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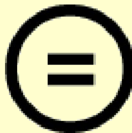

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A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Anti-inflammatory and anti-invasive effects of methanol extract of *Hydroclathrus clathratus*

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GRADUATE SCHOOL

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Anti-inflammatory and anti-invasive effects of methanol extract of *Hydroclathrus clathratus*

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A thesis submitted in partial fulfillment of the requirement for the degree of

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Chapter I

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

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, which finalize the restoration of cellular structure and function [1, 2]. However, prolonged inflammatory responses can be harmful, contributing to the pathogenesis of many diseases like cartilage disease, hyperosmolarity, ischemia-reperfusion, Alzheimer's, Parkinson's, diabetes, asthma, and heart disease [3-5]. Though macrophages are important players that 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]. Even though NO has beneficial microbicidal, immunomodulatory, anti-viral, and anti-tumor effects, it is well known that 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

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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 that is important for the stimulation of the secretion of other inflammatory cytokines [13]. According to these mechanisms, 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 sound background for the potential development of anti-inflammatory drugs.

NF-κB has often been termed a central mediator in the human immune response [14,15]. Active NF-κB, in turn, participates in the transcription control of many target genes, including iNOS, COX-2, and TNF-α [16]. Thus, many scientists 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 constitutes 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 affected the growth of human acute promyelocytic leukemia cells (HL-60), human breast carcinoma cells (MCF-7), and human hepatocellular carcinoma cancer cells [21]. Nevertheless, an evaluation of the anti-inflammatory effects of *H. clathratus* has not been reported yet.

In this study, we tried to evaluate the effects of methanol extract of *H. clathratus* (MEHC) on the expression of NO, PGE_2, and TNF-α in lipopolysaccharide (LPS)-stimulated BV2

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microglial cells. We found that MEHC downregulates LPS-induced expression of iNOS, COX-2, and TNF-α mRNA, as well as their gene products. In addition, the methanol extract of *H. clathratus* (MEHC) inhibited LPS-induced NF-κB activation by suppressing the translocation of p65 and p50 to the nucleus.

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Material and methods

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 -20℃. 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 Biotechnology, 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 obtained from Prof. I.W. Choi (Inje University, Busan, Republic of Korea). The cells was cultured at 37°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 pretreated 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.

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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 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 immunosolvent 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').

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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', 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 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 one-way ANOVA. Statistical significance was regarded at $P < 0.05$.

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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 of MEHC in BV2 microglial cells, regardless of the presence of LPS (data not shown). Therefore, the concentration of 100 μg/ml of MEHC was applied in further experiments.

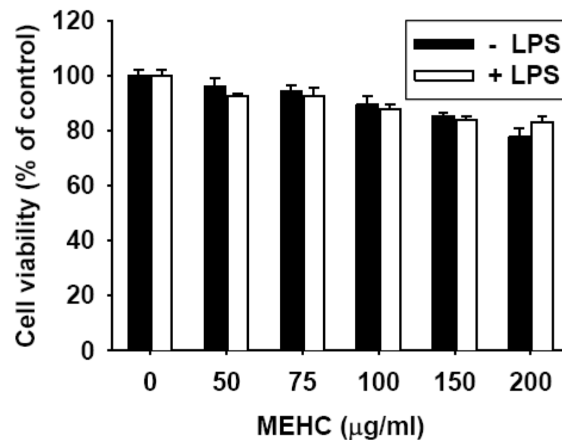

Figure 1: Effects of MEHC on the viability of BV2 microglial cells. Cells (1×10^5 cells/ml) were incubated with the indicated concentrations of MEHC (50-200 μg/ml) for 1 h before treatment with 1.0 μg/ml of LPS for 24 h. Cell viability was determined by MTT assay. ***Note:*** Each value indicates mean ± SE and is representative of results obtained from 3 independent experiments. $*P < 0.05$ was considered significantly different from the value in cells with an untreated control.

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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 of 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) as compared to that in untreated control group (5.9 ± 1.0 μM; Fig. 2A); however, pretreatment with MEHC significantly suppressed LPS-induced NO upregulation by 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 as 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. In all of these experiments, 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.

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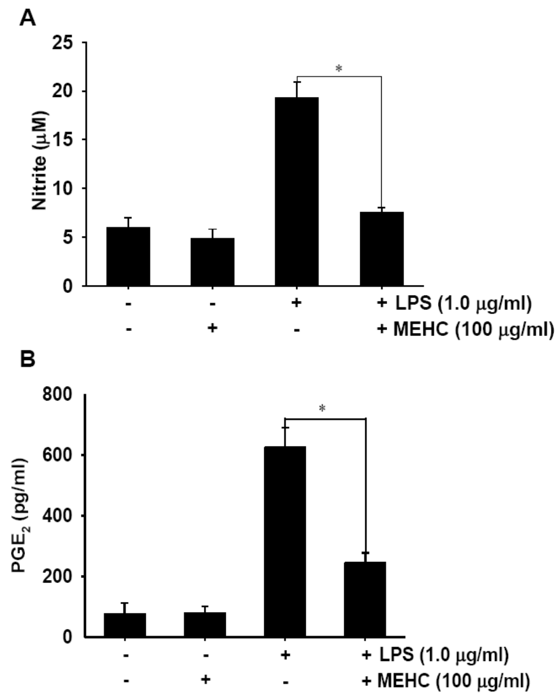

Figure 2: Effects of MEHC in LPS-induced NO (A) and PGE_2 (B) release in BV2 microglial cells. Cells (1×10^5 cells/ml) were incubated with 100 μg/ml of MEHC for 1 h before treatment with 1.0 μg/ml of LPS for 24 h. (A) The amounts of NO were determined using Griess reagent, and a standard curve was generated using $NaNO_2$ in the culture medium. (B) The levels of PGE_2 in the media were analyzed using a specific enzyme immunoassay according to the manufacturer's instruction. ***Note:*** Each value indicates mean ± SE and is representative of results obtained from 3 independent experiments. *$P < 0.01$ is significantly different from the value in cells treated with LPS alone.

