

Anti-inflammatory effects of egg white combined with chalcantinite in lipopolysaccharide-stimulated BV2 microglia through the inhibition of NF- κ B, MAPK and PI3K/Akt signaling pathways

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Abstract. Egg white-chalcantinite (EWCC) is a mixture of egg white and chalcantinite prepared by roasting chalcantinite (which is a natural mineral mainly composed of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) to the point of dehydration, pulverizing the dehydrated chalcantinite and then mixing the pulverized chalcantinite to react with egg white to trigger a reaction. When egg white-chalcantinite is prepared in this manner, the toxicity of chalcantinite is neutralized by the egg white, so that the toxicity is reduced or removed and the pharmaceutical properties are increased. However, the cellular and molecular mechanisms underlying the pharmacological activity of EWCC remain poorly understood. In this study, we investigated the inhibitory effects of EWCC on the production of lipopolysaccharide (LPS)-induced pro-inflammatory mediators in BV2 microglia. Our data indicated that the EWCC treatment significantly inhibited the excessive production of nitric oxide and prostaglandin E_2 in LPS-stimulated BV2 microglia in a concentration-dependent manner without causing cytotoxicity. It also attenuated the expression of inducible nitric oxide synthase, cyclooxygenase-2 and pro-inflammatory cytokines, including interleukin-1 β and tumor necrosis factor- α . Moreover, EWCC exhibited anti-inflammatory properties by the suppression of nuclear factor- κ B (NF- κ B) activation by blocking I κ B- α degradation, downregulation of extracellular signal-regulated kinase,

c-Jun N-terminal kinase, p38 mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/Akt pathways. Our results indicate that the inhibitory effects of EWCC on LPS-stimulated inflammatory mediator production in BV2 microglia are associated with the suppression of the NF- κ B, MAPK and PI3K/Akt signaling pathways. These findings suggest that EWCC may offer a substantial therapeutic potential for the treatment of neurodegenerative diseases that are accompanied by microglial activation.

Introduction

Minerals have been traditionally used in Oriental herbal medicine treatment, particularly for immune-related diseases in Korea, China and other Asian countries (1-3). Among them, chalcantinite is a richly colored blue water-soluble sulfate mineral, commonly found in the late-stage oxidation zones of copper deposits (4). The main ingredient of chalcantinite is CuSO_4 . CuSO_4 exists as a series of compounds that differ in their degrees of hydration. The anhydrous form occurs as a rare mineral known as chalcocyanite. The hydrated copper sulfate occurs in nature as chalcantinite ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) and two of the rarer ones include bonattite ($\text{CuSO}_4 \cdot 3\text{H}_2\text{O}$) and boothite ($\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$) (4). According to Oriental medicine, chalcantinite has sour, acrid, cold and toxic properties, and is affiliated with the liver meridian. Its main function is to eliminate toxins. Internally, it functions as a purgative and helps the body get rid of excess phlegm and toxins. Chalcantinite can also be applied externally to treat skin abscesses, reduce inflammation and remove necrotic tissue. The traditionally used dose of chalcantinite is between 0.3 and 0.6 g of powdered mineral, decocted in water for oral administration. Larger amounts can be used when chalcantinite is applied externally. However, chalcantinite is poisonous and should be used only with extreme caution. To date, due to safety issues concerning their cytotoxic mechanisms, many mineral medicines are rarely used.

In addition to being a quality protein source, egg whites contain many essential minerals including potassium, magne-

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sium, calcium, phosphorus, copper, zinc and iron. They are also a good source of riboflavin and selenium along with essential vitamins such as folate, B12, niacin betaine and choline (5-9). In Medieval times in the West, egg whites were used for medicinal purposes such as treating wounds, skin disorders and mending broken bones. At present, egg whites are considered one of the most nutrition-rich foods and are widely recommended for children, the elderly, athletes and patients with a variety of health conditions.

Egg white-chalcanthite (EWCC) is a mixture of egg white and chalcanthite and may be prepared by roasting the chalcanthite in order to dehydrate it. The dehydrated chalcanthite is then pulverized and the pulverized chalcanthite is mixed to react with the egg white in order to trigger a chemical reaction. In the egg white-chalcanthite prepared as noted above, the toxicity of the chalcanthite is neutralized by egg white, so that the toxicity is reduced or removed and the pharmaceutical properties are increased. Recently, it has been reported that EWCC inhibits the growth of human lung cancer NCI-H460 cells by inducing apoptotic cell death through caspase-3 activation (10). However, the precise biochemical mechanisms underlying EWCC-induced anti-inflammatory effects have not yet been clarified.

Microglia have conventionally been regarded as brain macrophages and have been proposed to play a major role in host defense and tissue repair in the central nervous system (CNS) (11). They are activated in response to environmental stress and exposure to lipopolysaccharide (LPS), interferon (IFN)- γ and β -amyloid (12,13). Activation of microglia results in the release of various bioactive molecules, including nitric oxide (NO), prostaglandin E₂ (PGE₂), reactive oxygen species (ROS), monocyte chemoattractant protein-1 (MCP-1) and pro-inflammatory cytokines, such as interleukin (IL)-1 β , IL-6 and tumor necrosis factor- α (TNF- α) (14,15). Overproduction of these inflammatory mediators causes various severe neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, cerebral ischemia, multiple sclerosis and trauma (16-18). Therefore, activated microglia have been shown to be a major cellular source of pro-inflammatory and/or cytotoxic factors that cause neuronal damage in the CNS. Previous studies have also demonstrated that a decrease in the number of pro-inflammatory mediators in microglia attenuates the severity of these disorders (19-21). Thus, control of microglial activation may be an effective therapeutic option for the treatment of various neurodegenerative diseases.

