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Role of ERK/MAPK signalling pathway in anti-inflammatory effects of *Ecklonia cava* in activated human mast cell line-1 cells

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ABSTRACT

Objective: The anti-inflammatory effects of *Ecklonia cava* (EC) and its mechanism of action were examined in phorbol-12 myristate 13-acetate (30 nmol/L) and A23187 (1 μ mol/L) (PMACI) stimulated human mast cell line-1 cells. **Methods:** Nitric oxide content, inducible nitric oxide synthase and cyclooxygenase-2 protein expression, pro-inflammatory cytokines including IL-1 β , TNF- α , and IL-6 mRNA and protein expressions were determined. In addition, extracellular regulated protein kinases/mitogen-activated protein kinase (ERK/MAPK) activation was examined. **Results:** EC dose-dependently suppressed inducible nitric oxide synthase and cyclooxygenase-2 protein expression and subsequently it reduces nitric oxide content in PMACI stimulated human mast cell line-1 cells. EC dose-dependently inhibited the mRNA as well as protein expression of TNF- α , IL-1 β , and IL-6 in the PMACI stimulated human mast cell line-1 cells without any cytotoxic effect. Furthermore, EC significantly inhibited PMACI induced phosphorylation of ERK1/2 in a dose-dependent manner without affecting the total protein levels. **Conclusions:** EC exert its anti-inflammatory actions via inhibition of ERK/MAPK signalling pathway, suggesting that EC is a potent and efficacious anti-inflammatory agent for mast cell-mediated inflammatory diseases.

1. Introduction

Mast cells play an important role in the mediation of inflammatory responses. Activated mast cells release pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, IL-8, and inflammatory mediators including nitric oxide (NO), cyclooxygenase (COX)-2, and histamine[1-3]. The release of these cytokines and mediators participates in the development of variety of inflammatory reactions. Therefore, inhibition of mast cell activation is becoming a new therapeutic approach to control chronic inflammation.

Activation of mast cells leads to phosphorylation of tyrosine kinase and mobilization of intracellular Ca²⁺. This is followed by activation of protein kinase C, mitogen-

activated protein kinase (MAPK), nuclear factor (NF)- κ B, and release of inflammatory cytokines[4-6]. MAPK signaling cascade plays an essential role in the initiation of inflammatory responses[7]. The induction of inflammatory cytokine genes requires activation of the MAPKs[8] and stimulation of extracellular regulated protein kinases/mitogen-activated protein kinase (ERK/MAPK) pathways are essential for IL-8 production in human mast cell line (HMC)-1 cells[9].

During the inflammatory processes, large amounts of mediators are released. Among these mediators, COX-2, a key enzyme for synthesizing prostaglandin, and inducible nitric oxide synthase (iNOS), a nitric oxide synthesis enzyme, have been implicated as mediators for variety of inflammatory reactions. NO exerted its inflammatory effects by stimulating iNOS and COX-2. Further activation of NO can initiate the transcription of downstream inflammatory genes such as pro-inflammatory cytokines (IL-1 β , TNF- α , and IL-6), and following stimulation of enzyme mediators (COX-2 and iNOS) mediated by signaling intermediation of the MAPK pathways[10-12]. In recent years, it has been demonstrated that both COX-2 and iNOS

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play important roles in various tumors and inflammatory diseases^[10,13]. COX-2 and iNOS were also strongly induced in activated mast cells^[14,15].

Marine algae have begun to attract attention as rich sources of diverse bioactive compounds with great pharmaceutical and biomedical potential. *Ecklonia cava* (EC) is an abundant brown algae used in the south-west coastal region of Japan and Korea. It has been reported that EC extract have numerous biological activities, including anti-oxidative, radical scavenging, immunomodulatory, and anti-mutagenic activities^[16–21]. Although several reports showed inhibitory effects of EC on inflammatory mediators from endothelial, microglial, and macrophage cells, the pharmacological actions of EC on pro-inflammatory cytokines and the underlying mechanisms in mast cells have not been investigated. In the present study, we examined the anti-inflammatory effect of EC on phorbol 12-myristate 13-acetate and calcium ionophore A23187 induced expression of pro-inflammatory cytokines and activation of MAPKs using human mast cell line, HMC-1.

