Regulatory effects and molecular mechanism of *Trigonostemon reidioides* on lipopolysaccharide-induced inflammatory responses in RAW264.7 cells

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Abstract. *Trigonostemon reidioides* (Kurz) Craib has been traditionally used for the treatment of vomiting and asthma in Cambodia. However, the underlying molecular mechanisms of the anti-inflammatory effect of *T. reidioides* extract remains unknown. The present study investigated the anti-inflammatory activity and molecular action of an ethanol extract of *T. reidioides* (ETR) in lipopolysaccharide (LPS)-induced RAW264.7 macrophage cells. Nitric oxide assays, ELISA, reverse transcription-quantitative polymerase chain reaction and western blot analysis were used. ETR treatment inhibited the production of nitric oxide by downregulating inducible nitric oxide synthase expression, while exhibiting no significant cytotoxicity compared with macrophages treated with LPS-alone. Consequently, ETR decreased the production of certain proinflammatory cytokines, including interleukin (IL)-1β, IL-6 and tumor necrosis factor-α. Additionally, ETR inhibited the activation of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase, c-Jun N-terminal kinase and p38 MAPK, as well as the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway. These effects were mediated by inhibition of the nuclear localization of nuclear factor κ-B (NF-κB). Taken together, the results of the present study demonstrate that ETR may exert an anti-inflammatory effect by inhibiting the expression of inflammatory mediators and cytokines via downregulation of the NF-κB, PI3K/Akt and the MAPK signaling pathways in LPS-stimulated macrophages. Based on these results, we hypothesize that ETR may be a potential therapeutic agent for the treatment of inflammatory disorders.

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Introduction

Trigonostemon reidioides (Kurz) Craib is a member of the Euphorbiaceae family that is native to areas in Southeast Asia, including Vietnam, Cambodia and Myanmar. The shrubs usually grow to 1.5 m tall and the leaves are simple and triangular. The flowers are white and sepals are obovated. The fruits are globose and 1-1.4 cm in diameter. The whole herbs have been traditionally used for vomiting diseases and asthma in Cambodia (1). Soonthornchareonnon *et al* (2) reported that an extract and rediocides isolated from *T. reidioides* roots exhibited acaricidal activity on *Dermatophagoides pteronyssinus*, the Thai common house dust mite. However, the underlying molecular mechanisms of the anti-inflammatory effect of *T. reidioides* extract or its components remain unknown.

Inflammation is a general defense response against a variety of stimulators. When inflammation occurs, several inflammatory mediators are produced, resulting in symptoms that include pain, fever, erythema and edema (3). One of the most potent stimuli for macrophages is the bacterial endotoxin lipopolysaccharide (LPS), which activates cells by causing the secretion of various proinflammatory mediators, including tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) (4,5). The expression of proinflammatory cytokines, including TNF-α and IL-6, is controlled by mitogen-activated protein kinases (MAPKs). MAPKs include extracellular signal-regulated kinase (ERK), p38 kinase (p38) and c-Jun N-terminal kinase (JNK) (6). Nuclear factor-κB (NF-κB) is involved in the expression of genes associated with immunity and inflammation, and it is also associated with the activation of MAPKs and the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway (3,7,8).

The present study investigated the anti-inflammatory effect and associated molecular mechanisms of an ethanol extract of *T. reidioides* (ETR) on LPS-stimulated inflammatory responses in RAW264.7 cells.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco

