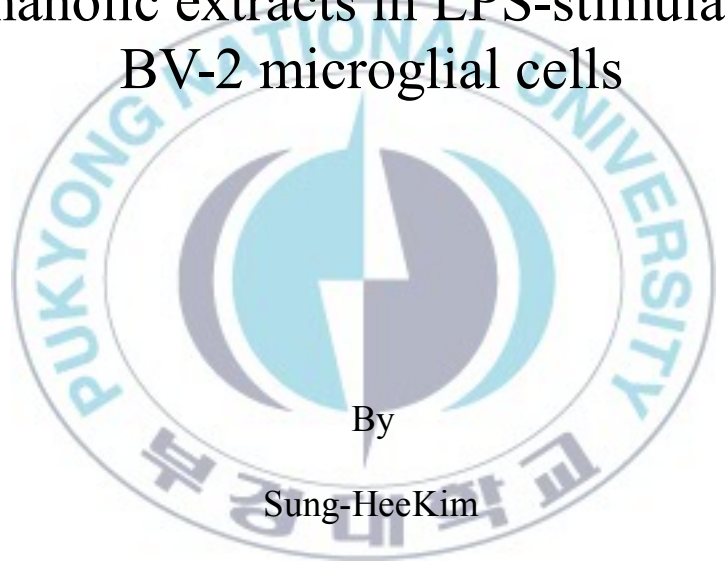
Thesis for the Degree of Master of Science

Anti-inflammatory effect of hexane fraction from *Myagropsis myagroides* ethanolic extracts in LPS-stimulated BV-2 microglial cells



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February 2013

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LPS로유도된 BV2 소교세포에서 외톨개모자반 헥산추출물의 항염증효과

Advisor: Prof. Hyeung-Rak Kim

By
Sung-Hee Kim

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요약

소교세포 (microglia)는 뇌의 면역을 담당하는 세포로서 중추신경계의 정상적인 활동을 유지하는 중요한 역할을 한다. 그러나 다양한 인자에 의해 자극을 받으면 소교세포가 활성화되고 여러 염증매개자들이 과분비하여 뇌에 손상을 주게 된다. 그러므로 본 연구에서는 이러한 염증반응을 억제할 수 있는 기능성 식품 재료로서, 외톨개모자반(*Myagropsis myagroides* (Mertens ex Turner) Fenshol, MMH)의 에탄올추출물에서 분리된 hexane fraction 의이용가능성을 기본적인 분자적 메커니즘 수준에서 확인하였다. 외톨개모자반은 개모자반과에 속하는 다년생 갈조류로 일본 연안 전체에 걸쳐 서식하며 우리 나라에서는 부산, 제주도 연안 등지에 분포한다. 외톨개모자반의 간보호 효과, 항균효과, 항응고 효과 등이 연구되어 왔다.

마우스 유래 소교세포주인 BV2 세포에 lipopolysaccharide (LPS)로 자극을 주어 염증성 매개체들의 발현을 유도하고, 이에 다양한 농도의

MMH 를 처리하였다. Inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2)와 같은 효소의 발현은 Western blot 으로 확인하였다. nitric oxide (NO)는 Griess reagent 를 사용하여 측정하였으며 prostaglandin E_2 (PGE_2)와 같은 염증성 매개체 및 interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α)와 같은 염증촉진성 cytokine 수준을 ELISA 를 통해 확인하였다. 또한 MMH 를 처리 하였을 때 이들 염증성 매개체들의 발현을 조절하는데 주된 역할을 하는 전사인자인 nuclear factor kappa B (NF-κB)의 활성화가 억제되는 것으로 관찰되었다. 이는 inhibitory kappa B (IκB)의 인산화 및 분해가 억제되어 NF-κB 의 핵으로의 이동이 감소한 것으로 확인할 수 있었다. 또한 NF-κB 의 promoter 활성을 luciferase assay 를 통해서 분석한 결과 이들의 활성이 MMH 에 의해서 저해되는 것이 나타났고, 이는 Extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK)의 활성 감소와 연관되어 있는 것으로 확인되었다.

이상의 결과에서 LPS 에 의해 자극된 소교세포주에서의 염증성 매개체들의 발현 및 생성은 MMH 처리에 의해 효과적으로 억제되는 것으로 관찰되었다. 이는 ERK, JNK 의 인산화의 억제 및 그에 따른 NF-κB 의 활성화가 저해되고 결과적으로 iNOS 와 COX-2 의 발현의 감소를 통해서 염증반응이 억제되는 것으로 판단된다. 따라서 본 결과는 MMH 가 소교세포의 활성화 및 염증성 반응을 억제할 수 있는 기능성 식품재료로 이용될 수 있음을 시사한다.

1. Introduction

Microglia are the primary immune cells in the brain, and play an important role in maintaining the integrity of central nerve system. They support synaptic integrity, but their main function is modulation of innate immune response (Graeber M, 2010). The developing brain is damaged by a variety of factors and mechanisms including inflammation, excytotoxicity, and oxidative stress. In these conditions, activated microglia secrete excessive levels of pro-inflammatory mediators such as nitric oxide (NO), prostaglandin E_2 (PGE_2), and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 (Silva *et al*., 2008; Bozinovski*et al*., 2007). These mediators and cytokines are known to exacerbate an injury and subsequent neurodegeneration. Therefore modulation of activated microglia is an effective strategy for therapeutics in the neuroinflammation.

NO and PGE_2 are primarily produced by enzymatic action of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively. Expressions of these enzymes as well as pro-inflammatory cytokines are regulated by the activation of nuclear factor-kappaB (NF-κB) (Bozinovski *et al*., 2007). iNOS and COX-2 gene promoters contain one and two NF-κB consensus sequence, respectively, which are responsible for binding of activated NF-κB. In most cell types, NF-κB is located

in the cytosol as an inactive complex by binding to inhibitory kappaB (IκB) family. NF-κB is activated by diverse external stimuli including inflammatory cytokines, bacterial components, and viral infection (Pahl *et al*., 1999). Lipopolysaccharide (LPS) stimulates the phosphorylation, ubiquitination, and proteasomal degradation of IκB by the activation of IκB kinase complex, resulting in translocation of NF-κB into the nucleus by dissociation of NF-κB-IκB-α complex (Janssen-Heininger*et al.*, 2000). The activation of NF-κB is also controlled by cellular signaling kinases such as mitogen-activated protein kinases (MAPKs) and phosphoinositide 3-kinase (PI3K)/AKT (Jang *et al.,* 2005; Cantley., 2002). MAPK family including extracellular signal regulated kinase (ERK), p38 MAPK, and c-Jun NH_2-terminal kinase (JNK) is associated with the transcriptional regulation of inflammatory genes (Jang *et al.,* 2005; Herlaar *et al.,* 1999). The elucidation of detailed molecular mechanisms involved in these regulations is important for dealing with the harmful effects of pro-inflammatory mediators (Jana *et al.,* 2007).

Marine macroalgae contain an abundance of polysaccharides, minerals, and polyunsaturated fatty acids, so they are known to be beneficial for human health. Recently, a variety of studies have revealed that the compounds, such as phlorotannins (Gupta. et al., 2012; Nwosu *et al.,* 2011), present in the extracts from *Sargassum horneri, Sargassum fulvellum, Undaria*

pinnatifida, and *Laminaria japonica* have biological activities including anti-oxidation (Airanthi *et al.,* 2011) and anti-inflammation (Kang *et al.,* 2007; Khan *et al.,* 2008*;* Lee *et al.,* 2012). *Myagropsis myagroides* (Mertens ex Turner) Fensholt, which grows in subtidal zone of the coast of East Asia, belongs to the family Sargassaceae in phaeophyta. It was shown to have hepatoprotective effect (Wong *et al.,* 2000), antimicrobial activity(Lee SY *et al*., 2011), and anticoagulant activity (Athukorala *et al*., 2007*)*. In addition, fucoxanthin isolated from *M. myagroides* inhibited the production of inflammatory mediators and pro-inflammatory cytokines in RAW 264.7 macrophage cells (Heo *et al.,* 2010).

