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Inhibitory Effects and Molecular Mechanism of Dieckol Isolated from Marine Brown Alga on COX-2 and iNOS in Microglial Cells

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To identify the neuroprotective effect of dieckol, a hexameric compound of phloroglucinol isolated from marine brown alga, *Ecklonia cava*, this study investigated the anti-inflammatory effect of dieckol on lipopolysaccharide (LPS)-stimulated murine BV2 microglia and elucidated the molecular mechanism. The results showed that dieckol suppresses LPS-induced production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) and expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in a dose-dependent manner, without causing cytotoxicity. It also significantly reduced the generation of proinflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α. Moreover, dieckol significantly reduced LPS-induced nuclear factor κB (NF-κB) and p38 mitogen-activated protein kinases (MAPKs) activation, as well as reactive oxygen species (ROS) production. Taken together, the inhibition of LPS-induced NO and PGE_2 production might be due to the suppression of NF-κB and p38 MAPK signal pathway and, at least in part, by inhibiting the generation of ROS. Hence, these effects of dieckol might assist therapeutic treatment for neurodegenerative diseases that are accompanied by microglial activation.

KEYWORDS: *Ecklonia cava*; dieckol; BV2 microglia; inducible nitric oxide synthase; cyclooxygenase-2

INTRODUCTION

Microglia, a specialized form of macrophage residing with a wide distribution in the brain, are believed to play a key role in host defense and tissue regeneration in the central nervous system (CNS) (*1*). Microglia function as macrophages in CNS; they migrate to the area of injured nervous tissue, and they engulf and destroy microbes and cellular debris. Chronic microglial activation and consequent overproduction of pro-inflammatory mediators such as nitric oxide (NO), prostaglandin E_2 (PGE_2), reactive oxygen, and pro-inflammatory cytokines [interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α] are pathological hallmarks of various neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), trauma, multiple sclerosis, and cerebral ischemia (*2*). Previous studies have demonstrated that reduction of pro-inflammatory mediators in microglia may attenuate the severity of these disorders (*3*). Therefore, mechanisms to regulate microglial activation may have important therapeutic potential for the treatment of many neurodegenerative diseases.

Lipopolysaccharide (LPS), a major constituent of Gram-negative bacteria, initiates a number of major cellular responses that play critical roles in the pathogenesis of inflammatory responses and has been employed to induce microglial activation. Therefore, LPS stimulation of the microglia is a powerful model for the study of mechanisms underlying neuron damage by various neurotoxic factors released from activated microglia (*4*). During the inflammatory processes, large amounts of neurotoxic factors are released. Among these mediators, PGE_2 and NO are products of the inducible isoforms of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) enzymes, respectively (*5*). COX is the enzyme catalyzing the conversion of arachidonic acid to prostaglandin H2, the precursor of a variety of biologically active mediators, such as PGE_2, prostacyclin, and thromboxane A2 (*6*). COX exists as two major isozymes: COX-1, a constitutive COX, and COX-2, an isoform induced in response to many stimulants and activated at the site of the inflammation (*5*). COX-2, which is induced in macrophages and endothelial

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cells by proinflammatory cytokines, may be responsible for the edema and vasodilation associated with inflammation. Several studies have reported that COX-2 is associated with cytotoxicity in brain diseases because inhibition of COX-2 induction and/or activity reduces brain injury after ischemia and the progression of AD and PD (*7*). NO is produced by immune-activated macrophages at various sites of inflammation. NO has been shown to be an important biological messenger molecule in diverse physiological functions, including vasodilation, neural communication, and host defense (*8*). It was reported that iNOS is not normally expressed in the brain, but LPS up-regulates iNOS expression in microglial cells, astrocytes, and possibly neurons (*9*). Excessive release of NO by activated microglia is correlated with the progression of neurodegenerative disorders. Taken together, the inflammatory mediators, including iNOS and COX-2, are responsible for the symptoms of much neuronal damage in neurodegenerative diseases, including stroke, cerebral ischemia, PD, and AD. Therefore, the inhibition of these inflammatory mediators is an important target in the treatment of inflammatory diseases including brain injury.

Brown algae, mostly used as sources for alginic acid in industry, are also widely consumed as food in many Asian and several European countries. Brown algae have two major chemical groups: polysaccharide and polyphenols. *Ecklonia cava* is an edible brown alga (Laminariaceae) that is present in the subtidal regions of Jeju Island in Korea. *E. cava* is used to produce food ingredients, animal feed, fertilizers, and folk medicine in gynecopathy. Recently, an increasing amount of evidence has demonstrated that *E. cava* exhibits radical scavenging, matrix metalloproteinase inhibitory, bactericidal, protease inhibitory, antioxidative, and anti-inflammatory activities (*10*−*12*). A number of medicinal plants in Asian countries contain tannins, which are regarded as active components. Phlorotannin components, which are oligomeric polyphenols of phloroglucinol units, are responsible for the pharmacological activities of *E. cava*, and phlorotannins such as eckol (a closed-chain trimer of phloroglucinol), 6,6′-bieckol (a hexamer), dieckol (a hexamer), phlorofucofuroeckol (a pentamer) were identified in *Ecklonia* species. Several studies using phlorotannins have been performed to examine their pharmacological actions including α2-antiplasmin activity (*13*), antioxidant activity (*14*), anti-inflammatory activity by inhibiting hyaluronidase (*15*), and bactericidal activity (*16*).

