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Ecklonia cava ethanolic extracts inhibit lipopolysaccharide-induced cyclooxygenase-2 and inducible nitric oxide synthase expression in BV2 microglia via the MAP kinase and NF-κB pathways

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ABSTRACT

Ecklonia cava (EC) is a brown alga that has demonstrated radical scavenging, bactericidal, tyrosinase inhibitory, and protease inhibitory activities. However, the molecular mechanisms underlying its anti-inflammatory action remain unclear. In the current study, we attempted to determine whether pretreatment with EC induces a significant inhibition of anti-inflammatory activity in lipopolysaccharide (LPS)-stimulated murine BV2 microglia. Our results indicate that EC inhibits LPS-induced nitric oxide (NO) and prostaglandin E_2 (PGE_2) production in a concentration-dependent manner and inhibits inducible nitric oxide (iNOS) and cyclooxygenase (COX)-2 in BV2 microglia without significant cytotoxicity. EC treatment significantly reduced nuclear factor-κB (NF-κB) translocation and DNA-binding in LPS-stimulated BV2 microglia. This effect was mediated through the inhibition of the degradation of the inhibitor κB and by inhibition of the mitogen-activated protein kinase (MAPK) phosphorylation, at least in part by inhibiting the generation of reactive oxygen species. Our data also indicate that EC extracts exert anti-inflammatory effects by suppressing proinflammatory cytokines. Collectively, these results suggest that EC suppresses the induction of cytokines by LPS, as well as iNOS and COX-2 expression, by blocking NF-κB and MAPK activation. These findings provide mechanistic insights into the anti-inflammatory and neuroprotective actions of EC in BV2 microglia.

1. Introduction

Ecklonia cava (EC) is a brown alga that grows plentifully in the subtidal regions of Jeju Island, Korea. EC contains a richer supply of total phenolic compounds, phlorotannins, than do other brown seaweeds (Heo et al., 2005). Recently, an increasing amount of evidence has demonstrated that EC exhibits radical scavenging, matrix metalloproteinase inhibitory, bactericidal, protease inhibitory, antioxidative, anti-inflammatory, immunomodulatory, and anti-asthmatic activities (Kang et al., 2004; Kim et al., 2006a, 2008; Ahn et al., 2004, 2008; Shin et al., 2006). Nevertheless, no reports have investigated the anti-inflammatory effects and molecular mechanisms of EC extracts in LPS-stimulated microglia.

Microglia enter the brain early in embryogenesis and develop in parallel with the maturation of the nervous system (Mosley et al., 2006). As such, these cells constitute up to 20% of the cell population in certain regions of the brain (Dobrenis, 1998; Lawson et al.,

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AD, Alzheimer's disease; COX, cyclooxygenase; DCFDA, 2′,7′-dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle medium; EC, *Ecklonia cava*; ECL, electrochemiluminescence; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; iNOS, inducible nitric oxide synthase; IκB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MS, multiple sclerosis; NF-κB, nuclear factor-κB; NO, nitric oxide; PD, Parkinson's disease; PBS, phosphate-buffered saline; PGE_2, prostaglandin E_2; ROS, reactive oxygen species; RT-PCR, reverse transcriptase-polymerase chain reaction; SAPK, stress-activated protein kinase; TNF-α, tumor necrosis factor-α.

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1990). Microglia are immune cells found in the central nervous system that are activated in response to brain injury and neurodegenerative disease, and are also induced following exposure to lipopolysaccharide (LPS), interferon-γ, or β-amyloid (Lehnardt et al., 2003; Meda et al., 1995; Zielasek and Hartung, 1996). Activated microglia release neurotoxic and proinflammatory factors, including nitric oxide (NO), prostaglandin E_2 (PGE_2), reactive oxygen species (ROS) and proinflammatory cytokines [interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α]. Therefore, microglial activation appears to play a pivotal role in the initiation and progression of several neurodegenerative diseases. Excessive amounts of each factor produced by activated microglia can result in brain injuries and diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), trauma, multiple sclerosis (MS) and cerebral ischemia. Thus, regulating microglial activation may have the therapeutic potential to reduce neuronal injury or death in neurodegenerative diseases.