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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 showed that treatment with LPS significantly increases the expression of iNOS and COX-2 at 6 h (Fig. 3A); however, the expression of these mRNAs was significantly downregulated after pretreatment with 100 μg/ml of MEHC. We further investigated the expression of iNOS and COX-2 proteins by western blot analysis. 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.

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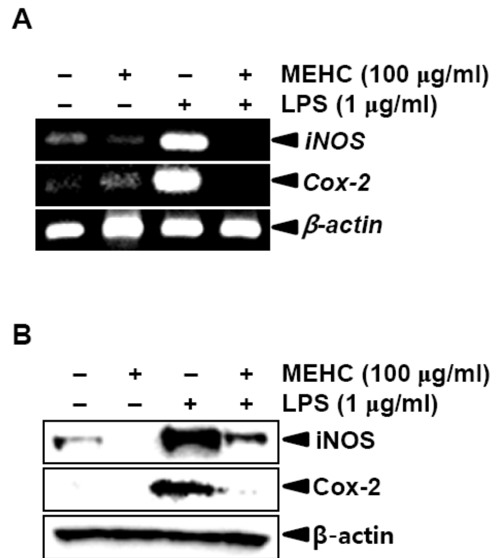

Figure 3: Effects of MEHC on LPS induced iNOS and COX-2 mRNA (A) and protein (B) expression in BV2 microglial cells. Cells (1×10^5 cells/ml) were incubated with 100 μg/ml of MEHC for 1 h before treatment with 1.0 μg/ml of LPS for 6 h. (A) Total RNA was isolated, and RT-PCR analyses of iNOS and COX-2 were performed. (B) In a parallel experiment, equal amounts of cell lysates were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with antibodies against iNOS or COX-2. ***Note:*** β-Actin was used as an internal control for RT-PCR and western blot analysis. The experiment was repeated 3 times, and similar results were obtained.

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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). To determine whether TNF-α production is regulated at the transcriptional levels, RT-PCR analysis was performed 6 h after LPS treatment in the presence of MEHC. 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.

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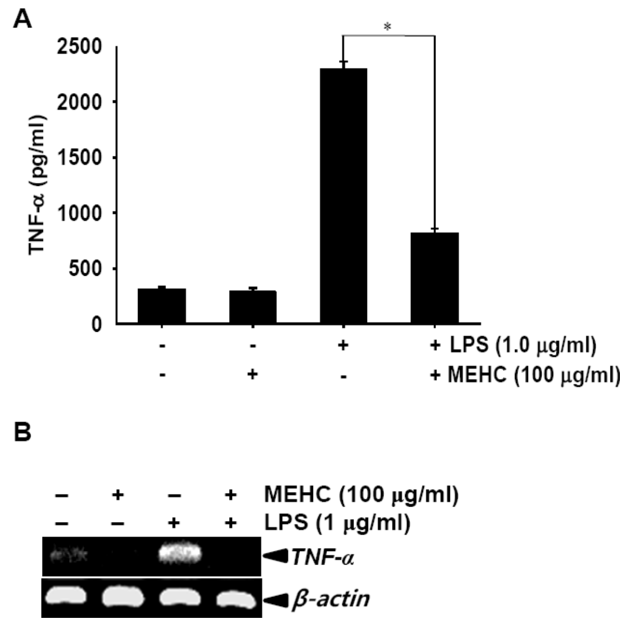

Figure 4: Effects of MEHC on LPS-induced TNF-α production and TNF-α mRNA expression in BV2 microglial cells. Cells (1×10^5 cells/ml) were incubated with 100 μg/ml of MEHC for 1 h before LPS (1.0 μg/ml) treatment for the indicated time. (A) After incubation for 24 h, the supernatants were collected, and the amount of TNF-α was measured by ELISA. (B) The level of TNF-α mRNA expression was determined by RT-PCR analysis for 6 h. β-Actin was used as an internal control for RT-PCR. ***Note:*** The experiment was repeated 3 times and similar results were obtained. *$P < 0.05$ was considered significantly different from cells treated with LPS alone.

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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). In a parallel experiment, the expression levels of p50 and p65 in the nuclear region were determined after stimulation with LPS. LPS significantly increased the protein expression of p50 and p65 in the nuclear compartment of BV2 microglial cells. However, treatment with MEHC suppressed LPS-induced nuclear translocation of p50 and p65 (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.

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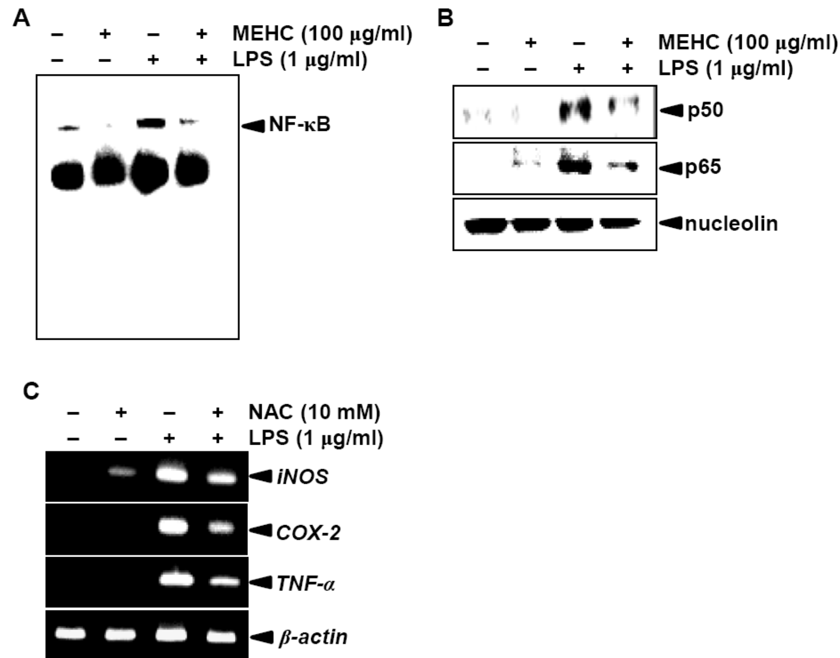

