JPP Journal of Pharmacy and Pharmacology

JPP 2008, 60: 917–924
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Received December 10, 2007
Accepted March 20, 2008
DOI 10.1211/jpp.60.7.0014
ISSN 0022-3573

Eutigoside C inhibits the production of inflammatory mediators (NO, PGE_2, IL-6) by down-regulating NF-κB and MAP kinase activity in LPS-stimulated RAW 264.7 cells

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Abstract

Eutigoside C, a compound isolated from the leaves of *Eurya emarginata*, is thought to be an active anti-inflammatory compound which operates through an unknown mechanism. In the present study we investigated the molecular mechanisms of eutigoside C activity in lipopolysaccharide (LPS)-stimulated murine macrophage RAW 264.7 cells. Treatment with eutigoside C inhibited LPS-stimulated production of nitric oxide (NO), prostaglandin E_2 (PGE_2) and interleukin-6 (IL-6). To further elucidate the mechanism of this inhibitory effect of eutigoside C, we studied LPS-induced nuclear factor (NF)-κB activation and mitogen-activated protein (MAP) kinase phosphorylation. Eutigoside C suppressed NF-κB DNA binding activity, interfering with nuclear translocation of NF-κB. Eutigoside C suppressed the phosphorylation of three MAP kinases (ERK1/2, JNK and p38). These results suggest that eutigoside C inhibits the production of inflammatory mediators (NO, PGE_2 and interleukin-6) by suppressing the activation and translocation of NF-κB and the phosphorylation of MAP kinases (ERK1/2, JNK and p38) in LPS-stimulated murine macrophage RAW 264.7 cells.

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Acknowledgements and funding: This work was supported by grants from the Jeju Bio-Co-Research Program, which was conducted by the Jeju Hi-Tech Industry Development Institute, and the post-Brain Korea 21 Program.

Introduction

Inflammation involves multiple processes that are mediated by activated inflammatory and immune cells such as monocytes/macrophages. Activation of monocytes and macrophages by lipopolysaccharide (LPS) leads to signalling cascades, resulting in production of various inflammatory mediators, including nitric oxide (NO), prostaglandins (PGs), inflammatory cytokines and others (Guha & Mackman 2001; Minagar et al 2002; Maruotti et al 2007). NO is an important mediator and effector molecule, with various biological functions (James 1995; Kapur et al 2000; Stepien et al 2005). Up-regulation of NO production by inducible nitric oxide synthase (iNOS) represents part of a prompt antibacterial response; however, NO has also been associated with acute and chronic inflammation (Macmicking et al 1997; Kolios et al 2004). PGs are produced by a variety of cell types, and the rate-limiting enzyme in the synthesis of PGs is cyclooxygenase (COX) (Harris et al 2002). COX-2 is induced by several stimuli, including growth factors, mitogens and cytokines. The uncontrolled activity of COX-2 is thought to play an important role in the pathogenesis of various chronic inflammatory diseases (Wadleigh et al 2000; Patrignani et al 2005; Harris & Breyer 2006).

LPS-induced expression of inflammatory genes is mediated by a series of signalling pathways such as nuclear factor (NF)-κB and mitogen-activated protein (MAP) kinases (Islam et al 2004; Lee & Schorey 2005). NF-κB is a major transcription factor involved in the release of proteins that mediate cardiovascular growth, stress response and inflammation by controlled gene expression. The NF-κB component p65 is located in the cytoplasm as an inactive complex bound to IκBα, which is phosphorylated and subsequently degraded. p65 then dissociates from IκBα to produce activated NF-κB (Murakami et al 2003; Kim et al 2006; Inoue et al 2007). Various upstream activators that are involved in LPS signalling may mediate activation of the MAP kinase pathways, which include extracellular signal-regulated kinase 1/2 (ERK1/2), p38 and stress-activated protein kinase/c-Jun NH_2-terminal

kinase (SAPK/JNK) in monocytes and macrophages. These kinases are important in controlling cellular responses to the environment and in regulating gene expression, cell growth and apoptosis related to many human diseases (Johnson & Lapadat 2002).

Eurya emarginata (Thumb) Makino (Theaceae) is distributed in coastal areas from southern China, along southern Korea and extending to central and southern Japan (Chung & Epperson 2000). The leaves of *E. emarginata* exhibit anticancer activities (Park et al 2004, 2005a). Moreover, eutigoside C (Figure 1), a new compound isolated from *E. emarginata*, is reported to be responsible for the anti-inflammatory activity of the leaves (Park et al 2005b). However, how eutigoside C exhibits this anti-inflammatory activity has not yet been elucidated. In this study, therefore, we investigated the anti-inflammatory mechanisms of eutigoside C. We demonstrate that eutigoside C inhibits the production of inflammatory markers (NO, PGE_2 and interleukin (IL)-6) by suppressing the activation of NF-κB and phosphorylation of MAP kinases (ERK1/2, JNK and p38) in LPS-stimulated murine macrophage RAW 264.7 cells.