LPS initiates a number of major cellular responses that play critical roles in the pathogenesis of inflammatory responses, which in turn may damage neurons. Therefore, LPS stimulation of microglia is a useful model to use studies of the mechanisms underlying neuron damage by various neurotoxic factors released from activated microglia (Bocchini et al., 1992; Woo et al., 2004). Among these mediators, PGE_2 and NO are the products of the inducible isoforms of COX-2 and iNOS enzymes, respectively (Vane et al., 1994). NO has been shown to be an important biological messenger molecule in diverse physiological functions, including vasodilation, neural communication and host defense (MacMicking et al., 1997; Mitchell et al., 1995). It was reported that iNOS is not normally expressed in the brain, but LPS upregulates iNOS expression in microglial cells, astrocytes and possibly in neurons (Vegeto et al., 2001; Murphy, 2000; Heneka and Feinstein, 2001). Excessive release of NO by activated microglia is correlated with the progression of neurodegeneration (Brown, 2007). COX is an enzyme that catalyzes the conversion of arachidonic acid to prostaglandin H2, a precursor for a variety of biologically active mediators, such as PGE_2, prostacyclin and thromboxane A2 (Hawkey, 1999; Picot et al., 1994). COX exists as two major isoenzymes: COX-1, which is a constitutive cyclooxygenase, and COX-2, which is an inducible cyclooxygenase. COX-2 is the predominant cyclooxygenase at sites of inflammation (Mitchell et al., 1995; Smith et al., 1996). COX-2 is produced in macrophages and endothelial cells in response to various stimuli, such as neuronal activity, proinflammatory cytokines and may also be responsible for the edema and vasodilation associated with inflammation. However, in the central nervous system, COX-2 is also expressed under normal conditions and contributes to many fundamental brain functions (Minghetti, 2004). Taken together, these data indicate that inflammatory mediators, including iNOS and COX-2, are responsible for the symptoms of many types of neuronal damage in neurodegenerative diseases, including stroke, cerebral ischemia, PD, MS and AD. Thus, inhibition of these inflammatory mediators is one target for the treatment of neurodegenerative diseases.

In this study, we investigated the effects and mechanisms of action of EC extract on LPS-stimulated various neurotoxic factors in murine BV2 microglia. The BV2 cell line has been shown to mimic many microglial responses and has been widely used as a model microglial system; thus, we used this cell line in this study. The present findings suggest that EC may contribute to the therapeutic potential of treating inflammatory diseases.

2. Materials and methods

2.1. Materials

LPS from *Escherichia coli* 026:B6, *p*-nitrophenyl phosphate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). RT-PCR reagents were purchased from Promega (Madison, WI). Reagents for Lightshift chemiluminescent electrophoretic mobility shift assays, nuclear and cytoplasmic extraction and biotin 3′ end labeling were purchased from Pierce (Rockford, IL). Specific antibodies against iNOS, COX-2 and p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκB), extracellular signal-regulated kinase (ERK), phosphorylated (p)-ERK 1/2, p38, p-p38, JNK/stress-activated protein kinase (SAPK) and p-JNK/SAPK were purchased from Cell Signaling Technology (Beverly, MA).

2.2. Preparation of EC extracts

The brown seaweed, *E. cava* (EC) was collected along the Jeju Island coast of Korea during the period from October 2004 to March 2005. EC extracts were prepared with ethanol treatment according to the method of Kim et al. (2008). Briefly, fresh EC was washed three times with tap water to remove salt, epiphyte and sand on the surface of the samples before storage at −20 °C. The frozen samples were lyophilized and homogenized using a grinder before extraction. The dried powder (1 kg) was extracted with 95% EtOH (1:10 w/v) and evaporated *in vacuo*.

2.3. Cell culture

The murine BV2 microglia were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, and were maintained in a humidified incubator with 5% CO_2. In all experiments, cells were pretreated with the indicated concentrations of EC for 60 min before the addition of LPS (1 μg/ml) in serum-free DMEM.

2.4. Cell viability assay

MTT was used as an indicator of cell viability as determined by mitochondrial-dependent reduction to formazan. In brief, the cells were seeded and then treated with the indicated concentrations of EC (50, 100 or 200 μg/ml) for 2 h before a 24-h treatment with LPS (1 μg/ml). After various treatments, the medium was removed and the cells were incubated with a solution of 0.5 mg/ml MTT. After incubation for 3 h at 37 °C and 5% CO_2, the supernatant was removed and the formation of formazan was observed by monitoring the signal at 540 nm using a microplate reader.

2.5. Nitrite assay

The concentrations of NO in culture supernatants were determined as nitrite, a major stable product of NO, using the Griess reagent as described previously (Coker and Laurent, 1998). After cells (5×10^5 cells/ml) were stimulated in 24-well plates for 24 h, 100 μl of each cultured medium was mixed with the same volume of the Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4). Nitrite levels were determined colorimetrically at 540 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Dynatech MR-7000; Dynatech Laboratories), and nitrite concentrations were calculated with reference to a standard curve of sodium nitrite generated by known concentrations.

2.6. Western blot analysis

Cells were washed three times with PBS and lysed with lysis buffer (1% Triton X-100, 1% deoxycholate, 0.1% NaN_3). Equal amounts of protein were separated on 10% SDS-polyacrylamide minigels and transferred to Immobilon polyvinylidenedifluoride membranes (Millipore). After incubation with the appropriate primary antibody, the membranes were incubated for 1 h at room temperature with a secondary antibody conjugated to horseradish peroxidase. Following three washes in Tris-buffered saline-Tween (TBST), immunoreactive bands were visualized using the electrochemiluminescence (ECL) detection system. In a parallel experiment, nuclear proteins were prepared using nuclear extraction reagents according to the manufacturer's protocol.

