BIO DESIGN

pISSN 2288-6982 | eISSN 2288-7105

RESEARCH ARTICLE P 108-115

Callicarpa rubella for. angustata C. P`ei shows anti-inflammatory effects by blocking p38 activation in murine macrophages

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Many attempts have been made to develop anti-inflammatory drugs by using natural products because natural products have been traditionally used to cure severe inflammatory diseases. In this study, we investigated the anti-inflammatory effects and the molecular mechanisms underlying these effects in murine macrophages by using an ethanol extract of *Callicarpa rubella* for. angustata C. P`ei (ECR). We found that ECR treatment significantly inhibited lipopolysaccharide (LPS)-stimulated nitric oxide (NO) production in macrophages and downregulated mRNA and protein expression levels of inducible nitric oxide synthase (iNOS). However, ECR did not modulate cyclooxygenase-2 (COX-2) expression at both mRNA and protein levels. Among the inflammatory cytokines, interleukin (IL)-1β production was reduced by ECR treatment whereas the level of IL-6 and tumor necrosis factor (TNF)-α was independent of ECR treatment. Western blot analysis revealed that ECR notably reduced the phosphorylation of p38 but had no effect on the activation of nuclear factor kappa B (NF-κB) and other mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK). These results imply that ECR exerts anti-inflammatory effects via selective inhibition of the production of inflammatory mediators including iNOS and IL-1β by inactivating p38.

INTRODUCTION

Lipopolysaccharide (LPS), the major constituent of the cell wall of gram-negative bacteria, induces inflammatory responses when administered to cells or animals. It induces the expression of various proinflammatory mediators such as nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS), prostaglandin E_2 (PGE_2) by cyclooxygenase-2 (COX-2), and various proinflammatory cytokines including interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α (Jones et al., 2001, Rhee and Hwang, 2000, Schroder et al., 2006, Stalinska et al., 2005, Szabo and Thiemermann, 1995). Although enhanced production of inflammatory mediators is important for host defense against external stimuli including LPS, excess amounts of these mediators cause severe inflammatory diseases, including septic shock, rheumatoid arthritis, systemic lupus erythematosus (SLE), and inflammatory bowel disease (IBD) (Clancy et al., 1998, Guadagni et al., 2007, Kroncke et al., 1998, Nathan and Xie, 1994, Nathan and Xie, 1994, Nishimoto and Kishimoto, 2006, Szabo and Thiemermann, 1995).

In various types of cells, including macrophages, LPS stimulates toll-like receptor 4 (TLR4) on the cell membrane and recruits myeloid differentiation factor 88 (MyD88) adaptor protein in cytoplasm. Recruited MyD88 adaptor protein in turn forms complexes with interleukin-1 receptor-associated kinase 2 (IRAK2), IRAK1 and IRAK4 (Kim and Lee, 2013). Subsequently, binding of TNF receptor associated factor 6 (TRAF6) and transforming growth factor-β activated kinase-1 (TAK1) leads to activation of nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs), which are important transcription factors for the production of various inflammatory mediators (Figure 1). Activation of NF-κB induced by LPS involves the phosphorylation of inhibitor κB alpha (IκBα) kinase (IKK) which phosphorylates IκBα leading to subsequent ubiquitination, degradation of IκBα, and translocation of NF-κB into the nucleus (Karin and Ben-Neriah, 2000, Karin and Delhase, 2000, Verma et al., 1995). The classical MAPKs are extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, which appear to regulate the production of proinflammatory cytokines and inflammatory mediators (Cario et al., 2000, Fujihara et al., 2003, Hanada and Yoshimura, 2002, Stalinska et al., 2005). In addition, it has also been reported that the Janus kinase-Signal Transducer and Activator of Transcription (JAK-STAT) pathway is involved in LPS-induced inflammatory responses (Lee et al., 2006, Samavati et al., 2009).

Ethnopharmacological uses of about 20 species of *Callicarpa* have been reported, and several members are well known in the traditional medical systems of China and South Asia (Chen et al., 2009, Huang et al., 2014, Tu et al., 2013). Ethnopharmacological reports indicate the use of *Callicarpa* species in the treatment of hepatitis, rheumatism, fever, headache, indigestion, and other ailments (Jones and Kinghorn, 2008). Several species of *Callicarpa* have been reported to be used against cancer (e.g., *Callicarpa*

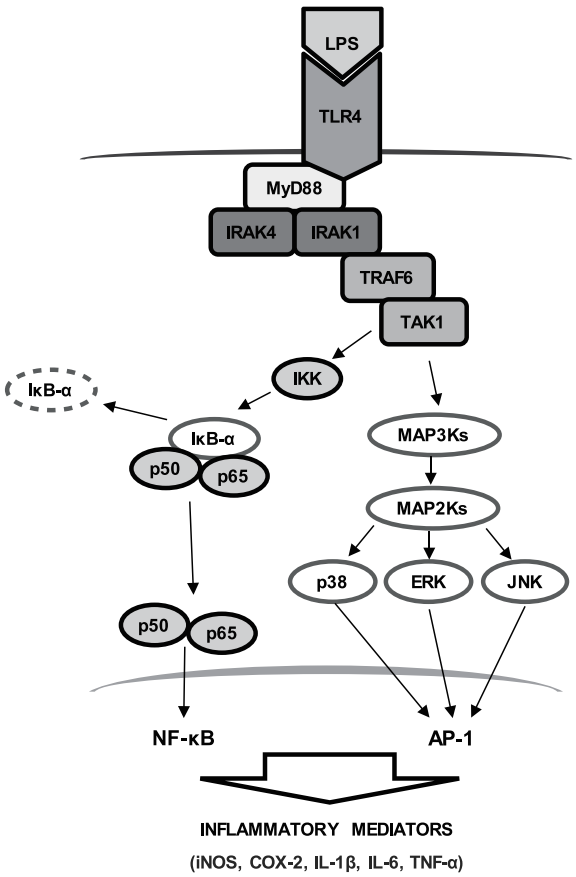

FIGURE 1 | Schematic representation of inflammatory responses induced by LPS stimulation in macrophages. TLR4 occupied with LPS assembles accessory molecules including MyD88, IRAK1, IRAK4, TRAF6, and TAK1. Subsequently, the production of inflammatory mediators including iNOS, COX-2, IL-1β, IL-6, and TNF-α is notably induced by the activation of signaling molecules, mainly NF-κB and MAPKs.