Materials and Methods

Reagents

LPS (*Escherichia. coli* 0111:B4) was purchased from Sigma Chemical Co. (St Louis, MO, USA). Fetal bovine serum (FBS) and DMEM were obtained from Gibco (Grand Island, NY, USA). The ELISA kit for IL-6 was obtained from BD Biosciences (San Diego, CA, USA) and the lactate dehydrogenase (LDH) cytotoxicity detection kit was from Promega (Madison, WI, USA). All other chemicals were of standard analytical grade (Sigma). Antibodies against iNOS, COX-2, IκB-α, p38, ERK1/2, phospho-ERK1/2, JNK and phospho-JNK were obtained from Cell Signaling Technology (Beverly, MA, USA); antibodies to phospho-p38 were from BD Biosciences (San Diego, USA).

Isolation of eutigoside C

Eutigoside C was isolated from the leaves of *E. emarginata*. The dried leaves (150 g) were extracted using 80% aqueous methanol to obtain the crude extract (48.8 g). Water was added to the extract, and then the aqueous layer was successively partitioned into hexane, ethyl acetate (EtOAc) and *n*-butanol. Part of the EtOAc fraction (7.7 g) was purified through a reverse-phase SiO_2 column using gradient elution with aqueous methanol (20–100%) to give five fractions (A–E). The polar fraction A (600 mg) was subjected to Sephadex LH-20 column chromatography using CH_2Cl_2/acetone/methanol (3/2/0 to 1/4/0 to 2/7/1) to give 32 fractions. Fraction 26 (13 mg) was further purified by HPLC to give eutigoside C (5.3 mg). The structure of eutigoside C was identified by comparing the spectroscopic data to that in the literature (Khan 1992).

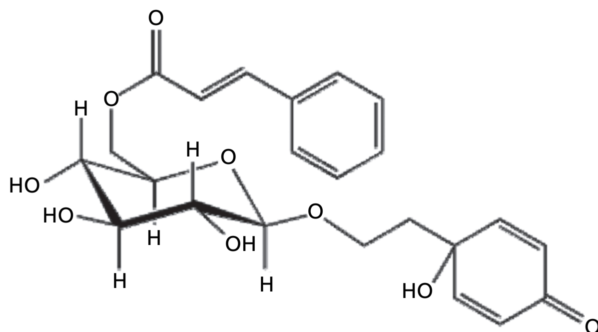

Figure 1 The chemical structure of eutigoside C isolated from *Eurya emarginata*.

Eutigoside C (Figure 1): Pale yellow powder: $[\alpha]^{20}_{D}$ −15 (MeOH, $c = 0.0038$); ^{1}H NMR (400 MHz, methanol-d_4) δ7.71 (1H, d, 16.0, H8″), 7.62 (2H, m, H2″ and H6″), 7.40 (3H, m, H3″, H4″, H5″), 6.97 (2H, m, H2, H6), 6.56 (1H, d, 16.0, H8″), 6.06 (2H, br d, 10.4, H3, H5), 4.50 (1H, dd, 11.8, 2.0, H6′), 4.32 (1H, dd, 11.8, 2.0, H6′), 4.25 (1H, d, 7.8, H1′), 3.92 (1H, dt, 10.2, 6.5, H8), 3.65 (1H, dt, 10.2, 6.5, H8), 3.51 (1H, dd, 9.0, 6.0, H5′), 3.34 (2H, m, H3′, H4′), 3.16 (1H, dd, 9.0, 7.8, H2′), 2.04 (2H, t, 6.5). ^{13}C NMR (100 MHz, methanol-d_4) 187.8 (C4), 168.5 (C9″), 154.4 (C2), 154.3 (C6), 146.5 (C7″), 135.7 (C1″), 131.6 (C4″), 130.1 (C2″, C6″), 129.3 (C3″, C5″), 128.0 (C3), 127.9 (C5), 118.7 (C8″), 104.4 (C1′), 77.9 (C3′), 75.4 (C2′), 75.0 (C5′), 71.7 (C4′), 69.2 (C1), 65.9 (C8), 64.8 (C6″), 41.0 (C7).