Figure 5: Effects of MEHC on NF-κB activity. BV2 microglial cells were preincubated with MEHC (100 μg/ml) for 1 h before stimulation of LPS (1.0 μg/ml) for 30 min, and (A) the nuclear extracts were assayed for NF-κB activity by EMSA and (B) the levels of p50 and p65 in the nuclear compartment by western blot analysis. (C) Cells (1×10^5 cells/ml) were incubated with 10 mM of NAC for 1 h before LPS (1.0 μg/ml) treatment for 30 min. After treatment with NAC for 6 h, total RNA was isolated, and RT-PCR analyses of iNOS, COX-2, and TNF-α were performed. ***Note:*** β-Actin and nucleoline were used as an internal control for RT-PCR and western blot analysis, respectively. The experiment was repeated 3 times and similar results were obtained.

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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 to secrete various inflammatory mediators [23]. Therefore, we analyzed whether MEHC inhibits the production of proinflammatory mediators in LPS-stimulated BV2 microglial cells. In this study, we revealed that MEHC inhibited LPS-induced NO and PGE_2 production by suppressing iNOS and COX-2 expression in BV2 microglial cells. MEHC also decreased 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]. Therefore, many researchers have tried to regulate NO production to overcome those imbalances within the immune system. In this regard, we found that MEHC is a good regulator to downregulate NO production. These results imply that MEHC may be a potent candidate for inhibiting NO production to prevent inflammatory diseases. Thus, activation of 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 evidences confirm the effects of COX-2 and PGE_2 as potential therapeutic targets for treating inflammatory diseases [27, 28]. Furthermore, TNF-α is a

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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. In this study, we found that MEHC inhibits the production of NO, PGE_2, and TNF-α in LPS-stimulated BV2 microglial cells. We also found that MEHC suppressed the LPS-induced iNOS and COX-2 protein expression, which leads to a reduced production of NO and PGE_2. Additionally, our results showed that MEHC attenuates the production of TNF-α as well as its expression at the transcription level in LPS-induced BV2 microglial cells.

NF-κB is a ubiquitous and rapidly responsive cellular transcription factor and 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. Additionally, we tested NF-κB activity in the presence of NAC, which is a proteasome and protease inhibitor of Rel/NF-κB activity [32], to confirm whether NF-κB directly regulates proinflammatory genes like iNOS, PGE2, and TNF-α. According to the RT-PCR analysis, iNOS, COX-2, and TNF-α mRNA expression was significantly suppressed in the presence of NAC. Taken together, these results suggest that

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MEHC suppressed LPS-induced NF-κB activity by suppressing the translocation of the NF-κB protein to the nucleus.

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CONCLUSION

Our results show that the methanol extracts of *H. clathratus* (MEHC) inhibits LPS-induced NO, PGE_2, and TNF-α production without any significant cytotoxicity. MEHC also inhibits the production of inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), and TNF-α mRNA in LPS-stimulated BV2 microglial cells. Additionally, MEHC significantly reduced nuclear translocation of the nuclear factor-κB (NF-κB) subunits, p50 and p65, and its DNA-binding activity in LPS-stimulated BV2 microglial cells. 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.

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Chapter II

Methanol Extract of *Hydroclathrus clathratus* Suppresses Matrix Metalloproteinase-9 in T24 Bladder Carcinoma Cells by Suppressing the NF-κB and MAPK Pathways

Introduction

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases involved in the breakdown of the extracellular matrix under normal physiological conditions (1). According to recent studies, MMPs are also closely related to tumor invasion and metastasis by their capacity for tissue remodeling via the extracellular matrix as well as degradation of the basement membrane and induction of angiogenesis (1,2). MMPs are mainly secreted as zymogens and cleaved to their active forms, and their functions are tightly regulated by several different mechanisms (3). One of them, MMP-9 is a key effector that promotes tumor cell invasion through type-IV collagen degradation-dependent extracellular matrix remodeling (4). MMP-9 expression has been observed in tumors of various organs, including the bladder, brain, liver, prostate and pancreatic carcinoma (5). In particular, elevated MMP-9 expression in bladder tumor tissues is correlated with tumor stage, grade and prognosis (6). Complications in most bladder cancers are attributed to metastasis to distant organs including the regional lymph nodes, liver, lungs, bone, adrenal glands and intestines (7). Therefore, a good strategy to treat bladder cancers is to target MMP-9 inhibition.

A recent study has shown that the nuclear factor kappa B (NF-κB) and mitogen activated protein kinase (MAPK) pathways tightly regulate MMP-9 expression in several types of cancer cells (8). NF-κB is normally located in the cytoplasm as an inactivated dimer composed of p65

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and p50 subunits (9). In response to pro-inflammatory stimuli, IκB is degraded by the phosphorylation and ubiqutination, and then NF-κB is released and translocated to the nucleus (10). Ultimately, NF-κB promotes the expression of tumor invasion-related genes such as MMP-9. The MAPKs are a group of signaling molecules that appear to play an important role in MMP expression. TNF-α stimulation is well known to result in the phosphorylation of p38, ERK and JNK leading to NF-κB activation in bladder cancer cells and induces tumor invasion (8). Therefore, the NF-κB and MAPK pathways are considered a good target to suppress MMP-9 expression to inhibit tumor invasion.