We found strong anti-inflammatory activity in hexane fraction from *M. myagroides* ethanolic extract (MMH) among various solvent fractions from *M. myagroides* ethanolic extract. To the best of our knowledge, there have not been any reports about the anti-inflammatory activity of hexane fraction from *M. myagroides*. In this regard, we investigated molecular mechanisms of anti-inflammatory activity of MMH in LPS-stimulated BV-2 microglial cells. The findings demonstrate that MMH may be used as a source of drug candidate for the prevention or treatment of cerebral inflammatory diseases.

2. Materials and Methods

2-1. Materials

LPS (*Escherichia coli* O55:B5), 2,2-diphenyl-1-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), and the specific kinase inhibitors (PD98059 and SP600125) were purchased from Sigma Chemical Corporation (St. Louis, MO, USA). CellTiter96 AQueous One Solution Cell Proliferation assay kit, dual luciferase assay kit, murine NF-κB promoter/luciferase DNA, pRL-TK DNA, and Moloney murine leukemia virus reverse transcriptase were obtained from Promega (Madison, WI, USA). Primary and secondary antibodies were purchased from Cell Signaling Biotechnology (Danvers, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. LipofectaminePlus Reagent and TRIzol reagent were purchased from Invitrogen (Carlbad, CA, USA).

2-2. Plant materials

M. myagroides was collected along the coast of Busan, South Korea in March 2011. The samples were rinsed using tap water to remove salt. They were air-dried under shade for 2 days and ground with hammer grinder. The resulting dried powder was stored at -20^{o}C until used.

Dried powder (2.38 kg) of *M. myagroides* was extracted

three times with 96% (v/v) ethanol for 3 h at 70°C. The combined extracts were concentrated using a rotary vacuum evaporator (Eyela, Tokyo, Japan) at 40°C and lyophilized to give a powder (378 g). The ethanolic extract was stepwise partitioned with the same volume of following solvents: *n*-hexane, ethyl acetate, *n*-butanol, and distilled water. The fractions were concentrated, lyophilized, and stored at -20°C until used. Each dried fraction was dissolved in DMSO and further diluted with culture media before use. The final concentration of DMSO in cell culture medium was less than 0.1%.

2-3. Total phenolic content and DPPH radical-scavenging activity of *M. myagroides* fractions

Total phenolic content (TPC) was measured according to the method of Singleton et al. (1999). The free radical scavenging activity was measured by DPPH scavenging assay. Forty microliters of 0.3 mM DPPH in methanol was mixed with 40 µl of extract or fraction at different concentrations and incubated at room temperature for 30 min in dark. Then the absorbance was measured at 517 nm. The concentration of DPPH radical was calculated by subtracting the absorbance of the sample from that of the control.

2-4.Cell cultures and sample treatment

The murine BV2 microglial cell line was grown and maintained in DMEM supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml) in a humidified atmosphere of 5% CO_2. Cells were stimulated with LPS (1μg/ml) in the presence or absence of MMH isolated from *myagroides* for the indicated periods. The stock solutions of MMH were prepared in DMSO.

2-5. Cell viability and proliferation

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using CellTiter96 AQueous One Solution Cell Proliferation assay kit according to the manufacturer's manual. Cells were inoculated at a density of 3×10^5 cells into 96-well plates and cultured at 37°C for 24 h. Cells were then treated with LPS (1 μg/ml) in the presence or absence of MMH in different concentration for 24 h. The final concentration of DMSO was less than 0.1% in the cell culture medium. The culture medium was removed and replaced by 95 μl of fresh culture medium and 5 μl of MTS solution. After 1 h, the absorbance at 490 nm was measured using a microplate reader (Glomax Multi Detection System, Promega).

2-6. Measurement of nitric oxide, $PGE_2$and cytokines

Cells (5×10^4 cells/well) were pre-treated with MMH (0-25 μg/mL) for 2 h prior to LPS treatment for 24 h. After treatment of LPS, cultured media of BV2 cells were collected and stored at -75°C until tested. For the measurement of NO, 100 μl of culture supernatant was mixed with the same volume of Griess reagent (0.1% naphthylethylenediaminedihydrochloride and 1% sulfanilamide in 5% phosphoric acid) and incubated at room temperature for 10 min. Absorbance of the mixture was measured with a microplate reader at 540 nm. Levels of PGE_2, TNF-α, IL-1β, and IL-6 in cultured media from each group were quantitatively determined by ELISA (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2-7. Western blotAnalysis

2-7-1. Preparation of total cell lysates

Proteins (30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto the nitrocellulose membranes (Whatman GmbH, Dassel, Germany). The membranes were washed with Tris-buffered saline (10 mM Tris-HCl, 150 mM NaCl, pH 7.5) supplemented with 0.05% Tween 20 [TBST] followed by blocking with TBST containing 5% non-fat dried milk. The membranes were incubated overnight with

primary antibodies. The membranes were then exposed to secondary antibodies coupled to horseradish peroxidase for 2 h at room temperature. The membranes were washed three times with TBST at room temperature. Immunoreactivities were detected by ECL reagents. Densitometric analysis of the data obtained from at least three independent experiments was performed using cooled CCD camera system EZ-Capture II (ATTO & Rise Co., Tokyo, Japan) and CS analyzer ver. 3.00 software (ATTO).

2-7-2. Preparation of cytosolic and nuclear extracts

BV2 cells plated in a 6-well cell culture plates at a density of 1×10^6 cells per well were pretreated with or without MME for 2 h and then treated with LPS for 0.5 h. Cells were washed two times with ice-cold PBS, scraped in PBS and centrifuged at 13000*g* for 5 min at 4°C. Pellets were suspended in 180 μl of hypotonic buffer A [10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.02 % NaN_3, 0.5 mM DTT and 1 mM phenylmethylsulfonyl fluoride (PMSF)] on ice, and afterward, 20 μl of 5 % Nonidet P-40 was added for 5 min. The mixture was centrifuged at 1800*g* for 5 min. Supernatant was collected as cytosolic extract. The pellets were washed with hypotonic buffer and resuspended in hypertonic buffer C [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 25 % glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2

mMethylenediaminetetraacetic acid, 0.02 % NaN_3, 0.5 mM DTT, and 1 mM PMSF] for 1 h on ice and centrifuged at 14000*g* for 10 min. The supernatant containing nuclear proteins was collected and stored at −70°C after determination of the protein concentration.

2-8. Reverse transcription-polymerase chain reaction (RT-PCR)

BV2 *c*ells plated in a 6-well cell culture plate at a dens ity of 3.0×10^5 cells/well were pretreated without or with MMH for 1 h and then treated with LPS for 6 h. Total RNA from each group was isolated with the TRIzol reagent. Five microgram of total RNA was used for reverse transcription using oligo-dT and M-MLV reverse transcriptase. PCR was carried out using the resulting cDNA as a template, with the following condition: 25 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The PCR products were visualized by agarose gel electrophoresis. Verification of PCR product of specific genes was established by their predicted sizes under ultraviolet light illuminator. The primer sequences were following. **Tabel 1**. GAPDH was used as an internal standard to evaluate relative expression of COX-2 and iNOS. Densitometric analysis of the data obtained from at least three independent experiments was performed using cooled CCD camera system EZ-Capture II and CS analyzer ver. 3.00 software.

Table 1.R T-PCR primers used this study.

