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Euscaphic Acid Isolated From Roots of *Rosa Rugosa* Inhibits LPS-Induced Inflammatory Responses Via TLR4-Mediated NF-κB Inactivation in RAW 264.7 Macrophages

In-Tae Kim,[1†] Suran Ryu,[1,2†] Ji-Sun Shin,[1,2†] Jung-Hye Choi,[3,4] Hee-Juhn Park,[5] and Kyung-Tae Lee[1,2,3*]

[1]*Department of Pharmaceutical Biochemistry, College of Pharmacy, Kyung Hee University, Seoul 130-701, Republic of Korea*

[2]*Department of Biomedical Science, College of Medical Science, Kyung Hee University, Seoul 130-701, Republic of Korea*

[3]*Department of Life and Nanopharmaceutical Science, College of Pharmacy, Kyung Hee University, Seoul 130-701, Republic of Korea*

[4]*Department of Oriental Pharmaceutical Science, College of Pharmacy, Kyung Hee University, Seoul 130-701, Republic of Korea*

[5]*Department of Pharmaceutical Engineering, Sanji University, Wonju, Republic of Korea*

ABSTRACT

As an attempt to search for bioactive natural products exerting anti-inflammatory activity, we have evaluated the anti-inflammatory effects of euscaphic acid (19α-hydroxyursane-type triterpenoids, EA) isolated from roots of *Rosa rugosa* and its underlying molecular mechanisms in lipopolysaccharide (LPS)-induced RAW 264.7 macrophages. EA concentration-dependently reduced the production of nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) induced by LPS in RAW 264.7 macgophages. Consistent with these data, expression levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein and iNOS, COX-2, TNF-α, and IL-1β mRNA were inhibited by EA in a concentration-dependent manner. In addition, EA attenuated LPS-induced DNA binding and transcriptional activity of nuclear factor-kappa B (NF-κB), which was accompanied by a parallel reduction of degradation and phosphorylation of inhibitory kappa Bα (IκBα) and consequently by decreased nuclear translocation of p65 subunit of NF-κB. Pretreatment with EA significantly inhibited the LPS-induced phosphorylation of IκB kinase β (IKKβ), p38, and JNK, whereas the phosphorylation of ERK1/2 was unaffected. Furthermore, EA interfered with the LPS-induced clustering of TNF receptor-associated factor 6 (TRAF6) with interleukin receptor associated kinase 1 (IRAK1) and transforming growth factor-β-activated kinase 1 (TAK1). Taken together, these results suggest that EA inhibits LPS-induced inflammatory responses by interference with the clustering of TRAF6 with IRAK1 and TAK1, resulting in blocking the activation of IKK and MAPKs signal transduction to downregulate NF-κB activations. J. Cell. Biochem. 113: 1936–1946, 2012.

KEY WORDS: EUSCAPHIC ACID; *ROSA RUGOSA*; INFLAMMATION; NUCLEAR FACTOR-κB; INHIBITORY κB KINASE; TRANSFORMING INHIBITOR OF κB KINASE COMPLEX

Inflammation is a one of the most important and ubiquitous defensive reaction to the stimuli, such as, toxins and pathogens, and its characteristics are redness, swelling, pain, and heat, which are localized at the site of infection. The mediators of inflammation include nitric oxide (NO), prostaglandin E_2 (PGE_2), and cytokines such as tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β).

[†]In-Tae Kim, Suran Ryu and Ji-Sun Shin contributed equally to this work.

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*Correspondence to: Kyung-Tae Lee, Ph.D., Department of Pharmaceutical Biochemistry, College of Pharmacy, Kyung Hee University, Dongdaemun-Ku, Hoegi-Dong, Seoul, 130-701, Republic of Korea. E-mail: ktlee@khu.ac.kr

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However, inflammation may aid microbial pathogenesis because the inflammatory response elicited by invading microbial pathogens can result in considerable host damage, making nutrients available, and providing access to host tissues [Lawrence et al., 2002].

Lipopolysaccharide (LPS) is recognized by toll-like receptor 4 (TLR4), thus leading to signaling activation via downstream signaling factors, which include the adaptor myeloid differentiation protein 88 (MyD88), IL-1 receptor-associated protein kinases (IRAKs), TNF receptor-associated factor 6 (TRAF6), and transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) [Barton and Medzhitov, 2003]. Upon LPS stimulation, IRAK1, a serine-threonine kinase, is phosphorylated within receptor complexes that include toll interacting protein (Tollip), MyD88, and TRAF6. Phosphorylation of IRAK1 presumably triggers IRAK1 to dissociate from the receptor complexes and translocate along with TRAF6 to the membrane-bound pre-associated TAK1-TAK1 binding protein (TAB)1-TAB2 complex, and then phosphorylated IRAK1 is eventually ubiquitinated and degraded. TAK1-TAB1-TAB2-TRAF6 dissociated from phosphorylated IRAK1 translocates to the cytosol, in which phosphorylated TAK1 is activated [Wang et al., 2001; Jiang et al., 2002]. TAK1 was later shown to be a pivotal factor for activation of inhibitor of κB (IκB) kinase complex (IKK) and mitogen-activating protein kinases (MAPKs) in response to TLR, IL-1R, and TNFR stimulations [Sato et al., 2005]. These signaling pathways in turn activate a variety of transcription factors that include nuclear factor-kappa B (NF-κB) (p50/p65), and AP-1 (c-Fos/c-Jun), which coordinate the induction of many gene-encoding inflammatory mediators. Thus, suppression of the above mentioned signaling molecules may have great potential for preventing and treating of inflammation-associated diseases.

NF-κB is one of the most ubiquitous transcription factors that regulate gene expressions involved in cellular proliferation, inflammatory responses, and cell adhesion. Activation of NF-κB induces gene transcription of multiple pro-inflammatory mediators including iNOS, COX-2, TNF-α, and IL-1β as well as others [Li and Verma, 2002]. Functionally active NF-κB exists mainly as a heterodimer consisting of subunits of the Rel family p50 and p65, which is normally sequestered in the cytosol as an inactive complex by binding to the IκBs in unstimulated cells [Baeuerle, 1998]. The activation mechanisms of NF-κB involve the phosphorylation of IκBs in two critical serine residues (Ser^{32}, Ser^{36}) via IKK signalosome complex [Brown et al., 1995; O'Connell et al., 1998]. Once IκBs are phosphorylated, they are ubiquitinated and degraded by the 26S proteasome [Sanchez-Perez et al., 2002]. The resulting free NF-κB is translocated into the nucleus, where it binds to the κB binding sites in the promoter region of target genes, and then induces transcription of pro-inflammatory mediators [Baeuerle and Baltimore, 1996].

