



Anti-allergic effects of *Lycopus lucidus* on mast cell-mediated allergy model

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Abstract

The current study characterizes the mechanism by which the aqueous extract of *Lycopus lucidus* Turcz. (Labiatae) (LAE) decreases mast cell-mediated immediate-type allergic reaction. The immediate-type allergic reaction is involved in many allergic diseases such as asthma and allergic rhinitis. LAE has been used as a traditional medicine in Korea and is known to have an anti-inflammatory effect. However, its specific mechanism of action is still unknown. LAE was orally administered to mice for high and fast absorption. LAE inhibited compound 48/80-induced systemic reactions in mice. LAE decreased the local allergic reaction, passive cutaneous anaphylaxis, activated by anti-dinitrophenyl (DNP) IgE antibody. LAE dose-dependently reduced histamine release from rat peritoneal mast cells activated by compound 48/80 or anti-DNP IgE. Furthermore, LAE decreased the secretion of TNF- α and IL-6 in phorbol 12-myristate 13-acetate (PMA) plus calcium ionophore A23187-stimulated human mast cells. The inhibitory effect of LAE on the pro-inflammatory cytokine was p38 mitogen-activated protein kinase (MAPK) and nuclear factor- κ B (NF- κ B) dependent. LAE attenuated PMA plus A23187-induced degradation of I κ B α and nuclear translocation of NF- κ B, and specifically blocked activation of p38 MAPK, but not that of c-jun N-terminal kinase and extracellular signal-regulated kinase. Our findings provide evidence that LAE inhibits mast cell-derived immediate-type allergic reactions and involvement of pro-inflammatory cytokines, p38 MAPK, and NF- κ B in these effects.

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Introduction

The extract of *Lycopus lucidus* Turcz. (Labiatae) has been used for centuries as an oriental traditional medicine. This crude drug is used for the management of menstrual

disorder and inflammatory disease (But et al., 1997; Park, 2004). It has been known to have anti-oxidative and anti-inflammation effects (Woo and Piao, 2004; Yun et al., 2003).

Mast cells, which are constituents of virtually all organs and tissue, are important mediators of inflammatory responses such as allergy and anaphylaxis. Anaphylaxis is mediated by histamine released in response to antigen cross-linking of immunoglobulin E (IgE) bound to Fc ϵ RI on mast cells. Mast cell activation causes the process of degranula-

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tion that results in releasing of mediators, such as histamine and an array of inflammatory cytokines (Church and Levi-Schaffer, 1997; Metcalfe et al., 1981; Miyajima et al., 1997). Among the inflammatory substances released from mast cells, histamine remains the best-characterized and most potent vasoactive mediator implicated in the acute phase of immediate hypersensitivity (Petersen et al., 1996). Mast cell degranulation can also be elicited by the synthetic compound 48/80, and it has been used as a direct and convenient reagent to study the mechanism of anaphylaxis (Ennis et al., 1980).

The signaling pathway leading to degranulation of mast cells has been extensively characterized (Beaven and Metzger, 1993; Beaven et al., 1984). Activation of mast cells leads to phosphorylation of tyrosine kinase and mobilization of internal Ca^{2+} . This is followed by activation of protein kinase C, mitogen-activated protein kinases (MAPKs), nuclear factor- κB (NF- κB), and releasing of inflammatory cytokines. Activated mast cells can produce histamine, as well as a wide variety of other inflammatory mediators such as eicosanoids, proteoglycans, proteases, and several pro-inflammatory and chemotactic cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-4, IL-13, and transforming growth factor- β (Bradding et al., 1993; Burd et al., 1989; Galli et al., 1991; Plaut et al., 1989). MAPKs and transcription factor NF- κB have important activities as mediators of cellular responses to extracellular signals. Some of the MAPKs important to mammalian cells include extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38. p38 MAPK and NF- κB are thought to play an important role in the regulation of pro-inflammatory molecules on cellular responses, especially TNF- α , IL-1 β , and IL-6 (Azzolina et al., 2003; Baldassare et al., 1999; Beyaert et al., 1996).