(Thermo Fisher Scientific, Inc., Waltham, MA, USA). Dimethyl sulfoxide (DMSO) and MTT were purchased from Duchefa Biochemie B.V (Haarlem, The Netherlands). *N*-(1-naphthyl) ethylenediamine dihydrochloride (Griess reagent) and sulfanilamide were purchased from Merck KGaA (Darmstadt, Germany). LPS from *Escherichia coli* serotype 0111:B4, bovine serum albumin, radioimmunoprecipitation assay (RIPA) buffer, protease inhibitor, phosphatase inhibitor and NG-methyl-L-arginine acetate salt (L-NMMA) were purchased from Sigma-Aldrich (Merck KGaA). LY294002, SP600125, rabbit monoclonal anti-β-actin (cat. no. 5125), anti-phospho-Akt (cat. no. 4060), anti-Akt (cat. no. 9272), anti-phospho-JNK (T183/Y185; cat. no. 4668), anti-JNK (cat. no. 9252), anti-phospho-ERK (T202/Y204; cat. no. 9101), anti-ERK (cat. no. 9102), anti-phospho-p38 (T180/Y182; cat. no. 9211), anti-p38 (cat. no. 9212), anti-NF-κB p65 (cat. no. 8242) and anti-NF-κB p50 (cat. no. 12540) primary antibodies (dilution, 1:1,000) and anti-rabbit horseradish peroxidase-conjugated immunoglobulin (Ig) G (cat. no. 7074; dilution, 1:2,000) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Rabbit polyclonal anti-iNOS antibody (ab3523; dilution, 1:500) was purchased from Abcam (Cambridge, UK). Goat polyclonal anti-Lamin B (cat. no. sc-6216; dilution, 1:1,000) and mouse anti-goat horseradish peroxidase conjugated IgG (cat. no. sc-2354; dilution, 1:5,000) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Plant material. The dried stems of *T. reidioides* were obtained from Siem Pang in north-east Cambodia in May 2015 and were identified by Dr Jae-Shin Kang (Biological Genetic Resources Utilization Division, National Institute of Biological Resources, Incheon, Korea). A voucher specimen (NIBRVP0000530014) was deposited at the National Institute of Biological Resources (Incheon, Korea).

Preparation of extract. Stems of *T. reidioides* (175 g) were pulverized and the dry material was extracted with 70% ethanol for 24 h at room temperature. The extract was filtered and concentrated in a vacuum under reduced pressure (temperature, 40˚C; pressure, 10 hPa) using a rotary flash evaporator (Büchi Labortechnik AG, Flawil, Switzerland), allowing for complete evaporation of the ethanol. The remaining aqueous solution was concentrated under vacuum (temperature, -85˚C; pressure, 5 mTorr) and freeze-dried. The yield of the crude ETR extract was 4% (w/w).

Cell culture. Murine macrophage RAW264.7 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured at 37˚C with 5% CO_2 in DMEM supplemented with 10% FBS and 1% penicillin (100 U/ml)-streptomycin (100 µg/ml) (Invitrogen; Thermo Fisher Scientific, Inc.).

Cell viability. RAW264.7 cells were plated at a density of $5x10^4$ cells/well in 96-well plates and treated with ETR (25-100 µg/ml) and L-NMMA (100 µM) for 1 h prior to LPS (1 µg/ml) stimulation for 24 h. Following culture for 24 h, 100 µl MTT solution (5 mg/ml in PBS, pH 7.4) was added to each well. After 2 h of incubation at 37˚C with 5% CO_2, the supernatant was removed and formazan crystals formed by viable cells were dissolved in DMSO. The absorbance of each well was measured at a wavelength of 540 nm using a SpectraMax 190PC microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). Data are presented as the mean ± standard deviation of three replicates.

Nitric oxide (NO) production assay. RAW264.7 cells were plated at a density of $5x10^4$ cells/well in 96-well plates and treated with ETR (25-100 µg/ml) and L-NMMA (100 µM) for 1 h prior to LPS (1 µg/ml) stimulation for 24 h. Equal volumes of cultured medium and Griess reagent were mixed and incubated at room temperature for 10 min. Absorbance was subsequently measured at 540 nm, using a SpectraMax 190PC microplate reader.

Enzyme-linked immunosorbent assay (ELISA). RAW264.7 cells were plated at a density of $1x10^6$ cells/well in 6-well plates and treated with ETR (25-100 µg/ml) for 1 h prior to LPS (1 µg/ml) stimulation for 24 h. Levels of the proinflammatory cytokines IL-1β (cat. no. BMS6002), TNF-α (cat. no. BMS607-3) and IL-6 (cat. no. BMS603-2) were measured in cell culture supernatants using ELISA kits (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

