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Ecklonia cava-derived Polysaccharide Prevent Hydro Peroxide-induced Oxidative Stress and Neurotoxicity in Human Microglial HMO6 Cells

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Abstract In the present study, we examined the beneficial effect of *Ecklonia cava*-derived polysaccharide (ECAP), which is residual product after bioactive polyphenol isolation form *Ecklonia cava*, on neurotoxicity and oxidative stress in hydro peroxide-treated human microglia HMO6 cells. We sought that ECAP inhibited the expression of p53 and Bcl-2 in hydro peroxide-induced HMO6 cells. ECAP dramatically suppressed the cleaved form of caspase-3 in hydro peroxide-treated HMO6 cells. Moreover, we observed that hydro peroxide stimulated the expression of glucose-6-phosphate dehydrogenase (G6PDH) and its downstream target NADPH oxidase 4 (NOX4) in HMO6 cells. In contrast, ECAP suppressed the expression of G6PDH and NOX4 in hydro peroxide-treated HMO6 cells. We also observed that anti-oxidant enzymes including superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), and catalase were stimulated by ECAP to protect the neurotoxicity in hydro peroxide-treated HMO6 cells. Taken together, we demonstrated that ECAP may inhibit hydro peroxide-mediated neurotoxicity through the regulation of p53 and Bcl-2 genes in HMO6 cells and ameliorate oxidative stress in hydro peroxide-treated HMO6 cells. Therefore we suggest that ECAP may hold the potential to prevent the incidence of neuronal damage-mediated neurodegenerative diseases.

Keywords: *Ecklonia cava, seaweed, polysaccharide, neurotoxicity, oxidative stress, microglia, HMO6, neuroprotection*

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1. Introduction

Oxidative stress cause many neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD), via changes in the levels of cytokine, neuronal cells loss, and microglial damage [1,2,3]. One of the central theories is that the chronic microglial activation promote to initiate the reactive oxygen species (ROS)-mediated neuronal degeneration and contribute to incidence of AD, PD, and HD [4,5].

Excessive amount of ROS is derived from NADPH oxidase in cells [6]. NADPH oxidase catalyzes the production of super oxide from two oxygen atoms, and it is the predominant source of extracellular ROS in microglia cells [7]. NADPH oxidase belong to the NOX family, which include NOX1, NOX2, NOX3, NOX4, and NOX5 [8]. In particular, NOX4 cooperates with glucose 6-phosphate dehydrogenase (G6PDH) lead to constitutive generation ROS production in microglia cells [9], which indicates that abnormality of ROS state is involved in the stimulation of neurodegenerative diseases such as AD, PD, and HD [10]. It has been suggested that edible plant derived polysaccharide suppress ROS production, which in turn ameliorates hydro peroxide- and chemical-mediated neurotoxicity through the regulation of multiple antioxidant enzymes superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) [11,12,13].

Ecklonia cava (*E. cava*) is one of the famous sea foods in the Asia-Pacific region [14]. Recently, it has been reported that *E. cava*-derived polyphenol including dieckol and seapolynol have the activity of ROS scavenging, anti-adipogenesis, and anti-inflammation [15,16]. However, it has not been evaluate whether *E. cava*-derived polysacharide, which is residual product after dieckol and seapolynol extraction from *E. cava*, has beneficial effect on neuroprotection in human cells. In the current study, we therefore determined whether *E. cava*-derived polysaccharide (ECAP) has beneficial effect on hydro peroxide-induced neurotoxicity in human microglia HMO6 cells.

2. Materials and Methods

2.1. Materials

E. cava-derived polysaccharide was obtained from Milaeml (St. Ogeum, Seoul, South Korea). Human

microglial HMO6 cells were kindly provided Dr. Byong-Hak Kim (Seoul National University, Seoul, South Korea). Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin (P/S), trypsin ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco (Grand Island, NY, USA). Hydro peroxide and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Phosphate-buffered saline (PBS) was purchased from RMS Bio-solution (Seoul, South Korea). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). Phosphatase inhibitor cocktails I and II were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Bio-Rad Protein Assay was purchased from Bio Rad, Inc. (Hercules, CA, USA). Antibodies against G6PDH, NOX4, SOD1, SOD2, catalase, and α-tubulin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). B-cell lymphoma 2 (Bcl-2), and cleaved caspase-3 were purchased from Cell Signaling Technologies (Danvers, MA, USA). Antibody against p53 from Abcam (Cambridge, MA, USA) was also used in our experiments.

