

Suppression of Sebum Production and Accumulation by β -Cryptoxanthin Due to the Inhibition of the Expression of Diacylglycerol Acyltransferase-1 and Perilipin in Hamster Sebocytes*

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Received December 13th, 2012; revised January 15th, 2013; accepted January 24th, 2013

ABSTRACT

Background: Acne vulgaris is characterized by the enhancement of sebaceous lipogenesis and sebum secretion, and apart from retinoids and some natural products there are few effective anti-acne agents that directly suppress sebum production and accumulation in sebaceous glands. **Objective:** We examined the effects of β -cryptoxanthin (β -CRX), which is a carotenoid pigment most abundant in *Citrus unshiu* Marcovich (Satsuma mandarin orange) and plays a role as a Vitamin A precursor on sebum production and accumulation in hamster sebaceous gland cells (sebocytes). **Materials and methods:** The regulation of sebum production was examined by the measurement of triacylglycerols (TGs), the major sebum component, and oil red O staining in insulin-differentiated hamster sebocytes. The expression of diacylglycerol acyltransferase-1 (DGAT-1), a rate-limiting enzyme of TG biosynthesis, and perilipin 1 (PLIN1), a lipid storage droplet protein, was analyzed using real-time PCR and Western blotting. **Results:** Hamster sebocytes constitutively produced TGs during cultivation and the production of TGs was enhanced by insulin treatment. Both constitutive and insulin-enhanced TG productions were dose- and time-dependently inhibited by β -CRX as well as 13-*cis* retinoic acid. In addition, the gene expression of DGAT-1 was suppressed by β -CRX in the sebocytes. Furthermore, the insulin-enhanced sebum accumulation as lipid droplets was reduced in the β -CRX-treated cells. Moreover, β -CRX was found to suppress the gene expression and production of PLIN1 in insulin-differentiated hamster sebocytes. **Conclusions:** These results provide novel evidence that β -CRX is an effective candidate for acne therapy by its ability to exert dual inhibitory actions against DGAT-1-dependent TG production and PLIN1-mediated lipid-droplet formation in hamster sebocytes.

Keywords: β -Cryptoxanthin; Sebocytes; Triacylglycerol Biosynthesis; Diacylglycerol Acyltransferase; Perilipin; Lipid-Droplet Formation; Sebum

1. Introduction

The pathogenesis of acne, a common inflammatory skin disease [1,2], is characterized by: 1) excess sebum production in sebaceous glands; 2) the formation of microcomedones, which is closely associated with the hyperkeratinization of the follicular wall and infundibulum; 3) the hyperproliferation of *Propionibacterium acnes* (*P. acnes*); and 4) the induction of inflammatory reactions such as the acceleration of cytokine production and the biosynthesis of arachidonic acid metabolites in keratinocytes, sebocytes, and invaded inflammatory cells [3,4]. The aggravation and duration of the inflammation are

likely to result in acne scar that causes a psychological and social impact in the patient's quality of life [4].

Sebum production in acne lesions has been reported to be increased by 5 α -dihydrotestosterone, insulin, insulin-like growth factor 1, and prostaglandins [5-9]. In addition, the biosynthesis of sebum components such as triacylglycerols (TGs) and sapienic acids is regulated by diacylglycerol acyltransferase (DGAT), a rate-limiting enzyme of TG synthesis [6,10], and Δ 6 desaturase (FADS2) [11], respectively, in human and hamster sebocytes. Furthermore, we have previously reported that the production of perilipin (PLIN), a lipid storage droplet protein, is augmented in differentiated hamster sebocytes and localized on the surface of intracellular lipid droplets,

*Conflict of interest: The authors have no conflict of interest to declare.