Gene	Direction	Sequence
iNOS	sense	5'-ACCACTCGTACTTGGGATGC-3′
	antisense	5'-CACCTTGGAGTTCACCCAGT-3′
COX-2	sense	5'-TGGGCAAAgAATGCAAACAT-3′
	antisense	5'-CAGCAAATCCTTGCTGTTCC-3′
TNF-α	sense	5′-GGCAGGTCTACTTTGGAGTCATTGC-3′
	antisense	5′-ACATTCGAGGCTCCAGTGAATTCGG-3′
IL-6	sense	5′-GTATGAACAACGATGATGCACTTGCAG-3′
	antisense	5′-GCATTGGAAATTGGGGTAGGAAGG-3′
GAPDH	sense	5'-GACCCCTTCATTGACCTCAA-3′
	antisense	5'-CTTCTCCATGGTGGTGAAGA-3′

2-9. Immunocytochemical analysis

To analyze nuclear localization of NF-κB in BV2 cells, cells were cultured on glass coverslips (SPL Lifesciences Co., Gyeonggi-do, Korea) in 24-well plates for 24 h. After preincubation with MME for 2 h, cells were stimulated with or without LPS (1 μg/ml). Cells were fixed in 4.0% paraformaldehyde in PBS for 15 min at room temperature, and then permeabilized with 0.5% Triton X-100 in PBS for 10 min. Permeabilized cells were washed with PBS and blocked with 3% BSA in PBS for 30 min. Thereafter, cells were incubated in an anti-NF-κB polyclonal antibody diluted in 3% BSA/PBS for 2 h, rinsed three times for 5 min with PBS, and incubated in Alexa

Fluor® 488-conjugated secondary antibody diluted in 3% BSA/PBS for 1 h. Cells were stained with 2 μg/ml DAPI and viewed, and images were captured using an LSM700 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

2-10. NF-κBpromoter/luciferase assay

Two microgram of pNF-κB promoter/luciferase DNA along with 40 ng of control pRL-TK DNA was transiently transfected into 2.0×10^5 BV2 microglia cells per well in a six-well plate using Lipofectamine Plus reagents for 40 h. Cells were treated with MMH for 2 h and stimulated with LPS (1 μg/ml) for 6 h. Luciferaseactivities of the cells were measured using dual-luciferase assay system according to the manufacturer's instructions. Each transfection was performed in triplicate, and all experiments were repeated at least three times. The luciferase activity was normalized with luciferase activity of control pRL-TK.

2-11. Statistical Analysis

Data were expressed as means ± standard deviations (SDs) of at least three independent experiments unless otherwise indicated. Data were analyzedusing one-way analysis of variance (ANOVA), followed by each pair of Student's *t*-test for multiple comparisons. Differences with a value of $p<0.05$ were considered statistically significant. All analyses were performed using SPSS for Windows,

version 10.07 (SPSS, Chicago, IL, USA).

3. Result

3-1. Total phenolic contents (TPCs), DPPH radical-scavenging activities, and NO-production inhibitory activities of solvent fractions from *M. myagroides* ethanolic extracts

Table 2 shows the yields, TPCs, DPPH radical-scavenging activities, and NO production inhibitory activities of various solvent fractions from *M. Myagroides* ethanolic extract. Hexane fraction showed the lowest level of TPC and the highest DPPH radical-scavenging activity compared to other fractions. Furthermore, hexane fraction showed the highest inhibitory activity on NO generation. Therefore, we further investigated the anti-inflammatory properties of the hexane fraction (MMH) and its underlying mechanisms.

Table 2.Biological properties of various solvent fractions from *M. myagroides* ethanolic extract

M. myagroides fractions	Weight (Yield)	Total phenolic content (mg/g)	DPPH* radical scavenging activity (EC_{50}, g/ml)	Inhibitory activity on NO generation** (EC_{50}, g/ml)
n-Hexane	56.9 g (15.05%)	43 ± 2	116.87 ± 1.22	9.98 ± 1.22
Ethyl acetate	3.1 g (0.82%)	210 ± 19	37.53 ± 2.51	25.07 ± 0.77
n-Butanol	1.8 g (0.48%)	ND***	> 200	> 200
Water	316.2 g (83.65%)	ND***	> 200	> 200
Ascorbic acid			1.79 ± 0.03	

*2,2-diphenyl-1-picrylhydrazyl

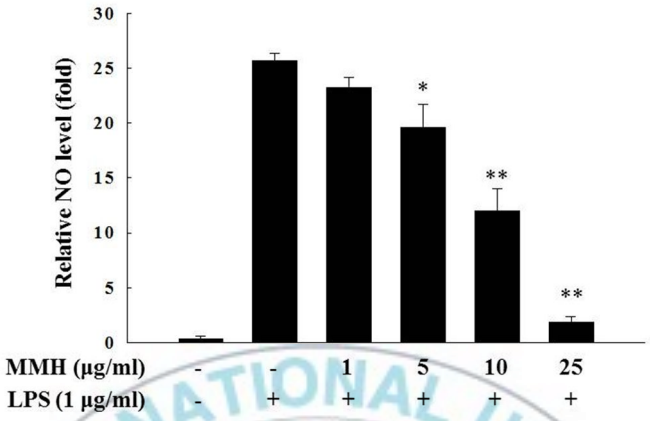
**Inhibitory activity of NO generation in LPS-stimulated BV-2 cells

***Not detected

3-2. MMH inhibits NO and PGE_2 productionin LPS-stimulatedBV-2 cells

We determined anti-inflammatory effect of MMH on LPS-induced production of inflammatory mediators, NO and PGE_2, in BV-2 cells. Cells pretreated with MMH were stimulated with or without LPS (1 μg/ml) for 24 h. As shown in Figure 1(A), MMH significantly suppressed LPS-induced NO production in a dose-dependent manner. In addition, PGE_2 concentration in the culture media was also decreased by MMH pretreatment in a dose-dependent manner (Figure 1(B)). Clearly, MMH at concentrations higher than 10 μg/ml strongly suppressed the production of NO and PGE_2 in LPS-stimulated BV-2 cells. MTS assay showed no cytotoxicity at the concentrations below 25μg/ml of MMH in BV-2 cells (Figure 1(C)). These data indicate that MMH inhibited LPS-induced NO and PGE_2 secretion in BV-2 cells at concentrations with no cytotoxicity.

(A)



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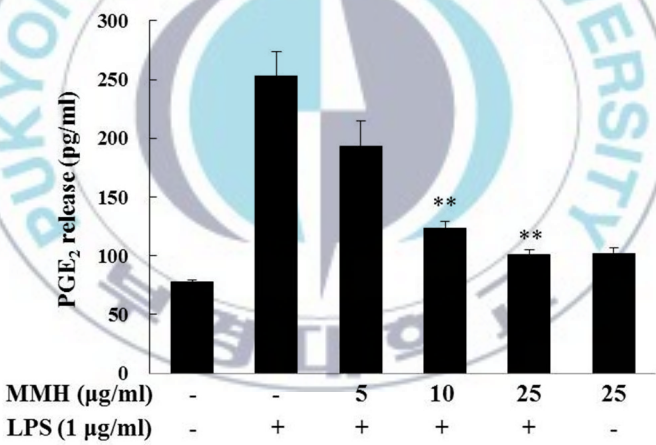

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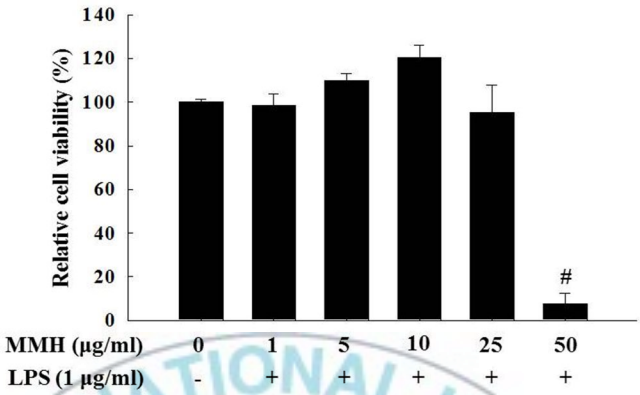


