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Research Article

Methanol Extract of *Hydroclathrus clathratus* Inhibits Production of Nitric Oxide, Prostaglandin E_2 and Tumor Necrosis Factor-α in Lipopolysaccharide-stimulated BV2 Microglial Cells via Inhibition of NF-κB Activity

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Abstract

Purpose: *Hydroclathrus clathratus is a brown marine seaweed known to possess anti-cancer, anti-herpetic, and anti-coagulant activities. The present study is aimed at investigating some anti-inflammatory effects of H. clathratus.*

Methods: *We investigated the anti-inflammatory effects of the methanol extract of H. clathratus (MEHC) by expression of mRNA and protein using RT-PCR and Western blot analysis in lipopolysaccharide (LPS)-stimulated BV2 microglial cells. The level of nitric oxide (NO) production was analyzed using Griess reaction. The release of prostaglandin E_2 (PGE_2) and tumor necrosis factor-α (TNF-α) were determined using sandwich ELISA. NF-κB activation was detected using EMSA methods.*

Results: *The results obtained indicate that the extract (MEHC) inhibited LPS-induced NO, PGE_2, and TNF-α production without any significant cytotoxicity ($p < 0.05$). MEHC also inhibited production of inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2) and TNF-α mRNA in LPS-stimulated BV2 microglial cells. In addition, MEHC significantly reduced ($p < 0.05$) nuclear translocation of the nuclear factor-κB (NF-κB) subunits, p50 and p65, and its DNA-binding activity in LPS-stimulated BV2 microglial cells.*

Conclusion: *These results suggest that MEHC suppresses the induction of TNF-α, as well as iNOS and COX-2 expression, by blocking LPS-induced NF-κB activation.*

Keywords: *Hydroclathrus clathratus, Nitric oxide, Prostaglandin E_2, Tumor necrosis factor-α, Nuclear factor-κB*

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INTRODUCTION

Inflammation is a beneficial host defense to external cellular injury or external challenge against infection and foreign substances such as bacteria and viruses that leads to the release of a variety of mediators [1,2]. However, prolonged inflammatory responses can be harmful, contributing to the pathogenesis of many diseases such as cartilage disease, hyperosmolarity, ischemia-reperfusion, Alzheimer's disease, Parkinson's disease, diabetes, asthma and heart disease [3-5]. Although macrophages provide immediate defense against foreign agents in the infection sites [6], microglia and macrophages release a different variety of mediators, including nitric oxide (NO), prostaglandin E_2 (PGE_2), cytokines [7], bioactive lipids, hydrolytic enzymes, and reactive oxygen intermediates, all of which have been implicated in the pathogenesis of tissue damage, when they were aberrantly expressed in the process of prolonged inflammation [8,9].

NO is generated by the inducible isoform of NO synthase (iNOS), which is primarily regulated at the expression level by transcriptional and post-transcriptional modifications [10]. Although NO has beneficial microbicidal, immunomodulatory, anti-viral, and anti-tumor effects, aberrant NO production can lead to inflammatory destruction of target tissues at the inflammation sites [11]. Prostaglandins also play a major role as mediators of the inflammatory response and are synthesized by cyclooxygenase (COX) [8]. In particular, inducible COX-2 is responsible for the production of large amounts of proinflammatory PGE_2 in the process of inflammation [12]. In addition, tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine important for the stimulation of the secretion of other inflammatory cytokines [13]. Thus, the inhibition of overproduction of NO, PGE_2 and TNF-α, by restricting iNOS, COX-2, and TNF-α gene expression, could serve as a promising potential in the development of anti-inflammatory drugs.

NF-κB has often been termed a central mediator in the human immune response [14,15]. Active NF-κB participates in the transcribed control of many target genes, including iNOS, COX-2 and TNF-α [16]. Thus, it has been reported that inhibitors of the NF-κB pathway suppress the initiation of the nuclear localization of the NF-κB subunits, p50 and p65, and control the switch-off of the expression of proinflammatory mediators such as NO, PGE_2, and TNF-α [17,18]. Therefore, modulation of NF-κB activity is a good strategy for therapy against inflammatory diseases.