2.2. Cell Culture

HMO6 cells were cultured and maintained in DMEM medium containing 3.7 g/L sodium bicarbonate, 1 % P/S, and 10 % FBS at 37°C in 5 % CO_2. HMO6 cells were seeded in 6 well plate (1×10^6 cells/well) and pretreated with 0, 3, and 6 μg/mL dried ECAP for 6 hours, and then stimulated with hydro peroxide at 500 μM for 6 hours. ECAP was dissolved in DMSO at a stock solution of 100 mg/mL, and was diluted to indicate concentrations with DMSO. These samples or vehicle were added to the media at volume percent concentration of 0.5 %.

2.3. RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from HMO6 cells using TRIzol reagent according to the manufacturer's protocol. Total RNA (1 μg) was used to produce cDNA with a Maxime RT PreMix KIT (iNtRON Biotechnology, Inc, Gyeonggi, South Korea). Target gene amplification was performed using specific oligonucleotide primers in a normal PCR system. The primers which we used in this study are shown in Table 1. PCR products were electrophoresed on 1.5 % (v/v) agarose gels, stained with ethidium bromide.

2.4. Western Blot Analysis

Cells were washed using PBS and lysed by lysis buffer (add as phosphatase inhibitor cocktail 2, phosphatase inhibitor cocktail 3). The protein content was determined using the Bradford assay. Protein extracts (35 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio Rad, Inc, Hercules, CA, USA). Membranes were immunoblotted with primary antibodies specific for indicated proteins at 4°C overnight. Membranes were then treated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000) for 1 hour. Bands were visualized using an enhanced chemiluminescence system (ECL, Thermo Fisher Scientific, MA, USA) and LAS image software (Fuji, New York, NY, USA).

3. Results and Discussion

3.1. *E. cava*-derived Polysaccharide (ECAP) on the Neurotoxicity in Hydro Peroxide-induced HMO6 Cells

We conducted the cell viability assay to analyze the cytotoxicity of HMO6 cells treated with ECAP for 6 hours. ECAP was not toxic up to 6 μg/mL in HMO6 cells (data not shown). Therefore, the concentration of 0, 3, and 6 μg/mL of ECAP were selected for the further examination. Hydro peroxide causes the apoptosis-mediated neurotoxicity via the regulation of p53 dependent signaling pathway in microglial cells [17]. Therefore, we next performed the examination whether ECAP regulates hydro peroxide-induced apoptosis-related genes in HMO6 cells. As shown in Figure 1A and 1B, hydro peroxide stimulated the expression of p53 in HMO6 cells compared to the corresponding control. Whereas ECAP dramatically suppressed the expression of p53 mRNA and protein in hydro peroxide-treated HMO6 cells. We also observed that hydro peroxide increased the cleaved caspase-3 in HMO6 cells. However, the cleaved caspase-3 was attenuated in hydro peroxide-treated HMO6 cells with presence of ECAP.

Table 1. Primers used in this study

Name	Forward (5' to 3')	Reverse (5' to 3')
SOD1	AGGGCATCATCAATTTCGAG	ACATTGCCCAAGTCTCCAAC
SOD2	TCCACTGCAAGGAACAACAG	TCTTGCTGGGATCATTAGGG
G6PDH	GCACAAGCTTCAGGTCTTCC	GAACAAGATCCGAGCGTAGC
NOX4	CTTCCGTTGGTTTGCAGATT	TGGGTCCACAACAGAAAACA
Catalase	AGCTTAGCGTTCATCCGTGT	TCCAATCATCCGTCAAAACA
p53	GCCCAACAACACCAGCTCCT	CCTGGGCATCCTTGAGTTCC
Bcl-2	CTGCACCTGACGCCCTTCACC	CACATGACCCCACCGAACTCAAAGA
Caspase-3	ACATGGCGTGTCATAA AATACC	CACAAAGCGACTGGATGAAC
18S	CTACCACATCCAAGGAAGGC	CTCGGGCCTGCTTTGAACAC

