pathway. Thus, studies investigated the inhibitory effects and the mode of action of eupatilin on mediator release from guinea pig lung mast cells activated by a specific antigen–antibody reactions.

MATERIALS AND METHODS

Animals

Hartley albino female guinea pigs, with body weight about 200 to 250 g, were used in this study. The animals were maintained in accordance with Principles of Laboratory Animal Care.

Drugs and Solutions

Eupatilin (molecular weight 344.3) was supplied by Dong-A Pharmaceutical (Kyunggi-do, South Korea), and ovalbumin (OVA), collagenase (type I), elastase (type I, porcine pancreatic), Fluo-3 AM, Percoll, phosphatidylserine, genistein, and apigenin were purchased from the Sigma Chemical Co. (St. Louis, Mo). Gelatin was purchased from Yakuri Pure Chemicals Co.; phenylmethylsulfonyl Fluoride (PMSF), aprotinin, and leupeptin from Roche (Mannheim, Germany); the polyvinyl difluoride (PVDF) membrane from Schleicher & Schuell (Dassel, Germany); phosphotyrosine-conjugated horseradish peroxidase (HRP) antibody from BD Transduction Laboratories (Chicago); HRP-conjugated goat anti-rabbit IgG from Zymed Laboratories Inc. (San Francisco, CA); anti-syk from Upstate Biotechnology (Lake Placid, NY); phosphotyrosine-conjugated HRP from BD Transduction Laboratories (Chicago); phospho-PKC α/β II, δ from Cell Signaling Technology (Beverly, MA); enhanced chemiluminescence (ECL) solution from Amersham Bioscience UK Ltd (Little Chalfont, Buckinghamshire,

England); LK6D silica gel plates from Whatman (Maidstone, Kent); phosphatidylbutanol (PBut) and phosphatidic acid from Avanti Polar Lipids (Albester, AL); [γ - ^{32}P]ATP (30 Ci/mmol) from NEN (Seoul, Korea); and [9,10(*n*) ^{3} . H]palmitic acid (53.0 Ci/mmol) from Amersham Biosciences Korea Ltd. (Seoul, Korea). All the chemicals used in these studies were of the best quality commercially available.

Active Sensitization Protocol (Anti-OVA Production)

Ten outbred female guinea pigs were first immunized by foot-pad injections of a mixture of 50 μg ovalbumin (OVA) and complete Freund's adjuvant. One week after immunization, the animals were injected intradermally with 100 μg OVA in one side of the back and with 200 μg OVA in the other side. Animals were sacrificed 1 wk later, and serum samples were stored in aliquots at -70°C until required. Serum antibody titers were determined by passive cutaneous anaphylaxis (PCA) as described previously (Andersson, 1980; Ro et al., 2000). IgG₁ antibody from guinea pig serum was applied and separated by anti-IgG₂ affinity column chromatography, washed with 0.1 M citric acid (pH 2.1). IgG₁ antibody was eluted with 0.1 M citric acid, and the absorbed IgG₂ antibody was then eluted with 0.2 M sodium carbonate (pH 11.3). The separated IgG₁ was concentrated using Amicon (Millipore, MA). The titers of anti-OVA in serum were in the range 1600–3200. Sera were used to prepare passively sensitized mast cells, as previously described (Ro et al., 2000).

Guinea Pig Lung Mast Cell Preparations

Guinea pig lung mast cells were isolated and purified using techniques similar to those previously reported by Ro et al. (2000). Briefly, the lungs obtained from 16 unsensitized guinea pigs (48 g/each lung) were each perfused with 50 ml of modified Tyrode buffer consisting of (millimolar): NaCl, 137; NaH₂PO₄, 0.36; KCl, 2.6; CaCl₂, 1; MgCl₂, 1.5; NaHCO₃, 25; and glucose, 1 g/L, and gelatin, 1 g/L, pH 7.4 (TGCM buffer). After removing large airways and blood vessels, the lungs were minced using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd, Gomershall, England). Pooled tissue was treated 3 times with 125 U/g tissue of collagenase and 5 U/g tissue of elastase. The lung fragments were treated with those enzymes for 15, 15 and 25 min, respectively. Freed cells were separated from residual tissue by filtration through metal mesh and a Nytex mesh (100 μm). The cells were then washed with CaCl₂- and MgCl₂-free Tyrode buffer containing gelatin (TG buffer), layered over gradients consisting of 10 ml Percoll (density 1.045 g/ml), and centrifuged at $800 \times g$ for 25 min. Pelleted cells (containing mast cells) were then resuspended in TG buffer and purified further using a discontinuous Percoll density gradient (consisting of densities 1.06, 1.07, 1.08, 1.09, and 1.10 g/ml). This gradient was centrifuged at $800 \times g$ for 25 min ($\sim 3.5 \times 10^7$ cells/gradient). The cell band between the 1.09 and 1.10 g/ml densities contained the highest purity and number ($1\text{--}2 \times 10^7$) of mast cells. This band was removed, washed with TGCM buffer, and designated the purified mast cell

preparation. Mast cell counts were obtained by Alcian blue staining, and cell viability was determined by trypan blue exclusion. The cell viability was consistently above 98%, and the purity range of the mast cells obtained was 80–90 %.

Mediator Release From Mast Cells

The purified mast cells (4×10^5) were passively sensitized with anti-OVA antibody (1 ml/ 10^6 cells) for 45 min at 37°C in a shaking water bath. The cells were then washed, resuspended in TGCM buffer (hereafter *sensitized mast cells*), challenged with 0.1 μ g/ml of OVA for 10 min, and then reaction was terminated by placing tubes in an ice bath. The supernatants obtained by centrifugation were used for histamine and leukotriene determinations. For leukotriene determination, 0.1% gelatin (final concentration) was added (because leukotrienes decompose in air) to the supernatants. In all experiments, eupatilin (5, 10, 30, or 50 μ M), apigenin (5 or 10 μ M), or genistein (30 or 50 μ M) was added to mast cells 10 min before OVA challenge (0.1 μ g/ml) (Ro et al., 2000).

