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Suppressive Effects of Amarouciaxanthin A on 3T3-L1 Adipocyte Differentiation through Down-regulation of PPARγ and C/EBPα mRNA Expression

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ABSTRACT: Fucoxanthin is converted to fucoxanthinol and amarouciaxanthin A in mice. It was previously reported that fucoxanthinol attenuated the adipogenesis of 3T3-L1 cells. However, the effects of amarouciaxanthin A on adipocyte differentiation have not been clarified. This study examined the effects of amarouciaxanthin A on 3T3-L1 adipogenesis by comparing the effects of fucoxanthinol, isofucoxanthinol, and amarouciaxanthin B. Amarouciaxanthin A significantly decreased glycerol-3-phosphate dehydrogenase (GPDH) activity, which was measured as an indicator of adipocyte differentiation. The suppressive effect of amarouciaxanthin A was stronger than that of fucoxanthinol, amarouciaxanthin B, and isofucoxanthinol. The mRNA expressions of adipocyte fatty acid-binding protein (aP2), lipoprotein lipase (LPL), and glucose-transporter 4 (Glut4) in 3T3-L1 cells were markedly down-regulated by amarouciaxanthin A compared to fucoxanthinol. Furthermore, the expression levels of peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT-enhancer-binding protein α (C/EBPα),, which are the key adipogenic transcriptional factors, were also decreased by amarouciaxanthin A during adipocyte differentiation. These results show that amarouciaxanthin A, which is a dominant metabolite of fucoxanthin in white adipose tissue, suppressed 3T3-L1 adipocyte differentiation.

KEYWORDS: Amarouciaxanthin A, PPARγ, C/EBPα, 3T3-L1, adipogenesis, GPDH activity

INTRODUCTION

Obesity is a serious health problem worldwide because it is a major risk factor for type 2 diabetes mellitus, hypertension, and dyslipidemia.[1,2] In obesity, adipocytes accumulate excessive fat and adipocytokine production is disrupted.[3] Adipose tissue development observed in obese individuals is closely related to hypertrophy and hyperplasia, the latter involving proliferation and differentiation of preadipocytes to adipocytes. The peroxisome proliferator-activated receptor (PPAR) and CCAAT-enhancer-binding proteins (C/EBP) families of transcription factors regulate this adipocyte differentiation.[4−7] These transcriptional factors regulate the expression of genes involved in the induction of adipocyte phenotypes, including adipocyte fatty acid finding protein and glucose transporter.[4,8]

We previously reported that a marine carotenoid, fucoxanthin, found in edible brown seaweeds such as *Undaria pinnatifida* and *Laminaria japanica*, suppresses body and white adipose tissue (WAT) weight gain and improves blood glucose concentration in diabetic/obese, KK-A^y mice, but not in normal C57BL/6J mice.[9] Dietary fucoxanthin is metabolized to fucoxanthinol and amarouciaxanthin A,[10] and accumulates in WAT as well as in the plasma and liver.[11] Furthermore, fucoxanthinol inhibits the differentiation of 3T3-L1 preadipocytes to adipocytes through the down-regulation of PPARγ mRNA expression. These results suggest that fucoxanthinol directly suppresses adipocyte differentiation. On the other hand, the effects of amarouciaxanthin A, a dominant metabolite of fucoxanthin that accumulates in the WAT, on adipocyte differentiation are not clear. Because the biological and physiological functions of carotenoids depend on their chemical structures, we examined the effects of amarouciaxanthin A on 3T3-L1 preadipocyte differentiation and compared them to those of amarouciaxanthin B and isofucoxanthinol, which are all similar in structure (Figure 1). Furthermore, we investigated the regulatory effects of amarouciaxanthin A on the mRNA and protein expression of transcriptional factors, PPARγ and C/EBPα, and on mRNA expression of markers for adipocyte differentiation. We found that amarouciaxanthin A suppressed 3T3-L1 differentiation through the down-regulation of PPARγ and C/EBPα mRNA expression. The suppressive effect of amarouciaxanthin A on 3T3-L1 differentiation was stronger than those of fucoxanthinol, isofucoxanthinol, and amarouciaxanthin B.