In our previous paper (*17*), the anti-inflammatory activity of *E. cava* extract was evaluated by a murine asthma model. To identify the components responsible for the above activity, 10 phloroglucinol derivatives from an ethyl acetate fraction of *E. cava* extract with the highest activity in comparison to the other fractions were purified, and their structures were identified successfully. Recently, our results showed that fucodiphloroethol G and phlorofucofuroeckol A could be the key effectors in the phlorotannins against allergic pathway on basophilic leukemia (KU812 and RBL-2H3) cell lines (*18*). In our preliminary investigation, we observed that dieckol, a hexameric compound of phloroglucinol with a dibenzo-1,4-dioxin unit in the molecular skeleton, possessed very strong neuroprotective activity. A recent study reported that dieckol was shown to inhibit the generation of reactive oxygen species (*19*). Nevertheless, the effects of dieckol on the expression of inflammation-related genes and molecular mechanisms in LPS-stimulated microgliahave not been demonstrated.

In this study, we investigated the inhibitory effects and mechanisms of action of dieckol isolated from *E. cava* extract on endotoxin-stimulated pro-inflammatory enzymes such as iNOS and COX-2, which reduce iNOS-derived NO and COX-2-derived PGE_2 production in the murine BV2 microglia, suggesting that dieckol might be considered as a possible candidate for controlling the neurodegenerative disorder, and the clinical relevance of the agent should be further elucidated.

MATERIALS AND METHODS

Materials and Chemicals. Marine brown alga *E. cava* was collected along the coast of Jeju Island, Korea, between October 2004 and March 2005. The samples were washed three times with tap water to remove the salt, epiphytes, and sand attached to the surface, then carefully rinsed with fresh water, and maintained in a medical refrigerator at −20 °C. Later, the frozen samples were lyophilized and homogenized with a grinder prior to extraction. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a JEOL JNM-ECP 400 NMR spectrometer (JEOL, Japan), using DMSO-d_6 solvent peak (2.50 ppm in ^{1}H and 39.5 ppm in ^{13}C NMR) as an internal reference standard. Sephadex LH-20 was obtained from Sigma, St. Louis, MO. Thin-layer chromatography (TLC) was run on precoated Merck Kieselgel 60 F254 plates (0.25 mm), and the spots on the TLC plate were detected under a UV lamp (254 and 365 nm) using $CHCl_3$/MeOH/H_2O/acetic acid (65:25:4:3, v/v/v/v) as a development solvent system. Vanillin-H_2SO_4 was employed as the detecting agent for phenolic compounds (*20*). All of the solvents for column chromatography were of reagent grade from commercial sources. LPS from *Escherichia coli* serotype 0111:B4 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma. Specific antibodies against iNOS, COX-2, and p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against IκB, ERK, JNK, p38, phosphorylated (p)-ERK, p-p38, and p-JNK were purchased from Cell Signaling Technology (Beverly, MA).

Isolation and Structural Conformation. The dried *E. cava* powder (3 kg) was extracted three times with 80% MeOH and then filtered. The filtrate was evaporated at 40 °C to obtain the methanol extract. After the extract had been suspended in distilled water, it was partitioned with ethyl acetate. The ethyl acetate fraction was mixed with Celite. The mixed Celite was dried and packed into a glass column and eluted with hexane, methylene chloride, diethyl ether, and methanol in that order. The diethyl ether fraction was subjected to Sephadex LH-20 column chromatography using stepwise gradient chloroform/methanol (2:1 → 0:1) solvent systems. Dieckol (10.95 mg) was obtained by further purification using a HPLC system, and the purified compound was identified by comparing their ^{1}H and ^{13}C NMR data to the literature. The chemical structure of dieckol is indicated in **Figure 1**.

Dieckol: ^{1}H NMR (400 MHz, methanol-d_4) δ 6.15 (1H, s), 6.13 (1H, s), 6.09 (1H, d, 2.9 Hz), 6.06 (1H, d, 2.9 Hz), 6.05 (1H, d, 2.9 Hz), 5.98 (1H, d, 2.8 Hz), 5.95 (1H, d, 2.8 Hz), 5.92 (3H, m); ^{13}C NMR (100 MHz, methanol-d_4) δ 161.8, 160.1, 157.8, 155.9, 154.5, 152.4, 147.3, 147.2, 147.1, 146.9, 144.3, 144.1, 143.4, 143.3, 138.6, 138.5, 126.5, 126.2, 125.6, 125.5, 124.9, 124.6, 124.5, 99.9, 99.7, 99.5, 99.4, 97.6, 96.2, 95.8, 95.7, 95.3.

Cell Culture. The murine BV2 cell line was maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified incubator under 5% CO_2. Confluent cultures were passed by trypsinization. For experiments, cells were washed twice with warm DMEM (without phenol red) and then treated in serum-free medium. In all experiments, cells were treated with dieckol for the indicated times after the addition of LPS (1 μg/mL).

Cell Viability Assay. The cell viability was measured with blue formazan that was metabolized from colorless MTT by mitochondrial dehydrogenases, which were active only in live cells. BV-2 cells were preincubated in 24-well plates at a density of 5×10^5 cells per well for 24 h and then washed. Cells with various concentrations of dieckol disolved in 20% (v/v) EtOH were treated with LPS for 24 h and incubated in 0.5 mg/mL MTT solution. Three hours later, the supernatant was removed, and formation of formazan was measured at 540 nm with a microplate reader (Dynatech MR-7000; Dynatech Laboratories).