LPS, the main component of endotoxins, initiates a number of major cellular effects that play critical roles in the pathogenesis of inflammatory responses and have been employed to induce microglial activation during infection by Gram-negative bacteria (22). Therefore, LPS stimulation of microglia is a useful model with which to study the mechanisms that underlie neuronal injury through various pro-inflammatory and neurotoxic factors released by activated microglia, such as NO, PGE₂, ROS, IL-1 β , IL-6 and TNF- α (14,15,23). In addition, inflammation is the organized response of an organism to injuries of multiple pathologies and the response involves the speedy upregulation and activation of many genes. Among them, nuclear factor- κ B (NF- κ B) is a critical transcription factor involved in this response and is normally present in the cytoplasm by virtue of

its association with the inhibitory protein, I κ B (24-26). Once activated by inflammatory stimulants such as LPS, I κ Bs are rapidly phosphorylated and degraded by I κ B kinase (IKK). I κ B degradation liberates NF- κ B to move into the nucleus, resulting in transcriptional induction of inflammation-associated genes (27,28).

Based on these previous observations, the present study was designed to evaluate the inhibitory effects of EWCC on the LPS-stimulated inflammatory mediator production in murine BV2 microglia and the related anti-inflammatory mechanisms. As a result of our findings, we suggest that EWCC may be a candidate for the treatment of various neurodegenerative disorders.

Materials and methods

Preparation of EWCC. For the preparation of EWCC, chalcanthite was heated and dehydrated. During the heating process, chalcanthite was uniformly heated while carefully being watched for a color change every 3-5 h until it turned completely grey. The heating duration (~10-24 h) varied with the amount and quality of the mineral used. Once the chalcanthite was dehydrated, it was cooled until the remaining heat was completely released. The moisture content of the chalcanthite ranged from 0 to 5%. After cooling, chalcanthite was finely pulverized. The pulverized chalcanthite was stored in a concealed airtight container in a dry place to prevent moisture from accumulating. The prepared chalcanthite powder was mixed with egg whites at ratios of 30:13 to 30:30 for 10 min. The egg whites needed to be homogeneously mixed with a wooden spatula in a ceramic vessel so the utensils would not interfere or cause any chemical reactions during the mixing process. After the chalcanthite and egg whites were sufficiently mixed, the mixture was cooled until the reaction heat was completely released. One hundred milligrams of the obtained substance was then dissolved in 1 liter of deionized water and centrifuged at 6,000 rpm for 10 min. A portion of the upper level concentration (0.80 μ m) was filtered by syringe filter after the centrifugation.

Cell culture. The murine BV2 cell line was maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in a humidified incubator with 5% CO₂. Confluent cultures were passed by trypsinization. The cells used in the experiments were washed twice with warm DMEM (without phenol red) and treated in a serum-free medium for at least 4 h before treatments. In all experiments, the cells were treated with various concentrations of EWCC at the indicated times before the addition of LPS (0.5 μ g/ml).

Cell viability assay. Cell viability was measured based on the formation of blue formazan that was metabolized from colorless MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, MO] by mitochondrial dehydrogenases, which are active only in live cells. BV2 cells were plated on 24-well plates at a density of 2x10⁵ cells/well for 24 h and then washed. Cells incubated with various concentrations of EWCC were treated with or without LPS (*Escherichia coli* 026:B6; Sigma) for 24 h and

Table I. Sequence of primers used for RT-PCR in this study.

Gene name	Sequences
iNOS	
Sense	5'-ATG TCC GAA GCA AAC ATC AC-3'
Antisense	5'-TAA TGT CCA GGA AGT AGG TG-3'
COX-2	
Sense	5'-CAG CAA ATC CTT GCT GTT CC-3'
Antisense	5'-TGG GCA AAG AAT GCA AAC ATC-3'
TNF- α	
Sense	5'-ATG AGC ACA GAA AGC ATG ATC-3'
Antisense	5'-TAC AGG CTT GTC ACT CGA ATT-3'
IL-1 β	
Sense	5'-ATG GCA ACT GTT CCT GAA CTC AAC T-3'
Antisense	5'-TTT CCT TTC TTA GAT ATG GAC AGG AC-3'
GAPDH	
Sense	5'-CGG-AGT-CAA-CGG-ATT-TGG-TCG-TAT-3'
Antisense	5'-AGC-CTT-CTC-CAT-GGT-GGT-GAA-GAC-3'

then incubated in 0.5 mg/ml MTT solution. Three hours later, the supernatant was removed and the formation of formazan was measured at 540 nm using a microplate reader (Dynatech MR-7000; Dynatech Laboratories).

NO production. Concentrations of NO in the culture supernatants were determined by measuring nitrite, which is a major stable product of NO, using the Griess reagent (Sigma). Cells (5×10^5 cells/ml) were stimulated in 24-well plates for 24 h and then 100 μ l of each culture medium was mixed with an equal volume of Griess reagent (1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4). Nitrite levels were determined using an ELISA plate reader at 540 nm and nitrite concentrations were calculated by reference to a standard curve generated by known concentrations of sodium nitrite (29).

Measurement of PGE₂ production. BV2 cells were incubated with purpurogallin in either the presence or absence of LPS (0.5 μ g/ml) for 24 h. Following the manufacturer's instructions, a volume of 100 μ l of culture-medium supernatant was collected for determination of PGE₂ concentration by ELISA (Cayman, Ann Arbor, MI) (29).