2. Materials and methods

2.1. Preparation of EC powder and extract

EC was obtained from a local market in Seosan, Korea. Fresh EC was washed, dried in the shade, and ground into powder. The dried powder was extracted 3 times with 10 volumes of ethanol at room temperature for 24 h. The combined extracts were centrifuged, filtered, concentrated under vacuum, lyophilized, and subsequently used for the experiment. The yield after vacuum evaporation was 14.2%.

2.2. Cell culture

HMC-1 cells were grown in Iscove's Modified Dulbecco's medium (Gibco BRL, Grand Island, NY, USA) with 10% (v/v) FBS, 100 U/mL of penicillin, and 100 μ g/mL of streptomycin at 37 °C in 5% (v/v) CO₂ atmosphere.

2.3. Cytotoxicity

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich Inc., St. Louis, MO, USA) assay. Briefly, HMC-1 cells (1×10^5 cells/well) in 96 well plates with various concentrations (100–1 000 μ g/mL) of EC extract were incubated for 24 h, and MTT (5 mg/mL) was added and further incubated for 4 h. The formazan crystals were dissolved in DMSO and the absorbance was measured at 570 nm with microplate reader (Bio-Tek Instrument Inc., Winooski, VT, USA).

2.4. Measurement of NO productions

HMC-1 cells were treated with phorbol-12 myristate 13-acetate (30 nmol/L) and A23187 (1 μ mol/L) (PMACI) in the presence of EC (0–100 μ g/mL) for 24 h in the 96 well plate. The concentration of NO in the culture supernatant was determined as nitrite, a major stable product of NO, using Griess reagent. Nitrite levels were determined colorimetrically at 540 nm.

2.5. Determination of cytokines

Release of the IL-1 β , TNF- α , and IL-6 cytokines in PMACI stimulated HMC-1 cells was measured using a commercial BD CBA human inflammatory cytokine kit (BD Biosciences, Franklin Lakes, NJ, USA). Serum free HMC-1 cells were treated with various doses of EC extract (0–100 μ g/mL) for 1 h before stimulation with PMACI and incubated for 8 h. After 3 h of incubation, samples were washed and analyzed using BD FACSTM flow cytometry (BD Biosciences).

2.6. Real-time PCR (RT-PCR)

Serum free HMC-1 cells were treated with EC extract for 1 h before stimulation with PMACI and incubated for 8 h. Quantitative RT-PCR was used to quantify the expression of mRNAs for IL-1 β , TNF- α and IL-6. Total RNA was isolated using RNeasy kit (Qiagen, Valencia, CA, USA). First-strand cDNA was synthesized using 1 μ g of total RNA and SuperScript First-Strand cDNA synthesis kit (Invitrogen, Korea). Primers used for PCR-assisted amplification were as follows: 5'-ACC CCC AGG AGA AGA TTC CAA AG-3' and 5'-CAT GTC TCC TTT CTC AGG GCT GAG-3' forward and reverse for IL-6, 5'-TTG ACC TCA GCG CTG AGT TG-3' and 5'-CCT GTA GCC CAC GTC GTA GC-3' forward and reverse for TNF- α , 5'-CAG GAT GAG GAC ATG AGC ACC-3' and 5'-CTC TGC AGA CTC AAA CTC CAC-3' forward and reverse for IL-1 β , and 5'-CAC TCA CGG CAA ATT CAA CGG CAC-3' and 5'-GAC TCC ACG ACA TAC TCA GCA C-3' forward and reverse for GAPDH. PCR amplification was carried out using SYBR RT-PCR kit (Takara, Japan). The PCR cycle was as follows: initial step at 95 °C for 10 min followed by second step at 95 °C for 30 s, 60 °C for 30 s for 40 cycles with melting curve analysis using Rotor Gene Q (Qiagen). The level of target mRNA was normalized to the level of GAPDH and compared with control (untreated sample) by $\Delta\Delta$ CT method^[22] using Rotor Gene Q software version 1.7.