*Hydroclathrus clathratu*s is a brown marine seaweed with anti-coagulant activity (11). The chemical constituents isolated from this seaweed are commonly polysaccharides H3-a1 and H3-b1, which have anti-viral and anti-cancer properties (12). Some components of *H. clathratus* affect the growth of human acute promyelocytic leukemia, human breast carcinoma, and human hepatocellular carcinoma cancer cell lines (13). In particular, phenolic compounds isolated various seaweeds predominantly possess anti-inflammatory and anti-proliferative activities through radical scavenging activity (14). Though the total polyphenol contents of seaweeds are interesting to assess anti-inflammatory and anti-proliferative activities, no reports have evaluated the effects of *H. clathratus* on MMP-9 gene expression in cancer cells.

In this study, we evaluated the effects of a methanol extract of *H. clathratus* (MEHC) on MMP-9 expression in TNF-α-stimulated T24 bladder carcinoma cells. We found that MEHC downregulated TNF-α-induced MMP-9 mRNA and protein expression by suppressing NF-κB activation and MAPK phosphorylation.

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Materials and methods

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 Republic 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 -20℃. 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

TNF-α and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louise, MO, USA). Polyclonal antibodies against MMP-9, p65, p50, IκBα, ERK, phospho (p)-ERK, JNK, p-JNK p38, p-p38, phosphatidyl-inositol 3 kinase (PI3K), p-PI3K, Akt and p-Akt were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against β-actin was obtained from Sigma. Roswell Park Memorial Institute medium (RPMI), antibiotics mixture and fetal bovine serum (FBS) were obtained from WelGENE Inc. (Daegu, Republic of Korea). Peroxidase-labeled goat anti-rabbit immunoglobulin was purchased from KOMA Biotechnology (Seoul, Republic of Korea). Other chemicals were purchased as Sigma grades.

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Total polyphenol contents

Total polyphenol contents of MEHC were quantified according to the methods of Yuan et al. (15). Sample absorbaces were read at 720 nm. Gallic acid was used as the standard for a calibration curve. Total pholyphenol contents of the seaweed extracts were represented as gallic acid equivalents.

Cell culture and cell viability assay

T24 bladder carcinoma cells (ATCC, Rockville, MD, USA) were cultured at 37°C in 5% CO_2 in RPMI supplemented with 10% FBS and antibiotics. In all experiments, cells were pretreated with MEHC 1 h before the addition of TNF-α (20 ng/ml) in serum-free RPMI. Cell viability was determined by an MTT assay. In brief, T24 bladder carcinoma cells (1×10^5 cells/ml) were plated onto 24 well plates and incubated overnight. The cells were treated with the indicated concentrations of MEHC for 1 h and then stimulated with TNF-α (20 ng/ml) for 24 h. Then, the cells were incubated with a solution of 0.5 mg/ml MTT and incubation for 45 min at 37℃ and 5% CO_2. Supernatant was removed and the formation of formazan was observed by monitoring the signal at 540 nm using a microplate reader.

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Isolation of total RNA and RT-PCR

Total RNA was extracted using easy-BLUE^{TM} total RNA extraction kit (iNtRON Biotechnology; Sungnam, Republic of Korea) according to the manufacturer's instruction. Two microgram RNA was reverse-transcribed using MMLV reverse transcriptase (Bioneer; Daejeon, Republic of Korea). cDNA was amplified by PCR using specific primer MMP-9 (forward 5'-gta ttt gtt caa gga tgg gaa ata c-3' and reverse 5'-gca gga tgt cat agg tca cgt ag-3') and GAPDH (forward 5'-cca ccc atg gca aat tcc at-3' and reverse 5'-tct aga cgg cag gtc agg tcc acc-3'). Reaction products were analyzed on 1.0% agarose gels, and the bands were visualized by ethidium bromide.

Western blot analysis

Total cell extracts were prepared using PRO-PREP protein extraction kit (iNtRON Biotechnology). The preparation of cytoplasmic or nuclear extracts was conducted using NE-PER cytosolic/nuclear extraction reagents (Pierce; Rockford, IL, USA). 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).

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Electrophoretic mobility assay (EMSA)

DNA-protein binding assays were carried out with nuclear extract. Synthetic complementary NF-κB (5'-agt tga ggg gac ttt ccc agg c-3') binding oligonucleotides (Santa Cruz Biotechnology) were 3'-biotinylated using the biotin 3'-end DNA labeling kit (Pierce) according to the manufacturer's instructions and annealed for 30 min at room temperature. The reaction mixture was electrophoretically separated on a 4% polyacrylamide gel in 0.5X Tris-borate buffer and transferred to a nylon membrane. The transferred DNAs were cross-linked to the membrane at 120 mJ/cm^2. Horseradish peroxidase-conjugated streptavidin was used according to the manufacturer's instructions to detect the transferred DNA.

Gelatin substrate gel zymography

T24 bladder carcinoma cells were incubated at 37°C in 5% CO_2 in serum-free RPMI medium supplemented with 10% FBS and antibiotics with or without MEHC for 24 h. Supernatants were collected and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) copolymerized with gelatin. After electrophoresis, the gels were washed several times with 2.5% Triton X-100 for 1 h at room temperature to remove the SDS and then incubated for 24 h at 37℃ in reaction buffer containing 5 mM $CaCl_2$ and 1 μM $ZnCl_2$. The gels were stained with 0.25% Coomassie blue for 30 min and then destained for 1 h in a solution of acetic acid and methanol. The proteolytic activity was evidenced as clear bands (zones of gelatin degradation) against the blue background of stained gelatin.