The roots of *Rosa rugosa* (Rosaceae) have traditionally been used to treat diabetes mellitus, pain, and chronic inflammatory diseases in Korea [Kyohaksa, 2003]. Previously, triterpenoid, such as, 28-*O*-glucosides of euscaphic acid (EA), tormentic acid, and arjunic acid were isolated from the underground parts of *R. rugosa* [Young et al., 1987]. Recently, it was reported that *R. rugosa* has anti-allergic [Jeon et al., 2009], hepatotoxic [Cheol Park et al., 2004], and anti-HIV protease effects [Park et al., 2005]. We previously reported on 19α-hydroxyursane-type triterpenoids for example, kaji-ichigoside F1 (EA 28-*O*-glucoside), rosamultin (tormentic acid 28-*O*-glucoside), EA, and tormentic acid from the roots of *R.rugosa* with antinociceptive and anti-inflammatory action [Jung et al., 2005]. Moreover, EA and tormentic acid isolated from the fresh leaves of *Rubus sieboldii* inhibited the 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced mouse ear edema, and DNA polymerase and cell cycle [Salazar et al., 2011]. In addition, EA isolated from *Folium eriobotryae* has hypoglycemic effect in normoglycemic and alloxan-diabetic mice [Chen et al., 2008] and antitumor-promoting activity in an in vivo two-stage carcinogenesis test of mouse tumor by using 7,12-dimethylbenz[a]anthracene (DMBA) as an initiator and TPA as a promoter [Banno et al., 2005]. We also recently reported the anti-inflammatory activity of tormentic acid via the inactivation of the NF-κB pathway in RAW 264.7 macrophages [Chen et al., 2008]. Based on chemical structure, EA has the same molecular formula as tormentic acid, but the configuration of the hydroxyl group at position 3 is different. However, no report has been issued on the molecular aspects of its anti-inflammatory effect in RAW 264.7 macrophages. Therefore, as a prelude to reveal the underlying mechanisms for the anti-inflammatory effects of EA (chemical structure shown in Fig. 1A), we evaluated variations in inflammatory proteins, mRNAs, and transcription factor in RAW 264.7 macrophages, which can be stimulated by LPS to mimic conditions of infection and inflammation.

MATERIALS AND METHODS

MATERIALS

EA used for this study was isolated from the roots of *R. rugosa* [Jung et al., 2005]. This isolated compound was determined by LC-MS and found to be 95% pure. Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY). COX-2, iNOS, p65, inhibitory kappa Bα (IκBα), p-IκBα, IKKβ, p-IKKβ, TAK1, IRAK1, ERK, p-ERK, JNK, p-JNK, p38, p-p38, β-actin, and PARP monoclonal antibodies and the peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The enzyme immunoassay (EIA) kits for PGE_2, TNF-α, and IL-1β were obtained from R&D Systems (Minneapolis, MN). RNA extraction kit was purchased from Intron Biotechnology (Seoul, South Korea). iNOS, COX-2, TNF-α, IL-1β, and β-actin oligonucleotide primers were purchased from Bioneer (Seoul, Korea). NS 398, a COX-2 enzyme inhibitor, was from Calbiochem (CA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tertazolium bromide (MTT), aprotinin, leupeptin, phenylmethylsulfonylfluoride (PMSF), dithiothreitol, L-N^6-(1-iminoethyl)lysine (L-NIL), LPS (*Escherichia coli*, serotype 0111:B4), protein A-Sepharose from *Staphylococcus aureus*, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

CELL CULTURE AND SAMPLE TREATMENT

The RAW 264.7 murine macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). These cells were grown at 37°C in DMEM medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml) in a humidified

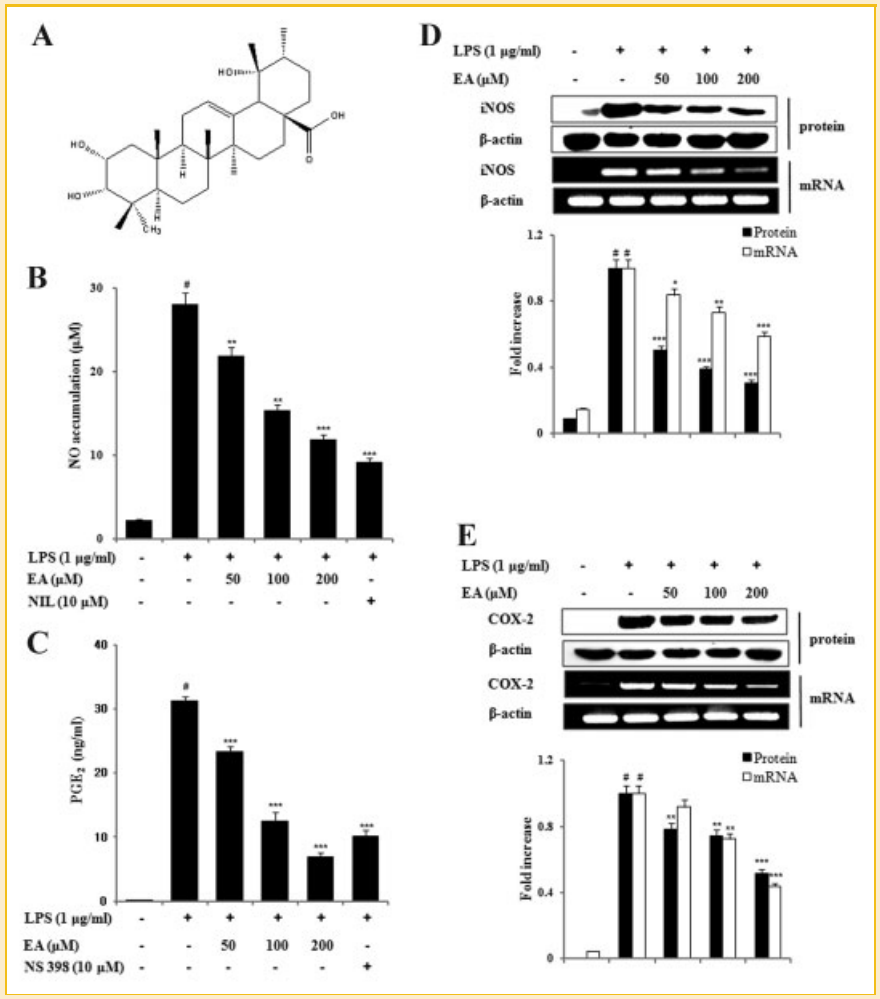

Fig. 1. Effects of EA on LPS-induced production of NO and PGE_2 and expressions of iNOS and COX-2 in RAW 264.7 macrophages. A: Chemical structure of EA. B,C: Effects of EA on LPS-induced NO and PGE_2 production. Following pretreatment with EA (50, 100, and 200 μM) for 1 h, cells were treated with LPS (1 μg/ml) for 24 h. Controls were not treated with LPS or EA. L-NIL (10 μM) and NS 398 (10 μM) were used as a PC, respectively. D,E: Effects of EA on LPS-induced iNOS and COX-2 protein and mRNA expressions. Lysates were prepared from control, LPS (1 μg/ml) alone or LPS plus with EA (50, 100, and 200 μM). Total cellular proteins (40 μg) were resolved by SDS-PAGE, transferred to PVDF membranes, and detected with specific antibodies. Total RNA was prepared for the RT-PCR analysis of iNOS and COX-2 from RAW 264.7 macrophages stimulated with LPS (1 μg/ml) with/without EA (50, 100, and 200 μM) for 4 h. The experiments were repeated three times and similar results were obtained. Western blotting and PCR of β-actin were performed to control similar initial protein or cDNA content of samples. Density ratios versus β-actin were determined by densitometry. The values are represented as the means ± SD from three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the LPS-only treated group.

atmosphere of 5% CO_2. Cells were incubated with various concentrations of EA (50, 100, and 200 μM) or with positive controls (PCs; L-NIL, NS 398, SB 203580, PD 98059, or SP 600125), and then stimulated with LPS (1 μg/ml) for the indicated time. Various concentrations of EA dissolved in DMSO were added to the medium.