MATERIALS AND METHODS

Materials. 3T3-L1 preadipocytes were obtained from Dainippon Sumitomo Pharma (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Thermo Scientific (Thermo Electron, Melbourne, Australia). Dulbecco's

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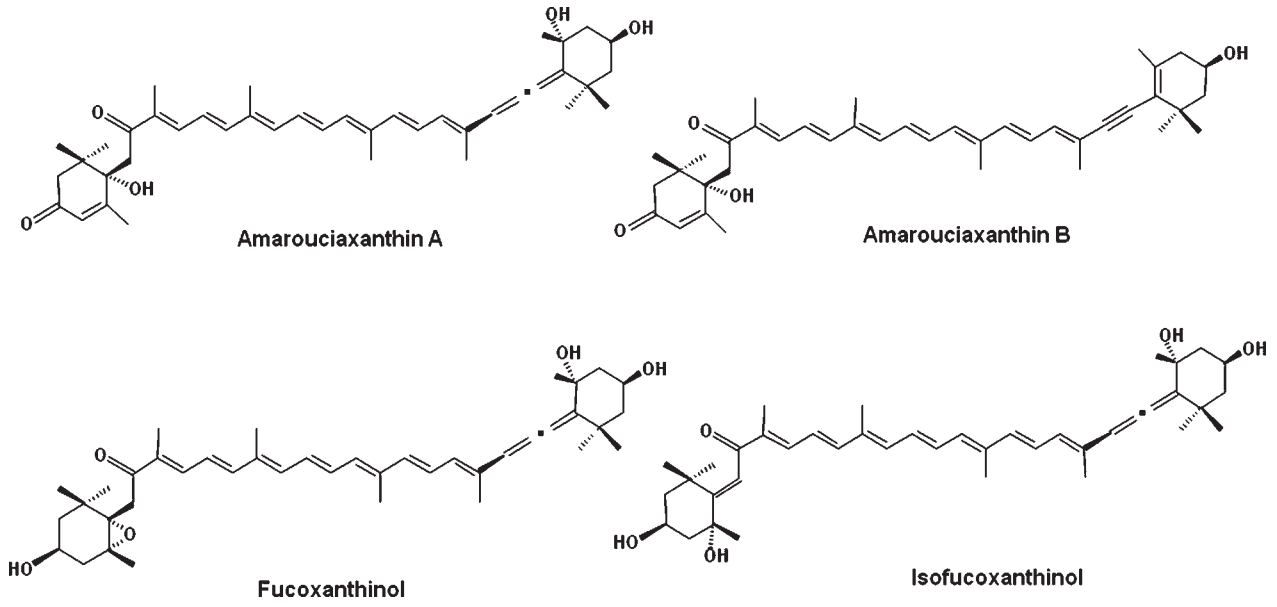

Figure 1. Structures of the carotenoids used in the present study.

modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Insulin, dexamethasone (DEX), and 1-methyl-3-isobutylxanthine (IBMX) were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Carotenoids. Amarouciaxanthin A, amarouciaxanthin B, fucoxanthinol, and isofucoxanthinol were used in this study (Figure 1). Crude seaweed lipids containing fucoxanthin were extracted from the commercially available, dry *U. pinnatifida* powder mix with acetone. Fucoxanthin was purified from crude seaweed lipids by silica gel column chromatography as previously described.[12] Fucoxanthinol was prepared from the isolated fucoxanthin by hydrolysis with porcine pancreas lipase (Sigma Chemical Co.).[12] Isofucoxanthinol was prepared from fucoxanthinol by alkaline treatment with 1% KOH for 35 min at room temperature. Amarouciaxanthin B and amarouciaxanthin A were separated from acetone extracts from *Amaroucium pliciferum* with silica gel column chromatography using acetone/*n*-hexane (1:9, v/v) and acetone/*n*-hexane (3:7, v/v), respectively. Finally, all of the carotenoids were purified using a high-performance liquid chromatography (HPLC, Hitachi, Tokyo, Japan) system equipped with an ODS column. The purities of carotenoids were >98% by HPLC analysis.

3T3-L1 Culture. 3T3-L1 preadipocytes were cultured in DMEM with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin until confluence and maintained for an additional 48 h at 37 °C in an atmosphere of 5% CO_2. Differentiation was induced by the addition of DMEM containing 10 μg/mL insulin, 0.5 mM IBMX, and 0.1 μM DEX (differentiation medium I) for 48 h. Cells were then incubated in DMEM containing 5 μg/mL insulin (differentiation medium II) for 48 h and further cultured in DMEM without insulin, which was replaced with fresh medium every 48 h. Carotenoids were added as ethanol solution to DMEM after a 48 h incubation period with differentiation medium II. The final ethanol concentration in the medium was adjusted to 0.1% without cytotoxicity.