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indicating that perilipin is a differentiation marker in hamster sebocytes [12]. On the other hand, retinoic acids such as tretinoin (all-*trans* retinoic acid; atRA) and isotretinoin (13-*cis* retinoic acid; 13-*cis*RA) have been topically and/or systemically used for acne therapy [13,14]. They have been reported to exhibit comedolytic, anti-inflammatory, and anti-lipogenetic actions in sebaceous glands and pilosebaceous units in humans, rats, and hamsters *in vivo* and *in vitro* [14-19]. However, the use of retinoids in acne therapy has been limited in acceptance because of adverse effects such as skin irritation, scaling, and teratogenicity [13].

β -Cryptoxanthin (β -CRX), a carotenoid pigment most abundant in *Citrus unshiu* Marcovich (Satsuma mandarin orange), has been reported to exhibit multiple disease preventive actions; e.g. anti-tumorigenic, anti-obesity, anti-atherogenic, and immunopotentiative ones [20-23]. In addition, carotenoids such as β -CRX and β -carotene have been reported to be present in the blood of people from different countries including Japan, which is associated with some health benefits [22,24]. Indeed, epidemiologic studies have shown that higher intakes or blood levels of β -CRX result in a reduced risk of lung cancer and rheumatoid arthritis development [25-27]. On the other hand, it has also been reported that low plasma level of Vitamin A is associated with the development and aggravation of acne [28]. In addition, β -CRX is a vitamin A precursor, which is oxidatively cleaved to vitamin A by β -carotene 15,15'-dioxygenase [29], and can be stably stored in some tissue for several months [30]. Taken together with a recent report by Shirakura *et al.* [31] where β -CRX inhibits the intracellular lipid-droplet formation in mouse 3T3-L1 adipocytes, we hypothesize that, instead of retinoids such as atRA and 13-*cis*RA, β -CRX is an effective candidate for acne therapy by modulating sebaceous lipogenesis. However, it is not fully understood whether β -CRX directly suppresses sebum production and accumulation in sebaceous gland cells (sebocytes) or not.

In the present study, we demonstrated that β -CRX dose- and time-dependently inhibited the production and intracellular accumulation of sebum in insulin-differentiated hamster sebocytes. Furthermore, the β -CRX-mediated inhibition of sebum production and accumulation is closely associated with the transcriptional suppression of DGAT-1 and PLIN1, respectively, in differentiated hamster sebocytes.

2. Materials and Methods

2.1. Cell Culture and Treatment

Hamster sebocytes (2.4×10^4 cells/cm²) [32] were plated onto 96-well multiplates, 35-mm or 100-mm diameter culture dishes (Becton Dickinson, Tokyo, Japan) and

then cultured for 24 h in DMEM/F12 (Invitrogen, Carlsbad, CA) supplemented with 6% heat-denatured fetal bovine serum (Nichirei Biosciences Inc., Tokyo, Japan), 2% human serum (C-C Biotech Co., Valley Center, CA), 0.68 mM *L*-glutamine (Invitrogen), and recombinant human epidermal growth factor (10 nM) (Progen Biotechnik GmbH, Heidelberg, Germany) to achieve complete cell adhesion as previously described [6,18]. The hamster sebocytes were treated every two days for up to 8 days with or without β -CRX (purity $\geq 95\%$; Shikoku Yashima Pure Chemicals, Tokushima, Japan) (**Figure 1**) or 13-*cis*RA (Sigma Chemical, St. Louis, MO) in the presence or absence of a sebocyte-differentiation inducer, insulin (10 nM) (Sigma Chemical) [12] in DMEM/F12 supplemented with heat-denatured fetal bovine serum, human serum, and *L*-glutamine. In this series of experiments, hamster sebocytes were used as far as the 3rd passage level.

