

## ***Lithospermi radix* Extract Inhibits Histamine Release and Production of Inflammatory Cytokine in Mast Cells**

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***Lithospermi radix* (LR, Boraginaceae, the root of *Lithospermum erythrorhizon* Siebold. et Zuccarinii) is used in herbal medicine to treat such conditions as eczema, skin burns and frostbite. This study investigates the effects of LR on the *anti*-allergy mechanism. LR inhibited the release of histamine from rat peritoneal mast cells by compound 48/80 in a dose-dependent manner. LR orally administered at 6.59 mg/100 g also inhibited the *anti*-DNP IgE-induced passive cutaneous anaphylaxis reaction. LR inhibited the PMA plus A23187-induced increase in IL-6, IL-8, and TNF- $\alpha$  expression in HMC-1 cells. In addition, LR also inhibited nuclear factor-kappa B (NF- $\kappa$ B) activation and I $\kappa$ B- $\alpha$  degradation. These results show that LR had an inhibitory effect on the atopic allergic reaction. Furthermore, the *in vivo* and *in vitro* *anti*-allergic effect of LR suggests possible therapeutic applications of this agent for inflammatory allergic diseases.**

**Key words:** *Lithospermi radix*; HMC-1 cell; passive cutaneous anaphylaxis; cytokine; nuclear factor-kappa B (NF- $\kappa$ B)

*Lithospermi radix* (LR, Boraginaceae, the root of *Lithospermum erythrorhizon* Siebold. et Zuccarinii) is an oriental herbal medicine that is used in Korea to treat such conditions, as eczema, skin burns and frostbite.<sup>1,2)</sup> The mast cell is the primary effector cell involved in the allergic or immediate hypersensitivity response.<sup>3)</sup> In addition to their role in atopic diseases, mast cells are the key mediators of antiparasitic responses and might also play an important role in such other inflammatory

states as immune-complex-mediated hypersensitivity.<sup>4)</sup> Mast cell activation occurs through Fc $\epsilon$ RI, which is the high-affinity receptor for IgE, and results in a series of responses. Calcium ionophores, neuropeptides, and compound 48/80 can also stimulate mast cell degranulation.<sup>5,6)</sup> Among the preformed and newly synthesized inflammatory substances released by the degranulation of mast cells, histamine has been the best characterized and most potent vasoactive mediator that has been implicated in the acute phase of the type I allergic reaction.<sup>7)</sup>

Rat passive cutaneous anaphylaxis (PCA), which is an animal model of the IgE-mediated immediate allergic reaction, is also induced by such mediators as histamine secreted from mast cells.<sup>8,9)</sup> Activated mast cells can produce histamine, as well as a wide variety of other inflammatory mediators such as leukotriene, proteoglycans, protease, and several proinflammatory and chemotactic cytokines such as tumor necrosis factor (TNF- $\alpha$ ), and interleukins (IL)-6, IL-4, IL-13 and IL-8.<sup>10,11)</sup>

Nuclear factor  $\kappa$ B (NF- $\kappa$ B) is a key transcription factor that regulates the expression of genes involved in immune and inflammatory responses.<sup>12)</sup> Unstimulated NF- $\kappa$ B is found in the cytoplasm bound to I $\kappa$ B- $\alpha$  upon stimulation. I $\kappa$ B- $\alpha$  is phosphorylated on one or more serine residues, ubiquitinated, and degraded, allowing the transport of NF- $\kappa$ B to the nucleus to bind recognition elements in the upstream promoter region of cytokine DNA.<sup>13)</sup>

We investigated in the present study the effect of LR on the compound 48/80-induced release of histamine from rat peritoneal mast cells, as well as on *anti*-

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Abbreviation: LR, *Lithospermi radix*

dinitrophenyl (DNP) IgE antibody-induced PCA. We show that LR inhibited phorbol 12-myristate 13-acetate (PMA) plus the A23187-induced production of cytokines (IL-6, TNF- $\alpha$  and IL-8) and activation of NF- $\kappa$ B in a human mast cell line.