Cell culture

The mouse macrophage RAW 264.7 cell line was purchased from ATCC (Rockville, MD, USA) and cultured in DMEM supplemented with 10% (v/v) heat-activated FBS, streptomycin (100 μg mL^{-1}) and penicillin (100 U mL^{-1}) at 37°C and 5% CO_2.

Cytotoxicity assay

LDH activity was determined by following the production of NADH during the conversion of lactate to pyruvate (Fernandez et al 2006). The optical density of the solution was then measured at 490 nm using an ELISA plate reader. Percentage cytotoxicity was determined relative to the control group.

Cell viability was determined by the 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, MTT was added to cells for the designed time. After 4 h, the formazan crystals were dissolved by adding DMSO. Metabolic activity was quantified by measuring absorbance at 540 nm. Cell viability was represented as relative absorbance compared with the controls.

Measurement of NO production

Nitrite, which is the end-point of NO generation by activated macrophages, was measured by a colorimetric assay. Briefly, 100 μL Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) were added to 100 μL samples of medium. The concentration of NO_2^- was calculated by comparison with a standard curve prepared using $NaNO_2$.

Measurement of IL-6 production

The inhibitory effect of eutigoside C on IL-6 production in LPS-treated RAW264.7 cells was determined as described previously (Cho et al 2000). Supernatants were harvested and assayed for cytokines by ELISA.

Western blot analysis

After incubation, the cells were collected and washed twice with cold phophate-buffered saline. The cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and kept on ice for 30 min. The cell lysates were centrifuged at 15000 rev min^{-1}pm at 4°C for 15 min and the supernatants were stored at −70°C until use. Protein concentration was measured using the Bradford method (Bradford 1976). Aliquots of the lysates were separated on a 10–12% SDS-polyacrylamide gel and transferred onto a PVDF membrane (Bio-Rad, HC, USA). After blocking the non-specific sites with 1% bovine serum albumin, the membrane was incubated with specific primary antibody at 4°C overnight. The membrane was further incubated for 60 min with peroxidase-conjugated secondary antibody at room temperature. The immunoactive proteins were detected using the WEST-ZOL (plus) Western Blot Detection System (iNtRON, Gyeonggi, Korea).

RNA preparation and RT-PCR

Total RNA was extracted from cells using the Tri-Reagent method (MRC, Cincinnati, OH, USA) according to the manufacturer's instructions. RNA extraction was carried out in an RNase-free environment. The reverse transcription of 1 μg RNA was carried out using M-MuLV reverse transcriptase (Promega), oligo $(dT)_{15}$ primer, dNTP (0.5 μM) and 1 U RNase inhibitor. PCR was performed with a DNA gene cycler (Bio-rad); the amplification programme included 30 cycles of 94°C for 45 s (denaturing), 50–58°C for 45 s (annealing) and 72°C for 1 min (extension). The PCR products were electrophoresed on a 1.2% agarose gel.

Transient transfection and luciferase assay

Cells were cotransfected with 50 ng NF-κB promoter-based luciferase reporter plasmid (Panomics, Redwood City, CA, USA) and 10 ng Renilla luciferase reporter plasmid (Promega), which served as the internal standard, using Trans-Fast^{TM} transfection reagent (Promega). After 24 h, cells were incubated with LPS (1 μg mL^{-1}) in the presence or absence of eutigoside C. After 20 h' incubation, luciferase activity in the cell lysate was determined using the Dual-Luciferase Reporter Assay Kit (Promega). Luciferase activity was normalized to transfection efficiency as monitored by Renilla luciferase expression. The level of luciferase activity was determined as a ratio compared with cells with no stimulation.

Electrophoretic mobility shift assay (EMSA)

NF-κB–DNA binding activity was measured by EMSA using a gel-shift assay system kit (Promega) according to the manufacturer's instructions. Briefly, double-stranded oligonucleotides containing the consensus NF-κB sequence 5′-AGTTGAGGGGACTTTCCCAGGC-3′ were end-labelled with [γ-^{32}P]ATP (2.5 μCi; Amersham Pharmacia Biotech, Piscataway, NJ, USA) using T4 polynucleotide kinase and were then used as probes for EMSA. Nuclear extract proteins (5 μg) were pre-incubated with gel-shift binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.05 mg mL^{-1} poly(deoxy-inosine-deoxycytosine)) for 10 min and then incubated with the labelled probe for 20 min at room temperature. Each sample was electrophoresed in a 6% non-denaturing polyacrylamide gel in 0.5 × TBE buffer at 250 V. The gel was dried and subjected to autoradiography.