Anal therapy is a drug delivery system through the anus. It is utilized in patients who have difficulty with oral administration. Absorbing of a drug in the rectum avoids the first-pass effect in the liver and allows it to circulate directly in the whole body. The absorption rate in the rectum is faster than that in the gastrointestinal tract. The rate and total amount absorption through the rectum are not very different from those with venous administration (Shin et al., 2004). Thus, anal therapy is expected to have a better effect than oral therapy due to the increased absorption rate and the strong medical action. In this study, we used anal therapy to enhance the effects of LAE.

The aim of this study is to evaluate the anti-allergic effect of LAE and to understand the mechanism of effect.

Materials and methods

Culture of HMC-1 cells. HMC-1 cells, a human mast cell line, were grown in Iscove's media supplemented with 10% FBS and 2 mM glutamine.

Animals. The original stock of male ICR mice and male Sprague–Dawley rats were purchased from Dae-Han Experimental Animal Center (Daejeon, Korea), and the animals were maintained in the College of Pharmacy, Woosuk University. The animals were housed 5–10 per cage in a laminar air flow room maintained at a temperature of 22 ± 2 °C and relative humidity of $55 \pm 5\%$ throughout the study. The care and treatment of the mice were in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Reagents. Compound 48/80, anti-DNP IgE, DNP-human serum albumin (HSA), α -minimal essential medium (α -MEM), o-phthalaldehyde, phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187, and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma (St Louis, MO). SB 203580 was purchased from Calbiochem (La Jolla, CA). rTNF- α and rIL-6 were purchased from R&D Systems Inc. (Minneapolis, MN).

Preparation of LAE. The plants of *L. lucidus* were collected in Soonchang (Jeonbuk, Korea) on July 16, 2000. A voucher specimen (number WSP-00-31) was deposited at the Herbarium of the College of Pharmacy, Woosuk University. The plant sample was extracted with purified water at 70°C for 5 h (two times). The extract was filtered, lyophilized, and kept at -4 °C. The yield of dried extract from starting crude materials was about 10.1%. The dried extract was dissolved in saline or Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl_2 , 1 mM MgCl_2 , 5.6 mM glucose, 0.1% bovine serum albumin) before use.

Compound 48/80-induced systemic reaction. Compound 48/80-induced systemic reaction was examined as previously described (Kim et al., 2005). Mice were given an intraperitoneal injection of 0.008 g/kg body weight (BW) of the mast cell degranulator compound 48/80. LAE was dissolved in saline and administered anally at doses ranging from 0.005 to 0.1 g/kg BW 1 h before the injection of compound 48/80 ($n = 10/\text{group}$). In the time-dependent experiment, LAE (0.1 g/kg) was anally administered ranging from 5, 10, 20, and 30 min after injection of compound 48/80 ($n = 10/\text{group}$). Mortality was monitored for 1 h after induction of anaphylactic shock.

PCA reaction. An IgE-dependent cutaneous reaction was generated by sensitizing skin with an intradermal injection of anti-DNP IgE followed 48 h later with an injection of DNP-HSA into the mouse tail vein. The anti-DNP IgE antibody and DNP-HSA were diluted in phosphate-buffered saline (PBS). The mice were injected intradermally with 0.5 μg of anti-DNP IgE into each of 2 dorsal skin sites that had been shaved 48 h earlier. The

sites were outlined with a water-insoluble red marker. After 48 h, each mouse received an injection of 1 μ g of DNP-HSA in PBS containing 4% Evans blue (1:4) via the tail vein. LAE (0.001 to 1 g/kg BW) was orally administered 1 h before the challenge. Thirty minutes after the challenge, the mice were killed and the dorsal skin was removed for measurement of the pigment area. The amount of dye was determined colorimetrically after extraction with 1 ml of 1 M KOH and 9 ml of a mixture of acetone and phosphoric acid (5:13) based on the previous report (Katayama et al., 1978). The intensity of absorbance was measured at 620 nm in a spectrophotometer (Shimadzu, UV-1201, Japan).