Reverse transcription polymerase chain reaction (RT-PCR). RAW264.7 cells were plated at a density of $1x10^6$ cells/well in 6-well plates and treated with ETR (25-100 µg/ml) for 1 h prior to LPS (1 µg/ml) stimulation for 24 h. Total RNA was prepared from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and 1 µg RNA was used as a template for each RT-PCR reaction. RT-PCR was performed using the SuperScript®III First-Strand Synthesis System (Thermo Fisher Scientific, Inc.) and Taq DNA polymerase (Invitrogen; Thermo Fisher Scientific, Inc.). The following conditions were used for each PCR reaction: 95˚C for 5 min (1 cycle); 95˚C for 30 sec, 55˚C for 40 sec and 72˚C for 1 min (30 cycles); and a final extension phase at 72˚C for 10 min. The following primers (Bioneer Corporation, Daejeon, Korea) were used for PCR amplification: IL-1β, 5'-CTTTGAAGAAGAGCCCATCC-3' (sense) and 5'-TTTGTCGTTGCTTGGTTCTC-3' (antisense); IL-6, 5'-CACTTCACAAGTCGGAGGCTT-3' (sense) and 5'-GCAAGTGCATCATCGTTGTTC-3' (antisense); TNF-α, 5'-CTGAGACAATGAACGCTACA-3' (sense) and 5'-TTCTTCCACATCTATGCCAC-3' (antisense); iNOS, 5'-GAGTTCGAGACTTCTGTGA-3' (sense) and 5'-GGCGATCTGGTAGTAGTG-3' (antisense); and GAPDH, 5'-CAGGTACCAGGAGAGTG-3' (sense) and 5'-GTAGACTCCACGACATCTC-3' (antisense). Bands of interest were visualized using a ChemiDoc XRS system and Quantity One software version 4.6.3 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Preparation of nuclear extract. Following treatment as indicated, cells were washed two times with PBS prior to trypsinization and centrifugation at 90 x g and 4˚C for 5 min. Cells were resuspended in PBS, centrifuged at 20,000 x g at 4˚C for 5 min and resuspended in 200 µl buffer (10 mM HEPES at pH 7.9; 10 mM KCl; 1 mM DTT; 0.5 mM PMSF; and 0.1 mM

EDTA). After incubation on ice for 10 min, cells were lysed by the addition of 12.5 μl 10% NP-40. Cells were then centrifuged at 20,000 x g for 2 min at 4˚C, and the supernatants were collected as a cytosolic extract. Pellets were resuspended in 50 μl extraction buffer (20 mM HEPES at pH 7.9; 0.4 M NaCl; 1 mM DTT; 1 mM PMSF; 1 mM EDTA; and 1% NP-40) and incubated on ice for 10 min. The nuclear extract was collected by centrifugation at 15,000 x g for 15 min at 4˚C.

Western blot analysis. RAW264.7 cells were plated at a density of 1x10^6 cells/well in 6-well plates and treated with ETR (25-100 μg/ml), SP600125 (25 μM) or LY294002 (25 μM) for 1 h prior to LPS (1 μg/ml) stimulation for different lengths of time, as indicated in the figure legends. Cells were washed twice with PBS and lysed with RIPA buffer containing protease inhibitors and phosphatase inhibitor. Cell lysates were centrifuged at 13,000 x g for 10 min at 4˚C, and the supernatants were subjected to western blot analysis as described previously (9,10). All western blots are representative of at least three independent experiments.

Statistical analysis. The statistical significance of the differences between groups was assessed using a Student's t-test for pair-wise comparisons or a one-way analysis of variance followed by a post hoc Dunnett's test for multiple comparisons. Statistical analysis was performed using Microsoft Excel software version 2007 (Microsoft Corporation, Redmond, WA, USA). Data are expressed as the mean ± standard deviation of at least three independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of ETR on cell viability and NO production in LPS-stimulated RAW264.7 cells. To investigate the effect of ETR treatment on LPS-induced inflammatory responses, the present study first evaluated cell viability following treatment with ETR, using the MTT assay. As demonstrated in Fig. 1A, ETR at concentrations up to 100 μg/ml did not cause significant cytotoxicity in LPS-induced RAW264.7 cells, compared with cells treated with LPS alone. Subsequently, the effect of ETR treatment on NO production in response to LPS stimulation was investigated. It is established that NO functions as a proinflammatory mediator in the pathogenesis of inflammation (11). In the present study, ETR treatment significantly inhibited LPS-induced NO production in a dose-dependent manner, with a half maximal inhibitory concentration (IC_{50}) of 38.58 μg/ml. Treatment with 100 μg/ml ETR resulted in an effect similar to that of treatment with 100 μM L-NMMA, which is an inhibitor of NOS (Fig. 1B). Therefore, these results demonstrated that ETR may exhibit an anti-inflammatory effect via suppression of NO production without cytotoxicity in LPS-stimulated macrophages.

Effect of ETR on expression of iNOS and proinflammatory cytokines in LPS-stimulated RAW264.7 cells. The effect of ETR on LPS-induced iNOS expression was investigated by RT-PCR and western blotting. iNOS has an important role in inflammation-induced NO production, and mediates immune system regulation and inflammatory reactions (12).