Statistical analysis

Analysis of variance in conjunction with Tukey's post-hoc test was used to determine the statistical significance of differences between values for a variety of experimental and control groups. Data are expressed as mean ± s.d. of at least three independent experiments performed in triplicate. $P < 0.05$ was considered statistically significant.

Results and Discussion

Cytotoxicity of eutigoside C

The cytotoxicity of eutigoside C was determined using the LDH release method and MTT assay. The result showed that eutigoside C did not affect cell toxicity at concentrations of 25–200 μM (Figure 2).

Effect of eutigoside C on the production of inflammatory mediators (NO, PGE_2 and IL-6)

In order to investigate the effect of eutigoside C on NO production, we measured the accumulation of nitrite in culture media. As shown in Figure 3A, eutigoside C dose-dependently

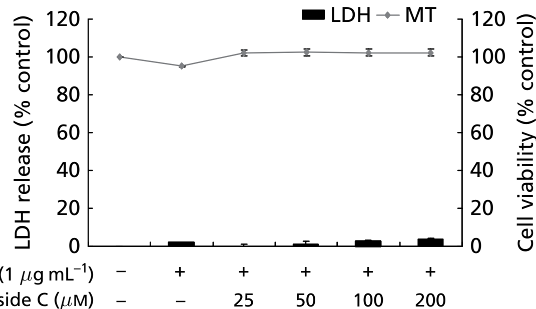

Figure 2 Cytotoxicity of eutigoside C in RAW 264.7 cells. The cells were stimulated with LPS (1 μg mL^{-1}) alone or with different concentrations of eutigoside C (25, 50, 100, 200 μM) for 24 h. Lactase dehydrogenase (LDH) release is expressed as a percentage of total cellular LDH. Cell viability is represented as relative absorbance compared with controls. Data represent mean ± s.d. of triplicate experiments. *$P < 0.05$; **$P < 0.01$ vs LPS alone.

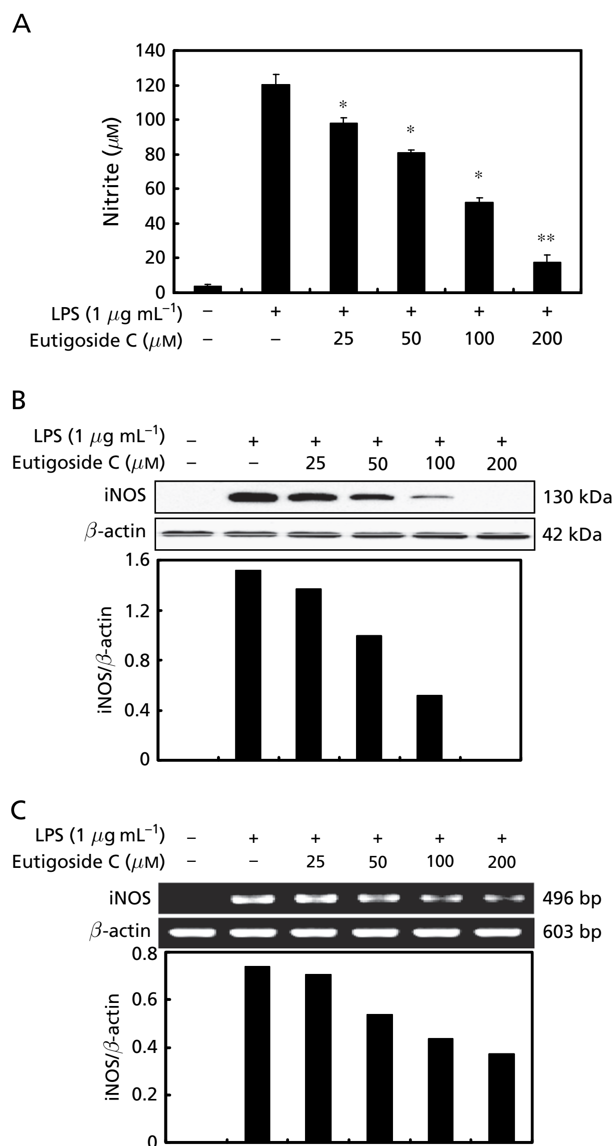

Figure 3 Effects of eutigoside C on nitric oxide (NO) production and expression of inducible NO synthase (iNOS) in LPS-stimulated RAW264.7 cells. (A) Cells were stimulated with LPS (1 μg mL^{-1}) alone or with various concentrations of eutigoside C (25, 50, 100, 200 μM) for 24 h. NO was determined by the Griess reagent method. Data are mean ± s.d. of triplicate experiments. *$P < 0.05$; **$P < 0.01$ vs LPS alone. (B) Cells were stimulated with LPS (1 μg mL^{-1}) alone or with various concentrations of eutigoside C (25, 50, 100, 200 μM) for 20 h. Whole-cell lysates (30 μg) were prepared and the protein subjected to 10% SDS-PAGE. Expression of iNOS was determined by Western blotting, using β-actin as the internal control. (C) Cells were stimulated with LPS (1 μg mL^{-1}) alone or with various concentrations of eutigoside C (25, 50, 100, 200 μM) for 18 h. Total RNA was isolated and the mRNA expression of iNOS determined by RT-PCR; β-actin was used as an internal control.