MEASUREMENT OF CELL VIABILITY BY MTT ASSAY

Cell viability studies were performed in 96-well plates. RAW 264.7 macrophages were mechanically scraped and plated at 1×10^5 cells/ml in 96-well plates containing 100 μl of DMEM medium with 10% FBS and incubated overnight. Cells were incubated with various concentrations of EA in absent or present of LPS for 24 h. After 4 h of incubation with MTT solution at 37°C, the medium was discarded and the formazan blue that formed in the cells was dissolved in 100 μl of DMSO. The optical density was measured at 540 nm.

MEASUREMENT OF NITRITE IN CULTURE MEDIA

RAW 264.7 macrophages were plated at 2.5×10^5 cells/ml in 24 well-plates and then incubated with or without LPS (1 μg/ml) in the absence or presence of various concentrations (50, 100, and 200 μM) of EA for 24 h. The nitrite accumulated in culture medium was measured as an indicated of NO production based on the Griess reaction. Briefly, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent [equal volumes of % (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylethylenediamine-HCl], incubated at room temperature for 10 min, and then the absorbance at 540 nm was measured in a microplate reader (Perkin

Elmer Cetus, Foster City, CA). Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was measured with the serial dilution standard curve of sodium nitrite.

DETERMINATION OF PGE_2, TNF-α, AND IL-1β PRODUCTION

RAW 264.7 macrophages were pretreated with EA (50, 100, and 200 μM) for 1 h and then stimulated with LPS (1 μg/ml) for 24 h. Levels of PGE_2, TNF-α, and IL-1β in the culture media were quantified using EIA kits (R&D Systems, Minneapolis, MN).

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

RAW 264.7 macrophages were collected by centrifugation and washed once with phosphate-buffered saline (PBS). The washed cell pellets were resuspended in PRO-PREP™ protein extraction solution (Intron Biotechnology, Seoul, Korea) and incubated with 20 min at 4°C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacture's instruction. Cellular protein from treated and untreated cell extracts was electroblotted onto a PVDF membrane following separation on a 10–12% SDS–polyacrylamide gel electrophoresis. The immune blot was incubated overnight with blocking solution (5% skim milk) at 4°C, followed by incubation for 4 h with a primary antibody. Blots were washed three times with Tween 20/Tris-buffered saline (TTBS) and incubated with a 1:1,000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Blots were again washed three times with TTBS, and then developed by enhanced chemiluminescence (Amersham Life Science).

RNA PREPARATION AND REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

Total cellular RNA was isolated using Easy Blue® kits (Intron Biotechnology). From each sample, 1 μg of RNA was reverse-transcribed (RT) using MuLV reverse transcriptase, 1 mM deoxyribonucleotide triphosphate (dNTP), and oligo (dT_{12-18}) 0.5 μg/μl. PCR analyses were performed on aliquots of the cDNA preparations to detect iNOS, COX-2, TNF-α, IL-1β, and β-actin (as an internal standard) gene expressions using a thermal cycler (Perkin Elmer Cetus, Foster City, CA). Reactions were carried out in a volume of 25 μl containing; 1 unit of Taq DNA polymerase, 0.2 mM dNTP, 10× reaction buffer, and 100 pmol of 5′ and 3′ primers. After an initial denaturation for 2 min at 95°C, 26 or 30 amplification cycles were performed for iNOS (1 min of 95°C denaturation, 1 min of 60°C annealing, and 1.5 min 72°C extension), COX-2 (1 min of 94°C, 1 min of 60°C, and 1 min 72°C), TNF-α (1 min of 94°C, 1 min of 55°C, and 1 min 72°C) and IL-1β (1 min of 94°C denaturation, 1 min of 60°C annealing, and 1 min 72°C extension). The PCR primers used in this study are listed below and were purchased from Bioneer (Seoul, Korea): sense strand iNOS, 5′-AATGGCAACATCAGGTCGGCCATCACT-3′, anti-sense strand iNOS, 5′-GCTGTGTGTCACAGAAGTCTCGAACTC-3′; sense strand COX-2, 5′-GGAGAGACTATCAAGATAGT-3′, anti-sense strand COX-2, 5′-ATGGTCAGTAGAC-TTTTACA-3′; sense strand TNF-α, 5′-ATGAGCACAGAAAGCATGATC-3′, anti-sense strand TNF-α, 5′-TACAGGCTTGTCACTCGAATT-3′; sense strand IL-1β, 5′-TGCAG-AGTTCCCCAACTGGTACATC-3′, anti-sense strand IL-1β, 5′-GTGCTGCCTAATG-TCCCCTTGAATC-3′; sense strand β-actin, 5′-TCATGAAGTGTGACGTTGACAT-CCGT-3′, anti-sense strand β-actin, 5′-CCTAGAAGCATTTGCGGTGCACGATG-3′. After amplification, the PCR reactions were electrophoresised on 2% agarose gel and visualized by ethidium bromide staining and UV irradiation.

NUCLEAR EXTRACTION AND ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

RAW 264.7 macrophages in 100 mm dishes (1×10^6 cells/ml) were preincubated with various concentrations of EA (50, 100, and 200 μM) and then stimulated with LPS (1 μg/ml) for 1 h. The cells were washed once with PBS, scraped into 1 ml of cold PBS, and pelleted by centrifugation. Nuclear extracts were prepared as described previously with slight modification [Kim et al., 2008]. Cell pellets were resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, 10 μg/ml aprotinin) and incubated on ice for 15 min. Cells were then lysed by adding 0.1% Nonidet P-40 and vortexed vigorously for 10 s. Nuclei were pelleted by centrifugation at 12,000*g* for 1 min at 4°C and resuspended in high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM NaF, 1 mM sodium orthovanadate). Nuclear extracts (10 μg) were mixed with double-stranded NF-κB oligonucleotide; 5′-AGTTGAGGGGACTTTCCCAGGC-3′ end-labeled with [γ-^{32}P] dCTP (underlying indicates a κB consensus sequence or a binding site for NF-κB homodimeric or heterodimeric complex). Binding reactions were performed at 37°C for 30 min in 30 μl of reaction buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 4% glycerol, 1 μg of poly (dI-dC), and 1 mM DTT. The specificity of binding was examined by competition with the 80-fold unlabeled oligonucleotide. DNA-protein complexes were separated from the unbound DNA probe on native 5% polyacrylamide gels at 100 V in 0.5× Tris Boric acid EDTA (TBE) buffer. Gels were vacuum-dried for 1 h at 60°C and exposed to X-ray film at 70°C for 24 h.

