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Lancemaside A Inhibits Lipopolysaccharide-Induced Inflammation by Targeting LPS/TLR4 Complex

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ABSTRACT

In our previous study, lancemaside A isolated from *Codonopsis lanceolata* (family Campanulaceae) ameliorated colitis in mice. In this study, the anti-inflammatory effects of lancemaside A was investigated in lipopolysaccharide (LPS)-stimulated mice and their peritoneal macrophage cells. Lancemaside A suppressed the production of pro-inflammatory cytokines, TNF-α and IL-1β, in vitro and in vivo. Lancemaside A also down-regulated inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), as well as the inflammatory mediators, nitric oxide (NO), and PGE_2. Lancemaside A also inhibited the expression of IL-1 receptor-associated kinase-4 (IRAK-4), the phosphorylation of IKK-β and IκB-α, the nuclear translocation of NF-κB and the activation of mitogen-activated protein kinases in LPS-stimulated peritoneal macrophages. Furthermore, lancemaisde A inhibited the interaction between LPS and TLR4, as well as IRAK-4 expression in peritoneal macrophages. Based on these findings, lancemaside A expressed anti-inflammatory effects by regulating both the binding of LPS to TLR4 on macrophages. J. Cell. Biochem. 111: 865–871, 2010.

KEY WORDS: LANCEMASIDE A; TLR4; LPS; IRAK-4; INFLAMMATION

There is increasing evidence that systemic inflammation is associated with increased risk of chronic diseases such as cardio-vascular disease, cancer, obesity, and insulin resistance [Libby, 2006; Schottenfeld and Beebe-Dimmer, 2006; Shoelson et al., 2007]. The mechanisms may involve macrophage and T lymphocyte activation, as well as the release of pro-inflammatory cytokines that induce the inflammatory activity [Tousoulis et al., 2005; Lin and Karin, 2007]. Inflammation can be mediated by inflammatory mediators, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, interferon (IFN)-γ, IL-12, IL-18, nitric oxide, and prostaglandins [Fairweather and Rose, 2005]. Among these inflammatory mediators, pro-inflammatory cytokines such as TNF-α and IL-1β are activated through nuclear factor-kappaB (NF-κB) but they also activate NF-κB [Collins et al., 1995; Baldwin, 1996]. Thus, these mediators stimulate the innate immune response but their over-expression causes acute phase endotoxemia leading to tissue injury, organ failure, shock, and even death [Astiz and Rackow, 1998]. Regulating expression of these inflammatory mediators can therefore be beneficial in reducing inflammatory diseases. Thus, to prevent chronic diseases associated with inflammation [O'keefe et al., 2008], the application of dietary constituents has recently become a focus of interest [Chan et al., 1998; Paradkar et al., 2004; Davis et al., 2006].

The rhizome of *Codonopsis lanceolata* (CL) is frequently used in Asian countries in food and/or herbal medicines for inflammatory diseases such as bronchitis and cough [Lee et al., 2002]. Its saponins exhibit anti-inflammatory and anti-tumor effects. We also reported that lancemaside A isolated from its BuOH fraction, which contains lancemaside A as a major constituent, potently inhibited colitis via TLR-linked NF-κB activation in mice [Joh et al., 2010].

Therefore, in the present study, to clarify the mechanism of lancemaside A, we investigated the anti-inflammatory effects of lancemaside A in LPS-stimulated peritoneal macrophages.

Abbreviations used: ANOVA, analysis of variance; CL, *Codonopsis lanceolata*; COX-2, cyclooxygenase-2; ECL, enhanced chemiluminescence; ERK, extracellular signal-regulated kinase; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; IRAK, interleukin-1 receptor-associated kinase; JNK, c-Jun NH_2-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappaB; PGE_2, prostaglandin E2; PVDF, polyvinylidene difluoride membranes; TLR, toll-like receptor; TNF, tumor necrosis factor.

The authors state no conflict of interest.