inhibited the production of NO in LPS-stimulated RAW 264.7 cells. Several studies have demonstrated that induction of iNOS produces a large amount of NO during endotoxaemia and under inflammatory conditions (Bove & van der Vliet 2006; Suh et al 2006). Therefore, to explain the mechanism of inhibition of NO production, we examined the effect of eutigoside C on iNOS gene expression. Eutigoside C inhibited iNOS protein and mRNA expression in a dose-dependent manner (Figures 3B and 3C). These results indicate that eutigoside C controlled NO production via iNOS gene expression, without producing cell toxicity.

Excessive PGE_2 produced by COX-2 in stimulated inflammatory cells modulates a variety of physiological processes in inflammatory disease (Williams & Shacter 1997). Therefore, compounds inhibiting COX-2 activity or its transcription might have anti-inflammatory or cancer chemopreventive effects (Kwon et al 2007). We evaluated the effect of eutigoside C on LPS-induced PGE_2 production and COX-2 expression. When the cells were treated with various concentrations of eutigoside C, PGE_2 production was inhibited in a dose-dependent manner (Figure 4A). Western blotting and RT-PCR analysis were carried out in order to determine if these effects on PGE_2 production were related to COX-2 levels. As shown in Figures 4B and 4C, eutigoside C inhibited COX-2 protein and mRNA expression. These results indicate that eutigoside C suppresses COX-2 expression at the transcriptional level, thereby decreasing the production of COX-2 protein and PGE_2.

IL-6 is a cytokine released by various types of cells and is known to trigger bone resorption and inflammatory diseases (Jones et al 2001; Kallen 2002). Thus, we studied the effect of eutigoside C on IL-6 production. The result revealed that eutigoside C inhibits IL-6 production and mRNA expression in a dose-dependent manner, with an IC50 of 30.4 μM (Figure 5). Inflammatory agents that induce IL-6 synthesis in macrophages also induce COX-2 expression (Hinson et al 1996; Anderson et al 1996; Chen & Lin 1999). In addition, IL-6 induces NO production via the JAK/STAT pathway during chronic inflammation (Wung et al 2005). Thus, the above results suggest that inhibition of IL-6 activity by eutigoside C might be responsible for modulating NO and PGE_2 production.

Effect of eutigoside C on the activation of NF-κB

NF-κB is essential for the expression of iNOS, COX-2 and inflammatory cytokines (Libermann & Baltimore 1990; Ohshima et al 2005). We therefore examined the effect of eutigoside C on NF-κB activation. Luciferase activity assays showed that eutigoside C inhibits NF-κB activation in a dose-dependent manner (Figure 6A). Inhibition of NF-κB activity can result from interference with various steps in the NF-κB activation pathway (Baldwin 1996; Karin 1999a). The transactivation of transcription factors can be modulated by changing the DNA-binding potential of the transcription factor (Wang et al 2002). To explore the mechanism by which eutigoside C inhibits NF-κB induction by LPS, we evaluated the DNA-binding activity of NF-κB by EMSA. In the presence of eutigoside C, LPS-induced NF-κB binding was suppressed in a dose-dependent manner (Figure 6B). Since p65 is the major component of NF-κB when activated by LPS in macrophages, we next determined the levels of p65 in nuclear extracts by Western blotting. Eutigoside C inhibited LPS-induced nuclear translocation of NF-κB-p65, similar to its inhibition of