Isolation of RNA and RT-PCR. The total RNA was isolated using TRIzol reagent (Invitrogen, CA). The total RNA (1.0 μg) obtained from the cells was reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI) to produce the cDNAs. RT-generated cDNAs encoding iNOS, COX-2, IL-1β, and TNF-α genes were amplified using PCR. PCR was performed using selective primers for the mouse iNOS (5′-ATGTCCGAAGCAAACATCAC-3′ and

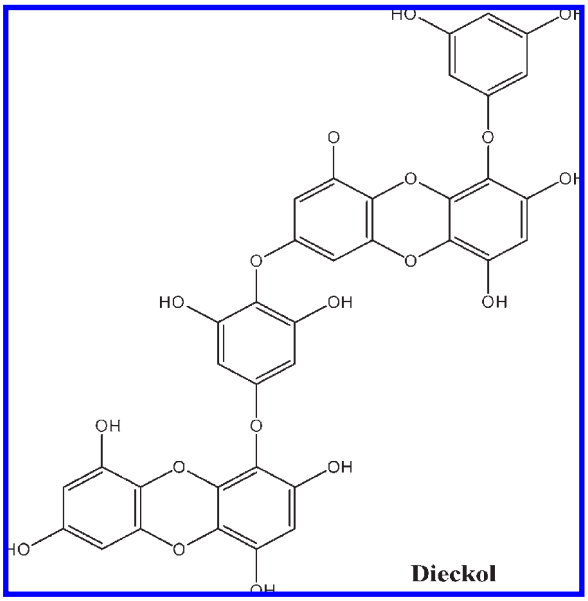

Figure 1. Chemical structure of dieckol isolated from *E. cava*.

5′-TAATGTCCAGGAAGTAGGTG-3′), COX-2 (5′-CAGCAAATC-CTTGCTGTTCC-3′ and 5′-TGGGCAAAGAATGCAAACATC-3′), IL-1β (5′-ATGGCAACTGTTCCTGAACTCAACT-3′ and 5′-TTTCC-TTTCTTAGATATGGACAGGAC-3′), and TNF-α (5′-ATGAGCA-CAGAAAGCATGATC-3′ and 5′-TACAGGCTTGTCACTCGAAT-T-3′). After amplification, portions of the PCR reactions were electrophoresed on agarose gel.

Nitrite Assay. The concentrations of NO in culture supernatants were determined as nitrite, a major stable product of NO, using the Griess reagent. After cells (5×10^5 cells/mL) had been 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 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.

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 poly(vinylidene difluoride) 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-20 (TBST), immunoreactive bands were visualized using the ECL detection system (Pierce). In a parallel experiment, nuclear protein was prepared using nuclear extraction reagents (Pierce) according to the manufacturer's protocol.

Cytokine Assays. The levels of cytokines were determined by enzyme-linked immunosorbent assay (ELISA). ELISA kits from R&D Systems (Minneapolis, MN) were employed for the measurement of IL-1β and TNF-α, and a kit from Cayman Chemical (Ann Arbor, MI) was employed for the measurement of PGE_2. The absorbance at 450 nm was determined using a microplate reader.

Measurement of Intracellular Reactive Oxygen Species (ROS) Generation. ROS were measured according to a method described previously with modification (*20*). BV2 microglia cells were washed with phosphate-buffered saline (PBS). To measure intracellular ROS, cells were incubated for 30 min at 37 °C with PBS containing 20 μM 2′,7′-dichlorofluorescein diacetate (DCFDA) (Molecular Probes, Eugene, OR) to label intracellular ROS. The cells were then immediately observed by fluorescence-activated cell sorting (FACS) analysis (BD Biosciences, Rutherford, NJ).

Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were prepared using NE-PER nuclear extraction reagent (Pierce). As a probe for the gel retardation assay, an oligonucleotide harboring the immunoglobulin κ-chain binding site (κB, 5′-CCGGTTAACAGAGG-GGGCTTTCCGAG-3′) was synthesized. A non-radioactive method whereby the 3′ end of the probe was labeled with biotin was employed in these experiments (Pierce). The binding reactions contained 10 μg of nuclear extract protein, buffer (10 mM Tris, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% Nonidet P-40, and 2.5% glycerol), 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 reaction mixture was electrophoretically analyzed on 5% polyacrylamide gel in 0.5× Tris−borate buffer. The reactions were transferred to nylon membranes. The biotin-labeled DNA was detected using a LightShift chemiluminescent electrophoretic mobility shift assay kit (Pierce). In all experiments, DNA-binding specificity was verified using a 50-fold excess of cold κB to the reaction mixture before the labeled probe was added.

Confocal Laser Scanning Microscopy Study. The NF-κB p65 nuclear localization was detected by indirect immunofluorescence assays using confocal microscopy. BV2 microglia cells were cultured directly on glass coverslips in 24-well plates for 24 h. After stimulation with 1 μg/mL LPS and/or 300 μg/mL dieckol, 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). Polyclonal antibodies to NF-κB p65 (1 μg/well) were applied for 1 h followed by 1 h of incubation with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laborities, Inc., West Grove, PA). After washing with PBS, the coverslips were mounted in Fluoromount-G (Southern Biotechnology Associates Inc., Birmingham, AL), and the fluorescence was visualized using a Zeiss LSM 510 laser scanning confocal device.

Statistical Analysis. The values presented represent means ± SEM. Statistical significance was determined by analysis of variance, followed by Scheffe's test. A value of $p < 0.05$ was deemed to be statistically significant.