Histamine Assay

Histamine was analyzed using an automated fluorometric method (with dialyzer) as previously described (Ro et al., 2000). The sensitivity of the assay was approximately 1–20 ng/ml of histamine. The amount of histamine released was expressed as a percentage of the total histamine present in unstimulated cells.

Leukotriene Immunoassay

The leukotrienes content of each of the cell supernatants was determined using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instruction. Briefly, 50- μ l samples were incubated with anti-leukotriene antiserum (diluted 1:120) and acetylcholinesterase-linked leukotriene (LT) D₄ (diluted 1:120) in wells that had been coated with mouse monoclonal antibodies for 18 h at room temperature. After rinsing with washing buffer, color was developed using Ellman's reagent, and the plates were read at 412 nm with a spectrophotometer. The concentrations of leukotrienes were then calculated using standard curves generated with specific leukotriene standards. The results were expressed in picograms per milliliter (4×10^5 cells). The measurable range of leukotriene D₄ was 10–1000 pg/ml. Anti-leukotriene antiserum has 100% cross-reactivity for LTC₄ and LTD₄, 67% for LTE₄, 61% for LTD₅, 54% for LTC₅, and 41% for LTE₅.

Estimation of Phosphotyrosine

Sensitized mast cells (4×10^5) were challenged with 0.1 μ g/ml of OVA for indicated times. The reaction was terminated by placing tubes in an ice bath. The cells were washed with PBS, and lysed with lysis buffer (1% Triton X-100, 20 mM Tris-Cl (pH 7.6), 150 mM NaCl, 200 mM sodium orthovanadate (Na₃VO₄), and protease inhibitor, 100 mM phenylmethylsulfonyl fluoride (PMSF), 1, μ g/ml aprotinin, 1 μ g/ml leupeptin). The cell lysate (15–20 μ g) obtained was subjected to 8% sodium dodecyl sulfate (SDS)–polyarylamid gel electrophoresis

and was electroblotted onto PVDF membrane, which was then washed with Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST), and blocked for 1 h in TBST containing 1% bovine serum albumin (BSA). After washing the membranes with TBST, the antibodies against phosphotyrosine-conjugated horseradish peroxidase (HRP) (dilution of 1:1000 in TBST) were added and incubated for overnight at 4°C. The membranes were then washed with TBST, and the protein bands were visualized using an enhanced chemiluminescence (ECL) solution. Alpha-tubulin was used as control. Jurket cell lysate was used as a positive control for Syk kinase (Fujii et al., 2003).

Immunoprecipitation of Syk

Sensitized and challenged mast cells (5×10^6) were resuspended with 1 ml lysis buffer (1% Nonidet-P40, 10 mM Tris-Cl (pH 8.6), 150 mM NaCl, 200 mM Na_3VO_4 , and protease inhibitor, 100 mM PMSF, 1 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ leupeptin) for 30 min on ice. The lysates were centrifuged and cleared with protein A/G agarose (Pierce, Rockford, IL). The cleared lysates were incubated with anti-Syk antibody (Upstate Biotechnology) overnight. Then immune complexes were precipitated with protein A/G agarose. The immunoprecipitates were washed five times with lysis buffer and suspended in sample buffer. Samples were denatured by boiling, separated on an 8% SDS-polyacrylamide gel, transferred to PVDF membrane, immunoblotted with phosphotyrosine antibody, and detected using an ECL solution (Fujii et al., 2003).

Measurement of $[\text{Ca}^{2+}]_i$ Level

Sensitized mast cells (4×10^5) were incubated for 30 min after adding Fluo-3 AM (5 μM) and fixed on a slide glass treated with poly-L-lysine; OVA antigen (0.1 $\mu\text{g}/\text{ml}$) was placed on the slide glass to stimulate the cells. The intracellular calcium ($[\text{Ca}^{2+}]_i$) levels of single mast cell were analyzed using an LSM 510 laser scanning microscope, and fluorescence intensity was quantified (Carl Zeiss, Germany). The results were expressed as relative values versus the fluorescence (F_0) of unstimulated cells ($F_0=1$). Eupatilin (5, 10, or 30 μM), genistein (30 or 50 μM), and apigenin (5 or 10 μM) were added to the mast cells 10 min before the OVA challenge (0.1 $\mu\text{g}/\text{ml}$) (Weintraub et al., 1994).

Measurement of Total PKC Activity

Sensitized mast cells (1×10^6) were preincubated with histone (0.2 mg/ml), phosphatidylserine (PS; 40 $\mu\text{g}/\text{ml}$), and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (0.03 μM ; 0.2 $\mu\text{Ci}/\text{ml}$) at 30°C for 10 min, and then stimulated with 0.1 $\mu\text{g}/\text{ml}$ OVA for 30 min. The reaction was terminated by adding 1 ml of 10% trichloroacetic acid (TCA) at 4°C. The precipitated proteins were filtered through a glass fiber disk (Whatman) to eliminate untreated $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, washed 4 times with 20 mM tetrasodium pyrophosphate, and washed once with absolute ethanol. The glass fiber disks were dried, and radioactivity was measured using a liquid scintillation counter (Altrichter et al., 1995). In order to determine Ca^{2+} -independent PKC activity, calcium-free assay buffer (Tyrode buffer containing MgCl_2 and gelatin (TGM

buffer) was used, instead of Tyrode buffer containing CaCl_2 and MgCl_2 (TGCM buffer).