TRANSIENT TRANSFECTION AND LUCIFERASE REPORTER ASSAY

RAW 264.7 macrophages were transfected with NF-κB-Luc reporter plasmid (BD Biosciences, San Jose, CA) plus the phRL-TK plasmid (Promega, Madison, CA) using Superfect reagent (Qiagen GmbH, Germany) according to the manufacturer's instructions. Cells were incubated for 2 h before adding 5 ml of DMEM containing 10% FBS. At 48 h after the start of transfection, cells were pretreated with EA for 1 h and then stimulated with LPS (1 μg/ml). After 3 h of stimulation, cells were lysed and luciferase activity was determined using the Promega luciferase assay system (Promega).

IMMUNOPRECIPITATION (IP)

Whole cell extracts were lysed with lysis buffer (10% glycerol, 1% triton X-100, 1 mM EGTA, 5 mM EDTA, 1 mM sodium pyrophosphate, 20 mM Tris–HCl, pH 7.9, 10 mM β-glycerophosphate, 137 mM NaCl, 1 mM PMSF, 10 mM NaF, 1 mM sodium orthovanadate, and protease inhibitor cocktail) for 15 min at 4°C. Equal amounts of total cellular protein were immunoprecipitated with anti-TRAF6 monoclonal antibody in TNT buffer (20 mM NaCl, 20 mM Tris–HCl, pH 7.5, 1% Triton X-100, 300 μM sodium

orthovanadate, 2 mM PMSF, and protease inhibitor cocktail). The TRAF6 antibody complex was precipitated with protein A/G-Sepharose beads for 4 h at 4°C, washed with wash buffer and subjected to Western blot analysis.

IKK KINASE ASSAY

Whole cell extracts were lysed with lysis buffer (10% glycerol, 1% Triton X-100, 1 mM EGTA, 5 mM EDTA, 1 mM sodium pyrophosphate, 20 mM Tris–HCl, pH 7.9, 10 mM β-glycerophosphate, 137 mM NaCl, 1 mM PMSF, 10 mM NaF, 1 mM sodium orthovanadate, and protease inhibitor cocktail) for 15 min at 4°C. Equal amounts of total cellular protein were immunoprecipitated with anti-IKKβ monoclonal antibody in TNT buffer (20 mM NaCl, 20 mM Tris–HCl, pH 7.5, 1% Triton X-100, 300 μM sodium orthovanadate, 2 mM PMSF, and protease inhibitor cocktail). The IKKβ–antibody complex was precipitated with protein A/G-Sepharose beads for 4 h at 4°C. The kinase assay was carried out in kinase buffer containing 5 μCi [γ-^{32}P]ATP and GST-IκBα (1–317) fusion protein (Santa Cruz, CA) as substrate and incubated for 30 min at 37°C. Each sample was mixed with Laemmli's loading buffer, heated for 10 min at 100°C, and subjected to 10% SDS–PAGE. The gels were dried and visualized by autoradiography.

STATISTICAL ANALYSIS

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. The data are reported as mean ± SD. All experiments were performed at least three times, each time with three or more independent observations.

RESULTS

EFFECTS OF EA ON NO AND PGE_2 PRODUCTION IN RAW 264.7 MACROPHAGES

We initially investigated the inhibitory effects of EA on LPS-induced production of the inflammatory mediators, such as NO and PGE_2 in RAW 264.7 macrophages. As shown in Figure 1B, LPS induced approximately 11-fold greater NO production than control group and EA significantly reduced LPS-induced NO production in a concentration-dependent manner. L-NIL (10 μM) was used as a positive inhibitor for NO inhibition. In addition, EA also concentration-dependently inhibited LPS-induced PGE_2 production in Figure 1C. The NS 398, COX-2 selective inhibitor, had a significant inhibitory effect on PGE_2 production. In addition, the cytotoxic effects of EA was evaluated in the presence or absence of LPS using the MTT assay, and this compound did not affect the viability of RAW 264.7 macrophages at least up to 300 μM in either in the presence or the absence of LPS after a period of 24 h (Supplementary. Fig. 1). Thus, the anti-inflammatory effects observed were not attributable to cytotoxic effects.

EFFECTS OF EA ON LPS-INDUCED INOS AND COX-2 PROTEINS AND MRNA EXPRESSIONS

To determine whether the inhibitory effects of EA on NO and PGE_2 production are related to the expressions of iNOS and COX-2, we examined their expression levels by Western blotting. In unstimulated RAW 264.7 macrophages, iNOS and COX-2 protein levels were undetectable. However, in response to LPS, the protein expression levels of iNOS and COX-2 were markedly upregulated, and EA significantly inhibited these iNOS and COX-2 expressions in a concentration-dependent manner (Fig. 1D,E). RT-PCR was performed to investigate whether EA suppressed the LPS-mediated inductions of iNOS and COX-2 at the transcriptional level, and showed that levels of iNOS and COX-2 mRNA expressions were also concentration-dependently reduced (Fig. 1D,E). However, EA did not affect the expression of β-actin, the housekeeping gene.

EFFECTS OF EA ON LPS-INDUCED TNF-α AND IL-1β PRODUCTION AND MRNA EXPRESSIONS

Because EA was found to potently inhibit the pro-inflammatory mediators, such as NO and PGE_2, we investigated the effects of EA on LPS-induced TNF-α and IL-1β release and expressions using EIAs and RT-PCR, respectively. Pretreatment with EA (50, 100, and 200 μM) for 1 h significantly reduced LPS-induced TNF-α and IL-1β production (Fig. 2A,B). Moreover, the mRNA levels of TNF-α and IL-1β were significantly up-regulated by LPS (1 μg/ml), and EA pretreatments at 50, 100, and 200 μM markedly and concentration-dependently inhibited these mRNA expressions (Fig. 2C,D). These results indicate that the inhibitory effect of EA on LPS-induced production of NO and PGE_2, and of pro-inflammatory cytokines take place at the transcription level.

EFFECTS OF EA ON LPS-INDUCED NF-κB ACTIVATION AND THE NUCLEAR TRANSLOCATION OF P65

Since NF-κB is a major transcription factor that modulates the expressions of pro-inflammatory proteins (e.g., iNOS and COX-2) and of pro-inflammatory cytokines (e.g., TNF-α and IL-1β) induced by LPS [Li and Verma, 2002], EMSA and luciferase reporter gene assays were performed to determine whether EA regulates LPS-induced NF-κB activity in LPS-stimulated RAW 264.7 macrophages. To examine the DNA-binding activity of NF-κB, nuclear extracts isolated from LPS-stimulated cells pretreated or not with EA were reacted with NF-κB specific ^{32}P-labeled oligonucleotides. As shown in Figure 3A, the DNA-binding activity of NF-κB was markedly increased by LPS alone (lane 2), whereas this binding was reduced by EA pretreatment (lanes 3–5). The specific interaction between DNA and p65 NF-κB was demonstrated by competitive inhibition with p65 antibody (lane 6). In addition, effects of EA on NF-κB transcription activity were measured by luciferase assay. EA significantly decreased transcription activity of NF-κB in a concentration-dependent manner (Fig. 3B). Furthermore, we also investigated whether EA prevents the translocation of the p65 subunit of NF-κB from cytosol into the nucleus using Western blotting. It was found that pretreatment with EA prior to LPS significantly attenuated p65 level in nuclear fraction (Fig. 3C).