2.2. Analyses of Sebum Production and Accumulation

After treating sebocytes with β -CRX, 13-*cis*RA, and/or insulin, the cells were subjected to the quantification of TGs, the major sebum component, using Liquitech TG-II (Roche Diagnostics, Tokyo, Japan) as previously described [12]. The amounts of intracellular TGs were calculated using an authentic trioleinate-standard solution (0.6 mg/ml). Intracellular DNA content was measured using salmon sperm DNA (6.25 - 100 mg/ml) and 3,5-diaminobenzoic acid dihydrochloride (Sigma Chemical). For the analysis of intracellular sebum accumulation, oil red O staining was performed. Briefly, the cells were washed once with Ca²⁺- and Mg²⁺-free phosphate-buffered saline [PBS(-)] and fixed with 4% paraformaldehyde (Wako Pure Chemicals, Osaka, Japan) diluted with PBS(-) for 1 h at room temperature. The cells were washed with distilled H₂O and then stained with 0.3% oil red O (Sigma Chemical) in isopropanol:distilled H₂O (3:2, vol:vol) at 37°C for 15 min. The stained cells were washed with distilled H₂O, and then viewed with a light microscope furnished with a digital camera (Olympus Optical Co., Tokyo, Japan).

2.3. Real-Time PCR

For the quantification of DGAT-1 and PLIN1 mRNA, total RNA was isolated from cells using ISOGEN (Nippon Gene, Toyama, Japan) and then the aliquot of RNA (500 ng) was subjected to reverse transcriptase reaction

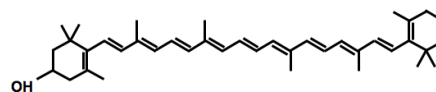


Figure 1. Chemical structure of β -cryptoxanthin (β -CRX).

for the synthesis of cDNA using a PrimeScript RT reagent Kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Aliquots (an equivalent of 2.5 ng of total RNA) of the transcript were subjected to real-time PCR using SYBR Premix Ex Taq II (Takara Bio) and the following specific primers: human DGAT-1 (NM_012079); 5'-TCTACAAGCCCATGCTTCGAC-3' (sense) and 5'-GGACGCTCACCAGGTA-3' (antisense), hamster PLIN1 (AB091681); 5'-ACCTTGCTGGATGGAGACC-3' (sense) and 5'-CCAGGACCTTGCTGAAGT-3' (antisense), and hamster glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (X52123); 5'-CAGAACATCATCCCTGCAT-3' (sense) and 5'-TAGGAACACGGAAGGCCAT-3' (antisense) as previously described [33]. The amplification cycle was performed at 94°C for 5 s and 60°C for 30 s using a Thermal Cycler Dice Real Time System TP-800 (Takara Bio). The obtained threshold cycle (CT) value for DGAT-1 and PLIN1 was normalized by that for GAPDH, and the relative expression level was expressed as the mean value of the control as 1.

2.4. Western Blot Analysis

The harvested cell lysate (50 μ g protein) was subjected to Western blot analysis using 12.5% acrylamide gel as previously described [33]. The membrane was reacted with rabbit anti-(human PLIN1) IgG, which was customized by Operon Biotechnologies (Tokyo, Japan). To evaluate the level of β -actin as an internal control, the harvested cell lysates (50 μ g protein) were similarly subjected to Western blot analysis using rabbit anti-(human β -actin) IgG (Medical & Biological Laboratories, Nagoya, Japan). Immunoreactive PLIN1 and β -actin were visualized with Amersham enhanced chemiluminescence-Western blotting detection reagents (GE Healthcare Bio-Sciences, Tokyo, Japan) according to the manufacturer's instructions. Relative amounts of PLIN1 protein against β -actin were quantified by densitometric scanning using an Image Analyzer LAS-1000 Plus (GE Healthcare), and the relative expression level was expressed as the mean value of the control as 1.

2.5. Statistical Analysis

A one-way ANOVA was used for the statistical analysis, and then the Fisher test was applied when multiple comparisons were performed.

