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The Green Algal Carotenoid Siphonaxanthin Inhibits Adipogenesis in 3T3-L1 Preadipocytes and the Accumulation of Lipids in White Adipose Tissue of KK-Ay Mice[1–3]

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Abstract

Background: Siphonaxanthin, a xanthophyll present in green algae, has been shown to possess antiangiogenic and apoptosis-inducing activities.

Objective: We evaluated the antiobesity effects of siphonaxanthin by using a 3T3-L1 cell culture system and in diabetic KK-Ay mice.

Methods: 3T3-L1 cells were differentiated with or without 5 μmol/L siphonaxanthin, and lipid accumulation and critical gene expressions for adipogenesis were examined. In vivo, 4-wk-old male KK-Ay mice were administered daily oral treatment of 1.3 mg siphonaxanthin for 6 wk and body weight, visceral fat weight, serum variables, and gene expressions involved in lipid metabolism were evaluated.

Results: Compared with the other carotenoids evaluated, siphonaxanthin potently inhibited adipocyte differentiation. Siphonaxanthin significantly suppressed lipid accumulation at noncytotoxic concentrations of 2.5 and 5 μmol/L by 29% and 43%, respectively. The effects of siphonaxanthin were largely limited to the early stages of adipogenesis. Siphonaxanthin significantly inhibited protein kinase B phosphorylation by 48% and 72% at 90 and 120 min, respectively. The expressions of key adipogenesis genes, including CCAAT/enhancer binding protein α (*Cebpa*), peroxisome proliferator activated receptor γ (*Pparg*), fatty acid binding protein 4 (*Fabp4*), and stearoyl coenzyme A desaturase 1 (*Scd1*), were elevated by 1.6- to 166-fold during adipogenesis. After 8 d of adipocyte differentiation, siphonaxanthin significantly lowered gene expression of *Cebpa*, *Pparg*, *Fabp4*, and *Scd1* by 94%, 83%, 95%, and 90%, respectively. Moreover, oral administration of siphonaxanthin to KK-Ay mice significantly reduced the total weight of white adipose tissue (WAT) by 13%, especially the mesenteric WAT by 28%. Furthermore, siphonaxanthin administration reduced lipogenesis and enhanced fatty acid oxidation in adipose tissue. Siphonaxanthin was observed to highly accumulate in mesenteric WAT, and the accumulation in the mesenteric WAT was almost 2- and 3-fold that in epididymal ($P = 0.14$) and perirenal ($P < 0.05$) WAT, respectively.

Conclusion: These results provide evidence that siphonaxanthin may effectively regulate adipogenesis in 3T3-L1 cells and diabetic KK-Ay mice. *J Nutr* 2015;145:490–8.

Keywords: siphonaxanthin, marine carotenoids, adipogenesis, 3T3-L1 cells, adipose tissue, KK-Ay mice

Introduction

Obesity, an abnormal condition in which excessive TG is accumulated in adipose tissue, is one of the biggest public health concerns today (1). The current obesity epidemic is largely caused by environmental and behavioral factors, including excessive food intake and physical inactivity (2). Adipocyte dysfunction, including an increase in the number and size of adipocytes, can cause adipose tissue expansion and dysfunction, and thereby promotes obesity. Dysregulation of adipocyte and adipose tissue function is linked to a number of health problems, including an increased risk of insulin resistance, type 2 diabetes, cardiovascular diseases, hypertension, dyslipidemia, and some cancers (3). Adipocytes reach maturity and become functional through adipogenesis, in which fibroblast-like preadipocytes differentiate into lipid-laden and insulin-responsive adipocytes

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[3] Supplemental Table 1 is available from the ''Online Supporting Material'' link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
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(4, 5). This process occurs in several stages and requires an elaborate network of transcription factors, among which PPARG and CCAAT/enhancer binding proteins (CEBPs)[6] are considered the principal determinants of cell fate (6). In addition, numerous signaling pathways, including the insulin/insulin-like growth factor I (IGF-I) signaling pathway, are known to play vital roles in directing adipogenesis (7). The 3T3-L1 preadipocyte culture system, which recapitulates most of the critical aspects of fat cell formation in vivo, has been widely used as an in vitro model to study the molecular mechanisms regulating adipogenesis (8). Accordingly, the establishment of animal models is valuable to assess the efficacy of potential therapeutic agents in the treatment of obesity. KK-Ay mice were established via transferring yellow obese gene (Ay) (9) into glucose-intolerant black KK mice. After feeding with a regular feed pellet diet, the KK-Ay mice spontaneously become obese, hyperinsulinemic, and hyperglycemic (10). Therefore, KK-Ay mice are considered to be a useful animal model system to study pathogenesis, therapy, and prevention of obesity and diabetes (11).

In recent years, marine algae have served as a valuable source for functional compounds with potentially beneficial health effects. Among these compounds from marine algae, natural carotenoids have attracted a great deal of attention because of their diverse and beneficial bioactivities, such as antioxidant, anticancer, anti-inflammatory, and antiobesity activities (12). Fucoxanthin, an extensively studied carotenoid from marine brown algae, was reported to have remarkable antiobesity properties, including decreased body weight gain and improved lipid metabolism in both obese mice (13, 14) and high-fat-diet–induced obese mice (15, 16).

Siphonaxanthin (**Figure 1**) is a xanthophyll present in green algae that can survive in deep water such as *Codium fragile*, *Caulerpa lentillifera*, and *Umbraulva japonica* (17). We previously found that siphonaxanthin possesses antiangiogenic and apoptosis-inducing activities (18, 19), and that this beneficial effect may result from its inhibitory effects on various kinases, including protein kinase B (Akt) and extracellular signal–regulated kinase (ERK) (20). Siphonaxanthin is one of the oxidative metabolites of lutein, and its structure contains a conjugated system of 8 C=C double bonds and 1 keto group located at C-8, similar to fucoxanthin (21). In addition to these structural groups, siphonaxanthin has an extra hydroxyl group at C-19 that might contribute to it being more beneficial than other carotenoids (19, 22). However, the potential role of siphonaxanthin in adipogenesis and its underlying mechanisms have not been studied.

In this study, we investigated the effect of siphonaxanthin on adipogenesis in 3T3-L1 preadipocytes by measuring lipid accumulation as well as the mRNA expression levels of various genes involved in adipocyte differentiation. Furthermore, the in vivo effect of orally administrated siphonaxanthin on the weight and gene expression of adipose tissue was evaluated in KK-Ay mice.

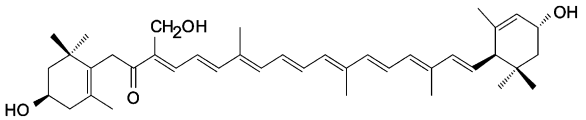

FIGURE 1 Chemical structure of siphonaxanthin.