Measurement of Glycerol-3-phosphate Dehydrogenase Activity. Glycerol-3-phosphate dehydrogenase (GPDH, EC 1.1.1.8) activity was measured using a commercial assay kit (Hokudo Co., Ltd., Sapporo, Japan) according to the manufacturer's instructions. 3T3-L1 cells were incubated in the carotenoid-containing DMEM for 2 days after incubation in the differentiation medium II. The cells were dissolved in enzyme extract solution after washing twice with PBS. The cell lysate was then homogenized on ice by supersonic waves and centrifuged at 2800*g* for 5 min at 4 °C. The supernatant was used for the measurement of GPDH activity. The protein content was measured with a DC protein assay kit (Bio-Rad Laboratories, Inc., Tokyo, Japan).

Total RNA Isolation and Analysis. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's protocol. For polymerase chain reaction (PCR) analysis, cDNA was first synthesized from the total RNA using the high-capacity cDNA archive kit (Applied Biosystems Japan Ltd., Tokyo, Japan). Then, real-time quantitative PCR analysis was performed using an ABI Prism 7500 (Applied Biosystems Japan Ltd.). After initial incubation at 50 °C for 2 min, the cDNA was denatured at 95 °C for 10 min, followed by 95 °C for 15 s and 60 °C for 1 min. The primers used were as follows: PPARγ, Mm01184322_m1; C/EBPα, Mm00514283_s1; aP2, Mm00445880_m1; Glut4, Mm 01245507_g1; LPL, Mm 00434764_m1; and GAPDH, Mm99999915_g1 (Applied Biosystems Japan Ltd.).

Western Blot Analysis. 3T3-L1 cells were incubated in DMEM containing 10 μM amarouciaxanthin A for 48 h after incubation with differentiation medium II for 48 h. Cells were lysed in ice-cold lysis RIPA buffer (20 mM Tris-HCl, pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.1 mg/mL phenylmethanesulfonyl fluoride (PMSF), 50 μg/mL aprotinin, and 1 mM sodium orthovanadate. Cell lysates were centrifuged at 15000 rpm for 20 min at 4 °C, and the supernatant was separated. The supernatant was estimated for protein measurement using a Bio-Rad DC protein assay kit. The extracted protein were separated by 10% SDS−polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane. After the membrane had been blocked with 0.5% skim milk in tris-buffered salt (TBS) containing 0.1% Tween-20 for 1 h, membranes were incubated with rabbit monoclonal antibody against PPARγ (1:200) for 1 h and then with secondary antibody rabbit IgG-conjugated horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. Immunoactive bands on membranes were visualized using Chemiluminescence detection kit (ECL system, Amersham GE Healthcare, USA) according to the manufacturer's instructions.

Molecular Simulation. The molecular structures of amaouciaxanthin A and amarouciaxanthin B were constructed based on Discovery Studio (DS) 2.5 software (Accelrys, Inc., San Diego, CA) on a PC terminal (Express; NEC, Tokyo, Japan) linked with Regatta (96 nodes; IBM). Energy minimization was achieved using a salvation model and calculated by the GBSW parameter with Minimization and Dynamics protocols

