Methanol Extract of *Polyopes lancifolius* Inhibits the Expression of Pro-inflammatory Mediators in LPS-stimulated BV2 Microglia Cells via Downregulation of the NF-κB Pathway

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Abstract

**Purpose:** This study is aimed at identifying the anti-inflammatory mechanisms of a methanol extract of Polyopes lancifolius (MEPL) in lipopolysaccharide (LPS)-stimulated BV2 microglial cells.

**Methods:** The expression of mRNA and protein were investigated RT-PCR and western blot analyses in LPS-stimulated BV2 microglial cells. The level of nitric oxide (NO) production was analyzed using Griess reaction. The release of prostaglandin E₂ (PGE₂) and tumor necrosis factor-α (TNF-α) were determined using sandwich ELISA. NF-κB activation was detected using EMSA methods.

**Results:** MEPL significantly suppressed NO production in LPS-stimulated BV2 cells without any cytotoxicity. The results also indicate that MEPL decreased the production of PGE₂ and TNF-α in LPS-stimulated BV2 cells. Furthermore, pretreatment with MEPL resulted in a downregulation of LPS-induced mRNA and protein expression of inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2) and TNF-α. Investigation of the effect of MEPL on nuclear factor-κB (NF-κB) activity, which is a potential transcriptional factor for regulating inflammatory genes such as iNOS, COX-2 and TNF-α, showed that MEPL substantially inhibited the LPS-induced DNA-binding activity of NF-κB. MEPL also suppressed the LPS-induced degradation and phosphorylation of IκBα, and it consequently blocked p65 translocation from the cytosol to the nucleus.

**Conclusion:** These data show that MEPL may regulate LPS-induced NO, PGE₂, and TNF-α production by suppressing NF-κB activity.

**Keywords:** Polyopes lancifolius, Nitric oxide, Prostaglandin E₂, Tumor necrosis factor-α, Nuclear factor-κB

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INTRODUCTION

Inflammation is an important host response to external challenge or cellular injury, which is mediated by a variety of cell signaling pathways to balance the restoration of tissue structure and function [1]. As a result of the activation of these signaling pathways, many genes are expressed that produce different kinds of mediators such as nitric oxide (NO) and prostaglandins, which are generated by inducible isoforms of NO synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively and cytokines such as tumor necrosis factor-α (TNF-α) [2]. According to recent studies, excessive and abnormal production of these mediators results in severe tissue damage, systemic inflammatory syndrome, septic shock, and atherosclerosis [3]. Therefore, many researchers have recently attempted to identify some food sources and chemicals to suppress the aberrant expression of inflammatory mediators.

Several well-characterized cell signaling pathways involve the production of different cytokines and inflammatory mediators. In particular, the nuclear factor-κB (NF-κB) pathway may play a key role due to its rapid activation and potency as a transcriptional activator [4]. NF-κB regulates several important processes such as cell growth, cancer, apoptosis, inflammation, immune responses, and developmental processes [5]. NF-κB is primarily regulated by its association with inhibitor κB (IκB) proteins in the cytoplasm. NF-κB normally exists in the cytoplasm in an inactive complex bound to IκB [6]. Most agents that activate NF-κB act through a common pathway based on phosphorylation-induced and/or proteasome-mediated degradation of IκB [7]. Removal of the inhibitor can initiate nuclear localization signals on NF-κB subunits. Free NF-κB translocates to the nucleus where it binds to target DNA elements and activates the transcription of genes encoding proteins involved in immune responses, inflammation, or cell proliferation [8]. Therefore, many NF-κB inhibitors, like non-steroidal anti-inflammatory drugs, cell-permeable peptides such as SN-50, and proteasome inhibitors, have been thought as chemotherapeutic agents, because they suppress a variety of inflammatory diseases [9].