EFFECTS OF EA ON THE LPS-INDUCED PHOSPHORYLATION OF IκBα, IKKβ, AND MAPKS

In unstimulated macrophage cells, NF-κB is sequestered in the cytosol by its inhibitor IκBα, and when cells are stimulated with LPS, IκB is phosphorylated by IKK complex, ubiquitinated, and rapidly

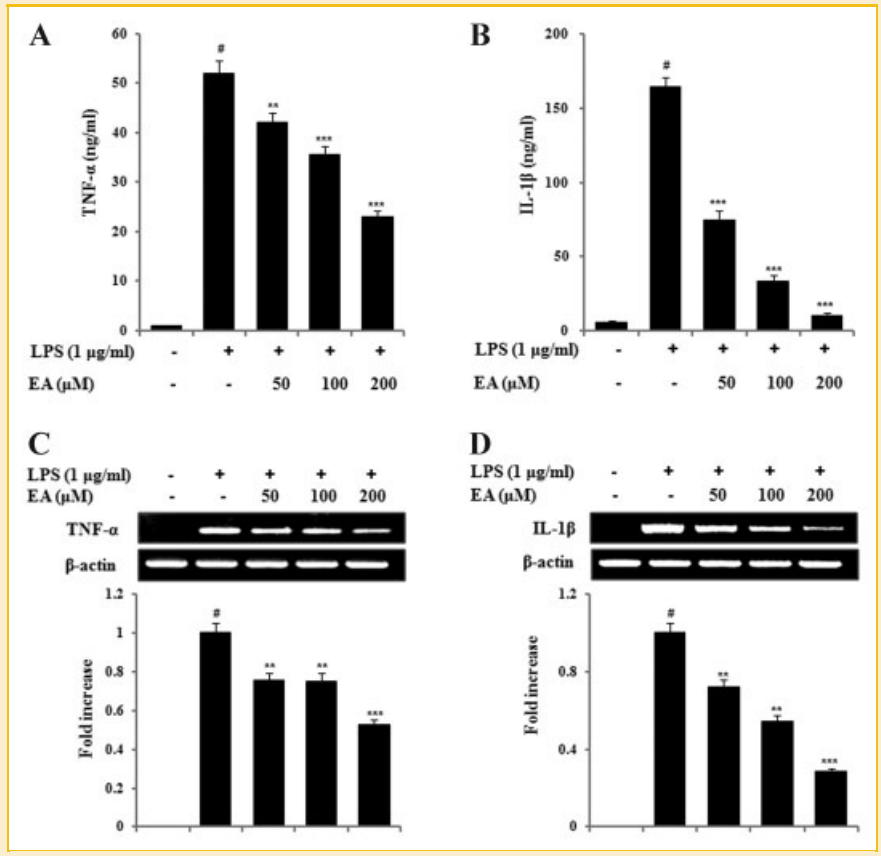

Fig. 2. Effects of EA on LPS-induced TNF-α and IL-1β production and their mRNA expressions in RAW 264.7 macrophages. A,B: Effects of EA on LPS-induced TNF-α and IL-1β production. Following pretreatment with EA (50, 100, and 200 μM) for 1 h, the cells were treated with LPS (1 μg/ml) for 24 h. Control values were obtained in the absence of LPS and EA. C,D: Effects of EA on LPS-induced TNF-α and IL-1β mRNA expressions. Total RNA was prepared for the RT-PCR analysis of TNF-α and IL-1β gene expressions from RAW 264.7 macrophages pretreated with different concentrations (50, 100, and 200 μM) of EA for 1 h followed by LPS (1 μg/ml) for 4 h. TNF-α-specific sequences (351 bp) and IL-1β specific sequences (387 bp) were detected by agarose gel electrophoresis, as described in Materials and Methods Section. The experiments were repeated three times and similar results were obtained. PCR of β-actin was performed to control similar initial cDNA content of samples. Density ratios versus β-actin were determined by densitometry. The values are represented as the means ± SD from three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the LPS-only treated group.

degraded via 26S proteasome, which results in the release of NF-κB [Sanchez-Perez et al., 2002]. Here, we examined the effects of EA on the LPS-induced phosphorylation and degradation of IκBα by Western blotting. After treatment with LPS (1 μg/ml) alone for 15 min, phosphorylated IκBα levels were markedly increased and EA significantly reduced LPS-induced IκBα phosphorylation in a concentration-dependent manner. Furthermore, EA pretreatment significantly blocked LPS-induced IκBα degradation (Fig. 4A). In an attempt to explore the effects of EA on the inhibition of IKK activity in RAW 264.7 macrophages, we performed Western blotting to measure the phosphorylation of IKKβ after treatment with EA. It was found that EA significantly inhibited the LPS-induced activation of IKKβ (Fig. 4B). In addition, we immunoprecipitated IKK from LPS-induced RAW 264.7 macrophages and then determined its kinase activity in the presence of EA (50, 100, and 200 μM). EA directly inhibited LPS-induced IKK activity in a concentration-dependent manner (Fig. 4C). These data indicates that EA is able to inhibit LPS-induced NF-κB activation by preventing upstream of IKK activation.

It has been known that activation of MAPKs (ERK1/2, p38, and JNK) through LPS-TLR4 signaling can activate various transcription factors, such as, NF-κB, activating transcription factor 2 (ATF2), and c-Jun, and these can modulate the expressions of cytokines and inflammatory mediator [Huang et al., 2010]. Maximal MAPK phosphoprotein expression levels are known to occur 10–30 min after LPS treatment in human and murine monocytes/macrophages [Bian et al., 2003]. Therefore, we carried out Western blotting for MAPK after treating with LPS (1 μg/ml) for 15 min in the presence or absence of EA. RAW 264.7 macrophages were pretreated with EA in the indicated concentrations for 1 h and then stimulated with LPS (1 μg/ml) for 15 min. Total cell lysates were then probed with phosphospecific antibodies for p38, ERK1/2, and JNK. The phosphorylations of p38, ERK1/2, and JNK were increased in cells treated with LPS alone. However, EA significantly inhibited phosphorylated p38 and JNK levels in LPS-induced RAW 264.7 macrophages in a concentration-dependent manner (Fig. 4D), but did not affect the phosphorylation of ERK1/2. Total ERK, JNK, and p38 expressions were unaffected by LPS or by LPS plus EA.

EFFECTS OF EA ON THE LPS-INDUCED ASSOCIATION OF TRAF6 WITH IRAK1 AND TAK1

LPS activates a TLR4-mediated downstream signaling cascade comprising adaptors MyD88 and TIR domain containing adapter-inducing IFN-β (TRIF) [Xiong et al., 2011]. Furthermore, MyD88 molecules cluster within TLR4 to bind with IRAK4, IRAK1, and IRAK2, and interact with TRAF6 and TAK1, resulting in activation of IKK and MAPKs [Qian et al., 2001; Takaesu et al., 2001]. To determine whether IRAK1, TRAF6, and TAK1 signalosome formation is involved in the effect of EA on LPS-induced activation of NF-κB and MAPKs, TRAF6 was immunoprecipitated and bindings of IRAK1 and TAK1 were evaluated by Western blotting (Fig. 5A). The association of TRAF6 with IRAK1 and TAK1 was observed after LPS treatment, while these bindings were significantly reduced after treatment with EA in a concentration-dependent manner. These findings suggest that EA inhibits LPS-induced NF-κB activation by attenuating IRAK1, TRAF6, and TAK1 signalosome formation.