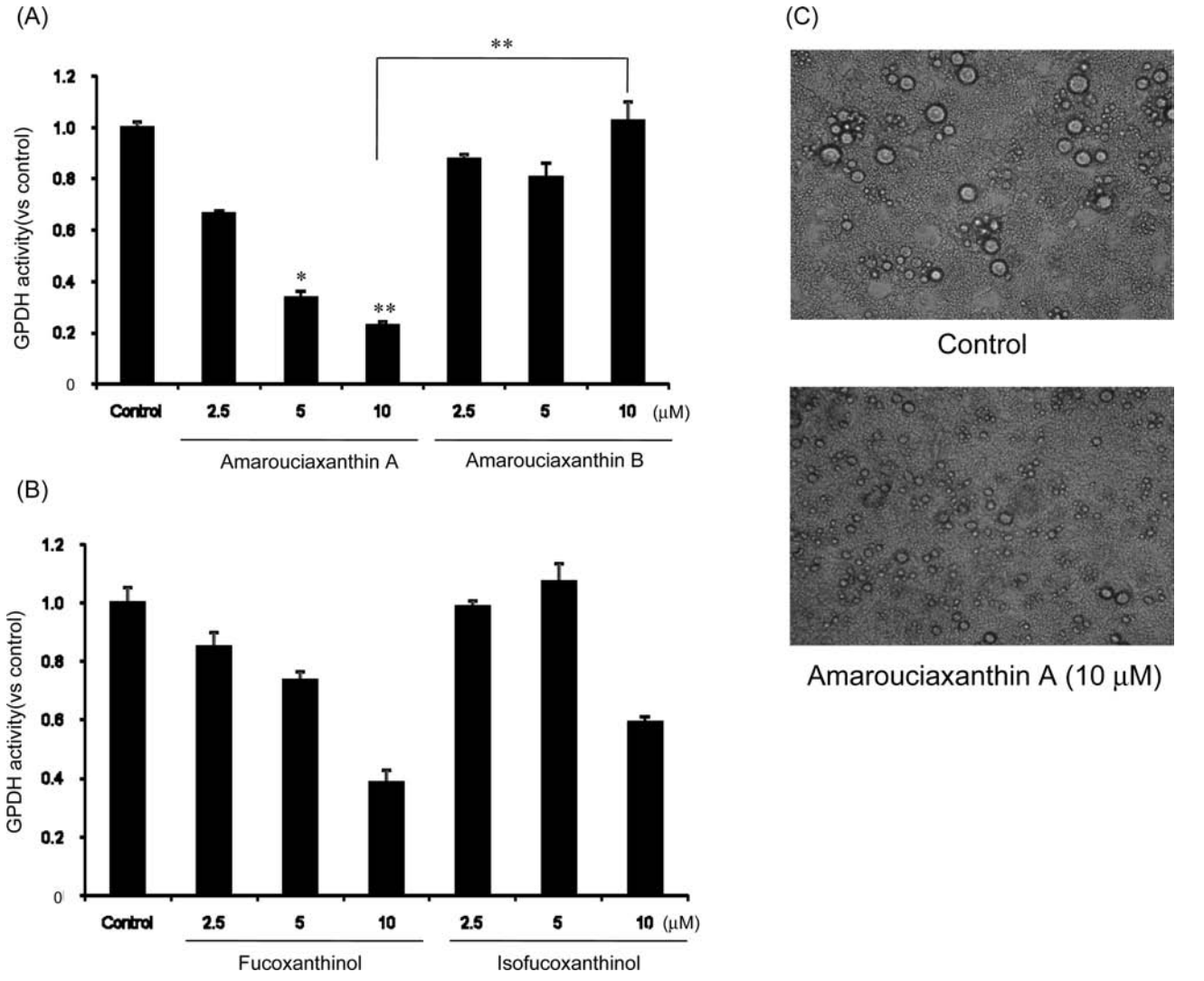

Figure 2. Effect of carotenoids on GPDH activity and oil droplets in 3T3-L1cells. (A) The cells were treated with (A) amarouciaxanthin A and amarouciaxanthin B or (B) fucoxanthinol and isofucoxanthinol for 2 days after incubation in differentiation medium II for 2 days. (C) Morphological observation of 3T3-L1 cells treated with ethanol (control) or 10 μM amarouciaxanthin A. Values represent the mean ± SD of three independent experiments. **, $P < 0.01$, *, $P < 0.05$, versus control or between two groups.

within DS. The calculation used a CHARMm (Chemistry at HARvard Macromolecular Mechanics) force field.

Statistical Analysis. Results are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA with the Tukey−Kramer test. P values of <0.05 and <0.01 were considered to be significant differences.

RESULTS

Effect of Carotenoids on GPDH Activity. GPDH has an important role in the conversion of glycerol to triglyceride during adipocyte differentiation. We, therefore, measured the GPDH activity of 3T3-L1 cells treated with carotenoids as an indicator of adipocyte differentiation. Amarouciaxanthin A possessing an allenic bond decreased GPDH activity in a dose-dependent manner (Figure 2A), although no toxicity was observed in 3T3-L1 cells treated with amarouciaxanthin A at 10 μM (data not shown). In 3T3-L1 cells incubated with 10 μM amarouciaxanthin A for 48 h, GPDH activity markedly decreased to 20% of that observed in control cells. Fucoxanthinol also decreased GPDH activity, as in our previous study.[13] Interestingly, GPDH activity was lower in 3T3-L1 cells treated with amarouciaxanthin A than with fucoxanthinol (Figure 2B). On the other hand, there was no significant change in the GPDH activity of 3T3-L1 cells treated with amarouciaxanthin B containing an acetylenic bond. Isofucoxanthinol decreased GPDH activity at 10 μM dose without cytotoxicity. In 3T3-L1 cells treated with 10 μM amarouciaxanthin A for 48 h, the sizes of the oil droplets were also smaller than those of the control cells (Figure 2C).

