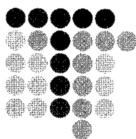

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Alaria esculenta Extract Protects against Oxidative Damage by Inducing Heme Oxygenase-1 Expression via Akt and Nrf2

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Abstract

Alaria esculenta is a brown seaweed found in the Arctic. This study investigated the protective effect of *A. esculenta* extract (AEE) against oxidant-mediated injury and its mode of action in RAW264.7 macrophages. The methyl thiazolyl tetrazolium (MTT) assay showed that H_2O_2 treatment reduced cell viability, whereas AEE protected cells from H_2O_2-mediated cytotoxicity in a dose-dependent manner. Because heme oxygenase-1 (HO-1) is known to protect cells against oxidative damage, we investigated the effect of AEE on *HO-1* gene expression and HO enzyme activity. The protective effect of AEE against H_2O_2-induced injury was correlated with increased HO enzyme activity. AEE also induced *HO-1* mRNA and protein expression, as determined RT-PCR and Western blotting, respectively. To characterize the mechanisms by which AEE induces *HO-1* gene expression, we examined the effect of AEE on the nuclear translocation of NF-E2-related factor-2 (Nrf2) and Akt phosphorylation. AEE treatment activated upstream signaling for *HO-1* gene expression, including the nuclear translocation of Nrf2 and Akt phosphorylation. Collectively, these results suggest that AEE has antioxidant activity that is mediated, at least in part, via the activation of Nrf2 and Akt and the subsequent induction of *HO-1* gene expression.

Keywords: *Alaria esculenta*, Heme Oxygenase-1, Nrf2, Akt

Reactive oxygen species (ROS), including superoxide radical, hydroxyl radical, and hydrogen peroxide, are generated in aerobic cells during normal metabolism. Oxidative stress can occur as a consequence of ROS/antioxidant imbalance via an overall increase in ROS generation, the depression of antioxidant systems, or both. An increase in intracellular ROS levels can cause cellular damage by oxidizing proteins, nucleic acids, and membrane lipids, and oxidative stress has been implicated in the pathogenesis of various diseases, including cancer, neurodegenerative disorders, inflammation, diabetes, and aging[1-3]. Therefore, it is valuable to identify agents with the ability to prevent oxidative stress-induced injury.

When cells are exposed to oxidative stress, antioxidative defense systems are induced to protect the cells against the stress. One of these systems is the induction of heme oxygenase (HO), which is the rate-limiting enzyme in the oxidative degradation of heme into bilirubin, iron, and carbon monoxide. While the heme oxygenases HO-2 and HO-3 are expressed constitutively, HO-1 expression is inducible[4]. Recent studies have demonstrated that HO-1 induction is an adaptive response that confers enhanced resistance to various oxidative stresses[5,6]. The expression of the *HO-1* gene is regulated via the phosphatidylinositol 3-kinase (PI3K) /Akt pathway and the NF-E2-related factor-2 (Nrf2) transcription factor[7,8].

Alaria esculenta is a brown alga known as dabberlocks or winged kelp that occurs mainly in the Arctic, including Greenland, Iceland, Norway, and Alaska. It is used for a variety of purposes, ranging from human consumption and alginate production to fodder and body care products[9]. It is rich in sugars, proteins, vita-

