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Fucoxanthin Protects Cultured Human Keratinocytes against Oxidative Stress by Blocking Free Radicals and Inhibiting Apoptosis

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Abstract

Fucoxanthin is an important carotenoid derived from edible brown seaweeds and is used in indigenous herbal medicines. The aim of the present study was to examine the cytoprotective effects of fucoxanthin against hydrogen peroxide-induced cell damage. Fucoxanthin decreased the level of intracellular reactive oxygen species, as assessed by fluorescence spectrometry performed after staining cultured human HaCaT keratinocytes with 2',7'-dichlorodihydrofluorescein diacetate. In addition, electron spin resonance spectrometry showed that fucoxanthin scavenged hydroxyl radical generated by the Fenton reaction in a cell-free system. Fucoxanthin also inhibited comet tail formation and phospho-histone H2A.X expression, suggesting that it prevents hydrogen peroxide-induced cellular DNA damage. Furthermore, the compound reduced the number of apoptotic bodies stained with Hoechst 33342, indicating that it protected keratinocytes against hydrogen peroxide-induced apoptotic cell death. Finally, fucoxanthin prevented the loss of mitochondrial membrane potential. These protective actions were accompanied by the down-regulation of apoptosis-promoting mediators (i.e., B-cell lymphoma-2-associated x protein, caspase-9, and caspase-3) and the up-regulation of an apoptosis inhibitor (B-cell lymphoma-2). Taken together, the results of this study suggest that fucoxanthin defends keratinocytes against oxidative damage by scavenging ROS and inhibiting apoptosis.

Key Words: Fucoxanthin, Carotenoid, Human keratinocyte, Oxidative stress, Apoptosis

INTRODUCTION

Fucoxanthin is an organic pigment, or carotenoid, found in the photosynthetic organs of edible brown seaweeds (Yan *et al.*, 1999). This compound has a unique structure that is characterized by an unusual allenic bond, a conjugated carbonyl group, an epoxide group, and an acetyl group (Mercadante and Egeland, 2004; Hu *et al.*, 2010). Fucoxanthin is used in indigenous herbal medicine to treat fever, urinary problems associated with swelling stomach ailments, and hemorrhoids (Khan *et al.*, 2007). The widespread application of fucoxanthin stems from its potent antioxidant activity (Sachindra *et al.*, 2007; Heo *et al.*, 2008; Heo and Jeon, 2009), anti-apoptotic activity (Das *et al.*, 2010), anti-obesity activity (Woo *et al.*, 2009), and anti-diabetic activity (Maeda *et al.*, 2005).

The antioxidant actions of fucoxanthin in cell-free systems include scavenging of the hydroxyl radical, the superoxide anion, singlet oxygen (Sachindra *et al.*, 2007; D'Orazio *et al.*, 2012), the 2,2-diphenyl-1-picrylhydrazyl radical, 12-doxyl-stearic acid, and the radical adduct of nitrobenzene with linoleic acid (Yan *et al.*, 1999). Moreover, fucoxanthin inhibits ultraviolet B (UVB)-mediated oxidative damage to human fibroblasts (Heo and Jeon, 2009) and hydrogen peroxide (H_2O_2)-mediated damage to monkey kidney fibroblasts (Vero line) (Heo *et al.*, 2008). In addition, fucoxanthin enhances heme oxygenase-1 and NAD(P)H dehydrogenase: quinone oxidoreductase-1 expression by activating the nuclear factor (erythroid-derived 2)-like 2/antioxidant response element pathway (Liu *et al.*, 2011).

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen, including the singlet oxygen, the hydroxyl radical (•OH), H_2O_2, and the superoxide anion

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($O_2^{\bullet-}$). ROS are natural byproducts of normal aerobic metabolism and play an important role in cell signaling and homeostasis (Devasagayam *et al.*, 2004). However, excessive ROS levels are a major cause of cell damage and cell death (Orrenius, 2007). In general, the harmful effects of ROS result in oxidatively-induced structural and functional alterations, not only to cellular components including bases in DNA, polyunsaturated fatty acids in lipids, amino acids in proteins, and the non-protein co-factors of certain enzymes (D'souza *et al.*, 2012; Licandro *et al.*, 2013; Sinha *et al.*, 2013), but also to components of the extracellular matrix (Bottai *et al.*, 2012). Furthermore, abnormally high intracellular ROS levels can cause pathological conditions, such as inflammation, atherosclerosis, diabetes, aging, and carcinogenesis (Loeb *et al.*, 2005; Schumacker, 2006).