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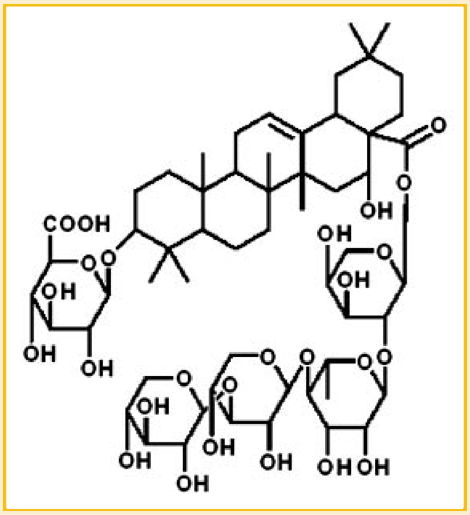

Fig. 1. The structure of lancemaside A.

MATERIALS AND METHODS

REAGENTS

Lancemaside A was isolated from CL as previously reported by Joh et al. [2010] (Fig. 1). RPMI 1640, penicillin–streptomycin, and LPS purified from *Escherichia coli* O111:B4 were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies for IRAK-4, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, LA). Antibodies for p-IKK-β, IκB-α, p65, p-p65, p38, p-p38, JNK, p-JNK, ERK, and p-ERK were purchased from Cell Signaling Technology (Beverly, MA). Cytokine ELISA kits were purchased from R&D Systems (Minneapolis, MN). Other chemicals were of the highest grade available.

ANIMALS

The male ICR mice (20–25 g, 5 weeks old) were supplied from Orient Animal Breeding Center (Sungnam, Korea). All animals were housed in wire cages at 20–22°C and 50 ± 10% humidity, fed standard laboratory chow (Samyang Co., Seoul, South Korea), and allowed water ad libitum. All experiments were performed in accordance with the NIH and Kyung Hee University guides for Laboratory Animals Care and Use and approved by the Committee for the Care and Use of Laboratory Animals in the College of Pharmacy, Kyung Hee University.

ISOLATION AND CULTURE OF PERITONEAL MACROPHAGES

Male ICR mice were intraperitoneally injected with 2 ml of 4% thioglycolate solution. Mice were sacrificed 4 days after injection and the peritoneal cavities were flushed with 10 ml of RPMI 1640. The peritoneal lavage fluids were centrifuged at 200*g* for 10 min and the cells were resuspended with RPMI 1640 and plated. After incubation for 1 h at 37°C, the cells were washed three times and non-adherent cells were removed by aspiration. Cells were cultured in 24-well plates (0.5×10^6 cells/well) at 37°C in RPMI 1640 plus 10% FBS. The attached cells were used as peritoneal macrophages [Park et al., 2009]. To examine the anti-inflammatory effects of lancemaside A, peritoneal macrophages were incubated in the absence or presence of lancemaside A with 50 ng/ml LPS.

DETERMINATION OF NITRIC OXIDE AND CYTOKINES

Nitrite was measured in culture media using Griess reagent [Bakker et al., 2009]. The culture medium (100 μl) was combined with 100 μl of Griess reagent [mixture of equal volume of 1% sulfanilamide in 5% H_3PO_4 and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in H_2O] in a 96-well plate, and then the absorbance was measured spectrophotometrically at 550 nm. Nitrite concentration was determined by using sodium nitrite as a standard.

Cytokines, TNF-α, IL-1β, and prostaglandin E2 (PGE_2), were assessed using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) according to the manufacturer's instructions.

IMMUNOBLOT ANALYSIS

The cell supernatant extracts prepared from macrophages were separated by 9% SDS–PAGE and transferred onto polyvinylidene difluoride membranes (PVDF). The membranes were blocked with 5% non-fat dried-milk proteins in PBST, then probed with COX-2, iNOS, IRAK-4, p-IKK-β, IκB-α, p65, p-p65, p38, p-p38, JNK, p-JNK, ERK, p-ERK, or β-actin antibody. After washing with PBST, proteins were detected with HRP-conjugated secondary antibodies for 1 h. Bands were visualized with enhanced chemiluminescence (ECL) reagent [Shin et al., 2005].