2.7. Western blot analysis for iNOS, COX-2, and MAPK

The levels of iNOS and COX-2 were determined in the cells which were stimulated with PMACI and pre-treated

with EC (0–100 $\mu\text{g/mL}$). For analysis of activation of MAPK pathway, cells were pre-treated with EC (0–200 $\mu\text{g/mL}$) for 30 min prior to stimulation with PMACI. Proteins were extracted with PRO-PREP Protein extraction solution (Intron biotechnology, Seongnam, Korea), and protein concentrations were measured using DC Protein assay kit (Bio-Rad, Hercules, CA, USA). Aliquots of samples were subjected to 10% SDS-PAGE and transferred onto PVDF membrane. After blocking, the membrane was incubated with the appropriate primary antibody (Santa Cruz Biotechnology, CA, USA) followed by incubation with secondary antibody conjugated with horseradish peroxidase. Immunoreactive bands were visualized using chemiluminescent imaging system (Fusion SL2, Vilber Lourmat, Marne-la-Vallée Cedex, France), and analyzed by Bio1d software (Vilber Lourmat, France).

2.8. Statistical analysis

Statistical analysis of the data obtained from all studies was implemented by the Statistical Package for the Social Sciences Software (SPSS, version 10.1 for windows, USA) and Student's *t*-test. Data expressed as mean \pm SD. $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Effects on NO production

PMACI significantly increased NO production to 1.95-fold of the basal level in HMC-1 cells, and PMACI induced NO production was dose-dependently decreased by EC treatment (Figure 1). NO productions were decreased by 25.4% and 46.6% at 50 and 100 $\mu\text{g/mL}$ EC treatments, respectively.

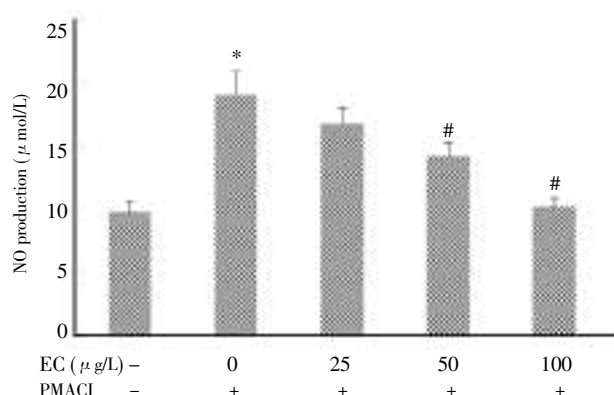


Figure 1. Effect of EC on PMACI induced NO production in HMC-1 cells.

Cells were treated with PMACI for 24 h in the presence of EC extract. Experiments were performed in triplicate using independent samples. Data are presented as mean \pm SD. *Significant difference with PMACI treatment ($P < 0.05$). #Significant difference with EC treatment in the presence of PMACI ($P < 0.05$).

To exclude the possibility that the inhibition of NO production was due to cytotoxicity caused by EC treatment, MTT assays were performed in HMC-1 cells treated with EC extract. Cell viability was not significantly affected by EC up to 1 000 $\mu\text{g/mL}$ exhibiting 96.3%-89.9% cell viability at 50–1 000 $\mu\text{g/mL}$ over 24 h period (data not shown). Thus, the inhibitory effect of EC on PMACI stimulated NO production was not due to any cytotoxic action on HMC-1 cells.

3.2. Effects on iNOS and COX-2 expression

To determine the mechanism by which EC reduced PMACI induced NO production, the effects of EC on PMACI induced iNOS and COX-2 protein expressions were determined by western blot analysis. As shown in Figure 2, PMACI induced iNOS and COX-2 production was dose-dependently attenuated in HMC-1 cells pre-treated with EC.

