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-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-picolrylhydrazyl 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 (H2O2)-mediated damage to monkey kidney (Vero line) (Heo et al., 2008). In addition, fucoxanthin enhances hemi 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), H2O2, and the superoxide anion.

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

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

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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 (Oren, 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 viable cells). Cells seeded on a 96-well plate at a density of $1 \times 10^5$ 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$_2$O$_2$ (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 $1 \times 10^6$ 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$_2$O$_2$ + 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$_2$O$_2$, 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% Triton 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