IMMUNOFLUORESCENT CONFOCAL MICROSCOPY

For the assay of p65, peritoneal macrophages were stimulated with LPS (50 ng/ml) in the presence or absence of lancemaside A (10 μM) for 60 min. The cells were then fixed with 4% formaldehyde and permeabilized with 0.2% Triton X-100. The cells were stained with goat polyclonal anti-p65 antibody for 2 h at 4°C and then incubated with secondary antibodies conjugated with Alexa 488 and propidium iodide (10 μg/ml, Calbiochem Co., San Diego, CA) for 1 h. Images were observed by confocal microscopy.

For the assay of LPS–TLR4 complex, peritoneal macrophages plated on cover slides were incubated at 37°C overnight. Macrophages were stimulated with Alexa Fluor 594-conjugated LPS (10 μg/ml, Invitrogen, CA) for 20 min in the presence or absence of lancemaside A. The cells were fixed with 4% formaldehyde and 3% sucrose for 20 min [Baldwin, 2001]. The cells were stained with rabbit polyclonal anti-TLR4 antibody for 2 h at 4°C and then incubated with secondary antibodies conjugated with Alexa Fluor 488 for 1 h [Park et al., 2008]. The stained cells were observed by confocal microscopy.

FLOW CYTOMETRY

Mouse peritoneal macrophages were incubated with or without Alexa Fluor 594-conjugated LPS (10 μg/ml, Invitrogen) for 30 min. The cells were then fixed in PBS containing 4% paraformaldehyde and 3% sucrose for 20 min. The cells were stained with rabbit polyclonal anti-TLR4 antibody for 2 h at 4°C and then incubated with secondary antibodies conjugated with Alexa Fluor 488 for 1 h and then analyzed by flow cytometry.

ASSAY OF SERUM TNF-α AND IL-1β IN LPS-STIMULATED MICE

Male ICR mice were intraperitoneally injected with LPS (4 mg/kg) in the absence or presence of lancemaside A (5 mg/kg). Mice were sacrificed 4 h after LPS injection, and whole blood was obtained by cardiac puncture. Serum was prepared by centrifugation at 12,000*g* for 20 min at 4°C. The levels of TNF-α and IL-1β in the serum were determined using ELISA kits (R&D Systems).

STATISTICAL ANALYSIS

Results are presented as the means ± standard deviation of at least three replicates. Analysis of variance (ANOVA) was used for comparisons between multiple groups The Student's *t*-test was used for the statistical analysis of the difference noted. *P* values of 0.05 or less were considered statistically significant.

RESULTS

EFFECT OF LANCEMASIDE A ON PROTEIN EXPRESSION OF PRO-INFLAMMATORY CYTOKINES

To investigate the anti-inflammatory effect of lancemaside A, we measured the inhibitory effect on protein expression of the pro-inflammatory cytokines, IL-1β and TNF-α, in LPS-stimulated macrophages. The stimulation of peritoneal macrophages with LPS increased IL-1β and TNF-α expression. When cells were treated with LPS in the presence of lancemaside A, IL-1β, and TNF-α expression were significantly decreased (Fig. 2). No cytotoxic effects of lancemaside A were observed in the cell viability test (crystal violet method) under the conditions used in these experiments.

We next examined the inhibitory effects of lancemaside A on serum IL-1β and TNF-α levels in mice intraperitoneally treated with LPS (Fig. 3). The treatment with LPS significantly increased serum IL-1β and TNF-α levels. However, treatment with LPS in the presence of lancemaside A significantly reduced these cytokine levels compared with those in control mice injected with LPS alone.

