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Research Paper

Anti-inflammatory effects of *Crataeva nurvala* Buch. Ham. are mediated via inactivation of ERK but not NF-κB

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ABSTRACT

Ethnopharmacological relevance: *Crataeva nurvala* Buch. Ham. is an important medicinal plant in India, and its extracts and components were used to treat various inflammatory diseases, such as urinary tract infection, rheumatoid arthritis, and colitis. However, no systemic studies about anti-inflammatory effects of *Crataeva nurvala* Buch. Ham. and its underlying mechanisms of action have been reported. This study aimed to explore the anti-inflammatory effects of ethanol extracts of *Crataeva nurvala* Buch. Ham. (ECN).

Materials and methods: The non-cytotoxic and maximal effective concentration of ECN was determined by measuring the formation of formazan from water-soluble tetrazolium salt in living cells. The inhibitory effect of ECN on nitric oxide (NO) synthesis was measured using Griess reagent, and Enzyme-linked immunosorbent assay (ELISA) was used to measure secreted tumor necrosis factor (TNF)-α and interleukin (IL)-6 protein levels. Furthermore, reverse transcription polymerase chain reaction (RT-PCR) and Western blotting analysis were used to assess the mRNA and protein expression of each inflammatory mediator or relating signaling protein, respectively.

Results: A non-cytotoxic concentration of ECN ($\leq$200 μg/ml) significantly reduced the production of NO and IL-6, but not TNF-α, in lipopolysaccharides (LPS)-stimulated RAW 264.7 macrophages. Decreased production of NO by ECN was correlated with reduced expression of iNOS at the mRNA and protein levels. However, cyclooxygenase (COX)-2 expressions at mRNA and protein level were not regulated by ECN. The mRNA expression of *IL-6* and *IL-1β*, but not *TNF-α*, was also inhibited by ECN treatment in LPS-stimulated RAW 264.7 macrophages. Reduced production of inflammatory mediators by ECN was followed by decreased activity of mitogen-activated protein kinases (MAPKs), especially extracellular signal-regulated kinase (ERK), but not nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).

Conclusions: These results indicate that ECN inhibits LPS-induced inflammatory responses via negative regulation of ERK in murine macrophages, suggesting that ECN is a candidate for alleviating severe inflammation.

1. Introduction

Inflammation is recognized as a localized, protective response to various pathological conditions, including tissue injury and microbial invasion, that are characterized by redness, swelling, heat, pain, and organ dysfunction (Kulinsky, 2007). Inflammation is also considered the major cause of most chronic diseases, including diabetes, Alzheimer's disease, asthma, and atherosclerosis. Many biological molecules, including reactive oxygen species (ROS), nitric oxide (NO), prostaglandin E_2 (PGE_2), and cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, are involved in the development of inflammation (Hanada and Yoshimura, 2002). Macrophages are major cells in the immune system that act as a first line of defense against invading agents (bacteria, viruses, and fungi) and respond to pathogen attacks by releasing cellular signaling molecules and anti-microbial agents (Bosca et al., 2005; Fujihara et al., 2003; MacMicking et al., 1997). However, the exaggerated production of inflammatory mediators by macrophages can damage the host; this presents as septic shock or autoimmune disease, such as rheumatoid arthritis, systemic lupus erythematosus, or dermatitis (Cutolo, 1999; Valledor et al., 2010). Therefore, an effective strategy for developing therapeutic agents to treat severe inflammation is the identification of agents that regulate the production of pro-inflammatory mediators.

Activated macrophages transcriptionally express inducible NO synthase (iNOS) in response to various pro-inflammatory cytokines and bacterial lipopolysaccharides (LPS), resulting in the production of NO via the oxidative deamination of L-arginine at the sites of inflammation (MacMicking et al., 1997; Nathan and Xie, 1994). NO regulates nearly all stages of inflammation development, particularly the early stages in which inflammatory cells transmigrate to the sites of inflammation. TNF-α and IL-6 are

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potent pro-inflammatory cytokines that stimulate the secretion of additional inflammatory cytokines (Schett, 2008). Therefore, inhibiting the excessive production of iNOS, TNF-α, and IL-6 in macrophages, by inhibiting the mRNA and protein expressions of these mediators, may be a viable strategy to develop novel anti-inflammatory agents.

Crataeva nurvala Buch. Ham. *(C. nurvala)* has been traditionally considered a valuable plant for treating fever, metabolic disorders, bile and phlegm secretions, and weak immune systems in India (Kirtikar et al., 2000). Many research groups reported its pharmacological effects for the treatment of diseases, including urinary tract infections (UTIs), diabetic mellitus, and benign prostatic hyperplasia (BPH) (Deshpande et al., 1982; Modi and Kohlapure, 2004; Shukla and Nayak, 2002; Sikarwar and Patil, 2010; Vijay and Agarwal, 2010). It was reported that symptoms of UTIs were diminished by treatment with *C. nurvala* decoction (Deshpande et al., 1982). Sikarwar and Patil observed that oral administration of the *C. nurvala* stem bark extract reduced blood glucose levels in alloxan-induced diabetic albino rat models (Sikarwar and Patil, 2010). Furthermore, PR-2000 and Himplasia, polyherbal formulations containing *C. nurvala* reduced a nonmalignant enlargement of the prostate gland (Modi and Kohlapure, 2004; Shukla and Nayak, 2002; Vijay and Agarwal, 2010).