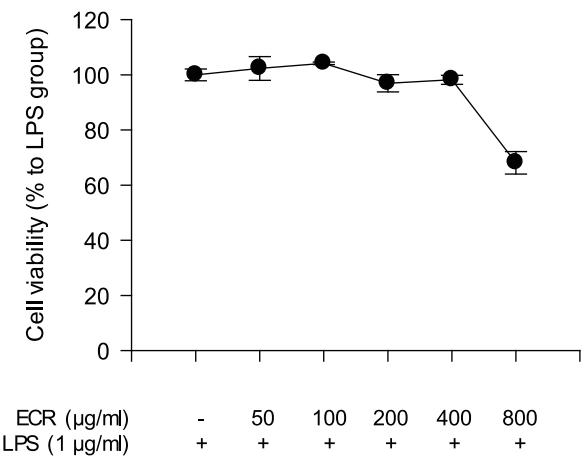

FIGURE 2 | Effect of ECR on cell viability. RAW 264.7 macrophages were treated with LPS in the presence of ECR (50, 100, 200, 400, and 800 μg/ml). After incubation for 24 h, cell viability was measured using the Ez-Cytox reagent. Cell viability was compared to that of the LPS-treated group. Data represent the mean ± S.D. (n = 3).

americana root to treat skin cancer and *Callicarpa rubella* bark to treat tumors of the large intestine) (Jones and Kinghorn, 2008, Jones et al., 2007). The essential oils of *Callicarpa americana* have recently been reported to have antialgal and phytotoxic activities, and several isolates from this species (and *Callicarpa japonica*) were identified as contributing to the mosquito bite-deterrent activity that was first indicated by folkloric usage (Cantrell et al., 2005, Tellez et al., 2000). Recent bioassay-guided investigations of *Callicarpa americana* extracts have resulted in the isolation of several active compounds, mainly of the clerodane diterpene structural type (Jones et al., 2007). However, the anti-inflammatory properties of *Callicarpa rubella* for. angustata C. P`ei were not elucidated.

In the present study, the anti-inflammatory effect of an ethanol extract of *Callicarpa rubella* for. angustata C. P`ei (ECR) in LPS-stimulated RAW 264.7 macrophages and its underlying mechanisms were investigated to evaluate the therapeutic potential of ECR for the treatment of abnormal inflammation.

RESULTS

ECR inhibited NO production but not COX-2 expression

LPS induces the production of inflammatory mediators in murine macrophages by activating of NF-κB and MAPKs (Figure 1). Many anti-inflammatory agents negatively regulate all of inflammatory mediators following inactivation of NF-κB and MAPKs. However, these agents exert adverse responses as side effects. Therefore, selective regulation of inflammatory mediators and identification of specific target signaling molecule are valuable strategy for the development of novel anti-inflammatory reagents. From these concepts, we investigated many natural plant extracts, which are traditionally known to have pharmacological effects, and evaluated the inhibitory effects on the inflammation by measuring the production of NO, a major inflammatory mediator, in LPS-stimulated RAW 264.7 macrophages. From many extracts which exert inhibitory effect on the NO production, we selected ECR since *Callicarpa* species have been traditionally used for many inflammatory diseases.

To determine the maximal effective concentration of ECR without inducing cytotoxicity, RAW 264.7 macrophages were treated with various concentrations of ECR (100, 200, 400, and 800 μg/ml) and subsequently stimulated with LPS. After incubation, the cells were further incubated for 1 h with 50 μg/ml Ez-Cytox solution and the supernatants were used to estimate cell viability. As shown in Figure 2, ECR at concentrations of ≤400 μg/ml did not reduce cell viability. However, at concentrations of >800 μg/ml, morphological changes (data not shown) and formation of soluble formazan indicative of cell death were observed. On the basis of these data, we determined that the maximal effective ECR concentration at which cell death is not induced to be 400 μg/ml in murine macrophages.

NO levels in media were measured, since inflammation is mediated by inflammatory mediators such as NO and PGE_2.