Methods

Preparation of carotenoids. Carotenoids, including β-carotene, zeaxanthin, lutein, fucoxanthin, and violaxanthin, were prepared as previously described (23). In addition, alloxanthin, diatoxanthin, and halocynthiaxanthin were extracted from the mantle of ascidians (*Halocynthia roretzi*). Bacterioruberin was extracted from halobacteria (*Halococcus thailandensis*; JCM 13552) obtained from the Japan Collection of Microorganisms (JCM) (RIKEN BioResource Center). Loroxanthin was extracted from green alga (*Chaetomorpha crassa Kützing*), and siphonaxanthin and siphonein were also extracted from green alga (*C. fragile*; donated by the NOF Corporation). The extracted carotenoids were purified by HPLC (LC-6; Shimadzu). The purified carotenoids (purity >98%) were used for the in vitro study.

For the in vivo study, crude lipid was extracted from dried *C. fragile* powder with acetone. The crude lipid extract was dissolved in dichloromethane and subjected to silica gel column chromatography. The siphonaxanthin fraction was eluted with dichloromethane/acetone (6:4) and rechromatographed by using a silica gel column, followed by elution with n-hexane/acetone (6:4) to obtain crude siphonaxanthin. The siphonaxanthin-rich fraction used for the in vivo study contained 73% siphonaxanthin and 27% phospholipids and glyceroglycolipids.

The absorption spectrum of each carotenoid was measured by using a photodiode array detector (SPD-M20A; Shimadzu) connected to the HPLC system. The mass spectra of the purified carotenoids were measured by an HPLC system connected to a mass detector (LCMC-2010EV; Shimadzu) equipped with an interface of atmospheric pressure chemical ionization. Carotenoids were stored at −80°C until use.

3T3-L1 cell culture and differentiation. The 3T3-L1 preadipocytes obtained from the Health Science Research Resources Bank (Osaka, Japan) were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. 3T3-L1 preadipocytes were seeded in a 24-well plate and cultured as described above. Two days postconfluence (defined as day 0), the cells were differentiated with differentiation medium (MDI; DMEM containing 1.0 μmol/L dexamethasone, 0.5 mmol/L methyl-isobutyl-xanthine, 170 nmol/L insulin, 10% FBS, and 1% penicillin-streptomycin) for 2 d. The cells were then cultured in DMEM containing 10% FBS, 1% penicillin-streptomycin, and 170 nmol/L insulin, replacing the medium every other day. To study the effect of various carotenoids on the differentiation of preadipocytes to adipocytes, the carotenoids (in DMSO) were added to the MDI differentiation medium throughout the differentiation process at 5 μmol/L. The final concentration of DMSO in the medium was adjusted to 0.1% without cytotoxicity. To test the cytotoxicity of siphonaxanthin, postconfluent 3T3-L1 preadipocytes were incubated in MDI with or without various concentrations of siphonaxanthin for 3 d, and the cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. On day 8, intracellular lipid accumulation was analyzed by Oil Red O staining as described previously (24). The stained lipid droplets within the cells were visualized by using a fluorescence microscope at 20× magnification and photographed with a digital camera (Keyence BZ-9000). To quantify the intracellular lipid content, the stained lipid droplets were dissolved in 2-propanol and the absorbance of extracted dye was then measured at 490 nm.

Uptake of siphonaxanthin in 3T3-L1 cells. Two days postconfluence, preadipocytes were cultured in MDI in the presence of siphonaxanthin

[6] Abbreviations used: *Acc*, acetyl-CoA carboxylase; *Acox1*, acyl-CoA oxidase 1; Akt, protein kinase B; BAT, brown adipose tissue; *Cdk2*, cyclin-dependent kinase 2; CEBP, CCAAT/enhancer binding protein; *Cebpa*, CCAAT/enhancer binding protein α; *Cebpb*, CCAAT/enhancer binding protein β; *Cpt1a*, carnitine palmitoyltransferase 1a; ERK, extracellular signal–regulated kinase; *Fabp4*, fatty acid binding protein 4; *Fas*, fatty acid synthase; *G6pd*, glucose-6-phosphate dehydrogenase; IGF-I, insulin-like growth factor I; MCE, mitotic clonal expansion; MDI, differentiation medium; *Me1*, malic enzyme 1; NEFA, nonesterified fatty acid; PGC1, peroxisome proliferator activated receptor γ coactivator 1; *Pgc1a*, peroxisome proliferator activated receptor γ coactivator 1α; *Pgc1b*, peroxisome proliferator activated receptor γ coactivator 1β; PI3K, phosphatidylinositol-3 kinase; *Scd1*, stearoyl CoA desaturase 1; SPX, siphonaxanthin; *Srebp1c*, sterol regulatory element binding protein 1c; *Ucp*, uncoupling protein; WAT, white adipose tissue.

for 24, 48, or 72 h. The cells were washed twice with PBS containing 10 mmol/L sodium taurocholate, and siphonaxanthin was extracted 3 times from the cells by using a mixture of dichloromethane/methanol/water (2:1:0.9, vol:vol:vol). The dichloromethane phase was collected, dried with nitrogen, and dissolved in methanol. An aliquot of each sample was subjected to HPLC analysis, and siphonaxanthin content was quantitated from the peak area by using a standard curve obtained with the use of purified siphonaxanthin (17).

RNA preparation and real-time qRT-PCR. Total RNA was extracted from 3T3-L1 cells or tissue by using sepasol reagent (Nacalai Tesque) according to the manufacturer's instructions and was treated with DNase (Wako Pure Chemical Industries). cDNA was synthesized from 2 μg of total RNA by using SuperScript RNase II reverse transcriptase kit (Invitrogen) with random hexamers. For RT-PCR, cDNA was diluted and mixed with iQ SYBR Green Supermix (Bio-Rad Laboratories) containing 1 μmol/L PCR primer (primer sequences are shown in **Supplemental Table 1**). Real-time qRT-PCR was performed by using a DNA Engine Option system (Bio-Rad Laboratories). The thermal cycling conditions were 15 min at 95°C for 1 cycle, followed by amplification for 43 cycles with melting for 15 s at 95°C and annealing and extension for 30 s at 60°C. The expression level of each gene was normalized by using *Gapdh* as an internal control.

Western blot analysis. Cells were washed with cold PBS twice and scraped into lysis buffer (20 mmol/L Tris-HCl, pH 8; 150 mmol/L NaCl, 1% Triton-X 100, protease inhibitor mixture, and phosphatase inhibitor mixture). The cell suspensions were centrifuged at 17,800 × *g* at 4°C for 10 min to collect the supernatant. Protein concentration was determined by using a commercial kit (DC protein assay, Bio-Rad Laboratories). Ten micrograms of protein was separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Immunoblots were incubated with the primary antibodies for Akt (1:1000; Cell Signaling), phospho-Akt (1:2000; Cell Signaling), or β-actin (1:1000; Cell Signaling) at room temperature for 1 h, and then incubated with HRP-conjugated anti-rabbit IgG secondary antibody at room temperature for 1 h. Signals were visualized with the substrate Chemi-lumi One (Nacalai Tesque) by using a Fujifilm visualizer (LAS-3000).