RESULTS

Effect of Dieckol on NO and PGE_2 Production in LPS-Stimulated BV2 Microglia. To examine the inhibitory effect of dieckol on LPS-stimulated NO production in BV2 microglia, we measured nitrite released into the culture medium using the Griess reagent. The amount of produced NO was determined by the amount of nitrite, a stable metabolite of NO. BV2 microglia were treated with various concentrations of dieckol (0, 50, 100, or 300 μg/mL) for 2 h before the addition of LPS (1 μg/mL). Pretreatment with different doses of dieckol led to a significant reduction in the formation of NO, as measured in the supernatants 24 h following LPS stimulation (**Figure 2A**). According to the NO detection assay, NO was significantly increased to 5.7 times the basal level in BV2 microglia after 24 h of LPS stimulation, and this increase was inhibited by dieckol treatment in a dose-dependent manner.

PGE_2 represents the most important inflammatory product of COX-2 activity and, thus, it was quantified in the supernatant. To assess whether dieckol could inhibit production of LPS-induced PGE_2 in BV2 microglia, the cells were pretreated with dieckol for 2 h and then stimulated with 1 μg/mL LPS. After incubation for 24 h, the cell culture medium was harvested, and the production of PGE_2 was measured using ELISA. Pretreatment of the cells with dieckol (50, 100, or 300 μg/mL) and LPS resulted in a significant dose-dependent reduction in PGE_2 production (**Figure 2B**). These results show that pretreatment with dieckol significantly suppresses the expression of LPS-stimulated pro-inflammatory mediators.

To exclude the possibility that the inhibition of NO and PGE_2 production was due to cytotoxicity caused by dieckol treatment, MTT assays were performed in BV2 microglia treated with dieckol for 24 h (**Figure 2C**). At the concentrations used (50−300 μg/mL), dieckol did not affect cell viability. Thus, the inhibitory

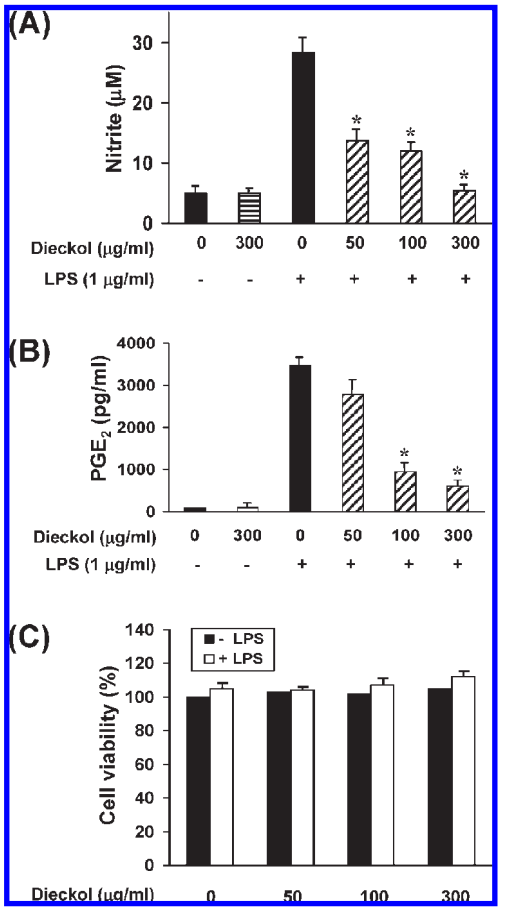

Figure 2. Effects of dieckol on NO (**A**) and PGE_2 (**B**) production and cell viability (**C**) in LPS-stimulated BV-2 microglia. Cells (5×10^5 cell/mL) were treated with the indicated concentration of dieckol (50, 100, or 300 μg/mL) 2 h before LPS (1 μg/mL) treatment for 24 h. Nitrite content was measured using the Griess reaction (**A**). The PGE_2 concentration was measured in culture media using a commercial ELISA kit (**B**). Cell viability was assessed by MTT reduction assays, and the results were expressed as percentage of surviving cells over control cells (no addition of LPS and dieckol) (**C**). Each value indicates the mean ± SEM from three independent experiments. * indicates a significant difference ($p < 0.05$) relative to cells treated with LPS in the absence of dieckol.

activity of dieckol on LPS-stimulated NO and PGE_2 production was not due to any cytotoxic action on BV2 microglia.

Effect of Dieckol on iNOS and COX-2 Expression in LPS-Induced BV2 Microglia. To confirm whether the inhibition of NO and PGE_2 production is due to a decreased level of iNOS and COX-2, the effect of dieckol on the level of iNOS and COX-2 protein and mRNA was determined by Western blot analysis and RT-PCR, respectively. The expression of iNOS and COX-2 protein was barely detectable in unstimulated BV2 microglia but was markedly increased after 24 h of LPS (1 μg/mL) treatment. However, dieckol significantly attenuated iNOS and COX-2 protein expression in LPS-stimulated BV2 microglia (**Figure 3A**). The effects of dieckol on *iNOS* and *COX-2* mRNA expression were also evaluated (**Figure 3B**). RT-PCR analysis also showed that *iNOS* and *COX-2* mRNA expression correlated with their protein levels. These results indicate that LPS exposure increased the expression of *iNOS* and *COX-2* mRNA and protein, but treatment with dieckol significantly suppressed the induction of LPS-stimulated mediators through transcriptional inhibition.