Effect of Amarouciaxanthin A on mRNA Expression of aP2, LPL, and Glut4. Because amarouciaxanthin A and fucoxanthinol decreased GPDH activity, we then examined the mRNA expressions of aP2, LPL, and Glut4, all late markers of adipocyte differentiation. Amarouciaxanthin A down-regulated aP2 mRNA expression in a dose-dependent manner (Figure 3A). The mRNA expression levels of Glut4 and LPL were also down-regulated by amarouciaxanthin A and fucoxanthinol (Figure 3B,C).

Effect of Amarouciaxanthin A and Amarouciaxanthin B on PPARγ and C/EBPα mRNA Expression. PPARγ and C/EBPα are principal regulators of adipogenesis.[14] To investigate whether

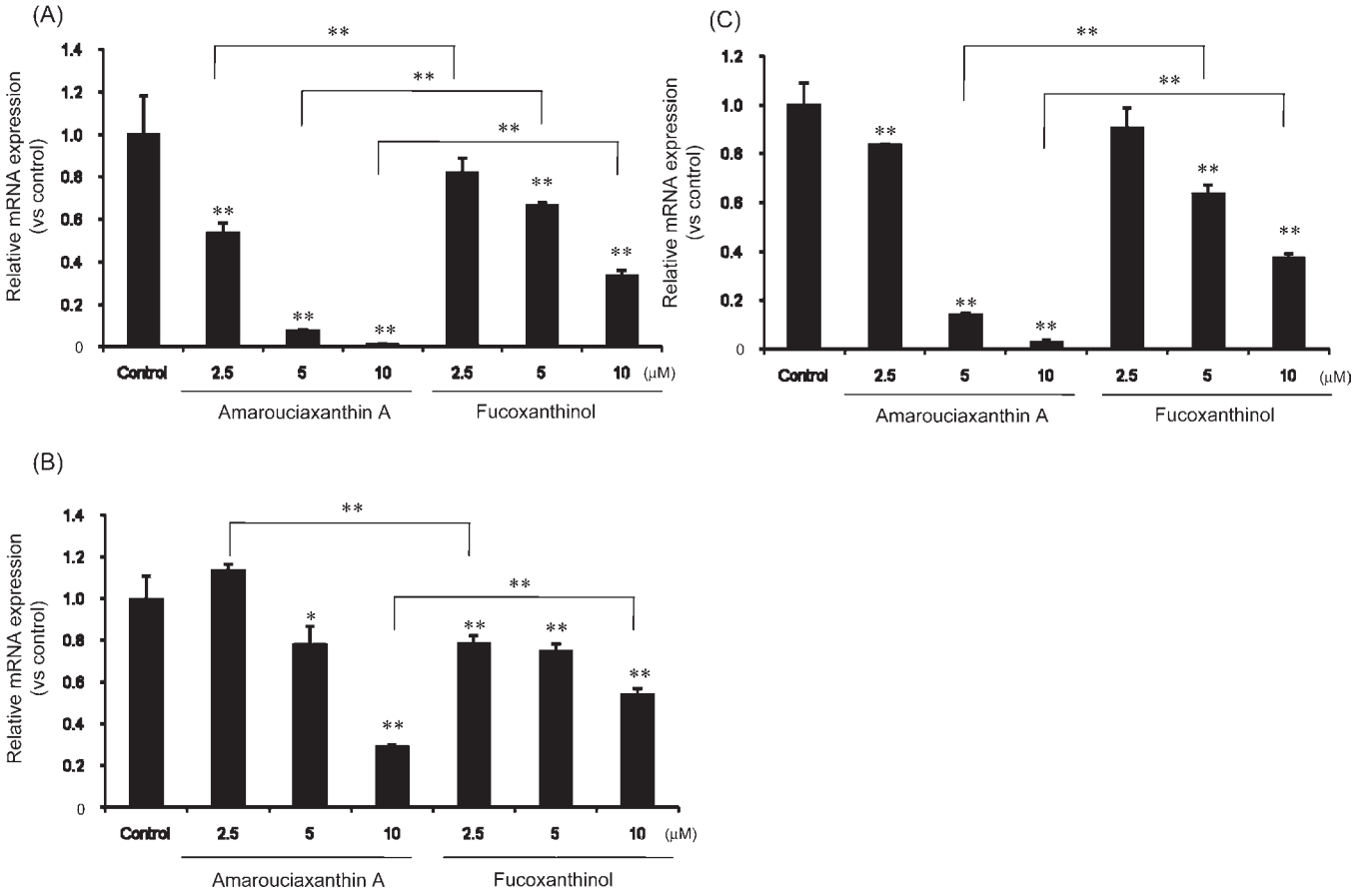

Figure 3. Effect of amarouciaxanthin A and fucoxanthinol on the mRNA expression of aP2, LPL, and Glut4. The cells were incubated with DMEM with amarouciaxanthin A or fucoxanthinol for 2 days after incubation with differentiation medium II for 2 days. The mRNA expression levels of aP2 (A), LPL (B), and Glut4 (C) were measured by quantitative real time PCR and expressed as a ratio to control cells levels (=1.0) after normalization using GAPDH mRNA expression levels. Values represent the mean ± SD of three independent experiments. **, $P < 0.01$, *, $P < 0.05$, versus control or between two groups.

amarouciaxanthin A suppresses adipogenesis through the PPARγ and C/EBPα pathway, we carried out quantitative real time PCR. As shown in Figure 4, amarouciaxanthin A significantly down-regulated mRNA expression of PPARγ and C/EBPα, which are the central determinants of the transcriptional factor during adipocyte differentiation. Amarouciaxanthin B also down-regulated PPARγ and C/EBPα mRNA expression in 3T3-L1 cells (Figure 4C,D). However, suppressive effects of amarouciaxanthin B on PPARγ and C/EBPα mRNA expression in 3T3-L1 cells were weaker than that of amarouciaxanthin A at 5 and 10 μM.

We further examined PPARγ and C/EBPα expression level by Western blotting. The expression level of PPARγ was lower than in control cells (Figure 5). The C/EBPα expression level also tended to decrease by amarouciaxanthin A. These results indicate that amarouciaxanthin A suppresses PPARγ and C/EBPα expression by down-regulating mRNA transcription.

