β-Cryptoxanthin Suppresses the Adipogenesis of 3T3-L1 Cells via RAR Activation

Yoshiyuki SHIRAKURA1, Katsuhiko TAKAYANAGI1, Katsuyuki MUKAI1,*
Hiroki TANABE2 and Makoto INOUÉ2

1Research and Development Center, Unitika Ltd., 23 Kozakura, Uji, Kyoto 611–0021, Japan
2Laboratory of Medicinal Resources, School of Pharmacy, Aichi Gakuin University,
1–100 Kussamoto-cho, Chikusa-ku, Nagoya 464–8650, Japan
(Received January 25, 2011)

Summary We recently reported that the oral intake of β-cryptoxanthin exerted anti-obesity effects by lowering visceral fat levels. In the present study, we characterized the molecular mechanisms underlying the lipid-lowering effects of β-cryptoxanthin on 3T3-L1 cells. Consistent with our previous findings, β-cryptoxanthin rapidly reduced the level of intracellular lipids in 3T3-L1 cells as assessed by Oil red O staining. Using an in vitro nuclear receptor binding assay, we demonstrated the ability of β-cryptoxanthin to bind to and activate members of the retinoic acid receptor (RAR) family. Accordingly, treatment of cells with LE540, an RAR antagonist, abolished the β-cryptoxanthin-dependent suppression of 3T3-L1 adipogenesis, suggesting that β-cryptoxanthin mediates its effects on 3T3-L1 cells via RAR activation. In addition, real-time RT-PCR analysis revealed that β-cryptoxanthin down-regulates mRNA expression of PPARγ, a key regulator of adipocyte differentiation, and that this inhibition was blocked by LE540 treatment. Taken together, these data indicate that RAR activation contributes to the molecular mechanism by which β-cryptoxanthin prevents obesity.

Key Words β-cryptoxanthin, carotenoid, adipocyte, RAR, Satsuma mandarin

Carotenoids are a group of more than 700 pigments that are biosynthesized de novo in plants, algae, fungi, and bacteria. Carotenoids display many beneficial effects, including the ability to act as provitamin A and precursors of retinol and retinoic acid. Accordingly, carotenoids are known to prevent common chronic diseases such as cardiovascular disease, age-related macular degeneration, and cancer (1). As animals are unable to synthesize carotenoids, they must obtain them from external sources.

Carotenoids are obtained from the diet in humans, and approximately 12 types are measurable in blood and other tissues, including lycopene, lutein, α-carotene, β-carotene, zeaxanthin, and β-cryptoxanthin (2, 3). Among these, the availability of β-cryptoxanthin, which is found in mango, papaya, persimmon, and Satsuma mandarin, is limited. Accordingly, the beneficial effects of β-cryptoxanthin have been less intensely investigated. β-Cryptoxanthin displays both structural and functional similarities to β-carotene (4). Recent studies have reported that β-cryptoxanthin exerts bone mineralization effects (5), whilst large-scale cohort studies have identified a negative correlation between serum β-cryptoxanthin and insulin resistance (6).

We previously reported that β-cryptoxanthin significantly reduced visceral fat levels, blood glucose, triglycerides, body weight, and waist circumference in placebo-controlled double-blind trials (7). β-Cryptoxanthin in these trials was extracted from the pulp of the Satsuma mandarin (Citrus Unshiu Marc.) after juicing with ethanol and emulsification to produce a β-cryptoxanthin-rich Emulsified Mandarin Extract (EME).

Adipocytes play important roles in energy homeostasis by storing excess energy as triglycerides, which are released as fatty acids in response to nutritional signals or starvation (8). Adipocytes additionally secrete hormones and adipokines, which regulate insulin sensitivity, energy balance, and blood pressure. Excessive fat accumulation in adipocytes disturbs adipokine secretion and increases the risk of disease, including type II diabetes, hypertension, and hyperlipidemia. Excessive fat also promotes adipocyte differentiation, which contributes in part to obesity and metabolic syndrome.

We assumed that the anti-obesity effects of β-cryptoxanthin are closely related to adipocyte function. In the present study, we investigated the molecular effects of β-cryptoxanthin on the differentiation of and lipid accumulation in mouse 3T3-L1 cells, a commonly used pre-adipocyte model that differentiates into mature triacylglyceride-storing adipocyte-like cells.