Effects of Dieckol on LPS-Induced TNF-α and IL-1β Production. We next attempted to determine the potential effects of

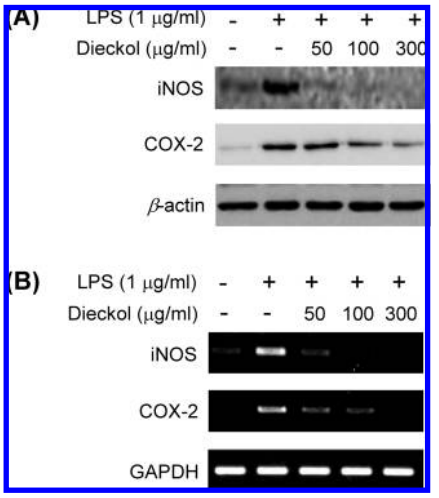

Figure 3. Inhibition of LPS-induced iNOS and COX-2 protein (**A**) and mRNA (**B**) expression by dieckol in BV2 microglia. (**A**) BV2 cells (5×10^5 cell/mL) were incubated with the indicated concentration of dieckol (50, 100, or 300 μg/mL) 2 h before LPS (1 μg/mL) treatment for 24 h. Cell lysates were 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 performed 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.

dieckol on the production of pro-inflammatory cytokines such as TNF-α and IL-1β. BV2 microglia were incubated with dieckol (0, 50, 100, or 300 μg/mL) in the presence or absence of LPS (1 μg/mL) for 24 h, and the cytokine levels were measured in the culture media by ELISA. As shown in **Figure 4A**, the TNF-α and IL-1β levels 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 dieckol. In a parallel experiment, we performed RT-PCR to determine whether dieckol inhibits the expression of these cytokines at a transcriptional level. As shown in **Figure 4B**, treatment of BV2 microglia with different concentrations of dieckol 2 h before LPS treatment resulted in a dose-dependent decrease in *IL-1β* and *TNF-α* mRNA. The results suggest that dieckol negatively regulates the accumulation of pro-inflammatory cytokines at the transcriptional level.

Effects of Dieckol on LPS-Induced Nuclear Translocation of NF-κB and I-κBα. NF-κB is one of the principal factors for COX-2 and iNOS expression mediated by LPS or proinflammatory cytokines. To characterize further the molecular mechanism of dieckol inhibition of iNOS and COX-2 protein level, NF-κB DNA-binding activity was determined by an electrophoretic mobility shift assay (**Figure 5A**). LPS treatment caused a significant increase in the DNA-binding activity of NF-κB. In contrast, the treatment of dieckol markedly suppressed the induced activity of NF-κB by LPS.

We also investigated the effect of dieckol on LPS-induced NF-κB p65 nuclear translocation as measured by Western blot analysis, because translocation of NF-κB to the nucleus has been shown to be required for NF-κB-dependent transcription following LPS stimulation. As shown in **Figure 5B**, significant levels of NF-κB p65 were localized to the nucleus at 1 h after LPS treatment. The p65 protein decreased in the nucleus of cells exposed to LPS in combination with dieckol, which verified that dieckol inhibited nuclear translocation of p65 protein.

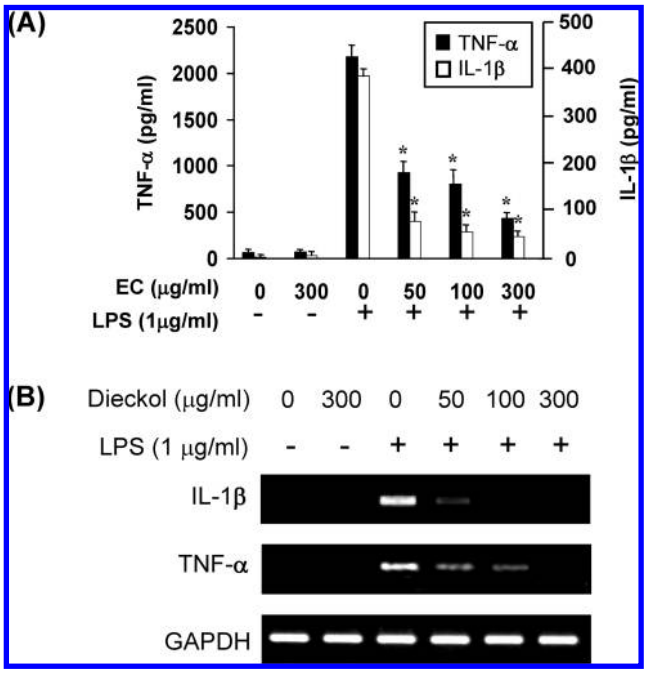

Figure 4. Effects of dieckol on LPS-induced TNF-α and IL-1β production in BV2 microglia. BV2 cells were incubated with dieckol (50, 100, or 300 μg/mL) for 2 h before LPS treatment (1 μg/mL), and total RNA and the supernatants were isolated at 6 and 24 h after LPS treatment, respectively. Extracellular levels of TNF-α and IL-1β were measured in culture media using commercial ELISA kits (**A**). After incubation for 6 h, the levels of TNF-α and IL-1β mRNAs were determined by RT-PCR (**B**). Each value indicates the mean ± SEM from three independent experiments. * indicates a significant difference ($p < 0.05$) relative to cells treated with LPS in the absence of dieckol.

Dieckol pretreatment significantly attenuated this nuclear translocation. The nuclear translocation and DNA binding of the NF-κB transcription factor are preceded by the degradation of inhibitory factor-κBα (I-κBα). To determine whether the inhibition of NF-κB DNA binding by dieckol is related to I-κBα degradation, cytoplasmic levels of I-κBα were examined by a Western blot analysis (**Figure 5B**). Pretreatment of BV2 microglia with dieckol blocked LPS-induced I-κBα degradation. The recovery of I-κBα protein in BV2 microglia provides strong evidence that dieckol inhibited the activation of NF-κB.