2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated using the TRIzol reagent (Invitrogen, CA). Total RNA (1.0 μg) from the cells was reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI) to produce cDNA. RT-generated cDNAs encoding iNOS, COX-2, TNF-α, IL-1β and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were amplified by PCR using selective primers. PCR was performed using selective primers for murine iNOS (5′-ATGTCCGAAGCAAACATCAC-3′ and 5′-TAATGTCCAGGAAGTAGGTG-3′), COX-2 (5′-CAGCAAATCCTTGCTGTTCC-3′ and 5′-TGGGCAAAGAATGCAAACATC-3′), IL-1β (5′-ATGGCAACTGTTCCTGAACTCAACT-3′ and 5′-TTTCCTTTCTTAGATATGGACAGGAC-3′), TNF-α (5′-ATGAGCACAGAAAGCATGATC-3′ and 5′-TACAGGCTTGTCACTCGAATT-3′). Following amplification, portions of the PCR reactions were electrophoresed on an agarose gel.

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2.8. Enzyme-linked immunosorbent assay (ELISA)

The levels of TNF-α, IL-1β and PGE_2 were determined by ELISA. ELISA kits from R&D Systems (Minneapolis, MN) were employed for the measurement of TNF-α and IL-1β, and a kit from Cayman Chemical (Ann Arbor, MI) was employed for the measurement of PGE_2.

2.9. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared using the NE-PER nuclear extraction reagent. As a probe for the gel retardation assay, an oligonucleotide harboring the immunoglobulin κ-chain binding site (κB, 5′-CCGGTTAACAGAGGGGGCTTTCCGAG-3′) was synthesized. A non-radioactive method was used whereby the 3′ end of the probe was labeled with biotin according to the manufacturer's protocol (Pierce). The binding reactions contained 10 μg of nuclear extract protein, buffer, 50 ng of poly(*dI–dC*) and 20 fM biotin-labeled DNA. The reactions were incubated for 20 min at room temperature in a final volume of 20 μl. The competition reactions were conducted by adding a 100-fold excess of cold κB to the reaction mix. The reaction mixture was electrophoretically separated on a 5% polyacrylamide gel in 0.5X Tris-borate buffer and transferred to a nylon membrane. The biotin-labeled DNA was detected using a LightShift chemiluminescent electrophoretic mobility shift assay kit (Pierce).

2.10. Confocal laser scanning microscopy

The nuclear localization of NF-κB p65 was examined by indirect immunofluorescence assay using confocal microscopy. BV2 cells were cultured directly on glass coverslips in 24-well plates for 24 h. After stimulation with 1 μg/ml LPS and/or 200 μg/ml EC, the cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS and blocked with 1.5% normal donkey serum (Sigma). A polyclonal antibody to NF-κB p65 (1 μg/well) was applied for 1 h, followed by 1 h of incubation with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG. After washing with PBS, the coverslips were mounted with Fluoromount-G, and fluorescence was visualized using a Zeiss LSM 510 Meta microscope.

2.11. Measurement of intracellular reactive oxygen species (ROS) generation

ROS were measured using a previously described method with modifications (Cho et al., 2000). BV2 cells were washed with phosphate-buffered saline (PBS). To measure intracellular ROS, the cells were incubated for 4 h at 37 °C with PBS containing 5 μM 2′,7′-dichlorofluorescein diacetate (DCFDA, Molecular Probes, Eugene, OR) to label intracellular ROS. The cells were then immediately subjected to fluorescence-activated cell sorting (FACS) analysis (BD Biosciences, Rutherford, NJ).

2.12. Statistical analysis

The values presented represent means ± S.E.M. and analyzed by SPSS for Windows, version 9 (SPSS, Chicago, IL, USA) for the possible significant interrelation between the various groups, the independent samples Scheffe's test. A value of $p < 0.05$ was deemed statistically significant.

3. Results

3.1. EC inhibits NO production in LPS-stimulated BV2 microglia in a dose-dependent manner

To evaluate the effects of EC on NO production in LPS-stimulated BV2 microglia, we measured nitrite released into the culture medium using the Griess reagent. BV2 microglia were treated with various concentrations of EC (0, 50, 100 or 200 μg/ml) for 2 h before adding LPS (1 μg/ml). The LPS-induced elevation in nitrite concentration in the medium decreased in EC dose-dependent manner (Fig. 1). According to the NO detection assay, NO was significantly increased to 5.1 times the basal level in BV2 microglia after 24 h of LPS stimulation, and this increase was inhibited by EC treatment in a dose-dependent manner.

To exclude the possibility that the inhibition of NO production was due to cytotoxicity caused by EC treatment, MTT assays were performed in BV2 microglia treated with EC for 24 h. At the concentrations used (50–200 μg/ml), EC did not affect cell viability (data not shown). Thus, the inhibitory activity of EC on LPS-stimulated NO production was not due to any cytotoxic action on BV2 microglia.

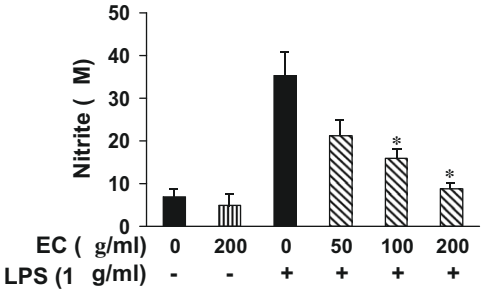

Fig. 1. Effect of EC on LPS-induced NO production in BV2 microglia. BV2 cells were pretreated with the indicated concentrations of EC for 2 h before being incubated with LPS (1 μg/ml) for 24 h. Culture supernatants were then isolated and analyzed for nitrite production. Each value indicates the mean ± S.E.M. from three independent experiments. * indicates a significant difference ($p < 0.05$) relative to cells treated with LPS in the absence of EC.