Preparation of RPMC. Mast cells were separated from rat peritoneal cavity cells according to the method described by Yurt et al. (1977). In brief, rats were anesthetized by ether and injected with 20 ml of Tyrode buffer B (137 mM NaCl, 5.6 mM glucose, 12 mM NaHCO₃, 2.7 mM KCl, 0.3 mM NaH₂PO₄, and 0.1% gelatin) into the peritoneal cavity and the abdomen was gently massaged for about 90 s. The peritoneal cavity was carefully opened, and the fluid-containing peritoneal cells were aspirated by Pasteur pipette. The peritoneal cells were sedimented at 150 $\times$ g for 10 min at room temperature and resuspended in Tyrode buffer B. Mast cells were separated from the major components of rat peritoneal cells, i.e., macrophages and small lymphocytes. Peritoneal cells were suspended in 1 ml of Tyrode buffer B, layered on 2 ml of metrizamide (22.5 w/v%), and centrifuged at room temperature for 15 min at 400 $\times$ g. The cells remaining at the buffer–metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 ml of Tyrode buffer A. Mast cell preparations were about 95% pure as assessed by toluidine blue staining. More than 97% of the cells were viable as judged by trypan blue uptake.

Inhibition of histamine release. Purified RPMCs were resuspended in Tyrode buffer A for treatment with compound 48/80. RPMC suspensions (2×10^5 cells/ml) were preincubated for 10 min at 37 $^{\circ}$ C before the addition of compound 48/80 (5 μ g/ml). The cells were preincubated with the LAE (0.001 to 1 mg/ml) preparations and then incubated for 10 min with compound 48/80. RPMC suspensions (2×10^5 cells/ml) were also sensitized with anti-DNP IgE (10 μ g/ml) for 16 h. The cells were preincubated with the LAE (0.001 to 1 mg/ml) at 37 $^{\circ}$ C for 10 min prior to the challenge with DNP-HSA (1 μ g/ml). The cells were separated from the released histamine by centrifugation at 400 $\times$ g for 5 min at 4 $^{\circ}$ C. The histamine content was measured by the o-phthaldialdehyde spectrofluorometric procedure (Shore et al., 1959). The fluorescence intensity was measured at emission of 438 nm and excitation of 353 nm using a spectrofluorometer (Shimadzu, RF-5301 PC).

Assay of TNF- α and IL-6 secretion. TNF- α and IL-6 secretion was measured by modification of an enzyme-linked immunosorbent assay (ELISA) as described previously (Scuderi et al., 1986). HMC-1 cells were cultured with α -MEM plus 10% FBS and resuspended in Tyrode buffer A. The cells were sensitized with PMA (20 nM) plus A23187 (1 μ M) for 6 h in the absence or presence of LAE. The ELISA was performed by coating 96-well plates with 6.25 ng/well of monoclonal antibody with specificity for TNF- α and IL-6, respectively. Before use and between subsequent steps in the assay, the coated plates were washed twice with PBS containing 0.05% Tween 20 and twice with PBS alone. For the standard curve, rTNF- α and rIL-6 were added to serum previously determined to be negative to endogenous TNF- α and IL-6. After exposure to the medium, the assay plates were exposed sequentially to biotinylated antihuman TNF- α and IL-6, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) tablet substrates. Optical density readings were made within 10 min of the addition of the substrate with a 405-nm filter.

Nuclear protein extraction. Preparation of nuclear extract was basically as described elsewhere (Jeong et al., 2002). Briefly, after cell activation for the times indicated, 1×10^7 cells were washed in 1 ml of ice-cold PBS, centrifuged at 1200 rpm for 5 min, resuspended in 400 μ l of ice-cold hypotonic buffer (10 mM HEPES/KOH, 2 mM MgCl₂, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 10 min, vortexed, and centrifuged at 15,000 $\times$ g for 30 s. Pelleted nuclei were gently resuspended in 50 μ l of ice-cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 20 min, vortexed, and centrifuged at 15,000 $\times$ g for 5 min at 4 $^{\circ}$ C. Aliquots of the supernatant that contain nuclear proteins were frozen in liquid nitrogen and stored at -70° C.