Mice and diets. All experimental animal protocols were approved by the Animal Experimentation Committee of Kyoto University for the care and use of experimental animals. Male KK-Ay/TaJcl mice (4 wk of age) were obtained from Japan Clea. All mice were housed individually and maintained on an alternating 12-h light/dark cycle at a temperature of 23 ± 1°C. After an acclimatization period of 1 wk, the KK-Ay mice were randomly divided into 2 groups (n = 5 per group), with ad libitum access to drinking water and the AIN-93G growth diet (25). The control and siphonaxanthin groups received daily oral treatments of 100 μL triolein or 100 μL triolein containing 1.3 mg siphonaxanthin, respectively. Body weight and food intake were monitored throughout the study. After 6 wk of administration, mice were feed-deprived for 12 h and killed by isoflurane anesthesia. Blood was collected, and organs were rapidly removed, weighed, and immediately frozen by liquid nitrogen. The white adipose tissue (WAT), brown adipose tissue (BAT), liver, and muscle were partly stored in RNA laterTM solution (Ambion) at −80°C until use for real-time RT-PCR analyses.

Biochemical analysis. The serum was prepared by centrifuging at 400 × *g* for 15 min at 4°C and stored at −80°C until use. Serum concentrations of TG, free cholesterol, total cholesterol, HDL cholesterol, nonesterified fatty acid (NEFA), and glucose were measured by using commercially available kits (TG E, F-Cho E, T-Cho E, HDL-C E, NEFA, and Glu C II, respectively; Wako Pure Chemical Industries). Serum aspartate aminotransferase and alanine aminotransferase concentrations were measured by using a commercial kit (GOT GPT C II; Wako Pure Chemical Industries). Liver TG and total-cholesterol concentrations were measured in the lipid fraction prepared from the liver by using commercial kits as mentioned above, respectively.

Siphonaxanthin analysis in the tissues of KK-Ay mice. Siphonaxanthin was extracted from the tissues and subjected to a quantitative HPLC analysis (19). The lipid extracts were dissolved in dichloromethane/methanol (1:1, vol:vol) for HPLC analysis. For the analysis of high-fat tissues (liver, brain, and adipose tissue), the extracts were loaded onto Sep-Pak Plus silica cartridges (Waters) to remove the TG fraction before HPLC analysis. The peak of siphonaxanthin was further confirmed from its characteristic UV-visible and mass spectra as described above.

Statistical analysis. Data analyses were performed by using the statistical program SPSS 16.0 for Windows. Comparisons were made between groups of normally distributed data by using a 1-factor ANOVA followed by Tukey's post hoc analysis. For in vivo experiments, Student's t test was used to analyze the differences between control and siphonaxanthin groups. Variance homogeneity was examined by using Levene's test. When the variances between groups were unequal, the data were transformed to logarithms before analysis by 1-factor ANOVA. Data with unequal variances between control and siphonaxanthin groups were transformed to logarithms or were subject to a Mann-Whitney U test. Analysis for siphonaxanthin accumulation in tissues showed unequal homogeneity even after transformation. Because of small variance in the testis, the other 13 tissues were log-transformed and subjected to the Tukey's post hoc analysis. The difference between mesenteric WAT and testis was analyzed by using Student's t test. For the change in siphonaxanthin content in cells, a 1-factor ANOVA with repeated measures was conducted. The changes in gene expression and Akt phosphorylation levels in cells were analyzed by using a repeated-measures 2-factor ANOVA (time × treatment) and then followed up by Student's t test to examine the differences between control and siphonaxanthin treatment groups at each time point. Data are represented as means ± SEMs. Significance was defined as $P < 0.05$.

Results

Effects of carotenoids on MDI-induced lipid accumulation in 3T3-L1 adipocytes. We first investigated the effects of 12 carotenoids on the differentiation of 3T3-L1 preadipocytes to adipocytes. The cells treated with alloxanthin, bacterioruberin, β-carotene, diatoxanthin, fucoxanthin, halocynthiaxanthin loroxanthin, lutein, and siphonein showed no difference in lipid droplet accumulation in adipocytes vs. the differentiated control. However, the lipid droplet accumulation in cells treated with siphonaxanthin, violaxanthin, and zeaxanthin was significantly decreased compared with differentiated control cells (**Figure 2**). Siphonaxanthin significantly suppressed adipogenesis compared with the other carotenoids evaluated, except for zeaxanthin (P = 0.27) (Figure 2). Lipid accumulation was

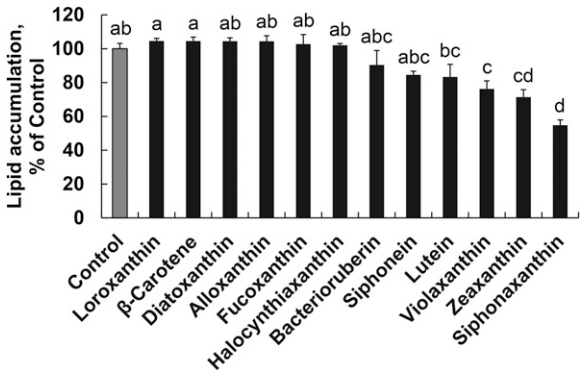

FIGURE 2 Effect of carotenoids on adipogenesis in 3T3-L1 cells. Lipid accumulation in adipocytes treated with MDI in the presence or absence of 5 μmol/L of various carotenoids for 8 d. Values are means ± SEMs, n = 3. Data were analyzed by Tukey's test. Labeled means without a common letter differ, $P < 0.05$. Control, differentiated adipocytes treated without carotenoids; MDI, differentiation medium.