## Materials and Methods

**Reagents.** Compound 48/80, anti-DNP IgE, DNP-human serum albumin (HAS), PMA, A23187, and Evans blue were purchased from Sigma (St. Louis, MO, USA). Recombinant IL-6 and IL-8, biotinylated IL-6 and IL-8, and anti-human IL-6 and IL-8 were purchased from BD Biosciences (San Diego, CA, USA). Recombinant TNF- $\alpha$ , biotinylated TNF- $\alpha$ , and anti-human TNF- $\alpha$  were purchased from R&D Systems (Minneapolis, MN, USA). MTT and the cell culture media, IMDM and fetal bovine serum (FBS), were purchased from Gibco BRL (Grand Island, NY, USA). The histamine kit was purchased from Oxford Biomedical Research (Oxford, MI, USA), anti-human NF- $\kappa$ B, I $\kappa$ B- $\alpha$  and actin were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA), and anti-mouse IgG-conjugated peroxidase was obtained from Amersham Pharmacia Biotech (Newark, NJ, USA).

**Animals.** The animal care and all experimental procedures were conducted in accordance with the Guide for Animal Experiments edited by the Korean Academy of Medical Sciences. The original stock of Sprague-Dawley (SD) rats was purchased from the Samtako Experimental Animal Center (Ossan, Gyeonggi-do, Korea), the animals then being maintained in the College of Oriental Medicine at Kyung Hee University. The rats were housed 4 per cage in a laminar air-flow room maintained at a temperature of  $22 \pm 1^\circ\text{C}$  and relative humidity of  $55 \pm 10\%$  throughout the study.

**Preparation of LR.** The plant sample was obtained from Kyung Hee University Medical Center (Seoul, Korea). LR was prepared by decocting a dried prescription of herbs in boiling distilled water. The extract decocted for approximately 3 h was filtered, lyophilized, and stored at  $4^\circ\text{C}$ . The LR water extract powder was dissolved in saline and passed through a 0.22- $\mu\text{m}$  syringe filter.

**Culture of the HMC-1 cells.** The human mast cell line (HMC-1) was grown in Iscove's modified Dulbecco's medium (IMDM) with 10% fetal bovine serum (FBS) at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  atmosphere at 95% humidity. The HMC-1 cells ( $3 \times 10^5$  cells) were pretreated with various concentrations of the LR extract (0.01–1 mg/ml) for 30 min prior to being stimulated with 25 nM PMA plus 1  $\mu\text{M}$  A23187, and then incubated at  $37^\circ\text{C}$  for 7 h.

**Preparation of the rat peritoneal mast cells (RPMC).** RPMC were isolated as previously described. Briefly,

the rats were anesthetized with ether, and injected into the peritoneal cavity with 10 ml of Tyrode buffer A (10 mM HEPES, 136 mM NaCl, 5 mM KCl, 2 mM  $\text{CaCl}_2$ , 2.75 mM  $\text{MgCl}_2$ , 5.6 mM glucose, 11 mM  $\text{NaHCO}_3$ , 0.56 mM  $\text{NaH}_2\text{PO}_4$ , and 0.1% bovine serum) containing 0.1% gelatin (Sigma, St. Louis, MO, USA). The abdomen was gently massaged for approximately 90 sec. The peritoneal cavity was carefully opened, and the fluid containing the peritoneal cells was aspirated with a Pasteur pipette. The peritoneal cells were then sedimented at  $150 \times g$  for 10 min at room temperature and resuspended in the Tyrode buffer. The mast cells were separated from the major components of the rat peritoneal cells (*i.e.*, macrophages and small lymphocytes) according to the method described elsewhere.<sup>14</sup> The mast cell preparation was approximately 95% pure, as assessed by toluidine blue staining. More than 97% of the cells were viable, as judged by the trypan blue exclusion.