Pharmaceutical-grade herbal extracts have different formulations based on their medicinal purposes. Approximately 80% of the drugs isolated from plants show a positive correlation between the therapeutic use and traditional use of the plant extracts from which they are derived (Fabricant and Farnsworth, 2001). Tinctures are alcoholic herbal extracts that contain more bioactive materials than herbal teas. *C. nurvala,* an important Ayurvedic herb, has been used as a tincture for the treatment of renal conditions in India.

C. nurvala was revealed to have lupeol, lupeol acetate, α-spinasterol acetate, Ψ-taraxasterol, 3-epi-lupeol, and β-sitosterol, as its major components and lupenone and β-sitosterol acetate as its minor components (Lakshmi and Chauhan, 1975). Some of these components are known to have pharmacological effects, including anti-inflammatory, anti-nociceptive, anti-tubercular, and anti-hyperlipidemic activities (Akihisa et al., 2005; Alappat et al., 2010; Chen et al., 2012; Dempsey et al., 1956). Lupeol, the major component in *C. nurvala* stem bark, was shown to have similar anti-arthritic activity to indomethacin, a commonly used nonsteroidal anti-inflammatory drug, in rheumatoid arthritis rat models (Geetha and Varalakshmi, 1999, 2001; Latha et al., 2001). It was reported that the inhibitory effects of lupeol in writhing, hyperalgesia, and post-operative pain models resulted from the inhibition of IL-1β and TNF-α production (de Lima et al., 2013; Saleem, 2009). In addition, *in vitro* and *in vivo* anti-inflammatory effects of β-sitosterol were reported (Lee et al., 2012; Mahajan and Mehta, 2011; Valerio and Awad, 2011).

The inhibitory effects of ethanol extracts from *C. nurvala* (ECN) on the production of inflammatory cytokines, including its underlying mechanism have not been elucidated, despite its use in treating many inflammatory diseases. In the present study, the anti-inflammatory effects of ECN on LPS-stimulated RAW 264.7 macrophages and its action mechanisms, such as the phosphorylation of inhibitor of κB (IκB) and mitogen-activated protein kinase (MAPK), were investigated to evaluate the therapeutic potential of ECN for the treatment of excessive inflammation.

2. Materials and methods

2.1. Cell culture and reagents

A 95% ethanol extract (Code No.: FBM124–022) of the *C. nurvala* (Capparaceae) was purchased from the International Biological Material Research Center (http://www.ibmrc.re.kr, Daejeon, Korea). The RAW 264.7 macrophages, a mouse monocytic cell line, were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 50 unit/ml penicillin, and 50 μg/ml streptomycin (GIBCO BRL, Grand Island, NY, USA) at 37 °C in humidified air containing 5% CO_2. The cytokines that were produced, including TNF-α and IL-6, were measured by using ELISA Ready-SET-Go! ® (Ebioscience, San Diego, CA, USA). Rabbit anti-IκBα and mouse anti-tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-iNOS, anti-cyclooxygenase (COX)-2, anti-phospho IκBα, anti-phospho p38, anti-p38, anti-phospho extracellular signal-regulated kinase (ERK), anti-ERK, anti-phospho c-Jun N-terminal kinase (JNK), and anti-JNK were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA).

2.2. Cell viability assay

The RAW 264.7 macrophages were seeded in 96 well plates (4×10^4 /well). After adhesion overnight, cells were incubated with ECN and LPS for 24 h. After incubation, cell viability was measured using EZ-Cytox cell viability assay kit (Daeil Lab, Seoul, Korea). Briefly, EZ-Cytox solution that contained a water soluble tetrazolium salt was added for 2 h at 37 °C and 100 μl of supernatants were transferred to 96 well plates. The absorbance was measured at 450 nm with a Synergy Microplate Reader (BioTek Instruments, Winooski, VT, USA).

2.3. Nitrite assay

The RAW 264.7 macrophages were incubated with ECN and LPS for 24 h. After incubation the levels of NO synthesis were determined by assaying the culture supernatants for nitrite by using the Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylenediamine dihydrochloride, and 2.5% phosphoric acid). Nitrate is the stable product of the reaction between NO and molecular oxygen. The absorbance was measured at 540 nm, by using a Synergy Microplate Reader, after incubation for 10 min.

2.4. Enzyme-linked immunosorbent assay (ELISA)

The RAW 264.7 macrophages were stimulated with LPS and ECN for 24 h. After stimulation, the supernatants were obtained, and a sandwich ELISA by using monoclonal antibodies specific to each mediator determined the quantities of TNF-α and IL-6 in culture supernatants. Before the application of samples, the plate was pre-coated with coating antibody in the supplied buffer. After incubation overnight at 4 °C, the plate was washed and treated with 1 × Assay Diluents for 1 h. Samples were loaded into each well and incubated for 2 h at room temperature (RT). After washing, the plate was treated with biotinylated secondary antibody solution and horseradish peroxidase (HRP)-streptavidin solution for 1.5 h, respectively, and the substrate solution was added to a washed-plate. After 10 min incubation under dark conditions, 1 N phosphoric acid (H_3PO_4) was added and optical density of the individual wells was determined to be 450 nm by a Synergy Microplate Reader.