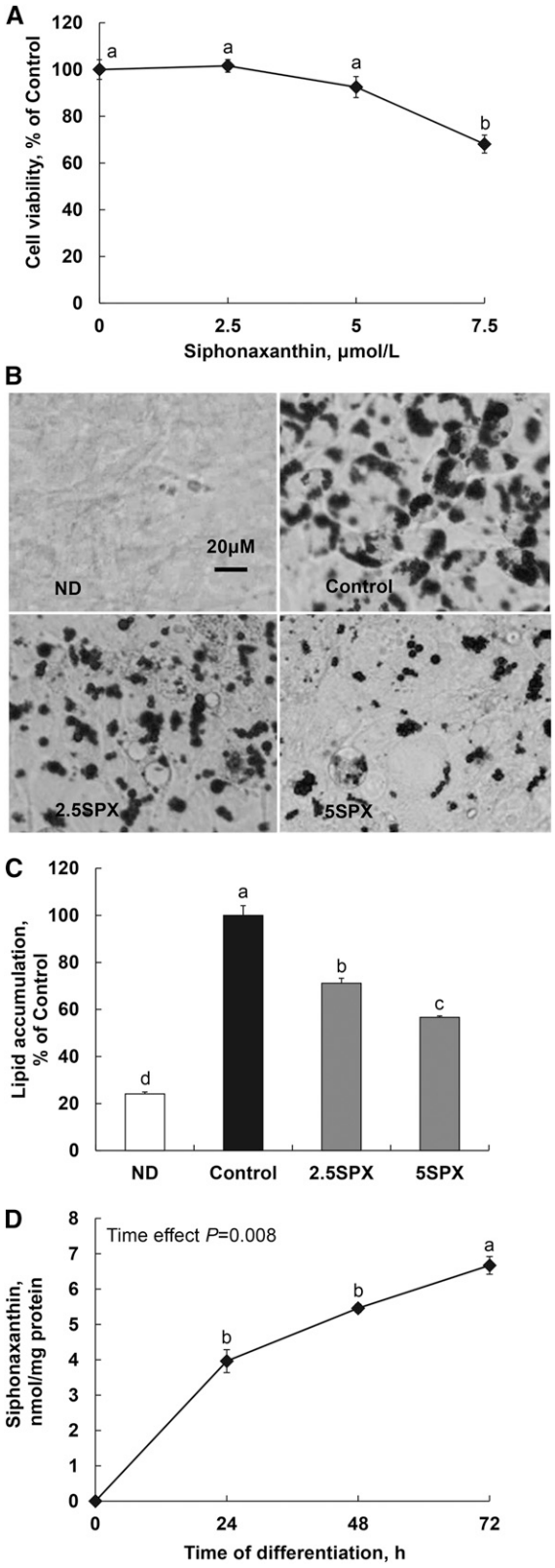

FIGURE 3 Effect of siphonaxanthin on adipogenesis in 3T3-L1 cells. (A) Cell viability in differentiated cells treated with siphonaxanthin (0–7.5 μmol/L) for 72 h. Oil Red O staining (B) and lipid accumulation (C) in adipocytes treated with MDI in the presence or absence of 2.5 or 5 μmol/L of siphonaxanthin for 8 d. Values are means ± SEMs; $n = 9$ for cell viability determination (A), $n = 6$ for lipid accumulation determination (C). Data were analyzed by Tukey's test. (D) Changes in siphonaxanthin content in 3T3-L1 cells treated with MDI in the presence of 5 μmol/L siphonaxanthin for 24, 48, and 72 h. Values are means ± SEMs, $n = 3$. Significance was determined by 1-factor ANOVA with repeated measures. Within each graph, labeled means without a common letter differ, $P < 0.05$. Control, differentiated adipocytes treated without siphonaxanthin; MDI, differentiation medium; ND, not differentiated 3T3-L1 preadipocytes; 2.5SPX, 2.5 μmol/L siphonaxanthin; 5SPX, 5 μmol/L siphonaxanthin.

decreased by ~43% after 5 μmol/L siphonaxanthin treatment (Figure 2).

Effects of siphonaxanthin on preadipocyte differentiation. Treatment with siphonaxanthin at a concentration of 7.5 μmol/L for 72 h significantly decreased cell viability. In contrast, at concentrations of 2.5 and 5 μmol/L, siphonaxanthin did not affect cell viability (**Figure 3**A). These data indicated that the inhibitory effect of 5 μmol/L siphonaxanthin during 3T3-L1 adipocyte differentiation was not due to cytotoxicity. The concentrations with no observed cytotoxicity were used in the following studies. Morphologic analysis of intracellular lipids by Oil Red O staining showed that the number and size of single lipid droplets in siphonaxanthin-treated cells were obviously decreased compared with the control cells (Figure 3B). Siphonaxanthin significantly reduced lipid accumulation in 3T3-L1 adipocytes in a dose-dependent manner (Figure 3C). The siphonaxanthin content in MDI-stimulated cells after 24 h of incubation with siphonaxanthin was 4.0 ± 0.3 nmol/mg protein, whereas the content reached 6.7 ± 0.3 nmol/mg protein after 72 h of incubation (Figure 3D).

Effects of siphonaxanthin on 3T3-L1 adipocyte differentiation at various stages during adipogenesis. Differentiating cells were treated with 5 μmol/L siphonaxanthin for various times periods (days 0–2, 0–4, 0–6, 0–8, 2–4, 2–6, 2–8, 4–6, 4–8, and 6–8). After 8 d of differentiation, these cells were subjected to Oil Red O staining. The cells treated with siphonaxanthin from days 0–8 exhibited ~45% inhibition of adipogenesis (**Figure 4**A). Moreover, the cells treated with siphonaxanthin on days 0–6 also showed a 40% decrease in lipid accumulation, similar to those treated for 8 d (Figure 4A). In addition, the presence of siphonaxanthin from days 0–2 and 0–4 caused a 25% decrease in lipid accumulation (Figure 4A). However, cells treated with siphonaxanthin on days 2–4, 2–6, 4–6, 4–8, and 6–8, including intermediate and late phases of adipogenesis, did not display significantly reduced levels of lipid accumulation compared with control cells (Figure 4A). These results indicated that the inhibitory effect of siphonaxanthin on adipogenesis occurred during the early phase. In addition, cells treated with siphonaxanthin from days 2 to 8 exhibited a significant decrease in lipid accumulation, implying a possible role for siphonaxanthin in regulating middle and later adipogenesis. Adipogenic stimuli strongly induced serine phosphorylation of Akt after 30 min, with a sustained level of phosphorylation up to 120 min; however, 5 μmol/L siphonaxanthin significantly inhibited MDI-induced Akt phosphorylation after 90 and 120 min (Figure 4B, C).

Effects of siphonaxanthin on adipogenic transcription factors and target genes. To identify the molecular mechanisms underlying the inhibitory effect of siphonaxanthin on adipocyte differentiation, the expression of key adipogenic transcription factors and target genes was analyzed at various time points during 3T3-L1 adipocyte differentiation. The gene expression of CCAAT/enhancer binding protein β (*Cebpb*) was increased after MDI stimulation up to 4 h, at which point it then