3.2. EC suppresses PGE_2 production in LPS-induced BV2 microglia

It is known that COX-2 produces large amounts of PGE_2 that induces inflammation involved in Alzheimer's disease, edema and fever (Coleman and Smith, 1994; Mitchell et al., 1995; Pasinetti and Aisen, 1998). Therefore, we investigated whether EC had any effect on the level of COX-2 mRNA and protein, as well as PGE2.

Because PGE_2 is the most prominent inflammatory product of COX-2 activity, we quantified PGE_2 in the supernatants. BV2 microglia were pretreated with EC for 2 h and then stimulated with 1 μg/ml LPS for 24 h. Pretreatment of the cells with EC (50, 100 or 200 μg/ml) and LPS resulted in a significant dose-dependent reduction in PGE_2 production (Fig. 2). These results show that pretreatment with EC significantly suppresses the expression of LPS-stimulated proinflammatory mediators.

3.3. EC represses the expression of iNOS and COX-2 in LPS-induced BV2 microglia

To determine the mechanism by which EC reduced LPS-induced NO and PGE_2 production, we studied the ability of EC (50, 100 or 200 μg/ml) to influence the LPS-induced production of iNOS or COX-2. As shown by Western blot analysis, LPS treatment significantly increased the expression of iNOS and COX-2. However, this expression was markedly attenuated in BV2 microglia pretreated with EC (Fig. 3). Western blots showed induction of iNOS and COX-2 proteins in BV2 microglia after 24 h of incubation with 1 μg/ml LPS (Fig. 3A). This induction was suppressed in a concentration-dependent manner by EC. RT-PCR analysis also showed

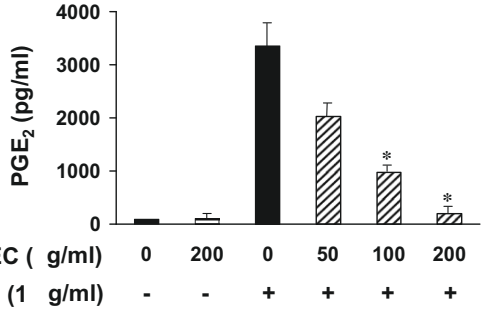

Fig. 2. Effects of EC on LPS-induced PGE_2 production in BV2 microglia. BV2 cells were incubated with EC (50, 100 or 200 μg/ml) in the presence or absence of LPS (1 μg/ml) for 24 h. PGE_2 concentration was measured in culture media using a commercial ELISA kit. Each value indicates the mean ± S.E.M. from three independent experiments. * indicates a significant difference ($p < 0.05$) relative to cells treated with LPS in the absence of EC.

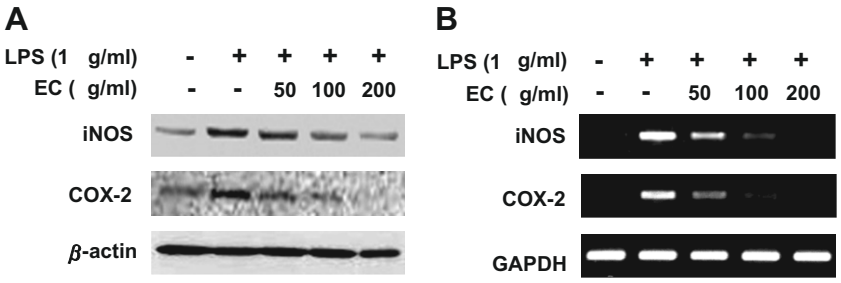

Fig. 3. Inhibition of LPS-induced iNOS and COX-2 protein (A) and mRNA (B) expression by EC in BV2 microglia. (A) BV2 cells (5×10^5 cell/ml) were incubated with the indicated concentrations of EC (50, 100 or 200 μg/ml) 2 h before LPS (1 μg/ml) treatment for 24 h. Cell lysates were electrophoresed, and the expression levels of iNOS and COX-2 were detected with specific antibodies. (B) After LPS treatment for 6 h, total RNA was prepared from BV2 microglia and RT-PCR was preformed for the *iNOS* and *COX-2* genes. β-Actin and GAPDH were used as internal controls for Western blot analysis and RT-PCR assays, respectively. This experiment was preformed in triplicate and similar results were obtained.

that iNOS and COX-2 mRNA levels were correlated with the levels of the corresponding proteins (Fig. 3B). These findings indicate that treatment with EC significantly suppressed the LPS-stimulated induction of iNOS and COX-2 through transcriptional inhibition.