MATERIALS AND METHODS

Materials. Mouse 3T3-L1 (CCL-92.1) pre-adipocytes were purchased from the American Type Culture Collection (Rockville, CT, USA). Fetal bovine serum, insulin, and dexamethasone were purchased from Invitrogen (Tokyo, Japan). Dulbecco’s modified Eagle’s medium
β-CryptoxanthinSuppressesAdipogenesisof3T3-L1

(DMEM), penicillin-streptomycin-amphotericin B suspension (100 U/mL), LE540, isobutyl-methylxanthine, dimethyl sulfoxide, trypsin-EDTA and Oil red O lipid assay kits were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). β-Cryptoxanthin was purchased from Shikoku Yashima Pure Chemicals (Toku-shima, Japan). Formaldehyde neutral buffered solution (10%) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Commercial in vitro nuclear receptor assay kits, RCAS kits, and Nu ligand kits were purchased from Enbiotech Laboratories (Tokyo, Japan) and Mycosystems (Kyoto, Japan), respectively. Isogen, an RNA extracting reagent, was purchased from Nippon Gene Co., Ltd. (Tokyo, Japan). Enzymes for reverse-transcription (PrimeScript RT reagent kit) and real-time PCR (Sybr Premix Ex taq) were purchased from TaKaRa Bio Inc. (Shiga, Japan).

3T3-L1 culture. 3T3-L1 cells were cultured in DMEM with 10% FBS, 50 μg/mL penicillin, 50 μg/mL streptomycin, and 125 ng/mL amphotericin at 37°C under 95% humidified air/5% CO2. Once the cells had reached confluency, they were incubated for an additional 48 h, then differentiated in differentiation medium I containing 10 μg/mL insulin, 0.5 mM isobutyloxanthin, and 1 μM dexamethasone. Next, the medium was replaced with differentiation medium II containing 10 μg/mL insulin and exchanged every 48 h. β-Cryptoxanthin and LE540 were dissolved in DMSO and added to media I and II accordingly.

Oil red O staining. Intracellular lipid accumulation was assessed by Oil red O staining during adipocyte differentiation. Briefly, 3T3-L1 cells were incubated with β-cryptoxanthin or β-cryptoxanthin and LE540-supplemented differentiation media I and II for 10 d. The cells were then washed with PBS and fixed in 10% formaldehyde neutral buffer solution at room temperature for 2 h. The fixed cells were washed with distilled water and stained using a lipid assay kit according to the manufacturer’s protocol. The level of lipid was represented by the absorbance of extracted Oil red O at 540 nm from a cell. Cell number was counted after trypsin-EDTA treatment with plates which were prepared with the same timing and operations as the stained ones.

Nuclear receptor binding assays. Nuclear receptor binding assays were performed using RCAS and Nu ligand kits according to the manufacturer’s protocol. The RCAS kit measures peroxisome proliferator-activated receptor (PPAR) γ and retinoid X receptor (RXR) α activation following β-cryptoxanthin treatment, whilst the Nu ligand kit assesses retinoic acid receptor (RAR) α and γ. All results were normalized to the positive controls; troglitazone, tributyltin chloride, and all-trans retinoic acid (atRA) for PPARγ, RXRα, and RARs, respectively.

RNA isolation and analysis. Total RNA from 3T3-L1 differentiated cell lysates at 48 and 92 h after initiation was isolated with Isogen according to the manufacturer’s protocol. For reverse transcription-polymerase chain reaction (RT-PCR) analysis, cDNA was synthesized from the total RNA using a PrimeScript RT reagent kit. Real-time quantitative RT-PCR analysis was performed using Sybr Premix Ex taq and automated sequence detection systems (StepOne: Applied Biosystems Japan Ltd., Tokyo, Japan). The cycling conditions were as follows: 40 cycles of 95°C for 15 s followed by 60°C for 1 min. The primers for PCR (PPARγ, c/EBPβ, aP2, RARγ2, and CRABP II) were purchased from Sigma Aldrich Japan (Tokyo, Japan). The sequences of the primers were PPARγ sense (5′-GATGCAGCTGCTAT-GAGCCTT-3′) and antisense (5′-AGAGGGTCCACAGAGCTGATTCC-3′) (9), c/EBPβ sense (5′-GCAAAGCGCGCGACAAG-3′) and antisense (5′-GGCTCGGGCAGCTGCTT-3′) (10), aP2 sense (5′-CAGAAATGGAATGAAAGTCT-3′) and anti-sense (5′-CGACTGACATGATGTGTTTGA-3′) (11), RARγ2 sense (5′-TGTTGTTCTCAGCACCAGTT-3′) and antisense (5′-AAAGAATCCAGTCAGCTGCT-3′) (12), and CRABP II sense (5′-CTGGAAGGCGAGAAGTCC-3′) and antisense (5′-TGGAGGGAGGTTGCTGTGTA-3′) (13).