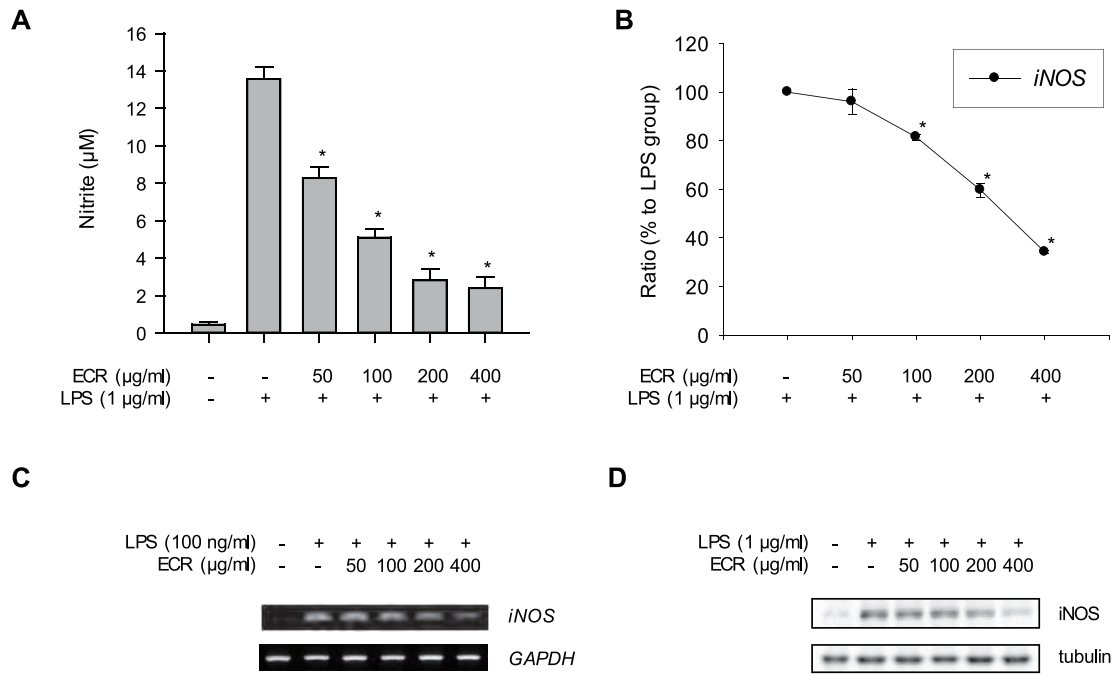

FIGURE 3 | Inhibitory effect of ECR on the production of NO. RAW 264.7 macrophages were stimulated with LPS in the presence of ECR (50, 100, 200, and 400 μg/ml) for the indicated times. (A) After stimulation for 24 h, NO secretion in the supernatants was measured using the Griess reagent. NO secretion was calculated using a standard curve prepared using a standard nitrite solution. Data represent the mean ± S.D. (n = 3). *p < 0.01 relative to the LPS-treated control group. (B and C) After stimulation for 6 h, total RNA was extracted and reverse transcribed to cDNA. (B) *iNOS* was amplified by real-time PCR, and the expression level in each group was compared to that in the LPS-treated group. Data represent the mean ± S.D. (n = 3). *p < 0.01 relative to the LPS-treated control group. (C) *iNOS* was amplified by PCR and detected using a gel documentation system. GAPDH served as an internal control. (D) Total cell lysates were prepared after stimulation for 24 h and subjected to western blot. The protein expression of iNOS was detected using an enhanced chemiluminescence reagent and was normalized to that of tubulin, a loading control.

As expected, LPS stimulation highly induced NO production in murine macrophages. ECR treatment notably inhibited NO secretion in a dose-dependent manner. In addition, cDNA amplification was performed using quantitative and semi-quantitative PCR to detect the relationship between NO secretion and *iNOS* expression. As shown in Figure 3, the LPS-induced increase in *iNOS* expression was inhibited by ECR treatment. Furthermore, ECR-mediated negative regulation of the expression of iNOS protein expression was confirmed by western blot analysis.

To investigate the effect of ECR on COX-2 expression, a responsible enzyme for the production of PGE_2, its expression at mRNA and protein levels were measured. As shown in Figure 4, LPS-stimulated RAW 264.7 macrophages showed clear induction of both mRNA and protein COX-2. However, ECR did not regulate the LPS-induced COX-2 expression. These results suggest that ECR regulates inflammatory responses by suppressing expression of iNOS but not COX-2.

ECR selectively inhibits the production of proinflammatory cytokines

To investigate whether ECR inhibits proinflammatory cytokines, including IL-1β, IL-6, and TNF-α, their protein and mRNA expression levels were measured in LPS-stimulated RAW 264.7 macrophages. As shown in Figure 5, the LPS-induced increase in *IL-6* and *TNF*-α was confirmed by ELISA. However, ECR treatment did not lead to any changes in the LPS-induced production of both cytokines. In conjunction with the ELISA results, quantitative and semi-quantitative PCR data revealed that mRNA expression levels of *IL-6* and *TNF*-α were not suppressed with ECR treatment although mRNA expression was induced by LPS. Unlike *IL-6* and *TNF*-α, the expression of *IL-1*β mRNA was alleviated by ECR in a dose-dependent manner. Taken together, ECR selectively inhibits LPS-mediated production of IL-1β in murine macrophages but not those of IL-6 and TNF-α.

ECR selectively inhibits the activation of p38

Since inflammatory responses activated by LPS are mainly mediated by the activation of NF-κB and MAPKs, including p38, ERK, and JNK, we attempted to elucidate the responsible targets for the anti-inflammatory functions of ECR by measuring the phosphorylation of IκB and MAPK. First, we detected the expression profile of IκB and the phosphorylation of IκB after ECR treatment. As shown in Figure 6A, the levels of LPS-stimulated p-IκB were not changed in response to ECR treatment. This result suggests that NF-κB signaling is not the major regulating

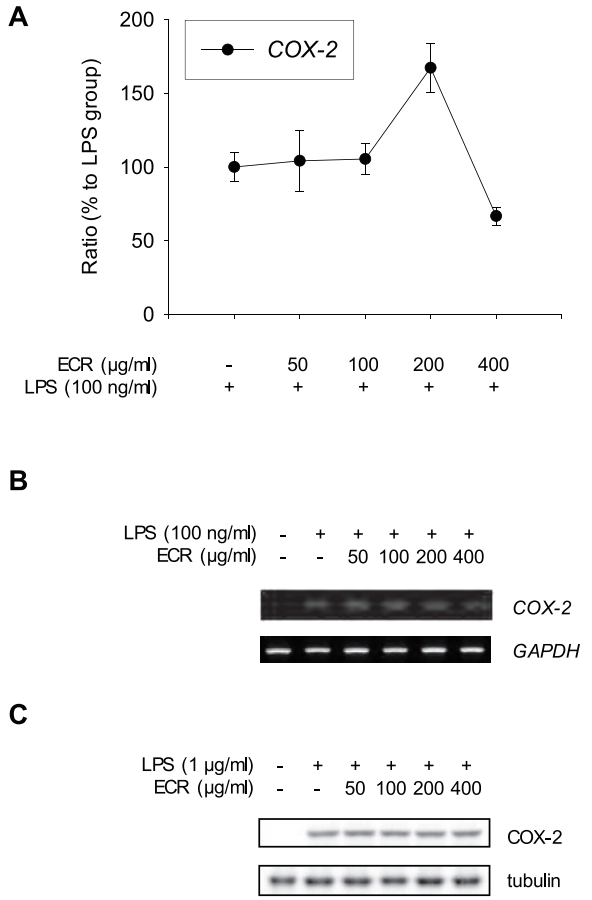

FIGURE 4 | Effect of ECR on the expression of COX-2. RAW 264.7 macrophages were stimulated with LPS in the presence of ECR (50, 100, 200, and 400 μg/ml) for the indicated times. (A and B) After stimulation for 6 h, total RNA was extracted and reverse transcribed to cDNA. (A) COX-2 was amplified by real-time PCR, and the expression in each group was compared to that in the LPS-treated group. Data represent the mean ± S.D. (n = 3). (B) *COX-2* was amplified by PCR and detected using a gel documentation system. GAPDH expression served as an internal control. (C) Total cell lysates were prepared after stimulation for 24 h and subjected to western blotting. The protein expression of COX-2 was detected using enhanced chemiluminescence reagent and was normalized to that of tubulin, a loading control.

mechanism of ECR-mediated inhibition of inflammatory responses.