RNA isolation and RT-PCR. Total RNA was prepared using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) and primed with random hexamers for the synthesis of complementary DNA using M-MLV Reverse Transcriptase (Promega, Madison, WI), according to the manufacturer's instructions using DNase I (1 U/ μ g RNA) pretreated total mRNA. Single stranded cDNA was amplified by a polymerase chain reaction (PCR) with primers for inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, IL-1 β , TNF- α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table I). The following PCR conditions were applied - GAPDH: 18 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C

for 30 sec; iNOS, COX-2, IL-1 β and TNF- α : 25 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for a 30-sec extension at 72°C for 30 sec. GAPDH was used as an internal control to evaluate the relative expression of COX-2, iNOS, IL-1 β and TNF- α (30).

Protein extraction and western blot analysis. Cells were washed with PBS three times, placed at a temperature of 4°C and lysed for 30 min in lysis buffer (20 mM sucrose, 1 mM EDTA, 20 μ M Tris-Cl, pH 7.2, 1 mM DTT, 10 mM KCl, 1.5 mM MgCl₂ and 5 μ g/ml aprotinin). Lysates were then centrifuged at 12,000 rpm at 4°C. The protein concentration was measured using a Bio-Rad protein assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Equal amounts of protein (30-50 μ g) were separated electrophoretically using 8-10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was then transferred to 0.45- μ m polyvinylidene fluoride (PVDF; Millipore, Bedford, MA) membranes. Membranes were soaked in blocking buffer (5% skimmed milk) and then incubated with the primary antibodies (Table II). After thorough washing with PBST, horseradish peroxidase conjugated antibodies were applied and immune complexes were then visualized using the enhanced chemiluminescence (ECL) detection system according to the recommended procedure (Amersham). In a parallel experiment, cells were washed with ice-cold PBS and scraped; and cytoplasmic and nuclear proteins were then extracted using NE-PER[®] Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Inc., Rockford, IL).

Cytokine assays. The levels of IL-1 β and TNF- α (R&D Systems, Minneapolis, MN) were measured by ELISA kits according to the manufacturer's instructions. Briefly, BV2 cells (5×10^5 cells/ml) were plated in 24-well plates and pretreated with indicated concentrations of EWCC for 1 h before treatment of 0.5 μ g/ml LPS for 24 h. One hundred microliters of culture-medium supernatants was collected to determine the IL-1 β and TNF- α concentrations by ELISA.

Immunofluorescence analysis. For detection of NF- κ B p65 translocation, cells were grown on glass coverslips for 24 h and then treated with 0.5 μ g/ml LPS, and were either pretreated or not pretreated with EWCC for 60 min. Cells were fixed with 3.7% paraformaldehyde, treated with 0.2% Triton X-100 and blocked with 2% BSA. Cells were then sequentially incubated with the anti-NF- κ B p65 antibody, FITC-conjugated donkey anti-rabbit IgG and DAPI solution and examined using a fluorescence microscope (Carl Zeiss, Germany).

Statistical analyses. Data values represent the means \pm SD. Statistical significance was determined using an analysis of variance that was followed by the Student's t-test. A value of P<0.05 was accepted as statistically significant.

Results

Effect of EWCC on NO and PGE₂ production in LPS-stimulated BV2 microglia. In the present study, we aimed to evaluate the

Table II. List of antibodies used in this study.

Antibody	Dilution	Product no.	Species of origin (supplier)
iNOS	1:1,000	610333	Rabbit polyclonal (BD Biosciences)
COX2	1:500	SC-1999	Mouse monoclonal (Santa Cruz Biotechnology)
NF- κ B p65	1:500	SC-109	Mouse monoclonal (Santa Cruz Biotechnology)
I κ B α	1:500	SC-847	Rabbit polyclonal (Santa Cruz Biotechnology)
PI3K	1:500	4257	Mouse monoclonal (Cell Signaling Technology)
p-PI3K	1:500	4228	Rabbit polyclonal (Cell Signaling Technology)
Akt	1:500	SC-8312	Rabbit polyclonal (Santa Cruz Biotechnology)
p-Akt	1:500	9271S	Rabbit polyclonal (Cell Signaling Technology)
p38	1:500	SC-728	Rabbit polyclonal (Santa Cruz Biotechnology)
ERK	1:2,000	SC-535	Rabbit polyclonal (Santa Cruz Biotechnology)
JNK	1:500	9252	Rabbit polyclonal (Cell Signaling Technology)
p-p38	1:500	9211S	Rabbit polyclonal (Cell Signaling Technology)
p-ERK	1:500	9106S	Mouse monoclonal (Cell Signaling Technology)
p-JNK	1:500	9255S	Mouse monoclonal (Cell Signaling Technology)
Lamin B	1:1,000	NA12	Mouse monoclonal (Oncogene Science)