3. Results

3.1. Inhibition of TG Production and DGAT-1 Gene Expression by β -CRX in Hamster Sebocytes

Since hamster sebocytes constitutively produce TGs dur-

ing cultivation *in vitro* [32], we first examined the effect of β -CRX on constitutive TG production in cultured hamster sebocytes. As shown in **Figure 2(A)**, β -CRX was found to decrease the level of TGs in a dose-dependent manner (70% inhibition at 10 μ M). In addition, the sebocyte differentiation inducer, insulin (10 nM), was found to enhance the production of TGs in hamster sebocytes, and the enhanced level of TGs was dose-dependently decreased by β -CRX (72% inhibition at 10 μ M). Furthermore, a similar decrease in TG level was time-dependently observed in hamster sebocytes treated with β -CRX as well as 13-*cis* RA (**Figure 3**), as we previously reported [18,34]. On the other hand, the gene expression of DGAT-1 was constitutively detectable, and was found to be increased in response to insulin treatment in hamster sebocytes (7.7 ± 3.9 fold, $p < 0.05$) (**Figure 2(B)**). In addition, β -CRX was found to suppress the gene expression of DGAT-1 in both the insulin-untreated and treated hamster sebocytes (52% and 88% inhibition, respectively) (**Figure 2(B)**). Therefore, these results suggest that β -CRX inhibits the production of TGs due to the suppression of DGAT-1 expression in hamster sebocytes.

3.2. Suppression of Sebum Accumulation by Decreasing Gene Expression and Production of Perilipin in Hamster Sebocytes

As the intracellular accumulation of sebum as lipid-droplets has been reported to be due to the increase of TG production in differentiated hamster sebocytes [32], oil red O staining revealed that the lipid-droplet formation was augmented in the insulin-differentiated hamster sebocytes (**Figures 4(B) vs. (A)**). In addition, the enhanced sebum accumulation was found to be abolished by adding β -CRX (10 μ M) (**Figures 4(C) vs. (B)**). On the other hand, perilipin, a lipid-droplet surface protein, has been reported to play an important role in the formation of intracellular lipid droplets in differentiated adipocytes, steroidogenic cells, and sebocytes [12,35]. As β -CRX inhibited sebum accumulation as lipid droplets (**Figure 4**), we examined whether β -CRX influenced the production of PLIN1 in hamster sebocytes. As shown in **Figure 5**, the production of PLIN1 was barely detectable in insulin-untreated sebocytes, but was augmented by insulin treatment. In addition, β -CRX was found to dose-dependently suppress the insulin-enhanced production of PLIN1 in hamster sebocytes. Furthermore, both basal and insulin-augmented levels of PLIN1 mRNA were found to be decreased in the β -CRX-treated cells (**Figure 6**). Thus, these results suggest that the suppression of PLIN1 production by β -CRX is associated with the inhibition of sebum accumulation in differentiated hamster sebocytes.