ROS generated by UVB radiation participate in the development of numerous cutaneous diseases and disorders (e.g., skin cancer, photoaging, and oxidative DNA damage in skin cells) (Fuchs *et al.*, 1989; Emerit, 1992). H_2O_2 is a major ROS generated by UVB; however, it is subsequently dissociated by exposure to ultraviolet radiation. Dissociated H_2O_2 forms hydroxyl radicals in the epithelium (Cadenas and Davies, 2000; Sander *et al.*, 2002). Hydroxyl radicals are highly reactive and destructive substances, which are largely responsible for the DNA damage and subsequent death that occurs in irradiated skin cells (Spencer *et al.*, 1995). It is therefore suggest that powerful antioxidants can exert preventive and/or therapeutic effects against ultraviolet radiation/ROS-mediated DNA injury (Arranz *et al.*, 2007; Plazar *et al.*, 2007). So far, there has been very little research on the keratinocyte-protective effects of fucoxanthin against oxidative stress. Therefore, the current study explored the utility of fucoxanthin as a new protectant against H_2O_2-induced oxidative stress in cultured human HaCaT keratinocytes.

MATERIALS AND METHODS

Reagents

The N-acetyl cysteine (NAC), 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) bromide (MTT), and Hoechst 33342 dye were purchased from Sigma Chemical Company (St. Louis, MO, USA). Fucoxanthin was purchased from Santa Cruz Biotechnolgy Inc (Santa Cruz, California, USA). All other chemicals and reagents were of analytical grade.

Cell culture

Human keratinocyte cell line HaCaT was obtained from the Amore Pacific Company (Gyeonggi-do, Republic of Korea) and maintained at 37°C in an incubator with a humidified atmosphere of 5% CO_2. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 μg/ml) and penicillin (100 units/ml).

Detection of intracellular ROS

The DCF-DA method was used to detect intracellular ROS levels generated by H_2O_2 oxidation in HaCaT keratinocytes (Rosenkranz *et al.*, 1992). To detect ROS in H_2O_2-treated HaCaT cells, the cells were seeded in plates at a density of 1.0 ×10^5 cells/well. Sixteen hours later, they were treated with fucoxanthin at a concentration of 2.5, 5, 10, 20 or 40 μM. 2 mM of NAC, an antioxidant, was used as a positive control. After 30 minutes incubation at 37°C, H_2O_2 (1 mM) was added to the wells and the plates were again incubated for 30 min at 37°C, after which DCF-DA solution (25 μM) was added. Ten minutes later, the fluorescence of 2',7'-dichlorofluorescein (DCF) was detected and quantified using a PerkinElmer LS-5B spectrofluorometer (PerkinElmer, Waltham, MA, USA).

Cell viability assay

The effect of fucoxanthin on the viability of HaCaT cells was determined by using MTT assay, which is based on the reduction of a tetrazolium salt by mitochondrial dehydrogenase in viable cells. Cells seeded on a 96-well plate at a density of 1 ×10^5 cells/ml were treated 16 h later with 2.5, 5, 10, 20, 40, or 80 μM. After incubation of 16 h, MTT stock solution (50 μl, 2 mg/ml) was added to each well to yield a total reaction volume of 200 μl. Four hours later, the supernatants were aspirated. The formazan crystals in each well were dissolved in dimethyl sulfoxide (DMSO, 150 μl), and the absorbance at 540 nm was read on a scanning multi-well spectrophotometer (Carmichael *et al.,* 1987).