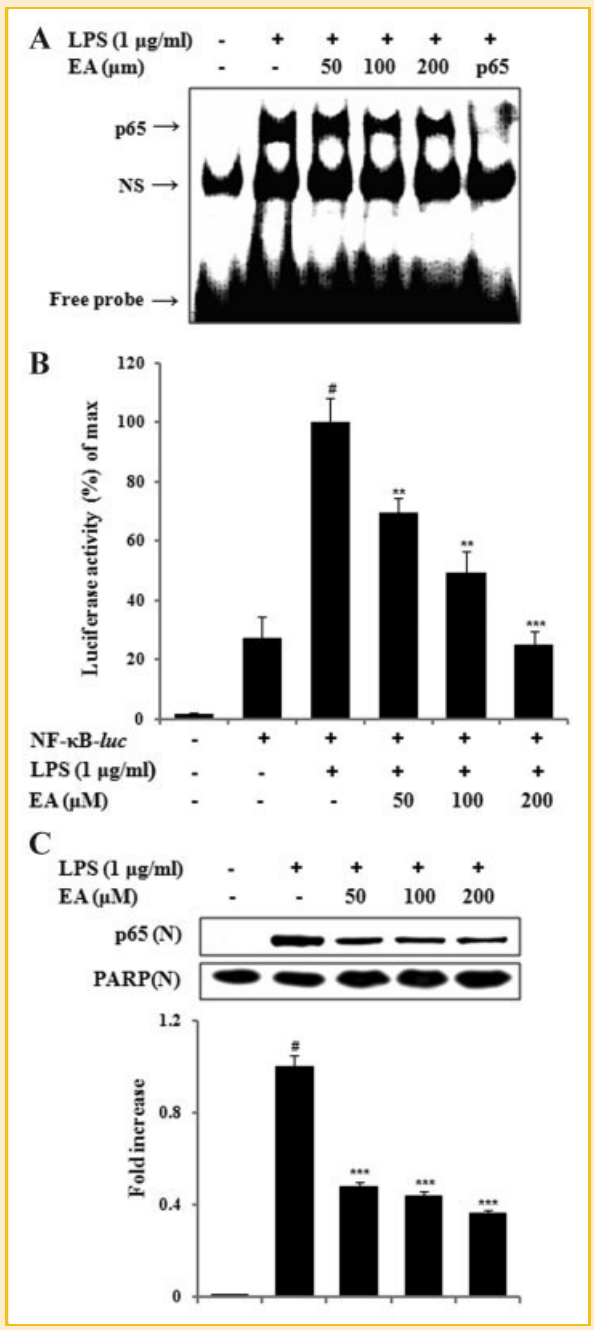

Fig. 3. Effects of EA on LPS-induced NF-κB activity and its translocation into the nucleus. A: Effects of EA on LPS-induced NF-κB-DNA binding activity. Nuclear extracts were prepared from control or pretreated with different concentrations (50, 100, and 200 μM) of EA for 1 h, then with LPS (1 μg/ml) for 1 h, and analyzed for NF-κB binding by EMSA. For supershift analysis, 2 μg of antibody against p65 subunits was included in the binding reaction and the arrow indicates the position of the p65 subunit of NF-κB. B: Effects of EA on LPS-induced NF-κB transcriptional activity. Cells were transiently cotransfected with pNF-κB-luc reporter and then either left untreated (Con) or were pretreated with different concentrations (50, 100, and 200 μM) of EA for 1 h. LPS (1 μg/ml) was then added and cells were further incubated for 3 h. Cells were then harvested and luciferase activities were determined using a Promega luciferase assay system and a luminometer. C: Effects of EA on LPS-induced nuclear translocation of NF-κB p65. Nuclear extracts, prepared from control or pretreated with different concentrations (50, 100, and 200 μM) of EA for 1 h, then with LPS (1 μg/ml) for 1 h, were prepared for the Western blotting of p65 of NF-κB using specific anti-p65 monoclonal antibody. PARP was used as internal controls. Density ratios versus PARP were determined by densitometry. The data shown are representative of three independent experiments. The values shown are means ± SD of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{**}P < 0.01$, $^{***}P < 0.001$ versus the LPS-only treated group.

DISCUSSION

Triterpenoids are the largest and most widespread class of secondary metabolites in plants. They exhibit low toxicity and a few of them have been used clinically worldwide [Shishodia et al., 2003; Cichewicz and Kouzi, 2004]. Recently, the triterpenoids were demonstrated to have a variety of biological activities, such as anti-HIV [Cichewicz and Kouzi, 2004], anti-malarial [Ma et al., 2006], anti-cancer [Ma et al., 2005], anti-inflammatory [de las Heras et al., 2003], as well as anti-atherosgenic properties [Pozo et al., 2006]. Tormentic acid and EA are belong to the 19-hydroxy ursane-type triterpenes and are epimers because they differ in steochemistry only at 3-hydroxyl group. In previous study, we demonstrated that tormentic acid containing 2α, 3β-dihydroxyl group exerts anti-inflammatory effects by inhibiting NF-κB activation in macrophages, and thus, prevents the expressions of iNOS, COX-2, and TNF-α [An et al., 2011]. In contrast to tormentic acid, EA has 2α, 3α-dihydroxyl group in A-ring. These polyhydroxytriterpenoids may have certain bioactivities based on the amphipathic structures and those unique physicochemical properties. Tormentic acid was found to have the more potent inhibitory effect on inflammatory activities than EA, indicating that configuration of 2,3-dihydroxy group of 19-hydroxy ursane-type triterpenes might affect the inhibitory potency on the inflammatory responses in LPS-induced RAW 264.7 macrophages. In accordance with our results, previous reports showed that corosolic acid, which is a naturally occruing triterpene acid, and its epimer on the configuration of 2,3-dihydroxyl group

