Published in final edited form as:

Arch Biochem Biophys. 2015 April 15; 572: 2–10. doi:10.1016/j.abb.2015.01.009.

Actions of β -Apo-Carotenoids in Differentiating Cells: Differential Effects in P19 Cells and 3T3-L1 Adipocytes

Cynthia X. Wang¹, Hongfeng Jiang², Jason J. Yuen², Seung-Ah Lee², Sureshbabu Narayanasamy^{3,4}, Robert W. Curley Jr.³, Earl H. Harrison⁴, and William S. Blaner²

¹Columbia College, Columbia University, New York, NY 10032

²College of Physicians and Surgeons, Columbia University, New York, NY 10032

³College of Pharmacy The Ohio State University, Columbus, Ohio 43210

⁴Department of Human Nutrition, The Ohio State University, Columbus, Ohio 43210

Abstract

β -Apo-carotenoids, including β -apo-13-carotenone and β -apo-14'-carotenal, are potent retinoic acid receptor (RAR) antagonists in transactivation assays. We asked how these influence RAR-dependent processes in living cells. Initially, we explored the effects of β -apo-13-carotenone and β -apo-14'-carotenal on P19 cells, a mouse embryonal carcinoma cell line that differentiates into neurons when treated with all-*trans*-retinoic acid. Treatment of P19 cells with either compound failed to block all-*trans*-retinoic acid induced differentiation. Liquid chromatography tandem mass spectrometry studies, however, established that neither of these β -apo-carotenoids accumulates in P19 cells. All-*trans*-retinoic acid accumulated to high levels in P19 cells. This suggests that the uptake and metabolism of β -apo-carotenoids by some cells does not involve the same processes used for retinoids and that these may be cell type specific. We also investigated the effects of two β -apo-carotenoids on 3T3-L1 adipocyte marker gene expression during adipocyte differentiation. Treatment of 3T3-L1 adipocytes with either β -apo-13-carotenone or β -apo-10'-carotenone, which lacks RAR antagonist activity, stimulated adipocyte marker gene expression. Neither blocked the inhibitory effects of a relatively large dose of exogenous all-*trans*-retinoic acid on adipocyte differentiation. Our data suggest that in addition to acting as transcriptional antagonists, some β -apo-carotenoids act through other mechanisms to influence 3T3-L1 adipocyte differentiation.

Keywords

retinoid; all-*trans*-retinoic acid; adipose differentiation; obesity; transcription; gene expression

Communicating Author: William S. Blaner, Ph.D. Department of Medicine, College of Physicians and Surgeons, Columbia University, 650 W. 168th St., New York, NY 10032, (212) 305-5429, wsb2@columbia.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Human diets contain both provitamin A and non-provitamin A carotenoids (1). Provitamin A carotenoids like β -carotene are taken up in the intestine by enterocytes and cleaved *in situ* by the enzyme β -carotene-15,15'-dioxygenase (BCO1¹) to retinoid (vitamin A and its metabolites) (2-4). This newly formed retinoid is metabolically indistinguishable from that acquired as dietary preformed vitamin A (2-4). Ultimately, all retinoid that is present in mammals is derived from provitamin A carotenoids, either directly from dietary provitamin A carotenoid or indirectly by an animal lower on the food chain. In humans, some dietary provitamin A carotenoid is absorbed intact into the body along with other dietary lipids in chylomicrons, and distributed primarily to the liver, but also other tissues (1-4). Many tissues express *Bco1* mRNA and protein and it has been proposed that this allows for the cleavage of provitamin A carotenoids to retinoids within these tissues (5-9). In addition to being cleaved to form retinoids, intact provitamin A carotenoids have many actions within tissues that are independent of their provitamin A activity, including actions as antioxidants (10). Non-provitamin A carotenoids, like lycopene, that are consumed in the diet are also taken up into the body in chylomicrons (1-4). These non-provitamin A carotenoids are widely distributed to tissues throughout the body (2-4).