3.4. EC attenuates TNF-α and IL-1β production in LPS-induced BV2 microglia

We next attempted to evaluate the potential effects of EC on the production of the proinflammatory cytokines TNF-α and IL-1β. BV2 cells were incubated with EC (50, 100 or 200 μg/ml) in the presence or absence of LPS (1 μg/ml) for 24 h, and TNF-α and IL-1β levels were measured in the culture media using an ELISA. The levels of both cytokines were increased in the culture media of LPS-stimulated BV2 microglia, and these increases were significantly decreased in a concentration-dependent manner by treatment with EC (Fig. 4A). In a parallel experiment, RT-PCR was performed to determine whether EC inhibited the expression of these cytokines at the transcriptional level. Treatment of BV2 microglia with different concentrations of EC for 2 h before LPS treatment resulted in a dose-dependent decrease in the mRNAs encoding IL-1β and TNF-α (Fig. 4B). These results suggest that EC acts primarily by preventing the accumulation of proinflammatory cytokines at the transcriptional level.

3.5. EC inhibits LPS-stimulated NF-κB activation in LPS-induced BV2 microglia

Activation of NF-κB is necessary for the induction of iNOS, COX-2 genes and cytokines. We next examined the activation of NF-κB in BV2 microglia in response to LPS (Fig. 5). It is known that NF-κB, when activated by LPS, enters the nucleus and induces gene expression. The heteromeric NF-κB complex is sequestered in the cytoplasm as an inactive precursor, complexed with an inhibitory IκB-like protein, and LPS induces NF-κB activation by increasing levels of the nuclear p65 protein, which is associated with decreased levels of cytosolic IκB-α protein. LPS treatment caused a significant increase in the DNA-binding activity of NF-κB, as measured using an electrophoretic mobility shift assay (Fig. 5A). In contrast, treatment with EC significantly suppressed the induced DNA-binding activity of NF-κB by LPS.

We also investigated the effect of EC on LPS-induced NF-κB p65 nuclear translocation. Significant levels of NF-κB p65 localized to the nucleus 15 min after LPS treatment (Fig. 5B). Levels of the p65 protein decreased in the nuclei of cells exposed to both LPS and EC, indicating that EC inhibits the nuclear translocation of the p65 protein. To determine whether the inhibition of NF-κB DNA-binding by EC was related to I-κBα degradation, the cytoplasmic levels of I-κBα were examined by Western blot analysis. Pretreatment of BV2 microglia with EC blocked LPS-induced I-κBα degradation. This finding provides evidence that EC inhibits the activation of NF-κB.

To clearly understand the influence of EC on NF-κB p65 nuclear translocation, the NF-κB p65 nucleus shift in BV2 microglia was analyzed using confocal microscopy (Fig. 5C). Confocal images revealed that NF-κB p65 was normally sequestered in the cytoplasmic compartment (Fig. 5C, Medium panel), and robust nuclear accumulation of NF-κB p65 was induced in BV2 microglia following stimulation with LPS (Fig. 5C, LPS panel). The LPS-induced translocation of NF-κB p65 was completely abolished by pretreatment with EC (Fig. 5C, LPS + EC panel). The translocation of NF-κB p65 was not induced in cells after pretreatment with EC alone in

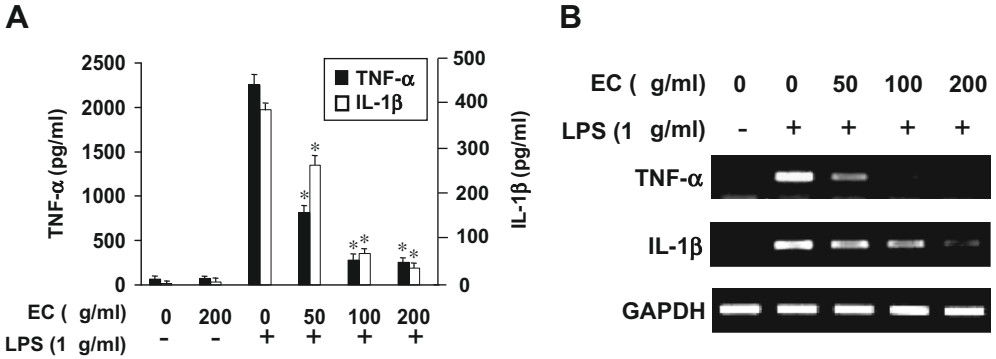

Fig. 4. Effects of EC on LPS-induced TNF-α and IL-1β production in BV2 microglia. BV2 cells were incubated with EC (50, 100 or 200 μg/ml) for 2 h before LPS treatment (1 μg/ml), and total RNA and the supernatants were isolated at 3 h and 24 h after LPS treatment, respectively. Extracellular levels of TNF-α and IL-1β were measured in culture media using commercial ELISA kits (A). After incubation for 3 h, the levels of TNF-α and IL-1β mRNAs were determined by RT-PCR (B). Each value indicates the mean ± S.E.M. from three independent experiments. * indicates a significant difference ($p < 0.05$) relative to cells treated with LPS in the absence of EC.