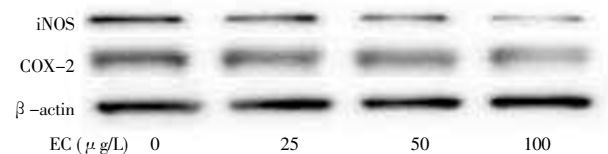


Figure 2. Effect of *Ecklonia cava* (EC) on PMACI induced iNOS and COX-2 protein expression.

Cells were pre-treated with EC prior to stimulation with PMACI, and expression of iNOS and COX-2 protein levels were determined by western blot. β -actin was used as an internal control.

3.3. Effects on pro-inflammatory cytokine production and mRNA expression

IL-1 β , TNF- α , and IL-6 were known to be pro-inflammatory cytokines that possessed a multitude of biological activities linked to the immune-pathology of acute or chronic inflammatory diseases. In order to assess the effect of EC on PMACI induced pro-inflammatory cytokine production, the HMC-1 cells were pre-treated with various concentrations of EC for 1 h prior to the addition of the stimulators. Figure 3A indicates that protein levels of IL-1 β , TNF- α , and IL-6 were markedly up-regulated in response to PMACI stimulation. Pre-treatment of EC (4–100 $\mu\text{g/mL}$) significantly inhibited IL-1 β , TNF- α , and IL-6 productions in a dose-dependent manner. The effects of EC on IL-1 β and TNF- α productions were greater than the effects on IL-6. The inhibitory effect of 100 $\mu\text{g/mL}$ EC on IL-1 β and TNF- α production were 75.0% and 60.6%, respectively, while IL-6 production was reduced by 23.0%.

The PMACI stimulated IL-1 β , TNF- α , and IL-6 mRNA expressions were also dose-dependently inhibited by EC,

and IL-1 β mRNA expression was most affected by EC treatment (Figure 3B). IL-1 β mRNA expression at 100 μ g/mL EC treatment exhibited 6.2% of control value, while TNF- α and IL-6 mRNA expressions were 57.8% and 78.6% of control value, respectively.

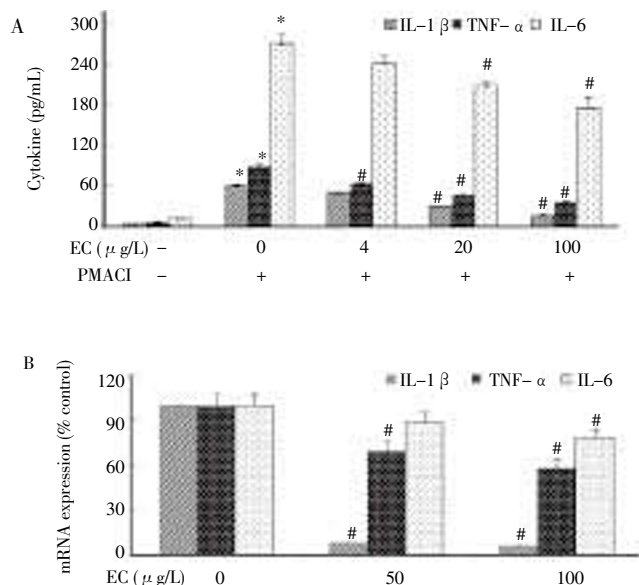


Figure 3. Effect of *Ecklonia cava* (EC) on cytokine expression.

Cells were pre-treated with EC for 1 h before stimulation with PMACI. (A) Protein levels of IL-1 β , TNF- α , and IL-6 were determined by ELISA. (B) The mRNA expression level of cytokines was determined by real-time PCR. Experiments were performed in triplicate using independent samples. Data are presented as mean \pm SD. *Significant difference with PMACI treatment ($P<0.05$). #Significant difference with EC treatment in the presence of PMACI ($P<0.05$).