**Histamine assay.** Purified RPMC were resuspended in the Tyrode buffer containing calcium for treatment with compound 48/80. The RPMC suspension ( $2 \times 10^5$  cells) was preincubated for 10 min at  $37^\circ\text{C}$  before adding compound 48/80 for stabilization. The cells were preincubated with the LR extract (0.01–1 mg/ml) for 10 min, and then incubated with compound 48/80 (5  $\mu\text{g}/\text{ml}$ ) for 20 min. The reaction was quenched by cooling the tubes on ice. The cells were separated from the released histamine by centrifugation at  $400 \times g$  for 10 min at  $4^\circ\text{C}$ . The histamine content was measured by using the enzyme immunoassay in the histamine kit. OD was measured at 450 nm with a microplate ELISA reader (Molecular Devices, CA, USA). The percentage inhibition was calculated as  $=(A - B) \times 100/A$ , where A was the amount of histamine released without the LR extract and B was the amount of histamine released with the LR extract.

**PCA reaction.** The IgE-dependent cutaneous reaction was generated by sensitizing the skin with an intradermal injection of anti-DNP IgE, this being followed 48 h later by an injection of DNP-HSA into the dorsal vein of the rat's penis. DNP-HSA was diluted in phosphate-buffered saline (PBS). The rats were injected intradermally with 0.5  $\mu\text{g}$  (50  $\mu\text{l}$ ) of anti-DNP IgE into each of three dorsal skin sites that had been shaved 48 h earlier, the sites being outlined with a water-insoluble red marker. Forty-eight hours later, each rat received an injection of 100  $\mu\text{g}$  (100  $\mu\text{l}$ ) of DNP-HSA in saline containing 4% Evans blue through the dorsal vein of the penis. One hour before the injection, the LR extract was orally administered (6.59 mg/100 g body weight). The rats were sacrificed 30 min after the intravenous challenge. The dorsal skin of the rats was removed and the pigment area was measured. The amount of dye was then colorimetrically measured after extraction with formamide. The absorbent intensity of the extract was

measured at a wavelength of 620 nm with a spectrophotometer (Beckman, IL, USA), the amount of dye being calculated by using the Evans blue measuring line.

**MTT assay for cell viability.** The viability of the cells was determined with an MTT colorimetric assay as described previously. Briefly, a HMC-1 cell suspension ( $3 \times 10^5$  cells) was cultured (24-well plates) with various concentrations of the LR extract (0.01–1 mg/ml). An MTT solution (5 mg/ml) was then added to each well, and the cells were cultured overnight. After washing out the supernatant, the insoluble formazan product was dissolved in DMSO. The OD value of the 96-well culture plates was measured with an ELISA reader at 540 nm. The OD value of formazan that formed in the untreated control cells was taken to represent 100% viability.

**TNF- $\alpha$ , IL-6, IL-8 assay.** The human mast cell line (HMC-1) was grown in IMDM with 10% FBS at 37°C in an atmosphere containing 5% CO<sub>2</sub> with 95% humidity. The HMC-1 cells were pretreated with various concentrations of the LR extract (0.01–1 mg/ml) for 1 h prior to being stimulated with PMA plus A23187. Each culture supernatant was then assayed for the TNF- $\alpha$ , IL-6 and IL-8 protein levels by an enzyme-linked immunosorbent assay (ELISA). The cytokines were measured by using a modified ELISA method as described elsewhere. Sandwich ELISA for TNF- $\alpha$ , IL-6 and IL-8 was carried out in duplicate in 96-well ELISA plates (Nunc, Denmark) coated with each of 100- $\mu$ l aliquots of the mouse *anti*-human TNF- $\alpha$ , IL-6 and IL-8 monoclonal antibodies in 1.0  $\mu$ g/ml of PBS at pH 7.4, and then incubated overnight at 4°C. The plates were washed with PBS containing 0.05% Tween-20 (Sigma, St. Louis, MO, USA) and blocked with PBS containing 1% BSA, 5% sucrose and 0.05% NaN<sub>3</sub> for 1 h. After additional washes, the sample or recombinant TNF- $\alpha$ , IL-6 or IL-8 standard was added and incubated at 37°C for 2 h. After incubating at 37°C for 2 h, the wells were washed, and 0.2  $\mu$ g/ml of biotinylated *anti*-human TNF- $\alpha$ , IL-6 or IL-8 was added, before incubating at 37°C for 2 h. After washing the wells, avidin-peroxidase was added, and the plates were incubated at 37°C for 30 min. The wells were again washed, and the ABTS substrate (Sigma, St. Louis, MO, USA) was added. Color development was measured at 405 nm with an automated microplate ELISA reader. A standard curve was run on each assay plate, using recombinant human TNF- $\alpha$ , IL-6 or IL-8 in a serial dilution.