Estimation of PKC α/β II or δ Phosphorylation

Sensitized mast cells (4×10^5) were challenged with $0.1 \mu\text{g/ml}$ of OVA for 10 min. The reaction was terminated by placing tubes in an ice bath. Eupatilin, genistein, or apigenin was added to mast cells 10 min before OVA challenge ($0.1 \mu\text{g/ml}$). The cells were washed with PBS and lysed with lysis buffer (1% Triton X-100, 20 mM Tris-Cl (pH 7.6), 150 mM NaCl, 200 mM Na_3VO_4 , and protease inhibitor, 100 mM PMSF, $1 \mu\text{g/ml}$ aprotinin, $1 \mu\text{g/ml}$ leupeptin). The cell lysate ($10 \mu\text{g}$) obtained was subjected to 10% SDS–polyacrylamide gel electrophoresis, electroblotted onto PVDF membrane, washed with Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST), and blocked for 1 h in TBST containing 1% BSA. After washing the membranes with TBST, the antibodies against phospho-PKC α/β II or δ (dilution of 1:1,000 in TBST) were added and incubated overnight at 4°C . The membranes were washed with TBST and treated with HRP-conjugated goat anti-rabbit IgG (dilution of 1:5,000 in TBST) in the TBST for 90 min. After washing, the protein bands were visualized using an ECL solution. Alpha-tubulin was used as control.

Measurement of PLD Activity and Phosphatidic Acid Production During Mast Cell Activation

Purified mast cells ($1\text{--}2 \times 10^7$) were prelabeled with [^3H]palmitic acid (final concentration $3.7 \mu\text{M}$; $200 \mu\text{Ci/ml}$) in 1 ml TGCM at 37°C for 1 h. The prelabeled cells were then washed twice and suspended in TGCM buffer. The prelabeled cells were sensitized with anti-OVA (IgG_1 , 1 ml antibody/ 10^6 cells) at 37°C for 45 min, washed, and suspended in 1 ml TGCM. PS ($15 \mu\text{g/ml}$) was added to cells (1×10^6 cells), followed by butanol (50 mM), and 5 min later the cells were stimulated with $0.1 \mu\text{g/ml}$ OVA at 37°C for 10 min or PS alone in final volume of $200 \mu\text{l}$. Reactions were stopped by adding 2 ml cold TGCM and the mixtures were centrifuged for 10 min at $800 \times g$. Cellular lipids were extracted from cell pellets using a modification of previously described procedure (Ro et al., 2000). Eupatilin, genistein, or apigenin was added 10 min before OVA challenge, respectively.

The standards phosphatidic acid and PBut (phosphatidylbutanol) plus extracted samples for the measurement of [^3H]PBut were applied to oxalated-coated thin-layer chromatography (TLC) plated (LK6D silica gel 60; Whatman) (5 g potassium oxalate dissolved in 250 ml H_2O , made up to 500 ml with methanol). The plates were then run in ethyl acetate/acetic acid/2,2,4-trimethylpentane (9:2:5) and visualized by iodine staining. The R_f values of PBut and phosphatidic acid were 0.81 and 0.58, respectively. The TLC plates were then scraped, and radioactivity was measured using a liquid scintillation counter.

Statistical Analysis

Experimental data are shown as means $\pm$ SEM. The analysis of significance between controls and experimental groups was carried out using the Student's

t-test for unpaired data; *p* values below 0.05 were considered significant as indicated.

RESULTS

Effect of Eupatilin on Mediator Release During Mast Cell Activation

In order to examine whether eupatilin itself a mediator (histamine and leukotrienes) releasing effect from allergic reaction, unsensitized mast cells were challenged with eupatilin (5, 10, 30, 50, 100, 150, or 200 μ M) alone, and were challenged by 0.1, 1, or 10 μ g/ml OVA (ovalbumin) alone with pretreatment of eupatilin. Mast cells sensitized with anti-OVA antibody were challenged with various concentrations of eupatilin (5, 10, 30, 50, 100, 150, or 200 μ M). The results obtained showed that eupatilin itself did not produce the release of mediators (data not shown). Dose (0.1 μ g/ml OVA) and time (10 min for histamine and 20 min for leukotrienes) of the OVA challenge were decided after preparing dose-response curves and examining time dependences.

When mast cells sensitized with anit-OVA antibody (1.0 ml/ 10^6 cells) were challenged with 0.1 μ g/ml OVA after pretreatment with eupatilin (5, 10, 30, or 50 μ M), histamine release was $27.9 \pm 1.10\%$, $24.4 \pm 1.01\%$, $19.7 \pm 0.70\%$, and $17.7 \pm 0.65\%$, respectively, whereas OVA alone released $33.6 \pm 1.05\%$. The corresponding degrees of histamine inhibition were 17.1%, 27.5%, 41.4%, and 47.5%, respectively (data for 50 μ M not shown in Table 1).

The amount of leukotrienes released by 0.1 μ g/ml OVA was 658.7 ± 9.32 pmole/ 4×10^6 cells. Eupatilin (5, 10, 30, or 50 μ M) inhibited leukotriene release by 21.9%, 36.5%, 51.1%, and 70.2% in a dose-dependent manner (Table 1).