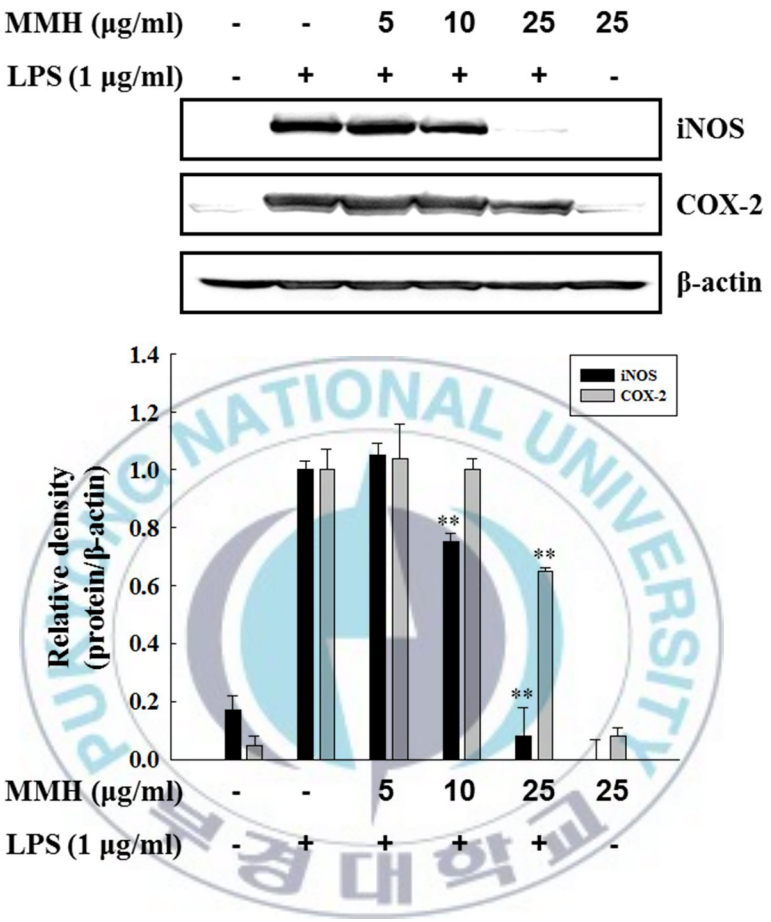
Figure 1.Effect of hexane fraction from *Myagropsis myagroides* ethanolic extract (MMH) on cell viability and the productions of NO and PGE_2 in LPS-stimulated BV-2 cells.

Cells pretreated with various concentrations of MMH for 2 h were stimulated with LPS (1 μg/ml) for 24 h.(A, B) Pretreatment of MMH inhibits NO and PGE_2 production in LPS-stimulated BV-2 cells. The culture media of the treated cells were used to measure the amount of nitrite to evaluate NO (A) or the production of PGE_2 (B). (C) Effect of MMH on cell viability analyzed by MTS assay. All data are presented as means ± SDs of three independent experiments. $^{*}p<0.05$ and $^{**}p<0.01$ indicated significant differences compared to LPS-only group. $^{\#}p<0.01$ indicate significant differences compared to MMH-untreated group.

3-3. MMH suppresses LPS-induced expressions of iNOS and COX-2 inBV-2 cells

To investigate whether the suppressions of NO and PGE_2 production by MMH treatment are related with the modulation of iNOS and COX-2 enzymes, we analyzed their protein and mRNA levels by Western blot and RT-PCR, respectively. As shown in Figure 2(A), treatment of BV-2 cells with LPS (1 μg/ml)for 16 h induced the expressions of iNOS and COX-2 protein. However, pretreatment with MMH led to a dose-dependent inhibition of the LPS-induced iNOS and COX-2 protein expressions in BV-2 cells. Twenty five μg/ml of MMH almost completely inhibited the LPS-induced iNOS expression. To further examine the effect of MMH on the transcriptional regulation of iNOS and COX-2 expression, RT-PCR analysis wasperformed. Figure 2(B) showed thatMMH suppressed the expressions of iNOS and COX-2 mRNA in a concentration-dependent manner in LPS-stimulated BV-2 cells. Similar to the result of Western blot, 25 μg/ml of MMH completely inhibited iNOS mRNA expression. These results indicate that MMH-mediated down-regulation of iNOS and COX-2 expressions in LPS-stimulated BV-2 cells is mainly due to their transcriptional repression.

(A)



(B)

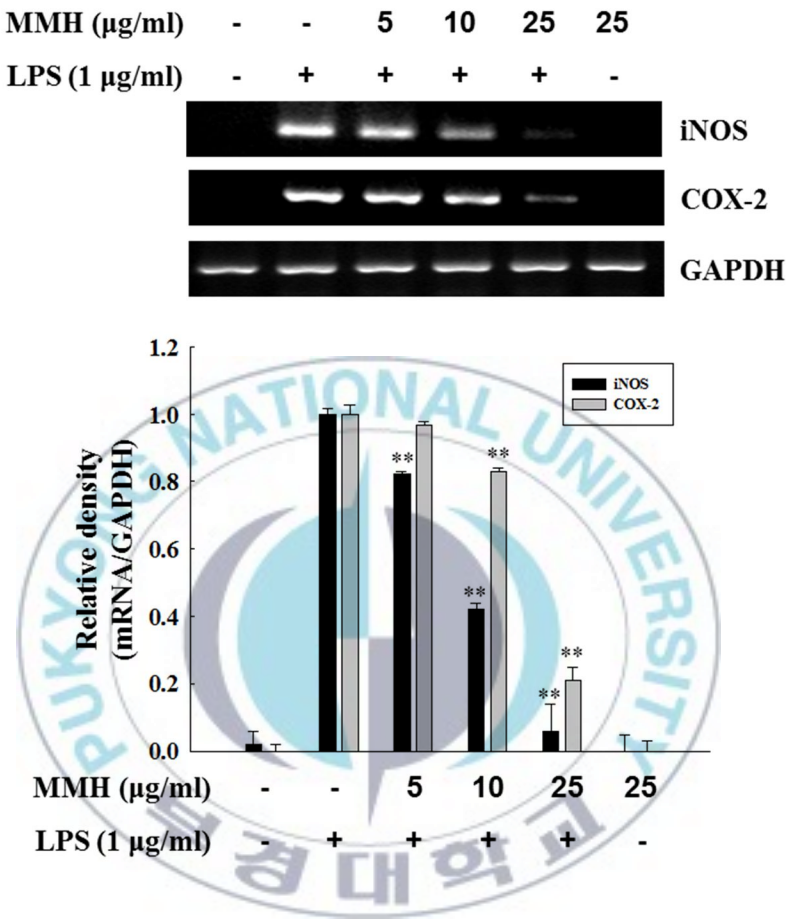


Figure 2.Effect of MMH on LPS-induced iNOS and COX-2 expressions in BV-2 microglial cells.

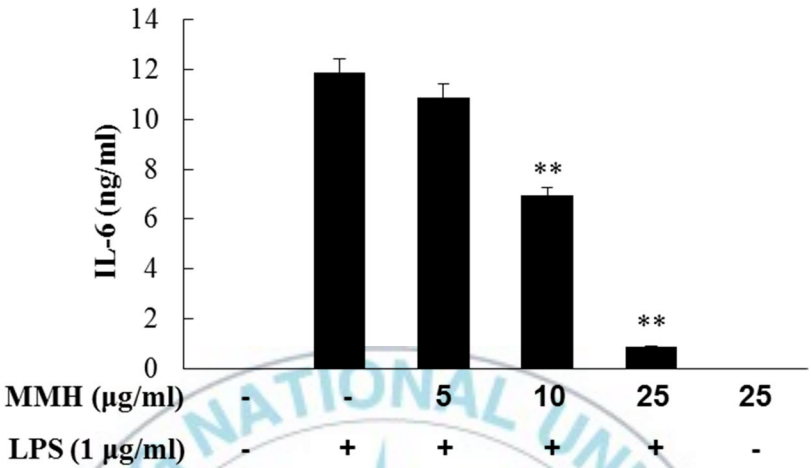
(A) Western blot analysis of iNOS and COX-2 protein expression. Cells pretreated with various concentrations of MMH for 2 hwere stimulated with or without LPS (1 μg/ml) for 16 h. (B) RT-PCR analysis of iNOS and COX-2 mRNA expression. Cells were incubated with various concentrations of MMH for 2 h, and

then stimulated with LPS (1 μg/ml) for 6 h. mRNA levels of iNOS, COX-2, and GAPDH were determined by RT-PCR analysis using corresponding gene-specific primers. The results presented are representatives of three independent experiments. Quantitative data represent means ± SDs of three independent experiments. $^{**}p<0.01$ indicate significant differences compared to LPS-only group.