We then investigated the effect of ECR on the phosphorylation states of MAPKs. As expected based on previous results, the phosphorylation of all MAPKs was highly induced after LPS treatment. ECR did not inhibit the LPS-induced phosphorylation of ERK and JNK, as in the case of NF-κB. However, ECR selectively inhibited the LPS-induced phosphorylation of p38 without affecting p38 protein levels (Figure 6B). This implies that the suppression of p38 activation by ECR is solely dependent on its phosphorylation state, not on its expression. Taken together, our results suggest that the anti-inflammatory effect of ECR is mediated by selective inhibition of p38, but not of ERK, JNK, or NF-κB.

DISCUSSION

Since steroidal drugs such as glucocorticoids have severe adverse effects, there is a need to develop new anti-inflammatory agents (Manelli and Giustina, 2000, Munstedt et al., 2004, Rubaltelli et al., 1997, van Raalte et al., 2009). Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and indomethacin were developed and many studies have focused on the development of new drugs that can function as anti-proinflammatory cytokine agents (Dinarello, 2010). Further, native plants are receiving greater attention nowadays because of anecdotal evidence from traditional medicine and their relatively fewer side effects (Bassett et al., 1990). Many phytochemicals have been identified as anti-inflammatory drug candidates and are under investigation for clinical use (Naksuriya et al., 2014, Ranjan et al., 2013). In this study, we investigated the anti-inflammatory effect of ECR and its underlying molecular mechanism. Although we elucidated the mechanism of action of ECR in murine macrophages, further studies are needed to clarify the major phytochemicals that mediate the anti-inflammatory effect of ECR.

iNOS and COX-2 are critical enzymes that induce inflammation. NO, synthesized from L-arginine by iNOS, is well known as an important mediator of acute and chronic inflammation (Huang et al., 2014, MacMicking et al., 1997, Moncada, 1999, Nathan and Xie, 1994). High concentrations of NO combine with superoxide to form OONO-peroxynitrite ion, which is responsible for severe cell damage and tissue destruction in inflammation (Bosca et al., 2005, Kroncke et al., 1998). PGE_2, produced by COX-2, is known to play key roles in inflammatory processes including pain, fever, swelling, and tenderness (Kawahara et al., 2014, Murakami and Kudo, 2006). In general, many of the reagents that represent anti-inflammatory properties inhibit the production of both iNOS and COX-2 (Nakamura et al., 2009, Noh et al., 2006, Uto et al., 2010). However, ECR selectively inhibited the production of iNOS, but not COX-2. In fact, inhibitory effects of some anti-inflammatory agents on PGE_2 production are independent on the expression of COX-2 (Barrios-Rodiles et al., 1996). Therefore, it is still of interest to investigate whether ECR regulates COX-2 activity even though majority of natural extracts that have anti-inflammatory effects have shown to suppress COX-2 expression but not its activity (Lee et al., 2014, Yu et al., 2012). There is some evidence that activation of NF-κB and MAPKs is essential for the induction of COX-2 (Jones et al., 2001, Jones et al., 2001). However, in this study, only p38 was regulated by ECR whereas other MAPKs and NF-κB were not. Moreover, the differential regulation of iNOS and COX-2 by JAK-STAT pathway was reported by a recent study in which a JAK3 inhibitor inhibited iNOS expression and NO production but not COX-2 induction in LPS-activated J774 macrophages (Sareila et al., 2008). Since no effect of ECR on the production of COX-2 and no involvement of NF-κB, ERK, and JNK were detected, further studies might be needed to clarify

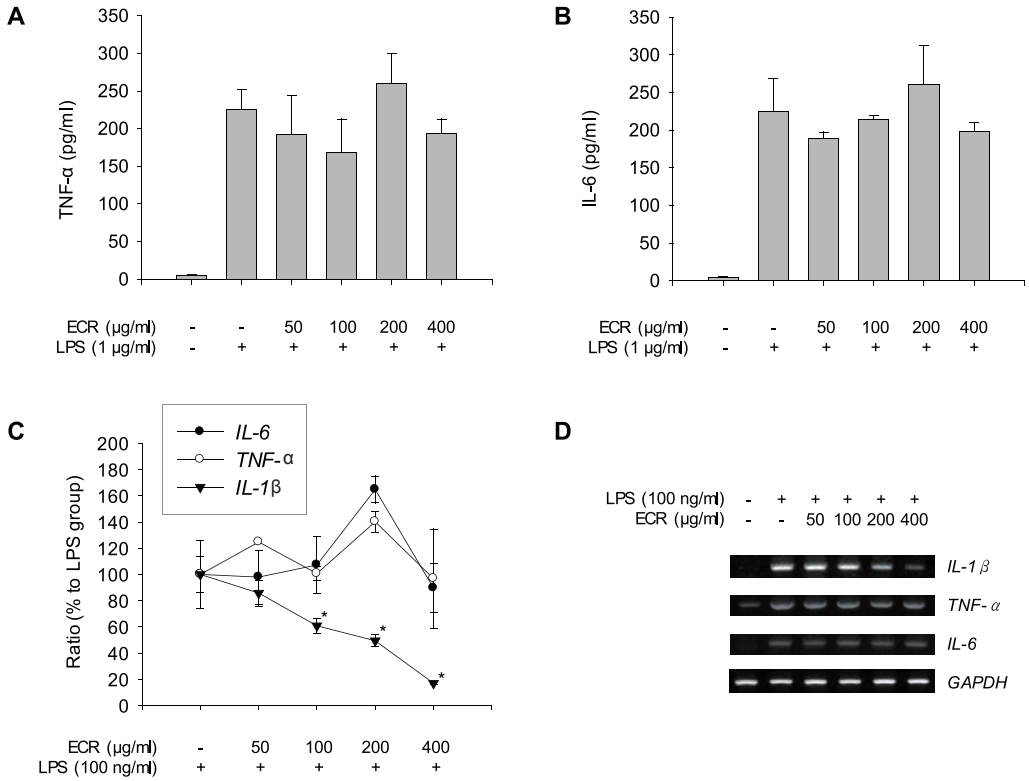

FIGURE 5 | Inhibitory effect of ECR on the production of proinflammatory cytokines. RAW 264.7 macrophages were stimulated with LPS in the presence of ECR (50, 100, 200, and 400 μg/ml) for indicated times. (A and B) After stimulation for 24 h, ELISA assays were used to measure TNF-α (A) and IL-6 (B) levels. The secretion of each cytokine was determined using a standard curve. Data represent the mean ± S.D. (n = 3). (C and D) After stimulation for 6 h, total RNA was extracted, and reverse transcribed to cDNA. (C) *IL-1*β, *TNF*-α and, *IL*-6 were amplified by real-time PCR, and the expression of each group was compared to that in the LPS-treated group. Data represent the mean ± S.D. (n = 3). *p < 0.01 relative to the LPS-treated control group. (D) mRNAs of *IL*-1β, *TNF*-α, and *IL*-6 were amplified by RT-PCR and detected using a gel documentation system. GAPDH was used as a loading control.

the intervention of JAK-STAT by ECR.