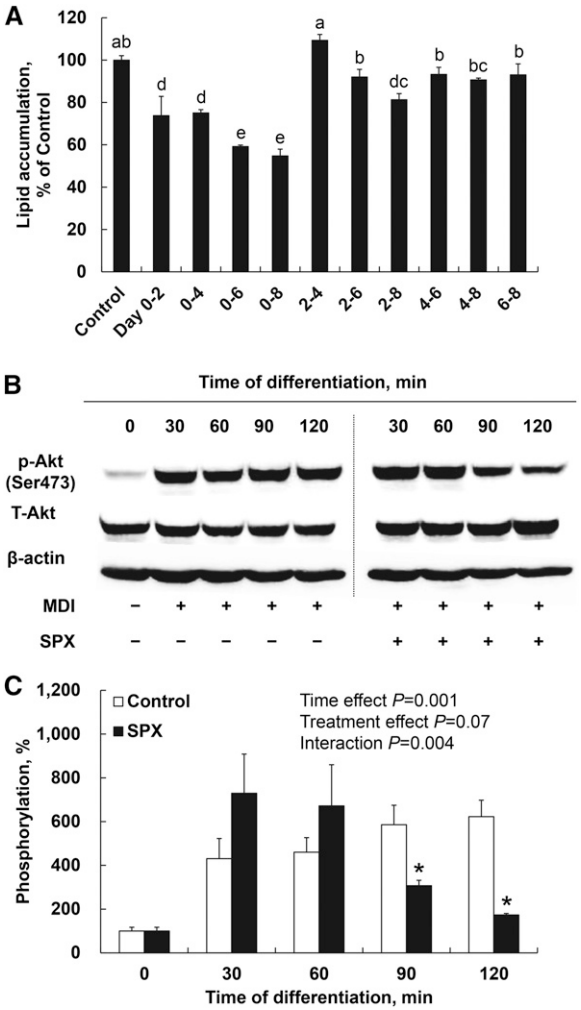

FIGURE 4 Effect of siphonaxanthin on 3T3-L1 adipocyte differentiation at various stages during adipogenesis. (A) Postconfluent 3T3-L1 preadipocytes were differentiated with MDI, and 5 μmol/L siphonaxanthin was added at various stages of the adipogenesis process. At day 8 of differentiation, intracellular lipid accumulation was determined. Values are means ± SEMs, $n = 3$. Data were analyzed by Tukey's test. Labeled means without a common letter differ, $P < 0.05$. (B, C) Akt phosphorylation in preadipocytes stimulated with MDI in the presence or absence of 5 μmol/L siphonaxanthin. Representative Western blots (B) and relative Akt phosphorylation (the ratio of p-Akt:T-Akt expression) expressed as percentages of undifferentiated 3T3-L1 preadipocytes (C) are shown. β-Actin was used as a reference. Values are means ± SEMs, $n = 3$. Significance was determined by repeated-measures 2-factor ANOVA (time × treatment), followed by Student's t test. *Different from control at each time, $P < 0.05$. Akt, protein kinase B; Control, differentiated adipocytes treated without siphonaxanthin; MDI, differentiation medium; p-, phosphorylated; SPX, siphonaxanthin; T-, total.

decreased (**Figure 5**A). Furthermore, *Cebpb* gene expression was not changed after siphonaxanthin treatment. Sterol regulatory element binding protein 1c (*Srebp1c*) is an insulin-regulated gene, and its gene expression was not altered in MDI-stimulated 3T3-L1 cells in the presence of siphonaxanthin (Figure 5B). mRNA expression of CCAAT/enhancer binding protein α (*Cebpa*) increased 1.8- to 4-fold in cells during the differentiation process, and the mRNA expression of *Pparg* also increased 1.6- to 2.6-fold compared with nondifferentiated 3T3-L1 preadipocytes; however, their expression was significantly suppressed in siphonaxanthin-treated cells (Figure 5C, D). Fatty acid binding protein 4 (*Fabp4*) (26) mRNA expression increased by 37- to 166-fold in cells undergoing adipogenesis; however, mRNA expression of *Fabp4* was almost completely inhibited by treatment with siphonaxanthin (Figure 5E). Stearoyl CoA desaturase 1 (*Scd1*) (27) mRNA expression increased by >1.8- to 8.9-fold during adipogenesis but was significantly inhibited after siphonaxanthin treatment (Figure 5F). Importantly, siphonaxanthin almost lowered the mRNA levels of these genes to the levels observed in undifferentiated cells after 6 or 8 d of adipocyte differentiation.

Effects of orally administrated siphonaxanthin on growth, serum, and liver variables in KK-Ay mice. On the basis of our in vitro observations, we performed an in vivo experiment using KK-Ay mice. Siphonaxanthin dissolved in triolein was orally administered to each mouse daily for 6 wk. There was no significant difference in the food intake, body weight gain, and liver weight between the control and siphonaxanthin groups (**Table 1**). Siphonaxanthin significantly reduced the weight of total WAT and mesenteric WAT compared with the control group, although the weights of the epididymal WAT, perirenal WAT, and BAT were not altered (Table 1). The serum concentrations of TG, free cholesterol, total cholesterol, HDL cholesterol, NEFA, glucose, and adiponectin were not significantly different between the control group and the siphonaxanthin group. Furthermore, serum aspartate aminotransferase and alanine aminotransferase concentrations in the siphonaxanthin group did not differ from those in the control group (Table 1), indicating that siphonaxanthin treatment at this dose did not cause damage to the liver. The concentrations of TG and total cholesterol in the liver did not differ between treatments (Table 1).

Effects of siphonaxanthin on the expression of mRNA related to lipid metabolism in KK-Ay mice. The effects of siphonaxanthin on the gene expression of adipogenic factors and lipid-regulating enzymes in the tissues of KK-Ay mice, including the liver, mesenteric adipose tissue, and skeletal muscle, were investigated by using real time RT-PCR analysis. In the liver, expression levels of FA synthesis–related genes, including *Srebp1c*, FA synthase (*Fas*), *Scd1*, and acetyl-CoA carboxylase (*Acc*) were measured; however, no significant difference was observed between treatments (data not shown). Siphonaxanthin-treated mice tended to have a lower gene expression of *Cebpa* ($P = 0.08$) and *Pparg* ($P = 0.06$) in the mesenteric adipose tissue compared with control mice, although this was not significant (**Figure 6**A). Siphonaxanthin significantly downregulated the mRNA expression of *Scd1* and glucose-6-phosphate dehydrogenase (*G6pd*) in mesenteric adipose tissue compared with the control group but did not affect the mRNA expression of *Srebp1c*, *Acc*, and malic enzyme 1 (*Me1*) (Figure 6A). In contrast to the lipogenic genes analyzed, the mRNA expression of the β-oxidation–related gene carnitine palmitoyltransferase 1a (*Cpt1a*) and the energy expenditure–related gene peroxisome proliferator activated receptor γ coactivator 1β (*Pgc1b*) was upregulated in the siphonaxanthin group, whereas the mRNA expression of peroxisome proliferator activated receptor γ coactivator 1α (*Pgc1a*) and acyl-CoA oxidase 1 (*Acox1*) was not altered (Figure 6B). Siphonaxanthin also significantly increased the mRNA expression of uncoupling protein (*Ucp*) 3 in skeletal muscle (Figure 6C).