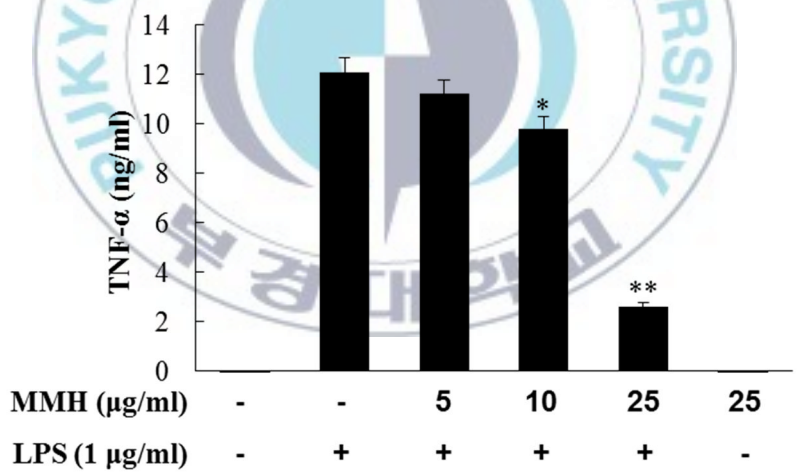
3-4. MMH inhibits LPS-induced TNF-αand IL-6productions in BV-2 cells

Pro-inflammatory cytokines such as TNF-α and IL-6 are secreted in the early stage of inflammatory responses, so they are the key markers of the inflammation. We determined the levels of these cytokines in the media of LPS-stimulated BV-2 cells. BV-2 cells were incubated with various concentrations of MMH for 2 h and then stimulated with LPS for 24 h. The levels of the cytokines in the culture media were quantitated by ELISA. The stimulation of BV-2 cells with LPS induced significant increase in thelevels of TNF-αand IL-6, and pre-treatmentwith MMH inhibited their increased productions in a dose-dependent manner (Figure 3(A) and 3(B)). In addition, we investigated whether the productions of these cytokines are regulated at transcriptional level. Figure 3(C) shows that expressions of TNF-αand IL-6 mRNA were induced by LPS, and that pretreatment with MMH inhibited the expressions of these cytokine genes in a dose-dependent manner. These results indicate that the inhibitory effect of MMH on the productions of pro-inflammatory cytokines is mediated by the suppression of their transcription in LPS-stimulated BV-2 cells.

(A)



(B)



(C)

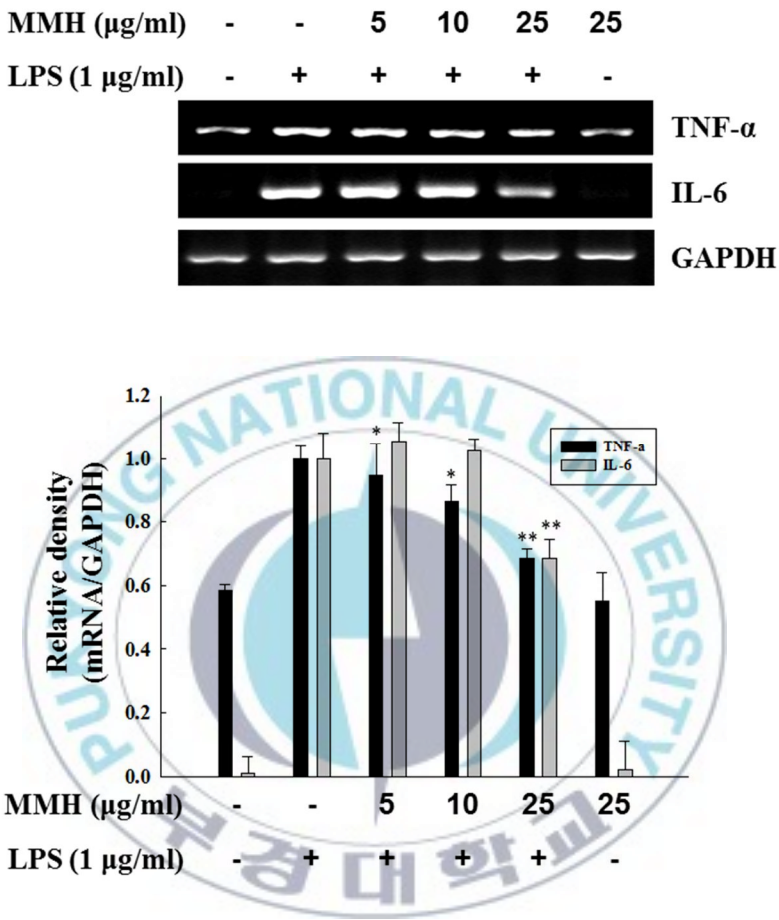


Figure 3.Effect of MMH on the secretion of pro-inflammatory cytokines in LPS-stimulated BV-2 cells.

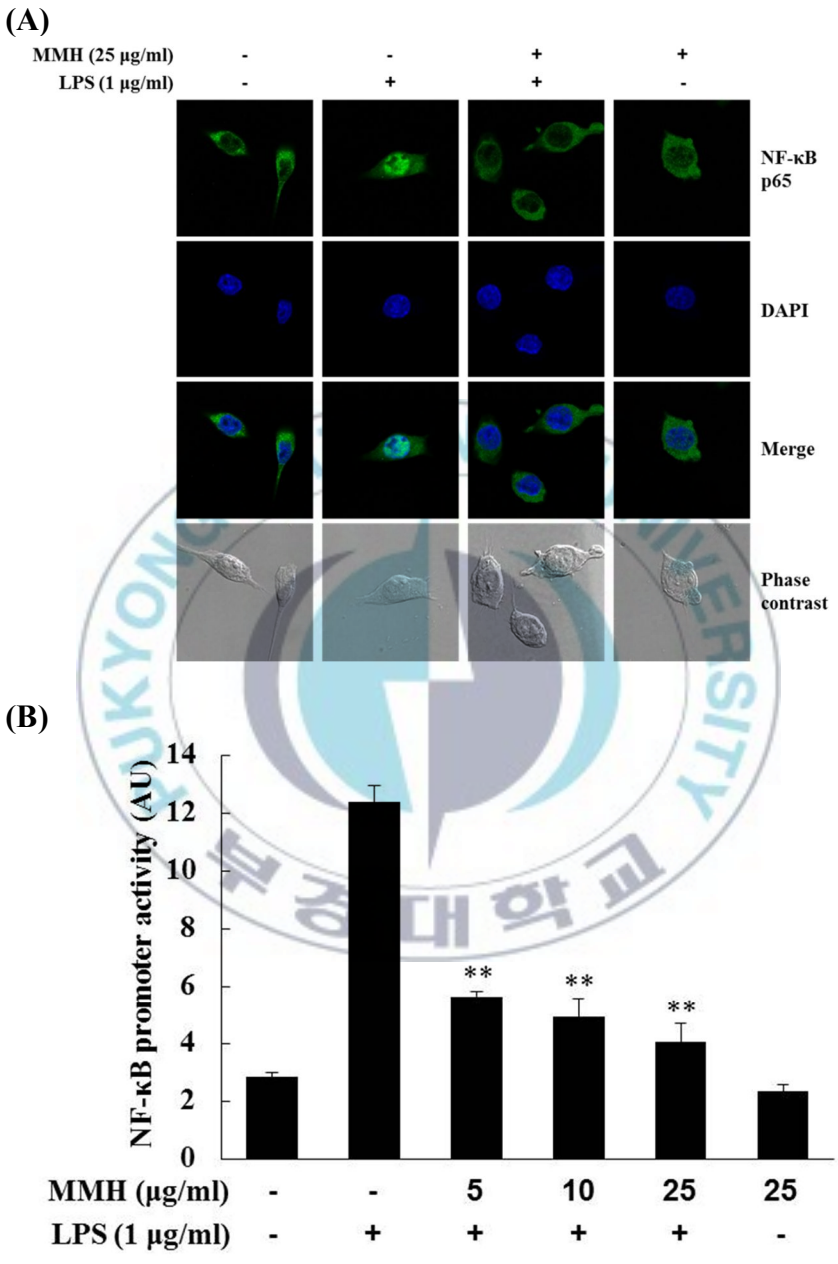
Cells pretreated with various concentrations of MMH were stimulated with or without LPS (1 μg/ml) for 24 h. TNF-α (A) and IL-6 (B) in the culture media were measured by ELISA. Data are means ± SDs of three independent experiments. (C) RT-PCR