The specific regulation of inflammatory molecular targets may lead to selective inhibition of the production of proinflammatory cytokines. As shown in figure 5, IL-1β production was inhibited by ECR in a dose-dependent manner. However, production of IL-6 and TNF-α was not regulated by ECR. Furthermore, ECR regulated only p38 among NF-κB and MAPKs in macrophages. The phosphorylation of p38 MAP kinase has been known to stimulate the expression of proinflammatory proteins and cytokines (Hope et al., 2009, Willemen et al., 2014). Many studies have reported that p38 is a key element of the proinflammatory signal transduction cascade responsible for activation of redox-sensitive transcription factor activator protein-1 (AP-1), which consists of either homo- or heterodimers between members of the Jun and Fos family (Lee et al., 2005, Yang et al., 2014). Recent findings also have suggested that AP-1 activation is closely related to up-regulation of iNOS, IL-1β, and IL-6 expression levels (Lee et al., 2006, Palanki, 2002). On the basis of these concerns, we propose that further studies are needed to clarify the molecular mechanism for the unregulation of IL-6 production by ECR.

In summary, this study demonstrates that ECR possesses anti-inflammatory activity, which selectively inhibits the production of NO and IL-1β by blocking of LPS-mediated p38 activation (Figure 7). Those effects suggest that ECR may be applied to the treatment of inflammatory diseases including rheumatoid arthritis, systemic lupus erythematosus, and atopic dermatitis. Further studies should be performed to clarify the effects of ECR on the animal inflammatory model and the signaling network between TLR4 activation and p38 phosphorylation.

METHODS

Cell culture and reagents

A 95% ethanol extract (Code No.: FBM124-021) from Callicarpa rubella for. angustata C. P`ei (Verbenaceae) was purchased from the International Biological Material Research Center (http://www.ibmrc.re.kr, Daejeon, Korea). The extract was diluted in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) and added directly to the culture media. The final concentrations of DMSO never exceeded over 0.1%, which did not affect the biological assays. The RAW 264.7 macrophages, a mouse monocytic cell line, were cultured in Dulbecco's Modified Eagle 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 a 5% CO_2 humidified air atmosphere. Rabbit anti-inhibitors of κB (IκB) and mouse

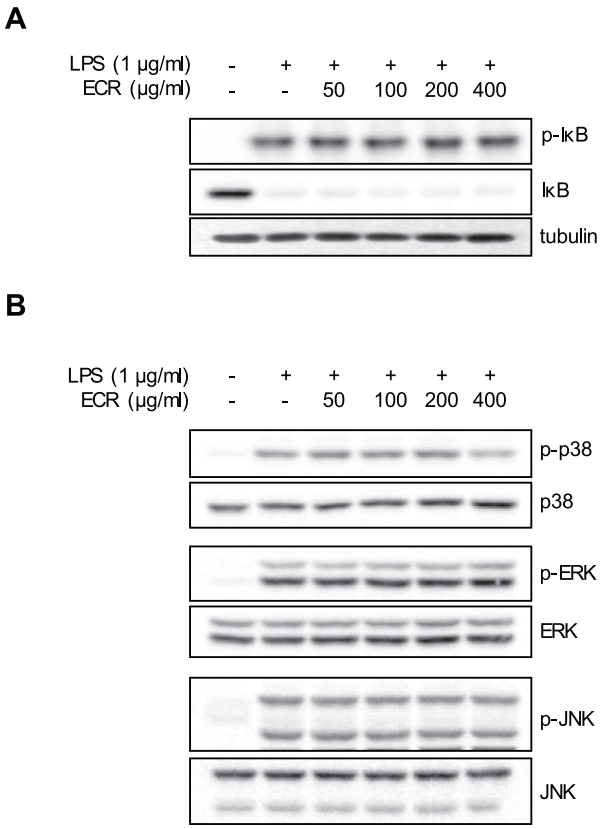

FIGURE 6 | Inhibitory effect of ECR on NF-κB and MAPK activation. RAW 264.7 macrophages were pretreated with various concentrations of ECR (50, 100, 200, and 400 μg/ml) for 1 h and stimulated with LPS for 15 min. Total cell lysates were prepared and subjected to western blot. The expressions of p-IκB, IκB (A), p-p38, p38, p-ERK, ERK, p-JNK, and JNK (B) were detected using specific antibodies and tubulin was used as a loading control.

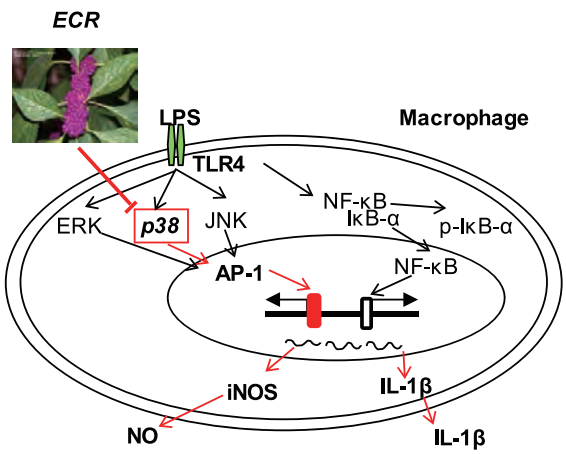

FIGURE 7 | Putative inhibitory pathway of ECR in LPS-activated inflammatory signaling. The production of proinflammatory mediators is induced in LPS-stimulated macrophages by activating NF-κB and MAPK signaling pathways. Of them, both NO and IL-1β productions are specifically alleviated by ECR through the inactivation of p38.

anti-tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-inducible iNOS, anti-phospho IκB, anti-phospho p38 mitogen-activated protein kinase (MAPK), 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).

