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Efficacy of fucoxanthin extract from *Sargassum horneri* on 3T3-L1 pre-adipocyte differentiation

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ABSTRACT

Obesity, a chronic disease characterized by excessive body fat accumulation, is associated with significant health risks. The state of being overweight or obese leads to a number of chronic diseases, including cardiovascular disease, type 2 diabetes, cancer, and osteoarthritis. Accordingly, the regulation of adipocyte proliferation and differentiation has been the focus of many studies. The goal of the present study was to investigate the function of fucoxanthin, extracted from *Sargassum horneri*, in adipocyte (3T3-L1 cells) differentiation. A quantitative real-time polymerase chain reaction was conducted to investigate the mRNA expression levels of adipocyte differentiation-related genes under fucoxanthin stimulation. All adipocyte-related genes responded to PIC stimuli. Additionally, using western blotting, we confirmed that fucoxanthin reduced adipocyte differentiation. These results indicate that fucoxanthin extracted from *Sargassum horneri* can regulate adipogenesis. Further studies are needed to reveal the signaling pathways that lead to reduced adipocyte differentiation induced by fucoxanthin.

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Introduction

Obesity, a chronic disease characterized by excessive body fat accumulation, often poses high risks to the health of the individual (1). Obesity has been shown to be a risk factor for type 2 diabetes, dyslipidemia, fatty liver disease, and cardiovascular diseases (2-3). According to the World Health Organization, the condition of being overweight or obese is the leading cause of death worldwide (4). Thus, obesity and overweight-related diseases and resulting deaths are areas, which have to solve in modern society along with epidemics around the world. Obesity is the result of a chronic positive energy balance that results in lipid deposition and an increase in the total number of adipocyte cells in individual adipocyte cells (5). Therefore, adipocyte differentiation (adipogenesis) is an important target for the regulation of obesity and obesity-related metabolic disorders (6-7). Adipogenesis is a complex process that is accompanied by changes in morphology, hormonal sensitivity, and adipogenesis-related gene expression. Preadipocytes undergo a series of steps to differentiate into mature adipocytes, and this process is controlled by increased expression of various transcription factors and adipogenesis-related genes (8).

Fucoxanthin is the most abundant carotenoid found in brown seaweeds (9). In studies of fucoxanthin, investigators have mainly focused on its anti-cancer effect, in particular in human leukemia cells, including the liver, colon, prostate, and bladder (10-14). It has been hypothesized the anti-cancer effect of fucoxanthin can be attributed to apoptosis induction and cell cycle termination (9, 13, 15). In addition, among the 15 carotenoids of food origin, neoxanthin and fucoxanthin have been reported to have the most potent growth-retarding activity in various human cancer cells (16). Therefore, validation of the anti-cancer activity of fucoxanthin in several cancer cell lines is important to clarify its potential use as a chemotherapeutic agent. Accordingly, in the present study, we aimed to confirm the effect of fucoxanthin extract from *Sargassum horneri* on the growth and delayed adipocyte differentiation activity in the 3T3-L1 cell line.

Materials and Methods

Cell culture and adipocyte differentiation

3T3-L1 cells, purchased from the Korean Cell Line Bank (KCLB No.10092.1), were maintained at 37°C in an atmosphere of 5% CO2 and 60–70% relative humidity in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1×antibiotic–antimycotic (Invitrogen, Carlsbad, CA, USA) by sub-culturing every 48 h. To induce adipocyte differentiation at 100% confluency, the cells were washed once in phosphate-buffered saline (PBS) and then placed in a differentiation medium containing 10% FBS, 1×antibiotic–antimycotic, and a hormonal cocktail containing 10 mg/mL insulin, 0.1 µM dexamethasone, and 0.5 mM IBMX. The differentiation medium was replaced

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every 2 days. Adipocyte differentiation was measured using Oil Red O staining. To test the anti-cancer effect on 3T3-L1 cells, the cells were washed once in PBS, treated with fucoxanthin at a dose of 1 mg/mL, and then incubated for 7 to 10 days during adipogenesis. Fucoxanthin product was provided by commercial company (Fucozen. Co., Ltd., Ansan city, Gyeonggi-do, Korea). All experiments were performed in triplicate with both the treated and untreated cell cultures.