Western blot analyses. Samples of protein were electrophoresed using 8–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, as described elsewhere (Kim and Sharma, 2004), and then transferred to nitrocellulose membrane. The p38 MAPK, ERK, and JNK activation was determined using anti-phospho-p38, -ERK, and -ERK antibodies (Cell Signaling, Beverly, MA). The I κ B α and p65 NF- κ B were assayed using anti-I κ B α and -NF- κ B (p65) antibodies (Santa Cruz Biotech, Santa Cruz, CA). Immunodetection was done using enhanced chemiluminescence detection kit (Amersham Pharmacia, Piscataway, NJ).

Electrophoretic mobility shift assays (EMSA). Nuclear protein (10 μ g) was incubated for 20 min at room temperature with 20 μ g of bovine serum albumin, 2 μ g of poly (dI-dC) from Pharmacia (Uppsala, Sweden), 2 μ l of buffer C (20 mM HEPES/KOH, 20% glycerol, 100 mM KCl, 0.5 mM PMSF, pH 7.9), 4 μ l of buffer F (20% ficoll-400, 100 mM

HEPES/KOH, 300 mM KCl, 10 mM DTT, 0.5 mM PMSF, pH 7.9), and 20,000 cpm of a ^{32}P -labeled probe encoding the κB consensus sequence (5'-CAG AGG GGA CTT TCC GAG AG-3') in a final volume of 20 μl . DNA–protein complexes were resolved at 180 V for 4 h in a native 4% polyacrylamide gel, dried, and visualized (with autoradiography using a Fuji X-ray film).

Statistical analysis. Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC). Treatment effects were analyzed using one-way ANOVA, followed by Duncan's multiple range tests. $P < 0.05$ was used to indicate significance.

Results

Effect of LAE on compound 48/80-induced systemic reaction

To determine the effect of LAE on allergic reaction, an in vivo model of systemic reaction was used. Compound 48/80 (0.008 g/kg) was used as a model of induction of a systemic fatal allergic reaction. After the intraperitoneal injection of compound 48/80, the mice were monitored for 1 h, after which the mortality rate was determined. As shown in Table 1, injection of compound 48/80 into mice induced fatal shock in 100% of animals. When LAE was administered anally at concentrations ranging from 0.005 to 0.1 g/kg BW for 1 h, the mortality with compound 48/80 was dose-dependently reduced. In addition, the mortality of mice administered anally with LAE (0.1 g/kg) 5, 10, 20, and 30 min after compound 48/80 injection increased time-dependently (Table 2).

Effect of LAE on anti-DNP IgE-mediated PCA

Another way to test anaphylaxis is to induce PCA. As described in Materials and methods, local extravasation was induced by local injection of IgE followed by an antigenic

Table 1
Effect of LAE on compound 48/80-induced systemic reaction

LAE treatment (g/kg BW)	Compound 48/80 (0.008 g/kg BW)	Mortality (%)
None (saline)	+	100
0.005	+	100
0.01	+	80
0.05	+	20
0.1	+	0
0.1	–	0

Groups of mice ($n = 10/\text{group}$) were anally pretreated with 200 μl saline or LAE. LAE was given at various doses 1 h before the compound 48/80 injection. The compound 48/80 solution was intraperitoneally given to the group of mice. Mortality (%) within 1 h following compound 48/80 injection was represented as the number of dead mice $\times$ 100/total number of experimental mice.