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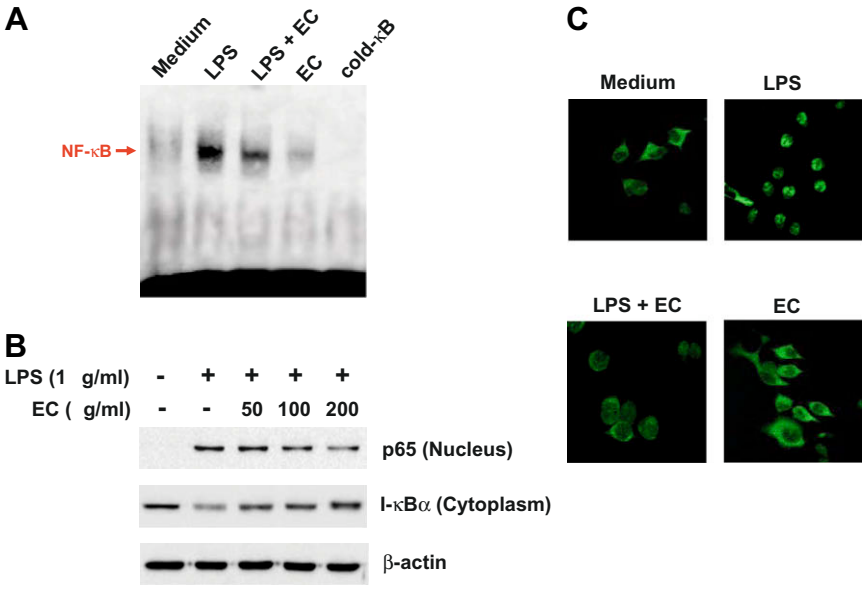

Fig. 5. Effects of EC on NF-κB activity in LPS-stimulated BV2 microglia. (A) Nuclear extracts (1 μg) were prepared and analyzed for DNA-binding activity of NF-κB using an electrophoretic mobility shift assay. BV2 microglia cells were pretreated with vehicle or the indicated concentrations of EC for 2 h before stimulation with LPS (1 μg/ml) for another 1 h. The result shown is representative of three independent experiments. (B) The p65 subunit of NF-κB in nuclear protein extracts and levels of I-κBα in the cytosolic protein were determined by a Western blot analysis. BV2 cells were treated with LPS (1 μg/ml) for 0.5 h, and p65 protein and I-κBα were detected using specific antibodies. (C) BV2 microglia cells were pretreated with 200 μg/ml EC for 2 h before stimulation with LPS (1 μg/ml) for 20 min. The p65 protein localization in cells was determined with an anti-p65 antibody and a FITC-labeled anti-rabbit IgG antibody, and cells were viewed with laser confocal scanning microscopy. A representative of three to five independent experiments is shown.

the absence of LPS stimulation (Fig. 5C, EC panel). These results show that EC inhibits the translocation of NF-κB p65. Taken together, these results suggest that the inhibition of NF-κB activation by EC is the mechanism responsible for the EC-mediated suppression of NO, PGE_2 and proinflammatory cytokines in BV2 microglia.

3.6. EC reduces LPS-induced ROS production in LPS-induced BV2 microglia

To assess the mechanism responsible for the inhibitory effect of EC on NF-κB activation, we examined the effect of EC on the LPS-induced production of ROS in BV2 microglia. It has been reported that ROS are involved in the activation of NF-κB (Gloire et al., 2006). Cells were incubated with EC (50, 100 or 200 μg/ml) in the presence or absence of LPS (1 μg/ml) for 24 h. As shown in Fig. 6, LPS-stimulated BV2 microglia showed increased ROS production. In contrast, pretreatment of cells with EC resulted in a significant reduction in ROS production in the presence of LPS.

3.7. EC inhibits LPS-stimulated phosphorylation of MAPKs in BV2 microglia

Subsequent experiments were designed to elucidate the signaling cascades that induce the expression of the iNOS and COX-2 genes in BV2 microglia in response to stimulation by LPS. There is evidence that MAP kinases play a key role in the regulation of cell growth and differentiation and in the control of cellular responses to cytokines and stresses, as well as in the activation of NF-κB. Moreover, MAP kinase is known to be important in iNOS and COX-2 expression.

To investigate whether the inhibition of NF-κB activation by EC was mediated via the MAP kinase pathway, we examined the effect of EC on the LPS-induced phosphorylation of ERK-1/2, p38 and JNK/SAPK in BV2 microglia using Western blot analysis (Fig. 7). We have shown that these proteins are phosphorylated following stimulation with LPS. Thus, we examined the effects of EC on the LPS-induced activation of ERK-1/2, p38 and JNK/SAPK MAP kinase. EC (200 μg/ml) markedly inhibited p38, ERK-1/2 and JNK/SAPK MAP kinase activation. The amount of non-phosphorylated p38, ERK-1/2 and JNK/SAPK was unaffected by LPS or EC treatment. Therefore, it appears that the MAP kinase pathways are important in the LPS-mediated expression of iNOS and COX-2. These results suggest that phosphorylation of ERK-1/2, JNK/SAPK, and p38 MAPK is involved in the inhibitory effect of EC on LPS-induced NF-κB binding in BV2 microglia.