analysis of cytokine mRNA expression. Cells were incubated with various concentrations of MMH for 2 h, and then stimulated with LPS (1 μg/ml) for 6 h. mRNA levels of TNF-α, IL-6, and GAPDH were determined by RT-PCR analysis using corresponding gene-specific primers. The results presented are representatives of three independent experiments. Quantitative data represent means ± SDs of three independent experiments. $^{*}p<0.05$ and $^{**}p<0.01$ indicate significant differences compared to LPS-only group.

3-5. MMH inhibits degradation of IκB-α and nuclear translocation of NF-κB in LPS-stimulated BV-2 cells

To analyze the effect of MMH on the regulation of transcriptional factor during the inflammatory response, we investigated the effect of MMH on the intercellular localization of NF-κB/p65 in LPS-stimulated BV-2 cells. Immunofluorescence microscopy (Figure 4(A)) showed that NF-κB protein was mainly localized in the cytoplasm of unstimulated cells. After stimulation with LPS, NF-κB was translocated into the nucleus; but pretreatment with MMH remarkably suppressed this translocation regardless of LPS treatment in BV-2 cells. Considering the inhibitory effect of MMH on the LPS-induced NF-κB translocation, we next examined the effect of MMH on the NF-κB promoter activity in LPS-stimulated BV-2 cells. The luciferase assay showed that the treatment with MMH at concentrations higher than 5μg/ml significantly suppressed the LPS-induced NF-κB promoter-driven luciferase expression (Figure 4(B), $p<0.01$). This result indicates that inhibitions of LPS-induced iNOS, COX-2, and pro-inflammatory cytokine expressions by MMH are mediated at least in part via blocking NF-κB promoter activity. To further examine the inhibition of NF-κB translocation by MMH, we analyzed the subcellular distributions of NF-κB and IκB-α by Western blot. As shown in Figure 4(C), LPS treatment induced translocation of NF-κB into the nucleus as well as degradation and

phosphorylation of IκB-α in BV-2 cells. However, MMH pretreatment strongly suppressed the nuclear translocation of NF-κB in a dose-dependent manner, which is consistent with the result of the immunofluorescence microscopy (Figure 4(A)). Furthermore, MMH treatment inhibited the LPS-induced phosphorylation and degradation of IκB-α protein in a dose-dependent manner (Figure 4(C)). These results suggest that MMH attenuated the expressions of iNOS, COX-2, and pro-inflammatory cytokines at least in part by affecting NF-κB pathway.


(A)
MMH (25 μg/ml) - - + +
LPS (1 μg/ml) - + + -
NF-κB p65
DAPI
Merge
Phase contrast
(B)
NF-κB promoter activity (AU)
0
2
4
6
8
10
12
14
**
**
**
MMH (μg/ml) - - 5 10 25 25
LPS (1 μg/ml) - + + + + -

(C)

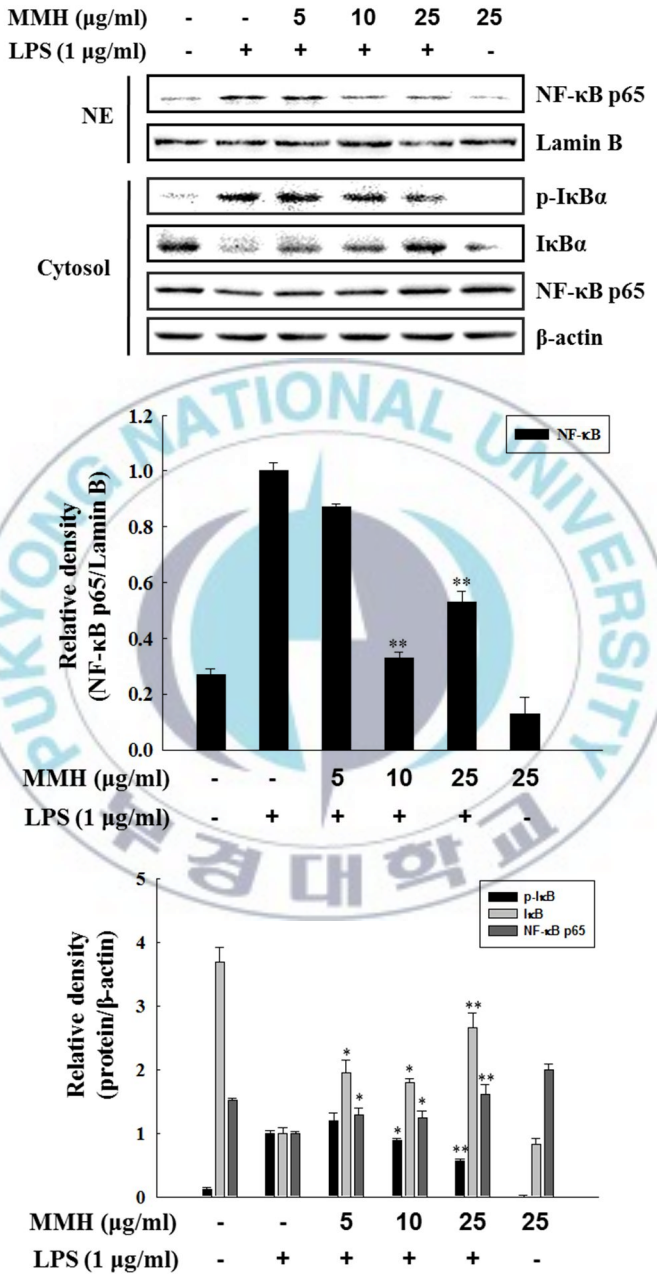


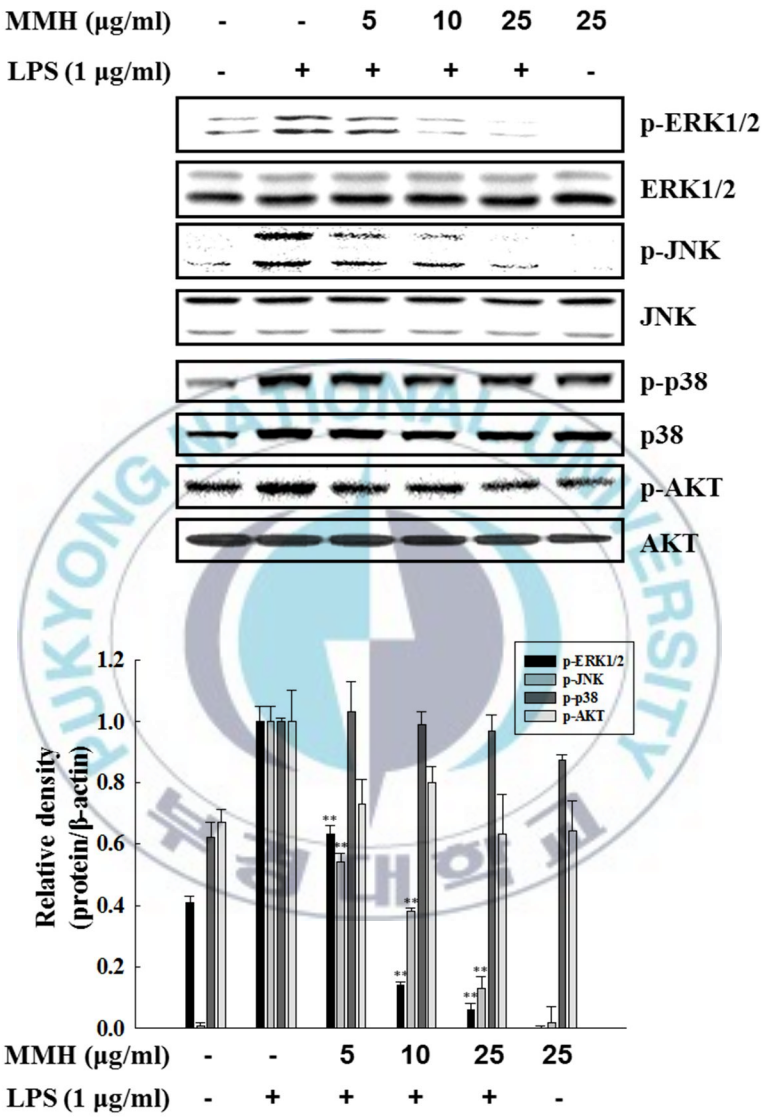
Figure4. Effect of MMH on the nuclear translocation of NF-κB and the degradation of IκB-α in LPS-stimulated BV-2 cells.