TABLE 1. Effect of eupatilin on the release of mediators from passively sensitized guinea pig (anit-OVA antibody) lung mast cells activated by 0.1 μ g/ml OVA

Treatment		Histamine (%)	Leukotrienes (pg/ 4×10^5 cells)
OVA		33.6 ± 1.05	658.7 ± 9.32
Eupatilin (μ M)	5	27.9 ± 1.10 (17.1) ^a	514.2 ± 36.12 (21.9) ^a
	10	24.4 ± 1.01 (27.5) ^a	418.5 ± 33.90 (36.5) ^a
	30	19.7 ± 0.70 (41.4) ^a	321.9 ± 21.27 (51.1) ^a
Apigenin (μ M)	5	27.9 ± 0.25 (17.1) ^a	537.5 ± 8.18 (18.4) ^a
	10	26.9 ± 0.75 (20.1) ^a	403.1 ± 11.74 (38.8) ^a
Genistein (μ M)	30	26.0 ± 0.80 (22.6) ^a	443.8 ± 6.01 (32.6) ^a
	50	24.6 ± 0.25 (26.9) ^a	384.5 ± 12.93 (41.6) ^a

Note. Guinea pig lung mast cells were purified by enzyme digestion, rough and discontinuous Percoll density gradient method. Mast cells (4×10^5 cells) were passively sensitized by anti-OVA antibody (1 ml/ 10^6 cells), and challenged by 0.1 μ g/ml OVA. Inhibitors were added 10 min before OVA challenge. Histamine and leukotrienes were determined as described in Materials and Methods. The amount of histamine released was expressed as percent of total histamine content. Parentheses were expressed as a decreasing percentage evoked by inhibitor pretreatment. Experiments were performed in blocks of eight.

^aSignificant at *p* < 0.05 compared with OVA alone.

The tyrosine kinase inhibitors apigenin (5 or 10 μM) and genistein (30 or 50 μM), also flavonoids, inhibited histamine and leukotriene release in a dose-dependent manner (Table 1). Of the flavonoids, eupatilin showed a greater inhibitory effect than apigenin or genistein. The tyrosine kinase inhibitors (eupatilin, apigenin, or genistein) also showed a stronger effect on leukotriene inhibition than on histamine release.

Effects of Eupatilin on Phosphorylations of Tyrosine or of syk During Mast Cell Activation

Flavonoids (apigenin and genistein) exert inhibitory effects of protein tyrosine kinase (Shichijo et al., 2003). In our experiment, flavonoids also inhibited the release of mediators (histamine and leukotrienes) during mast cells activation. Therefore it can be inferred that tyrosine kinases are involved in the signaling events induced by mast cells activated by OVA/anti-OVA antibody reaction. The tyrosine phosphorylation of cellular proteins was investigated in the mast cell activation. When sensitized mast cells (1×10^6 cells) were stimulated with 0.1 $\mu\text{g}/\text{ml}$ OVA, tyrosine phosphorylation occurred within 30 s and showed a peak at 30 s–1 min (Figure 1A). The phosphorylated proteins were detected in the 53-, 56-, and 72-kD ranges, but both bands of 53 and 56 kD (Lyn) were very weak (data not shown) and it was difficult to obtain large amounts of protein from primary purified guinea pig lung mast cells. For this reason we examined Syk kinase rather than Lyn kinase. We further analyzed the phosphorylation of Syk protein using immunoprecipitation assays. Syk phosphorylation was increased in mast cells activated with OVA/anti-OVA antibody (Figure 1C).

Eupatilin, genistein, and apigenin all inhibited the phosphorylation of Syk kinase (72 kD proteins) in a dose-dependent manner; eupatilin (30 μM) completely inhibited the phosphorylation of tyrosine kinase (Figure 1B). Moreover, they also inhibited the phosphorylation of Syk (Figure 1C); eupatilin had a more potent inhibitory effect than the specific tyrosine kinase inhibitors apigenin and genistein (Figure 1, B and C).

Effect of Eupatilin on Ca^{2+} Influx During Mast Cell Activation

Increased intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) is necessary for the degranulation of preformed mediators and newly synthesized mediators in activated mast cell. Therefore, studies examined the effect of eupatilin on $[\text{Ca}^{2+}]_i$ in single mast cell by confocal laser scanning microscopy; a relative intensity ratio of 1 was used for the fluorescence intensity of the unstimulated cells. The $[\text{Ca}^{2+}]_i$ level in single activated mast cell reached a plateau at 200 s (from 1.00 ± 0.002 to 1.96 ± 0.102 relative intensity) in the presence of external Ca^{2+} , as compared with unsensitized single mast cell (1.00 ± 0.002 intensity). Eupatilin significantly reduced $[\text{Ca}^{2+}]_i$ level in a dose-dependent manner (to 1.05 ± 0.103 to 1.58 ± 0.198 at 5 μM ; to 1.16 ± 0.094 to 1.33 ± 0.085 at 10 μM ; 1.00 ± 0.002 to 1.26 ± 0.095 at 30 μM) (Figure 2). Genistein (10 μM) and apigenin (5 μM) also inhibited $[\text{Ca}^{2+}]_i$, but showed less than 5 μM eupatilin (Figure 2).