**Western blotting analysis.** The HMC-1 cells were pretreated with various concentrations of the LR extract (0.01–1 mg/ml) for 1 h, and then stimulated with PMA plus A23187 for 2 h. The cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS). The washed cell pellets were resuspended in ice-cold buffer A (10 mM PEPES at pH 7.9, 10 mM KCl,

1.5 mM MgCl<sub>2</sub>, 0.5 mM DTT, 0.2 mM PMSF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, a proteinase inhibitor, and 0.1% NP 40), and after immediately vortexing for 10 sec, subjected to 15 min of incubation at 4°C. The nuclei were harvested by centrifugation at  $10,000 \times g$  for 5 min, resuspended in ice-cold buffer B (20 mM PEPES at pH 7.9, 300 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, and 1 mM Na<sub>3</sub>VO<sub>4</sub>, a proteinase inhibitor), and after vortexing for 10 sec, subjected to 20 min of incubation at 4°C. Nucleus fractions were obtained as supernatants after centrifugation at  $10,000 \times g$  and 4°C for 10 min. The protein concentration was determined by using the Bio-Rad protein assay reagent according to the manufacturer's instruction. Each sample was separated on 10% SDS-polyacrylamide gel, and then the proteins were electrotransferred to a nitrocellulose membrane at 4°C. The membrane was blocked with 5% skim milk in PBS containing 0.1% Tween-20 (PBST) for 2 h at room temperature. After lightly washing with PBST, the membrane was incubated overnight at 4°C with *anti*-NF- $\kappa$ B Ab or *anti*-I $\kappa$ B- $\alpha$  Ab (1:500 or 1:1000 dilutions in the blocking solution) and then washed three times with PBST. Horseradish peroxidase-conjugated secondary Ab (1:2000 dilution in the blocking solution) was incubated for 1 h at room temperature, and the antibody-specific proteins were visualized by an enhanced chemiluminescence procedure, using an ECL detection reagent (Amersham Pharmacia, NJ, USA).

**Statistical analysis.** Each measured value is presented as the mean  $\pm$  standard error. A statistical analysis was performed by using SAS statistical software (SAS Institute, Cary, NC). Treatment effects were analyzed by using one-way ANOVA, this being followed by Duncan's multiple-range test, and  $P < 0.05$  was used to indicate significance.

## Results

### *Effect of the LR extract on histamine release from RPMC*

Figure 1 shows the inhibitory effect of the LR extract on the compound 48/80-induced release of histamine from RPMC. The LR extract inhibited the compound 48/80-induced histamine release at concentrations of 0.01–1 mg/ml in a dose-dependent manner. At a concentration of 1 mg/ml, the level of inhibition reached 92.25%. In particular, the LR extract significantly inhibited the compound 48/80-induced release of histamine at concentration of 1 mg/ml ( $P < 0.001$ ).

### *Effect of the LR extract on PCA*

PCA is one of the most important *in vivo* models for anaphylaxis in a local allergic reaction. The LR extract (6.59 mg/100 g) orally administered to the SD Rats significantly inhibited the PCA reaction (Fig. 2 and Table 1).