(A) Cellular distribution of NF-κB/p65 (green) protein. Cells were pretreated with MMH (25 μg/ml) for 2 h, followed by LPS stimulation for 30 min. DAPI (blue) was used for staining nuclei. (B) Effect of MMH on NF-κB promoter activity. Cells were transfected with 2 μg of NF-κB promoter-containing luciferase DNA for 40 h. Transfected cells pretreated with MMH for 2 h were stimulated with LPS for additional 6 h. Data are means ± SDs of three independent experiments.(C) Nuclear localization of NF-κB and the regulation of IκB were determined by Western blot. Cells pretreated with MMH for 2 h were stimulated with LPS for 30 min. The results presented are representatives of three independent experiments. Quantitative data represent means ± SDs of three independent experiments. $^{*}p<0.05$ and $^{**}p<0.01$ indicate significant differences compared to LPS-only group.

3-6. MMH inhibits activation of JNK andERK, but not AKT and p38, in LPS-stimulated BV-2cells

To evaluate the effect of MMH on the signaling pathway associated with NF-κB activation, we examined the changes in the activation of intracellular signaling proteins in BV-2 cells. As shown in Figure 5(A), MMH inhibited the phosphorylations of JNK and ERKin a dose-dependent manner in LPS-stimulated BV-2 cells, whereas there were no marked effects of MMH on the activations of AKT and p38 MAPK. Next we compared the production of NO and the protein levels of iNOS and COX-2 in the presence of ERK inhibitor (PD98059) or JNK inhibitor (SP600125). Figure 5(B) shows that the level of NO secretion from the LPS-stimulated BV-2 cells was remarkably inhibited by MMH as well as by ERK or JNK inhibitors. In addition, the protein levels of iNOS and COX-2 were also decreased by the pretreatments with MMH or inhibitors in LPS-stimulated cells (p<0.01 compared to LPS-only treated group). The protein level of iNOS coincided with the level of NO secretion (Figure 5(B)). These data suggest that the reduction of iNOS and COX-2 by MMH is mediated by blocking the activations of ERK and JNK via NF-κB pathway.

(A)


MMH (μg/ml) - - 5 10 25 25
LPS (1 μg/ml) - + + + + -
p-ERK1/2
ERK1/2
p-JNK
JNK
p-p38
p38
p-AKT
AKT
Relative density (protein/β-actin)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
p-ERK1/2
p-JNK
p-p38
p-AKT
**
MMH (μg/ml) - - 5 10 25 25
LPS (1 μg/ml) - + + + + -

(B)

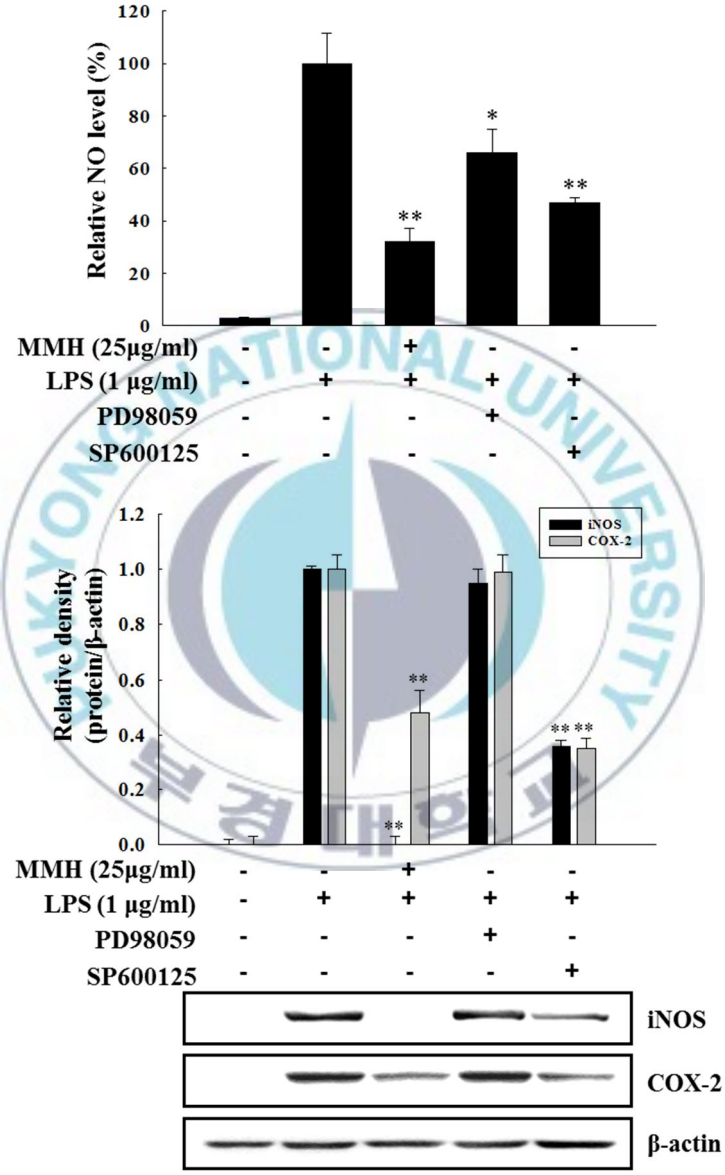

Relative NO level (%)
120
100
80
60
40
20
0
MMH (25μg/ml)
LPS (1 μg/ml)
PD98059
SP600125
Relative density (protein/β-actin)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
iNOS
COX-2
β-actin

Figure 5. Effects of MMH on the phosphorylations of MAPKs and AKT in LPS-stimulated BV-2 cells.

(A) Western blot analysis of MAPK and AKT phosphorylations. Cells were incubated with various concentrations of MMH for 2 h, and then stimulated with LPS (1 μg/ml) for 30 min. Whole cell lysates were prepared and analyzed by Western blot for total and phosphorylated proteins of AKT, ERK, JNK, or p38 MAPK using corresponding antibodies. Quantitative data shows relative density ratios of pERK1/2, pAKT, pJNK, and pp38 over ERK1/2, AKT, JNK, and p38, respectively (means ± SDs of three independent experiments).(B) MMH mimics the effects of specific kinase inhibitors in LPS-stimulated BV-2 cells. Cells were pretreated with MMH (25 μg/ml) for 2 h or with specific kinase inhibitor (PD98059 for ERK and SP600125 for JNK) for 30 min, and then treated with LPS (1 μg/ml) for 16 h. The culture media were collected for the measurement of NO, and total cell lysates were prepared for Western blot analysis of iNOS and COX-2. NO concentrations are shown as means ± SDs of three independent experiments. Relative density ratios of iNOS and COX-2 over β-actin are shown in the graph (means ± SDs of three independent experiments). $^{*}p<0.05$ and $^{**}p<0.01$ indicate significant differences compared to the LPS-only group.