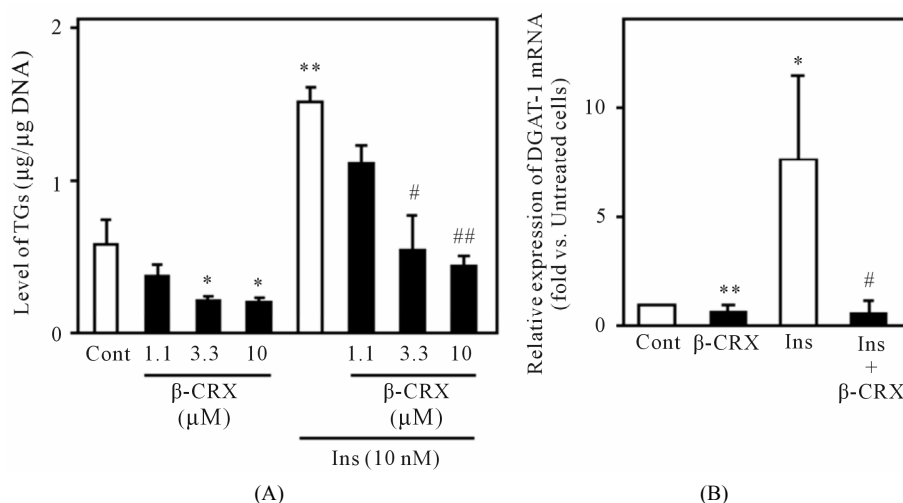


Figure 2. The decrease of intracellular TG level by β -CRX in hamster sebocytes. Hamster sebocytes at the 3rd passage were treated every two days for 8 days with or without β -CRX (1.1 - 10 μ M) in the presence or absence of insulin (Ins) (10 nM). **A:** The harvested cell lysate was subjected to the measurement of intracellular TG level as described in the Materials and Methods. **B:** Isolated RNA (an equivalent of 2.5 ng of total RNA) from the cells was subjected to the analysis of DGAT-1 mRNA expression as described in the Materials and Methods. Data are shown as mean \pm SD of three dishes. * and **, significantly different from untreated cells (Cont) ($p < 0.05$ and 0.01 , respectively). # and ##, significantly different from insulin (Ins) (10 nM)-treated cells ($p < 0.05$ and 0.01 , respectively).

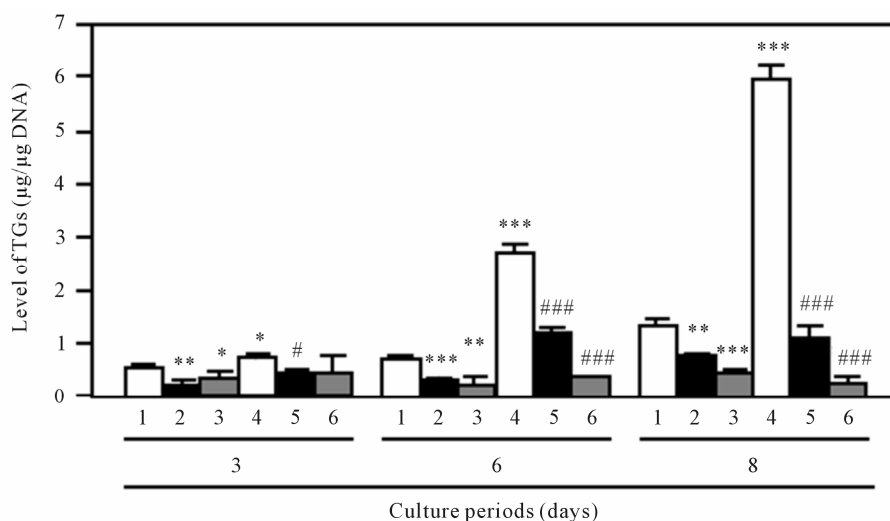


Figure 3. Time-dependent inhibition of TG production by β -CRX in hamster sebocytes. Hamster sebocytes at the 3rd passage were treated every two days for up to 8 days with or without β -CRX (10 μ M) or 13-*cis*RA (1 μ M) in the presence or absence of insulin (10 nM), and then the intracellular level of TGs was measured. Data are shown as mean \pm SD of three dishes. Lane 1, untreated cells; Lane 2, β -CRX (10 μ M)-treated cells; Lane 3, 13-*cis*RA (1 μ M)-treated cells; Lane 4, insulin (10 nM)-treated cells; Lane 5, cells treated with insulin (10 nM) and β -CRX (10 μ M); and Lane 6, cells treated with insulin (10 nM) and 13-*cis*RA (1 μ M). *, **, and ***, significantly different from untreated cells ($p < 0.05$, 0.01 , and 0.001 , respectively). # and ###, significantly different from insulin (10 nM)-treated cells ($p < 0.05$ and 0.001 , respectively).