2.5. Quantitative real time polymerase chain reaction (qPCR)

Total RNA was prepared from the cells and reverse-transcribed into complementary DNA (cDNA) by using a TOPscript™ cDNA synthesis kit (Enzynomics, Daejeon, Korea). Then PCR amplification of the cDNA was performed. Quantification of mRNA was also performed by using a real-time reverse transcription polymerase chain reaction (RT-PCR), iTaq™ Universal SYBR Green Supermix (Bio-rad, Hercules, CA, USA), according to the manufacturer's instructions. The

PCR was run for 40 cycles of denaturation at 94 °C (5 s) and annealing/extension at 60 °C (30 s) by using a CFX Connect™ real-time thermal cycler (Bio-Rad). The results were normalized with multi reference genes, β-actin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and were expressed as the ratio of gene expressions to LPS treated group (100%). PCR primers were designed by using Beacon designer 7.0 (Premier Biosoft, Palo Alto, CA, USA). The sequences of PCR primers used in this study were: mouse iNOS (sense, 5′-TGG CCA CCA AGC TGA ACT-3′; antisense, 5′-TCA TGA TAA CGT TTC TGG CTC TT-3′), COX-2 (sense, 5′-GAT GCT CTT CCG AGC TGT G-3′; antisense, 5′-GGA TTG GAA CAG CAA GGA TTT-3′), TNF-α (sense, 5′-CTG TAG CCC ACG TCG TAG C-3′; antisense, 5′-TTG AGA TCC ATG CCG TTG-3′), IL-6 (sense, 5′-TCT AAT TCA TAT CTT CAA CCA AGA GG-3′; antisense, 5′-TGG TCC TTA GCC ACT CCT TC-3′), IL-1β (sense, 5′-TTG ACG GAC CCC AAA AGA T-3′; antisense, 5′-GAT GTG CTG CTG CGA GAT T-3′), β-actin (sense, 5′-CGT CAT ACT CCT GCT TGC TG-3′; antisense, 5′-CCA GAT CAT TGC TCC TCC TGA-3′), and GAPDH (sense, 5′-GCT CTC TGC TCC TCC TGT TC-3′; antisense, 5′-ACG ACC AAA TCC GTT GAC TC-3′).

2.6. Semi-quantitative RT-PCR

PCR primers were designed using the Primer3 program. The sequences of PCR primers used in this study were: mouse iNOS (sense, 5′-GCA TGG AAC AGT ATA AGG CAA ACA-3′; antisense, 5′-GTT TCT GGT CGA TGT CAT GAG CAA-3′), COX-2 (sense, 5′-GCA TGG AAC AGT ATA AGG CAA ACA-3′; antisense, 5′-GTT TCT GGT CGA TGT CAT GAG CAA-3′), TNF-α (sense, 5′-GTG CCA GCC GAT GGG TTG TAC C-3′; antisense, 5-′AGG CCC ACA GTC CAG GTC ACT G-3′), IL-6 (sense, 5′-TCT TGG GAC TGA TGC TGG TGA C-3′; antisense, 5′-CAT AAC GCA CTA GGT TTG CCG A-3′), IL-1β (sense, 5′-AGC TGT GGC AGC TAC CTG TG-3′; antisense, 5′-GCT CTG CTT GTG AGG TGC TG-3′), and GAPDH (sense, 5′-GTC TTC ACC ACC ATG GAG AAG G-3′; antisense, 5′-CCT GCT TCA CCA CCT TCT TGC C-3′). The PCR was run for 20–25 cycles of 94 °C (30 s), 60 °C (30 s), and 72 °C (30 s) by using a Bioer thermal cycler (Bioer Technology Co., Hangzhou, China). After amplification, 10 μl of the RT-PCR products were separated in 1.5% (w/v) agarose gels and stained with ethidium bromide.

2.7. Preparation of total cell lysates

LPS-stimulated RAW 264.7 cells were treated with ECN and LPS for 15 min and washed with ice-cold phosphate buffered saline (PBS). The cells were allowed to lyse in lysis buffer containing 0.5% NP-40, 0.5% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA), 1% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 μg/ml aprotinin, collected in microtubes, and centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatants were prepared in new microtubes.

2.8. Western blotting

Protein concentration was measured by using the Bradford method. Aliquots of the cell lysates were separated in a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel in a Mini-Protein II gel apparatus (Bio-Rad) and transferred onto nitrocellulose membranes (GE Healthcare, Milwaukee, WI, USA) with transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), and 20% MeOH (v/v)]. After blocking non-specific sites with 5% bovine serum albumin (BSA) solution, the membrane was incubated overnight at 4 °C with the primary antibodies (1:1000). Each membrane was incubated for 1 h more with secondary peroxidase-conjugated goat immunoglobulin G (IgG, 1:5000). The target proteins were detected by using an enhanced chemiluminescence (ECL) solution. Protein levels were quantified by scanning the immunoblots and analyzing them with LabWorks software (UVP Inc., Upland, CA, USA).