Detection of hydroxyl radical

Hydroxyl radical generated by the Fenton reaction (H_2O_2 + $FeSO_4$) were reacted with DMPO. The resultant DMPO/•OH adducts were detected using an ESR spectrometer (Li *et al.,* 2004). The ESR spectrum was recorded 2.5 min after phosphate-buffered saline (PBS, pH 7.4) was mixed with 0.2 ml each of 0.3 M DMPO, 10 mM $FeSO_4$, 10 mM H_2O_2, and fucoxanthin (20 μM). The ESR spectrometer parameters were: a magnetic field of 336.8 mT, power at 1.00 mW, a frequency of 9.4380 GHz, a modulation amplitude of 0.2 mT, gain at 200, a scan time of 0.5 min, a scan width of 10 mT, a time constant of 0.03 sec, and a temperature of 25°C.

Single-cell gel electrophoresis (Comet assay)

The degree of oxidative DNA damage was determined in a Comet assay (Rajagopalan *et al.*, 2003). The cell suspension was mixed with 75 μl of 0.5% low-melting agarose (LMA) at 39°C and the mixture was spread on a fully frosted microscopic slide pre-coated with 200 μl of 1% normal melting agarose (NMA). After solidification of the agarose, the slide was covered with another 75 μl of 0.5% LMA and then immersed in a lysis solution (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 1% Trion X-100, and 10% DMSO, pH 10) for 1 h at 4°C. The slides were subsequently placed in a gel electrophoresis apparatus containing 300 mM NaOH and 10 mM Na-EDTA (pH 13) for 40 min to allow for DNA unwinding and the expression of alkali-labile damage. An electrical field was then applied (300 mA, 25 V) for 20 min at 4°C to draw the negatively charged DNA towards the anode. The slides were washed three times for 5 min at 4°C in a neutralizing buffer (0.4 M Tris, pH 7.5), stained with 75 μl of propidium iodide (20 μg/ml) and observed under a fluorescence microscope and an image analyzer (Kinetic Imaging, Komet 5.5, UK). The percentage of the total fluorescence in the comet tails and the tail lengths of 50 cells per slide were recorded.

Western blot

Cells were harvested, washed twice with PBS, lysed on ice for 30 min in 100 μl of lysis buffer (120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP 40) and then centrifuged at 13,000×*g* for 15 min. The supernatants were collected from the lysates and the protein concentrations were determined. Aliquots of the lysates (40 μg of protein) were boiled for 5 minutes and electrophoresed in 10% sodium dodecylsulfate-polyacrylamide gel. The proteins in the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), which were then incubated with the primary antibodies. The membranes were subsequently incubated with the secondary immunoglobulin G-horseradish peroxidase conjugates (Pierce, Rockford, IL, USA). Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK), and then exposed to X-ray film.

Nuclear staining with Hoechst 33342

Cells were treated with fucoxanthin at a concentration of 20 μM or NAC (2 mM) and H_2O_2 (1 mM) was added into plate 1 h later. After an additional 24 h incubation at 37°C, the DNA-specific fluorescent dye Hoechst 33342 (1.5 μl of a 10 mg/ml stock) was added to each well and the cells were incubated for 10 min at 37°C. The stained cells were visualized under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera. The degree of nuclear condensation was evaluated and the apoptotic cells were quantified.

Effect of fucoxanthin on H_2O_2-induced $\Delta\psi_m$ depolarization

Cells were seeded in a 6-well plate at 1×10^5 cells/well. At 16 h after plating, the cells were treated with 20 μM of fucoxanthin, and 1 h later, 1 mM H_2O_2 was added to the plate. The cells were incubated for an additional 12 h at 37°C. After adding 2.5 μM of JC-1 solution for 30 min, $\Delta\psi_m$ was analyzed by flow cytometer after staining cells with JC-1 (Becton Dickinson, Mountain View, CA, USA).

Statistical analysis

All measurements were performed in triplicate and all values are expressed as the mean ± the standard error. The results were subjected to an analysis of variance (ANOVA) using Tukey's test to analyze differences between means. In each case, a $p<0.05$ was considered statistically significant.