4. Discussion and Conclusion

This study was designed to investigate anti-inflammatory activity of various solvent fractions from *M. myagroides* ethanolic extracts and its detailed molecular mechanisms in LPS-stimulated BV-2 microglial cells. We found that hexane fraction from *M. myagroides* ethanolic extracts (MMH) showed the highest anti-inflammatory activity among solvent fractions. Since fucoxanthin, one of the active compounds present in *M. myagroides*, was shown to be rapidly oxidized under oxygen and sunlight during its dryness, an MMH's anti-inflammatory effect may not be attributed to fucoxanthin (Prokhorova LI and Revina AA., 2001).

NO and PGE_2 are key inflammatory and neurotoxic mediators in neuroinflammation. They are responsible for the detrimental effects under injury or disease state of central nervous system, including ischemia, Alzheimer's disease, and neuronal damage (Meda*et al*., 1995). Many studies revealed that abnormally high levels of NO and PGE_2, found in various types of brain injuries and neurodegenerative diseases, are caused by the excessive expressions of iNOS and COX-2 enzymes (Teismann *et al*., 2003;Brown GC and Neher JJ., 2010; Cunningham C., 2012). The microglial cells that overexpress iNOS and COX-2 proteins were found in rodent brains treated with LPS(Minghetti *et al*., 1995). The treatment with inhibitors of iNOS and COX-2 resulted

in neuroprotection against LPS-induced neurotoxicity, suggesting that NO and PGE_2 are important mediators in neurotoxicity(Teismann *et al*., 2003;Oh *et al*., 2009). We showed that MMH has a potential to inhibit the production of both NO and PGE_2 in LPS-stimulated BV-2 cells. In addition, we provided evidence that the inhibition of NO and PGE_2 production by MMH results from the suppression of both mRNA and protein expressions of iNOS and COX-2 in LPS-stimulated BV-2 cells. Moreover, we found that the inhibition of iNOS gene expression by MMH was more marked than that of iNOS protein, indicating that inhibition of NO by MMH is associated with downregulation of iNOS at both transcriptional and translational levels in the LPS-stimulated cells. These data suggest the possibility that MMH has a protective effect on the neuroinflammatory response in neurodegenerative diseases.

Activated microglia produce high levels of proinflammatory cytokines including IL-6 and TNF-α, which are involved in the pathogenesis of several neurodegenerative disorders (Lee JY *et al*., 2012; Wong CK *et al*., 2000; Lee SY *et al*., 2011).We showed that MMH suppressed both secretion and transcription of these proinflammatory cytokines in LPS-stimulated BV-2 cells; the inhibitory effect of MMH was more significant for the secretion of the cytokines. These results support the potential of MMH to be a neuroprotective agent against neuroinflammation.

Transcriptions of iNOS, COX-2, and pro-inflammatory cytokine genesare mainly controlled by NF-κB transcription factor, which binds to respective *cis*-acting elements in iNOS and COX-2 promoter region (Marks-Konczalik *et al*., 1998;Xie *et al*., 1993).A variety of external stimuli induce the phosphorylation of IκB-α in the NF-κB-IκB-α complex, resulting in proteasomal degradation of IκB-α and subsequent translocation of NF-κB from the cytosol into the nucleus (Janssen-Heininger *et al., 2000;* Jang *et al*., 2005). In this study, we showed that MMH inhibited the phosphorylation and subsequent degradation of IκB-α, resulting in the suppression of activation and nuclear translocation of NF-κB p65 subunit. The results in this study suggest that transcriptional repressions of iNOS, COX-2, and pro-inflammatory cytokines genes by MMH are primarily due to the inactivation of NF-κB in LPS-stimulated microglial cells. It is likely that NF-κB is an important target transcription factor for anti-inflammatory effect of MMH.

Moreover, it was shown that intercellular signaling proteins such as MAPKs and AKT are activated by LPS and involved in transcription of iNOS, COX-2, and pro-inflammatory cytokine genes in microglial cells (Jang *et al.*, 2005; Herlaar *et al*., 1999;Jung *et al*., 2010; Skaper, S.D., 2007).We found that the phosphorylations of ERK and JNK are decreased by MMH treatment in a concentration-dependent manner, but not those of p38 and AKT in LPS-stimulated BV-2 cells. In addition, secretion

of NO was markedly inhibited by the MMH pretreatment; similar inhibitory effect was also observed when specific ERK or JNK inhibitor was pretreated. Given the roles of ERK and JNK in inflammatory processes, these kinases can be another target of MMH's anti-inflammatory activity in LPS-stimulated BV-2 cells. We also demonstrated that MMH down-regulated NF-κB promoter activities in LPS-stimulated BV-2 cells in concentration-dependent manner. These data indicate that MMH showed anti-inflammatory effects at least partly by affecting ERK, JNK, and NF-κB pathway.

In the present study, MMH showed anti-inflammatory effect in LPS-stimulated BV-2 cells. Although BV-2 cells are immortalized from murine microglial cells, they have been used as a valid substitute for primary microglia in a variety of experiments including innate immune response study using LPS (Henn A*et al., 2009;* Lund S *et al*., 2005; Yang CS *et al*., 2007). However, the culture of LPS-stimulated BV-2 may not be the same condition as *in vivo* neuroinflammatory response. Further studies are required to investigate anti-inflammatory and neuroprotective effects of MMH in the animal models of neurodegenerative diseases. In addition, isolation and analysis of anti-inflammatory compounds from MMH are also necessary. Taken together, this study suggests that MMH has a strong therapeutic agent for the treatment or prevention of neuroinflammation in neurodegenerative diseases.

Among the solvent fractions from *M.myagroides* ethanolic

extract, MMH has the highest anti-inflammatory activity in LPS-stimulated BV2 microglial cells. MMH inhibits the production of inflammatory mediators(NO and PGE_2) and suppresses expression of the inflammatory genes (iNOS, COX-2, IL-6, and TNF-α) in LPS-stimulated BV-2 cells. These inhibitions are associated with the activity of MMH to block activation of NF-κB, JNK and ERK.

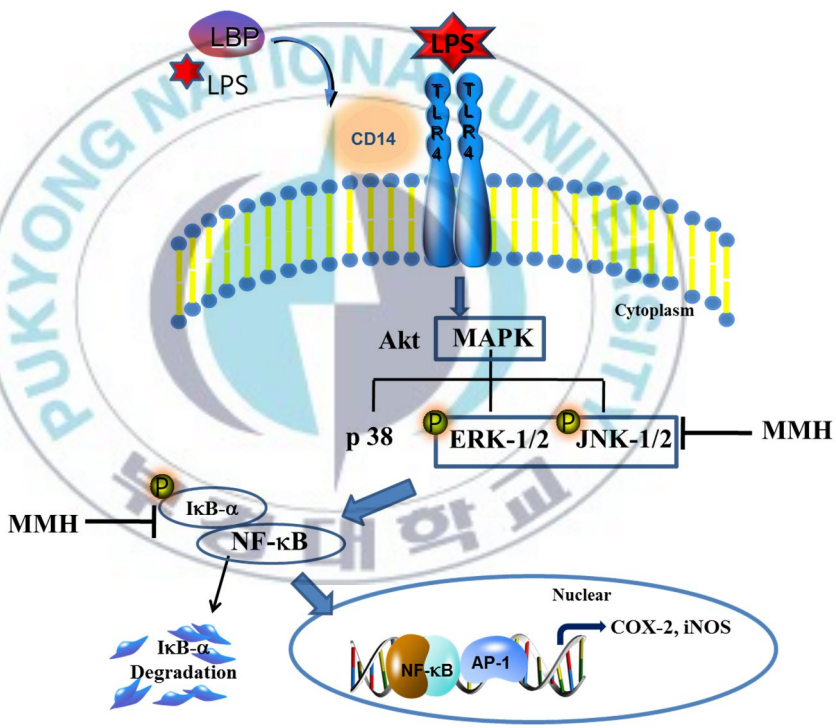


Figure 6. Possible regulatory mechanisms of MMH in the NF-κB pathway.

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