Statistical analyses. All data are given as the mean±SD. Statistical significance was determined using Student’s t-test and Tukey’s test; p<0.05 was considered to be statistically significant.

RESULTS

β-Cryptoxanthin inhibits lipid accumulation in 3T3-L1 cells

We first assessed the ability of β-cryptoxanthin to inhibit lipid accumulation in 3T3-L1 cells by measuring the intensity of Oil red O staining in treated cells. As shown in Fig. 1, 3T3-L1 cells incubated with 1 μM β-cryptoxanthin showed a 25% reduction in intracellular lipids compared to untreated cells, whilst a reduction of 40% was observed at 10 μM β-cryptoxanthin. No cytotoxic effect of β-cryptoxanthin to 3T3-L1 cells was observed by counting cell number at the time of staining. These results indicate that β-cryptoxanthin potently inhibits intracellular lipid accumulation in 3T3-L1 cells.

β-Cryptoxanthin is a ligand of the RAR α and γ

Previous reports have demonstrated the ability of β-cryptoxanthin to act as an RAR ligand (14). We next sought to investigate the mechanism by which β-cryptoxanthin inhibits intracellular lipid accumulation by assessing the binding ability of β-cryptoxanthin to PPARγ and RXRα (RCAS kit) (Fig. 2A) and RARα and γ (Nu ligand kit) (Fig. 2B). As shown in Fig. 2A, we observed no binding of β-cryptoxanthin to PPARγ or RXRα. In contrast, Fig. 2B demonstrates the efficient binding of β-cryptoxanthin to RXRα and γ. These results confirm the ability of β-cryptoxanthin to act as a ligand of RXRα and γ. On the other hand, the addition of β-cryptoxanthin to the RCAS kit did not affect PPARγ activation by troglitazone (data not shown). This result indicates that β-cryptoxanthin cannot act as an antagonist to PPARγ.

LE540 inhibits the effects of β-cryptoxanthin on 3T3-L1 adipogenesis

To confirm whether β-cryptoxanthin affects 3T3-L1
Fig. 1. β-Cryptoxanthin inhibits lipid accumulation in 3T3-L1 cells. The accumulation of intracellular lipids was determined by Oil red O staining 10 d following the initiation of differentiation. There were two plates for each experiment which were prepared with the same timing and operation. One plate was used for the staining; the other was used for cell counting. The staining was performed and then the intensity was measured following extraction by isopropyl alcohol at 540 nm. Cell number was counted with a hemocytometer after trypsin treatment. Panels A and B are the results of the control and β-cryptoxanthin-supplemented cultures, respectively. Panel C shows the relative levels of accumulated lipid compared to the control. Panel D shows cell viability of each sample. Each value is the ratio of absorbance at 540 nm to the cell number. Each value represents the mean±SD (n = 3). **p<0.01 vs control (DMSO).

Fig. 2. Nuclear receptor binding of β-cryptoxanthin. Agonist-dependent interactions between nuclear receptors and enzyme-binding (A) antibodies and (B) co-activators were determined as (A) horseradish peroxidase and (B) alkaline phosphatase activity. Gray lines represent the results for the positive-agonists while dark lines represent the results for β-cryptoxanthin. The agonists of PPARγ, RXRα, and RARs were troglitazone, tributyltin chloride, and atRA, respectively. Each value represents the mean±SD (n = 3).
Cryptoxanthin Suppresses Adipogenesis of 3T3-L1 via RAR and RXR Activation

We assessed the effects of cryptoxanthin in the presence of LE540, an RAR pan-antagonist (15). As shown in Fig. 3, whilst cryptoxanthin inhibited lipid accumulation at a concentration of 10 μM, inhibition was blocked in the presence of 5 μM LE540. No cytotoxic effect was observed by treatments of cryptoxanthin and LE540. These results indicate that cryptoxanthin inhibits 3T3-L1 adipogenesis via RARα and γ activation.