2.9. Statistical analysis and experimental replicates

The data are represented as mean ± standard error of the mean (SEM). Differences between experimental conditions were assessed by the Mann–Whitney *U* test. Mann–Whitney *U* test was performed by using Prism 3.0 (GraphPad Software, San Diego, CA, USA) and $p < 0.01$ was considered statistically significant. The data from nine replicates were analyzed, including three independent experiments with three replicates in each.

3. Results

3.1. Effects of ECN on the viability of activated macrophages

To determine the maximal effective concentration of ECN, with minimal cytotoxicity, RAW 264.7 macrophages were treated with the indicated concentrations of ECN for 24 h in the presence of LPS. Cell viability was determined by the ability of the cells to metabolically reduce a tetrazolium salt to a formazan dye. As shown in Fig. 1, ECN treatment did not influence cell viability at a dose of ≤200 μg/ml in the presence of 0.5 μg/ml LPS. However, approximately half of the cells (52.14 ± 12.06%) were viable when the cells were treated with higher concentration of ECN (400 μg/ml). This data indicated that low doses of ECN (200 μg/ml) do not affect RAW 264.7 macrophage viability. Therefore, concentrations of ≤200 μg/ml were used in subsequent experiments.

3.2. Effects of ECN on LPS-induced iNOS and NO production

NO release was examined after treating the RAW 264.7 macrophages with ECN in the absence or presence of LPS to determine the anti-inflammatory effects of ECN. Culture supernatants were collected after 24 h incubation. The amount of nitrite accumulated in the culture media was estimated, by using the Griess reagent, as an indicator of NO release. As shown in Fig. 2A, the nitrite concentration in the media increased in LPS-activated RAW 264.7 macrophages as compared to unstimulated cells. When RAW 264.7 macrophages were treated with various concentrations of ECN, LPS-stimulated nitrite production significantly decreased in a dose-dependent manner (Fig. 2A). Since nitrite is a product of iNOS activation, the effect of ECN on *iNOS* mRNA and protein in RAW 264.7 macrophages was measured by using RT-PCR and Western blotting analyses, respectively. As shown in Fig. 2B, treatment of RAW 264.7 macrophages with various concentrations of ECN decreased

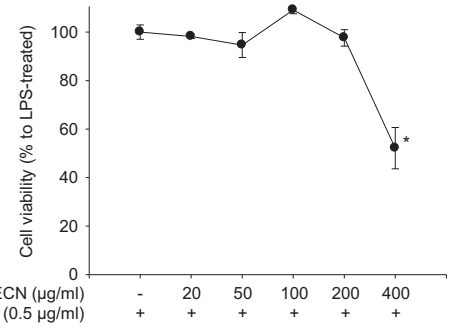

Fig. 1. Effects of ECN on cell viability. RAW 264.7 macrophages were simultaneously treated with LPS (0.5 μg/ml) and ECN (20, 50, 100, 200, and 400 μg/ml). After incubation for 24 h, cell viability was measured by using the EZ-Cytox reagent. Cell viability was compared to the LPS-treated group. Data represent the mean ± SEM. *$p < 0.01$ relative to the LPS-treated control group.