In addition to BCO1, one other mammalian enzyme is able to metabolize carotenoids, β -carotene-9',10'-oxygenase 2 (BCO2) (11-13). Unlike BCO1 which catalyzes β -carotene cleavage about its central 15-15' double bond, BCO2 catalyzes the asymmetric cleavage of β -carotene at the 9'-10'-double bond, forming β -apo-10'-carotenal, one of the structurally distinct products that are collectively known as β -apo-carotenals (3, 5, 11, 12). These β -apo-carotenals can either undergo enzymatic oxidation to corresponding β -apo-carotenoic acids or reduction to corresponding β -apo-carotenols that can subsequently undergo esterification or conversion to retinoid (3, 5, 11-14). Some β -apo-carotenoids are formed non-enzymatically (3). They are also formed in plants and can be found in various plant food sources (3).

Retinoids, which ultimately must be derived from provitamin A carotenoids, are potent transcriptional regulators (15-17) affecting expression levels of more than 500 genes (17). All-*trans*- and 9-*cis*-retinoic acid are transcriptionally active retinoids, regulating transcription upon binding to one of 3 distinct retinoic acid receptors (RARs) or 3 distinct retinoid X receptors (RXRs) (15-17). At the cellular level, retinoids regulate cell differentiation, proliferation, and apoptosis (18, 19). They therefore are needed for maintaining immunity, male and female reproduction, pre- and post-natal development, barrier function, and vision (18, 19). There is also considerable research interest in retinoid actions in both the prevention and the development of metabolic disease, including obesity, insulin resistance, liver disease and cardiovascular disease (20-26).

¹**Abbreviations:** BCO1, β -carotene-15,15'-dioxygenase; BCO2, β -carotene-9',10'-oxygenase 2; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal calf serum; LC/MS/MS, liquid chromatography tandem mass spectrometry; NBS, newborn calf serum; peroxisome proliferator-activated receptor, PPAR; qPCR, quantitative real time-polymerase chain reaction; RAR, retinoic acid receptor; RXR, retinoid X receptor.

It has been known for several decades that β -apo-carotenoids, which comprise both enzymatic and non-enzymatic by-products formed from β -carotene, are present in human and animal blood and tissues (27, 28). It is likely that many or most of these will be eliminated from the body, but the extent of this is not established (29). In 2007, Plutzky and colleagues reported that one of these compounds, β -apo-14'-carotenal, interacts with the retinoid X receptors (RXRs) and both peroxisome proliferator-activated receptors- α and - γ (PPAR α and - γ) to oppose known effects of both synthetic and natural ligands of these receptors, thereby preventing target gene induction *in vitro* and functional cellular responses *in vivo* (30, 31). More recently, Harrison and colleagues reported that some β -apo-carotenals and β -apo-carotenoic acids, specifically β -apo-14'-carotenal, β -apo-14'-carotenoic acid and β -apo-13-carotenone, are very potent RAR antagonists, with binding affinities in the low nM range (3, 32). These binding affinities are similar in magnitude to that of all-*trans*-retinoic acid for the RARs (32). In addition, β -apo-13-carotenone is reported to be an antagonist of RXR α transactivation (33). Collectively, this published work suggests that some β -apo-carotenoids may modulate the normal actions of all-*trans*-retinoic acid within the body. The objective of our studies was to investigate how β -apo-carotenoids may affect all-*trans*-retinoic acid- and RAR-dependent functions in living cells, using murine P19 cells and 3T3-L1 cells as cell models. We focused specifically on β -apo-13-carotenone and β -apo-14'-carotenal since these compounds have been shown to be potent RAR antagonists (3, 32).