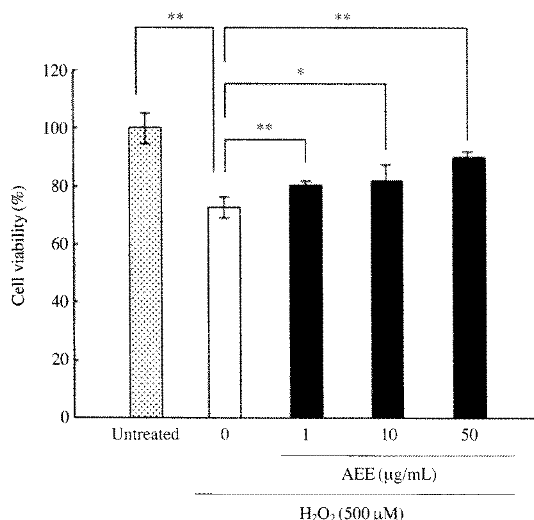

Figure 1. AEE protects against H_2O_2-induced oxidative damage in RAW264.7 cells. RAW264.7 cells (1×10^6 cells/mL) were pre-treated with vehicle (0.1% DMSO) or AEE (1, 10, or 50 μg/mL) for 1 h, and then treated with H_2O_2 (500 μM) for 18 h. Cell viability was measured using the MTT assay, as described in the Materials and Methods. Unstimulated cells were designated as having 100% viability. The values are presented as the mean ± standard deviation (SD) for triplicate samples from each group. $^{*}P<0.05$ and $^{**}P<0.01$, as determined using Dunnett's t-test and compared to the vehicle control group.

mins, and other trace metals and contains up to 42% alginic acid, although the physiological and pharmacological activities of the alga are unknown[9]. This study investigated the protective effects of *A. esculenta* extract (AEE) against oxidative stress-induced injury. We also examined the underlying protective mechanism of AEE relative to *HO-1* mRNA expression and subsequent enzyme activity in RAW264.7 macrophages.

Protective Effect of AEE against H_2O_2-induced Cytotoxicity in RAW264.7 Macrophages

To investigate the protective effect of AEE on H_2O_2-induced cell death, we examined cell viability using the methyl thiazolyl tetrazolium (MTT) assay. The cell survival rate decreased after treatment with H_2O_2 (500 μM), whereas pre-treatment with AEE (1, 10, or 50 μg/mL) protected RAW264.7 cells against oxidative damage. The protective effect of AEE against H_2O_2-induced cellular toxicity was dose-dependent (Figure 1).

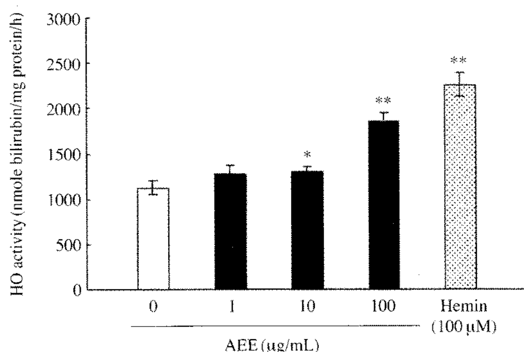

Figure 2. Effect of AEE on HO enzyme activity in RAW-264.7 cells. RAW264.7 cells (1×10^6 cells/mL) were treated with vehicle (0.1% DMSO), AEE (1, 10, or 100 μg/mL), or hemin (100 μM) for 18 h. HO enzymes were isolated and HO activity was measured, as described in the Materials and Methods. The values are presented as the mean ± standard deviation (SD) for triplicate samples from each group. $^{*}P<0.05$ and $^{**}P<0.01$, as determined using Dunnett's t-test and compared to the vehicle control group.

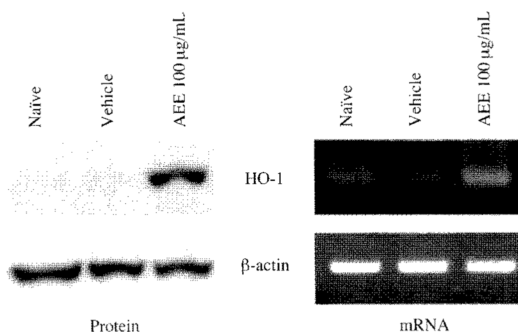

Figure 3. Effects of AEE on the expression of HO-1 protein and mRNA in RAW264.7 cells. RAW264.7 cells (1×10^6 cells/mL) were treated with vehicle (0.1% DMSO) or AEE 100 μg/mL. (A) After 18 h, the whole-cell lysates were isolated and analyzed using Western blot analysis. (B) After 6 h, total RNA was isolated and *HO-1* mRNA expression was determined using RT-PCR. The results presented are representative of three independent experiments.