Many seaweed extracts are well-known as regulators of immune and inflammatory responses [10]. Therefore, many researchers are looking for more potent compounds with anti-inflammatory characteristics. Polypedes lancifolius, mainly found in the Republic of Korea and Japan, is such a seaweed with medicinal value [11]. Only one study has been conducted and this revealed that bromophenol purified from P. lancifolia, which was thought to be the same species as P. lancifolius, may have potential as a natural nutraceutical for treating type 2 diabetes [11]. Nevertheless, little evidence exists regarding the anti-inflammatory properties of this seaweed.

In this study, we investigated the effects of a methanol extract of P. lancifolius (MEPL) on the expression of NO, PGE2, and TNF-α in lipopolysaccharide (LPS)-stimulated BV2 microglia cells. Our data showed that MEPL downregulated iNOS, COX-2, and TNF-α mRNA expression in LPS-stimulated BV2 cells. MEPL also inhibited LPS-induced NF-κB activation by suppressing the degradation and phosphorylation of IκBα in the cytoplasm and p65 translocation to the nucleus.

EXPERIMENTAL

Preparation of MEPL

MEPL was purchased from Jeju HI-Tech Industry Development Institute (extract NO. 1340; Jeju, Republic of Korea). The red alga, P. lancifolius (stock NO. AR038) was collected along the Jeju Island coast of Republic of Korea in April, 2005. Briefly, fresh P. lancifolius 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 was
extracted with 80% methanol and evaporated in vacuo.

Reagents

Lipopolysaccharide (LPS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were obtained from WelGENE Inc., Daegu, Republic of Korea. Antibodies against iNOS, COX-2, p-p65, IκBα and phospho (p)-IκBα polyclonal antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. The antibody against β-actin was from Sigma. Peroxidase-labeled goat anti-rabbit immunoglobulin was purchased from KOMA Biotechnology, Seoul, Republic of Korea. Other chemicals were purchased as Sigma grade.

Cell culture and sample treatment

BV2 cell lines were cultured at 37 °C in 5% CO2 in DMEM supplemented with 10% FBS and antibiotics. For the analysis of cell viability, the cells (1 × 10^5 cells/ml) were incubated with the various concentrations of MEPL 1 h before stimulation with LPS (1.0 µg/ml) for the indicated time.

Analysis of cell viability

Cell viability was determined by an MTT assay. BV2 cells (1 × 10^5 cells/ml) were plated on 24-well plates and incubated overnight. The cells were pretreated with varying concentrations of MEPL, 1 h before stimulation with LPS (1.0 µg/ml) for 24 h. MTT assay was used to determine cell viability.

Nitric oxide determination

BV2 cells (1 × 10^5 cells/ml) were plated onto 24-well plates and pretreated with the indicated concentrations of MEPL, 1 h before treatment with LPS (1.0 µg/ml) for 24 h. Supernatants were collected and assayed for NO production using Griess reagent. Nitrite concentration was determined using a dilution of sodium nitrite as a standard.

Measurement of PGE_2 and TNF-α

Expression levels of PGE_2 and TNF-α were measured using enzyme linked immunosorbert assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

Isolation of total RNA and RT-PCR

Total RNA was extracted using easy-BLUE™ total RNA extraction kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instruction. Two microgram RNA was reverse-transcribed using MMLV reverse transcriptase (Promega, Madison, WI, USA). cDNA was amplified by PCR using specific primer iNOS (forward 5’-ctt cct cca ccc tac cca gtt-3’ and reverse 5’-ctt cta tgc tgc tgc tgc-3’), COX-2 (forward 5’-aag act tgc cag gct gaa ctg ccc tcc ttc-3’), TNF-α (forward 5’-ggc aag tgg aac tgg ccc tcc ccc tcc-3’), and β-actin (forward 5’-gtt ggt ggt ggt ggt ggt ggt-3’ and reverse 5’-ggg ggt ggt ggt ggt ggt ggt-3’). The following PCR conditions were applied: COX-2 and iNOS, 25 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, and extended at 72 °C for 30 s; β-actin, 23 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extended at 72 °C for 30 s. β-actin was used as an internal control to evaluate relative expression of iNOS, COX-2, and TNF-α.