4. Discussion

We demonstrated that β -CRX suppresses the production of TGs and the gene expression of DGAT-1 in differentiated hamster sebocytes. Our previous study showed that the decrease of TG production by both 13-*cis*RA and atRA is closely related to that of DGAT activity as well

as the DGAT-1 transcript in differentiated hamster sebocytes [18]. In addition, Harris *et al.* (2011) [36] reported that DGAT is required for not only TG synthesis but also lipid droplet formation in adipocytes from DGAT knockout mice. Therefore, these results provide novel evidence that β -CRX inhibits *de novo* synthesis of TGs

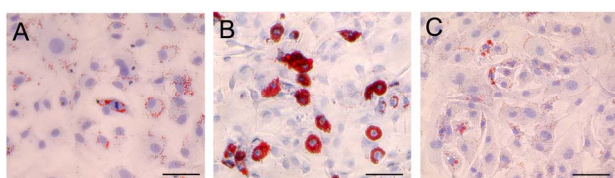


Figure 4. β -CRX decreases sebum accumulation in insulin-differentiated hamster sebocytes. Hamster sebocytes treated with insulin (10 nM) and/or β -CRX (10 μ M) as shown in Figure 2 were subjected to oil red O staining for the analysis of intracellular sebum accumulation as described in the Materials and Methods. A: untreated cells; B: insulin (10 nM)-treated cells; and C: cells treated with insulin (10 nM) and β -CRX (10 μ M). Bars: 50 μ m.

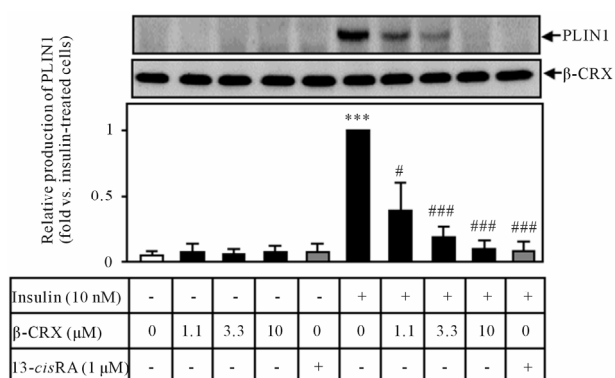


Figure 5. β -CRX and 13-*cis*RA suppress the production of PLIN1 in insulin-differentiated hamster sebocytes. Cell lysate (50 μ g protein) prepared from hamster sebocytes treated every two days for 8 days with insulin (10 nM), β -CRX (1.1 - 10 μ M), and/or 13-*cis*RA (1 μ M) was subjected to Western blot analysis for PLIN1 and β -actin as described in the Materials and Methods. Data are shown as mean \pm SD of four independent experiments. ***, significantly different from untreated cells ($p < 0.001$). # and ###, significantly different from insulin (10 nM)-treated cells ($p < 0.05$ and 0.001, respectively).

by suppressing DGAT-1 expression, which may in turn participate in the inhibition of lipid droplet formation in differentiated sebocytes. Furthermore, taken together with previous reports that TGs from sebaceous glands are a source of nutrition of *P. acnes* [1,3], β -CRX-decreased TG production is very likely to indirectly result in the prevention of *P. acnes* proliferation in acne lesions.

The PAT-family of intracellular lipid storage droplet proteins such as PLIN1-5 has been reported to play important roles in the regulation of TG storage and lipolysis in adipocytes and steroidogenic cells [35]. Our previous study showed that PLIN1 localizes to the surface of intracellular sebum-droplets in differentiated hamster sebocytes [12]. In the present study, we found that not only intracellular lipid-droplet formation but also the gene expression and production of PLIN1 was suppressed by β -CRX in differentiated hamster sebocytes. A similar

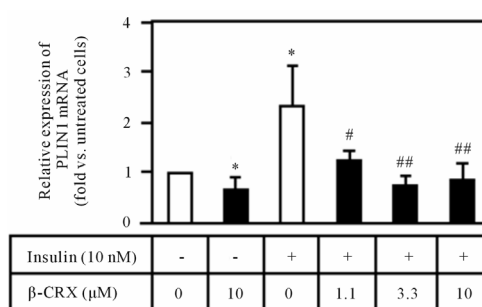


Figure 6. Suppression of PLIN1 mRNA expression by β -CRX in insulin-differentiated hamster sebocytes. Isolated RNA (an equivalent of 2.5 ng of total RNA) from the cells treated with insulin (10 nM), β -CRX (1.1 - 10 μ M), and/or 13-*cis*RA (1 μ M) as shown in Figure 5 was subjected to the analysis of PLIN1 mRNA expression as described in the Materials and Methods. Data are shown as mean \pm SD of four different experiments. *, significantly different from untreated cells ($p < 0.05$). # and ##, significantly different from insulin (10 nM)-treated cells ($p < 0.05$ and 0.01, respectively).