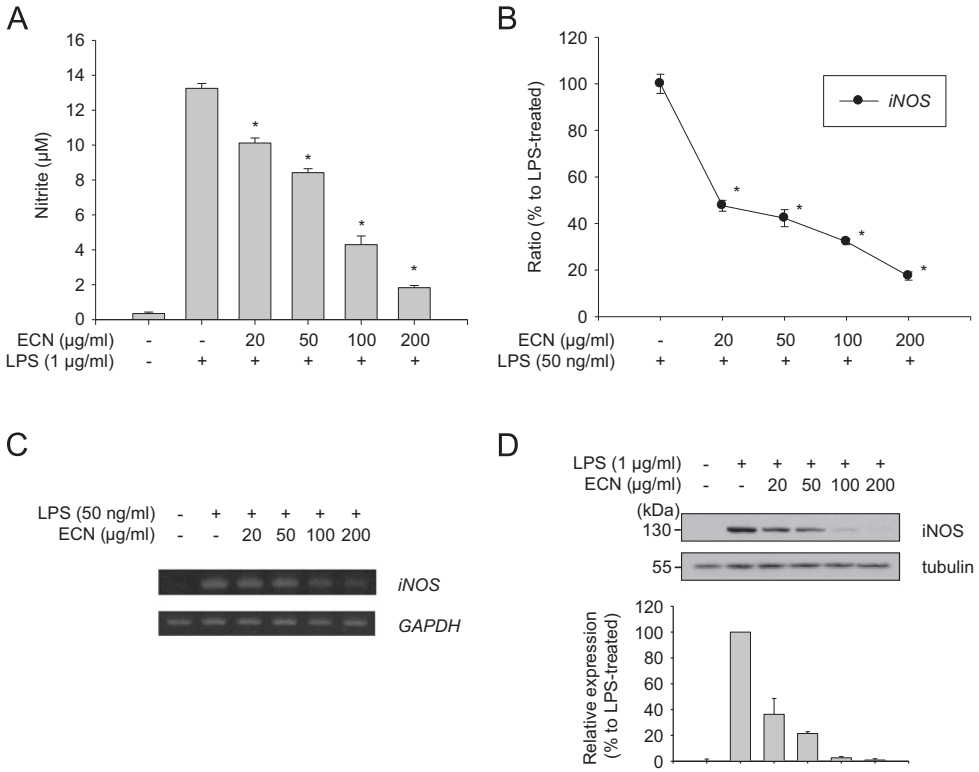

Fig. 2. Inhibitory effects of ECN on the production of NO. RAW 264.7 macrophages were stimulated with simultaneous treatments of LPS and ECN (20, 50, 100, and 200 μg/ml) for the indicated times. (A) After 24 h stimulation, NO secretion in the supernatants was measured by using the Griess reagent. NO secretion was calculated by using a standard curve according to a nitrite standard solution. Data represent the mean ± SEM. $^{*}p < 0.01$ relative to the LPS-treated control group. (B and C) After 6 h stimulation, total RNA was extracted and reverse transcribed to cDNA. (B) *iNOS* was amplified by qPCR and the expression of each group was compared to the LPS-treated group. Data represent the mean ± SEM. $^{*}p < 0.01$ relative to the LPS-treated control group. (C) *iNOS* was amplified by PCR and detected by using a gel documentary system. GAPDH served as an internal control. (D) Total cell lysates were prepared after 24 h stimulation and subjected to Western blotting analysis. The protein expression of iNOS was detected by using an enhanced chemiluminescence reagent. Those levels were quantified by analysis with LabWorks software and normalized to corresponding tubulin levels. Relative expression levels of iNOS are represented as a bar graph (lower panel).

the LPS-stimulated increase in *iNOS* mRNA expression in a dose-dependent manner. Semi-quantitative PCR data verified the inhibitory effect of ECN on the *iNOS* mRNA expression level (Fig. 2C). Furthermore, ECN treatment decreased the LPS-induced increase in iNOS protein expression (Fig. 2D). These results indicated that ECN reduces NO production by inhibiting the expression of iNOS in LPS-activated macrophages.

3.3. Effects of ECN on LPS-induced COX-2 expression

Since the production of PGE_2, another mediator that induces fever in inflammatory states, is mainly regulated by COX-2, we measured the effects of ECN on COX-2 mRNA and protein expression in macrophages. Quantitative and semi-quantitative PCR data revealed that *COX-2* mRNA was dramatically increased following treatment with LPS. However, LPS-induced *COX-2* mRNA and protein expression was not regulated by ECN (Fig. 3A–C). These data suggested that ECN does not regulate COX-2 expression, and it might not change the secretion of PGE_2, despite its effects on iNOS and NO.

3.4. Differential inhibitory effects of ECN on pro-inflammatory cytokine production in activated macrophages

Because LPS induces the production of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β, we evaluated the anti-inflammatory effects of ECN on cytokine production in LPS-stimulated macrophages. As shown in Fig. 4A and B, LPS-stimulated RAW 264.7 macrophages produced large amounts of TNF-α and IL-6. ECN treatment reduced LPS-stimulated production of IL-6 in a dose-dependent manner but did not affect TNF-α production. Quantitative RT-PCR analyses revealed that the LPS-induced increase in *IL-6* and *IL-1β* mRNA expression was inhibited by ECN treatment, whereas the mRNA level of *TNF-α* was not affected (Fig. 4C). Similar results were obtained by using semi-quantitative PCR analysis (Fig. 4D). These results indicated that ECN selectively inhibits the production of pro-inflammatory cytokines, especially IL-6 and IL-1β, at the transcriptional level.

3.5. ECN selectively inhibits MAPK phosphorylation in activated macrophages

To elucidate the mechanisms underlying the anti-inflammatory effect of ECN, we investigated the activation of major signaling pathways that produce the inflammatory mediators, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and MAPKs, in macrophages. We measured the phosphorylation and expression of IκBα, because NF-κB activation is a consequence of IκBα degradation in cytosol. LPS treatment increased the phosphorylation of IκBα and decreased IκBα protein levels; however, ECN did not affect the phosphorylation and expression of IκBα (Fig. 5A). Next, we assessed the phosphorylation of MAPKs in response to ECN treatment. LPS stimulation of macrophages induced the phosphorylation of MAPKs, including JNK, ERK, and p38, without affecting total protein, and ECN inhibited the increase in ERK phosphorylation in a dose-dependent manner. In contrast, LPS-stimulated phosphorylation of JNK and p38 was not affected by ECN treatment (Fig. 5B). These results indicated that the inflammatory response activated through the MAPK signaling pathway, especially ERK, is inhibited by ECN in macrophages.