3.4. Effects on MAPK activation

MAPK signalling cascade plays an essential role in the cytokine production and inflammatory response. Therefore, in order to elucidate the mechanism underlying the effects of EC, activation of ERK1/2 was examined using western blot analysis (Figure 4). PMACI treatment significantly increased phosphorylated ERK1/2 expression to 4-fold of the control value, and EC pre-treatment significantly inhibited PMACI induced phosphorylation of ERK1/2 in a dose-dependent manner without affecting the total protein levels (Figure 4A, 4B and 4E). The effect of EC on ERK1/2 activation was not significantly up to 50 μ g/mL concentration, and the ERK1/2 activation was decreased by 18.4% and 35.5% at 100 and 200 μ g/mL of EC concentration, respectively. Total ERK1/2 protein expression levels were not affected by PMACI or EC treatment (Figure 4C and 4D). The results indicated that EC exerted its anti-inflammatory actions via inhibition of ERK/MAPK signalling pathway.

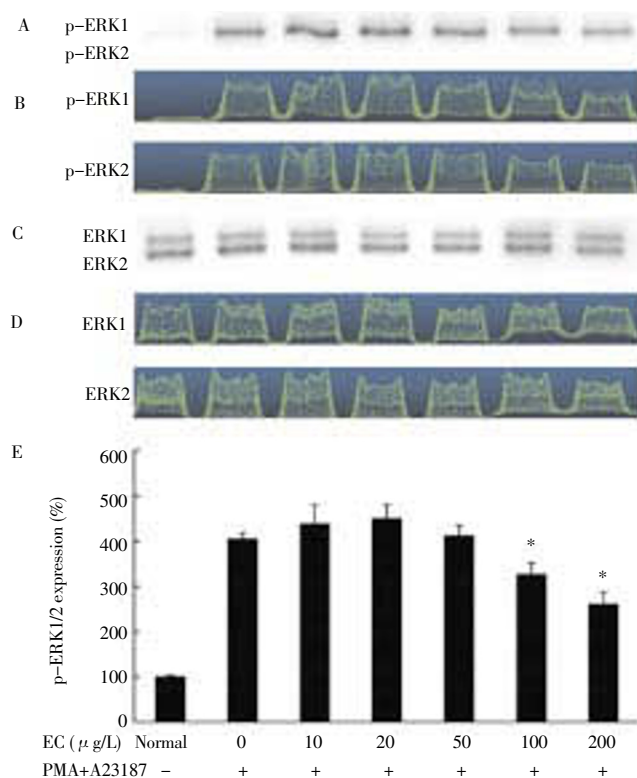


Figure 4. Effects of *Ecklonia cava* (EC) on activation of the ERK/MAPK in HMC-1 cells.

Phosphorylation of ERK1/2 was analyzed by western blot. (A and C) Western blot results of the phosphorylation of ERK1/2 and total ERK1/2. (B, D and E) Densitometric results of the phosphorylation of ERK1/2. Data are presented as mean \pm SD. #Significant difference with EC treatment in the presence of PMACI ($P<0.05$).

4. Discussion

Present study examined the pharmacological and biological effects of EC on the production of inflammatory mediators in human mast cells stimulated with PMACI. It has been reported that HMC-1 cells are immature cells unable to bind specific IgE due to a lack of Fc ϵ R1 receptors[23]. Hence, we used PMACI as a substitute for the IgE/anti-IgE reaction in stimulating HMC-1 cells. To further understand the molecular mechanism of EC, the effects of EC on the production of NO, the expression levels of iNOS, COX-2, and cytokines (IL-1 β , TNF- α , and IL-6), and the activation of ERK/MAPK were investigated. The results indicate that EC effectively inhibits PMACI induced pro-inflammatory cytokine and NO production through a blockade of the MAPK pathways in HMC-1 cells.

During the inflammatory processes, large amounts of pro-inflammatory mediators, NO, and prostaglandin E2 is generated and suppression of these mediators may be a useful tool to reduce inflammatory symptoms[24]. The regulation of PMACI induced iNOS and COX-2 expression, which

is sensitive prime components of intracellular signalling pathways responsible for inflammatory events, by EC was elucidated using western blot analysis. In the present study, EC dose-dependently suppressed iNOS protein expression and subsequently it reduces COX-2 expression and NO content in PMACI stimulated HMC-1 cells.