Western blot analysis

Total cell extracts were prepared using PRO-PREP protein extraction kit (iNITRON Biotechnology; Sungnam, Republic of Korea). The preparation of cytoplasmic and nuclear extracts was conducted using NE-PER nuclear and cytosolic extraction reagents (Pierce, Rockford, IL, USA). Total cell
extracts were separated on polyacrylamide gels and then standard procedures were used to transfer them to the nitrocellulose membranes. The membranes were developed using an ECL reagent (Amersham, Arlington Heights, IL, USA).

**Electrophoretic mobility assay (EMSA)**

DNA-protein binding assays were carried out with nuclear extract. Synthetic complementary NF-κB binding oligonucleotides (5'-AGT TGA GGG GAC TTT CCC AGG C-3', Santa Cruz Biotechnology) were 3'-biotinylated using the biotin 3'-end DNA labeling kit (Pierce) according to the manufacturer’s instructions. Assays were performed using a Lightshift EMSA Optimization kit (Pierce) according to the manufacturer’s protocol.

**Statistical analysis**

All data were derived from at least three independent experiments. The images were visualized with Chemi-Smart 2000 (Vilber Lourmat, Marine; Cedex, France). Images were captured using Chemi-Capt (Vilber Lourmat) and transported into Photoshop. All bands were quantified by Scion Imaging software (http://www.scioncorp.com). Statistical analyses were conducted using SigmaPlot software (version 11.0). Values were presented as mean ± SE. Significant differences between the groups were determined using two-way ANOVA test. Statistical significance was set at $p < 0.05$.

**RESULTS**

**Effect of MEPL on cell viability**

To determine the effect of MEPL on BV2 cells, an MTT assay was performed at 24 h after treatment with MEPL (50 – 200 µg/ml) in the presence or absence of LPS. MEPL (50 – 150 µg/ml) had no cytotoxic effect on BV2 cells. However, cell viability was reduced when 200 µg/ml of MEPL was used with LPS. MEPL as well as LPS (1 µg/ml) alone did not show any cytotoxic effect on BV2 cells (Fig 1). Therefore, 50–100 µg/ml MEPL was applied in subsequent experiments.

![Fig 1: Effect of MEPL on BV2 cell viability. Cells (1 × 10^5 cells/ml) were incubated with the indicated concentrations of MEPL (50 – 200 µg/ml) for 1 h before lipopolysaccharide (1.0 µg/ml) treatment for 24 h; n = 3; *p < 0.05 compared with untreated control.](image)

**Effect of MEPL on LPS-induced NO and PGE_2 production**

Cells were stimulated with LPS (1.0 µg/ml) for 24 h after pretreatment with MEPL (100 µg/ml) for 1 h. NO and PGE_2 production were analyzed using the Griess reaction assay and ELISA, respectively. Stimulating the cells with LPS resulted in a significant increase in NO production (19.3 ± 0.7 µM) compared to the untreated control (5.1 ± 0.2 µM; Fig 2A). Importantly, MEPL treatment significantly inhibited LPS-induced NO production to the level of the untreated control (5.1 ± 0.2 µM). Consistent with the inhibition of NO production, MEPL treatment (134 ± 11 pg/ml) markedly attenuated LPS-induced PGE_2 production (465 ± 35 pg/ml; Fig 2B). Our data also revealed that MEPL alone (83 ± 19 pg/ml) sustained PGE_2 production compared to the untreated control (80 ± 21 pg/ml). Taken together, these results indicate that MEPL significantly suppressed the release of NO and PGE_2 in LPS-stimulated BV2 cells.
Effect of MEPL on LPS-induced nitric oxide (NO) and prostaglandin (PG) E$_2$ production in BV2 cells. Cells (1 x 10$^5$ cells/ml) were incubated with 100 µg/ml of MEPL for 1 h before LPS (1.0 µg/ml) treatment for 24 h; n = 3; *p < 0.05 compared with the value in cells treated with LPS alone.