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Matrigel invasion assay

T24 bladder carcinoma cells were trypsinized and 5×10^4 cells were placed onto matrigel-coated transwell for 3 h. The cells were treated with 100 μg/ml of MEHC for 1 h and then stimulated with TNF-α (20 ng/ml) for 24 h. Then, the cells in the upper chamber were removed with a cotton swab. The cells that adhered on the outer surface of the transwell insert were fixed, stained with Coomassie blue, and counted under a light microscope.

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. 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 $p < 0.05$.

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Results

Effects of MEHC on cell viability

As assayed by gallic acid equivalents, total phenol contents represented about 2.7 ± 0.2% of MEHC. Next, in order to determine the effects of MEHC on cell viability in T24 bladder carcinoma cells, an MTT assay was performed at 24-h after treatment with the indicated concentrations of MEHC in the presence or absence of TNF-α. MEHC alone at 25–150 μg/ml showed no cytotoxic effects (Fig. 6), but significant cytotoxicity was found at 200 μg/ml MEHC, regardless of the presence of TNF-α. DMSO (0.1%) as a solvent had no cytotoxic effect (data not shown). Therefore, the concentration of MEHC was applied within this range for further experiments.

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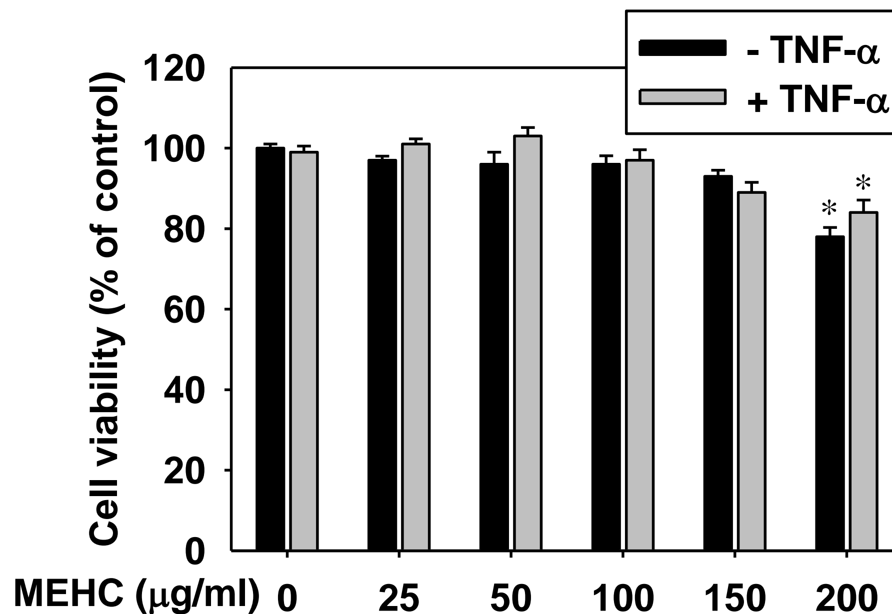

Figure 6. Effects of a methanol extract of *Hydroclathrus clathratus* (MEHC) on T24 cell viability. T24 bladder carcinoma cells (1×10^5 cells/ml) were incubated with the indicated concentrations of MEHC 1 h before tumor necrosis factor (TNF)-α (20 ng/ml) treatment for 24 h. Cell viability was determined by the MTT assay. Each value indicates the mean ± SE, and is representative of results obtained from three independent experiments. *$P < 0.05$ was considered significantly different from the values in cells with an untreated control.

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Suppressive effect of MEHC on MMP-9 activity

Zymography, the real time polymerase chain reaction (RT-PCR) and western blot assays were conducted to assess whether MEHC regulates MMP-9 expression. The zymography data showed that MEHC significantly suppressed TNF-α-induced MMP-9 activity (Fig. 7A). We also found that MEHC significantly downregulated TNF-α-induced MMP-9 mRNA and protein expression. In the Western blot analysis, TNF-α stimulation of cells resulted in a significant increase in MMP-9 expression compared to that in the untreated control (Fig. 7B). However, MEHC reversed TNF-α-induced MMP-9 activity to the levels of the untreated control. Moreover, *MMP-9* gene expression was confirmed by RT-PCR analysis. Pretreatment with MEHC significantly suppressed TNF-α-induced *MMP-9* upregulation at the mRNA level (Fig. 7C). Taken together, these results indicate that MEHC suppressed upregulation of TNF-α-stimulated MMP-9 expression at the transcriptional level.

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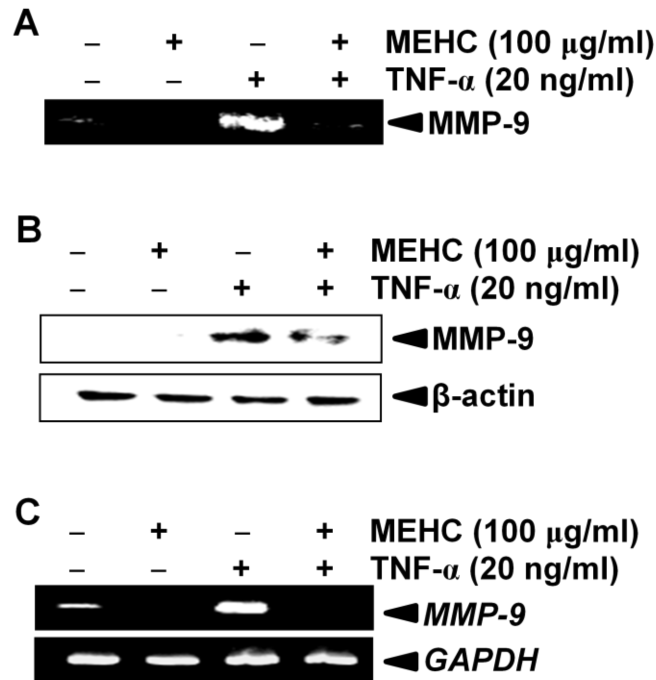

Figure 7. Effects of a methanol extract of *Hydroclathrus clathratus* (MEHC) on tumor necrosis factor (TNF)-α-induced matrix metalloproteinase-9 (MMP-9) expression in T24 bladder carcinoma cells. (A) T24 cells were treated with 100 μg/ml MEHC 1 h before TNF-α (20 ng/ml) treatment for 24 h. Conditioned medium was collected after 24-h, followed by gelatin zymography. (B) Equal amounts of cell lysates were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with antibodies against MMP-9. Cells were incubated with the indicated concentrations of MEHC 1 h before the TNF-α (20 ng/ml) treatment for 24 h. (C) Total RNA was isolated, and RT-PCR analysis of MMP-9 was performed. GAPDH and β-actin were used as internal controls for the RT-PCR and Western blot analysis, respectively. The experiment was repeated three times, and similar results were obtained.