Table 2

Time-dependent effect of LAE on compound 48/80-induced systemic reaction

LAE treatment (g/kg BW)	Time (min)	Compound 48/80 (0.008 g/kg BW)	Mortality (%)
None (saline)		+	100
0.1	5	+	0
0.1	10	+	40
0.1	20	+	40
0.1	30	+	100

Groups of mice ($n = 10/\text{group}$) were anally pretreated with 200 μl saline or LAE. LAE (0.1 g/kg) was given at 5, 10, 20, and 30 min after the compound 48/80 injection. The compound 48/80 solution was intraperitoneally given to the group of mice. Mortality (%) within 1 h following compound 48/80 injection was represented as the number of dead mice $\times$ 100/total number of experimental mice.

challenge. LAE dose-dependently inhibited PCA reaction (Table 3).

Effect of LAE on histamine release from RPMC

The inhibitory effects of LAE on compound 48/80- or anti-DNP IgE-mediated histamine release from RPMC are shown in Fig. 1. LAE dose-dependently inhibited compound 48/80- or anti-DNP IgE-mediated histamine release at concentrations of 0.01–1 mg/ml.

Effect of LAE on intracellular Ca^{2+}

Calcium movements across membranes of mast cells are critical to histamine release (Beaven et al., 1984). To investigate the mechanisms of LAE on the reduction of histamine release, we assayed the intracellular Ca^{2+} . Fig. 2 shows the stimulation of intracellular Ca^{2+} when RPMCs are treated with compound 48/80 (2 $\mu\text{g}/\text{ml}$). Preincubation of LAE (0.1 mg/ml) with RPMC decreased compound 48/80-induced intracellular Ca^{2+} levels.

Effect of LAE on TNF- α and IL-6 secretion from HMC-1 cells

TNF- α and IL-6 are the most important proinflammatory cytokines. Therefore, we tested the effect of LAE on TNF- α and IL-6 secretion from HMC-1 cells. HMC-1 cell line is a

Table 3
Effect of LAE on the 48 h PCA

LAE treatment (g/kg BW)	Anti-DNP IgE plus DNP-HSA	Amount of dye ($\mu\text{g}/\text{site}$)
None (saline)	+	5.980 $\pm$ 0.424
0.001	+	4.891 $\pm$ 0.516
0.01	+	4.318 $\pm$ 0.397
0.1	+	3.364 $\pm$ 0.197*
1	+	2.616 $\pm$ 0.201*

LAE was anally administered 1 h prior to challenge with antigen. Each data represents the mean $\pm$ SEM of three independent experiments. * $P < 0.05$, significantly different from the saline value.

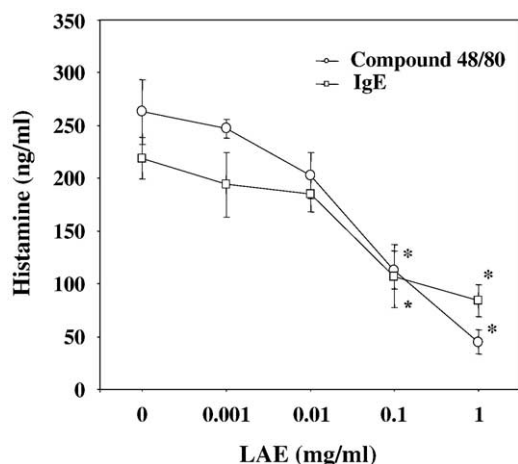


Fig. 1. Effect of LAE on compound 48/80- or IgE-induced histamine release from RPMC. The cells (2×10^5 cells/ml) were preincubated with LAE at 37 °C for 10 min prior to incubation with compound 48/80 or DNP-HSA. Each data represents the mean $\pm$ SEM of three independent experiments. * $P < 0.05$ (significantly different from the saline value).

useful cell for studying cytokine activation pathway (Sillaber et al., 1993). Culture supernatants were assayed for each cytokines level by ELISA methods. As shown in Table 4, LAE inhibited the secretion of TNF- α and IL-6 in PMA plus A23187-stimulated HMC-1 cells.