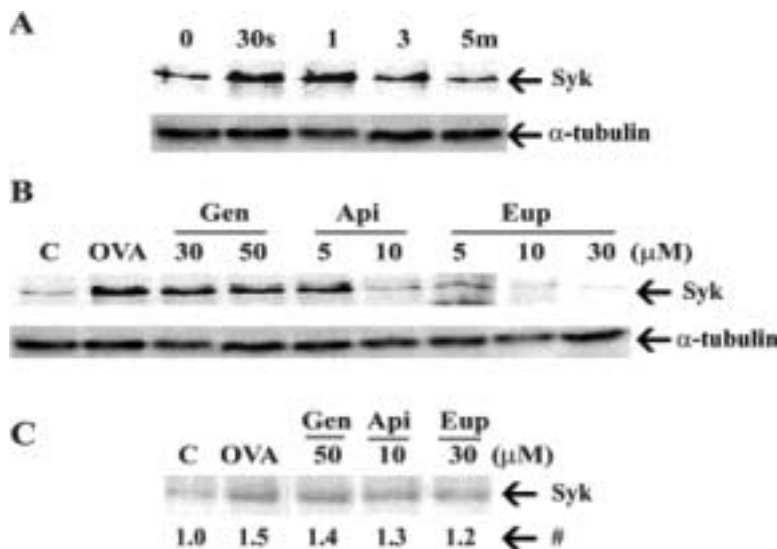


FIGURE 1. Inhibitory effect of eupatilin on the tyrosine phosphorylation in purified guinea pig lung mast cells activated using an OVA/anti-OVA antibody reaction. Purified and sensitized mast cells (1×10^6 cells) were challenged with 0.1 μ g/ml OVA for the indicated times. Eupatilin or tyrosine kinase inhibitors were administered to mast cells 10 min before OVA challenge. Tyrosine phosphorylation was determined by immunoblotting as described in Materials and Methods. (A) Time-dependent phosphotyrosine by OVA challenge. (B) A dose-dependent manner in phosphotyrosine in inhibitor pretreatment for 1 min. (C) Immunoprecipitation of Syk in inhibitor treatment for 1 min. Arrows indicate the position of 72 kD. Juket cell lysate represents standard Syk kinase. Data are representative of six independent experiments; s, second; m, minute; C, control; OVA, ovalbumin; Gen, genistein; Api, apigenin; Eup, eupatilin; #, relative intensity versus the control. α -Tubulin was used as control for Syk.

Effect of Eupatilin on PKC Activity During Mast Cell Activation

It can be inferred that PKC activity is also influenced by eupatilin because the $[Ca^{2+}]_i$ level was reduced by eupatilin treatment. It was of interest to examine total phosphorylated protein in order to measure the effects of eupatilin on PKC activity. The activity of phosphorylated protein activated by 0.1 μ g/ml OVA/anti-OVA antibody ($1 \text{ ml}/10^6$ cells) was significantly increased by 88.4% from $23,386 \pm 1229$ cpm to $44,066 \pm 832$ cpm. Eupatilin (5, 10, or 30 μ M) decreased PKC activity to 5.4%, 11.3%, and 16.3%, respectively (from $44,066 \pm 832$ cpm to $41,686 \pm 761$ cpm at 5 μ M; to $39,087 \pm 636$ cpm at 10 μ M; to $36,885 \pm 1157$ cpm to 30 μ M). Eupatilin (5, 10, or 30 μ M) inhibited the PKC activity less than mediator release. However, a high concentration of eupatilin (100 μ M) decreased it by 40.2% (from $44,066 \pm 832$ cpm to $26,333 \pm 1326$ cpm) (Figure 3A). Apigenin (5 or 10 μ M) significantly inhibited the PKC activity by up to 28.0% and 47.3% (to $31,710 \pm 1941$ cpm at 5 μ M; to $23,221 \pm 1104$ cpm at 10 μ M), and genistein (30 or 50 μ M) by up to 40.7% and 63.8% (to $26,111 \pm 1315$ cpm at 30 μ M; to $15,947 \pm 2565$ cpm at 50 μ M). Apigenin and genistein at the concentration used for histamine and leukotriene

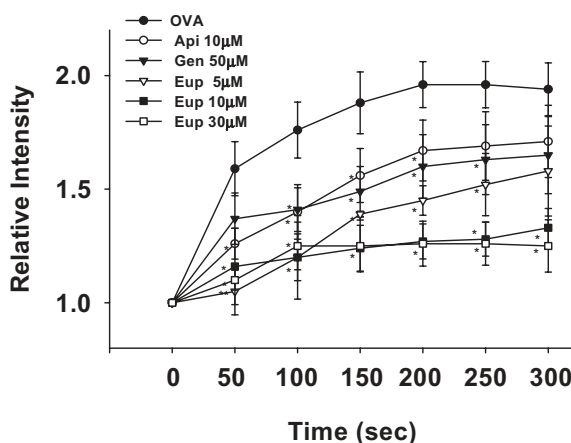


FIGURE 2. Effect of eupatilin on the Ca^{2+} influx in a single mast cell activated by OVA/anti-OVA reaction. Flu-3 AM ($5.0 \mu\text{M}$) was added to sensitized mast cells (4×10^5 cells) and incubated for 30 min. The mast cells were fixed on a glass slide and activated with $0.1 \mu\text{g/ml}$ OVA in the absence or presence of eupatilin and tyrosine kinase inhibitors, and visualized by using a confocal laser scanning fluorescence microscopy. Relative intensity versus the fluorescence values (F_0) of unstimulated cells ($F_0 = 1$). Experiments were performed in blocks of eight. OVA, ovalbumin; Eup, eupatilin; Api, apigenin; Gen, genistein. Asterisk indicates significant at $p < 0.05$ compared with OVA alone.

releases were more potent than a high concentration of eupatilin ($100 \mu\text{M}$) (Figure 3A).