Total RNA extraction and cDNA synthesis

3T3-L1 cells from the initial culture were plated in a 35-mm culture dish and incubated for 48–72 h. Once differentiation was induced, cells were incubated for 7–10 days and then harvested. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Total RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA quantity was determined using a spectrophotometer. For cDNA synthesis, 2 μg of RNA was applied using random primers, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

Quantitative real-time polymerase chain reaction (qPCR)

To quantify the expression levels of adipogenesis marker genes in 3T3-L1 cells under fucoxanthin stimulation, a quantitative real-time polymerase chain reaction (qPCR) was conducted using the Bio-Rad CFX-96 apparatus (Bio-Rad, Hercules, CA, USA). Primers for gene amplification (Table 1) were designed using PRIMER3 software (http://bioinfo.ut.ee/primer3-0.4.0/). Each reaction was performed in a 25-μL mixture containing 14 μL of SYBR Green Master Mix, 2 μL of forward primer (5 pmol), 2 μL of reverse primer (5 pmol), 5 μL of distilled water, and 2 μL (50 ng/μL) cDNA. PCR conditions were as follows: a predenaturation step at 94°C for 5 min; 39 cycles of 94°C for 20 s, 56°C for 20 s, 72°C for 30 s, and a final step at 72°C for 10 min. All measurements were performed in triplicate for all specimens, and the 2−ΔΔCt method was used to compare data (17). The relative expression of each target gene was calculated by normalizing its expression level to that of glyceraldehyde-3-phosphate dehydrogenase.

Western blotting

Total protein was extracted using RIPA buffer and separated on a 10% polyacrylamide gel, followed by transfer to a PVDF membrane. The primary antibodies used were rabbit anti-PPARγ (Invitrogen, Carlsbad, CA, USA), anti-C/EBPα (Novus Biologicals, Centennial, CO, USA), anti-UCP1 (Abcam, Cambridge, UK), and anti-β-actin (Novus Biologicals, Centennial, CO, USA). HRP-conjugated anti-mouse IgG or anti-rabbit IgG (Bio-Rad) were used as the secondary antibodies. The blots were treated with ECL substrate solutions and exposed to a ChemiDoc XRS System (Bio-Rad) to detect chemiluminescence.

Statistical analysis

Both the T-test and analysis of variance statistical tests were conducted to determine significance levels. Data were shown by mean± standard error of the mean. Duncan multiple range tests followed by one-way ANOVA were used for comparison among different incubation times in each group.

Results

The 3T3-L1 cells were treated with different concentrations of fucoxanthin (31.25, 62.5, 125, 250, 500, 1,000, 2,000, and 4,000 μg/mL), as shown in Figure 1A. In the WST-1 assay, specific concentrations (125, 250, 500, 1,000, 2,000, and 4,000 μg/mL) showed cytotoxicity in 3T3-L1 cells, whereas the lower concentrations of fucoxanthin (31.25 and 62.5 μg/mL) did not reduce adipocyte differentiation (Figure 1B). Cytotoxicity and cell viability tests confirmed that 0, 500, and 1,000 μg/mL fucoxanthin are reasonable concentrations to achieve the inhibitory effects in 3T3-L1 cells. Thus, we conducted a functional analysis with 0, 500, and 1,000 μg/mL fucoxanthin at the cellular level.