Materials and Methods

Cells and Cell Culture

P19 mouse embryonal carcinoma cells were obtained from American Type Culture Collections (ATCC) (ATCC CRL-1825, Manassas, VA) and cultured as recommended by the supplier in α -Minimal Essential Medium (α -MEM) supplemented with 0.1% Pen-Strep, 7.5% newborn calf serum (NCS), and 2.5% fetal bovine serum (FBS). Cells were plated and studied on plastic 6-well dishes (Corning). The plated P19 cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Media were changed at 48 h intervals. To explore the effects of all-*trans*-retinoic acid, β -apo-13-carotenone and/or β -apo-14'-carotenal on P19 cells, concentrated stocks of each (1 mM) were made in sterile absolute ethanol or DMSO immediately prior to each experiment. Depending on experimental need, an appropriate quantity of each stock was added to the complete medium, mixed vigorously, and then placed over the P19 cell cultures. The final volume of ethanol or DMSO added to the culture medium accounted for 0.1% or less of the total volume of the medium. All-*trans*-retinoic acid was obtained from Sigma-Aldrich (St. Louis, MO). β -Apo-13-carotenone and β -apo-14'-carotenal were chemically synthesized as previously described (32). The purities of the stocks for each of these three compounds were checked by liquid chromatography tandem mass spectrometry (LC/MS/MS) immediately prior to the start of cell culture experiments (see below). For some experiments, P19 cells were treated with LE 540, a synthetic RAR pan-antagonist employed in other published studies (34). LE 540 was kindly provided by Dr. Hiroyuki Kagechika of the Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Tokyo, Japan.

3T3-L1 cells were obtained from ATCC (ATCC CL-173) and maintained as preadipocytes in 90% Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 0.1% Pen-Strep (referred to in the text as growth medium). The 3T3-L1 preadipocytes were always passaged before the cultures reached 70% confluence. To induce differentiation of the 3T3-L1 preadipocytes to adipocytes, we employed procedures recommended by ATCC. When the 3T3-L1 preadipocytes had reached 100% confluence, the medium was changed to 90% DMEM supplemented with 10% FBS, 1.0 μ M dexamethasone, 0.5 mM methylisobutylxanthine and 1.0 μ g/mL bovine insulin (referred to as differentiation medium). After 48 h of culture in this medium, the medium was again changed to 90% DMEM supplemented with 10% FBS and 1.0 μ g/mL bovine insulin (referred to as maintenance medium). Both preadipocytes and adipocytes were cultured in plastic 6-well dishes, at 37 °C, in a humidified atmosphere consisting of 95% air and 5% CO₂. Concentrated stocks of all-*trans*-retinoic acid, β -apo-13-carotenone and β -apo-10'-carotenoid acid were made in DMSO and diluted at the time of the experiment, as described above for studies of P19 cells.

Liquid chromatography tandem mass spectrometry (LC/MS/MS)

LC/MS/MS analyses of β -apo-13-carotenone and β -apo-14'-carotenal were conducted using a Waters Xevo TQ MS ACQUITY UPLC system (Waters, MA, USA) controlled by MassLynx Software 4. 1. Samples were maintained at 4 °C in the autosampler and 5 μ L was loaded onto a Waters ACQUITY UPLC BEH Phenyl column (3.0 $\times$ 100 mm; 1.7 μ m particle size) for analysis of β -apo-13-carotenone and β -apo-14'-carotenal. The column was maintained at 40 °C throughout analysis. The UPLC flow rate was continuously 500 μ L/min in a binary gradient mode with the following mobile phase: initial flow conditions were 8% solvent A (H₂O, containing 0.2% formic acid and 1 mM ammonium formate) and 92% solvent B (methanol, containing 0.2% formic acid and 1 mM ammonium formate). Solvent B was increased linearly to 100% over a 5 min period and maintained in the subsequent 3 min. This was followed by a reduction of solvent B to 92% starting at 8.2 min and continuing through 9 min followed by next injection. β -Apo-carotenoids of interest eluted between 1.2 and 5.5 min. Positive ESI-MS/MS mass spectrometry with MRM (multiple reaction monitoring) mode was performed using the following parameters: capillary voltage, 4 kV; source temperature, 150 °C; desolvation temperature, 500 °C; desolvation gas flow, 1000 L/hr; and collision gas flow, 0.15 mL/min. The transitions employed for the MRM analysis were 259.4 $\rightarrow$ 175.2 for β -apo-13-carotenone and 311.3 $\rightarrow$ 92.5 for β -apo-14'-carotenal.

Cellular all-*trans*-retinoic acid concentrations were determined by LC/MS/MS exactly as we have previously reported (35, 36).