It can be inferred that eupatilin selectively inhibits Ca^{2+} -dependent PKCs, and that apigenin and genistein nonselectively inhibit Ca^{2+} -dependent and Ca^{2+} -independent PKCs. Therefore, it was of interest to examine whether Ca^{2+} -dependent PKC $\alpha/\beta\text{II}$ or δ activities are inhibited by eupatilin using Western blots. The phosphorylation of Ca^{2+} -dependent PKC $\alpha/\beta\text{II}$ or δ were significantly increased in mast cells activated with $0.1 \mu\text{g/ml}$ OVA/anti-OVA antibody (Figure 3B). Eupatilin ($30 \mu\text{M}$) showed inhibition of increased PKC $\alpha/\beta\text{II}$ phosphorylation less than apigenin ($10 \mu\text{M}$) or genistein ($50 \mu\text{M}$). However, the phosphorylation of PKC δ was not inhibited by a low or a high concentration of eupatilin, but completely inhibited by genistein (Figure 3B).

Studies also examined indirectly whether Ca^{2+} -independent PKCs are inhibited by eupatilin in a calcium free buffer. The activity of phosphorylated protein in calcium free buffer increased up to 63.5% from $19,441 \pm 146$ cpm to $31,794 \pm 227$ cpm. Eupatilin ($5, 10, 30$, or $100 \mu\text{M}$) did not inhibit PKC activity in a calcium-free buffer (i.e., from $19,441 \pm 146$ cpm to $31,503 \pm 228$ cpm). The Syk inhibitor apigenin (5 or $10 \mu\text{M}$) inhibited PKC activity up to 13.6% and 19.2%, respectively (to $27,467 \pm 1348$ cpm at $5 \mu\text{M}$; to $25,677 \pm 792$ cpm at $10 \mu\text{M}$). The nonspecific Syk inhibitor genistein (30 or $50 \mu\text{M}$) inhibited PKC activity by up to 17.4% and 25.2%, respectively (to $26,257 \pm 2064$ cpm at $30 \mu\text{M}$; to $23,792 \pm 557$ cpm at $50 \mu\text{M}$) in a calcium-free buffer system. Those results suggest that eupatilin inhibits Ca^{2+} -dependent PKC $\alpha/\beta\text{II}$

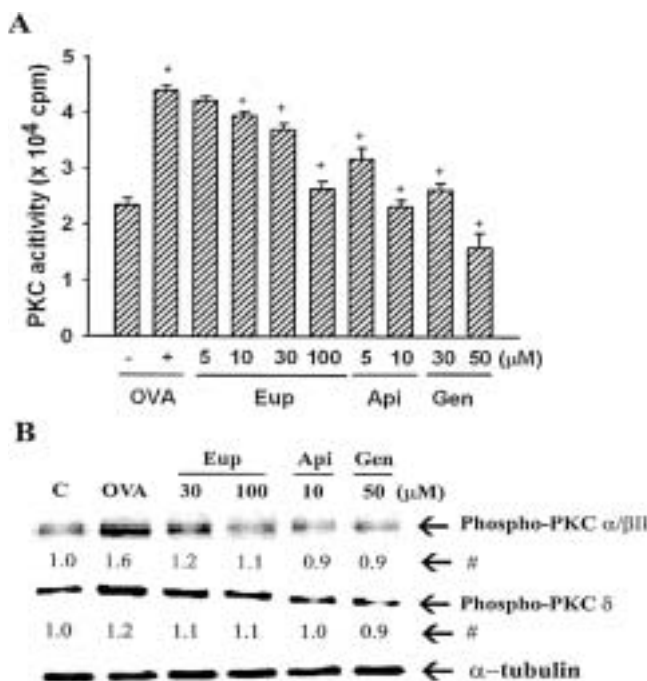


FIGURE 3. Effect of eupatilin on the PKC activity in the purified guinea pig lung mast cells activated with OVA/anti-OVA antibody reaction. Purified and sensitized mast cells (1×10^6 cells) were treated with histone (0.2 $\mu\text{g/ml}$), PS (40 $\mu\text{g/ml}$), and [$\gamma\text{-}^{32}\text{P}$]ATP (0.03 μM ; 0.2 $\mu\text{Ci/ml}$) and then challenged with OVA (0.1 $\mu\text{g/ml}$) for 10 min as described in Materials and Methods. (A) Total PKC activity in the presence of calcium. (B) The phosphorylation of PKC $\alpha/\beta\text{II}$ or δ activities using Western blots. Experiments were performed in blocks of eight. OVA, ovalbumin; Eup, eupatilin; Api, apigenin; Gen, genistein. Asterisk indicates significant at $p < 0.05$ compared without OVA challenge; plus sign, significant at $p < 0.05$ compared with OVA challenge; #, relative intensity versus the control.

activities more than both other inhibitors. However, inhibitory degrees of PKC $\alpha/\beta\text{II}$ activities by eupatilin were less than our expectation. Therefore, it can be inferred that eupatilin partially inhibits Ca^{2+} -dependent PKCs activities more than Ca^{2+} -independent PKCs activities. However, eupatilin might affect other signaling cascades.

Effect of Eupatilin on PLD Activity and Phosphatidic Acid Production During Mast Cell Activation

There have been reports that PKC activity or increased $[\text{Ca}^{2+}]_i$ activate membranous PLD activity occurs during mast cell activation (Lin et al., 1994; Ro et al., 2000), and that the activated PLD produces phosphatidic acid and choline. Phosphatidic acid was hydrolyzed by PAPase to yield diacylglycerol. The diacylglycerol formed induces histamine release via the activation of PKC by Ca^{2+} , and also induces the synthesis of leukotrienes via the formation of