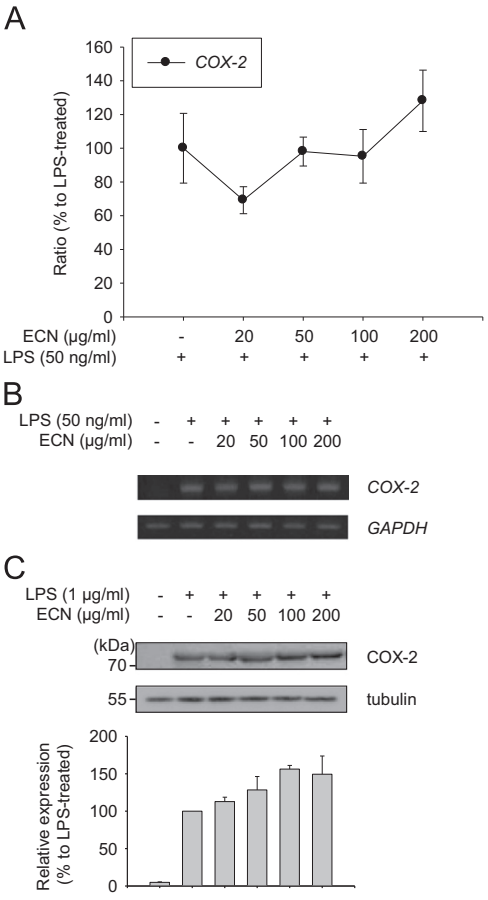

Fig. 3. Effects of ECN on the expression of COX-2. RAW 264.7 macrophages were stimulated with simultaneous treatments of LPS and ECN (20, 50, 100, and 200 μg/ml) for the indicated times. (A and B) After 6 h stimulation, total RNA was extracted and reverse transcribed to cDNA. (A) *COX-2* was amplified by qPCR, and the expression of each group was compared to the LPS-treated group. Data represent the mean ± SEM. (B) *COX-2* was amplified by PCR and detected using a gel documentary system. GAPDH expression served as an internal control. (C) Total cell lysates were prepared after 24 h stimulation and subjected to Western blotting analysis. The protein expression of COX-2 was detected by using an enhanced chemiluminescence reagent, and those levels were normalized to levels of tubulin, a loading control. Relative expression levels of COX-2 are represented as a bar graph (lower panel).

4. Discussion

In this study, we used mouse macrophage cells to evaluate the anti-inflammatory effects of ECN. Mouse RAW 264.7 macrophage cells have been frequently used to evaluate inflammatory effects of a compound or extract because of the following reasons: 1) the immunological functions of humans are conserved in mice. This is supported by reports about sequencing of both humans and mice (Church et al., 2009; Rossant and McKerlie, 2001). 2) Mouse macrophages produce the same inflammatory mediators as human macrophages (Bogdan, 2001; Weinberg, 1998), although there are minor differences in signal transductions for the production of inflammatory mediators. 3) Compounds, which are known to be effective in humans, were primarily studied in mouse cells to determine their inflammatory effect and underlying mechanism (Kim et al., 2005; Pugazhenthi et al., 2007). The results obtained from studies with mouse cells have been applied to human disease models and used for clinical applications (Chuengsamarn et al., 2012; Tome-Carneiro et al., 2013).

Since the regulation of iNOS/NO is central to the inflammatory process, many studies have attempted to identify novel anti-inflammatory agents that inhibit iNOS expression and elucidate their mechanism of action. In the present study, we demonstrated that ECN inhibits NO production in RAW 264.7 macrophages by inhibiting the expression of iNOS in a non-cytotoxic manner. These results suggested that ECN contains anti-inflammatory phytochemicals.

Similar to NO, an excessive production of PGE_2 is correlated with many inflammatory disorders (Rampton et al., 1980; Robinson et al., 1975). Therefore, molecules that reduce PGE_2 production are being actively investigated to develop anti-inflammatory drugs. In the present study, we evaluated the inhibitory effect of ECN on the production of COX-2, which is the enzyme responsible for the secretion of PGE_2, in LPS-stimulated macrophages; however, we observed no significant decrease in COX-2 expression at the mRNA and protein levels. Furthermore, ECN only regulated ERK activation, not NF-κB or other MAPKs in macrophages. It was reported that the activation of NF-κB and MAPKs is essential for COX-2 induction (Jones et al., 2001a; Jones et al., 2001b); however, only ERK was regulated by ECN in the present study. The differential regulation of iNOS and COX-2 by the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is supported by a recent report showing that a JAK3 inhibitor reduced iNOS expression and NO production but not COX-2 in LPS-activated J774 macrophages (Sareila et al., 2008). Taken together, this data suggested that ECN has no effect on COX-2 production because it does not regulate NF-κB, JNK, or p38. Further studies are needed to clarify whether ECN regulates the JAK/STAT pathway.