DISCUSSION

Fucoxanthin is an important marine carotenoid that is found in edible brown seaweeds and microalgae. We previously reported that dietary fucoxanthin suppressed body and WAT weight gain in diabetic/obese KK-A^y mice.[9,15] Furthermore, we also indicated that fucoxanthinol, one of the fucoxanthin metabolites in the body, suppresses 3T3-L1 cell differentiation.[13] In the present study, we demonstrated for the first time that amarouciaxanthin A, which is another fucoxanthin metabolite detected in WAT, decreased GPDH activity, thus playing an important role in the triglyceride synthesis pathway during adipocyte differentiation. It is noteworthy that the GPDH activity of 3T3-L1 cells treated with 5 μM amarouciaxanthin A with an allenic bond was lower than that of amarouciaxanthin B with an acetylenic bond. The suppressive effect of amarouciaxanthin A on GPDH activity also tended to be stronger than those of fucoxanthinol and isofucoxanthinol. The incidence of obesity is related to the differentiation of preadipocytes to adipocytes and enlargement of adipocytes in adipose tissue.[16] Because amarouciaxanthin A is a dominant fucoxanthin metabolite that accumulates in WAT, the present results show that amarouciaxanthin A might be the molecule responsible for the antiobesity effect of fucoxanthin in vivo. We previously found that neoxanthin with an allenic bond suppresses 3T3-L1 cell differentiation, whereas lutein and β-carotene do not.[17] Therefore, allenic bonds may be important for the suppression of 3T3-L1 cell differentiation.

To obtain information regarding the structure of amarouciaxanthin A, computational analysis was performed using molecular simulation and surface analysis software. Amarouciaxanhin A shows the energy-minimized, three-dimensional structure of Figure 6A. The structure of the allenic bond of the side in the molecule is bulky compared to lutein, which did not show an inhibitory effect on 3T3-L1 differentiation in a previous study.[18] The three-dimensional structure of amarouciaxanthin A may be important for the inhibitory effect on 3T3-L1 differentiation. On the other