Effect of MEPL on LPS-induced iNOS and COX-2 protein and mRNA

To assess whether MEPL regulates NO and PGE$_2$ production at the transcriptional level, iNOS and COX-2 protein and mRNA expression were determined by Western blot and RT-PCR analyses, respectively. Although iNOS and COX-2 protein expression increased significantly in the presence of LPS alone, Western blot analysis data showed decreasing iNOS and COX-2 protein expression following the MEPL pretreatment (Fig 3A). We further investigated these results by conducting an RT-PCR analysis for iNOS and COX-2 mRNA expression. The RT-PCR analysis showed that LPS treatment alone significantly increased iNOS and COX-2 expression at the transcriptional level at 6 h (Fig 3B). But these expressions were downregulated after pretreatment with MEPL at 100 µg/ml, similar to the western blot analysis data. Taken together, these data indicate that MEPL suppressed the upregulation of LPS-stimulated iNOS and COX-2 expression at the transcriptional level.

Effect of MEPL on LPS-induced TNF-α production and mRNA expression

Next, we investigated TNF-α and TNF-α mRNA production in LPS-induced BV2 cells. Cells were pretreated with MEPL (100 µg/ml) for 1 h before LPS stimulation for 24 h, and the level of TNF-α in the culture supernatant was determined by ELISA. Consistent with the NO and PGE$_2$ data, treating the cells with LPS alone significantly increased TNF-α production approximately 10-fold (3533 ± 65 pg/ml) compared to the untreated control (332 ± 39 pg/ml); however, the LPS-induced TNF-α increase was restored to a 50% reduction in the presence of MEPL (1540 ± 35 pg/ml; Fig 4A). To determine the effect of MEPL on TNF-α gene expression, an RT-PCR analysis was performed at 6 h after LPS treatment. The RT-PCR data showed that MEPL suppressed TNF-α mRNA expression in LPS-stimulated BV2 cells (Fig 4B). These
data indicate that MEPL regulates TNF-α production in LPS-stimulated BV2 microglia cells by regulating TNF-α gene expression.

**Fig 4:** Effect of MEPL on LPS-induced TNF-α production (A) and TNF-α mRNA expression (B) in BV2 cells. Cells \( (1 \times 10^5 \text{ cells/ml}) \) were incubated with 100 µg/ml MEPL for 1 h before LPS (1.0 µg/ml) treatment for the indicated time; \( n = 3 \); *\( p < 0.05 \) compared with the value in cells treated with LPS alone.

**Inhibitory effect of MEPL in LPS-induced NF-κB activity**

Activating NF-κB induces the expression of pro-inflammatory mediators such as iNOS, COX-2 and TNF-α [5]. Therefore, we used Western blot analysis and an electrophoretic mobility shift assay (EMSA) to investigate how MEPL regulates the specific DNA-binding activity of NF-κB. BV2 cells were preincubated with MEPL for 1 h and then stimulated with LPS for 30 min. The EMSA was conducted to determine whether MEPL inhibits the DNA-binding activity of NF-κB. LPS caused a remarkable increase in binding complexes between NF-κB and specific-binding DNA; however, pretreatment with MEPL significantly reduced LPS-induced NF-κB binding activity (Fig 5A). In a parallel experiment, LPS significantly induced the phosphorylation and degradation of IκBα and increased p65 expression in the nuclear compartment of BV2 cells (Fig 5B). However, nuclear translocation of p65, and phosphorylation and degradation of IκBα induced by LPS were inhibited in cells preincubated with MEPL (Fig 5B). Furthermore, N-acetyl-L-cysteine (NAC) was used as a potent proteasome and proteases inhibitor of Rel/NF-κB activity to confirm those results. We conducted an RT-PCR analysis to detect iNOS, COX-2, and TNF-α mRNA expression in the presence of NAC. Interestingly, NAC inhibited the expression of LPS-stimulated iNOS, COX-2, and TNF-α expression at the transcriptional level (Fig 5C). These data indicate that MEPL reduced NF-κB activity in LPS-stimulated BV2 cells by suppressing IκBα phosphorylation and degradation.