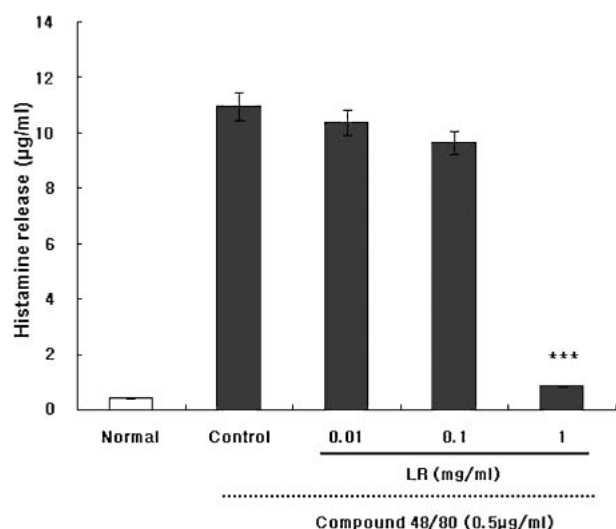


**Fig. 1.** Effects of LR on Compound 48/80-Induced Histamine Release of Rat Peritoneal Mast Cells.

The cells ( $2 \times 10^5$  cells) were pretreated with various concentrations of LR at  $37^\circ\text{C}$  for 10 min prior to being incubated with compound 48/80. Each data value represents the mean  $\pm$  S.E.M. of three independent experiments. \*\*\* $P < 0.01$ , significantly different from the control value.

#### Effect of the LR on the cytotoxicity in HMC-1 cells

The HMC-1 cells were incubated with LR at  $37^\circ\text{C}$ . After incubating the cells for 24 h, the cell viability was measured by using an MTT assay. LR (0.01–1 mg/ml) had no cytotoxic effect over a 24-h period (data not shown).

#### Effect of the LR extract on TNF- $\alpha$ , IL-6, and IL-8 secretion from the HMC-1 cells

The HMC-1 cells were stimulated with PMA and the calcium ionophore, A23187. In order to assess the effect of LR in PMA and the A23187-induced TNF- $\alpha$ , IL-6 and IL-8 secretion, the cells were pretreated with various LR concentrations for 1 h prior to adding the stimulators. The results show that pretreating the cells with the LR extract resulted in the inhibition of TNF- $\alpha$  secretion, the inhibitory effect of the LR extract being significant at doses of 0.01 and 1 mg/ml (Fig. 3A). The inhibitory effect of the LR extract on IL-6 secretion was significant at doses of 0.01 and 1 mg/ml (Fig. 3B).

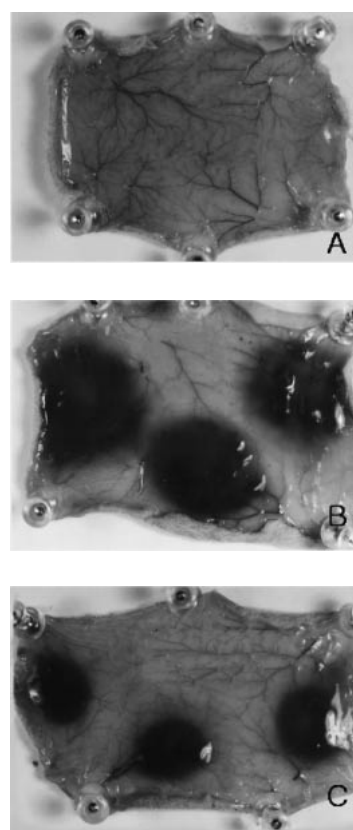


**Fig. 2.** Challenge with the Antigen (DNP-HAS) after a Photogram of the Internal Surface of Rat Skin.

A, Normal; B, Control; C, *Lithospermi Radix* (LR, 6.59 mg/100 g).

The inhibitory effect of the LR extract on IL-8 secretion was also significant at doses of 0.01 and 1 mg/ml (Fig. 3C).