*Hydroclathrus clathratu*s is a brown marine seaweed with anti-coagulant activities [19]. Chemical constituents commonly isolated from this seaweed are the polysaccharides H3-a1 and H3-b1, which have anti-viral and anti-cancer properties [20]. Some components of *H. clathratus* specifically affect the growth of human acute promyelocytic leukemia cells (HL-60), human breast carcinoma cells (MCF-7), and human hepatocellular carcinoma cancer cells [21]. However, some studies have been focused on the antiviral and anti-cancer property of *H. clathratus*.

In this study, the effects of methanol extract of *H. clathratus* (MEHC) on the expression of NO, PGE_2 and TNF-α in lipopolysaccharide (LPS)-stimulated BV2 microglial cells were investigated.

EXPERIMENTAL

Preparation of MEHC

MEHC was purchased from Jeju HI-Tech Industry Development Institute (Jeju, Republic of Korea). *H. clathratus* (stock No.; AP060) was collected along the Jeju Island coast of Korea in July, 2006. Briefly, fresh *H. clathratus* was washed three times with tap water to remove salt, epiphyte and sand on

the surface of the samples before storage -20ºC. The frozen samples were lyophilized and homogenized using a grinder before extraction. The dried powder was extracted with 80 % methanol and evaporated *in vacuo*.

Chemicals

Antibodies against iNOS, COX-2, p65, and p50 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against β-actin was from Sigma (St. Louise, MO, USA). Peroxidase-labeled goat anti-rabbit immunoglobulin was purchased from KOMA Biotechnology (Seoul, Republic of Korea). Lipopolysaccharide (LPS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA).

Cell culture and MTT assay

BV2 murine microglial cells were cultured at 37^{o}C in 5% CO_2 in DMEM in supplemented with 5% FBS and antibiotics (WelGENE Inc., Daegu, Republic of Korea). In all experiments, cells were pre-treated with the indicated concentrations of MEHC for 1 h before the addition of LPS (1 μg/ml) in serum-free DMEM. MTT assays were used to determine cell viability.

Nitric oxide assay

Griess reagent assay was used for analyzing NO production. Briefly, the samples were mixed with equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride) and then incubated at room temperature for 5 min. The absorbance was measured at 540 nm on a microplate reader. Nitrite concentration was determined from a sodium nitrite standard curve

Measurement of PGE_2 and TNF-α

Expression levels of PGE_2 and TNF-α were measured using enzyme immunosorbent 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 isolated using easy-BLUE^{TM} total RNA extraction kit (iNtRON Biotechnology; Sungnam, Republic of Korea). The total RNAs were amplified by PCR using the following primers: iNOS (forward 5'-cct cct cca ccc tac caa gt-3' and reverse 5'-cac cca aag tgcttc agt ca-3'), COX-2 (forward 5'-aag act tgc cag gct gaa ct-3' and reverse 5'-ctt ctg cag tcc agg ttc aa-3'), TNF-α (forward 5'-gcg acg tgg aac tgg cag aa-3' and reverse 5'-tcc atg ccg ttg gcc agg ag-3'), and β-actin (forward 5'-tgt gat ggt ggg aay ggg tc-3' and reverse 5'-ttt gat gtc acg cac gat tt-3').

Western blot analysis

Total cell extracts were prepared using PRO-PREP protein extraction solution (iNtRON Biotechnology). 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') were 3'-biotinylated using the biotin 3'-end DNA labeling kit (Pierce; Rockford, IL, USA) 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 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. Statistical significance was regarded at $p < 0.05$.

RESULTS

Effects of MEHC on cell viability

To determine the effects of MEHC on cell viability in BV2 microglial cells, an MTT assay was performed 24 h after treatment with the indicated concentrations of MEHC in the presence or absence of LPS. MEHC alone in the range of 50 – 200 µg/ml showed no cytotoxic effect (Fig 1), but significant cytotoxicity was found at over 250 µg/ml MEHC in BV2 microglial cells, regardless of the presence of LPS. Therefore, 100 µg/ml MEHC was applied in further experiments.