arachidonic acid. Therefore, studies examined the effect of eupatilin on PLD activity and phosphatidic acid levels in activated mast cells. Production of PBut (phosphatidylbutanol), which is converted by PLD activation, was determined. PBut increased approximately 2.5 times from 4790 ± 238 cpm to $11,757 \pm 330$ cpm. On the other hand, the production of PBut was significantly reduced by eupatilin pretreatment in a dose-dependent manner (from $11,757 \pm 330$ cpm to 9122 ± 277 cpm at $5 \mu\text{M}$; to 7909 ± 314 cpm at $10 \mu\text{M}$; 5942 ± 271 cpm at $30 \mu\text{M}$; 5329 ± 350 cpm at $50 \mu\text{M}$). The inhibitory effects of PLD activity by eupatilin (at 5, 10, 30, or $50 \mu\text{M}$) were 24.4%, 32.7%, 49.5%, and 54.7%, respectively (Figure 4). PLD activity was inhibited by genistein (to 9472 ± 220 cpm at $30 \mu\text{M}$; to 9321 ± 1004 cpm at $50 \mu\text{M}$) and apigenin (9832 ± 224 cpm at $5 \mu\text{M}$; to 8022 ± 237 cpm at $10 \mu\text{M}$) in activated mast cells (Figure 4). The inhibition of PLD activity by genistein (30 or $50 \mu\text{M}$) was 19.4% and 20.7%, and by apigenin 16.4% and 28.8%, respectively.

Phosphatidic acid was also increased in activated mast cells. The increased phosphatidic acid (3398 ± 227 cpm) was inhibited by eupatilin in a dose-dependent manner. Inhibitions of phosphatidic acid production by eupatilin (at 5, 10, 30, or $50 \mu\text{M}$) were 22.7%, 34.3%, 54.7%, and 57.3%, respectively (to 2626 ± 139 cpm at $5 \mu\text{M}$; to 2233 ± 51 cpm at $10 \mu\text{M}$; to 1538 ± 66 cpm at $30 \mu\text{M}$; to 1450 ± 164 cpm at $50 \mu\text{M}$) (Figure 5). Apigenin inhibited phosphatidic acid production up to 22.9% (to 2621 ± 96 cpm at $5 \mu\text{M}$) and 29.4% (to 2399 ± 139 cpm at $10 \mu\text{M}$). Genistein (30 or $50 \mu\text{M}$) also inhibited it up to 27.9% and 35.7% (to 2451 ± 236 cpm at $30 \mu\text{M}$; to 2185 ± 70 cpm at

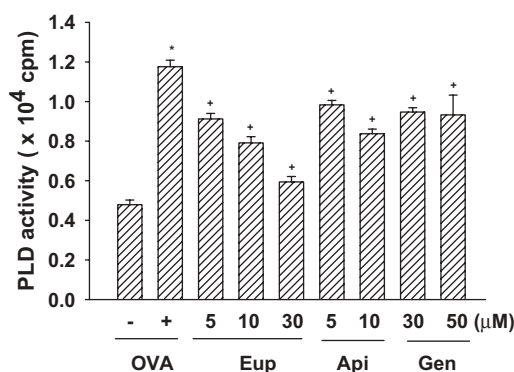


FIGURE 4. Effect of eupatilin on the PLD activity in the purified guinea pig lung mast cells activated with OVA/anti-OVA antibody reaction. Purified mast cells ($1-2 \times 10^7$ cells) were prelabeled with [^3H]palmitic acid ($3.3 \mu\text{M}$; $200 \mu\text{Ci/ml}$) and washed. The prelabeled cells were sensitized with anti-OVA antibody ($1 \text{ ml}/10^6$ cells) for 45 min. Ten minutes before OVA challenge, 50 mM butanol and inhibitors were added and then cells were challenged with OVA ($0.1 \mu\text{g/ml}$) for 10 min. PBut levels were determined as described in Materials and Methods. Experiments were performed in blocks of eight. OVA, ovalbumin; Eup, eupatilin; Api, apigenin; Gen, genistein. Asterisk indicates significant at $p < 0.05$ compared without OVA challenge; plus sign, significant at $p < 0.05$ compared with OVA challenge.

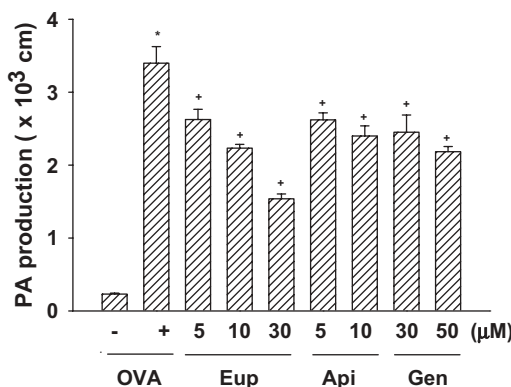


FIGURE 5. Effect of eupatilin on the production of phosphatidic acid in the purified guinea pig mast cells activated with OVA/anti-OVA antibody reaction. Purified mast cells ($1-2 \times 10^7$ cells) were prelabeled with [^3H]palmitic acid ($3.3 \mu\text{M}$; $200 \mu\text{Ci/ml}$) and washed. The prelabeled cells were then sensitized with anti-OVA antibody ($1 \text{ ml}/10^6$) for 45 min. Ten minutes before OVA challenge, 50 mM butanol and inhibitors were added and challenged with OVA ($0.1 \mu\text{g/ml}$) for 10 min. The production of phosphatidic acid was determined as described in Materials and Methods. Experiments were performed in blocks of eight. OVA, ovalbumin; Eup, eupatilin; Api, apigenin; Gen, genistein. Asterisk indicates significant at $p < 0.05$ by comparison without challenge; plus sign, significant at $p < 0.05$ compared with OVA challenge.

$50 \mu\text{M}$) (Figure 5). The inhibitory effect of PLD on phosphatidic acid induction by eupatilin was greater than by apigenin and genistein.