Cell viability assay

The RAW 264.7 macrophages were seeded at 96 well plate (4 x 10^4/well). After adhesion for overnight, cells were incubated with ECR and LPS for 24 h. After incubation, Ez-cytox solution (Daeil Lab, Seoul) was added for 2 h at 37°C and 100 μl supernatants carefully transferred to new 96 well plate. The absorbance was measured at 595 nm with a Synergy Microplate Reader (BioTek Instruments, Winooski, VT, USA).

Nitrite assay

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

ELISA (Enzyme-linked immunosorbent assay)

The RAW 264.7 macrophages were stimulated with LPS and ECR for 24 h. After stimulation, the supernatants were obtained and the quantities of TNF-α and IL-6 in culture supernatants were determined by a sandwich ELISA using monoclonal antibodies specific to each mediator (Ebioscience, San Diego, CA, USA). Before the application of samples, the plate was pre-coated with coating antibody in supplied buffer. After overnight incubation at 4°C, the plate was washed, and 1X Assay diluents were treated for 1 h. And then, samples were loaded to each well and incubated for 2 h at RT. Biotinylated secondary antibody solution and horseradish peroxidase (HRP)-streptavidin solution were treated for 1 h and 30 min, respectively, and substrate solution was added to washed-plate. After 10 min incubation in dark condition, 1N H_3PO_4 was treated and optical density of the individual wells was determined at 450 nm by Synergy Microplate Reader.

Quantitative Real-Time PCR

Total RNA was prepared from the cells and reverse-transcribed into complementary DNA (cDNA) using TOPscript™ cDNA synthesis kit (Enzynomics, Daejeon), and then PCR amplification of the cDNA was performed. Quantification of mRNA was also performed using real-time RT-PCR with manufacturer's instructions of iTaq™ Universal SYBR Green Supermix (Bio-rad, Hercules, CA, USA). The PCR was run for 40 cycles of denaturation at 94°C (5 s) and annealing/extension at 60°C (30 s) using a CFX Connect™ real-time thermal cycler (Bio-Rad). The results were normalized with multi reference genes, β-actin and GAPDH, and were expressed as the ratio of gene expressions to LPS treated group (100%). The sequences of PCR primers used in this study are: mouse iNOS (sense, 5'TGG CCA CCA AGC TGA ACT3′; antisense, 5'TCA TGA TAA CGT TTC TGG CTC TT3′), COX-2 (sense, 5'GAT GCT CTT CCG AGC TGT G3′; antisense, 5′GGA TTG GAA CAG CAA GGA TTT3′), TNF-α (sense, 5'CTG TAG CCC ACG TCG TAG C3′; antisense, 5'TTG AGA TCC ATG CCG TTG3′), IL-6 (sense, 5'TCT AAT TCA TAT CTT CAA CCA AGA GG3′; antisense, 5'TGG TCC TTA GCC ACT CCT TC3′), IL-1β (sense, 5'TTG ACG GAC CCC AAA AGA T3′; antisense, 5′GAT GTG CTG CTG CGA GAT T3′), β-actin (sense, 5'CGT CAT ACT CCT GCT TGC TG3′; antisense, 5′CCA GAT CAT TGC TCC TCC TGA3′), and GAPDH (sense, 5′GCT CTC TGC TCC TCC TGT TC3′; antisense, 5′ACG ACC AAA TCC GTT GAC TC3′).

Semi-quantitative RT-PCR (Reverse transcription polymerase chain reaction)

The sequences of PCR primers used in this study are: mouse iNOS (sense, 5'GCA TGG AAC AGT ATA AGG CAA ACA3′; antisense, 5′GTT TCT GGT CGA TGT CAT GAG CAA3′), COX-2 (sense, 5'GCA TGG AAC AGT ATA AGG CAA ACA3′; antisense, 5′GTT TCT GGT CGA TGT CAT GAG CAA3′), TNF-α (sense, 5'GTG CCA GCC GAT GGG TTG TAC C3′; antisense, 5′AGG CCC ACA GTC CAG GTC ACT G3'), IL-6 (sense, 5'TCT TGG GAC TGA TGC TGG TGA C3'; antisense, 5'CAT AAC GCA CTA GGT TTG CCG A3'), IL-1β (sense, 5'AGC TGT GGC AGC TAC CTG TG3′; antisense, 5′GCT CTG CTT GTG AGG TGC TG3′), and GAPDH (sense, 5′GTC TTC ACC ACC ATG GAG AAG G3′; antisense, 5′CCT GCT TCA CCA CCT TCT TGC C3′). The PCR was run for 20-25 cycles of 94°C (30 s), 60°C (30 s), and 72°C (30 s) using a Bioer's 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.

Preparation of total cell lysates

LPS-stimulated RAW 264.7 cells were treated with ECR and LPS for 15 min and subsequently 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 to microtubes, and then centrifuged at 13,000 rpm for 30 min at 4°C. The supernatants were prepared in new microtubes.

Western blotting

Protein concentration was measured by the Bradford method. Aliquots of the cell lysates were separated on 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) 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 (1:1,000). Each membrane was further incubated for 1 h with secondary peroxidase-conjugated goat immunoglobulin G (IgG, 1: 5,000). The target proteins were detected using an enhanced chemiluminescence (ECL) solution.

Statistical analysis

Differences between experimental conditions were assessed by the Student's t-test. $p < 0.01$ considered to be statistically significant. In all instances, the means of data from three independent experiments were analyzed.

ACKNOWLEDGEMENTS

This study was supported by the National Research Foundation of Korea grant funded by the Korean government (Ministry of Education, Science and Technology; grant no. NRF-2012R1A2A2A01047338 and NRF-2013R1A1A2062389).

AUTHOR INFORMATION

The authors declare no potential conflicts of interest.

Original Submission: Aug 10, 2014
Revised Version Received: Aug 25, 2014
Accepted: Aug 26, 2014

REFERENCES

Barrios-Rodiles M., Keller K., Belley A., and Chadee K. (1996). Nonsteroidal antiinflammatory drugs inhibit cyclooxygenase-2 enzyme activity but not mRNA expression in human macrophages. *Biochem Biophys Res Commun* **225**, 896-900.