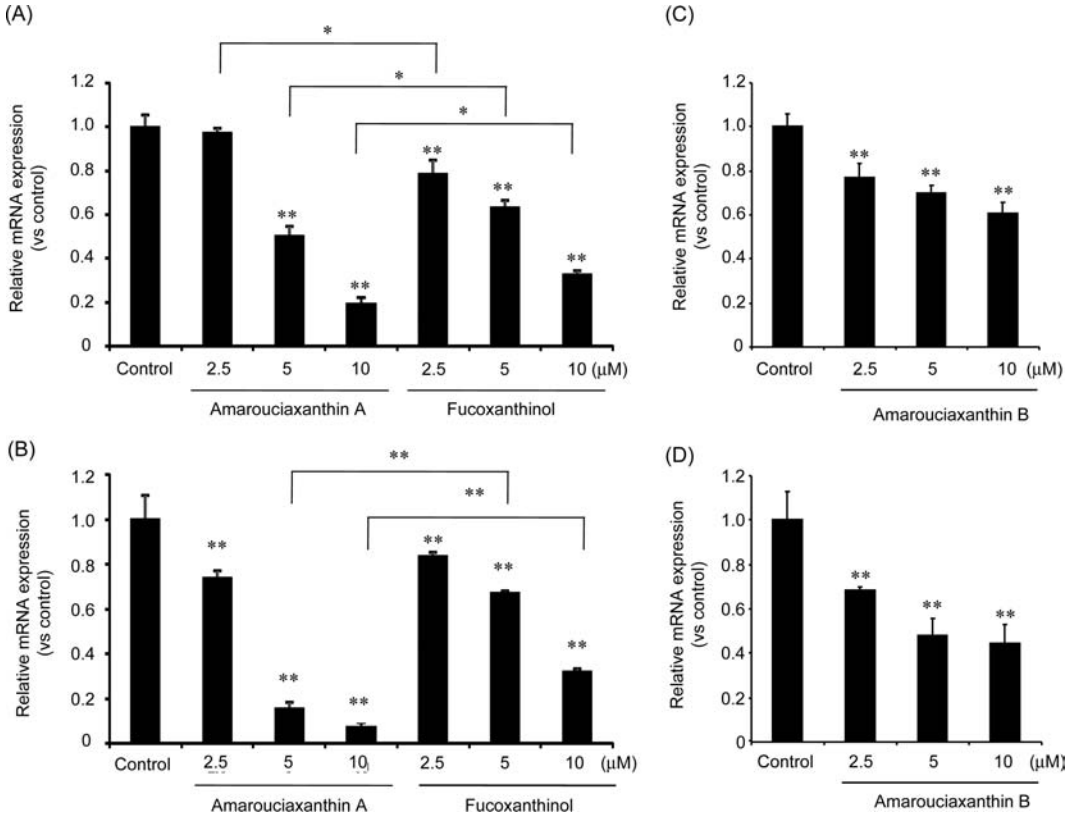

Figure 4. Effect of carotenoids on the mRNA expression of PPARγ and C/EBPα in 3T3-L1 cells. The cells were treated with amarouciaxanthin A, fucoxanthinol, or amarouciaxanthin B for 2 days after induction with differentiation medium II for 2 days. The mRNA expression levels of PPARγ (A, C) and C/EBPα (B, D) were measured by quantitative real time PCR and expressed as a ratio to control cells levels (=1.0) after normalization using GAPDH mRNA expression levels. Values represent the mean ± SD of three independent experiments. **, $P < 0.01$, *, $P < 0.05$, versus control or between two groups.