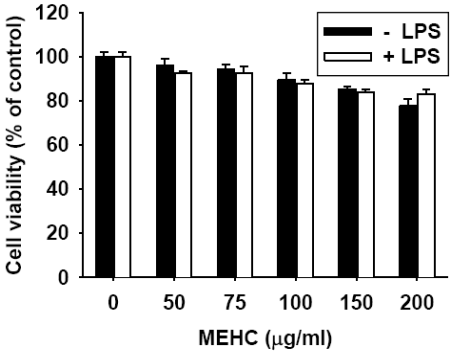

Fig 1: Effect of MEHC on the viability of BV2 microglial cells. Each value is Mean ± SE (n=3).

Effects of MEHC on LPS-induced NO and PGE_2 Production

In order to evaluate the anti-inflammatory effects of MEHC, cells were stimulated with LPS (1.0 µg/ml) for 24 h after pretreatment with 100 µg/ml MEHC for 1 h. The production of NO and PGE_2 was analyzed using the Griess reaction assay and ELISA, respectively. NO production in cells stimulated with LPS was significantly higher (19.3 ± 1.7 µM) compared to that in untreated control group (5.9 ± 1.0 µM; Fig 2A). However, pretreatment with MEHC significantly suppressed LPS-induced NO upregulation (7.5 ± 0.6 µM). Consistent with the data of NO production, stimulation with LPS increased PGE_2 production (625 ± 65 pg/ml) by approximately 9-fold when compared to that in the control group (77 ± 35 pg/ml). However, LPS-induced PGE_2 expression was decreased to approximately 40% (245 ± 65 pg/ml) in the presence of MEHC. MEHC alone had no influence on the production of NO and PGE_2. These results indicate that MEHC suppresses LPS-induced NO and PGE_2 production in BV2 microglial cells.

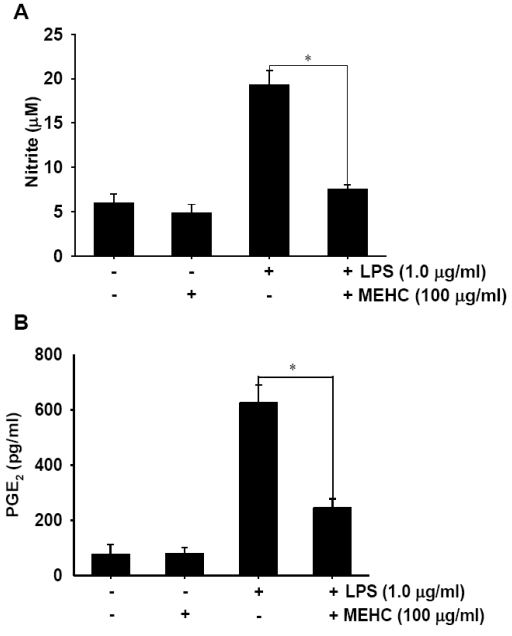

Fig 2: Effect of MEHC in LPS-induced NO (A) and PGE_2 (B) release in BV2 microglial cells. Each value is Mean ± SE (n=3). * Significantly different was compared with cells treated with LPS alone ($p < 0.05$).

Effects of MEHC on LPS-induced iNOS and COX-2 mRNA and protein

To investigate whether MEHC regulates the expression of iNOS and COX-2 genes, we treated cells with 100 µg/ml of MEHC for 6 h in the presence of LPS, and the protein and mRNA expression of both iNOS and COX-2 were determined by RT-PCR and western

blot analysis, respectively. RT-PCR analysis shows that treatment with LPS significantly increases the expression of iNOS and COX-2 at 6 h (Fig. 3A), but the expression of these mRNAs was significantly downregulated after pretreatment with 100 µg/ml of MEHC. Although LPS significantly increased the expression of iNOS and COX-2 proteins, western blot analysis showed a decrease of these expression patterns in a manner similar to mRNA expression in the presence of MEHC (Fig. 3B). Taken together, these results indicate that MEHC suppresses the upregulation of LPS-stimulated iNOS and COX-2 expression at the transcriptional level.