Assessment of the effects of cryptoxanthin on mRNA expression in 3T3-L1 adipocytes

To gain further insight into the role of cryptoxanthin inhibition on lipid accumulation in 3T3-L1 cells, real-time RT-PCR was performed following cryptoxanthin treatment. As shown in Fig. 4, cryptoxanthin decreased mRNA levels of PPARG but not C/EBPβ at concentrations of 1 and 10 μM. This inhibition did not occur in the presence of 5 μM LE540. Taken together with our in vitro binding assays, these data demonstrate that cryptoxanthin activates CRABP II and RARγ2 in response to RAR activation (12, 13). From these results, we conclude that cryptoxanthin affects 3T3-L1 differentiation via the down-regulation of PPARG expression through RAR activation.

Discussion

We previously reported that the oral intake of EME-supplemented juice reduces not only visceral fat but also blood glucose, triglycerides, body weight, and waist circumference. Adipocytes, through the secretion of adipokines, regulate many important physiological processes, including insulin sensitivity, energy balance between storage and consumption, and blood pressure. We therefore hypothesized that the effects of EME intake are based on adipocyte modulation.

In the present study, we found that cryptoxanthin, a major carotenoid found in blood, suppresses 3T3-L1 adipogenesis. Several nuclear receptors were reported to be involved in the adipocyte differentiation (16). Among them, PPARγ, RXRα, RARα, and RARγ were important. For example, PPARγ is the master regulator of adipocyte differentiation to control adipocyte-specific gene expressions (17). RXRα forms a hetero-dimer with PPARγ and the hetero-dimer is required for early differentiation of adipocytes (18). These reports indicate PPARγ and RXRα were involved in the adipocyte differentiation. On the other hand, all-trans retinoic acid also suppresses the adipocyte differentiation together with RARs. RAR has three isoforms, named RARα/β/γ. Among them, RARα and RARγ are reported to be expressed in preadipocytes predominantly (19), suggesting their involvement in the adipocyte differentiation. To identify the functional contribution of these nuclear receptors to the adipocyte differentiation and
the ligand activity of β-cryptoxanthin, we performed a nuclear receptor binding assay.

From the result of the binding assay, we discovered the ability of β-cryptoxanthin to affect 3T3-L1 differentiation through its function as a ligand of RARα and γ; moreover, we showed that this effect could be blocked through the application of LE540, an RAR antagonist. Real-time RT-PCR analysis showed that β-cryptoxanthin down-regulates mRNA expression of PPARγ, a key regulator of differentiation via C/EBPβ. These results strongly indicate that β-cryptoxanthin inhibits 3T3-L1 adipogenesis via the down-regulation of PPARγ transcription, through RAR activation. Like β-cryptoxanthin, atRA, an original RAR ligand in vivo, affects cellular differentiation. AtRA has been widely applied as a medicine for specific types of blood cancer; however, serious side effects following its application have been reported (20). As shown in Fig. 4, the RAR-ligand activity of β-cryptoxanthin did not reach the potency of atRA. However, as the serum concentrations of β-cryptoxanthin are higher and show longer-lived RAR activity in the absence of side effects, β-cryptoxanthin may present an attractive substitute for atRA therapy. The serum concentration of β-cryptoxanthin reached 1–2 μM after the oral intake of EME-supplemented juice for 8 wk (7), enough to activate RARs.

Regarding RAR activation and adipocyte differentiation, a working model has previously been reported. Studies have indicated that RAR combined CCAAT (a c/EBP binding element) is present in specific gene promoters and prevents transcription (21). Other reports have demonstrated that Smad3 (which binds to c/EBPβ and inhibits its transcriptional activation) is upregulated by atRA (22), whilst the down-regulation of PPARγ, a key regulator of adipocyte differentiation, following atRA treatment has been reported (19). Taken together, these studies indicate that RAR, when activated by β-cryptoxanthin, most likely binds to the c/EBP responsive element in the PPARγ promoter or inhibits PPARγ tran-}

![Fig. 4. Gene expression modulation following β-cryptoxanthin treatment. Panels A and B represent adipocyte differentiation-related genes and RAR-responsible genes in 3T3-L1 cells treated with β-cryptoxanthin, respectively. Each value represents the mean±SD (n = 3). (*p<0.05, **p<0.01 vs control (β-CRX 0 μM, LE540 0 μM).)](image-url)
strated in this study. β-Cryptoxanthin is abundantly contained in Satsuma mandarin and no other fruit or vegetable contains as much β-cryptoxanthin as Satsuma mandarin. Since Satsuma mandarin is the fruit produced most in Japan and has been eaten for more than 400 y, its safety is epidemiologically proven. We conclude that β-cryptoxanthin is a safe and natural compound for the prevention of obesity.

REFERENCES