INHIBITORY EFFECTS OF LANCEMASIDE A ON INFLAMMATORY ENZYME PRODUCTION IN LPS-STIMULATED PERITONEAL MACROPHAGES

Mouse peritoneal macrophages were stimulated with LPS in the presence or absence of lancemaside A to confirm whether they down-regulate inflammatory enzymes such as COX-2 and iNOS. When these macrophages were stimulated with LPS in the absence of lancemaside A, PGE_2, and NO levels were significantly induced. Co-treatment with LPS and lancemaside A significantly reduced PGE_2 and NO expression in a dose-dependent manner (Fig. 4). In contrast, their expression in control LPS groups did not decrease. Lancemaside A (10 μM) inhibited the LPS-induced production of PGE_2 by approximately 70%, respectively, and the LPS-induced production of NO by 58%, respectively.

To determine whether lancemaside A suppress protein expressions of COX-2 and iNOS to regulate the production of these pro-inflammatory mediators, we examined the protein levels of COX-2 and iNOS in peritoneal macrophages stimulated with LPS in the presence or absence of lancemaside A (Fig. 4A). LPS induced these enzymes. However, lancemaside A significantly inhibited the expression of these enzymes.

INHIBITORY EFFECTS OF LANCEMASIDE A ON IKK-β PHOSPHORYLATION, IκB-α DEGRADATION AND NF-κB ACTIVATION IN LPS-STIMULATED PERITONEAL MACROPHAGES

Phosphorylation of IKK-β and IκB-α (through ubiquitination, and proteolytic degradation) lead to the nuclear translocation of NF-κB, which exists as a complex of NF-κB·IκB-α in the cytoplasm [Wang et al., 2001]. To investigate the inhibitory effects of lancemaside A on the phosphorylation of IKK-β and degradation of IκB-α in LPS-induced peritoneal macrophages, the cells were treated with LPS in the presence and absence of lancemaside A (Fig. 5A). Exposure to LPS increased phosphorylation of IKK-β and degradation of IκB-α in the cells. The treatment with LPS and lancemaside A decreased the LPS-induced phosphorylation of IKK-β and degradation of IκB-α.

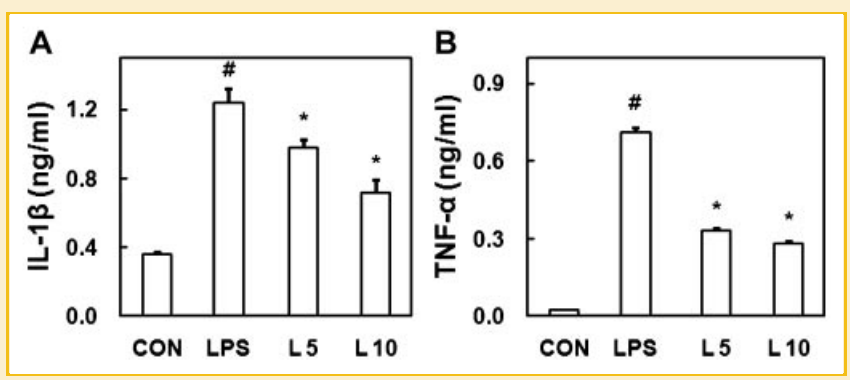

Fig. 2. Inhibitory effects of lancemaside A on the production of pro-inflammatory cytokines in LPS-induced peritoneal macrophages. The peritoneal macrophages (0.5×10^6 cells) were treated with 50 ng/ml LPS in absence (LPS) or presence of lancemaside A (L5, 5 μM lancemaside A; L10, 10 μM lancemaside A) for 20 h. Levels of IL-1β (A) and TNF-α (B) in culture supernatants were measured by ELISA. Normal control (CON) was treated with vehicle alone instead of LPS and lancemaside A. All data are expressed as mean ± SD (n = 4 in a single experiment). $^{\#}P < 0.05$, significantly different versus CON group. $^{*}P < 0.05$ versus LPS group.