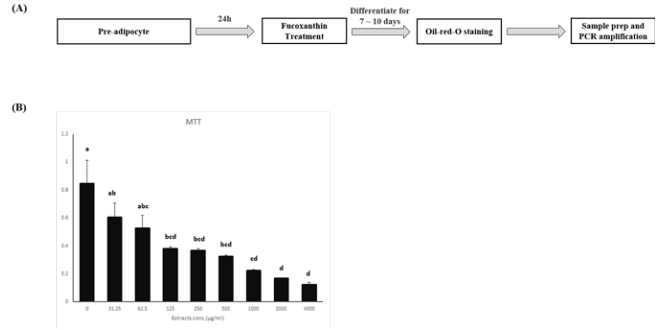

Figure 1. A: Schematic of addition of fucoxanthin on day 9 of differentiation. **B**: Viability test of murine pre-adipocyte cells (3T3-L1) treated with fucoxanthin. Comparison of 3T3-L1 cell viabilities treated with the Fucoxanthin extract depending on the different concentrations (0−4,000 ug/mL).

Table 1. Primer sets used in this study.

Gene symbol	Description	Accession No.	Primer sequences		Product size
			Forward (5'->3')	Reverse (5'->3')	
PPAR-g	peroxisome proliferator activated receptor gamma	NM_001127330	GCCCTTTGGTGACTTTATGG	CAGCAGGTTGTCTTGGATGT	169
C/EBP-a	CCAAT/enhancer binding protein, alpha	NM_001287514	GGCAGGAGGAAGATACAGGA	GCAAAAAGCAAGGGATTAGG	160
UCP1	uncoupling protein 1 (mitochondrial, proton carrier)	NM_009463	TGGCTTCTTTTCTGCGACT	TTGGTTTTATTCGTGGTCTCC	177
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	NM_001289726	CGAGACCCCACTAACATCAA	GGTTCACACCCATCACAAAC	172

To validate the effect of fucoxanthin extract from *Sargassum horneri* on adipocyte differentiation, we conducted qRT-PCR and western blotting on fucoxanthin-treated 3T3-L1 cells. Before quantitative analysis, Oil-Red-O staining was conducted to identify adipocyte differentiation. Under differentiation conditions, fucoxanthin-treated 3T3-L1 cells were found to be less differentiated than untreated 3T3-L1 cells (Figure 2A). In addition, quantitative analysis using absorbance measurements (Figure 2B) showed that absorbance of Oil-Red-O was reduced by fucoxanthin treatment. Collectively, these results indicate that fucoxanthin extracted from *Sargassum horneri* reduced adipocyte differentiation.

We then conducted qRT-PCR on fucoxanthin-treated 3T3-L1 cells to investigate the effect of fucoxanthin on adipocyte differentiation. Quantitative expression analysis was performed on PPAR-γ, CREB, and UCP1 genes by fucoxanthin reactivity based on the treatment conditions of fucoxanthin (Figure 3A). As a result, it was confirmed that the expression of adipogenesis-related marker genes decreases with the treatment of fucoxanthin. These findings demonstrate a decrease in adipogenesis following fucoxanthin treatment. Similar to the qRT-PCR results for adipogenesis marker genes, western blot data revealed the function of fucoxanthin in adipocyte differentiation (Figure 3B). Although western blotting showed that the adipogenesis marker increased at the point of 0.5 mg/mL, the results indicated that adipocyte differentiation was downregulated by fucoxanthin.

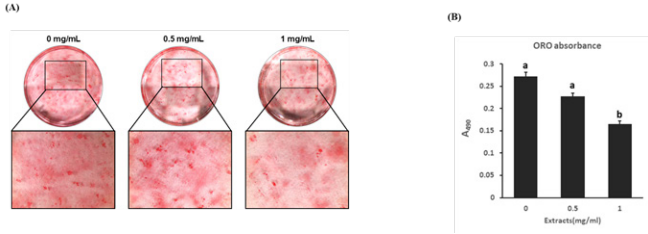

Figure 2. A: Oil-Red-O (ORO) staining by murine preadipocytes under fucoxanthin extract treatment during differentiation. ORO staining of 3T3-L1 cells incubated for 7–10 days with 0.5 and 10 mg/mL fucoxanthin. **B**: Bar graph shows the quantitative analysis of ORO absorbance.