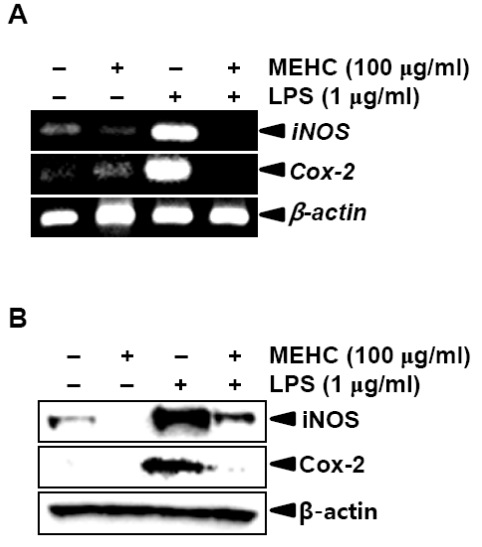

Fig. 3: Effect of MEHC on LPS induced iNOS and COX-2 mRNA (A) and protein (B) expression in BV2 microglial cells.

Effects of MEHC on LPS-induced TNF-α production and mRNA expression

We investigated whether MEHC inhibits the production of TNF-α and the expression of TNF-α mRNA in LPS-stimulated BV2 microglial cells. Cells were pretreated with 100 µg/ml of MEHC for 1 h before LPS stimulation for 24 h. The level of TNF-α in the culture supernatant was determined by ELISA. TNF-α production in cells stimulated with LPS was 7-fold higher (2294 ± 68 pg/ml) than that in the control group (311 ± 21 pg/ml; Fig. 4A). However, MEHC restored TNF-α production to a little above control level (815 ± 45 pg/ml). Consistent with the data of NO and PGE_2 production, treatment with MEHC alone has no influence on TNF-α production (291 ± 35 pg/ml). RT-PCR data showed that MEHC significantly suppresses the expression of TNF-α mRNA in LPS-stimulated BV2 microglial cells (Fig. 4B). These results indicate that MEHC regulates LPS-stimulated TNF-α release at the transcriptional level.

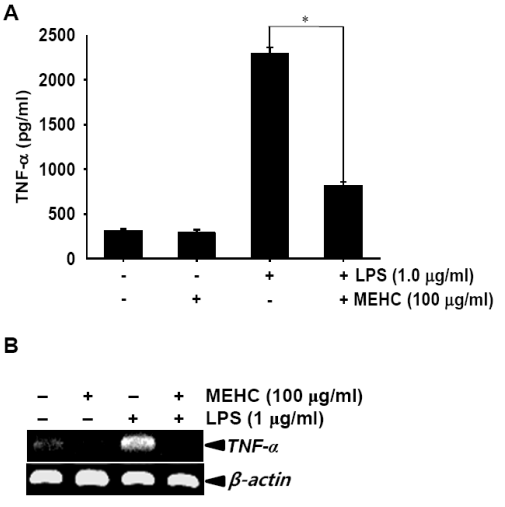

Fig 4: Effect of MEHC on LPS-induced TNF-α production and TNF-α mRNA expression in BV2 microglial cells. * Significantly different was compared with cells treated with LPS alone ($p < 0.05$).

Inhibitory effects of MEHC on LPS-induced NF-κB activity

In order to investigate whether MEHC inhibits proinflammatory genes such as iNOS, COX-2, and TNF-α via suppression of NF-κB activity, we analyzed the specific DNA-binding activity of NF-κB by EMSA assay. Stimulation with LPS caused a remarkable increase in binding complexes between NF-κB and specific-binding DNA. However, pretreatment with MEHC for 1 h significantly reduced LPS-induced NF-κB activity (Fig. 5A). LPS significantly increased the protein

expression of p50 and p65 in the nuclear compartment of BV2 microglial cells, but this was suppressed by treatment with MEHC (Fig 5B). Furthermore, *N*-acetyl-L-cysteine (NAC) was used as a potent proteasome and proteases inhibitor of Rel/NF-κB activity to re-confirm those results. We conducted an RT-PCR analysis to detect iNOS, COX-2, and TNF-α mRNA expression in the presence of NAC. As we expected, NAC inhibited the expression of LPS-stimulated iNOS, COX-2, and TNF-α expression at the transcription level (Fig 5C). These results indicate that MEHC inhibits the expression of proinflammatory genes via suppression of NF-κB activity.