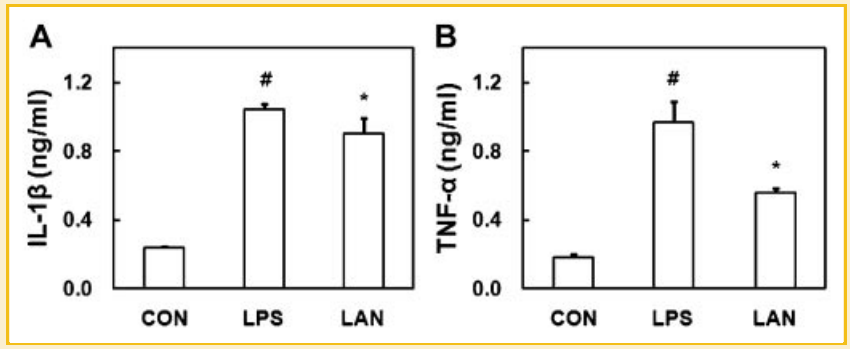

Fig. 3. Inhibitory effects of lancemaside A on the production of inflammatory cytokines in mice. ICR mice were intraperitoneally injected with LPS (4 mg/kg) and/or lancemaside A (5 mg/kg; LAN, lancemaside A). Normal control (CON) was treated with vehicle alone instead of LPS and lancemaside A. Mice sacrificed 4 h after LPS injection, whole blood was obtained by cardiac puncture and serum was obtained by centrifugation at 12,000*g* for 20 min. A: The serum levels of IL-1β (A) and TNF-α (B) were measured using ELISA kit. All data shown are the mean ± SD (n = 6). $^{\#}P < 0.05$, significantly different versus CON group. $^{*}P < 0.05$ versus LPS group.

We next examined whether lancemaside A could inhibit the phosphorylation and the nuclear translocation of NF-κB in LPS-stimulated peritoneal macrophages according to immunoblot (Fig. 5B) and confocal analysis (Fig. 5C). The p65 levels in the cytosol of LPS-treated cells were decreased compared to that in cells treated with LPS alone. Lancemaside A significantly inhibited the level of p65 in the cytosol. As further confirmation, p65 translocation into the nuclear was detected by confocal analysis using p65 antibody. Lancemaside A significantly inhibited the translocation of the p65 subunit of NF-κB into the nucleus.

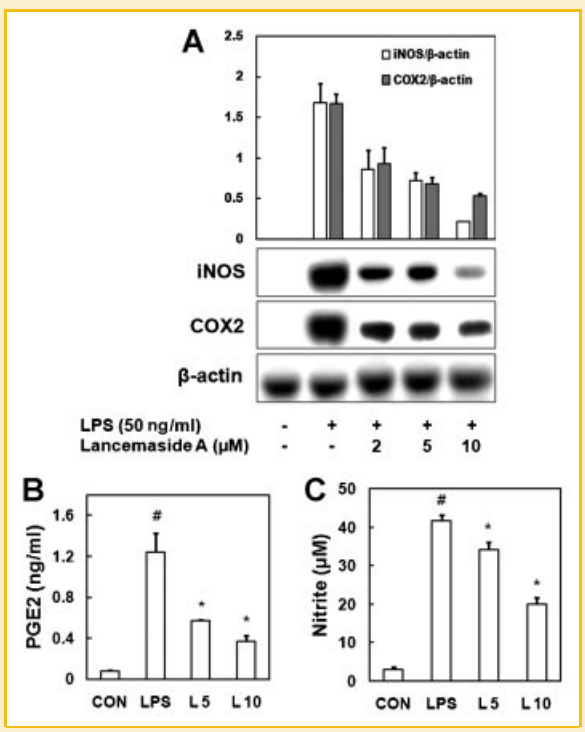

Fig. 4. Effects of lancemaside A on COX-2 and iNOS expression (A) and their inflammatory mediators, PGE_2 (B) and NO (C), in LPS-stimulated peritoneal macrophages. Peritoneal macrophages isolated from mice were incubated with LPS in absence (LPS) or presence of lancemaside A (L5, 5 μM lancemaside A; L10, 10 μM lancemaside A) for 20 h, and then the levels of PGE_2 and nitrite in the culture medium were measured using ELISA kit and Griess reagents, respectively. Normal control (CON) was treated with vehicle alone instead of LPS and lancemaside A. All data are expressed as mean ± SD (n = 4 in a single experiment). $^{\#}P < 0.05$, significantly different versus CON group. $^{*}P < 0.05$ versus LPS group. Protein expression of COX-2 and iNOS levels was measured by immunoblot analysis. Peritoneal macrophages isolated from mice were incubated with LPS in absence or presence of lancemaside A (10 μM) for 20 h.