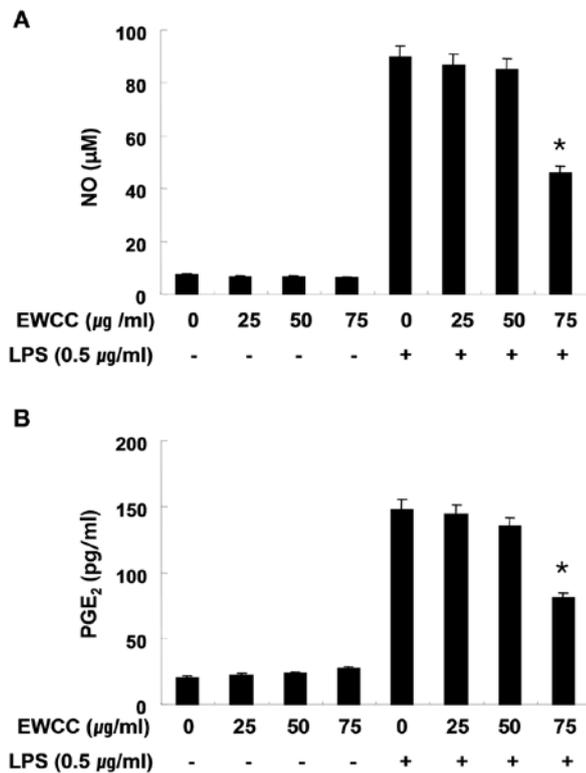


Figure 1. Inhibition of NO and PGE₂ production by EWCC treatment in LPS-stimulated BV2 microglia. BV2 cells were pretreated with various concentrations of EWCC (25, 50 and 75 µg/ml) for 1 h before incubation with LPS (0.5 µg/ml) for 24 h. (A) Nitrite content was measured using the Griess reagent and (B) PGE₂ concentration was measured in culture media using a commercial ELISA kit. Each value indicates the means \pm SD and is representative of the results obtained from three independent experiments. *P<0.05 indicates a significant difference from the value obtained for cells treated with LPS in the absence of EWCC.

potential anti-inflammatory properties of EWCC regarding the production of two major inflammatory mediators, NO and PGE₂, in LPS-stimulated BV2 microglia. To determine

the level of NO production, we measured the nitrite released into the culture medium using Griess reagent. BV2 cells were pretreated with various concentrations of EWCC (25, 50 and 75 µg/ml) for 1 h before being stimulated with LPS (0.5 µg/ml). According to the NO detection assay, LPS alone was able to markedly induce NO production from the cells as compared to those generated by the control. However, pretreatment with EWCC significantly repressed the levels of NO production in LPS-stimulated BV2 microglia in a concentration-dependent manner of up to 75 µg/ml (Fig. 1A).

PGE₂ represents another important inflammatory mediator that is produced from the conversion of arachidonic acid by COXs. Therefore, we next evaluated the inhibitory effects of EWCC on PGE₂ levels present in the supernatant. To assess whether EWCC inhibits production of LPS-induced PGE₂ in BV2 microglia, cells were pretreated with various concentrations of EWCC for 1 h and then stimulated with 0.5 µg/ml LPS. After incubation for 24 h, the cell culture medium was harvested and the production of PGE₂ was measured using an ELISA. As shown in Fig. 1B, the amount of PGE₂ present in the culture medium increased from initial levels after 24 h of exposure to LPS alone, however a marked repression was observed after administration of EWCC in a concentration-dependent fashion.

Effect of EWCC on the cell viability in LPS-stimulated BV2. In order to exclude the cytotoxic effect of EWCC on the cell growth of BV2 cells, the cells were exposed to various concentrations of EWCC for 24 h in the presence or absence of LPS, and cell viability was then measured by an MTT assay. Under the same condition, we also analyzed whether chromatin condensation, a hallmark of apoptosis, was induced by EWCC treatment. As indicated in Fig. 2, the concentrations of EWCC (25-75 µg/ml) used to inhibit NO and PGE₂ production did not affect cell viability. These results clearly indicated that the inhibition of NO and PGE₂ production in LPS-stimulated BV2 cells was not due to the cytotoxic action of EWCC.

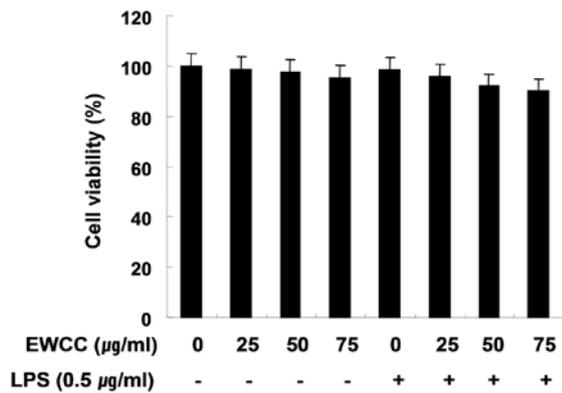


Figure 2. Effects of EWCC and LPS on cell viability of BV2 microglia. BV2 cells were treated with the indicated concentrations of EWCC and LPS (0.5 µg/ml) alone, or pretreated with the indicated concentrations of EWCC for 1 h before LPS treatment. After 24 h, cell viability was assessed using an MTT reduction assay and the results are expressed as the percentage of surviving cells over control cells (no addition of EWCC). Each value indicates the means ± SD and is representative of the results obtained from three independent experiments.