inhibition of lipid-droplet formation has been reported to be observed in β -CRX-treated mouse 3T3-L1 adipocytes [31]. Therefore, the β -CRX-inhibition of lipid-droplet formation is likely to include the transcriptional suppression of PLIN1 production in hamster sebocytes. Moreover, intracellular lipid-droplet formation has been reported to be associated with the protein kinase A (PKA)-dependent phosphorylation of PLIN, which facilitates the hormone-sensitive lipase-mediated lipolysis of neutral lipid droplets in adipocytes [37,38]. We previously reported that neither the level of cyclic AMP (cAMP) nor PKA activity was augmented by 13-*cis*RA [18], of which its suppressive action against sebum accumulation is similar to that of β -CRX in differentiated hamster sebocytes. However, β -carotene, which is structurally and functionally similar to β -CRX [39], has been reported to increase the level of cAMP and PKA activity in human pulmonary adenocarcinoma cells and epithelial cells from small airways and pancreatic ducts [40,41]. Thus, β -CRX may also influence the phosphorylation of PLIN1, which is coordinately associated with the abolishment of lipid-droplets in differentiated hamster sebocytes. Further experiments are needed to clarify these hypotheses.

The development of comedones has been related to the enhancement of sebum production in sebaceous glands, under which various lipogenetic signaling through androgen and/or insulin-like growth factor 1/insulin pathways is activated [42,43]. In the inflamed acne lesions resulting from comedogenesis, the expression and activation of PPAR γ have been reported to be augmented and closely associated with the aggravation of acne pathology [44,45]. Shirakura *et al.* [31] reported that β -CRX does not affect peroxisome proliferators activating receptor γ

(PPAR γ) activation. We have preliminarily demonstrated that β -CRX did not alter the PPAR γ ligand, troglitazone-augmented production of TGs and PLIN1 production in hamster sebocytes (Sato T and Ito A, unpublished data). Therefore, the inhibitory actions of β -CRX against sebum production and storage in sebocytes may at least partially account for the beneficial efficacy in the prevention of comedogenesis in acne lesions under non-inflammatory conditions.

Carotenoids split into two groups; xanthophylls and carotenes [21]. Xanthophylls such as β -CRX exist as an ester form with fatty acids in fruits or vegetables [46,47]. In addition, xanthophyll esters have been reported to be present in human skin, where the xanthophylls are re-esterified following absorption [48]. Since the blood concentration of β -CRX has been reported to be higher than that of β -carotene or lycopene [47], it is suggested that β -CRX is easily absorbed and accumulated in the skin, where it may behave as Vitamin A for the control of cutaneous functions. Taken together with a report of El-Akawi *et al.* [28] that low plasma levels of Vitamin A are associated with acne development and aggravation, therefore, the supplementation of β -CRX is likely to be effective for the prevention of acne or acne maintenance.

In conclusion, our findings provide novel evidence that β -CRX is an effective candidate for acne therapy by inhibiting not only sebaceous lipogenesis but also sebum accumulation against insulin-differentiated hamster sebocytes, in which acne pathology is at least partly mimicked *in vitro*. Furthermore, these findings may contribute to a novel understanding of the molecular mechanisms of dietary carotenoids in the maintenance of skin barrier functions.

5. Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (C) (#22590506). We wish to thank Dr. K. Kitamura and Miss. M. Kuwata for their technical assistance.

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