Accumulation of siphonaxanthin in the tissues of KK-Ay mice. Siphonaxanthin was detected in each tissue of

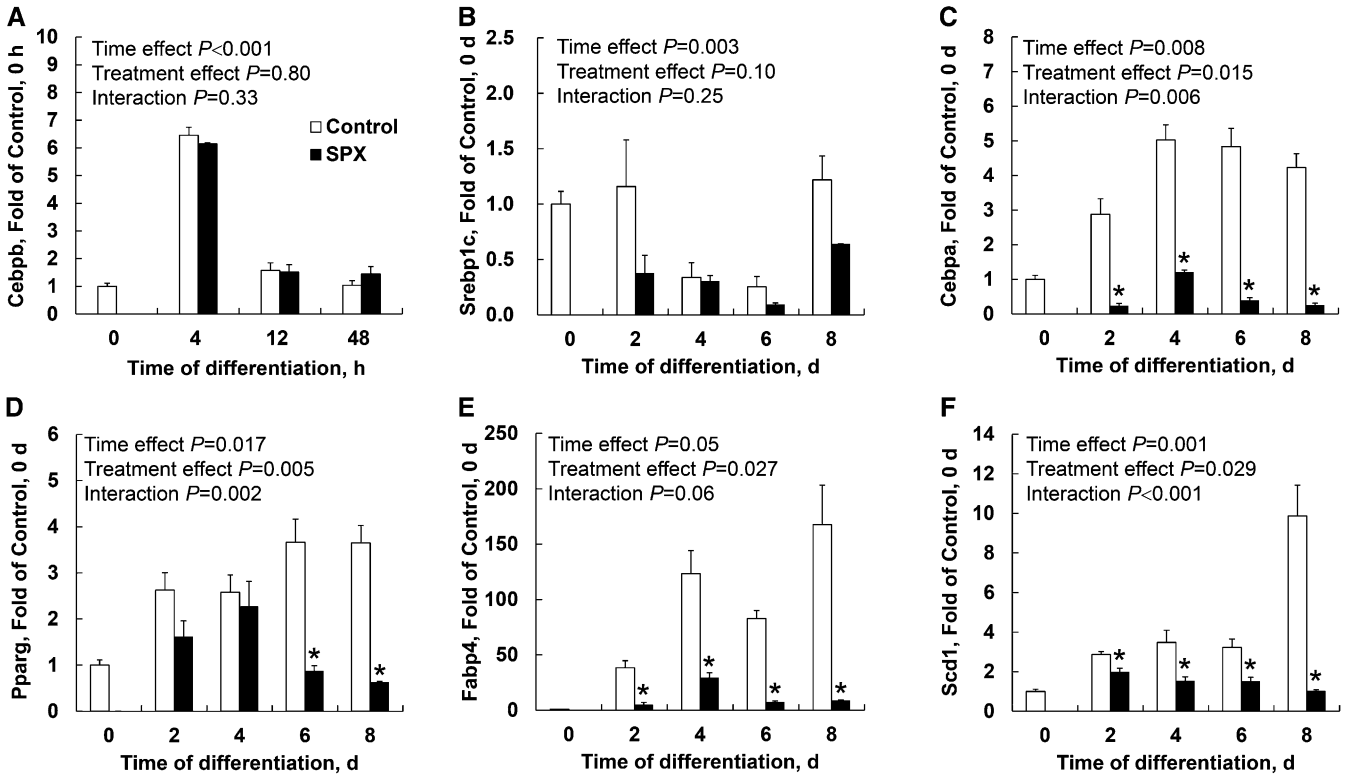

FIGURE 5 Effects of siphonaxanthin on *Cebpb* (A), *Srebp1c* (B), *Cebpa* (C), *Pparg* (D), *Fabp4* (E), and *Scd1* (F) mRNA levels of genes involved in adipogenesis. Preadipocytes were differentiated with MDI in the presence or absence of 5 μmol/L siphonaxanthin, and mRNA levels were determined at various time points. Values were normalized to *Gapdh*. Values are means ± SEMs, $n = 3$. Significance was determined by repeated-measures 2-factor ANOVA (time × treatment), followed by Student's t test. *Different from control at each time, $P < 0.05$. *Cebpa*, CCAAT/enhancer binding protein α; *Cebpb*, CCAAT/enhancer binding protein β; Control, differentiated adipocytes treated without siphonaxanthin; *Fabp4*, fatty acid binding protein 4; *Scd1*, stearoyl CoA desaturase 1; SPX, siphonaxanthin; *Srebp1c*, sterol regulatory element binding protein 1c.

KK-Ay mice after oral administration for 6 wk by HPLC analysis, except for the bladder, brain, and small intestine (**Figure** 7). Siphonaxanthin was highly accumulated in WAT and digestion-associated tissues (pancreas, large intestine, and stomach) (Figure 7). Notably, siphonaxanthin was observed to highly accumulate in mesenteric WAT, and the accumulation in the mesenteric WAT was almost 2- and 3-fold that in epididymal ($P = 0.14$) and perirenal ($P < 0.05$) WAT, respectively (Figure 7). The siphonaxanthin accumulation in mesenteric WAT was significantly higher than that in the other tissues, including the pancreas, large intestine, stomach, BAT, heart, liver, testis, muscle, kidney, lung, and spleen (3- to 12-fold) (Figure 7). In addition, some unidentified peaks near the peak of siphonaxanthin appeared in the chromatograms of the tissues in which siphonaxanthin was detected. These peaks might be oxidation metabolites of siphonaxanthin.

Discussion

In the present study, we examined the effect of siphonaxanthin on adipocyte differentiation in 3T3-L1 cells and obesity in a diabetic/obese KK-Ay mouse model. We observed that siphonaxanthin significantly suppressed 3T3-L1 adipocyte differentiation via the downregulation of the transcription factors *Cebpa* and *Pparg*, which strictly govern adipocyte differentiation. Furthermore, the development of total and mesenteric adipose tissue was significantly decreased in siphonaxanthin-treated KK-Ay mice.

In this study, we screened 12 carotenoids to evaluate their antiadipogenesis activity and identified siphonaxanthin as a compound that potently inhibits adipogenesis. It is assumed that the antiadipogenic activity of siphonaxanthin is likely associated with its unique structure. Treatment with carotenoids without extra functional groups, including keto groups and hydroxyl groups, on the carbon skeleton (β-carotene) did not result in a significant inhibition of lipid accumulation in adipocytes. Maeda et al. (24) reported that fucoxanthin at a higher concentration (10–25 μmol/L) can decrease lipid accumulation in 3T3-L1 cells. Although both fucoxanthin and siphonaxanthin are keto-carotenoids, fucoxanthin has an epoxide group and an allenic bond in its molecular structure, whereas siphonaxanthin has a hydroxyl group at C-19. An analog of siphonaxanthin, loroxanthin, which has no keto group, did not inhibit adipocyte differentiation. Likewise, siphonein, which is 12:1-esterified at the C-19 hydroxyl group of siphonaxanthin, had no significant inhibitory effect on adipocyte differentiation. We assume that the presence of both the keto group and the hydroxyl group may be important for the inhibitory effect of siphonaxanthin on adipocyte differentiation.