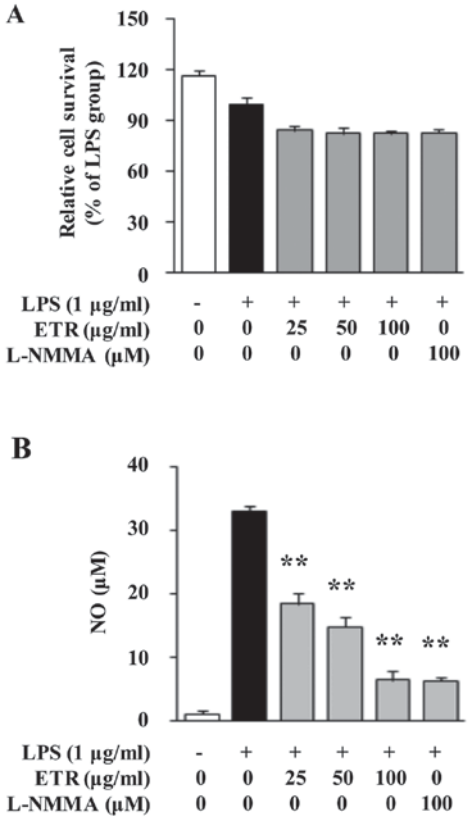

Figure 1. Effect of ETR on cytotoxicity and NO production in LPS-stimulated RAW264.7 cells. (A) Cytotoxicity of ETR was determined by MTT assay. (B) NO production was determined by measuring the concentrations of NO_2^- and NO_3^- in cell culture supernatants. L-NMMA (100 μM) was used as a positive control. Data are presented as the mean ± standard deviation of three independent replicates. **P<0.01 vs. LPS-treated cells. ETR, ethanol extract of *Trigonostemon reidioides* (Kurz) Craib; NO, nitric oxide; LPS, lipopolysaccharide; L-NMMA, NG-methyl-L-arginine acetate salt.

As demonstrated in Fig. 2A, ETR treatment at concentrations of 50 and 100 μg/ml inhibited LPS-induced iNOS expression in a dose-dependent manner at the mRNA and protein levels in RAW264.7 cells. These results indicated that ETR may suppress the LPS-induced NO production by downregulating iNOS expression in RAW264.7 cells.

Proinflammatory cytokines, including TNF-α, IL-1β and IL-6, are released from macrophages upon stimulation with LPS or other inflammatory stimuli (13,14). To evaluate whether the reduction in NO affects the inflammatory response, the expression of these proinflammatory cytokines were examined by RT-PCR and ELISA. As presented in Fig. 2B, ETR treatment inhibited the expression of TNF-α, IL-1β and IL-6 in a dose-dependent manner at the mRNA level. In addition, protein levels of IL-1β and IL-6 as determined by ELISA were significantly reduced by ETR treatment compared with LPS-treated cells; ETR treatment at 100 μg/ml inhibited IL-1β and IL-6 production by 85.47 and 96.24%, respectively (Fig. 2C). These results indicated that ETR may regulate the transcription and protein secretion of proinflammatory cytokines in LPS-activated macrophages.

Effect of ETR on NF-κB nuclear translocation in LPS-stimulated RAW264.7 cells. NF-κB activation leads to the production and release of pro-inflammatory proteins (15-17).