Pro-inflammatory cytokines are key mediators of apoptosis and innate immune reactions, and excessive levels of pro-inflammatory cytokines can induce tissue injury and potentiate septic shock (Guadagni et al., 2007; Nishimoto and Kishimoto, 2006). Therefore, agents that inhibit the production and action of pro-inflammatory cytokines may inhibit the progression of inflammatory diseases. The pro-inflammatory cytokines, TNF-α and IL-6, are major pathogenic factors for many inflammatory diseases, including rheumatoid arthritis; an anti-IL-6 receptor antibody is currently used as a therapeutic agent for the clinical treatment of this disease (Kavanaugh, 2007; Nishimoto and Kishimoto, 2006; Straub et al., 2006). In this study, we found that ECN significantly inhibited IL-6 production but not TNF-α, in LPS-stimulated RAW 264.7 macrophages. Several cases indicate that natural compounds cannot simultaneously inhibit both of these cytokines (Samavati et al., 2009; Zhu et al., 2013). One possibility for the differential regulation of IL-6 and TNF-α by ECN is that IL-6 and TNF-α possess different promoter binding regions for transcription factors. Because previous studies revealed that the STAT binding region is present in the IL-6 promoter but not the TNF-α promoter (Lee et al., 2006), ECN might regulate STAT signaling.

Many intracellular signaling pathways are associated with increased expression of iNOS and pro-inflammatory cytokines (Schroder et al., 2006; Stalinska et al., 2005; Szabo and Thiemermann, 1995). In particular, MAPKs, such as p38, ERK, and JNK, play pivotal roles in the production of various inflammatory mediators (Hanada and Yoshimura, 2002; Stalinska et al., 2005). LPS stimulation of murine macrophages significantly enhanced the production of inflammatory mediators via MAPK phosphorylation and stimulation of downstream signaling pathways (Cario et al., 2000; Fujihara et al., 2003; Schroder et al., 2006). These reports imply that inhibition of p38, ERK, and JNK phosphorylation represents potential target pathways to oppose severe inflammatory states. However, several reports suggested that MAPK signaling cascades might be differently involved in the response of anti-inflammatory compounds in macrophages (Burk et al., 2009; Liu et al., 2010; Shan et al., 2009). Among them, ERK activity is elevated by external stimuli in various inflammatory cells, including macrophages. For this reason, several components from natural plants that inhibit ERK activity, such as curcumin, quercetin, and resveratrol, exhibit various pharmacological effects in inflammatory diseases (Lin et al., 2008; Min et al., 2010; Wang and Dong, 2012). In this study, ECN inhibited LPS-induced phosphorylation of ERK, but not p38 and JNK, in a dose-dependent manner, and total MAPK levels were unchanged. Collectively, these results suggested that ECN inhibits inflammatory

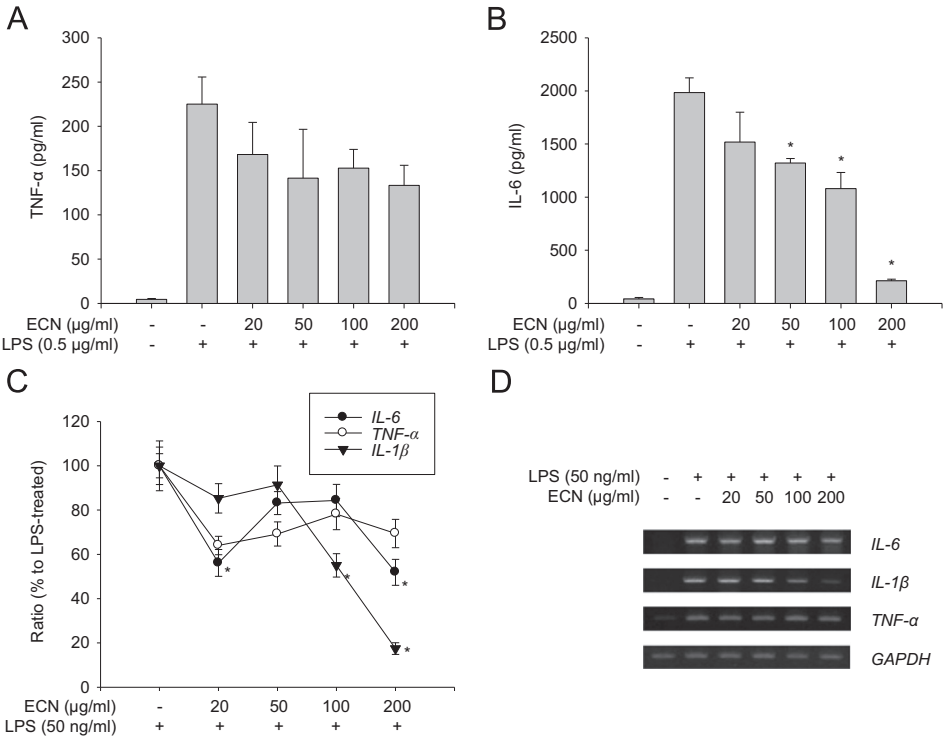

Fig. 4. Inhibitory effect of ECN on the production of pro-inflammatory cytokines. RAW 264.7 macrophages were stimulated with simultaneous treatment of LPS and ECN (20, 50, 100, and 200 μg/ml) for the indicated times. (A and B) After 24 h stimulation, ELISA were used to measure TNF-α (A) and IL-6 (B) levels. The secretion of each cytokine was determined by using a standard curve. Data represent the mean ± SEM. $^{*}p < 0.01$ relative to the LPS-treated control group. (C and D) After 6 h stimulation, total RNA was extracted and reverse transcribed to cDNA. (C) *IL-1β*, *TNF-α* and, *IL-6* were amplified by qPCR, and the expression of each group was compared to the LPS-treated group. Data represent the mean ± SEM. $^{*}p < 0.01$ relative to the LPS-treated control group. (D) *IL-1β*, *TNF-α* and, *IL-6* were amplified by PCR and detected by using a gel documentary system. GAPDH was used as a loading control.