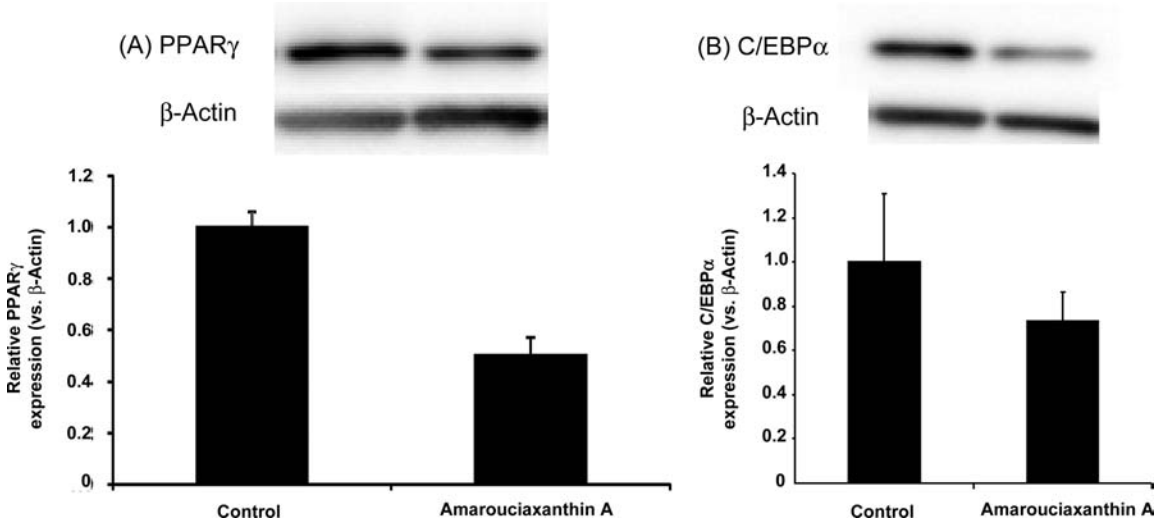

Figure 5. PPARγ and C/EBPα expression of 3T3-L1 cells treated with amarouciaxanthin A. The cells were treated with 10 μM amarouciaxanthin A for 2 days after induction with differentiation medium II for 2 days. PPARγ (A) and C/EBPα (B) protein levels were detected by Western blot analysis using the ECL detection reagent. The PPARγ and C/EBPα expression levels are represented as a ratio to control cells levels (=1.0) after normalization using β-actin levels. Values represent the mean ± SD of three independent experiments.

hand, amarouciaxanthin B with an acetylenic bond also showed a three-dimensional structure similar to that of amarouciaxanthin A, although the suppressive effect on 3T3-L1 differentiation was different between amarouciaxanthin A and amarouciaxanthin B. Furthermore, in the present study, we observed that the suppressive effects of allenic carotenoids such as amarouciaxanthin A, fucoxanthinol, and isofucoxanthinol on adipocyte differentiation were not similar. These results show that other structures

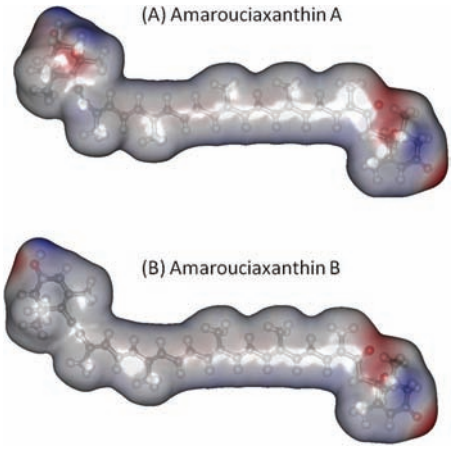

Figure 6. Three-dimensional structures of (A) amarouciaxanthin A and (B) amarouciaxanthin B. Carbon, oxygen, and hydrogen atoms are indicated in gray, red, and white, respectively. Blue areas are positively charged, red areas are negatively charged, and white areas are neutral.

and chemical properties are also important in regulating adipocyte differentiation. Further studies are required to clarify active structures and properties of carotenoids to inhibit adipocyte differentiation.

The differentiation process of 3T3-L1 cells is coordinated by various transcription factors, including these belonging to PPAR and C/EBP families.[19] PPARγ and C/EBPα, in particular, are the principal regulators of terminal adipogenesis. Amarouciaxanthin A markedly down-regulated PPARγ and C/EBPα mRNA in 3T3-L1 cells. These results suggest that the suppression of 3T3-L1 differentiation by amarouciaxanthin A is, at least partly, involved in the down-regulation of PPARγ and C/EBPα mRNA. In addition, aP2, LPL, and Glut4 mRNA were also down-regulated by amarouciaxanthin A. LPL and aP2 mRNA are adipocyte-specific genes involved in fatty acid metabolism regulated by PPARγ and C/EBPα.[20−22] In addition, Glut4 also creates and maintains the adipocyte phenotype.[23] From these results, the suppressive effects of amarouciaxanthin A on adipocyte-specific mRNA expression are suggested to be mediated by the down-regulation of PPARγ and C/EBPα mRNA expression.

In conclusion, amarouciaxanthin A, which is a dominant metabolite of fucoxanthin accumulated in the WAT, suppressed 3T3-L1 adipocyte differentiation through the down-regulation of PPARγ and C/EBPα mRNA expression. The suppressive effect of amarouciaxanthin A on 3T3-L1 adipogenesis was stronger than those of fucoxanthinol, isofucoxanthinol, and amarouciaxanthin B.

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ABBREVIATIONS USED

GPDH, glycerol-3-phosphate dehydrogenase; aP2, adipocyte fatty acid binding; LPL, lipoprotein lipase; Glut4, glucose transporter 4; PPARγ, peroxisome proliferator-activated receptor γ; C/EBP, CCAAT enhancer binding protein; WAT, white adipose tissue; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; DEX, dexamethasone; IBMX, 1-methyl-3-isobutylxanthine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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