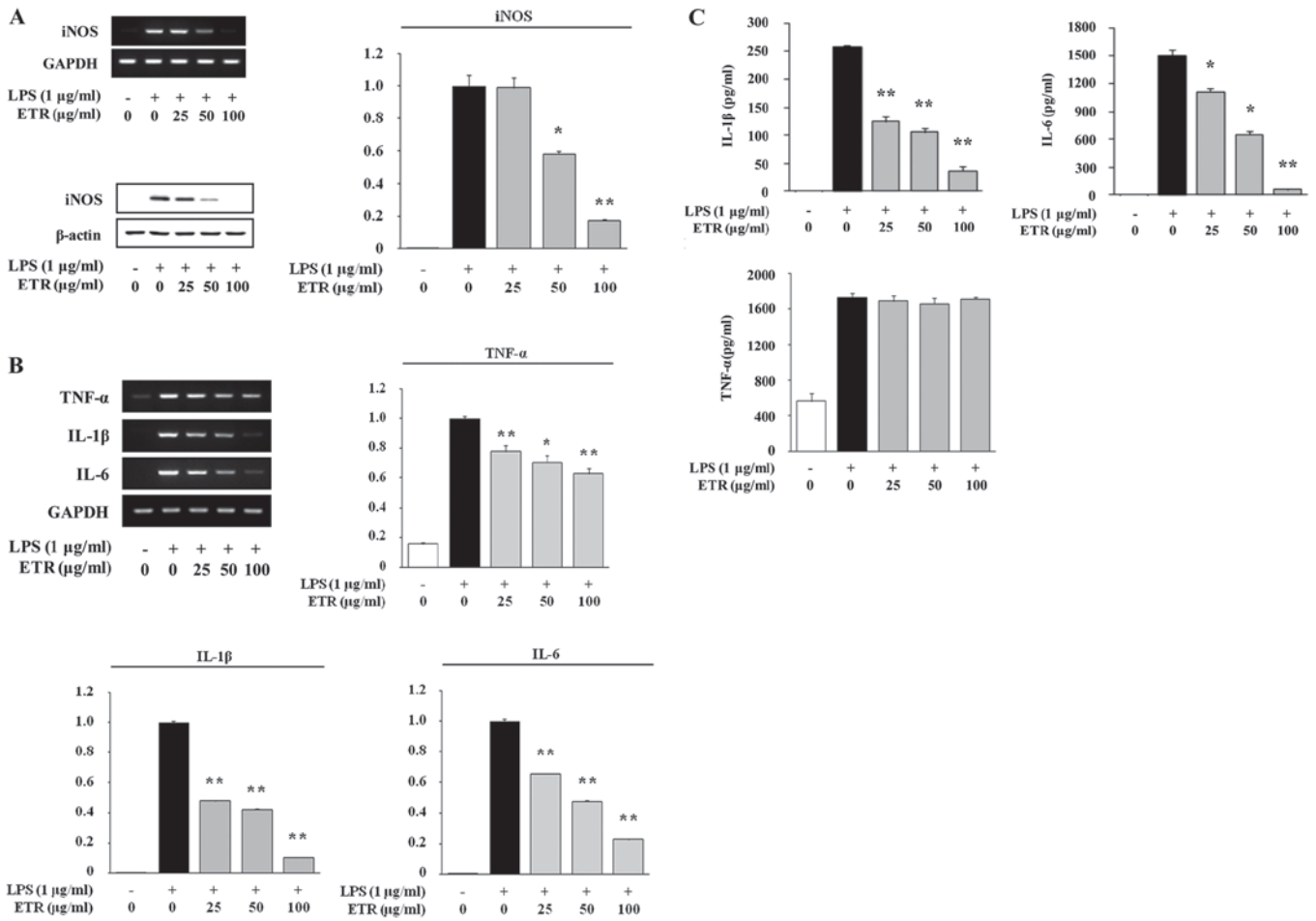

Figure 2. Effect of ETR on the expression of iNOS and proinflammatory cytokines in LPS-stimulated RAW264.7 cells. (A) Expression levels of iNOS mRNA (top) and protein (bottom) were determined by RT-PCR and western blotting. GAPDH and β-actin served as internal controls for RT-PCR and western blotting, respectively. (B) Expression levels of TNF-α, IL-1β and IL-6 mRNA were determined by RT-PCR. (C) Concentrations of IL-1β, TNF-α and IL-6 cytokines in cell culture supernatants were determined by ELISA. Data are presented as the mean ± standard deviation of three independent replicates. *P<0.05 and **P<0.01 vs. LPS-treated cells. ETR, ethanol extract of *Trigonostemon reidioides* (Kurz) Craib; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; RT-PCR, reverse transcription-polymerase chain reaction; TNF, tumor necrosis factor; IL, interleukin.

NF-κB is normally present in the cytoplasm of non-stimulated cells, and nuclear translocation of NF-κB is required for it to initiate transcription of inflammation-associated target genes (18). The present study investigated whether ETR inhibits the translocation of NF-κB (p65 and p50 subunits) into the nucleus. As demonstrated in Fig. 3, ETR treatment (100 μg/ml) markedly suppressed the nuclear translocation of p65 and p50 subunits in LPS-stimulated RAW264.7 cells. This result indicated that ETR may regulate LPS-induced inflammatory mediators and the expression of proinflammatory cytokines by inhibition of the NF-κB signaling pathway.