4. Discussion and conclusions

The present study was undertaken to examine the pharmacological and biological effects of EC on the production of inflammatory mediators in murine macrophage BV2 microglia stimulated with LPS. To further understand the molecular mechanism of EC activity in microglia, we investigated the effects of EC on the production of NO and PGE_2, the expression levels of iNOS, COX-2 and cytokines (TNF-α, IL-1β), the activation of MAPKs, and the activation of the transcription factor NF-κB. The results of this study indicate that EC effectively inhibits LPS-induced production of TNF-α, IL-1β, NO and PGE_2 through a blockade of the NF-κB and MAPK pathways in BV2 microglia. In addition, treatment with EC did not result in significant cytotoxicity in LPS-stimulated BV2 microglia. The inhibitory effect of EC on inflammatory mediator expression suggests one of the mechanisms responsible for its anti-inflammatory action and its potential for use as a therapeutic agent for treating LPS-stimulated brain injury.

It has been reported that abnormalities in the production or function of cytokines, such as TNF-α and IL-1β, may play roles in many inflammatory lesions (De Nardin, 2001). Cytokines are released in the brain following many different neuropathological stimuli. Activated microglia contribute secondarily to inflammation-mediated tissue destruction via the release of cytokines such as IL-1β and TNF-α. TNF-α is primarily produced by monocytes,

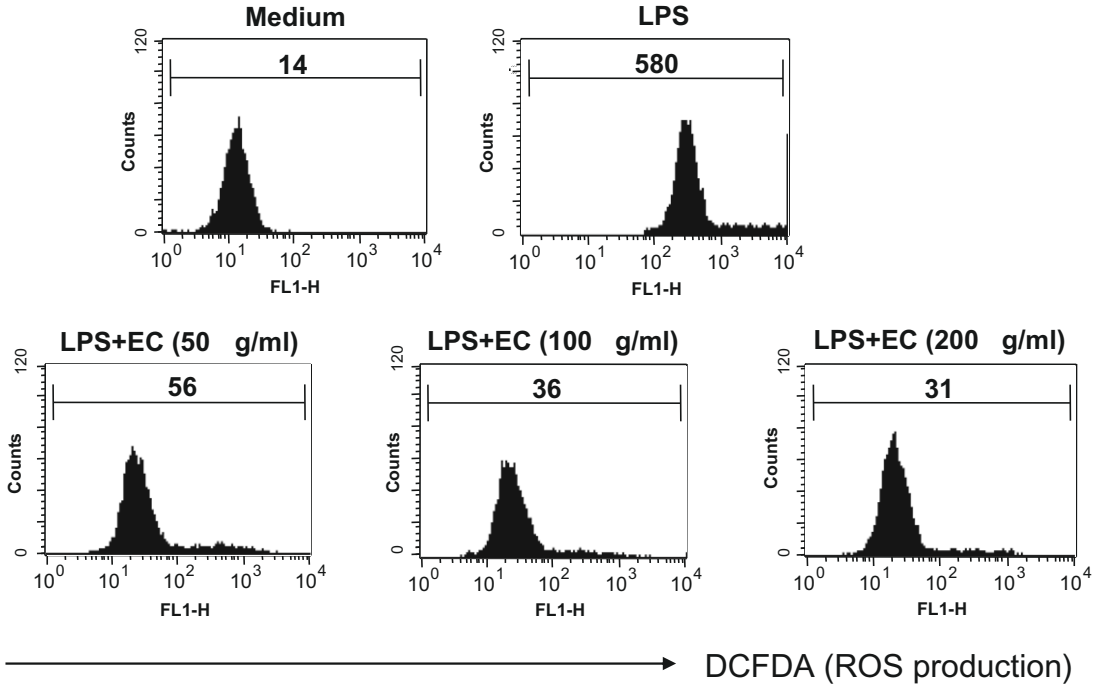

Fig. 6. Effects of EC on LPS-induced ROS production in BV2 microglia. BV2 cells were pretreated with the indicated concentrations of EC (50, 100 or 200 μg/ml) 2 h before LPS (1 μg/ml) treatment for 24 h. Cells were resuspended and mean fluorescence intensity (MFI) was measured using flow cytometry. Each value represents the results of three independent experiments.

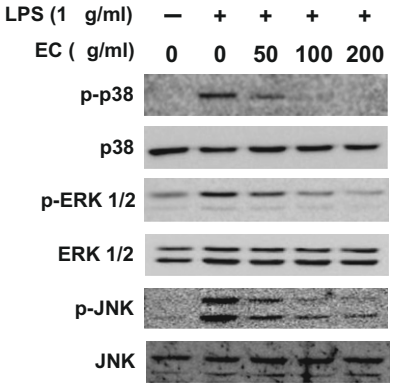

Fig. 7. Effects of EC on LPS-induced phosphorylation of ERK-1/2, SAPK/JNK and p38 MAP kinase in BV2 microglia cells. BV2 cells were treated with vehicle or the indicated concentrations of EC (50, 100 or 200 μg/ml) for 2 h before incubation with LPS (1 μg/ml). Cell lysates were then prepared and subjected to Western blotting with antibodies specific for phosphorylated forms of ERK-1/2, SAPK/JNK and p38. Results represent three independent experiments.