Effect of LAE on MAPKs and NF- κ B activation

Stimulation of HMC-1 cells with PMA plus A23187 resulted in increased phosphorylation of all three MAPKs peaking at 15–30 min (data not shown). Treatment of LAE attenuated the PMA plus A23187-induced p38 MAPK activation but did not affect the phosphorylation of ERK and JNK (Fig. 3A). Stimulation of HMC-1 cells with PMA plus A23187 induced the degradation of I κ B α and nuclear translocation of p65 NF- κ B after 2 h of incubation. LAE inhibited the PMA plus A23187-induced degradation of I κ B α and nuclear translocation of p65 NF- κ B (Fig. 3B).

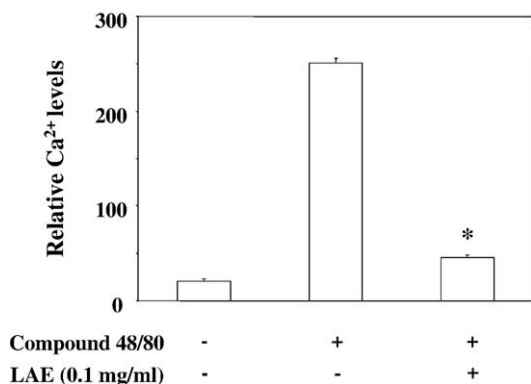


Fig. 2. Effect of LAE on the intracellular Ca²⁺ levels in RPMC. Cells were preincubated for 10 min with LAE (0.1 mg/ml) before adding compound 48/80 (2 μ g/ml), and then another 10 min with compound 48/80. Each bar represents the mean $\pm$ SEM of three independent experiments. * $P < 0.05$ (significantly different from the compound 48/80 value).

Table 4

Effect of LAE on PMA plus A23187-stimulated TNF- α and IL-6 secretion from HMC-1 cells

Treatment	Concentration (mg/ml)	Content (ng/ml)	
		TNF- α	IL-6
None (saline)		0.192 $\pm$ 0.017	0.169 $\pm$ 0.036
PMA + A23187		1.176 $\pm$ 0.014	1.682 $\pm$ 0.241
PMA + A23187 + LAE	0.01	1.155 $\pm$ 0.180	1.476 $\pm$ 0.252
PMA + A23187 + LAE	0.1	0.782 $\pm$ 0.262*	1.056 $\pm$ 0.116*
PMA + A23187 + LAE	1	0.462 $\pm$ 0.091*	0.781 $\pm$ 0.072*

PMA (20 nM) plus A23187 (1 μ M)-stimulated HMC-1 cells were incubated for 6 h in the absence or presence of LAE. TNF- α and IL-6 secreted into the medium are presented as the mean $\pm$ SEM of three independent experiments. * $P < 0.05$, significantly different from the PMA + A23187 value.

LAE also decreased NF- κ B/DNA binding. PDTC, a potent NF- κ B inhibitor, was used as a positive control. HMC-1 cells pretreated with LAE or PDTC attenuated PMA plus A23187-induced NF- κ B binding activity (Fig. 3C). Treatment of cells with specific p38 MAPK inhibitor, SB 203580 (5 μ M), and NF- κ B inhibitor, PDTC (10 μ M), decreased PMA plus A23187-induced TNF- α and IL-6 secretion (Table 5).

Discussion

Immediate-type allergic reaction (anaphylactic allergic reaction) is a life-threatening syndrome induced by the sudden systemic release of inflammatory mediators, such as histamine, heparin, and various cytokines from mast cells. Mast cells are located throughout the human body, and upon allergen exposure, they are stimulated via the IgE receptor (Kemp and Lockey, 2002). The results of this study demonstrated that LAE has anti-allergic properties. LAE inhibited compound 48/80-induced systemic allergic reaction and IgE-mediated local allergic reaction. LAE attenuated compound 48/80-induced or IgE-mediated histamine release from RPMC. These results indicate that mast cell-mediated immediate-type allergic reactions are inhibited by LAE.