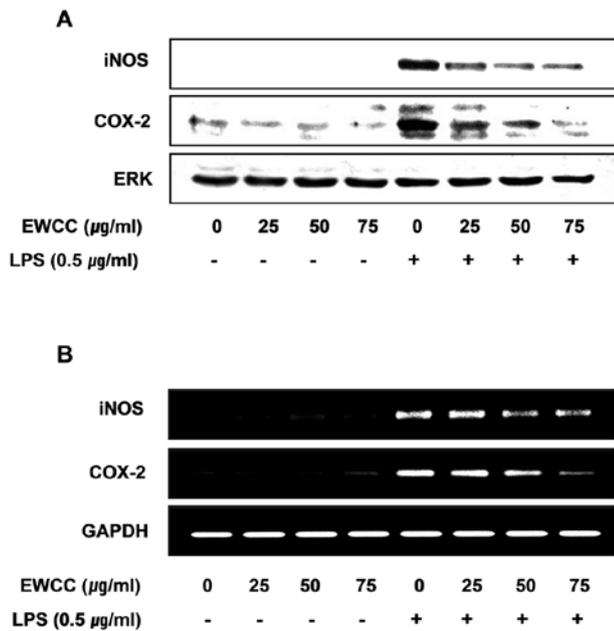


Figure 3. Inhibition of iNOS and COX-2 expression by EWCC in LPS-stimulated BV2 microglia. (A) BV2 cells were pretreated with the indicated concentrations of EWCC 1 h prior to incubation with LPS (0.5 µg/ml) for 24 h. Cell lysates were then prepared and western blotting was performed using antibodies specific for murine iNOS and COX-2. (B) After LPS treatment for 6 h, total RNA was prepared for RT-PCR analysis of iNOS and COX-2 gene expression in LPS-stimulated BV2 microglia. Actin and GAPDH were used as internal controls for western blot analysis and RT-PCR assays, respectively. The experiment was repeated three times and similar results were obtained.

Effect of EWCC on LPS-stimulated levels of iNOS and COX-2 mRNA and protein. To ascertain whether the inhibition of NO and the PGE₂ production by EWCC treatment is associated with decreased levels of iNOS and COX-2, we performed RT-PCR and western blot analysis for detection of mRNA and protein levels. The levels of iNOS and COX-2 protein were markedly upregulated after 24 h of LPS (0.5 µg/ml) treatment,

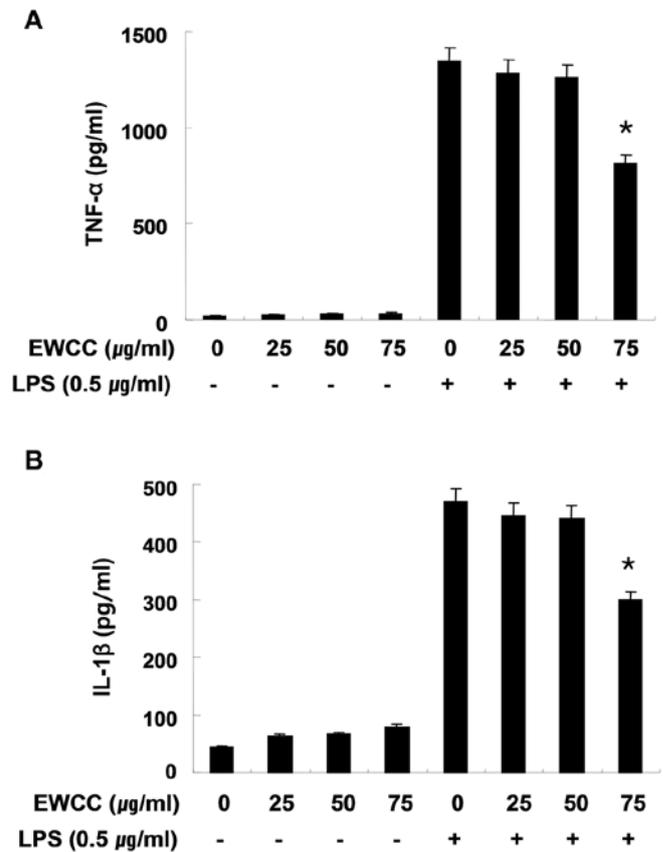


Figure 4. Effect of EWCC on the production of LPS-stimulated pro-inflammatory cytokines in BV2 microglia. BV2 cells were pretreated with various concentrations of EWCC for 1 h before LPS treatment (0.5 µg/ml). After incubation for 24 h, the levels of (A) TNF-α and (B) IL-1β present in the supernatants were measured. Each value indicates the means ± SD and is representative of the results obtained from three independent experiments. *P<0.05 indicates a significant difference from the value obtained for cells treated with LPS in the absence of EWCC.

and EWCC significantly inhibited iNOS and COX-2 protein expression in LPS-stimulated BV2 microglia in a concentration-dependent manner (Fig. 3A). Next, to investigate whether or not EWCC suppresses the LPS-mediated induction of iNOS and COX-2 via a pretranslational mechanism, the effects of EWCC on iNOS and COX-2 mRNA expression were evaluated (Fig. 3B). RT-PCR analysis showed that the reduction in iNOS and COX-2 mRNA correlated with the reduction in the corresponding protein levels. The results suggest that EWCC-induced reduction in the expression of iNOS and COX-2 resulted in the inhibition of NO and PGE₂ production.