#### Effect of the LR extract on NF- $\kappa$ B activation from HMC-1 cells

To determine whether the inhibitory action of LR was due to an effect on NF- $\kappa$ B activation, the nucleus levels of NF- $\kappa$ B were examined by a Western blot analysis after stimulating with PMA plus A23187. Stimulation of the HMC-1 cells with PMA plus A23187-induced the degradation of I $\kappa$ B- $\alpha$  and nuclear translocation of p65 NF- $\kappa$ B after 2 h of incubation.

**Table 1.** Effect of Administering LR on PCA in Rats

| Group <sup>a</sup> | Amount of dye (µg/ml) <sup>b</sup> | Area/min (mm) <sup>c</sup> | Weight (g) <sup>d</sup> | Inhibition (%) |
|--------------------|------------------------------------|----------------------------|-------------------------|----------------|
| None               | 330.2 $\pm$ 42.9                   |                            |                         |                |
| Control            | 8846.0 $\pm$ 649.1                 | 183.2 $\pm$ 12.2           | 1.6 $\pm$ 0.1           |                |
| LR                 | 4637.6 $\pm$ 828.1***              | 90.8 $\pm$ 11.4***         | 0.8 $\pm$ 0.1***        | 52.4%          |

<sup>a</sup>Anti-DNP IgE was injected into dorsal skin sites. After 48 h, LR (6.59 mg/100 g) was administered orally 1 h prior to challenge with the antigen (DNP-HAS).

<sup>b</sup>Each amount of dye is presented as the mean  $\pm$  S.E.M. from three independent experiments.

<sup>c</sup>The patch of Evans blue was measured 30 min after the antigen (DNP-HAS) injection.

<sup>d</sup>The weight of the Evans blue patch was measured 30 min after the antigen (DNP-HAS) injection.

\*\*\* $p < 0.001$  compared with the control group (anti-DNP IgE plus DNP-HAS).

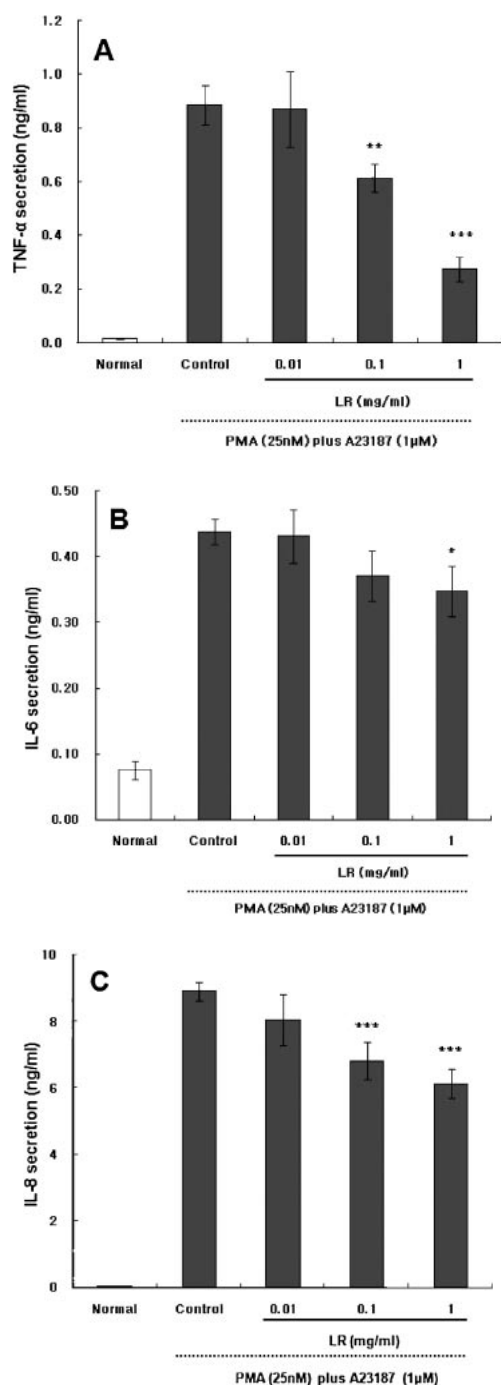


**Fig. 3.** Effects of LR on PMA Plus A23187-Induced Cytokine Production (A, TNF- $\alpha$ ; B, IL-6; C, IL-8) in HMC-1 Cells.