The intracellular calcium pathways are critical to the degranulation of mast cells. Agents that stimulate an intracellular calcium level have been shown to induce mast cell degranulation (Tasaka et al., 1986). Calcium movements in mast cells represent a major target for effective anti-allergic drugs, as this is an essential event linking stimulation to secretion (Beaven et al., 1984). The transduction pathways modulating intracellular Ca²⁺ are modified by ADP-ribosylates G-protein binding protein (Alfonso et al., 2000). Our result showing an attenuation of compound 48/80-induced intracellular Ca²⁺ in mast cells with LAE treatment is consistent with other reports. From these observations, we strongly speculate that the decreased intracellular Ca²⁺ is involved in the inhibitory effect of LAE

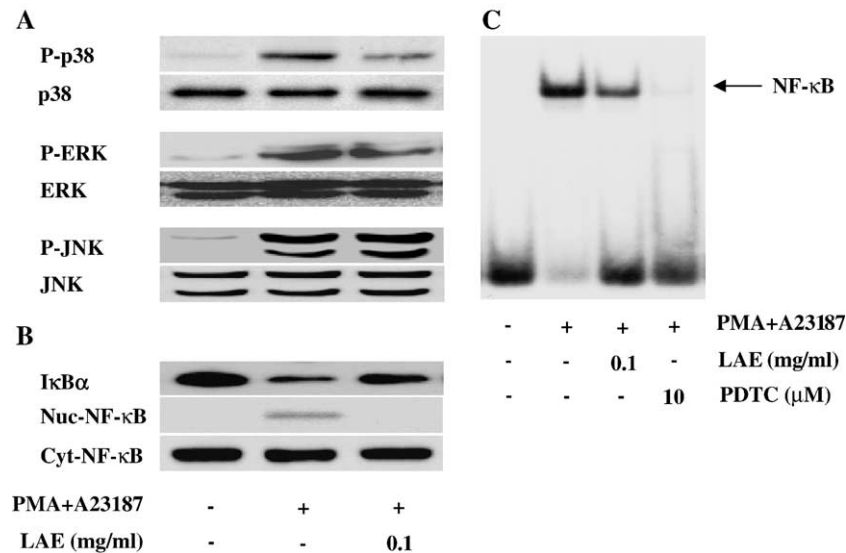


Fig. 3. Effect of LAE on the MAPKs and NF-κB. After pretreatment of LAE (0.1 mg/ml) for 30 min, HMC-1 cells were stimulated by PMA (20 nM) plus A23187 (1 μM) for 15 min for MAPK activation or 2 h for NF-κB activation. (A) Phosphorylation of MAPKs was analyzed by Western blot. (B) Cell extract was analyzed by Western blot for IκBα in cytoplasmic and translocation of NF-κB in nuclear extracts. (C) Inhibition of NF-κB binding activity by LAE was analyzed by EMSA. PDTC (10 μM) was pretreated to cells for 30 min prior to PMA plus A23187 stimulation.

on histamine release, and LAE might have membrane-stabilizing activity through the G-protein.

Numerous reports established that stimulation of mast cells with compound 48/80 or IgE initiates the activation of signal-transduction pathway, which leads to histamine release. Several recent studies have shown that compound 48/80 and other polybasic compounds are able, apparently directly, to activate G-proteins (Mousli et al., 1990). Compound 48/80 increases the permeability of the lipid bilayer membrane by causing a perturbation in the membrane. This result indicates that the increase in membrane permeability may be an essential trigger for the release of the mediator from mast cells. In this sense, anti-allergic agents having a membrane-stabilizing action may be desirable. LAE might stabilize the lipid bilayer membrane, thus preventing the perturbation being induced by compound 48/80.