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Inhibitory effect of MEHC on invasion of T24 bladder carcinoma cells

MMP-9 is thought to be critically involved in the processes of tumor invasion and angiogenesis[5]. Because MEHC inhibited MMP-9 expression and activity, we examined the effects of MEHC on invasion of T24 bladder carcinoma cells (Fig. 8A). When the cells were treated with TNF-α alone, a remarkable 3-fold higher increase in cell invasion was observed compared to that in the untreated control. However, MEHC pretreatment resulted in an approximate 50% reduction in penetration through a matrigel-coated membrane compared to that in the TNF-α-treated group (Fig. 8B). These results confirm that MEHC inhibited TNF-α-induced invasion in T24 bladder carcinoma cells.

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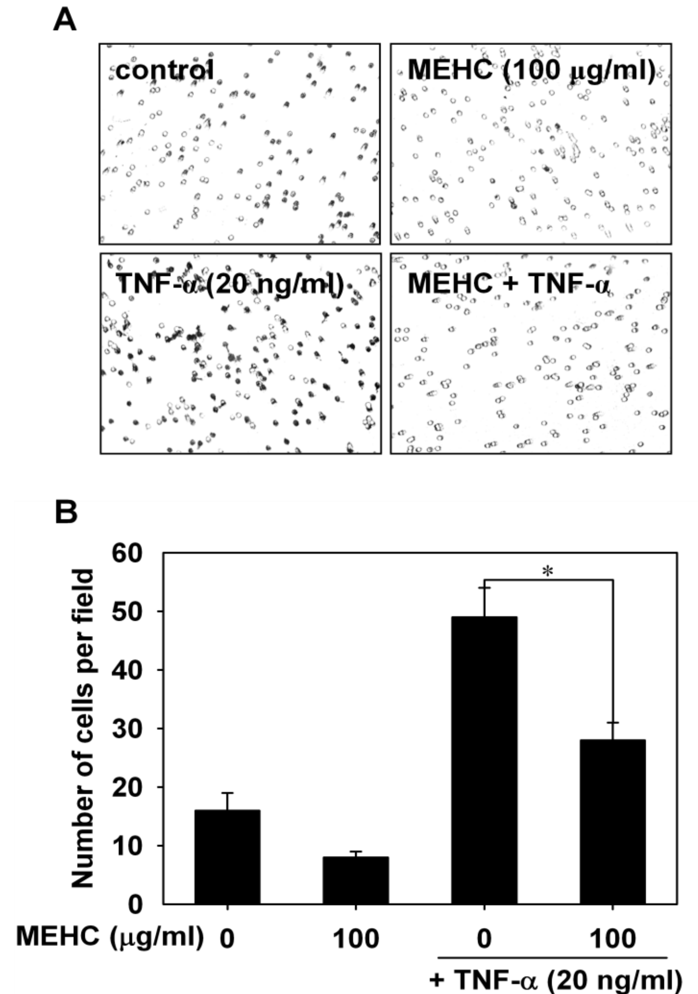

Figure 8. Suppressive effects of a methanol extract of *Hydroclathrus clathratus* (MEHC) on invasion of T24 bladder carcinoma cells. (A) The upper parts of the transwells were coated with matrigel for the invasion assay. Then, the cells were cultured in serum-free media for 1 h before treatment with MEHC (100 μg/ml) in the absence or presence of tumor necrosis factor (TNF)-α (20 ng/ml). After 24-h, the numbers of cells passing through the matrigel to the membrane were dyed using 0.125% Coomassie blue in ethanol. (B) Data are expressed as overall mean ± SE from three independent experiments, and the numbers of cells per field are estimated. Statistical significance was determined by two-way ANOVA test (* $P < 0.05$ vs. TNF-α-treated group).

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Inhibitory effects of MEHC on TNF-α-induced NF-κB activity

We assessed the specific DNA-binding activity of NF-κB using an electrophoretic mobility shift assay (EMSA) to investigate whether MEHC inhibits MMP-9 activity by suppressing NF-κB activity. TNF-α stimulation caused a remarkable increase in binding complexes between NF-κB and specific-binding DNA at 30 min; however, pretreatment with MEHC for 1-h significantly reduced TNF-α- induced NF-κB activity (Fig. 9A). In a parallel experiment, p65, p50, and IκBα expression levels in the cytoplasmic region were determined after TNF-α stimulation in the absence or presence of MEHC (Fig. 9B). TNF-α significantly decreased p65, p50, and IκBα expression in the cytoplasmic compartment of T24 bladder carcinoma cells. However, treatment with MEHC sustained the TNF-α-induced expression of p65, p50 and IκBα (Fig. 9B). Because the PI3K and Akt signal pathways regulate NF-κB activity upstream, we evaluated whether MEHC regulates TNF-α-induced phosphorylation of this signaling pathway. As expected, stimulation with TNF-α significantly induced PI3K and Akt phosphorylation; however, pretreatment with MEHC shifted the expression level of the untreated control (Fig. 9C). These results indicate that MEHC inhibited the suppression of NF-κB activity by suppressing the PI3K/Akt signal pathway.