The cells ( $3 \times 10^5$  cells) were pretreated with various concentrations of LR (0.01–1 mg/ml) for 1 h and then stimulated with PMA (25 nM) plus A23187 (1  $\mu$ M) for 7 h. The level of cytokine in the supernatant was measured by using ELISA. Each data value represents the mean  $\pm$  S.E.M. of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , significantly different from the control value.

LR inhibited the PMA plus A23187-induced degradation of I $\kappa$ B- $\alpha$  and nuclear translocation of p65 NF- $\kappa$ B (Fig. 4). Figure 4B shows the NF- $\kappa$ B and  $\beta$ -actin protein levels quantified by Scion image software.

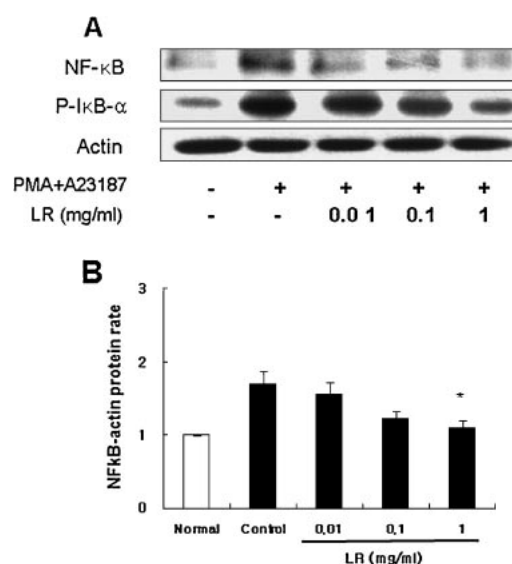


**Fig. 4.** Effect of LR on the Activation of NF- $\kappa$ B in HMC-1 Cells.

The cells ( $4 \times 10^6$  cells) were pretreated with various concentrations of the LR extract (0.01–1 mg/ml) for 1 h and then stimulated by PMA (25 nM) plus A23187 (1  $\mu$ M) for 2 h. A, Cell extract analyzed by western blotting for I $\kappa$ B- $\alpha$  in cytoplasmic and translocation of NF- $\kappa$ B in nuclear extracts. B, NF- $\kappa$ B protein levels quantified by Scion image software. Each bar represents the mean  $\pm$  S.E.M. of four independent experiments. \* $P < 0.05$ , significantly different from the control value.

## Discussion

Mast cells are located throughout the human body, and the important role in the immediate-type allergic reaction is well recognized.<sup>15)</sup> Compound 48/80 increases the permeability of the lipid bilayer membrane by causing a perturbation in the membrane.<sup>16)</sup> Thus, the increase in membrane permeability may be an essential trigger for the release of the mediator from mast cells. As shown by previous studies, the family of 48/80 basic compounds can activate the release of histamine from mast cells.<sup>17,18)</sup>

This response is preceded by methylation of the membrane phospholipids, phosphorylation of tyrosin kinase and mobilization of internal  $\text{Ca}^{2+}$ . This is followed by the activation of protein kinase C, nuclear factor- $\kappa$ B (NF- $\kappa$ B), and the release of inflammatory cytokines.<sup>18,19)</sup>

We observed that LR inhibited the compound 48/80-induced release of histamine from RPMC. LR might have stabilized the lipid bilayer membrane, thus preventing the perturbation being induced by compound 48/80. In addition, when the drugs were administered orally to the rats, LR potently inhibited the IgE-mediated PCA reaction. PCA is one of the most important *in vivo* models for anaphylaxis in a local allergic reaction.<sup>20)</sup> This finding suggests that LR might be useful in the treatment of allergic skin reactions.