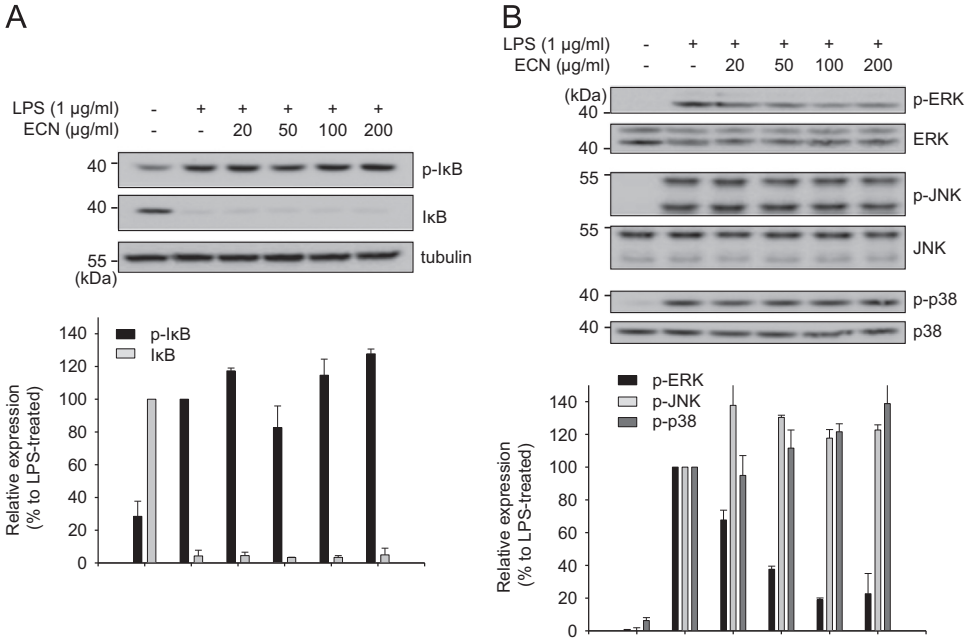

Fig. 5. Inhibitory effects of ECN on NF-κB and MAPK activation. RAW 264.7 macrophages were pretreated with various concentrations of ECN (20, 50, 100, and 200 μg/ml) for 1 h and stimulated with LPS for 15 min. Total cell lysates were prepared and subjected to Western blotting analysis. The expression of p-IκBα, IκBα (A), p-ERK, ERK, p-p38, p38, p-JNK, and JNK (B) were detected by using specific antibodies. Relative expression levels of IκBα and p-IκBα were normalized to tubulin levels. Levels of phosphorylated MAPKs were normalized to corresponding total MAPK levels. Quantitative analyses of phosphorylated or total protein levels are shown after normalization (lower panel).

mediators by inhibiting the activity of MAPKs, especially ERK, rather than their expression.

NF-κB is the other major regulatory signaling molecule for inflammation. After LPS-induced phosphorylation and degradation of IκBα in the cytosol, NF-κB subunits are free to translocate into the nucleus (Karin and Ben-Neriah, 2000; Karin and Delhase, 2000). After nuclear translocation, the NF-κB subunits, p65 and p50, regulate the production of various inflammatory mediators, including TNF-α, IL-6, and NO (Surh et al., 2001; Verma et al., 1995). In this study, LPS stimulation of macrophages activated NF-κB and

MAPKs. ECN did not alter NF-κB activity or the degradation of IκBα in LPS-stimulated RAW 264.7 macrophages. Both NF-κB and MAPK signaling transductions share TLR4 adapter molecules and accessory molecules, including myeloid differentiation primary response gene 88 (MyD88), TIR-domain-containing adapter-inducing interferon-β (TRIF), TNF receptor associated factor 6 (TRAF6), and interleukin-1 receptor-associated kinase 1 (IRAK1). These signaling and accessory molecules are quite different in both signaling pathways. Therefore, ECN might selectively regulate ERK, but not regulate TLR4 accessory molecules.

This study examined the regulation of inflammatory mediators by ECN in activated macrophages. Because macrophages play a key role in the pathogenesis of many inflammatory diseases, ECN-mediated selective regulation of inflammatory mediators suggests that this compound may have therapeutic potential for inflammatory diseases. In therapeutic aspects, it might be necessary to investigate how efficiently ECN regulates acute and chronic inflammation mechanisms in animal models. In addition, further studies are needed to analyze the major components responsible for the ECN-mediated reduction of inflammatory mediators and to elucidate the exact mechanism involved.

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