We also investigated the effect of lancemaside A on LPS-induced MAPKs (ERK, JNK, and p38 MAP kinases) activation. While LPS activated MAPKs, lancemaside A inhibited this activation (Fig. 5D). Of the MAPKs, p-ERK was most potently inhibited.

INHIBITORY EFFECTS OF LANCEMASIDE A ON INTERACTION BETWEEN LPS AND TLR4

We examined whether lancemaside A inhibit the interaction between LPS and TLR4 in peritoneal macrophages using flow cytometry and confocal microscopy analyses. According to flow cytometry analysis, lancemaside A treatment with LPS significantly prevented the binding of LPS to the macrophages (Fig. 6A). Lancemaside A at 10 μM inhibited the binding by 85%.

When macrophages were treated with Alexa Fluor 594-conjugated LPS alone, the fluorescence intensity of LPS as well as TLR4 was observed in the outside of the cell membrane by confocal microscope analysis (Fig. 6B). In the presence of lancemaside A, the binding of Alexa Fluor 594-conjugated LPS to peritoneal macrophages was inhibited.

Interaction between LPS and TLR4 leads to the activation of IRAK-4, important mediators in the signal transduction of Toll/IL-1 receptor (TIR) family members [Li et al., 2002]. To investigate the inhibitory effects of lancemaside A on the activation of IRAK-4 in peritoneal macrophages, the cells were treated with LPS in the presence and absence of lancemaside A (Fig. 6C). While LPS activated IRAK-4, lancemaside A inhibited activation of IRAK-4.

DISCUSSION

In the previous study, we found that lancemaside A inhibited colitis via TLR-linked NF-κB activation in mice [Joh et al., 2010]. Therefore, to understand the anti-inflammatory mechanism of