RESULTS

Fucoxanthin attenuates ROS generation

The fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) was used to detect the ROS scavenging activity of fucoxanthin in HaCaT cells treated with H_2O_2. The fluorescence spectrometric data revealed that the intracellular ROS scavenging activity of fucoxanthin was 16% at 2.5 μM, 20% at 5 μM, 24% at 10 μM, 41% at 20 μM, and 52% at 40 μM. This may be compared with 74% for N-acetylcysteine (NAC) (2 mM), a well-known ROS scavenger used as the positive control (Fig. 1A). The results from the MTT (thiazoyl blue tetrazolium bromide) assay showed that fucoxanthin itself was not cytotoxic towards human HaCaT keratinocytes at concentrations up to 20 μM. However, the compound showed significant cytotoxicity at concentrations above 40 μM (Fig. 1B). Therefore, 20 μM was chosen as the optimal concentration for further study.

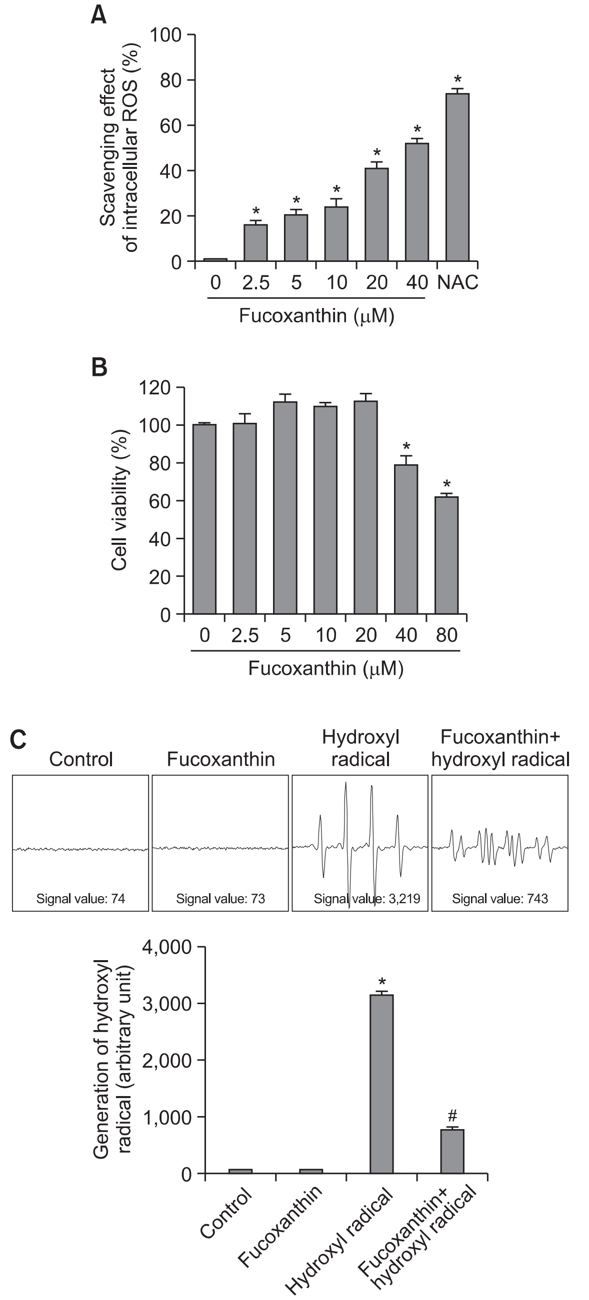

Fig. 1. Fucoxanthin attenuates ROS generation. (A) HaCaT cells were treated with fucoxanthin a concentration of 2.5, 5, 10, 20, or 40 μM, or with NAC at a concentration of 2 mM. H_2O_2 (1 mM) was added to the plate 1 h later. After incubating for 30 min, intracellular ROS were detected by spectrofluorometry after DCF-DA staining. *Indicates significantly different from the control ($p<0.05$). (B) Fucoxanthin was added to cells at a concentration of 0, 2.5, 5, 10, 20, 40, or 80 μM. Cell viability was determined after 16 h by the MTT assay. *Indicates significantly different from the control ($p<0.05$). (C) The hydroxyl radical generated by the Fenton reaction (H_2O_2+$FeSO_4$) was reacted with DMPO and the resulting DMPO/•OH adducts were detected by ESR spectrometry. The results are expressed as representative peak data. *Indicates significantly different from the control ($p<0.05$) and #Indicates significantly different from H_2O_2-treated cells ($p<0.05$).