Mast cell-derived pro-inflammatory cytokines, especially TNF-α and IL-6, have a critical biological role in the

allergic reaction. Mast cells are a principal source of TNF-α in human dermis, and degradation of mast cells in the dermal endothelium is abrogated by the anti-TNF-α antibody (Walsh et al., 1991). IL-6 is also produced from mast cells and its local accumulation is associated with a PCA reaction (Mican et al., 1992). These reports indicate that reduction of pro-inflammatory cytokines from mast cell is one of the key indicators of reduced allergic symptom. In our present study, LAE inhibited the secretion of TNF-α and IL-6 in PMA plus A23187-stimulated HMC-1 cells. This result suggests that the anti-allergic effect of LAE results from its reduction of TNF-α and IL-6 generation from mast cells. Intracellular Ca^{2+} plays an important role in the expression of TNF-α and IL-6. Depletion of intracellular Ca^{2+} blocked the IgE-induced TNF-α and IL-6 expression through NF-κB signaling pathway in RBL-2H3 mast cells (Jeong et al., 2002). Because of the reducing effect of LAE on the intracellular Ca^{2+} , we suggest that one possible pathway of the inhibitory effect of LAE on the TNF-α and IL-6 expression is mediated by the reduction of intracellular Ca^{2+} in HMC-1 cells.

To evaluate the mechanisms of effect of LAE on the pro-inflammatory cytokine expression, we examined the effect of LAE on MAPK activation. The MAPK cascade is one of the important signaling pathways in immune responses. The exact signaling pathways among three types of MAPKs such as p38, ERK, and JNK are still unclear; however, p38 MAPK is thought to play an important role in the regulation of inflammatory responses. Phosphorylation of p38 MAPK is essential for the expression of the pro-inflammatory cytokines (Manthey et al., 1998; Shapiro and Dinarello, 1995). According to present results, PMA plus A23187 simultaneously activated

Table 5
p38 MAPK and NF-κB regulate TNF-α and IL-6 secretion from HMC-1 cells

Treatment	Content (ng/ml)	
	TNF-α	IL-6
None (saline)	0.187 ± 0.009	0.176 ± 0.042
PMA + A23187	1.199 ± 0.054	1.203 ± 0.146
PMA + A23187 + SB 203580	0.542 ± 0.077*	0.612 ± 0.098*
PMA + A23187 + PDTC	0.399 ± 0.022*	0.454 ± 0.071*

HMC-1 cells were pretreated with SB 203580 (5 μM) or PDTC (10 μM) for 30 min prior to PMA (20 nM) plus A23187 (1 μM) stimulation. TNF-α and IL-6 secreted into the medium are presented as the mean ± SEM of three independent experiments. **P* < 0.05, significantly different from the PMA + A23187 value.

all three MAPKs in HMC-1 cells. Among the MAPKs, LAE only inhibited the activation of p38 MAPK but not of ERK or JNK. Furthermore, the specific p38 MAPK inhibitor, SB 203580, decreased TNF- α and IL-6 production. These data suggest that LAE has an inhibitory activity on p38 MAPK activation and downstream TNF- α and IL-6 production. Expression of TNF- α and IL-6 gene is also dependent on the activation of transcription factor NF- κ B. Activation of NF- κ B requires phosphorylation and proteolytic degradation of the inhibitory protein I κ B α (Azzolina et al., 2003). In PMA and A23187-stimulated mast cells, LAE decreased the degradation of I κ B α and nuclear translocation of p65 NF- κ B. Additionally, the specific NF- κ B inhibitor, PDTC, reduced PMA plus A23187-induced TNF- α and IL-6 secretion. These data demonstrate that LAE attenuates activation of NF- κ B and downstream TNF- α and IL-6 production.

Because we used the whole water extract of *L. lucidus*, not a purified component, the active components that are responsible for the biological effect are not clear at this time. The effort to identify active components from *L. lucidus* in the immediate-type allergic reaction is ongoing in our laboratory. Recently, the anti-inflammatory activity of the extracts of *L. lucidus* has been reported (Yun et al., 2003). Among the various components from *L. lucidus*, betulinic acid, a pentacyclic triterpene, modulates the production of TNF- α and IL-6 from monocytes and macrophages in inflammatory and immune responses. This report might shed light on our future study. In conclusion, the results obtained in the present study provide evidence that anal therapy of LAE contributes importantly to the prevention or treatment of allergic diseases.

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