Effects of EWCC on LPS-induced IL-1β and TNF-α production and mRNA expression. We next investigated whether EWCC suppresses the production of pro-inflammatory cytokines, IL-1β and TNF-α, in LPS-stimulated BV2 cells. BV2 microglia were incubated with EWCC in the absence or presence of 0.5 µg/ml of LPS for 24 h and the cytokine levels were evaluated in the culture supernatants. As indicated in Fig. 4A and B, induction of IL-1β and TNF-α by LPS treatment was significantly decreased by EWCC in cell culture media. In a parallel experiment, using RT-PCR, we studied the effects of EWCC on LPS-induced IL-1β and

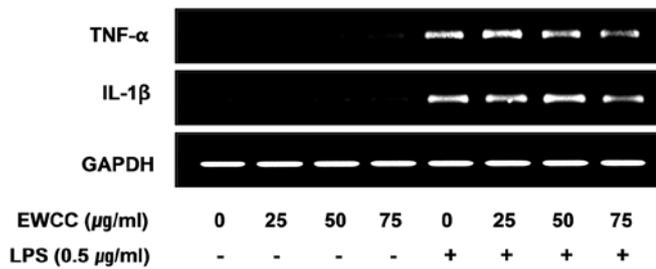


Figure 5. Effect of EWCC on LPS-stimulated IL-1 β and TNF- α expression in BV2 microglia. BV2 cells were pretreated with various concentrations of EWCC for 1 h before LPS treatment (0.5 $\mu\text{g/ml}$) and the total RNA was isolated at 6 h after LPS treatment. The levels of IL-1 β and TNF- α mRNA were determined using RT-PCR. GAPDH was used as an internal control.

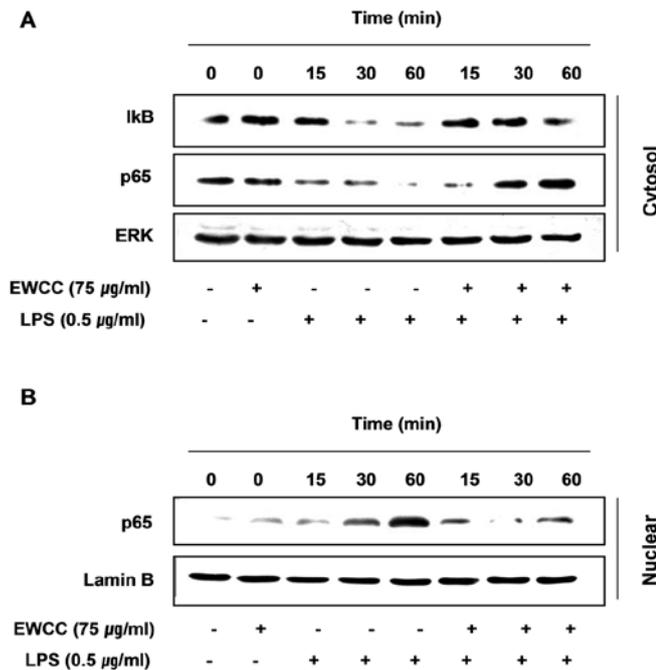


Figure 6. Effects of EWCC on LPS-induced NF- κ B translocation and I κ B degradation in BV2 microglia. Cells were treated with EWCC (75 $\mu\text{g/ml}$) for 1 h before LPS treatment (0.5 $\mu\text{g/ml}$) for the indicated times. (A) Cytosolic and (B) nuclear proteins were subjected to 10% SDS-polyacrylamide gels followed by western blotting using anti-NF- κ B p65 and anti-I κ B- α antibodies. The results are representative of those obtained from two independent experiments. ERK and lamin B were used as internal controls for cytosolic and nuclear fractions, respectively.

TNF- α mRNA expression. As shown in Fig. 5, IL-1 β and TNF- α mRNA transcription also decreased following EWCC treatment. These results suggest that EWCC is effective in the suppression of pro-inflammatory cytokine production through the alteration of the transcription levels of IL-1 β and TNF- α in activated microglia.

Effect of EWCC on LPS-induced NF- κ B activity. NF- κ B is one of the important transcription factors that regulate gene expression of pro-inflammatory mediators including iNOS, COX-2, TNF- α and IL-1 β in activated BV2 microglia (24-26); therefore, to further characterize the mechanism through which EWCC inhibits pro-inflammatory and/or cytotoxic factor expression, we examined whether EWCC prevents the

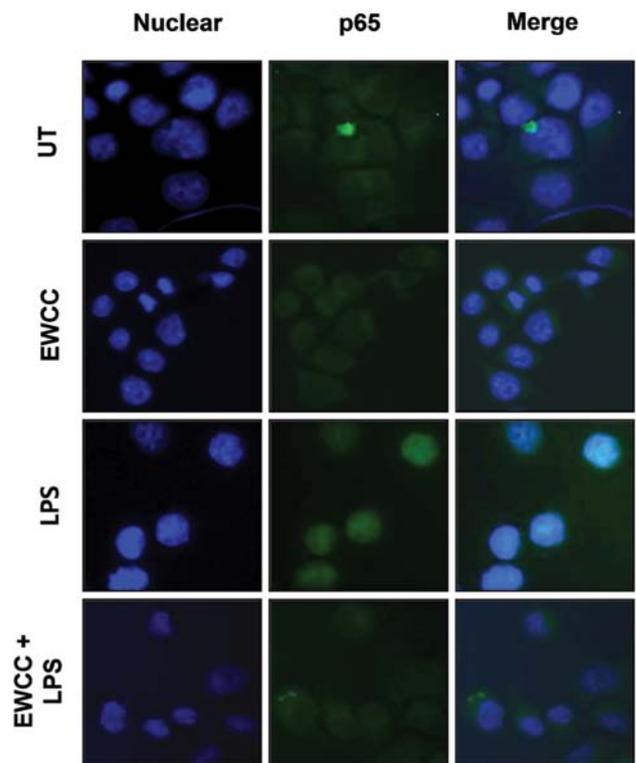


Figure 7. Inhibition of NF- κ B translocation by EWCC in LPS-stimulated BV2 microglia. Cells were treated with EWCC (75 $\mu\text{g/ml}$) for 1 h before LPS treatment (0.5 $\mu\text{g/ml}$). After 1 h incubation, localization of NF- κ B p65 was visualized with fluorescence microscopy after immunofluorescence staining with NF- κ B p65 antibody (green). Cells were also stained with DAPI for visualization of the nuclei (blue). Results are representative of those obtained from three independent experiments.