The antiadipogenic effect of siphonaxanthin is likely due to its ability to modulate cellular events during the early stage of adipocyte differentiation. The insulin-dependent phosphatidylinositol-3 kinase (PI3K)-Akt signaling pathway is thought to be important for the initiation of molecular events in adipogenesis. Previous work demonstrated that Akt activated in response to insulin plays a critical role in the development and maintenance of proper adipose tissue (28). The phosphorylation of Akt occurs within the first several hours after insulin stimulation, and it is shown to be necessary for 3T3-L1 adipocyte differentiation (29, 30). The

TABLE 1 Effect of siphonaxanthin on body weight, adipose tissue mass, plasma variables, and lipid metabolism in KK-Ay mice[1]

	Control	Siphonaxanthin
Body weight gain, g/6 wk	13.6 ± 0.7	13.8 ± 1.0
Food intake, g/d	4.34 ± 0.04	4.35 ± 0.05
Liver weight, g	4.20 ± 0.25	4.04 ± 0.19
BAT, g/100 g body weight	0.81 ± 0.05	0.78 ± 0.07
WAT, g/100 g body weight		
Total	10.3 ± 0.1	8.99 ± 0.6*
Mesenteric	3.08 ± 0.12	2.22 ± 0.05*
Epididymal	4.57 ± 0.12	4.51 ± 0.21
Perirenal	2.69 ± 0.06	2.26 ± 0.36
Plasma TG, mg/dL	139 ± 14.9	123 ± 22.8
Plasma free cholesterol, mg/dL	31.1 ± 1.9	26.2 ± 2.2
Plasma total cholesterol, mg/dL	126 ± 7.2	105 ± 9.1
HDL cholesterol, mg/dL	83.1 ± 4.7	67.3 ± 6.6
NEFAs, mEq/L	0.65 ± 0.04	0.67 ± 0.05
Glucose, mg/dL	268 ± 36.1	193 ± 36.4
AST, U/L	33.0 ± 1.85	33.2 ± 5.00
ALT, U/L	8.33 ± 0.37	8.89 ± 0.58
Adiponectin, μg/mL	4.66 ± 0.20	4.18 ± 0.23
Liver TG, mg/g liver	54.2 ± 12.9	40.0 ± 2.6
Liver cholesterol, mg/g liver	7.61 ± 0.44	9.45 ± 1.20

[1] Values are means ± SEMs, $n = 5$ per group. Data were analyzed by Student's t test. *Different from control, $P < 0.05$. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BAT, brown adipose tissue; Control, mice treated without siphonaxanthin; NEFA, nonesterified fatty acid; WAT, white adipose tissue.

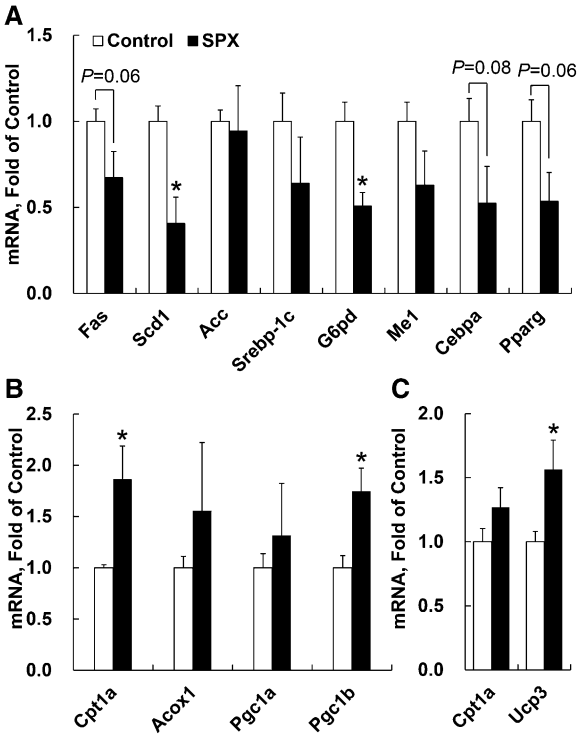

FIGURE 6 Effects of siphonaxanthin on the mRNA expression level of genes involved in lipid metabolism in mesenteric adipose tissue and skeletal muscle. KK-Ay mice were treated with siphonaxanthin for 6 wk. (A) Lipogenesis markers (*Fas*, *Scd1*, *Acc*, *Srebp1c*, *G6pd*, *Me1*, *Cebpa*, and *Pparg*) mRNA expressions in the mesenteric adipose tissue. (B) FA oxidation markers (*Cpt1a*, *Acox1*, *Pgc1a*, and *Pgc1b*) mRNA expressions in the mesenteric adipose tissue. (C) mRNA expression of *Cpt1a* and *Ucp3* in the skeletal muscle. Values are means ± SEMs, $n = 5$ per group. Data were analyzed by Student's t test. *Different from control, $P < 0.05$. *Acc*, acetyl-CoA carboxylase; *Acox1*, acyl-CoA oxidase 1; *Cebpa*, CCAAT/enhancer binding protein α; Control, mice treated without siphonaxanthin; *Cpt1a*, carnitine palmitoyltransferase 1a; *Fas*, fatty acid synthase; *G6pd*, glucose-6-phosphate dehydrogenase; *Me1*, malic enzyme 1; *Pgc1a* peroxisome proliferative activated receptor γ coactivator 1α; *Pgc1b* peroxisome proliferative activated receptor γ coactivator 1β; *Scd1*, stearoyl CoA desaturase 1; SPX, siphonaxanthin; *Srebp1c*, sterol regulatory element binding protein 1c; *Ucp3*, uncoupling protein 3.

expression and function of PPARG and CEBPs are regulated by transcription factors activated by the Akt signaling cascade, including GATA2 and cAMP response element-binding protein (7). Our observation is in accordance with a previous report in which siphonaxanthin inhibited the phosphorylation of Akt at low concentrations (0.1 and 0.5 μmol/L) in vascular endothelial cells (20). In this study, we speculate that siphonaxanthin may have no effect on the upstream components of the Akt signaling cascade, including the insulin receptor, insulin receptor substrate, and PI3K. Further studies are needed to determine how intracellular siphonaxanthin modulates the insulin signaling pathway in the early stage of adipogenesis.

Mitotic clonal expansion (MCE) is known to occur in the early stage of adipogenesis, and it is a prerequisite for terminal differentiation. Numerous studies demonstrated that inhibition of MCE prevents adipogenesis in preadipocytes (31, 32). We evaluated the gene expression of cyclin-dependent kinase 2 (*Cdk2*) as a cell cycle marker, because *Cdk2* regulates the proliferation and DNA replication of preadipocytes during MCE. The gene expression of *Cdk2* was significantly suppressed by siphonaxanthin treatment at 12 h of adipocyte differentiation (data not shown). Further studies are also needed to investigate the effect of siphonaxanthin on cell cycle progression of 3T3-L1 preadipocytes during the MCE process.