AEE-induced *HO-1* Gene Expression and Enzyme Activity in RAW264.7 Macrophages

Because the *HO-1* gene contributes to enhanced resistance to oxidative stress[5], the effect of AEE on HO enzyme activity was examined in RAW264.7 cells. AEE treatment significantly increased HO enzyme activity in a dose-dependent manner, as shown in Figure 2. Hemin, a *HO-1* inducer, also strongly induced HO enzyme activity. The effect of AEE on the produc-

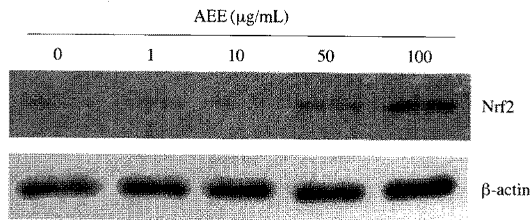

Figure 4. Effect of AEE on the nuclear translocation of Nrf2 in RAW264.7 cells. RAW264.7 cells (1×10^6 cells/mL) were treated with vehicle (0.1% DMSO) or AEE (1, 10, 50, or 100 μg/mL) for 2 h. Nuclear proteins from the cells were subjected to Western blot analysis using anti-Nrf2 antibody. The blots were re-probed with anti-β-actin antibody as a loading control. The results are representative of two separate experiments.

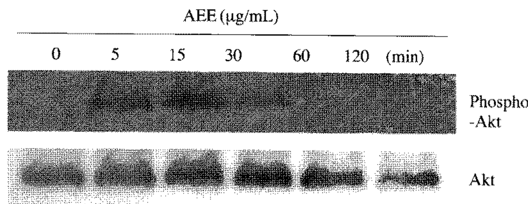

Figure 5. Effect of AEE on the phosphorylation of Akt in RAW264.7 cells. RAW264.7 cells (1×10^6 cells/mL) were treated with vehicle (0.1% DMSO) or AEE 100 μg/mL for various times (5, 15, 30, 60, or 120 min). Whole-cell lysates were analyzed using Western blot analysis with anti-phospho-Akt antibody. The blots were re-probed with anti-Akt antibody as a control. The results are representative of two experiments.

tion of HO-1 protein was determined by Western blot analysis. The expression of the HO-1 protein was barely detectable in naive RAW264.7 cells or vehicle-treated cells, but increased markedly after 18 h of AEE (100 μg/mL) treatment (Figure 3). In addition, the effects of AEE on *HO-1* mRNA expression were evaluated using RT-PCR. RAW264.7 cells were harvested and mRNA was isolated after a 6-h incubation with 100 μg/mL AEE. AEE treatment significantly increased the *HO-1* mRNA level, which was consistent with the Western blot analysis (Figure 3).

Involvement of Nrf2 Nuclear Translocation in AEE-induced *HO-1* Expression

Under normal conditions, Nrf2 is sequestered in the cytoplasm by Keap1 protein. The translocation of Nrf2 into the nucleus is essential for the transactivation of various target genes, including *HO-1*[7,10]. To determine whether the upregulation of *HO-1* expression is mediated by Nrf2, we examined the effect of AEE on the nuclear translocation of Nrf2 using Western blot analysis. As shown in Figure 4, the Nrf2 protein level in the nucleus increased in response to treatment with AEE in a dose-dependent manner.

AEE induced Akt Phosphorylation

The protein kinase Akt is involved in the activation of Nrf2 and the upregulation of several antioxidant genes, including *HO-1*[11,12]. Because the Akt-mediated phosphorylation of Nrf2 facilitates the nuclear translocation of Nrf2, we examined the effects of AEE on the Akt signaling pathway. A transient increase in Akt phosphorylation was observed in AEE-treated RAW264.7 cells, whereas the level of unphosphorylated Akt remained unchanged (Figure 5).

Discussion

Oxidative stress refers to disrupted redox equilibrium between the production of ROS and the ability of cells to protect against damage caused by these molecules. ROS-antioxidant imbalance can damage cellular components directly and disturb cellular function, both of which are implicated in the pathogenesis of various diseases. Therefore, compounds that prevent oxidative stress-induced injury may have many potential medical applications.