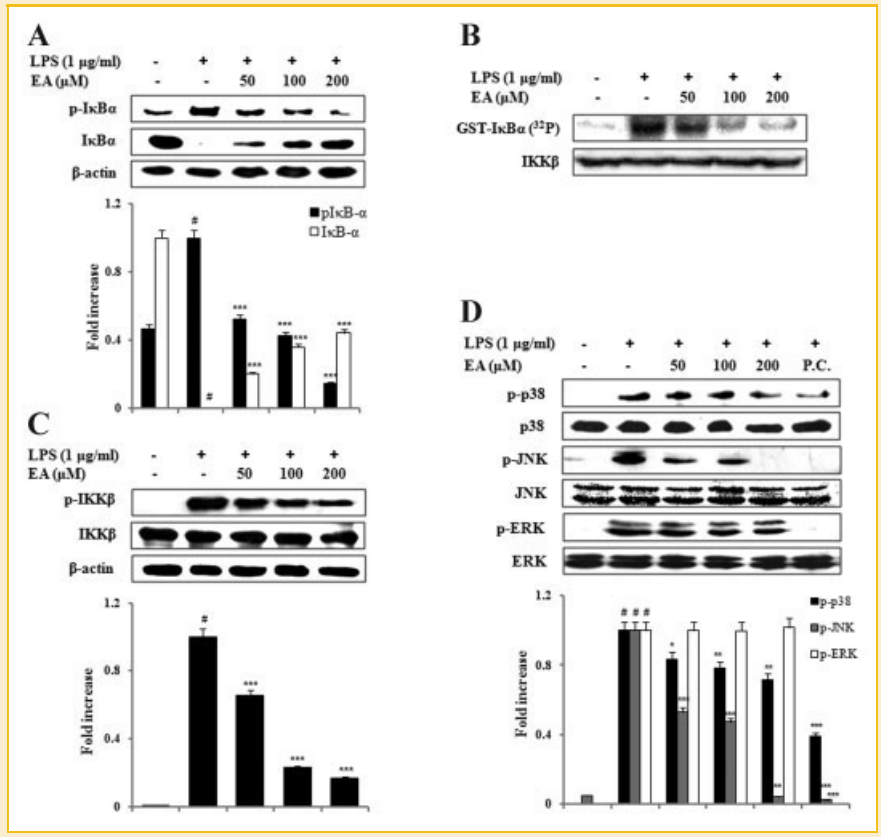

Fig. 4. Effect of EA on the LPS-induced the phosphorylationof IκBα, IKKβ, and MAPK. A: Effects of EA on IκBα protein phosphorylation and degradation. Following pretreatment with EA (50, 100, and 200 μM) for 1 h, cells were treated with LPS for 15 min. Total proteins were prepared and Western blotting was performed using specific p-IκBα and IκBα antibodies. B: Effects of EA on IKKβ phosphorylation. Following pretreatment with EA (50, 100, and 200 μM) for 1 h, cells were treated with LPS (1 μg/ml) for 5 min. Total cellular proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and detected with specific p-IKKβ and IKKβ antibodies. C: Effects of EA on IKKβ kinase activity. Lysates were prepared from control, 10 min LPS (1 μg/ml) alone or LPS plus with EA (50, 100, and 200 μM). IKKβ protein was then immunoprecipitated with IKKβ primary antibody and IKKβ-antibody complex was precipitated with protein A/G-Sepharose beads. IKKβ kinase activity was determined using [γ-^{32}P] ATP and GST-IκBα fusion protein as substrate and incubated for 30 min at 37°C. Each sample was mixed with Laemmli's loading buffer, heated for 10 min at 100°C, and subjected to 10% SDS-PAGE. The gels were dried and visualized by autoradiography. D: Effects of EA on MAPKs phosphorylation. Following pretreatment with EA (50, 100, and 200 μM) for 1 h, cells were treated with LPS (1 μg/ml) for 15 min. Whole cell lysates were analyzed by Western blotting using antibodies against activated MAPKs. p38 specific inhibitor (SB 203580, 10 μM), ERK specific inhibitor (PD 98059, 10 μM), and JNK specific inhibitor (SP 600125, 10 μM) were treated as a PCs. Density ratios versus pan-p38, JNK, ERK respectively were determined by densitometry. The data shown are representative of three independent experiments. The values are represented as the means ± SD from three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the LPS-only treated group.

showed different potency of hypoglycemic activity [Wen et al., 2007]. The inhibitory potency of these compounds on glycogen phosphorylase activities decreased sharply when both 2-hydroxy and 3-hybroxy groups were in the same side of A-ring. Although we already published the anti-inflammatory activity of tormentic acid in LPS-induced RAW 264.7 cells, little information is available with respect to the molecular mechanisms underlying the anti-inflammatory effect of EA. Thus, this study was aimed to evaluate anti-inflammatory effect of EA by screening the inhibitory effects on LPS-induced pro-inflammatory molecules in macrophages and to further explore the possible underlying mechanisms of these inhibitions.

The pathology of inflammation is initiated by complex processes triggered by microbial pathogens such as LPS, which is a prototypical endotoxin [West et al., 1995]. LPS can directly activate macrophages, which trigger the production of inflammatory mediators, such as, NO, PGE_2, TNF-α, ILs, and leukotrienes [Molloy et al., 1993; Hinz and Brune, 2002]. The pharmacological reduction of LPS-inducible inflammatory mediators is regarded as one of the attractive therapeutic strategy for many acute and chronic inflammatory diseases. In the present study, it was found that EA is a potent inhibitor on the LPS-induced pro-inflammatory molecules, including NO, PGE_2, TNF-α, and IL-1β (Fig. 1B,C, Fig. 2A,B). To further explore the possible mechanisms underlying these inhibitions by EA, we examined the expression levels of iNOS, COX-2, TNF-α, and IL-1β. The inhibitions of iNOS and COX-2 gene expression were evidenced by reductions in their protein and mRNA levels (Fig. 1D,E). Thus, observed inhibitions of the production of NO and PGE_2 might be attributed to the suppressions of the transcriptions of iNOS and COX-2 mRNA and subsequent reductions in their protein expressions. In addition, MTT assays showed that pretreatment of macrophages with EA did not change cell growth or cytotoxicity, indicating that the suppressions of pro-inflammation mediators by EA were not attributable to any nonspecific cytotoxic

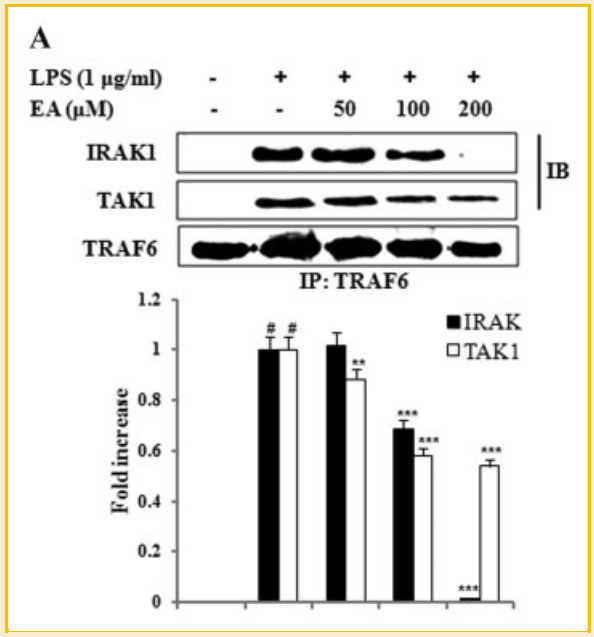

Fig. 5. Effects of EA on association of TLR adaptor protein, IRAK1 and TAK1, and phosphorylation of TAK1. A: Effects of EA on association of TLR adaptor protein, IRAK1 and TAK1. Following pretreatment with EA (50, 100, and 200 μM) for 1 h, cells were treated with LPS (1 μg/ml) for 10 min. TRAF6 immunoprecipitated using TRAF6 primary antibody and then precipitated with protein A/G-Sepharose beads. IRAK1 and TAK1 antibodies were added, and then each protein was electroblotted. B: Effects of EA on phosphorylation of TAK1. Following pretreatment with EA (50, 100, and 200 μM) for 1 h, cells were treated with LPS for 5 min. Total proteins were prepared and Western blotting was performed using specific p-TAK1 and TAK antibodies. Density ratios versus TRAF6 or TAK1 were determined by densitometry. The data shown are representative of three independent experiments. The values are represented as the means ± SD from three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{**}P < 0.01$, $^{***}P < 0.001$ versus the LPS-only treated group.