translocation of the p65 subunit of NF- κ B to the nucleus. Western blot analysis showed that the amount of NF- κ B p65 in the nucleus was markedly increased after exposure to LPS alone, but the LPS-induced p65 level in the nuclear fractions was reduced by EWCC pretreatment (Fig. 6). Similar results were also found using immunofluorescence microscopy (Fig. 7). In addition, we investigated whether EWCC blocks the LPS-stimulated degradation of I κ B- α using western blotting. As shown in Fig. 6, I κ B- α was markedly degraded at 30 min after LPS treatment. However, this LPS-induced I κ B- α degradation was significantly reversed by EWCC. These results suggest that EWCC inhibits NF- κ B activation in BV2 microglia cells by suppression of I κ B degradation and nuclear translocation of NF- κ B. Taken together the above findings show the anti-inflammatory effect of EWCC in the LPS-stimulated BV2 cells involved the NF- κ B pathway.

Effects of EWCC on the LPS-induced activation of the PI3K/Akt pathway in BV2 microglia. Findings from recent studies have indicated that the phosphoinositide 3-kinase (PI3K)/Akt signaling molecule is able to trigger NF- κ B activation through I κ B degradation (31,32). Therefore, we examined the effect of EWCC on LPS-induced PI3K/Akt activation. As shown in Fig. 8, phosphorylation of PI3K and Akt showed a marked increase within 15 min after LPS stimulation; however, EWCC pretreatment resulted in a significant

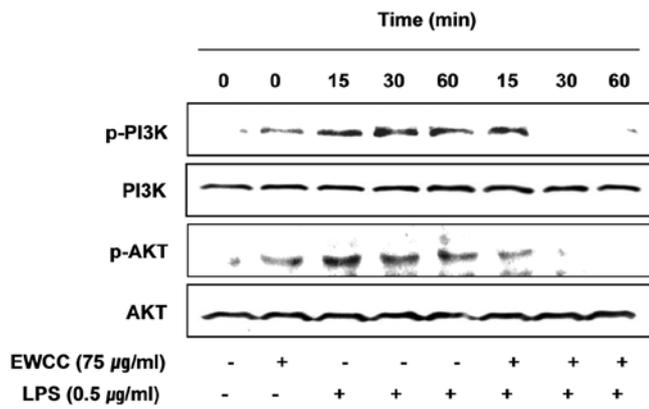


Figure 8. Effects of EWCC on PI3K and AKT activation induced by LPS in microglia. BV2 cells were treated with the indicated doses of EWCC 1 h before LPS treatment (0.5 $\mu\text{g/ml}$) for the indicated times. The total protein (50 μg) was subjected to 10% SDS-PAGE, followed by western blotting using anti-p-PI3K, anti-PI3K, anti-p-Akt and anti-Akt antibodies. The results are representative of those obtained from three independent experiments.

blockage of LPS-induced PI3K and Akt phosphorylation. The results show that inhibition of pro-inflammatory mediator expression by EWCC in LPS-stimulated BV2 microglia was associated with the inactivation of the PI3K/Akt signaling pathway.

Effects of EWCC on the LPS-induced activation of MAPKs in BV2 microglia. Mitogen-activated protein kinases (MAPKs) are the most important signaling molecules with involvement in activated microglia (33,34). Therefore, we determined the activation levels of the MAPK pathway at various times after LPS stimulation of BV2 cells. Stimulation of BV2 cells with LPS led to the rapid activation of p38MAPK, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), with peak levels of each phospho-MAPK occurring 15-60 min after the addition of LPS. However, EWCC pretreatment significantly inhibited phosphorylation of MAPKs in LPS-stimulated BV2 microglia (Fig. 9). This finding suggests that EWCC is capable of disrupting key signal transduction pathways activated by LPS in BV2 microglia, which subsequently prevents the production of pro-inflammatory mediators.

Discussion

Microglia are brain macrophage cells that reside within the CNS. They play a pivotal role in the innate immune response of the CNS and serve as the first line of defense against invading pathogens (35). Activation of microglia by LPS induces the release of various pro-inflammatory mediators, such as IL-1 β , IL-6, TNF- α , NO and PGE₂, which have been implicated as important intermediaries of neuronal injury (14-16). In that regard, the inhibition of a variety of pro-inflammatory enzymes and cytokines would be an effective therapeutic approach against many neurodegenerative diseases. Many recent studies have reported the neuroprotective effects of natural extracts that are useful therapeutic agents for the protection of neurons from toxicities associated with exposure to activated microglia.

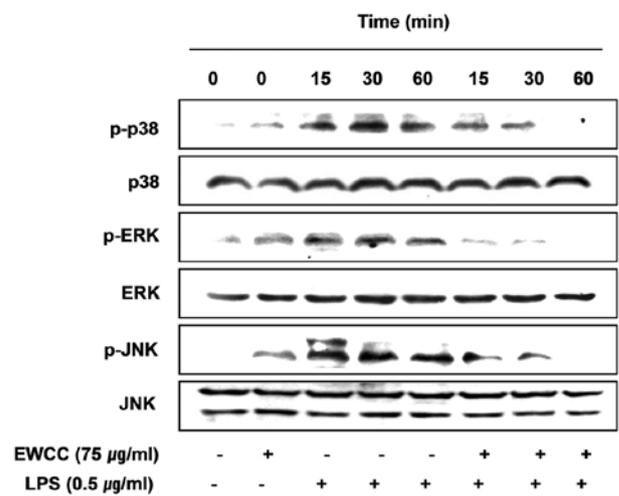


Figure 9. Effects of EWCC on MAPK activation induced by LPS in microglia. BV2 cells were treated with the indicated concentrations of EWCC 1 h before LPS treatment (0.5 $\mu\text{g/ml}$) for the indicated times. The total protein (50 μg) was subjected to 10% SDS-PAGE, followed by western blotting using the indicated antibodies. Results are representative of those obtained from three independent experiments. Actin was used as the internal control.