We next investigated the scavenging effects of fucoxanthin against hydroxyl radical in a cell-free system using electron spin resonance (ESR) spectrometry. ESR analysis showed that the hydroxyl radical signal generated in the Fenton reaction (H_2O_2+$FeSO_4$) system increased to 3,219 compared to 74 of control, however, fucoxanthin treatment restricted the increase in the hydroxy radical signal to 743 (Fig. 1C).

Fucoxanthin reduces H_2O_2-mediated DNA damage

We next examined H_2O_2-mediated damage to HaCaT cell DNA using the alkaline comet assay and Western blotting analysis. The comet assay measures global DNA injury, including breaks in double stranded and single stranded DNA, as well as oxidative damage to DNA bases. Fig. 2A shows fluorescence microscopy images of nuclei and the percentage of cellular fluorescence in the comet tails. Treatment with H_2O_2 significantly increased comet parameters such as tail length (assessed by visual inspection) and the percentage of damaged DNA in the nuclear tails. As shown in Fig. 2A, H_2O_2 increased the fluorescence in the tail to 49% compared with ~5% in both the untreated control group and the fucoxanthin-treated group. However, the H_2O_2-treated cells with fucoxanthin led to a significant decrease in the percentage of DNA in the tails (to 27%).

The phosphorylation of nuclear histone H2A.X is an indication of breaks in double stranded DNA (Rogakou *et al.*, 1998). H_2O_2 treatment increased the expression of phospho-H2A.X, whereas pre-treatment with fucoxanthin decreased the phospho-H2A.X level (Fig. 2B). Taken together, these suggest that fucoxanthin inhibits oxidative stress-induced damage to DNA in HaCaT cells.

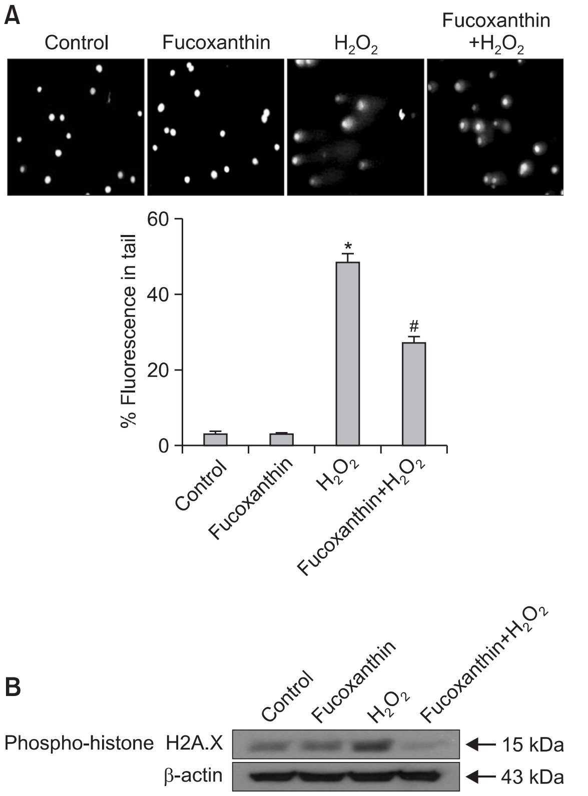

Fig. 2. Fucoxanthin protects against H_2O_2-induced DNA damage. (A) Representative images and the extent of cellular DNA damage were detected by the comet assay. *Indicates significantly different from the control (p<0.05) and #Indicates significantly different from H_2O_2-treated cells (p<0.05). (B) Cell lysates were subjected to Western blot analysis with a specific antibody against phospho-histone H2A.X. β-actin was used as a loading control.