To clearly understand the influence of dieckol on the NF-κB p65 nuclear translocation, the NF-κB p65 nucleus shift situation in BV2 microglia was determined by immunofluorescence analysis (**Figure 5C**). After fixation, the cells were stained with anti-p65 antibody and observed at 400× magnification. Confocal images revealed that NF-κB p65 was normally sequestered in the cytoplasmic compartment (**Figure 5C**, Medium panel), and nuclear accumulation of NF-κB p65 was strongly induced after stimulation of BV2 microglia with LPS (**Figure 5C**, LPS panel). The LPS-induced translocation of NF-κB p65 was completely abolished by pretreatment of the cells with dieckol (**Figure 5**C, LPS + Dieckol panel). The translocation of NF-κB p65 was not induced in the cells after pretreatment with dieckol alone in the absence of LPS stimulation (**Figure 5C**, Dieckol panel). The results showed that dieckol inhibited the translocation of NF-κB p65. These results clearly showed that the inhibition of NF-κB activation by dieckol may be the mechanism responsible for the suppression of NO, PGE_2, and pro-inflammatory cytokines in BV2 microglia.

Effect of Dieckol on the Phosphorylation of MAPKs in LPS-Stimulated BV2 Microglia. The subsequent experiments were designed to elucidate the signaling cascades, which turn on the expression of iNOS and COX-2 gene in BV2 microglia cells in response to stimulation by LPS. Evidence has accumulated that the mitogen-activated protein (MAP) kinases play a critical role in the regulation of cell growth and differentiation and in the control of cellular responses to cytokines and stresses. They play a critical role for the activation of NF-κB. Moreover, MAP kinase has been known to be important for the expression of iNOS and COX-2 expression. To investigate whether the inhibition of NF-κB activation by dieckol is mediated through the MAP kinase pathway, we examined the effect of dieckol on the LPS-induced phosphorylation of ERK-1/2, JNK, and p38 kinase in BV2 microglia using Western blot analyses (**Figure 6**). In our study, we have shown that ERK-1/2, JNK, and p38 kinase were phosphorylated by stimulation with LPS. Therefore, we examined the effect of dieckol on LPS-induced activation of MAP kinase. As shown in **Figure 6**, dieckol (300 μg/mL) markedly inhibited p38 kinase activation, whereas phosphorylation of ERK-1/2, JNK was not affected by dieckol treatment. These results suggest that phosphorylation of p38 is involved in the inhibitory effect of dieckol on LPS-induced iNOS and COX-2 expression in BV2 microglia.

Effects of Dieckol on LPS-Induced ROS Production. Several lines of investigation suggest that suppression of LPS-induced ROS results in diminution of NF-κB activity and subsequent inhibition of NF-κB gene expression (*21*). To assess whether dieckol inhibits ROS generation, the BV2 microglia were incubated with dieckol (0, 50, 100, or 300 μg/mL) in the presence or absence of LPS (1 μg/mL) for 30 min. As shown in **Figure 7**, the LPS-stimulated BV2 microglia shows an increase in ROS production. In contrast, pretreatment of cells with dieckol resulted in a significant scavenging of ROS production by LPS. When the cells were incubated with dieckol (300 μg/mL) alone, the concentration of ROS was maintained at a background level similar to that in the unstimulated samples.

DISCUSSION

Microglia play an important role in neuroinflammatory conditions. Activated microglia produce various pro-inflammatory and neurotoxic mediators. In the present study, we evaluated dieckol, which is a phlorotannin component of *E. cava* having pharmacological and biological effects on the production of inflammatory mediators in BV2 microglia stimulated with LPS. We investigated the effect of dieckol on the production of NO, PGE_2, and ROS, expression of iNOS and COX-2, and cytokines (TNF-α, IL-1β). The results indicate that dieckol is an effective inhibitor of LPS-induced cytokines and expression of iNOS and COX-2 through blockade of NF-κB and MAPK pathway and/or antioxidative activity in BV2 microglia. Therefore, inhibition of these mediators may have beneficial effects in the treatment of microglia-mediated oxidative stress and neuroinflammatory reaction.

Activation of microglia was observed to induce brain injury by release of pro-inflammatory mediators and neurotoxic compounds, such as IL-1β, and TNF-α, reactive oxygen species, NO, and PGE_2. These pro-inflammatory mediators and cytokines are thought to be responsible for pathological conditions of neurodegenerative disease, such as AD, cerebral ischemia, and multiple sclerosis. In this regard, the inhibition of those pro-inflammatory mediators and cytokines would be an effective therapeutic approach to relieve the progression of neurodegenerative diseases.