The present results demonstrated that EWCC exhibits pharmacological and biological activities via significant inhibitory effects on the production of LPS-induced inflammatory mediators and cytokines in BV2 microglial cells. These mediators and cytokines include NO, PGE₂, IL-1 β and TNF- α . The inhibitory effects were also mediated through the inhibition of NF- κ B activation and the PI3K/Akt and MAPK pathways. These data suggest that EWCC may be a possible therapeutic agent for the treatment of inflammatory disease, including neurodegenerative disorders.

NO is an important regulatory molecule in a wide range of physiological and pathological processes. NO is produced from L-arginine by NO synthase (NOS), a three member enzyme family, including iNOS, which is produced mainly by activated glial cells (36). NO production can lead to inflammatory, infectious, ischemic, traumatic and neurodegenerative diseases (13,37-39). Thus, inflammatory diseases could be prevented through the inhibition of iNOS and NO. Similarly, PGE₂ is also a well-known inflammatory mediator derived from arachidonic acid via the action of cyclooxygenases. The overproduction of PGE₂ in response to growth factors, cytokines and pro-inflammatory molecules is associated with the upregulation of COX-2 (40,41). COX-2 has emerged as one of the major players in brain inflammation and increased COX-2 expression is believed to contribute to neurodegeneration (42,43). Therefore, intense research has been undertaken in order to search for selective COX-2 inhibitors. In the present study, EWCC inhibited both PGE₂ and NO production in a concentration-dependent manner (Fig. 1) and these inhibitory effects were mediated through the downregulation of COX-2 and iNOS expression at the mRNA and protein levels (Fig. 3) without causing cytotoxicity (Fig. 2). Chronic microglial activation and consequent overproduction of pro-inflammatory cytokines, such as IL-1 β and TNF- α , are a histopathological hallmark of various neurological diseases (14,15). The data

also show that EWCC inhibits LPS-induced cytokine expression at the transcriptional level in BV2 microglia (Fig. 5).

NF- κ B, as a result of its key role in several pathologic conditions, is a major drug target in a variety of diseases. Activation of the NF- κ B protein plays a pivotal role in inflammation due to its ability to induce transcription of pro-inflammatory genes. The activity of NF- κ B is suppressed in the cytoplasm, while it is complexed with an inhibitory I κ B protein. The phosphorylation of I κ B subsequently leads to its ubiquitination and degradation (27,28). NF- κ B dimers are then free to translocate to the nucleus and activate target genes, including iNOS, COX-2 and pro-inflammatory cytokines. It is well known that the blockade of NF- κ B transcriptional activity in the microglial nucleus can also suppress the expression of iNOS, COX-2 and pro-inflammatory cytokines, such as IL-1 β , IL-6 and TNF- α (24,44). Recently, involvement of the PI3K/Akt pathway in the expression of inflammatory mediators in microglia through the activation of NF- κ B has also been demonstrated (31,32,45). Therefore, many current anti-inflammatory therapies seek to block NF- κ B activity. Our study indicated that EWCC inhibited the LPS-induced I κ B- α degradation and that it inhibited the nuclear translocation of the p65 subunit of NF- κ B in BV2 microglia (Figs. 6 and 7). Therefore, inhibition of NF- κ B signaling pathways in microglia by EWCC might result in the downregulation of pro-inflammatory mediators, resulting in an anti-inflammatory effect. Furthermore, EWCC significantly inhibited PI3K/Akt activation in LPS-stimulated BV2 microglia (Fig. 8), indicating that EWCC inhibits LPS-induced NF- κ B activation via the inactivation of the PI3K/AKT signaling pathway.

The involvement of various intracellular signaling pathways, such as MAPK, in the induction of inflammatory mediators has also been reported (33,34). Therefore, we determined whether EWCC tightly regulates the expression of MAPKs to induce anti-inflammatory effects in LPS-stimulated microglia. Our results indicated that EWCC is a potent inhibitor of the activation of MAPKs, such as p38MAPK, ERK and JNK, induced by LPS stimulation in BV2 microglia (Fig. 9), suggesting that the anti-inflammatory effects were also due to the inhibition of the MAPK signaling pathway.

In summary, the present results demonstrate that EWCC significantly attenuated the release of inflammatory mediators such as NO, PGE₂, TNF- α and IL-1 β in a concentration-dependent manner; in particular, it acted at the level of transcription in activated BV2 microglia. The inhibitory action of EWCC was mediated by the prevention of NF- κ B activation and the inhibition of I κ B degradation. In addition, the activation of PI3K/Akt and MAPKs was significantly decreased after pretreatment with EWCC in LPS-stimulated BV2 microglial cells. As a result of the findings presented in this study, EWCC may provide an effective treatment for many neurodegenerative diseases, and the pharmacology and mode of action of its active components must be further investigated.

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