This study investigated the involvement of HO-1 in the antioxidant activity of *A. esculenta*, an Arctic seaweed. We demonstrated that AEE has protective activity against H_2O_2-induced cytotoxicity, which was mediated, at least in part, via the induction of *HO-1* gene expression and subsequent enzyme activity in RAW264.7 macrophages. We also found that the nuclear translocation of Nrf2 and Akt phosphorylation were involved in AEE-induced *HO-1* gene expression.

Several groups have demonstrated that HO-1 participates in the mechanism that protects cells against oxidative damage. Soares *et al.*[13] showed that the overexpression of *HO-1* in cells resulted in a marked reduction in oxidative stress-induced injury and cytotoxicity. Furthermore, *HO-1* null mice and *HO-1*-deficient humans show a reduced host defense against oxidative stress[14,15]. We found that AEE decreased H_2O_2-induced cytotoxicity and induced HO-1 protein production and enzyme activity in RAW264.7 cells. Therefore, it is possible that the induction of *HO-1* expression is one of the mechanisms responsible for the antioxidant activity of AEE. However, we cannot exclude the possibility of other mechanisms, such as the induction of other phase II detoxifying and antioxidant enzymes, including glutathione S-transferase (GST) and

NAD(P)H:quinone oxidoreductase (NQO1). Further studies are required to clarify this issue.

Nrf2 plays an important role in the upregulation of antioxidants and detoxifying enzymes, including HO-1 and GST[7,16]. The expression of these genes is regulated by a *cis*-acting element present in the promoter, the antioxidant response element (ARE), and Nrf2 is involved in the activation of ARE[7,10]. Under normal conditions, Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) protein, and must be translocated into the nucleus for the transactivation of various target genes[7,10]. In our study, the nuclear level of Nrf2 increased in RAW264.7 cells after treatment with AEE. Therefore, it is possible that the induction of *HO-1* gene expression by AEE is due to the increased nuclear translocation of Nrf2. Because Nrf2 regulates the gene expression of other antioxidant and detoxifying enzymes[7,16], these results should be considered in future investigations into the role of AEE in the regulation of GST and NQO1.

One of the most plausible mechanisms for the activation of Nrf2 involves the phosphorylation of Nrf2 by protein kinases, which promotes the nuclear translocation of Nrf2 and subsequent ARE binding. Several reports demonstrated that *HO-1* expression and Nrf2 nuclear translocation were correlated with Akt signaling[8,12,17]. In this study, AEE induced Akt phosphorylation, suggesting that the Akt signaling pathway is involved in AEE-induced Nrf2 activation and the subsequent expression of *HO-1* in RAW264.7 cells. Many cell surface receptors induce the production of second messengers that activate PI3K, which may then lead to the downstream activation of Akt. Therefore, investigation into the role of upstream signaling molecules, such as PI3K, is required to further elucidate the AEE-mediated regulation of *HO-1* gene expression.

In summary, this study demonstrated that AEE protects RAW264.7 cells against oxidative stress-induced damage, and that the effect is mediated, at least in part, via the induction of HO-1 protein expression and enzyme activity. In addition, AEE induced Akt phosphorylation and the nuclear translocation of Nrf2, which may play critical roles in *HO-1* expression. These findings imply that AEE is a potential natural antioxidant, and further studies are required to define the functional compounds in the extract that regulate Akt-Nrf2 signaling and subsequent *HO-1* induction.

Materials & Methods

Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. *A. esculenta* was collected from July to August 2005 at Kongsfjorden, in the Svalbard Islands. The taxonomic identification of seaweed specimens was confirmed by an alga taxonomist, J. H. Kim, at the Korea Polar Research Institute (KOPRI). Shade-dried seaweed was extracted using a volumetrically equal mixture of acetone and methylenechloride for 24 h at room temperature. This step was repeated twice. The extracted solution was then evaporated to dryness under vacuum, yielding a dark, sticky residue. Then, the residue was extracted once more with methanol, using the same procedure. Each of the crude extracts was combined and used as experimental material. The prepared samples were stored in a refrigerator at −20°C, for later study. The combined crude extract of *A. esculenta* was reconstituted in dimethylsulfoxide (DMSO) and aliquots were stored at −80°C. Working solutions were prepared immediately before adding to cell cultures.