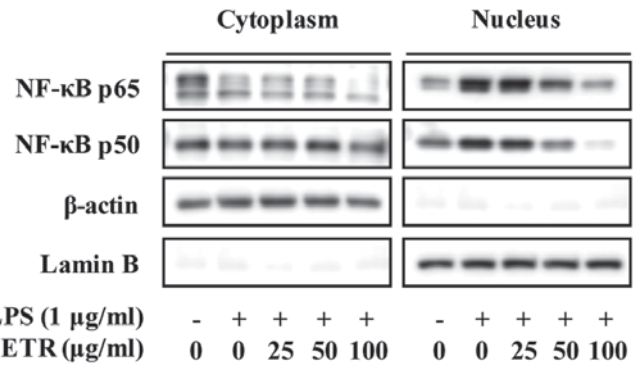

Figure 3. Effect of ETR on the nuclear translocation of NF-κB in LPS-stimulated RAW264.7 cells. Cells were pretreated with ETR (25-100 μg/ml) for 1 h, followed by LPS (1 μg/ml) stimulation for 30 min. Cytosolic and nuclear extracts were analyzed via western blotting with anti-NF-κB p65, anti-NF-κB p50, anti-β-actin (cytosolic loading control) and anti-Lamin B (nuclear loading control) antibodies. Images are representative of three independent replicates. ETR, ethanol extract of *Trigonostemon reidioides* (Kurz) Craib; NF-κB, nuclear factor-κB; LPS, lipopolysaccharide.

Effect of ETR on the phosphorylation of MAPKs and Akt in LPS-stimulated RAW264.7 cells. MAPKs and Akt are involved in signaling pathways that contribute to the regulation of macrophage inflammatory mediators via activation of transcription factors, particularly NF-κB (13,19,20). To determine the underlying molecular mechanism by which ETR acts on the LPS-induced inflammatory response, the present study investigated the phosphorylation of Akt, ERK, JNK and p38 MAPK by western blot analysis. As presented in Fig. 4A, ETR treatment reduced the phosphorylation of Akt, JNK and p38 MAPK in LPS-stimulated RAW264.7 cells, however, no effect on the phosphorylation of ERK was