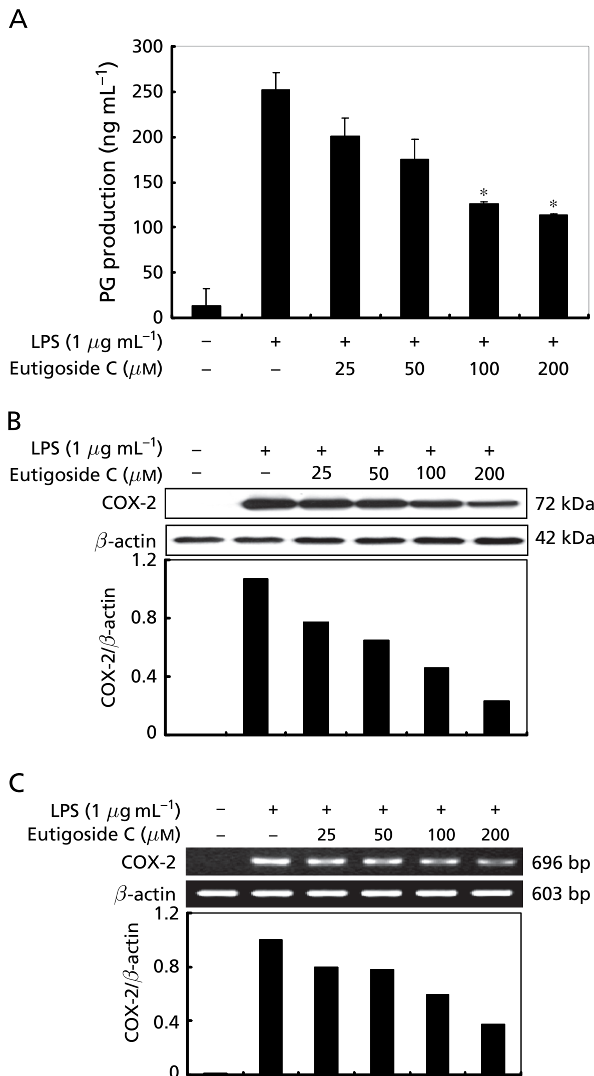

Figure 4 Effect of eutigoside C on prostaglandin (PG)E_2 production and cyclooxygenase (COX)-2 expression in LPS-stimulated RAW 264.7 cells. (A) Cells were stimulated with LPS (1 μg mL^{-1}) alone or with various concentrations of eutigoside C (25, 50, 100, 200 μM) for 24 h. PGE_2 produced and released into the culture medium was assayed by enzyme immunoassay. Data are mean ± s.d. of triplicate experiments. *$P < 0.05$; **$P < 0.01$ vs LPS alone. (B) Cells were stimulated with LPS (1 μg mL^{-1}) alone or with various concentrations of eutigoside C (25, 50, 100, 200 μM) for 20 h. Whole-cell lysates (30 μg) were prepared and the protein was subjected to 10% SDS-PAGE. Expression of COX-2 was determined by Western blotting; β-actin was used as an internal control. (C) Cells were stimulated with LPS (1 μg mL^{-1}) alone or with various concentrations of eutigoside C (25, 50, 100, 200 μM) for 18 h. Total RNA was isolated and the mRNA expression of COX-2 was determined by RT-PCR; β-actin was used as an internal control.

NF-κB–DNA binding (Figure 6C). The nuclear translocation and DNA binding of NF-κB are induced by the degradation of the inhibitory protein IκB-α (Karin 1999b). In order to determine whether the inhibition of NF-κB–DNA binding is related to IκB-α degradation, we examined the cytoplasmic levels of IκB-α. Stimulation of cells with LPS