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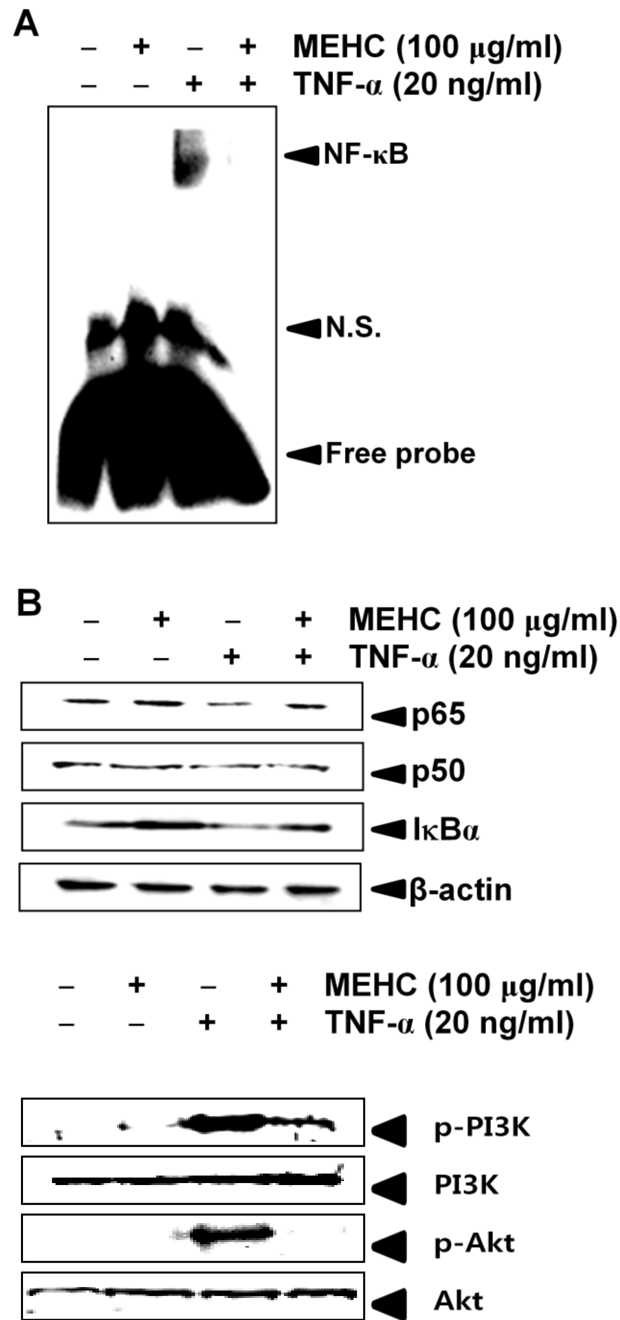

Figure 9. Effects of a methanol extract of *Hydroclathrus clathratus* (MEHC) on nuclear factor (NF)-κB DNA binding activity. Cells were preincubated with MEHC (100 µg/ml) 1-h before TNF-α (20 ng/ml) stimulation for 30 min. (A) Nuclear extracts were assayed for NF-κB activity

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using an electrophoretic mobility shift assay and (B) the levels of p50, I B, and p65 were analyzed by Western blot analysis. (C) In a parallel experiment, protein lysates were prepared, subjected to SDS-PAGE and immunoblotted using antibodies against PI3K, Akt, and their phosphorylated forms. β-Actin was used as an internal control for western blot assays. The experiment was repeated three times, and similar results were obtained. N.S., non-specific.

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Inhibitory effects of MEHC on TNF-α induced MAPK phosphorylation

We next evaluated the effects of MEHC on TNF-α-induced phosphorylation of ERK, p38, and JNK in T24 bladder carcinoma cells. The phosphorylation levels of the various MAPKs increased dramatically 30 min after stimulation with TNF-α. MEHC significantly reduced TNF-α-induced phosphorylation of ERK, p38, and JNK. However, non-phosphorylated ERK, p38, and JNK expression was unaffected by TNF-α and/or MEHC alone. These results indicate that MEHC may be involved in inhibiting MAPK activity to suppress TNF-α-induced invasion.

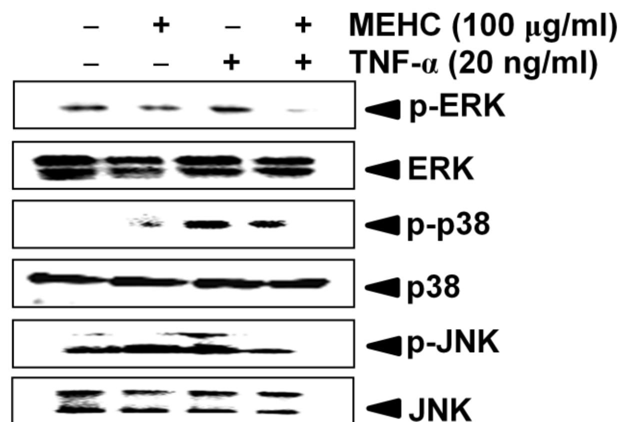

Figure 10. Effects of a methanol extract of *Hydroclathrus clathratus* (MEHC) on mitogen activated protein kinases (MAPKs) in TNF-α-stimulated T24 bladder carcinoma cells. T24 cells were pretreated with 100 μg/ml MEHC for 1 h and then incubated with TNF-α (20 ng/ml) for 30 min. Total cellular protein was resolved by SDS-PAGE, transferred to nitrocellulose membranes, and detected with specific antibodies against ERK, JNK, p38, and their phosphorylated forms. The experiment was repeated three times, and similar results were obtained. Statistical significance was determined by two-way ANOVA test (*$P < 0.05$ vs. TNF-α-treated group).