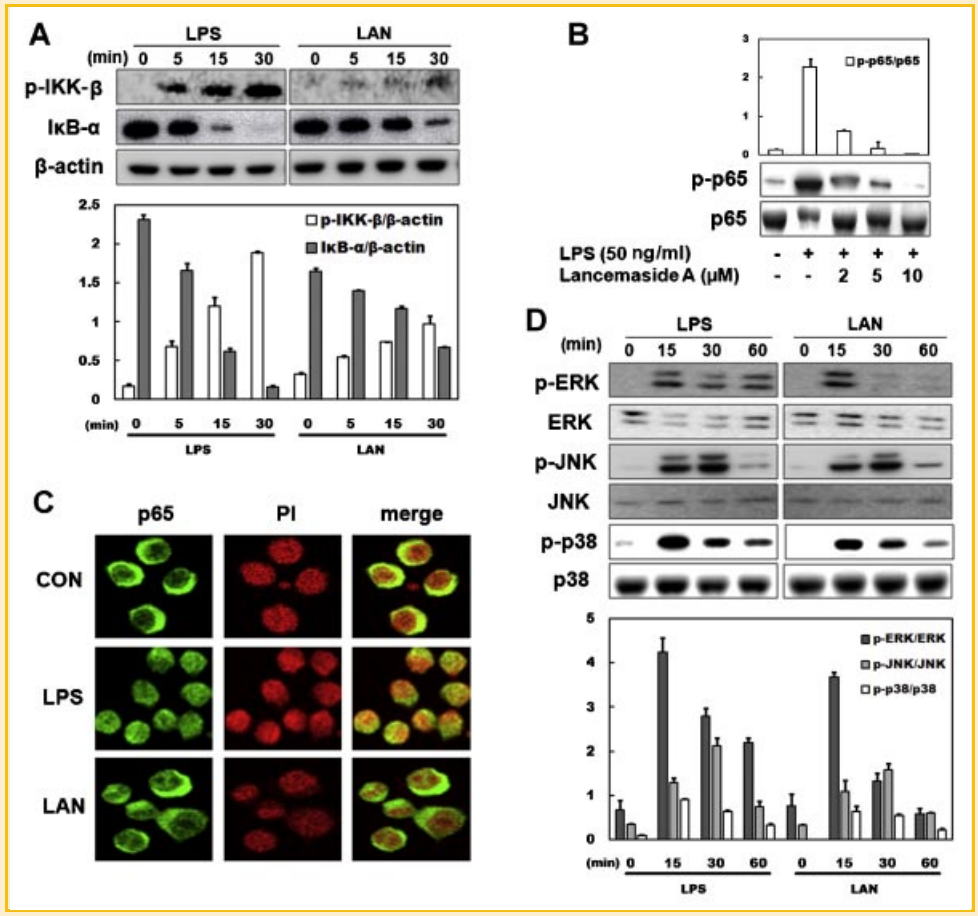

Fig. 5. Effects of lancemaside A for IKK-β phosphorylation, IκB-α degradation, and NF-κB activation. The peritoneal macrophages isolated from mice were treated with 50 ng/ml LPS in absence (LPS) or presence of lancemaside A (LAN, 10 μM). Normal control (CON) was treated with vehicle alone instead of LPS and lancemaside A. A: Effect in IKK-β phosphorylation and IκB-α degradation. Cells were treated with LPS for 0, 5, 15, and 30 min. Phosphorylation of IKK-β and degradation of IκB-α were determined by immunoblot analysis. β-actin was used as a control. B: Effect in phosphorylation of NF-κB. It was determined 30 min after the treatment with LPS. C: Effect in NF-κB nuclear translocation. It was detected by confocal analysis using an antibody for p65 subunit. D: Effects in MAP kinase expression. The peritoneal macrophages were incubated with 50 ng/ml LPS in absence (LPS) or presence of lancemaside A (LAN, 10 μM) for 60 min.

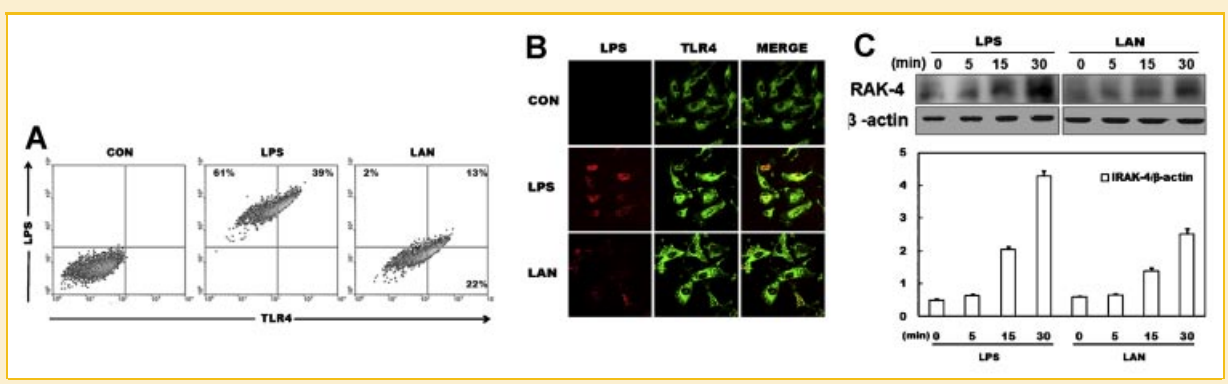

Fig. 6. Inhibitory effects of lancemaside A on interaction between LPS and TLR4 in peritoneal macrophages. Peritoneal macrophages isolated from mice were incubated with Alexa Fluor 594-conjugated LPS for 30 min in absence (LPS) or presence of lancemaside A (LAN, 10 μM), and then the TLR4 and LPS binding in the surface of peritoneal macrophages (A) were measured by flow cytometry. CON was treated with vehicle alone instead of LPS and lancemaside A. B: Interaction between Alexa Fluor 594-conjugated LPS and TLR4 was detected by confocal analysis using an antibody for TLR4. C: Effect in expression of IRAK-4. It was determined 0, 5, 15, and 30 min after the treatment with 50 ng/ml LPS.