Human mast cells have become recognized as a source of many cytokines. Although the importance and role of mast cell-derived cytokines in diseases is

uncertain, it is conceivable they may play an important role under both physiological and pathological conditions.<sup>21)</sup> HMC-1 cells can serve as a useful mast cell system for examining the expression of chemokines during inflammation.<sup>22)</sup> The mast cell-derived pro-inflammatory cytokines, particularly TNF- $\alpha$ , IL-6 and IL-8, play a key biological role in the allergic reaction. TNF- $\alpha$  is an inflammatory mediator with a wide variety of biological functions. It is also clear that the production of TNF- $\alpha$  has an detrimental role in allergies and asthma, as a mediator of the late-phase inflammatory reaction.<sup>23,24)</sup> TNF- $\alpha$  in mast cells has been found to be prestored in the cytoplasmic granules and is released with histamine and other preformed mediators within minutes of antigenic stimulation. This suggests a potential role in early inflammation as well.<sup>25)</sup>

IL-6 is a pleiotropic inflammatory cytokine produced by T cells, monocytes, macrophages and synovial fibroblasts, and plays a major role in cellular activation.<sup>26,27)</sup> IL-6 is also produced from mast cells in released allergic inflammatory diseases such as rheumatoid arthritis (RA) and asthma.<sup>28,29)</sup> Moreover, its local accumulation is associated with a PCA reaction.<sup>30)</sup> IL-8 acts on such inflammatory effector cells as neutrophils, T lymphocytes, B lymphocytes, and eosinophils.<sup>31)</sup> The role of mast cells in IL-8-induced neutrophil migration has been confirmed by other studies.<sup>32,33)</sup> The amount of IL-8 released has also been reported to be higher in the bronchoalveolar large fluid of asthmatic patients.<sup>34)</sup>

This study has demonstrated that LR inhibited the production of TNF- $\alpha$ , IL-6 and IL-8 secretion in PMA plus A23187-stimulated HMC-1 cells. This suggests that the *anti*-allergic effect of LR was the result of its ability to decrease the level of TNF- $\alpha$ , IL-6 and IL-8 generation from mast cells.

We also experimented on NF- $\kappa$ B activation to recognize the mechanism for the effect of LR on pro-inflammatory cytokine expression. The nuclear translocation of NF- $\kappa$ B follows the degradation of its inhibitor, I $\kappa$ B- $\alpha$ , an event coupled with stimulation-dependent inhibitor phosphorylation. In PMA plus A23187-induced stimulated mast cells, LR inhibited NF- $\kappa$ B activation by suppressing I $\kappa$ B- $\alpha$  degradation. Consequently, we could demonstrate that the TNF- $\alpha$ , IL-6 and IL-8 inhibitory mechanism of LR involved the suppression of NF- $\kappa$ B activation.

Other studies have shown that treating with LR significantly inhibited the carcinogen, N-butyl-N-butanolnitrosoamine (BBN),-induced suppression of chemotactic activity and the production of IL-1 and TNF- $\alpha$  by macrophages.<sup>35)</sup> LR has also inhibited the lipopolysaccharide (LPS) and interferon- $\gamma$ -induced expression of inducible nitric oxide synthase and TNF- $\alpha$  in mouse peritoneal macrophages.<sup>36)</sup> The color components of LR are composed of the naphthoquinone compound, shikonin, and its derivatives.<sup>37,38)</sup> Shikonin has been reported to confer many medicinal properties such as

anti-inflammatory and anti-tumor effects.<sup>2,39)</sup> we therefore assumed that the effects of LR in this study would be related to shikonin or its derivatives.

In conclusion, LR inhibited the mast cell-derived inflammatory allergic reactions by blocking the release of histamine and the activation of the pro-inflammatory cytokine, NF- $\kappa$ B, by suppressing I $\kappa$ B- $\alpha$  degradation. Furthermore, the *in vivo* and *in vitro* anti-allergic effect of LR suggests a possible therapeutic application of this agent to control inflammatory allergic diseases.

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