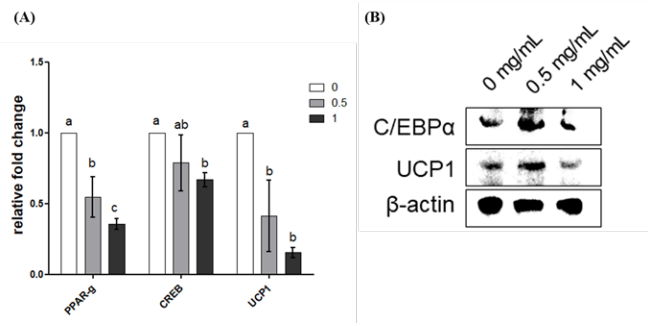

Figure 3. A: Characterization of adipocyte differentiation after treatment with fucoxanthin extract. Relative expression profiles of PPARγ, CREB, and UCP1 between the control 3T3-L1 cells and fucoxanthin extract-treated 3T3-L1 cells by qRT-PCR analysis. **B:** Western blotting.

Discussion

The chronic disease of obesity can be categorized into two main types: hyperplasia, in which the number of fat cells increases, and hypertrophy, in which the volume of fat cells increases (18-19). Hypertrophy is the most prominent feature in most obese patients, but it has a stronger correlation with severe obesity and is highly characteristic of patients with severe obesity (20). At the cellular level, obesity was originally considered a hypertrophic disease, in which both the number and size of adipose cells increases (21). In addition, fat cells constantly arise from undifferentiated progenitor cells or mature adipocytes, which are re-differentiated pre-adipocyte from de-differentiated adipocyte (22). Both types of adipose tissue generation play an important role in obesity. Most of our understanding of adipocyte differentiation and adipogenesis comes from *in vitro* studies of fibroblasts and pre-adipocytes (8). Adipogenesis involves multiple steps that regulate transcription factors and cell-cycle proteins that regulate gene expression, leading to adipocyte development. Recently, positive and negative regulators of adipogenesis have been identified (23).

In the present study, we determined the effect of fucoxanthin extracted from *Sargassum horneri* on adipocyte differentiation. In previous studies, different species of edible seaweeds have been used to study the effect of fucoxanthin, including *Undaria pinnatifida, Hijikia fusiformis, Sargassum fulvellum,* and *Chaetoseros* sp. (24-25). It has been confirmed that fucoxanthin has anti-cancer effects on various cancer cells (26-29). Previous studies have also revealed that fucoxanthin regulates apoptosis, proliferation, and differentiation by regulating cell cycle arrest (30-33).

With regard to the regulation of adipogenesis, it was confirmed that fucoxanthin extracted from *Undaria pinnatifida* inhibited the production of white adipose tissue in mice (34), and researchers in another study showed that fucoxanthin regulates adipocytokines (35). Additionally, *in vivo* experiments on the effect of fucoxanthin on adipose metabolism were conducted in obese mice (36). As a result, a high-fat diet containing fucoxanthin decreased adipocyte size, body weight gain, and visceral fat pads. In addition, extracts from *Petalonia binghamiae* reduce the accumulation of lipid droplets in the liver (37). Similarly, extracts from *Undaria pinnatifida* decreased visceral fat deposits, adipocyte size, and hepatic lipid droplet accumulation as a result of β-oxidation of activated fatty acids (38). Based on these findings, it is reasonable to conclude that fucoxanthin may regulate adipocyte differentiation. Further studies are required to delineate the signaling pathways that lead to reduced adipocyte differentiation by fucoxanthin.

In the present study, we investigated the function of fucoxanthin extracted from *Sargassum horneri* on 3T3-L1 adipocyte differentiation and further studied their target genes and signaling cascades in adipocyte cells. Fucoxanthin was found to decrease adipocyte differentiation, and the expression of adipogenesis marker genes also decreased with fucoxanthin treatment. However, further studies are necessary to uncover the biological function(s) of fucoxanthin extracted from *Sargassum horneri* in adipocytes. The present study was intended to provide basic information that can be used to understand the molecular mechanisms of fucoxanthin extracted from *Sargassum horneri* and applied to fat tissue and to enable the evaluation of *Sargassum horneri* as a potential treatment of

adipogenesis.

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