Bassett I.B., Pannowitz D.L., and Barnetson R.S. (1990). A comparative study of tea-tree oil versus benzoylperoxide in the treatment of acne. *Med J Aust* **153**, 455-458.

Bosca L., Zeini M., Traves P.G., and Hortelano S. (2005). Nitric oxide and cell viability in inflammatory cells: a role for NO in macrophage function and fate. *Toxicology* **208**, 249-258.

Cantrell C.L., Klun J.A., Bryson C.T., Kobaisy M., and Duke S.O. (2005) . Isolation and identification of mosquito bite deterrent terpenoids from leaves of American (Callicarpa americana) and Japanese (Callicarpa japonica) beautyberry. *J Agric Food Chem* **53**, 5948-5953.

Cario E., Rosenberg I.M., Brandwein S.L., Beck P.L., Reinecker H.C., and Podolsky D.K. (2000). Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. *J Immunol* **164**, 966-972.

Chen J.J., Wu H.M., Peng C.F., Chen I.S., and Chu S.D. (2009). seco-Abietane diterpenoids, a phenylethanoid derivative, and antitubercular constituents from Callicarpa pilosissima. *J Nat Prod* **72**, 223-228.

Clancy R.M., Amin A.R., and Abramson S.B. (1998). The role of nitric oxide in inflammation and immunity. *Arthritis Rheum* **41**, 1141-1151.

Dinarello C.A. (2010). Anti-inflammatory Agents: Present and Future. *Cell* **140**, 935-950.

Fujihara M., Muroi M., Tanamoto K., Suzuki T., Azuma H., and Ikeda H. (2003). Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. *Pharmacol Ther* **100**, 171-194.

Guadagni F., Ferroni P., Palmirotta R., Portarena I., Formica V., and Roselli M. (2007). Review. TNF/VEGF cross-talk in chronic inflammation-related cancer initiation and progression: an early target in anticancer therapeutic strategy. *In Vivo* **21**, 147-161.

Hanada T., and Yoshimura A. (2002). Regulation of cytokine signaling and inflammation. *Cytokine Growth Factor Rev* **13**, 413-421.

Hope H.R., Anderson G.D., Burnette B.L., Compton R.P., Devraj R.V., Hirsch J.L., Keith R.H., Li X., Mbalaviele G., Messing D.M., Saabye M.J., Schindler J.F., Selness S.R., Stillwell L.I., Webb E.G., et al. (2009). Anti-inflammatory properties of a novel N-phenyl pyridinone inhibitor of p38 mitogen-activated protein kinase: preclinical-to-clinical translation. *J Pharmacol Exp Ther* **331**, 882-895.

Huang B., Fu H.Z., Chen W.K., Luo Y.H., and Ma S.C. (2014). Hepatoprotective Triterpenoid Saponins from Callicarpa nudiflora. *Chem Pharm Bull (Tokyo)* **62**, 695-699.

Jones B.W., Heldwein K.A., Means T.K., Saukkonen J.J., and Fenton M.J. (2001). Differential roles of Toll-like receptors in the elicitation of proinflammatory responses by macrophages. *Ann Rheum Dis* **60 Suppl 3**, iii6-12.

Jones B.W., Means T.K., Heldwein K.A., Keen M.A., Hill P.J., Belisle J.T., and Fenton M.J. (2001). Different Toll-like receptor agonists induce distinct macrophage responses. *J Leukoc Biol* **69**, 1036-1044.

Jones W.P., and Kinghorn A.D. (2008). Biologically Active Natural Products of the Genus Callicarpa. *Curr Bioact Compd* **4**, 15-32.

Jones W.P., Lobo-Echeverri T., Mi Q., Chai H.B., Soejarto D.D., Cordell G.A., Swanson S.M., and Kinghorn A.D. (2007). Cytotoxic constituents from the fruiting branches of Callicarpa americana collected in southern Florida. *J Nat Prod* **70**, 372-377.

Karin M., and Ben-Neriah Y. (2000). Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol* **18**, 621-663.

Karin M., and Delhase M. (2000).The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. *Semin Immunol* **12**, 85-98.

Kawahara K., Hohjoh H., Inazumi T., Tsuchiya S., and Sugimoto Y. (2014). Prostaglandin E-induced inflammation: Relevance of prostaglandin E receptors. *Biochim Biophys Acta.*

Kim Y.J., and Lee J.-O. (2013). Recognition of Lipopolysaccharides by TLR4 and its Accessory Proteins. *Biodesign* **1**, 3-12.

Kroncke K.D., Fehsel K., and Kolb-Bachofen V. (1998). Inducible nitric oxide synthase in human diseases. *Clin Exp Immunol* **113**, 147-156.

Lee C., Lim H.K., Sakong J., Lee Y.S., Kim J.R., and Baek S.H. (2006).

Janus kinase-signal transducer and activator of transcription mediates phosphatidic acid-induced interleukin (IL)-1beta and IL-6 production. *Mol Pharmacol* **69**, 1041-1047.

Lee C.W., Park S.M., Kim Y.S., Jegal K.H., Lee J.R., Cho I.J., Ku S.K., Lee J.Y., Ahn Y.T., Son Y., S A.J., Kim S.C., and An W.G. (2014). Biomolecular evidence of anti-inflammatory effects by Clematis mandshurica Ruprecht root extract in rodent cells. *J Ethnopharmacol* **155**, 1141-1155.

Lee J.S., Oh T.Y., Kim Y.K., Baik J.H., So S., Hahm K.B., and Surh Y.J. (2005). Protective effects of green tea polyphenol extracts against ethanol-induced gastric mucosal damages in rats: stress-responsive transcription factors and MAP kinases as potential targets. *Mutat Res* **579**, 214-224.

MacMicking J., Xie Q.W., and Nathan C. (1997). Nitric oxide and macrophage function. *Annu Rev Immunol* **15**, 323-350.

Manelli F., and Giustina A. (2000). Glucocorticoid-induced osteoporosis. *Trends Endocrinol Metab* **11**, 79-85.

Moncada S. (1999). Nitric oxide: discovery and impact on clinical medicine. *J R Soc Med* **92**, 164-169.