Extraction of β -apo-carotenoids and all-*trans*-retinoic acid from cell pellets

For analysis of β -apo-carotenoid and all-*trans*-retinoic acid levels in P19 or 3T3-L1 cells, frozen cell pellets (flash frozen in liquid N₂ and stored at -80 °C) were resuspended and homogenized in 1 ml of ice cold phosphate buffered saline. Internal standards (retinyl acetate for the β -apo-carotenoids and penta-deuterated all-*trans*-retinoic acid (Toronto Research Chemicals, North York, Ontario) were then added in 1 ml of ethanol containing

0.1% (w/v) butylated-hydroxytoluene as an antioxidant. Six volumes of HEAT (10:6:7:7 Hexane/Ethanol/Acetone/Toluene) were subsequently added along with saturated NaCl solution (200 μ l) to facilitate phase separation. Samples were vortexed for 60 sec and centrifuged at 5,000 g for 5 min at 4 °C. The upper organic layer was removed and the aqueous phase was extracted two more times by adding HEAT/NaCl. The three organic phases were combined and dried under a stream of nitrogen. The residue was reconstituted in 40 μ l of 1:1 (v/v) methyl t-butyl ether/methanol before LC/MS/MS.

RNA Isolation and Quantitative Real Time-Polymerase Chain Reaction (qPCR) Analysis of mRNA Levels

Total RNA was isolated from cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA samples were DNase I-digested employing the RNeasy mini kit (Qiagen) to remove residual genomic DNA and subsequently quantified using a NanoDrop spectrophotometer. For cDNA synthesis, 1 μ g of total RNA was reverse-transcribed using the High Capacity Reverse Transcription Kit (Applied Biosystems). cDNA synthesis was carried out for 10 min at 20 °C and 120 min at 37 °C. The reaction was stopped by incubation at 85 °C for 5 min using a thermal cycler (Eppendorf). Sequences for the primers used in this study were published earlier (37). qPCR was performed in a total volume of 25 μ l, containing diluted cDNA template (1:40), forward and reverse primers (1 μ M each), and LightCycler 480 SYBR Green I Master Mix (Roche) on a LightCycler 480 II instrument (Roche). Expression levels of target genes are presented relative to the mRNA levels for the reference gene *Gapdh* or *18s* rRNA. Since normalization with either reference gene gave the same results, we will only report data normalized for *Gapdh*. A dissociation curve program was employed after each reaction to verify the purity of the PCR products.

Statistical Analyses

Quantitative gene expression data from the qPCR analyses were normalized to the control gene *Gapdh* or *18s* rRNA. Data were analyzed using Excel (Microsoft) and GraphPad (Prism) and are presented as mean $\pm$ SE. To assess statistically significant differences between treatments, we first carried out an analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test. Data were considered significantly different when $P < 0.05$.

Results

P19 cells are an established mouse pluripotent embryonal carcinoma cell line that can differentiate into neurons and glial cells when cultured in the presence of all-*trans*-retinoic acid (38). Since expression levels for a number of developmentally important genes are known to increase very rapidly in P19 cells upon exposure to all-*trans*-retinoic acid (38), we explored the effects that β -apo-13-carotenone and β -apo-14'-carotenal have on all-*trans*-retinoic acid-induced P19 cell differentiation. We first investigated the effects of β -apo-13-carotenone on expression of 3 differentiation-related genes (*Hoxa1*, *Hoxa2*, and *Hoxa3*) and 2 canonical retinoic acid-responsive genes (*Cyp26a1* and *Rarb*). As shown in Figure 1, all-*trans*-retinoic acid treatment of the P19 cells brought about, within 2 h of treatment, marked inductions of 7-, 28-, 8-, 6- and 5-fold respectively for *Hoxa1*, *Hoxa2*, *Hoxa3*, *Cyp26a1* and

Rarb. Further increases in expression levels for all 5 genes were noted 4 h after treatment (Figure 1). When P19 cells were exposed to only β -apo-13-carotenone, no inductions in expression levels for *Hoxa1*, *Hoxa2*, *Hoxa3*, *Cyp26a1*, or *Rarb* were observed (Figure 1), indicating that β -apo-13-carotenone is unable to induce P19 cell differentiation. When added in combination with all-*trans*-retinoic acid, 2 h after treatment, β -apo-13-carotenone significantly diminished, albeit to a relatively small degree, the effects of all-*trans*-retinoic acid on inducing *Hoxa1* and *Cyp26a1* expression in P19 cells. However, by 4 h, these small treatment effects were no longer significant. For all 5 genes at 4 h, the extents of gene induction upon all-*trans*-retinoic acid treatment were identical in the presence or absence of β -apo-13-carotenone. Based on these data, it would appear that β -apo-13-carotenone has only a small effect on antagonizing all-*trans*-retinoic acid induction of P19 cell differentiation.