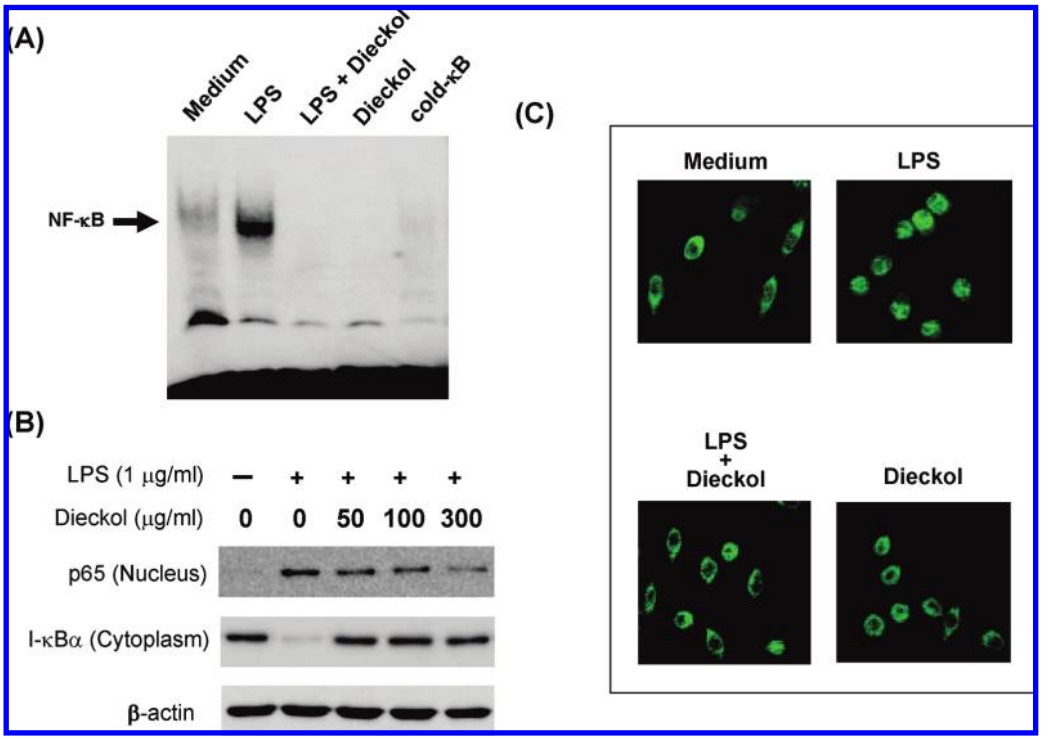

Figure 5. Effect of dieckol on NF-κB activity in LPS-stimulated BV2 microglia. (**A**) Nuclear extracts (1 μg) were prepared and analyzed for DNA binding activity of NF-κB using an electrophoretic mobility shift assay. BV2 microglia cells were pretreated with vehicle or the indicated concentrations of dieckol 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 1 h, and p65 protein and I-κBα were detected using specific antibodies. (**C**) BV2 microglia cells were pretreated with 300 μg/mlL dieckol for 2 h before stimulation with LPS (1 μg/mL) for 1 h. 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.

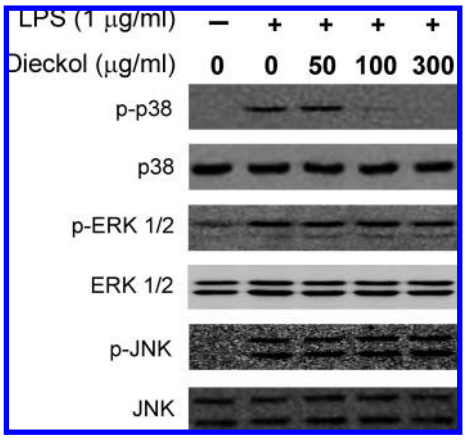

Figure 6. Effects of dieckol on LPS-induced phosphorylation of MAP kinase in BV2 microglia cells. BV2 cells were treated with vehicle or the indicated concentration of dieckol (50, 100, or 300 μg/mL) for 2 h before incubation with LPS (1 μg/mL). Cell lysates were then prepared and subjected to Western blotting with antibodies specific for phosphorylated forms of ERK-1/2, SAPK/JNK, and p38. Results represent three independent experiments.

Marine algae provide a source of chemical diversity that can be used to develop new chemopreventive agents and have recently been identified as an underexploited plant resource and functional food (*22*). Polyphenols are one of the most common classes of secondary metabolites in algae. Among several categories of marine algae polyphenols, phlorotannins are pharmacologically prominent compounds, which are found in the form of organic polymers, such as phloroglucinol (1,3,5-trihydroxybenzene). Dieckol is one of the major phlorotannins isolated from *Ecklonia* species and exhibits a variety of biological and pharmacological activities such as free radical scavenging activity, antiplasmin inhibiting activity, antimutagenic activity, bactericidal activity, HIV-1 reverse transcriptase and protease inhibiting activity, and tyrosinase inhibitory activity. In the present study, we report that dieckol decreases NO and PGE_2 overproduction in LPS-activated BV2 microglia. Furthermore, we found that dieckol attenuated the expression iNOS and COX-2 mRNA and protein, indicating the action of dieckol occurs at the transcriptional level. Our data also indicate that dieckol suppresses the production of proinflammatory cytokines, such as IL-1β and TNF-α, which in turn induces neuronal cell damage; suppressing the production of these cytokines is important for the prevention of neurodegenerative diseases. The inhibition was concentration dependent without any cytotoxic effect. Our results indicate that nontoxic concentrations (100 and 300 μg/mL) of dieckol could be promising therapeutic candidates for neurodegenerative diseases caused by microglial activation in brain.

NF-κB is well-known as a critical regulator of various genes involved in cellular proliferation and immune and inflammatory responses. It has been shown that NF-κB activation is a factor critical to the expression of various cytokines and enzymes. Inducers of NF-κB activation include pro-inflammatory cytokines, growth factors, microbial infections, endotoxin, and oxidant stress (*23*). 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 (*24*). The heteromeric NF-κB complex is sequestered in the cytoplasm as an inactive precursor complexed with an inhibitory protein, an IκB-like protein, and LPS