Munstedt K., Borces D., Bohlmann M.K., Zygmunt M., and von Georgi R. (2004). Glucocorticoid administration in antiemetic therapy: is it safe? *Cancer* **101**, 1696-1702.

Murakami M., and Kudo I. (2006). Prostaglandin E synthase: a novel drug target for inflammation and cancer. *Curr Pharm Des* **12**, 943-954.

Nakamura T., Kodama N., Arai Y., Kumamoto T., Higuchi Y., Chaichantipyuth C., Ishikawa T., Ueno K., and Yano S. (2009). Inhibitory effect of oxycoumarins isolated from the Thai medicinal plant Clausena guillauminii on the inflammation mediators, iNOS, TNF-alpha, and COX-2 expression in mouse macrophage RAW 264.7. *J Nat Med* **63**, 21-27.

Naksuriya O., Okonogi S., Schiffelers R.M., and Hennink W.E. (2014). Curcumin nanoformulations: a review of pharmaceutical properties and preclinical studies and clinical data related to cancer treatment. *Biomaterials* **35**, 3365-3383.

Nathan C., and Xie Q.W. (1994). Nitric oxide synthases: roles, tolls, and controls. *Cell* **78**, 915-918.

Nathan C., and Xie Q.W. (1994). Regulation of biosynthesis of nitric oxide. *J Biol Chem* **269**, 13725-13728.

Nishimoto N., and Kishimoto T. (2006). Interleukin 6: from bench to bedside. *Nat Clin Pract Rheumatol* **2**, 619-626.

Noh E.J., Ahn K.S., Shin E.M., Jung S.H., and Kim Y.S. (2006). Inhibition of lipopolysaccharide-induced iNOS and COX-2 expression by dehydroevodiamine through suppression of NF-kappaB activation in RAW 264.7 macrophages. *Life Sci* **79**, 695-701.

Palanki M.S. (2002). Inhibitors of AP-1 and NF-kappa B mediated transcriptional activation: therapeutic potential in autoimmune diseases and structural diversity. *Curr Med Chem* **9**, 219-227.

Ranjan A.P., Mukerjee A., Helson L., Gupta R., and Vishwanatha J.K. (2013). Efficacy of liposomal curcumin in a human pancreatic tumor xenograft model: inhibition of tumor growth and angiogenesis. *Anticancer*

Res **33**, 3603-3609.

Rhee S.H., and Hwang D. (2000). Murine TOLL-like receptor 4 confers lipopolysaccharide responsiveness as determined by activation of NF kappa B and expression of the inducible cyclooxygenase. *J Biol Chem* **275**, 34035-34040.

Rubaltelli F.F., Chiti G., and Dani C. (1997). Adverse effects of prenatal glucocorticoid treatment in the preterm infant. *Acta Biomed Ateneo Parmense* **68 Suppl 1**, 35-38.

Samavati L., Rastogi R., Du W., Huttemann M., Fite A., and Franchi L. (2009). STAT3 tyrosine phosphorylation is critical for interleukin 1 beta and interleukin-6 production in response to lipopolysaccharide and live bacteria. *Mol Immunol* **46**, 1867-1877.

Sareila O., Korhonen R., Karpanniemi O., Nieminen R., Kankaanranta H., and Moilanen E. (2008). Janus kinase 3 inhibitor WHI-P154 in macrophages activated by bacterial endotoxin: differential effects on the expression of iNOS, COX-2 and TNF-alpha. *Int Immunopharmacol* **8**, 100-108.

Schroder K., Sweet M.J., and Hume D.A. (2006). Signal integration between IFNgamma and TLR signalling pathways in macrophages. *Immunobiology* **211**, 511-524.

Stalinska K., Guzdek A., Rokicki M., and Koj A. (2005). Transcription factors as targets of the anti-inflammatory treatment. A cell culture study with extracts from some Mediterranean diet plants. *J Physiol Pharmacol* **56 Suppl 1**, 157-169.

Szabo C., and Thiemermann C. (1995). Regulation of the expression of the inducible isoform of nitric oxide synthase. *Adv Pharmacol* **34**, 113-153.

Tellez M.R., Dayan F.E., Schrader K.K., Wedge D.E., and Duke S.O. (2000). Composition and some biological activities of the essential oil of Callicarpa americana (L.). *J Agric Food Chem* **48**, 3008-3012.

Tu Y., Sun L., Guo M., and Chen W. (2013). The medicinal uses of Callicarpa L. in traditional Chinese medicine: an ethnopharmacological, phytochemical and pharmacological review. *J Ethnopharmacol* **146**, 465-481.

Uto T., Suangkaew N., Morinaga O., Kariyazono H., Oiso S., and Shoyama Y. (2010). Eriobotryae folium extract suppresses LPS-induced iNOS and COX-2 expression by inhibition of NF-kappaB and MAPK activation in murine macrophages. *Am J Chin Med* **38**, 985-994.

van Raalte D.H., Ouwens D.M., and Diamant M. (2009). Novel insights into glucocorticoid-mediated diabetogenic effects: towards expansion of therapeutic options? *Eur J Clin Invest* **39**, 81-93.

Verma I.M., Stevenson J.K., Schwarz E.M., Van Antwerp D., and Miyamoto S. (1995). Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. *Genes Dev* **9**, 2723-2735.

Willemen H.L., Campos P.M., Lucas E., Morreale A., Gil-Redondo R., Agut J., Gonzalez F.V., Ramos P., Heijnen C., Mayor F., Jr., Kavelaars A., and Murga C. (2014). A novel p38 MAPK docking-groove-targeted compound is a potent inhibitor of inflammatory hyperalgesia. *Biochem J* **459**, 427-439.

Yang Y., Kim S.C., Yu T., Yi Y.S., Rhee M.H., Sung G.H., Yoo B.C., and Cho J.Y. (2014). Functional roles of p38 mitogen-activated protein kinase in macrophage-mediated inflammatory responses. *Mediators Inflamm* **2014**, 352371.

Yu T., Lee S., Yang W.S., Jang H.J., Lee Y.J., Kim T.W., Kim S.Y., Lee J., and Cho J.Y. (2012). The ability of an ethanol extract of Cinnamomum cassia to inhibit Src and spleen tyrosine kinase activity contributes to its anti-inflammatory action. *J Ethnopharmacol* **139**, 566-573.