**DISCUSSION**

Inhibitors of inflammatory mediators and cytokines have been considered an approach to anti-inflammatory drugs. Our data revealed that MEPL inhibited LPS-induced NO and PGE₂ production by suppressing iNOS and COX-2 mRNA expression in BV2 cells without cytotoxicity. MEPL also decreased TNF-α production by suppressing its mRNA...
expression. Furthermore, we showed that these effects occurred by inhibiting NF-κB activity. These data indicate that MEPL alleviates pro-inflammatory mediators such as NO, PGE$_2$, and TNF-α via suppression of NF-κB activity.

Expression of NO, PGE$_2$, and TNF-α plays a critical role in macrophage activation and is also associated with acute and chronic inflammatory diseases [12]. Particularly, NO normally contributes to the control of replication or killing of intracellular microbial pathogens and cancer cells [13]. However, its uncontrolled release can result in inflammatory destruction of target tissues during an infection [14]. The release of iNOS-mediated NO is one of the major factors during inflammatory processes. PGE$_2$ is another pro-inflammatory mediator involved in inflammatory responses, which is generated by metabolism of arachidonic acid by cyclooxygenase (COX) [15]. Activation of the COX-2 gene is particularly responsible for various inflammatory diseases by inducing PGE$_2$ overexpression [2]. Accumulating evidence confirms COX-2 as a potential therapeutic target for treating inflammatory diseases [2]. Additionally, TNF-α is a pleiotropic inflammatory cytokine involved in systemic inflammatory diseases [16]. Overproduction of TNF-α switches on signaling pathways that change the cell functions of LPS-stimulated macrophages [17]. As TNF-α plays a role in several diseases, a substantial amount of research has been conducted concerning TNF-α blockers and anti-TNF-α therapies. Recently, anti-TNF-α therapy has been broadly introduced to treat various inflammatory diseases [16]. Therefore, suppressing NO, PGE$_2$, and TNF-α production by inhibiting their regulatory genes could be a very important therapeutic target for developing anti-inflammatory agents. Our results showed that MEPL attenuated the production of the anti-inflammatory mediators such as NO, PGE$_2$, and TNF-α in LPS-induced BV2 cells. These data suggest the possibility of developing anti-inflammatory chemicals using MEPL. Nevertheless, further study is needed to elucidate what MEPL component suppresses the expression of anti-inflammatory mediators.

NF-κB has often been referred to as a central mediator and is strongly implicated in a variety of inflammatory diseases [9]. Many stimulants including LPS, bacteria, and viruses can lead to NF-κB activation, which leads to the control of the expression of many inflammatory cytokines, chemokines, immune receptors, and cell surface adhesion molecules [18]. Once NF-κB is fully activated, it participates in the regulation of various target genes in different cells and is involved in different functions [4]. In this study, we showed that MEPL inhibited p65 protein translocation via suppression of IκBα degradation. As a result, MEPL suppressed the LPS-induced DNA-binding activity of NF-κB. Because the expression of many inflammatory genes, iNOS, COX-2, and TNF-α are modulated by NF-κB binding to its specific promoter regions, it is a potential target for suppressing NF-κB activity and regulating LPS-induced inflammation [5]. We tested NF-κB activity with NAC, which is a proteasome and protease inhibitor of Rel/NF-κB activity [19]. According to the RT-PCR analysis, iNOS, COX-2, and TNF-α mRNA expression was suppressed in the presence of NAC. Taken together, these results suggest that MEPL suppresses inflammatory mediators such as NO, PGE$_2$, and TNF-α via LPS-induced NF-κB activity by suppressing p65 translocation and IκB phosphorylation.

**CONCLUSION**

This study showed that MEPL has anti-inflammatory activity which depends on its ability to regulate NO, PGE$_2$, and TNF-α production by suppressing NF-κB activation in LPS-stimulated BV2 microglia cells. The data suggest that MEPL has a high potential to treat LPS-induced inflammatory diseases.
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