Cell Culture

RAW264.7 cells (ATCC TIB71) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 5% fetal bovine serum. All cells were cultured at 37°C and 5% CO_2 in a humidified incubator.

Cell Viability Assay

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[18]. RAW264.7 cells (1×10^6 cells/mL) were pre-treated with vehicle (0.1% DMSO) or various concentrations of AEE for 1 h, and then treated with H_2O_2 (500 μM) for 18 h. The cells were rinsed and incubated with MTT (2 mg/mL) for 4 h. The reagent was discarded and the formazan blue that formed in the cells was dissolved with DMSO. The absorbance at 540 nm was measured in a microplate reader. The absorbance of the formazan formed in untreated cells was considered to represent 100% viability.

HO Activity Assay

The method used for determining HO activity via bilirubin formation was according to Pae *et al.*[19]. Briefly, RAW264.7 macrophages were plated at 1×10^6 cells/mL and treated with various concentrations of AEE for 18 h. After incubation, the macrophages were washed twice with phosphate-buffered saline (PBS), gently scraped off the dish, and centrifuged (10,000 × g, 5 min, 4°C). The cell pellet was suspended in 2 mM $MgCl_2$ phosphate buffer (100 mM, pH 7.4), frozen at −70°C, thawed three times and finally sonicated on

ice before centrifugation at 13,000 rpm for 15 min at 4°C. The supernatant (400 μL) was added to a NADPH-generating system containing 0.8 mM NADPH, 2 mM glucose-6-phosphate, 0.2 U glucose-6-phosphate-1-dehydrogenase, and 2 mg of rat liver cytosol protein as a source of biliverdin reductase, potassium phosphate buffer (100 mM, pH 7.4), and hemin (10 μM) in a final volume of 200 μL. The reaction was incubated for 1 h at 37°C in the dark and terminated by adding 600 μL of chloroform. The extracted bilirubin was calculated from the difference in light absorption between 464 and 530 nm wavelengths. HO activity was measured as nanomoles of bilirubin formed per milligram of macrophage cell protein per hour.

Western Immunoblot Analysis

Whole-cell lysates (40 μg for HO-1 and Akt) or nuclear extracts (40 μg for Nrf2) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). The membranes were pre-incubated for 1 h at room temperature in Tris-buffered saline (pH 7.6) containing 0.05% Tween-20 and 3% fatty acid-free bovine serum albumin. Then, the nitrocellulose membranes were incubated with specific antibodies against HO-1 (Santa Cruz Biotechnology, Santa Cruz, CA), Nrf2 (Cell Signaling Technology, Beverly, MA), and the phosphorylated form of Akt (Cell Signaling Technology). Immunoreactive bands were detected by incubation with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Cell Signaling Technology) and the bands were visualized using the ECL system (Amersham Biosciences).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RAW264.7 cells (1×10^6 cells/mL) were pre-treated with vehicle (0.1% DMSO) or 100 μg/mL AEE for 6 h. After treatment, the cells were harvested, and total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH). Total RNA was reverse-transcribed into cDNA using oligo $(dT)_{15}$ as primers. The following forward and reverse primer sequences were used: for *HO-1*, 5′-TGAAGGAGGCCACCAAGGAGG-3′ and 5′-AGAGGTCACCCAGGTAGCGGG-3′; for *β-actin*, 5′-AGAGGGAAATCGTGCGTGAC-3′ and 5′-CAATAGTGATGACCTGGCCGT-3′. A PCR master mixture consisting of PCR buffer, 4 mM $MgCl_2$, 6 pmole each of *HO-1* forward and reverse primers, and 2.5 U of *Taq* DNA polymerase was added to the cDNA samples. Then, the samples were heated to 94°C for 5 min and cycled 25 times at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by an additional 5-min extension at 72°C. The PCR products were electrophoresed in 1% agarose gels and visualized using ethidium bromide staining.

Statistical Analysis

The mean ± standard deviation (SD) was determined for each treatment group in the individual experiments. Homogeneous data were evaluated using a parametric analysis of variance, and Dunnett's two-tailed *t*-test was used to compare treatment groups with the vehicle control when significant differences were observed[20].

Acknowledgements

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