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Discussion

Our previous study suggested that MEHC considerably suppresses inflammatory responses such as lipopolysaccharide-induced pro-inflammatory cytokines and nitric oxide expression in BV2 microglia cells (unpublished data). It has also been reported that MEHC inhibits the proliferation of a variety of human cancer cells, including human breast carcinoma (MCF-7) and human hepatocellular carcinoma (13). Nevertheless, it is not fully known how MEHC regulates anti-cancer activity during the invasion process. Therefore, we determined that MEHC suppressed TNF-α-induced NF-κB activity and this downregulation lead to decreased invasion of bladder cancer cells through the regulation of MMP-9. This study provides substantial evidence that MEHC contained common levels of phenol content compared to other seaweed (approximate 3% in extracts) and inhibited TNF-α-induced MMP-9 expression in T24 bladder carcinoma cells by suppressing NF-κB activity through the PI3K/Akt and MAPK pathways.

Tumor metastasis is a multistep process by which a subset of individual cancer cells disseminates from a primary tumor to distant secondary organs or tissues. MMPs play a major role promoting tumor metastasis (16,17). One of them, MMP-9, is regarded as a critical molecule to eliminate the progression of tumor invasion. Thus, the inhibitory effect of MMP-9 expression is important as a therapeutic experimental model of tumor invasion. Our results demonstrated that MEHC inhibited TNF-α-induced MMP-9 activity accompanied by the suppression of MMP-9 gene transcription in T24 bladder carcinoma cells. Interestingly, the matrigel assay showed that MEHC suppressed cell invasion. MMP-9 is synthesized and secreted into the extracellular matrix as a late pro-enzyme, and the pro-peptide domain, containing a cysteine switch, interacts with zinc at the active site for enzyme activation by regulatory proteins such as tissue inhibitor of MMP (TIMP) and MT1-MMP (18□20). Therefore, whether MEHC-induced MMP9

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downregulation is regulated by TIMPs and MT1-MMP in the extracellular matrix needs to be investigated.

Several studies have identified the signal transduction pathways that are involved in regulating MMP-9 expression in tumor cells (21,22). In particular, NF-κB is an important transcription factor for regulating the *MMP-9* gene promoter and contains NF-κB binding sites (23). Once NF-κB is fully activated, it participates in the regulation of various target genes in different cells and is involved in various functions (24,25). NF-κB is a heterodimer consisting of p65 and p50 proteins; it is located in the cytosol and is complexed with the IκBα inhibitory protein (26). A variety of extracellular signals including TNF-α and growth factors activate IκB kinase, which results in ubiquitination and degradation of IκBα by proteasomes and eventual dissociation of IκBα from NF-κB (27). The activated NF-κB is then translocated to the nucleus where it binds to specific DNA sequences. Ultimately, NF-κB promotes the expression of various genes including MMP-9 (28). In this study, we showed that MEHC inhibited p65 and p50 protein translocation by suppressing IκBα degradation. However, the human MMP-9 promoter also contains two other transcription factors such as AP-1 and Sp1 (29,30). In particular, AP-1 binding to the MMP-9 promoter region is thought to be important for regulating MMP-9 expression in response to phorbol 12-myristate 13-acetate (PMA); however, many researchers have reported that AP-1 activation is not involved in TNF-α-stimulated MMP-9 expression (31,32). MMP-9 expression is also regulated by Sp1 in response to PMA and TNF-α (30,32). Therefore, further study is required to determine which transcriptional factors are inhibited in MEHC-induced MMP-9 downregulation.

We investigated whether MEHC inhibits PI3K and Akt phosphorylation, because PI3K and Akt are upstream regulators of NF-κB (34,35). Treatment with MEHC suppressed PI3K and Akt

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phosphorylation. These data indirectly support the result that the TNF-α-induced PI3K/Akt/NF-κB/MMP-9 pathway was inhibited by treatment with MEHC. In a previous study, Zhu et al. reported that these signaling pathways are highly activated during the progression of chronic myeloid leukemia (36). Additionally, the role of MAPKs in the regulation of MMP-9 expression is particularly well understood in TNF-α-stimulated cancer cells (37,38). Some researchers have reported that TNF-α-induced p38 is a MAPK effector that induces MMP-9 expression regardless of ERK1/2 and JNK (37,38). In contrast, other have shown that TNF-α induces MMP-9 expression through AP-1-dependent ERK1/2 and/or JNK activation (38). In this study, we found that MEHC suppressed TNF-α-induced ERK, p38, and JNK phosphorylation, whereas non-phosphorylated ERK, p38, and JNK kinase expression was unaffected by MEHC treatment. Presumably, this discrepancy with previous studies was due to the cell types and specificity of the chemicals.

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Conclusion

Our findings showed that MMP-9 activity was significantly increased in response to tumor necrosis factor-α (TNF-α). However, treatment with MEHC substantially reversed TNF-α-induced MMP-9 activity. A matrigel invasion assay also showed that MEHC reduced TNF-α-induced invasion of T24 bladder carcinoma cells. We also found that MEHC significantly downregulated the expression of the *MMP-9* gene induced by TNF-α stimulation. Furthermore, we investigated the effects of MEHC on nuclear factor (NF)-κB activity, which is a potential transcriptional factor for regulating many invasive genes including MMP-9. MEHC suppressed NF-κB activity by suppressing IκB degradation and nuclear translocation of the NF-κB p65 and p50 subunits. TNF-α-induced phosphorylation of phosphatidyl-inositol 3 kinase (PI3K)/Akt and mitogen activated protein kinase (MAPK) was significantly downregulated in the presence of MEHC. Taken together, these results indicate that MEHC is a potential anti-invasive agent by suppressing TNF-α-induced cancer cell invasion and by specifically inhibiting NF-κB and MAPKs, as well as downstream target genes such as MMP-9.

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