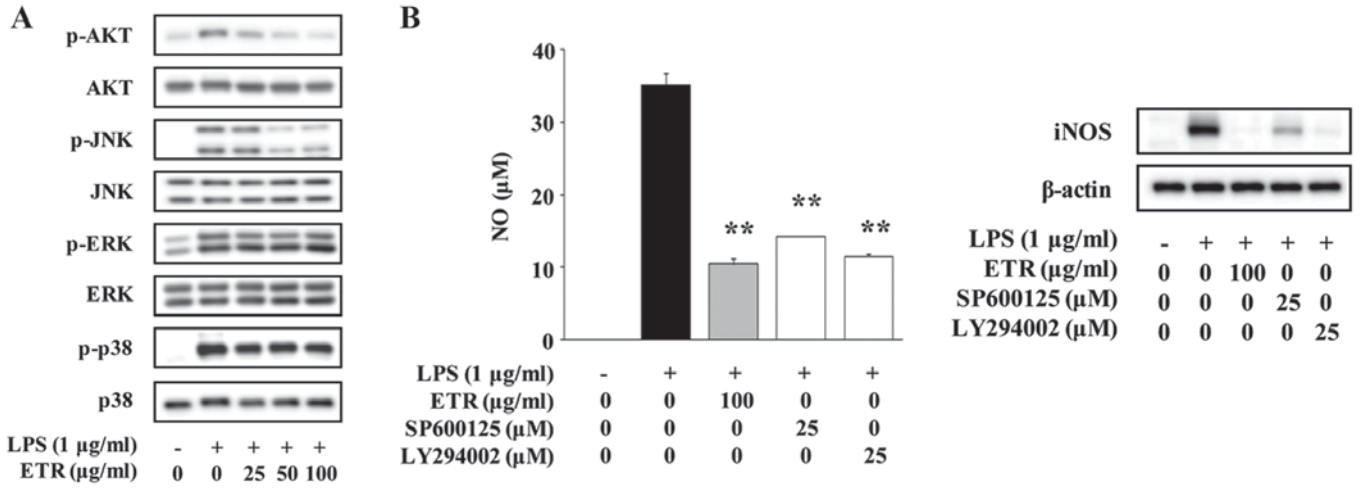

Figure 4. Effect of ETR on the phosphorylation of Akt and mitogen-activated protein kinases in LPS-stimulated RAW264.7 cells. Cells were pretreated with ETR (25-100 μg/ml) for 1 h, followed by LPS (1 μg/ml) stimulation for 30 min. (A) Cell lysates were subjected to western blot analysis with anti-p-Akt, anti-Akt, anti-p-ERK, anti-ERK, anti-p-JNK, anti-JNK, anti-p-p38 and anti-p38 antibodies. (B) Cells were pretreated with ETR (100 μg/ml), LY294002 (25 μM) or SP600125 (25 μM) for 1 h, followed by LPS (1 μg/ml) stimulation for 24 h. NO production was determined by NO assay using Griess reagent, and iNOS expression was analyzed by western blot analysis. Data are presented as the mean ± standard deviation of three independent replicates. **P<0.01 vs. LPS-treated cells. ETR, ethanol extract of *Trigonostemon reidioides* (Kurz) Craib; LPS, lipopolysaccharide; p-, phosphorylated-; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; NO, nitric oxide; iNOS, inducible nitric oxide synthase.

observed. Notably, the phosphorylation of Akt and JNK was almost completely inhibited by ETR treatment at 50 and 100 μg/ml. Kyriakis and Avruch (21) and Jung *et al* (22) have reported that iNOS expression was promoted by the PI3K/Akt and MAPK signal transduction pathways in LPS-stimulated macrophages. To confirm that the MAPKs and the PI3K/Akt pathway are involved in the anti-inflammatory effect of ETR on LPS-activated macrophages, the inhibitory effect of ETR on NO production and iNOS expression was investigated in the presence of SP600125, a JNK-specific inhibitor, and LY294002, an Akt-specific inhibitor. NO production and iNOS expression in the LPS-activated macrophages were significantly suppressed by treatment with either ETR, SP600125 or LY294002 compared with macrophages treated with LPS alone (Fig. 4B). These data indicated that the inhibitory effect of ETR on LPS-induced inflammation may involve the PI3K/Akt and MAPK signaling pathways.

Discussion

Inflammation is a physiological response that is mediated by activating various immune cells, including macrophages and monocytes. Inflammation serves a key role in the host defense system. Excessive inflammation causes a variety of diseases, including arthritis, inflammatory bowel disease, neurodegenerative disorders and septic shock syndrome (23,24). Inflammatory responses are regulated by NO, which is synthesized by NOS. In particular, iNOS is regarded as the primary regulator of NO production in macrophages (25,26).

T. reidioedes has been described as exhibiting potential anticancer activity by inducing cytotoxicity in several cancer cell lines, including liver, cervical, oral, colon, lung and gastric cancers (1). Recently, several studies have reported that natural products such as *T. reidioedes* may be effective in the treatment and prevention of inflammatory diseases (27,28). However, there have been few reports investigating the molecular mechanisms of the inflammatory effect of *T. reidioedes*. In the present study, to determine these underlying mechanisms, the anti-inflammatory activity of ETR was investigated. The results demonstrated that ETR treatment reduced NO production and the expression of various proinflammatory mediators, including iNOS, IL-1β, IL-6 and TNF-α in LPS-induced RAW264.7 cells.

The transcription factor NF-κB has a critical role in the regulation of inflammatory gene expression (27). It has been previously established that NF-κB activation is related to the MAPK and the PI3K/Akt signaling pathway, through LPS stimulation (28-30). In the present study, ETR treatment inhibited NF-κB activation and the phosphorylation of JNK and Akt. Of note, treatment with SP600125 and LY294002, inhibitors of JNK and Akt phosphorylation respectively, significantly inhibited the expression of NO and iNOS in the LPS-activated macrophages, an effect that was similar to the ETR treatment. These data suggested that the ETR anti-inflammatory effect may involve the PI3K/Akt and MAPK signaling pathways.

In conclusion, the current study demonstrated that ETR may exert an anti-inflammatory effect by suppressing the expression of inflammatory mediators and cytokines, via the downregulation of NF-κB, Akt and the MAPK signaling pathways in LPS-stimulated macrophages. To the best of our knowledge, this is the first report of the anti-inflammatory activity of ETR in LPS-induced RAW264.7 cells. These results indicate that ETR may be a promising candidate for development as an anti-inflammatory drug.

Acknowledgements

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