DISCUSSION

Studies showed that the aggregation of Fc γ RI in the membrane of guinea pig lung mast cells results in the transduction of a signal, that elevates $[\text{Ca}^{2+}]_i$, the activation of various enzymes and second messengers, and the release of inflammatory mediators (Ro et al., 2000). In this experiment, eupatilin significantly inhibited histamine release and leukotriene synthesis induced by mast cells activation. These inhibitory responses were greater than those of apigenin or genistein, which were used as positive controls (Table 1). There are reports that some flavonoids inhibit leukotrienes through an inhibition of phospholipase A₂ (PLA₂) and 5-lipoxygenase, but some flavonoids do not (Kim et al., 2001; Hirano et al., 2004). However, in our experiments, it was confirmed that eupatilin does not inhibit PLA₂ activity (data not shown).

Eupatilin reduced the phosphorylation of Syk kinase in the initial stage of Fc γ RI-mediated events in mast cells more than apigenin or genistein (Figure 1, B and C). This finding supports previous reports that the aggregation of Fc γ RI or Fc ϵ RI activates the common sets of tyrosine kinases, such as Lyn and Syk through ITAM (immunoreceptor tyrosine-based activation motif), through which flavonoids such as genistein and apigenin inhibit tyrosine kinases (Shichijo et al., 2003; Yamamoto et al., 2003). Moreover, it was shown that Syk-null

cells and a rat model of asthma aerosolized with Syk antisense showed a loss of degranulation (Stenton et al., 2002; Siraganian, 2003). In our experiment, 72-kD tyrosine kinase was markedly phosphorylated in activated mast cells and was inhibited by eupatilin (Figure 1A). The inhibitory effect of eupatilin on Syk kinase was also greater than those of genistein and apigenin (Figure 1C). Data suggest that eupatilin inhibits the signal pathway via the tyrosine phosphorylation of Syk in the Fc γ R-dependent activated mast cells.

Increases in $[Ca^{2+}]_i$ require the induction of degranulation in activated mast cells. Several investigators reported that $[Ca^{2+}]_i$ increased in activated mast cells (Hide & Beaven, 1991; Weintraub et al., 1994; Ro et al., 2000; Kempuraj et al., 2003). This increase in $[Ca^{2+}]_i$ is blocked by eupatilin (Figure 2). Results showed that genistein and apigenin also inhibit $[Ca^{2+}]_i$. These results are in agreement with previous reports in a human basophilic leukemia cell line KU812F (Fujii et al., 2003).

A number of studies found that PKC is an essential transducer of signals for secretion in various mast cells. Some PKC isoforms (α , β 1, β 2) are activated by an increase $[Ca^{2+}]_i$ level, whereas other PKC isoforms (δ , ϵ , ζ) are activated without $[Ca^{2+}]_i$ increase (Gould et al., 2001). It was previously reported that various proteins are phosphorylated by PKC in activated guinea pig lung mast cells (Ro et al., 2000). However, eupatilin at a concentration inhibiting mediator releases inhibited total PKC activity less than apigenin or genistein (Figure 3A). Therefore, both directly and indirectly it was confirmed that eupatilin inhibiting Syk kinase selectively blocked Ca^{2+} -dependent PKC activity, whereas apigenin and genistein inhibited Ca^{2+} -dependent and Ca^{2+} -independent PKC activities. It can be inferred that eupatilin inhibits mediator release by partial inhibition of Ca^{2+} -dependent PKC activity via Ca^{2+} influx.

Many have found that Syk kinase plays a significant role in signal transduction in various cell types, probably by stimulating the downstream phosphorylation of PLC. It has also been widely reported that tyrosine kinases and other kinases play an important role in the signal transduction evoked by mast cell activation (Cockcroft, 2001; Seow et al., 2002; Akin et al., 2004; Shichijo et al., 2003). However, few studies found that the aggregation of Fc ϵ RI on mast cells is regulated by PLD activity associated with tyrosine kinases in the initial stage (Kumada et al., 1993; Lin et al., 1994). It was also observed that PLD activity was higher than PLC activity in activated guinea pig lung mast cells (Ro et al., 2000). The activation of PLD is regulated by both PKC activity and an increase in $[Ca^{2+}]_i$ and induces the degranulation and newly synthesized mediators. Therefore, studies focused on the PLD activity associated with tyrosine kinases. Eupatilin, which blocked Syk kinase, reduced PLD activity and phosphatidic acid production in activated mast cells (Figures 4 and 5). Moreover, the degree of inhibition of eupatilin was similar to that of apigenin, but greater than genistein (Figure 4). The activation of membranous PLD enzyme during mast cell activation hydrolyzes phospholipids, especially phosphatidylcholine, to yield choline and phosphatidic acid. Phosphatidic

acid, which was shown as a cellular second messenger, was enzymatically converted to diacylglycerol, which triggers free arachidonic acid, which in turn is used for the synthesis of leukotrienes. Diacylglycerol also induces histamine release via PKC activation (Gould et al., 2001). It can be inferred that eupatilin inhibits PLD activity by acting on Syk kinase. Therefore, it is essential that the inhibition of Syk kinase by eupatilin inhibits $[Ca^{2+}]$, PLD enzyme, and the production of phosphatidic acid, which is followed by the inhibition of Ca^{2+} -dependent PKC α/β II activities and the production of arachidonic acid, leading to mediator release inhibition.

Our data suggest that eupatilin inhibits cellular protein tyrosine kinases (Syk), and inhibits the downstream multisignaling pathways and Ca^{2+} influx during mast cell activation induced by a specific antigen–antibody reaction. Thus, it is proposed that eupatilin has potential use in the therapy of inflammatory disorders associated with allergic diseases.

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