Mast cell derived pro-inflammatory cytokines are important mediators of inflammation and have received much attention over the past decade. TNF- α , IL-6, and IL-1 play a major role in triggering and sustaining the inflammatory response in mast cells[25], and the inhibition of these pro-inflammatory cytokine productions by mast cells may be a key to suppress the inflammatory symptoms. Recently Yang *et al.* reported that 6,6'-bieckol isolated from EC exhibited anti-inflammatory activity through the inhibition of TNF- α and IL-6 by negative regulation of the NF κ B pathway in LPS-stimulated macrophages[20]. Also the inhibitory effect of EC extracts on the production of TNF- α in LPS-induced human endothelial cells[21], and on the release of TNF- α and histamines mediated by IgE in murine asthma model[19] have been reported. IL-1 β transcriptionally activates TNF- α and IL-13 mRNA, and protein production, and probably modulates TNF- α mediated inflammatory diseases[26,27]. As an initiator of inflammation, IL-1 β cooperates with arachidonic acid metabolites to recruit inflammatory immune cells, leading to the development of the adaptive immune response[26]. To gain further insights into the molecular mechanisms of EC-mediated inhibition of inflammatory mediators, the regulatory effects of EC on IL-1 β , TNF- α , and IL-6 productions were evaluated. The results of the present study indicate that EC dose-dependently inhibited the mRNA expression and production of IL-1 β , TNF- α , and IL-6 in PMACI stimulated HMC-1 cells without any cytotoxic effect, suggesting its anti-inflammatory properties by regulating the cytokine production at the level of transcription. The inhibitory effect of IL-1 β was greater than TNF- α and IL-6, suggesting that EC affects the initiation step of the inflammatory response.

The effect of EC was further investigated to determine whether EC has an inhibitory effect on signal transduction of mast cell stimulation. The MAPK cascade is one of the important signalling pathways in immune responses, and several recent studies reported that suppression of MAPKs in mast cells can be an appropriate target for pharmacological treatment of inflammatory disorder[7,28]. The MAPK pathway is also required for the expression of inflammatory mediator genes, including COX-2, iNOS, IL-1 β , and TNF- α . It was reported that ERK and/or p38 MAPK are involved in up-regulation of IL-1 β and IL-8 production[4,9], and p38 MAPK inhibitor suppressed TNF- α and IL-6 production in

monocytic or mast cells[6,8]. Inhibition of p38 MAPK and ERK also attenuated COX-2 activity[29]. In addition, ERK, p38, and JNK are known to participate in the pathway of iNOS expression[30]. The effect of EC on the activation of MAPK members in selected cell lines has also been reported. Dieckol isolated from EC inhibited LPS-induced activation of p38 MAPK pathway in microglial cells[31]. However, the regulation of MAPK signalling pathway in mast cells has not been reported. Therefore, the inhibitory effects of EC on the intracellular signalling network in pro-inflammatory cytokine expression by activation of MAPKs were examined in HMC-1 cells. EC dose-dependently blocked the activation of ERK1/2 induced by PMACI, suggesting that the anti-inflammatory effect of EC, at least in part, might be derived through regulation of the MAPK (ERK) pathway. Although EC attenuated ERK activation, the effects of EC on other pathway-involved MAPK and its upstream/down stream are not elucidated in the present study and remain to be clarified.

In conclusion, this study clearly demonstrated that EC exerts significant anti-inflammatory potential due to activated HMC-1 cells through the reduction of NO production by suppression of iNOS and COX-2 production as well as pro-inflammatory cytokines, including IL-1 β and TNF- α , at the transcriptional level. In addition, EC also inhibits the activation of the ERK/MAPK pathway. These results suggest that EC could contribute the prevention or treatment of mast cell-mediated inflammatory diseases.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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