macrophages and T cells, and may play a role in innate immune response. As such, it is a potent activator of macrophages and can stimulate the production or expression of IL-6, IL-1β, PGE_2, collagenase and adhesion molecules. TNF-α has been implicated in the pathogenesis of many neurological conditions, gliosis, cerebral edema and exacerbation of amyloid neurotoxicity (Arvin et al., 1995; Selmaj and Raine, 1988; Shohami et al., 1993). The *IL-1* gene is associated with an increased risk of developing clinical AD (Licastro et al., 2000) and is overexpressed in activated astroglia cells from AD brains (Griffin et al., 1989). Following CNS damage, IL-1 is rapidly released from activated microglia. Thus, the inhibition of cytokine production or function is a key mechanism in the control of inflammation. In the present study, we found that EC significantly inhibited the production of the proinflammatory cytokines TNF-α and IL-1β in BV2 microglia stimulated by LPS. These findings provide evidence that EC possesses potentially useful anti-inflammatory activities.

NF-κB is a pleiotropic regulator of various genes involved in immune and inflammatory responses. It has been shown that NF-κB activation is a key transcription factor which activates several cellular signal transduction pathways that are implicated in the production of iNOS, COX-2 and various cytokines (Baeuerle and Henkel, 1994; Baldwin, 1996). The promoter region of the murine gene encoding iNOS and COX-2 contains NF-κB binding motifs. It has been reported that binding of NF-κB to the NF-κB sites upstream of the *iNOS* and *COX-2* promoters plays an important role in the LPS-induced upregulation of the *iNOS* and *COX-2* genes (Lee et al., 2003). Because the expression of these proinflammatory mediators is modulated by NF-κB, our findings suggest that EC treatment blocks the degradation of IκB and activation of NF-κB in BV2 microglia. In this study, we describe novel anti-inflammatory mechanisms mediated by EC based on the inhibition of the LPS-mediated activation of NF-κB.

It has been shown that several natural antioxidant compounds directly inhibit the expression of the NF-κB-dependent cytokines iNOS and COX-2, and thus reduce inflammation (Ma et al., 2003; Surh et al., 2001), and the activation of the NF-κB complex is related with the cellular redox state (Hirota et al., 1999). The suppressive effects of these antioxidant compounds on the production of the associated inflammatory mediators are associated with their antioxidant activities. The antioxidant NF-κB inhibitors restrict the production of inflammatory mediators by suppressing the expression of the corresponding genes, and also prevent inflammatory diseases. Moreover, changes in intracellular ROS can regulate signal transduction pathways, leading to the modulation of NF-κB activity. ROS are associated with neuroinflammatory and neurodegenerative processes (Brown and Bal-Price, 2003; Floyd, 1999). Recently, EC has been reported to exhibit antioxidant and anti-inflammatory activity (Shin et al., 2006). However, the biological activity and molecular mechanisms of EC's actions have not yet been shown in LPS-stimulated microglia.

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In the present study, we demonstrated that EC has intracellular radical scavenging activity in BV2 microglia, suggesting a possible mechanism for the inhibitory effect of EC on NF-κB activation. Therefore, the potential inhibition of ROS generation by EC is consistent with the inhibition of NF-κB-dependent cytokines and iNOS and COX-2 expression, and thus reduced inflammation.

Various intracellular signaling pathways are involved in the modulation of NF-κB activity and inflammatory cytokine expression. The MAPKs are a group of signaling molecules that appear to play important roles in inflammatory processes. LPS regulates iNOS and COX-2 expression through a MAPK signaling pathway. As such, LPS treatment results in the phosphorylation of p38, ERK-1/2 and JNK, leading to NF-κB activation in microglia (Hou et al., 2006; Kim et al., 2006b; Moon et al., 2007). In addition, activation of MAPK has been demonstrated to be important in the regulation of iNOS and COX-2 expression through control of the activation of NF-κB (Pergola et al., 2006; Suh et al., 2006). Thus, we investigated the effect of EC on the LPS-stimulated phosphorylation of MAPKs in BV2 microglia. The present study indicates that EC is a potent inhibitor of MAPKs such as ERK, p38 and JNK induced by LPS stimulation in BV2 microglia. These results suggest that MAPKs are involved in the inhibitory effect of EC on LPS-induced iNOS and COX-2 expression, and NF-κB activation.

In summary, we have demonstrated that treatment of BV2 microglia with EC can decrease levels of pro-inflammatory mediators following LPS stimulation. EC significantly inhibited the release of NO, PGE_2, TNF-α and IL-1β in a concentration-dependent manner, acting at the transcriptional level. The anti-inflammatory properties of EC were mediated by the downregulation of MAPKs, NF-κB, and the inhibition of ROS accumulation. The inhibition of the microglial inflammatory response is considered a promising target for the treatment of many neuropathologies. Thus, we conclude that EC possesses potential anti-inflammatory activity and beneficial characteristics for the treatment of neurodegenerative diseases under many neuroinflammatory conditions. However, additional studies are needed to determine which component of EC may contribute to its anti-inflammatory activity.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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