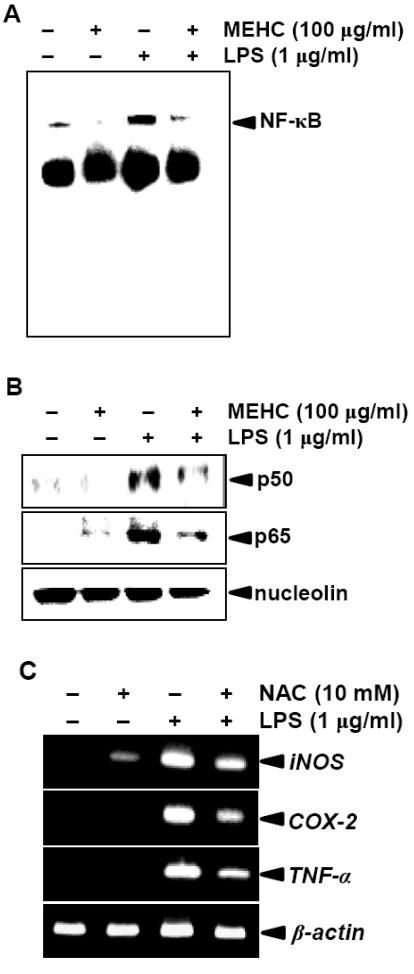

Fig. 5: Effect of MEHC on NF-κB activity in BV2 microglial cells.

DISCUSSION

Pathological agents like bacteria and fungi stimulate microglia and macrophages leading to the induction of mediators relevant to the inflammatory process [22]. In particular, LPS strongly leads to the activation of several transduction pathways and secretion of various inflammatory mediators [23]. The results obtained in this study revealed that MEHC inhibits LPS-induced NO and PGE_2 production by suppressing iNOS and COX-2 expression in BV2 microglial cells. MEHC also decreases TNF-α production by suppressing its mRNA expression. Furthermore, we found that these effects were induced via suppression of NF-κB activity. Taken together, these data indicate that MEHC inhibits LPS-induced proinflammatory mediators such as NO, PGE_2, and TNF-α via suppression of NF-κB activity.

NO is significantly produced in microglia and macrophages, and it normally is an important regulator and mediator of numerous processes, including smooth muscle relaxation and neurotransmission [24]. However, experimental studies have also confirmed that NO overproduction causes severe inflammatory diseases such as septic shock [25]. Thus, many researchers have tried to regulate NO production so as to overcome these imbalances within the immune system. In the present study, we found that MEHC is a good regulator for downregulation of NO production. These results imply that MEHC may be a potent candidate for inhibiting NO production and inflammatory diseases. PGE_2, which results from overproduction of the COX-2 gene, is particularly responsible for various biological events such as neuronal function, female reproduction, vascular hypertension, tumorigenesis, kidney function and inflammation [26]. Accumulating evidence confirms the effects of COX-2 and PGE_2 as potential therapeutic targets for treating

inflammatory diseases [27, 28]. Furthermore, TNF-α is a cytokine involved in the inflammatory and destructive process common to several human inflammatory diseases, and overproduction of TNF-α switches on the signaling pathways that change the cell functions of LPS-stimulated microglia and macrophages [29]. Due to this signaling activity, anti-TNF-α therapy has been broadly introduced to treat various inflammatory diseases [13]. 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.

NF-κB is a ubiquitous and rapidly responsive cellular transcription factor which is strongly activated by LPS and TNF-α under various pathological conditions [30]. In non-stimulated cells, NF-κB is present in the cytosol where it is complexed with inhibitor IκB. Activation of NF-κB depends on the signal-induced phosphorylation of IκB by specific IκB kinases, which initiates the inhibitor's conjugation to ubiquitin and subsequent degradation by the proteasome [31]. In this study, we found that MEHC suppresses LPS-induced nuclear translocation of p65 and p50 as well as the specific DNA-binding activity of NF-κB. In addition, the expression of iNOS, COX-2 and TNF-α mRNA significantly suppressed in the presence of NAC. These results suggest that MEHC inhibits LPS-induced NF-κB activity by suppressing the translocation of the NF-κB protein to the nucleus.

CONCLUSION

This study confirmed that MEHC has anti-inflammatory activity, which has an influence on the regulation of NO, PGE_2, and TNF-α expression by suppressing NF-κB activity. Therefore, MEHC may be a safe and effective therapeutic agent for the regulation of LPS-associated inflammatory disease.

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