Fucoxanthin reduces H_2O_2-induced apoptosis

To evaluate the cytoprotective effects of fucoxanthin on apoptosis induced by oxidative stress, HaCaT cell nuclei were stained with Hoechst 33342 and then visualized by fluorescence microscopy. H_2O_2-treated cells showed significant nuclear fragmentation (apoptotic index 8.3), which is characteristic of programmed cell death (Fig. 3). On the other hand, intact nuclei were observed in untreated control cells and fucoxanthin-treated cells. Pre-treatment of cells with fucoxanthin or NAC significantly attenuated nuclear fragmentation following exposure to H_2O_2; the apoptotic index was 5.6 and 4.1 in fucoxanthin- or NAC-with H_2O_2-treated cells, respectively. Thus, fucoxanthin protected cells against apoptosis induced by oxidative stress.

Fucoxanthin decreases H_2O_2-induced mitochondrial damage

Changes in mitochondrial membrane potential ($\Delta\psi_m$) were assessed to further examine the mechanism by which fucoxanthin protects HaCaT cells against H_2O_2-induced apoptosis. To do this, we used JC-1, a cationic dye that reveals mitochondrial membrane polarization (Perelman *et al.*, 2012). Flow cytometry data demonstrated that the loss of $\Delta\psi_m$ was

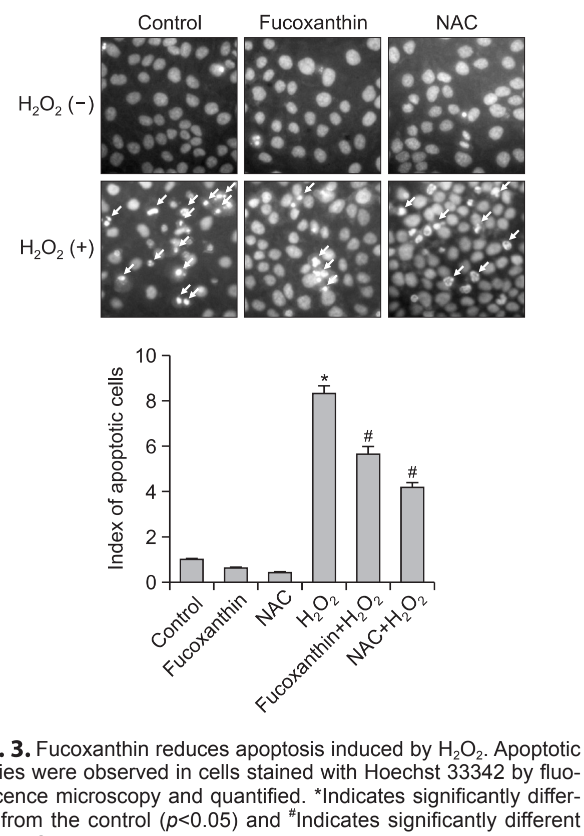

Fig. 3. Fucoxanthin reduces apoptosis induced by H_2O_2. Apoptotic bodies were observed in cells stained with Hoechst 33342 by fluorescence microscopy and quantified. *Indicates significantly different from the control (p<0.05) and #Indicates significantly different from H_2O_2-treated cells (p<0.05).

augmented in H_2O_2-treated cells relative to that in control cells, as shown by an increase in JC-1 fluorescence. However, treatment with fucoxanthin partially reversed the loss of $\Delta\psi_m$ in response to oxidative stress (Fig. 4A). We next investigated the expression of mitochondrial proteins related to apoptosis, in particular, Bcl-2, an anti-apoptotic protein, and Bax, a pro-apoptotic protein. Fig. 4B illustrates that pre-treatment with fucoxanthin increased the expression of Bcl-2 and decreased the expression of Bax in H_2O_2-treated cells. Furthermore, oxidative stress increased the levels of activated caspase-9 and caspase-3, the major executive caspases in caspase-dependent programmed cell death pathways (Fig. 4B). The increased activation of caspase-9 and caspase-3 in H_2O_2-treated cells was partially reversed by pre-treatment with fucoxanthin. These observations suggest that fucoxanthin protects against cell death by reducing H_2O_2-induced apoptosis.