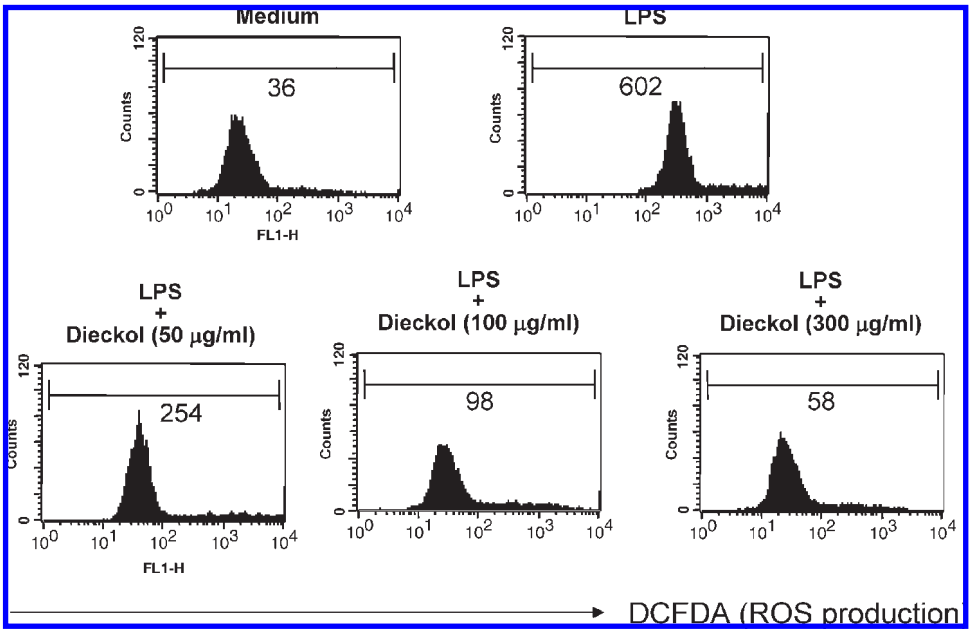

Figure 7. Effect of dieckol on LPS-induced ROS production in BV2 microglia. BV2 cells were pretreated with the indicated concentration of dieckol (50, 100, or 300 μg/mL) 2 h before LPS (1 μg/mL) treatment for 30 min. Cells were resuspended, and mean fluorescence intensity (MFI) was measured using flow cytometry. Each value represents the results of three independent experiments.

induced NF-κB activation through increasing nuclear p65 protein associated with decreased cytosolic IκB protein, followed by the translocation of NF-κB into the nucleus to activate genes with NF-κB binding sites. NF-κB is an important transcription factor for iNOS, COX-2, and pro-inflammatory cytokines such as IL-1β and TNF-α in LPS-stimulated microglia. Because the expression of pro-inflammatory mediators and cytokines is known to be modulated by NF-κB, we performed EMSA to investigate the possibility that dieckol inhibits NF-κB activity. Our findings suggest that dieckol treatment blocks the degradation of IκB and, therefore, NF-κB activation induced by LPS in BV2 microglia. In this study, we describe novel anti-inflammatory mechanisms mediated by dieckol, which are based on the inhibition of LPS-mediated activation of NF-κB signaling pathway.

ROS are commonly produced during inflammatory processes, are involved in signal transduction and gene activation, and can contribute to host cell and organ damage (*25*). Excessive generation of ROS stimulated the production of pro-inflammatory cytokines via activation of NF-κB. Several lines of evidence indicate that neuroprotective effects of antioxidants are due to increasing antioxidant enzyme and lowering of ROS (*26*). Moreover, changes in intracellular ROS can regulate the signal transduction pathway, leading to modulation of NF-κB activity. Scavenging of ROS by antioxidants could inhibit the NF-κB-dependent production of pro-inflammatory mediators, thereby preventing LPS toxicity (*27*). ROS, including superoxide anion, hydroxyl radical, and hydrogen peroxide, may have multiple roles in the pathogenesis of a number of neurodegenerative diseases. Thus, ROS could have roles in microglia as signaling molecules. In previous studies, dieckol was shown to possess significant anti-inflammatory and antioxidant properties (*28*). However, the effect of dieckol on inflammation-mediated neurodegeneration has not been elucidated nor have the molecular mechanisms by which this is effected. In the present study, we demonstrate that dieckol has an intracellular ROS scavenging activity in BV2 microglia, suggesting a possible mechanism responsible for the inhibitory effect of dieckol on NF-κB activation. Thus, potential inhibition of ROS generation by dieckol is in accord with inhibition of NF-κB-dependent cytokines, 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. MAPKs are proposed in response to LPS stimulation. It has been previously demonstrated that activation of MAPK is significant in the regulation of iNOS and COX-2 expression via controlling the activation of NF-κB in microglia (*29*). It is possible that neuroprotective mechanisms are related to MAP kinases or the inhibition of NO and PGE_2 production. Therefore, we investigated the effect of dieckol on LPS-stimulated phosphorylation of ERK-1/2, JNK, and p38 kinase in BV2 microglia. Interestingly, the phosphorylation of p38 kinase in response to LPS was decreased only by dieckol treatment; however, no significant changes by dieckol in the LPS-induced phosphorylation of ERK-1/2 and JNK were observed. Hence, these results suggest that p38, but not ERK-1/2 or JNK, is involved in the inhibitory effect of dieckol on LPS-induced iNOS and COX-2 expression and NF-κB activation.

In conclusion, the results obtained in this study indicate that dieckol treatment of BV2 microglia results in decreased pro-inflammatory cytokines and mediator following LPS stimulation. Dieckol significantly inhibited the release of NO, PGE_2, TNF-α, and IL-1β in a concentration-dependent manner, and it acts at the transcription level. The anti-inflammatory properties of dieckol are mediated by down-regulation of NF-κB, p38 kinase activation, and/or inhibition of ROS signal in BV2 microglia. These studies indicate that dieckol appears to have the potential to target p38 kinase and NF-κB in microglia and inhibit iNOS and COX-2 expression; such inhibition may contribute to the pathogenesis of neurodegenerative human brain diseases and to therapeutic efficacy.

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