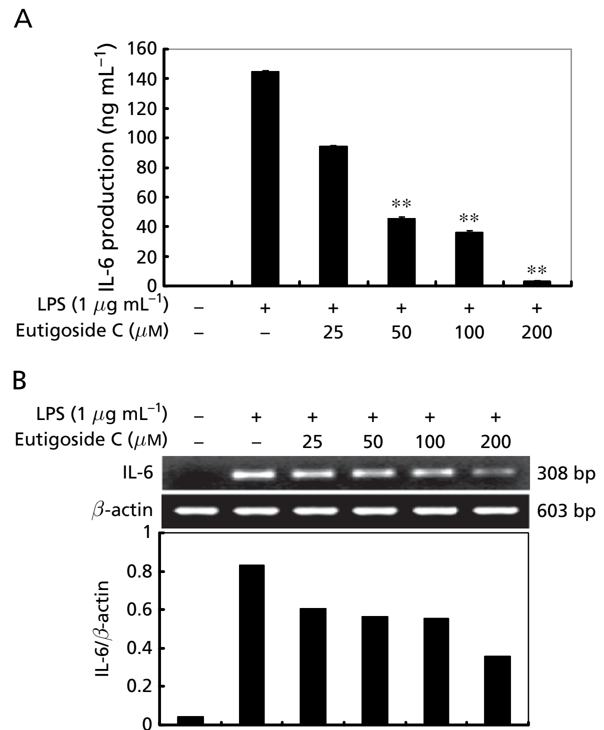

Figure 5 Effect of eutigoside C on interleukin (IL)-6 production in LPS-stimulated RAW 264.7 cells. (A) Cells were stimulated with LPS (1 μg mL^{-1}) alone or with various concentrations of eutigoside C (25, 50, 100, 200 μM) for 24 h. IL-6 produced and released into the culture medium was assayed by ELISA. Data represent mean ± s.d. of triplicate experiments. *$P < 0.05$; **$P < 0.01$ vs LPS alone. (B) Cells were stimulated with LPS (1 μg mL^{-1}) alone or with various concentrations of eutigoside C (25, 50, 100, 200 μM) for 18 h. Total RNA was isolated and the mRNA expression of IL-6 was determined by RT-PCR; β-actin was used as an internal control.

for 20 min decreased the intensity of the IκB-α band, and the intensity recovered to basal level in the presence of eutigoside C in a dose-dependent manner (Figure 6D). These results indicate that inhibition of pro-inflammatory mediators by eutigoside C in LPS-stimulated RAW 264.7 cells may involve transcriptional regulation through suppression of NF-κB–DNA binding potential and interference with nuclear translocation of NF-κB.

Effect of eutigoside C on the phosphorylation of MAP kinases

Three families of MAP kinases induce activated macrophages (ERK, JNK and p38 MAP kinases). These kinases play key roles in the regulation of cell growth, differentiation and stress-induced gene expression (Raingeaud et al 1995; Cobb 1999). The pro-inflammatory cytokines that are released from macrophages are also involved in many inflammatory events, including expression of iNOS and COX-2 involving the MAP kinase signalling pathway (Murakami et al 2005; Rafi et al 2007). Thus, we investigated the effect of eutigoside C on the phosphorylation of