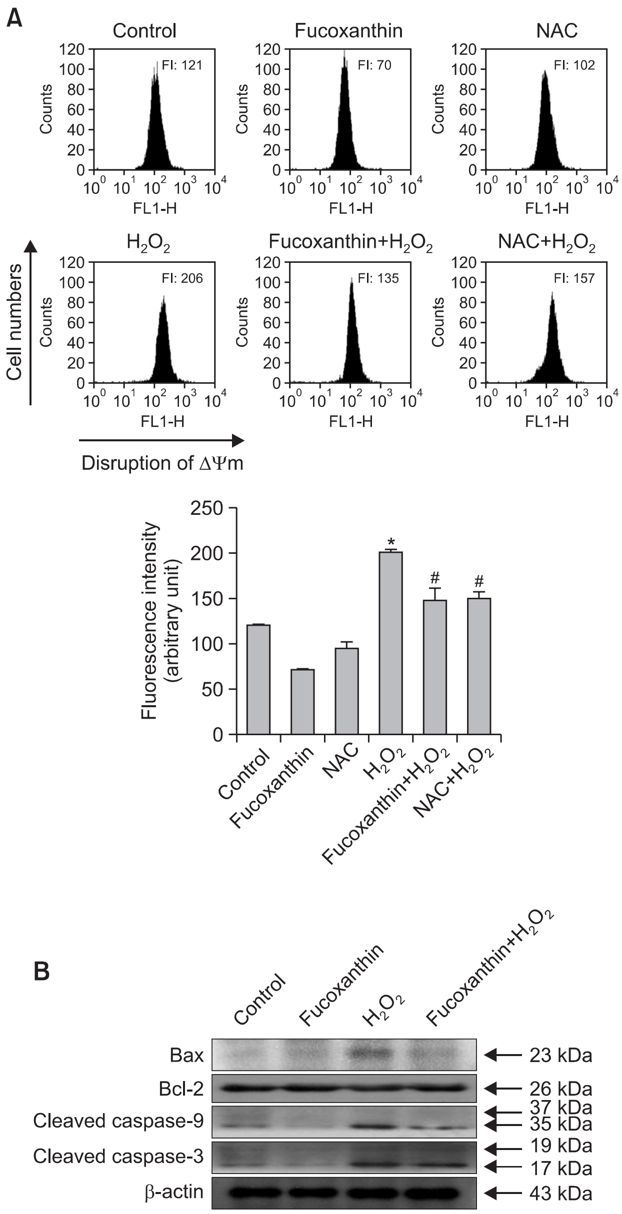

Fig. 4. Fucoxanthin reduces the oxidative stress-induced loss of mitochondrial membrane potential and alters the balance of apoptosis regulatory proteins after exposure to H_2O_2. (A) Alterations in $\Delta\psi_m$ were analyzed by flow cytometry after staining cells with JC-1, while the expression levels of mitochondrial apoptosis regulatory proteins were analyzed by Western blotting. *Indicates significantly different from the control ($p<0.05$) and #Indicates significantly different from H_2O_2-treated cells ($p<0.05$). (B) Cell lysates were subjected to Western blot analysis with primary antibodies specific for Bax, Bcl-2, caspase-9, and caspase-3. Activated (cleaved) caspase-9 and caspase-3 are represented by the 35 kDa and 17 kDa bands, respectively. β-actin was used as a loading control.

DISCUSSION

Oxidative stress is implicated in numerous pathological conditions, including malignancy, cardiovascular disease, metabolic disease, inflammatory reactions, and aging (Chakravarti and Chakravarti, 2007; Sun, 2007; Roberts and Sindhu, 2009; Klaunig *et al.*, 2010). Oxidative stress is caused by an imbalance between ROS production and antioxidant activity. Fucoxanthin is a well-known antioxidant, not only because of its singlet oxygen-quenching capability but also because of its free radical scavenging properties (Yan *et al.*, 1999; Sachindra *et al.*, 2007; D'Orazio *et al.*, 2012). The current study showed that fucoxanthin also scavenged intracellular ROS in H_2O_2-treated HaCaT keratinocytes, in addition to quenching the hydroxyl radical generated in the cell-free Fenton reaction system (Fig. 1).