During adipogenesis, transient activation of CCAAT/enhancer binding protein β (CEBPB) expression and its phosphorylation by insulin-activated MAPK and glycogen synthase kinase 3β are thought to be involved in the initiation of MCE and the induction of pleiotropic transcription factors, such as CCAAT/enhancer binding protein α (CEBPA) and PPARG (33–35). Our results showed that *Cebpb* gene expression was not influenced by siphonaxanthin treatment. Similar results were also described in 3T3-L1 cells treated with other antiadipogenic substances, such as β-cryptoxanthin, curcumin, and neoxanthin (31, 36, 37). Moreover, it is reported that genistein and dehydrodiconiferyl alcohol did not affect CEBPB protein concentrations, although they inhibited the activity of CEBPB, such as its phosphorylation, DNA-binding, and centromeric localization (32, 38). Interestingly, siphonaxanthin reduced the mRNA levels of *Cebpa* and *Pparg*, which is likely a consequence of CEBPB function in the early phase of adipogenesis. Therefore, we hypothesize that siphonaxanthin may interfere with the activity or the downstream target of CEBPB during adipogenesis.

Maeda et al. (39) reported that dietary fucoxanthin (0.2% of diet) significantly reduced the weight of abdominal WAT and blood glucose in KK-Ay mice fed a high-fat diet, but weight was not altered with <0.1% fucoxanthin. Our observations implied that siphonaxanthin was more effective in inhibiting adipogenesis than fucoxanthin in 3T3-L1 cells and that the weights of the total and mesenteric WAT in KK-Ay mice fed a normal diet were significantly reduced by oral administration of siphonaxanthin at a low dosage (equal to 0.03% dietary siphonaxanthin). Because the mRNA expression levels of *Cebpa* and *Pparg* tended to be lowered in mice treated with siphonaxanthin, similar to the results in the cultured cell model, the reduction in adipose tissue

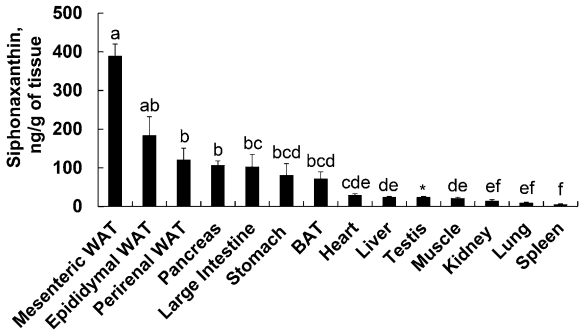

FIGURE 7 Accumulation of siphonaxanthin in the tissues of KK-Ay mice treated with siphonaxanthin for 6 wk. Values are means ± SEMs, $n = 5$. Data were analyzed by Tukey's test except for the testis. Labeled means without a common letter differ, $P < 0.05$. The difference between mesenteric WAT and testis was analyzed by using Student's t test. *Different from mesenteric WAT, $P < 0.05$. BAT, brown adipose tissue; WAT, white adipose tissue.

in siphonaxanthin-treated mice may be partly due to the inhibition of adipocyte differentiation. Another possibility is that lipid accumulation was suppressed in pre-existing adipocytes via the reduction of lipid synthesis and the enhancement of FA oxidation. Sterol regulatory element binding protein 1c (SREBP1C) and its target genes *Scd1*, *Fas*, and *Acc* are involved in adipogenesis and lipogenesis, and they are thought to have an important role in regulating FA synthesis (40). Our results indicated that siphonaxanthin significantly downregulated the gene expression of *Scd1* and *G6pd* in mesenteric adipose tissue and had a tendency to lower the gene expression of *Fas*. In addition, acyl-CoA oxidase 1 (ACOX1) and carnitine palmitoyltransferase 1A (CPT1A) are the key enzymes involved in FA oxidation systems, and the peroxisome proliferator activated receptor γ coactivator 1 (PGC1) family has emerged as a key player in the control of mitochondrial biogenesis and oxidative metabolism in adipose tissue (41, 42). The gene expression of *Cpt1a* and *Pgc1b* in mesenteric adipose tissue was significantly upregulated by siphonaxanthin treatment in KK-Ay mice. Uncoupling proteins (UCPs) in BAT are known to have a major role in whole-body thermogenesis, and the downregulation of their expression contributes to rapid weight gain (43). In particular, UCP3, which is predominantly expressed in skeletal muscle, supports a role in energy balance and lipid metabolism (44). Siphonaxanthin did not modulate the gene expression of *Ucp*s in BAT (data not shown); however, it significantly enhanced the mRNA expression of *Ucp3* in skeletal muscle. These data indicated that the antiobesity effect of siphonaxanthin observed in this study may be caused by the combination of reduction in lipid synthesis and enhancement in FA oxidation.

It was reported that *Pparg*-deficient models and *Pparg* antagonists induce insulin resistance, which is caused by a lack of adequate adipose tissue mass contributing to impaired glucose metabolism and reduction in leptin secretion (45). The antiadipogenic effect of siphonaxanthin would be due to suppression of *Pparg* gene expression, so it is considered whether siphonaxanthin would lead to other deleterious effects, such as a reduction in insulin sensitivity in vivo. In our results, however, plasma glucose concentration tended to decrease by administration of siphonxanthin. Even though further studies are needed, antiadipogenic effects of siphonaxanthin with fewer side effects might be desirable for the prevention of obesity.

In our study, plasma glucose, TG, and cholesterol concentrations of siphonaxanthin-treated mice were 72% ($P = 0.24$), 89% ($P = 0.21$), and 84% ($P = 0.13$) of those in control mice, respectively, but not significant as expected. Compared with the results of the cultured cell study, the modest response of siphonaxanthin in vivo may be due to bioavailability, metabolic fate, and tissue distribution in mice of siphonaxanthin after oral administration. There had been no information about the distribution and accumulation of siphonaxanthin and its metabolites after oral ingestion. In the current study, we found for the first time the selective accumulation of siphonaxanthin in WAT, especially mesenteric WAT. This observation may partly explain the specific effect to mesenteric WAT by oral administration of siphonaxanthin. In addition, some unknown metabolites of siphonaxantin were detected in each tissue including the mesenteric WAT. The biological effect of these metabolites on adipose tissue should be important for the understanding of our findings. As an animal model in this study, we used KK-Ay mice, which spontaneously become obese, hyperinsulinemic, and hyperglycemic, caused by a very strong genetic tendency toward obesity. In addition, the dose of siphonaxanthin was relatively low compared with the effective dose of fucoxanthin in previous reports (14, 16, 39). It would be worthwhile therefore to evaluate the dose response of siphonaxanthin in vivo with the use of other models, such as a high-fat-diet–induced obesity model.

Our results indicate that siphonaxanthin may be a useful dietary supplement for the regulation of lipid accumulation in adipose tissue. To our knowledge, we are the first to suggest such a role for siphonaxanthin in adipogenesis, although further studies are required to understand its mechanism and metabolism.

Acknowledgments

Z-SL and TS designed the experiment; Z-SL and KN conducted the research and analyzed the data; EF provided the methods for critical experiments; Z-SL wrote the manuscript; YM participated in revisions of the manuscript; TH provided instruments and reagents for analyses; and TS had primary responsibility for the final content. All authors read and approved the final manuscript.

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