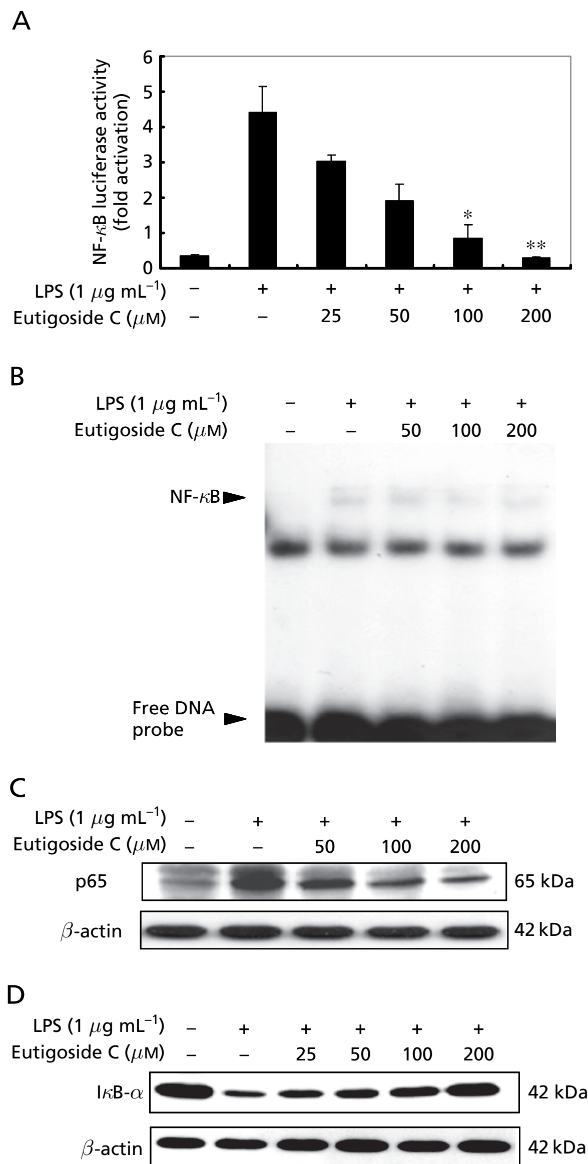

Figure 6 Effects of eutigoside C on nuclear factor (NF)-κB activation in LPS-stimulated RAW264.7 cells. (A) Cells were transiently cotransfected with NF-κB-promoter-based luciferase reporter plasmid (pNF-κB-Luc) and Renilla luciferase reporter plasmid (pRL-null) (internal control) for 30 h. The cells were then stimulated with LPS (1 μg mL^{-1}) alone or with different concentrations of eutigoside C (25, 50, 100, 200 μM) for 16 h. The luciferase activity was measured and data were normalized to Renilla luciferase expression. Data are mean ± s.d. of triplicate experiments. *$P < 0.05$; **$P < 0.01$ vs LPS alone. (B) Cells were stimulated with LPS (1 μg mL^{-1}) alone or with various concentrations of eutigoside C (50, 100, 200 μM) for 1 h. Nuclear extracts (10 μg) were prepared and assayed for NF-kB binding activity by electrophoretic mobility shift assay. (C) Cells were stimulated with LPS (1 μg mL^{-1}) alone or with various concentrations of eutigoside C (50, 100, 200 μM) for 1 h. Nuclear extracts (20 μg) were prepared and the protein was subjected to 10% SDS-PAGE; expression of NF-κB–p65 was determined by Western blotting; β-actin was used as an internal control. (D) Cells were stimulated with LPS (1 μg mL^{-1}) alone or with various concentrations of eutigoside C (50, 100, 200 μM) for 20 min. Whole-cell lysates (30 μg) were prepared and the protein level was subjected to 12% SDS-PAGE. Expression of IκB-α was determined by Western blotting; β-actin was used as an internal control.

these three MAP kinases. The results showed that eutigoside C inhibited LPS-induced phosphorylation of ERK1/2, JNK and p38 (Figure 7). These results indicate that eutigoside C has an inhibitory effect through the inhibition of MAP kinase phosphorylation.

The iNOS gene promoter contains binding sites for several homologous factors, such as NF-κB, activation protein (AP)-1 and CCAAT/enhancer binding protein (C/EBP) (Baeuerle & Baltimore 1996). Pharmacological approaches have demonstrated that p38 MAP kinase and/or ERK are required for transcription factor NF-κB activation in response to LPS (Lee et al 2006; Suh et al 2006) and JNK is believed to be essential for activation of transcription factor AP-1 (Ma et al 2004). Our results showed that eutigoside C inhibited the phosphorylation of p38 MAP kinase and ERK. Eutigoside C also inhibited the phosphorylation of JNK (Figure 7). It is possible that eutigoside C inhibits pro-inflammatory mediators by suppressing AP-1 activation signalling.

LPS binds to the serum protein LPS-binding protein and is delivered to the cell surface receptor CD14. LPS then interacts with the transmembrane signalling receptor toll-like receptor 4 (TLR4) to activate a series of signalling proteins that includes myeloid differentiation primary response gene (88) (MyD88), IL-1 receptor-associated kinase (IRAK) and tumor necrosis factor (TNF)-receptor-associated factor 6 (TRAF6) (Fujihara et al 2003; Pailsson-McDermott & O'Neill 2004). These pathways directly or indirectly phosphorylate and activate various transcription factors and signalling molecules, including NF-κB and MAP kinases (Guha & Mackman 2001). Our results showed that eutigoside C inhibited activation of NF-κB and the three MAP kinases (Figures 6 and 7). It is possible that eutigoside C inhibits inflammatory genes and transcription factors by blocking the signalling receptors, such as CD14 and TLR4, or accessory proteins, such as MyD88 and TRAF6. However, further studies are needed to support this conclusion.

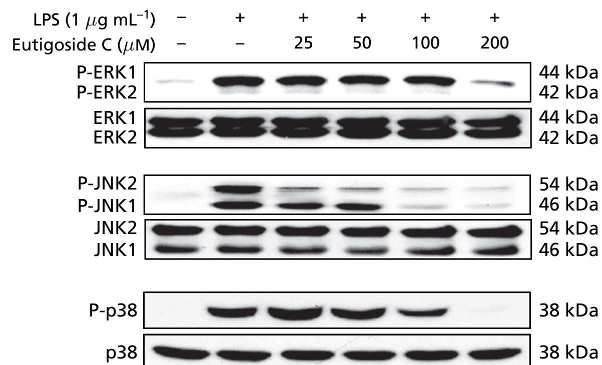

Figure 7 Effects of eutigoside C on the phosphorylation of MAP kinases in LPS-stimulated RAW264.7 cells. Cells were stimulated with LPS (1 μgmL^{-1}) alone or with various concentrations of eutigoside C (25, 50, 100, 200 μM) for 30 min. Whole-cell lysates (30 μg) were prepared and the protein was subjected to 12% SDS-PAGE. Expression of MAP kinases was determined by Western blotting. β-actin was used as an internal control.

Conclusions

In this study, we demonstrated that the anti-inflammatory effects of eutigoside C may be caused by modulation of macrophage-mediated inflammatory functions such as overproduction of NO, PGE_2 and IL-6, but without producing cell toxicity. Furthermore, these inhibitory effects involved suppression of the activation of NF-κB and phosphorylation of MAP kinases (ERK1/2, JNK and p38).

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