effect (Supplementary Fig. 1). Several inflammatory cytokines, particularly TNF-α and IL-1β, are known to play key roles in the induction and perpetuation of inflammation in macrophages. In the present study, we found that TNF-α and IL-1β release and mRNA expressions, which were highly stimulated by LPS, were inhibited by EA (Fig. 2). In particular, inhibitions of IL-1β production and mRNA expressions by EA were more enhanced than those of TNF-α.

NF-κB is a mammalian transcription factor that controls a number of genes, such as, iNOS, COX-2, TNF-α, IL-1β, and IL-6, which are important for immunity and inflammation, and NF-κB is activated by LPS [Li and Verma, 2002]. Accordingly, inhibition of NF-κB has shown to be effective at controlling inflammatory diseases in several animal models. For example, blocking NF-κB activity by the overexpression of IκBα has been reported to inhibit both inflammatory response and tissue destruction in rheumatoid synovium [Li and Verma, 2002]. In colitis model, it was demonstrated that the inhibition of NF-κB by IKK inhibitor ameliorates colonic inflammatory injury via down-regulation of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6) mediated by NF-κB [Shibata et al., 2007]. Based on these reports, we tested whether EA inhibited NF-κB activity in RAW 264.7 macrophages by using EMSA and reporter gene assays. Our results indicate that EA inhibits the LPS-induced DNA binding and transcriptional activities of NF-κB (Fig. 3A,B). To identify the mechanisms of arvelexin involved in the inhibition of NF-κB activity, we tested the effect of EA on NF-κB activation signal. Upon stimulation with LPS, IκB become phosphorylated and undergoes proteolytic degradation, and finally, NF-κB becomes activated and translocate to the nucleus. We also found that the translocation of p65 protein to the nucleus and that the degradation and phosphorylation of IκBα were also inhibited by EA in a concentration-dependent manner (Fig. 3C, Fig. 4A). We provided further evidence that inhibition of IκBα phosphorylation by EA result from disturbance of IKK activation. As IKKs have remarkable responsibility for NF-κB activation, this kinase may become a target for pharmacological intervention in a number of inflammatory disease [Karin and Delhase, 2000]. We observed the inhibitory effects of EA on LPS-induced IKKβ phosphorylation and kinase activity of IKKβ complex precipitated in a concentration-dependent manner (Fig. 4B,C). In recent publications, it has been demonstrated that IKKα exhibits a catalytic activity of p65 phosphorylation (at Ser536), whereas IKKβ is largely responsible for phosphorylation of both, IκBα (at Ser32) and p65 [Perkins, 2007], which is consistent with our finding that EA had no effect on IKKα activation (data not shown).

The activation of NF-κB is regulated by cellular kinases, including MAPKs [Guha and Mackman, 2001]. MAPKs are a highly conserved family of protein serine/threonine kinases and include the p38, ERK1/2, and JNK subgroups [Ruland and Mak, 2003]. Moreover, it is important to establish the relative contribution of these MAPKs to LPS-induced signaling in macrophages [Chen and Wang, 1999]. Therefore, to further confirm the inhibitory mechanisms of NF-κB activation by EA, we investigated the effects of EA on p38, ERK1/2, and JNK phosphorylation in RAW 264.7 macrophages stimulated with LPS for 15 min, and it was found that p38 and JNK phosphorylations were suppressed by EA in a concentration-dependent manner (Fig. 4D). p38 activated by LPS stimulation has been postulated to play an important role in the control of NF-κB-dependent iNOS and TNF-α gene expressions [Bhat et al., 1998]. On the other hand, JNK can phosphorylate several transcription factors, such as c-Jun, JunB, c-fos, ATFs, and regulates the expression of several stress-responsive genes, including TNF-α and IL-2 [Hambleton et al., 1996; Roy et al., 2008; Huang et al., 2010]. We observed that EA significantly and dose-dependently decreased AP-1 transcriptional activation (Supplementary Fig. 3). Our findings suggest that inhibition of pro-inflammatory mediators by EA may be regulated by either both IKK-NF-κB and JNK-AP-1 separately or together.

It is generally known that the activation of TLR4 can eventually lead to the activation of a signalosome consisting of TRAF6, IRAK1, and TAK, and subsequently, to activation of TAK1 by autophosphorylation, resulting in activation of TAK1, IKK, and MAPKs [Sakurai et al., 2000; Prickett et al., 2008; Brown et al., 2011]. Our data demonstrated that EA has inhibitory effects on the binding of TRAF6 with IRAK1 or TAK1 (Fig. 5), indicating the possible target of EA on the interference with the clustering of TRAF6 with IRAK1 and TAK1. In accordance with these results, EA also inhibited the

LPS-induced TAK1 phosphorylation (Supplementary Fig. 2). In addition, TLR4 and IL-1R have a common TIR motif in their cytoplasmic domain and share adaptor proteins, such as MyD88, TRAF6, and IRAKs, for NF-κB activation [Akira and Takeda, 2004]. So, we investigated whether EA might affect IL-1β-induced NF-κB activation through the inhibition of binding of TRAF6 and IRAK1 by immunoprecipitation. The association between TRAF6 and IRAK1 was observed after IL-1β treatment, but this binding was significantly reduced by EA. Furthermore, EA inhibited IL-1 β-induced NF-κB translocation to nucleus (Supplementary Fig. 4). These findings indicated that EA inhibits NF-κB activation by attenuating IRAK1, TRAF6, and TAK1 signalosome formation, and subsequent TAK1 phosphorylation, which pivotal upstream signal for NF-κB activation.

Finally, to verify the in vivo relevance of our in vitro results demonstrating the anti-inflammatory effects of EA, we evaluated the production of pro-inflammatory mediators in isolated mouse peritoneal macrophages (Supplementary Fig. 5). Consistent with our findings in RAW 264.7 macrophages, EA significantly inhibited the LPS-induced NO and TNF-α by attenuating the expression of iNOS and TNF-α ex vivo. Moreover, EA significantly inhibited the LPS-induced transcriptional activity of NF-κB (Supplementary Fig. 6).

In conclusion, the present data reveal that EA inhibited LPS-induced iNOS, COX-2, TNF-α, and IL-1β expressions by interference with the clustering of TRAF6 with IRAK1 and TAK1, resulting in blocking the activation of IKK and MAPKs signal transduction to down-regulate NF-κB activations in RAW 264.7 macrophages. These findings provide a partial molecular description of the mechanism that underlies the anti-inflammatory properties of EA.

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