lancemaside A, the anti-inflammatory effects of lancemaside A was investigated in lipopolysaccharide (LPS)-stimulated mice and their peritoneal macrophage cells.

In normal mice without any stimuli or treatment, blood TNF-α and IL-1β levels are barely detectable [Blanque et al., 1996]. Treatment with LPS increases serum TNF-α and IL-1β levels and causes inflammation [Kotanidou et al., 2002]. Suppression of TNF-α and/or IL-1β production by other constituents, such as luteolin, quercetin, salvianolic acid B, ginsenosides and platycodins, is suggested to ameliorate several inflammatory disorders, including endotoxemia, colitis, scratching and rheumatoid arthritis [Chen et al., 2001; Kotanidou et al., 2002; Kwon et al., 2005; Shin and Kim, 2005; Chung et al., 2008].

Their potential mechanisms have been proposed to inhibit signal transduction pathways, such as the activation of NF-κB [Huang et al., 1999; Chen et al., 2001]. Interestingly, IL-1β and/or TNF-α level are also negatively correlated with life span [Yaraee et al., 2009]. This implies that inhibiting the subsequent production of pro-inflammatory cytokines (the later stage) still benefits animal survival.

In the present study, we found that lancemaside A inhibited TNF-α and IL-1β in LPS-stimulated peritoneal macrophages and mice. Lancemaside A also reduced the expression levels of COX-2 and iNOS, as well as their products, PGE_2 and NO_2, in LPS-treated peritoneal macrophages. Lancemaside A inhibited the LPS-induced phosphorylation of IKK-β and degradation of IκB-α as well as the translocation of the p65 subunit of NF-κB into the nucleus. This agent also inhibited JNK, ERK, and p38 MAPK activations. Among them, ERK activation was most potently inhibited. Lancemaside A also potently inhibited the interaction between LPS and its receptor, TLR4, on the cell membrane of peritoneal macrophages. TLR-4 is a pattern recognition receptor that responds to LPS, a constituent of gram-negative bacteria that activates secretion of pro-inflammatory mediators from monocytes and dendritic cells, leading to activation of the acquired immune response [Hoshino et al., 1999]. TLR-4 is up-regulated in various inflammatory diseases induced by LPS [Cario and Pldolsky, 2000]. The binding of LPS to TLR-4 induces the activation of IRAK-4, which is involved in host defense mechanisms, either by the recognition of pathogens or as receptors for pro-inflammatory cytokines [Li et al., 2002]. Various inflammatory diseases involve over-expression of the pro-inflammatory cytokines, TNF-α and IL-1β, and inflammatory mediators such as NO and PGE_2 via NF-κB and MAPK pathways in macrophages [Tak and Firestein, 2001; Moynagh, 2005]. The anti-inflammatory activity of lancemaside A inhibits MAPK, as well as the NF-κB pathway, like timosaponin BII and ginsenoside Rh2 [Park et al., 2005; Ahn et al., 2006; Kim et al., 2007; Lu et al., 2009]. However, the inhibitory effects of timosaponin BII and ginsenoside Rh2 against the binding of LPS to TLR-4 were not investigated. Lancemaside A potently prevented the binding of LPS to macrophages, as well as the IRAK-4 activation. These results suggest that lancemaside A may inhibit NF-κB and MAPKs activation by regulating the binding of LPS to TLR-4 on macrophages.

Based on these findings, lancemaside A may express an anti-inflammatory effect by regulating the binding of LPS to TLR-4 on macrophages.

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