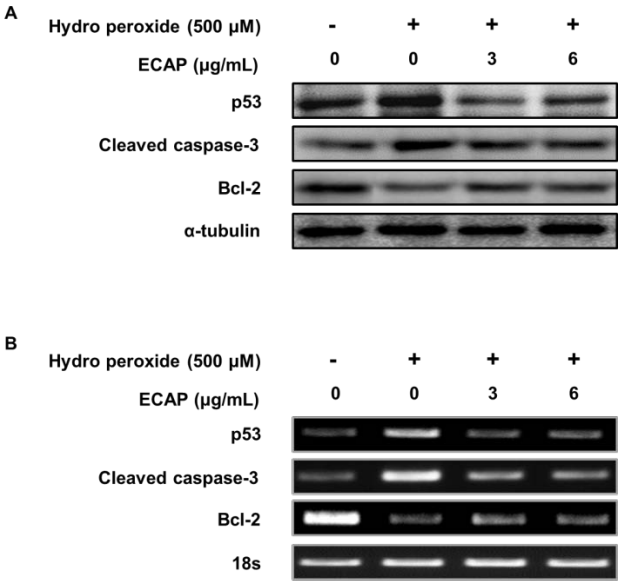

Figure 1. Effect of ECAP on cellular markers of apoptosis in HMO6 cells. Cells were treated with indicated concentrations of ECAP for 6 hours before treatment with 500 μM hydro peroxide for 6 hours. (A) Western blot analysis for detecting the levels of p53, cleaved caspase-3, and Bcl-2. (B) The expression levels of p53, caspase-3, and Bcl-2 mRNA were assessed by RT-PCR

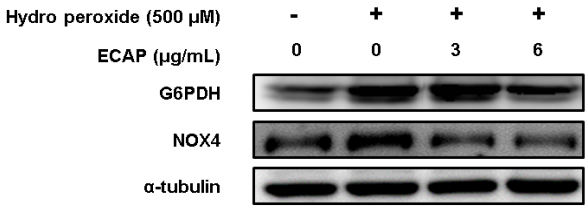

Figure 2. ECAP regulates hydro peroxide-mediated oxidative stress-associated proteins and mRNA levels in HMO6 cells. (A) Western blot were performed by incubating total lysates were with G6PDH, NOX4, and α-tubulin specific antibodies. (B) The expression levels of NOX4, G6PDH, and α-tubulin mRNA were evaluated by RT-PCR.

Bcl-2 is normally expressed in human microglia cells [19] and has been reported a key regulator of apoptosis as a downstream of p53 gene [20]. It has been showed that hydro peroxide treatment markedly increase apoptosis via inhibit the expression of Bcl-2 in neuronal and normal cells [21,22]. Previous report showed that polysaccharide isolated from *E. cava* inhibits radiation-mediated cellular damage via the up-regulation of Bcl-2 in splenocyte [23]. Our data showed that hydro peroxide suppressed the expression of Bcl-2 mRNA and protein in HMO6 cells. Whereas ECAP enhanced the expression of Bcl-2 in hydro peroxide-treated microglia HMO6 cells. These results indicated that ECAP may prevent hydro peroxide-mediated neuronal cell apoptosis and neurotoxicity through the regulation of p53 and Bcl-2 in HMO6 cells.