Previous work suggests a direct link between ROS signaling and oxidative DNA damage (Ditch and Paull, 2012). Other studies show that DNA is susceptible to ROS-induced oxidative injury at the nuclear level (Frenkel, 1992; Marnett, 2000) and that DNA is the most frequent cellular target of oxidative stress stemming from ROS production (Neofytou *et al.*, 2012). Moreover, recent experimental evidence shows that severe ROS-induced oxidative stress leads to the induction of apoptosis in affected cells due to high levels of DNA damage (Deavall *et al.*, 2012). The present study showed that H_2O_2 treatment increased DNA tail length in the comet assay, as well as the expression of phospho-H2A.X, both of which were attenuated by fucoxanthin (Fig. 2). Thus, fucoxanthin protected cellular DNA against the destructive impact of oxidative stress.

Mitochondrial dysfunction following oxidative DNA damage and exposure to other genotoxic factors leads to an irreversible event: apoptotic cell death (Green and Reed, 1998). The mitochondrial electron transport system in the mitochondrial membrane is one of major sources of intracellular ROS (Santos *et al.*, 2011). Excessive ROS levels activate the mitochondrial apoptotic pathway (also termed the intrinsic apoptotic pathway) (Li *et al.*, 2012), as illustrated by alterations in the permeability of the mitochondrial membrane and release of cytochrome *c* into the cytoplasm, which then activates caspase-dependent signaling cascades. The present study showed that H_2O_2-treated HaCaT cells exhibited distinct features of apoptosis, such as nuclear fragmentation. However, fucoxanthin decreased the amount of nuclear fragmentation in these cells (Fig. 3). Furthermore, apoptosis is associated with a collapse in $\Delta\psi_m$ (Jeong and Seol, 2008). Although $\Delta\psi_m$ was markedly decreased in H_2O_2-treated cells relative to that in control cells, it was rescued by fucoxanthin (Fig. 4A).

Mitochondrial functions are regulated by Bcl-2 family proteins. Bcl-2 family proteins function at the core of the apoptotic pathway, ultimately leading to caspase activation (Jeong and Seol, 2008). The Bcl-2 family comprises anti-apoptotic members (e.g., Bcl-2, Bcl-xL, and myeloid leukemia cell differentiation protein Mcl-1), pro-apoptotic multi-BH domain members (BH1-3; e.g., Bax and BCL-2-homologous antagonist/killer (Bak)), and pro-apoptotic BH3-only members (e.g., BH3 interacting domain death agonist (Bid), Bim, Noxa, and p53 upregulated modulator of apoptosis (Puma)) (Cory *et al.*, 2003). In response to an array of apoptotic stimuli, cells increase their expression of Bax to activate the mitochondrial apoptosis pathway via the inhibition of Bcl-2. By doing so, Bax controls the release of cytochrome *c* from the mitochondrial membrane into the cytoplasm (which forms the apoptotic protease activating factor-1 (Apaf-1)-based apoptosome), resulting in the induction of caspase-9/caspase-3-dependent programmed cell death (Nys and Agostinis, 2012). The present study showed that fucoxanthin protected cells against H_2O_2-induced apoptosis by increasing Bcl-2 expression and inhibiting Bax expression (Fig. 4B). Taken together, the results of the current study show that fucoxanthin protects human HaCaT keratinocytes from oxidative stress by blocking free radicals and mitigating apoptosis.

It has been reported that fucoxanthin did not show any side effect. Maeda *et al.* (2005) demonstrated that rats did not show body weight loss when fed fucoxanthin rich fraction for 4 weeks. Beppu *et al.* (2009b) demonstrated that single and repeated oral administration of fucoxanthin showed no mortality and no abnormalities in appearance. And Beppu *et al*. (2009a) also reported that orally administered fucoxanthin is a safe compound in terms of mutagenicity under the *in vitro* and *in vivo* system.

Therefore, fucoxanthin might be a useful pharmacological agent that reduces the detrimental effects of oxidative stress on the skin.

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