3.2. *E. cava*-derived Polysaccharide (ECAP) Stimulates the Expression of Antioxidant Enzymes in Hydro Peroxide-treated HMO6 Cells

To determine the effect of ECAP on oxidative stress, HMO6 cells were stimulated to oxidative stress with hydro peroxide in the absence of presence of ECAP for 6 hours. The effect of ECAP on the anti-oxidative stress (G6PDH, NOX4) of microglia HMO6 cells were measured by western blot analysis. As shown in Figure 2, we observed that ECAP reduced the expression of G6DPH and its downstream target NOX4 in hydro peroxide-treated HMO6 cells.

Previous studies have suggested that increasing the activities of anti-oxidant enzymes such as SOD1, SOD2, and catalase have a protective property against oxidative stress in neurodegenerative disease [24]. It have been shown that seaweed-derived polysaccharide prevent oxidative stress through the regulation of anti-oxidant enzymes [25,26]. To explore the possible anti-oxidant activity of ECAP, microglia HMO6 cells were cultured with 0, 3, and 6 μg/mL of ECAP for 6 hours followed by 6 hours of hydro peroxide treatment. As shown in Figure 3A, Hydro peroxide inhibited the expression of SOD1, SOD2, and catalase mRNA in HMO6 cells. In contrast, ECAP in concentration of 6 μg/mL dramatically increased the mRNA expression of SOD1, SOD2, and catalase by 2.34, 1.36, and 3.13 folds, respectively, compared to HMO6 cells treated with hydro peroxide. We also observed that hydro peroxide greatly suppressed the protein expression of SOD1, SOD2, and catalase in HMO6 cells. Consistent with previous observation, ECAP prevented the hydro peroxide-medicated changes of anti-oxidant enzymes expression in HMO6 cells as shown in Figure 3B, revealing that hydro peroxide promoted the oxidative stress through the altering the expression of antioxidant enzymes SOD1, SOD2, and catalase, while ECAP stimulated the expression of antioxidant enzymes and subsequently prevented hydro peroxide-mediated oxidative stress in HMO6 cells.

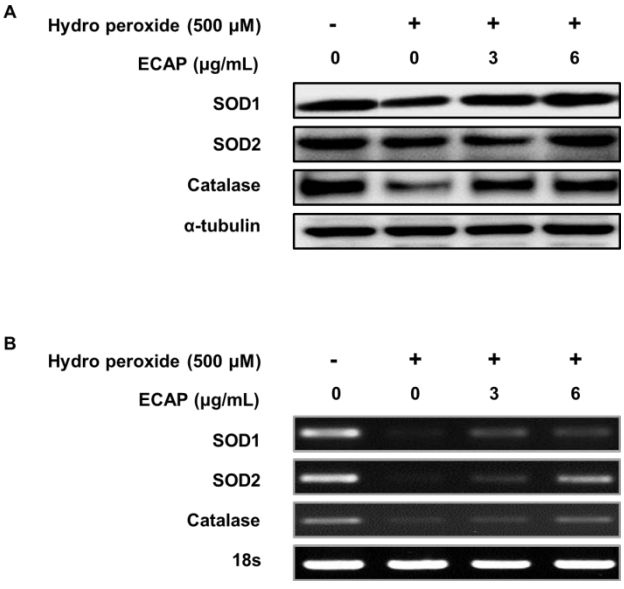

Figure 3. ECAP suppressed the hydro peroxide-mediated alteration of anti-oxidant enzymes including SOD1, SOD2, and catalase in HMO6 cells. Total cellular protein was extracted and subjected to SDS-PAGE. (A) Western blots are shown for antioxidant enzymes such as SOD1, SOD2, and catalase. (B) The expression levels of SOD1, SOD2, and catalase mRNA were evaluated by RT-PCR

4. Conclusion

Taken together, we here showed that ECAP repress hydro peroxide-mediated neurotoxicity through the regulation of p53 and Bcl-2 genes in microglia HMO6 cells. Moreover, ECAP ameliorated oxidative stress by increasing SOD1, SOD2, and catalase in hydro peroxide-treated HMO6 cells. These finding suggested that ECAP may act as a potential natural ingredient to prevent the incidence of neuronal damage-medicated neurodegenerative diseases.

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Conflict of Interest

The authors declare no conflict of interest.

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