Since we did not obtain evidence that β -apo-13-carotenone markedly disrupted all-*trans*-retinoic acid-induced P19 cell differentiation, we carried out similar experiments using β -apo-14'-carotenal in place of β -apo-13-carotenone (Figure 2). As part of this experiment, though, we also studied the effects on the synthetic RAR pan-antagonist LE 540 (34) on P19 cell gene expression patterns. Our data fail to establish any effect for β -apo-14'-carotenal on the all-*trans*-retinoic acid-induced increases in expression of *Hoxa1*, *Hoxa2*, *Hoxa3*, *Cyp26a1* or *Rarb*, either at 2 or 4 h after treatment (Figure 2). LE 540, a well studied RAR pan-antagonist, had a small but statistically significant effect on blocking all-*trans*-retinoic acid induction of *Hoxa1* and *Rarb* gene expression (Figure 2). For the other three genes, LE 540 had no effect on expression. Thus, treatment of P19 cells with either of these two RAR pan-antagonists had little effect on the P19 cell differentiation program induced by all-*trans*-retinoic acid.

We next asked whether cultured P19 cells were able to accumulate either β -apo-13-carotenone or β -apo-14'carotenal from the medium. Initially, we established an LC/MS/MS protocol that could separate and identify the family of β -apo-carotenals and β -apo-carotenoids that theoretically could be formed from β -carotene. A LC/MS/MS profile showing the separations for these compounds as well as β -apo-13-carotenone, β -carotene, and the internal standard retinyl acetate is provided in Figure 3A. Interestingly, after 4 h incubation in medium containing 1 μ M β -apo-13-carotenone, no β -apo-13-carotenone could be detected in extracts from P19 cells (Figure 3B). The P19 cells similarly failed to accumulate β -apo-14'-carotenal (data not shown). The low limits of detection of our LC/MS/MS protocol for each of these β -apo-carotenoids were less than 0.1 pmol. Thus, we should have been able to detect each compound in P19 cells if the cells had accumulated only 0.001% of the β -apo-carotenoid that had been added to the culture medium. This is unlike all-*trans*-retinoic acid, for which substantial intracellular concentrations could be detected when P19 were incubated for 4 h in medium supplemented with 0.1 μ M all-*trans*-retinoic acid (Figure 3C).

Because P19 cells failed to accumulate β -apo-carotenoids in detectable quantities, it became clear that the P19 cell line was not amenable to the study of β -apo-carotenoid modulation of all-*trans*-retinoic acid action. Consequently, we chose to continue our investigation with a different cell line: 3T3-L1 cells. 3T3-L1 cells are an established mouse preadipocyte line that can be induced to differentiate into adipocytes under appropriate conditions. As has

been well established in previous studies, differentiation of 3T3-L1 cells is blocked by all-*trans*-retinoic acid (39, 40), and therefore we wondered whether β -apo-carotenoid antagonism of all-*trans*-retinoic acid could inhibit this effect. We first established by LC/MS/MS that β -apo-13-carotenone was taken up and accumulated by 3T3-L1 preadipocytes when it was provided in the culture medium at a concentration of 5 μ M. We then investigated the effects of β -apo-13-carotenone on expression of the adipocyte marker genes lipoprotein lipase (*Lpl*), *Ap2*, and adiponectin (*Adipoq*) in 3T3-L1 cells after induction of adipocyte differentiation. The cells were exposed to β -apo-13-carotenone (5 μ M), all-*trans*-retinoic acid (0.1 μ M), a combination of these, or vehicle alone, for a total of 7 days. This involved adding each compound 1 day before differentiation was induced, through exposure for 2 days in differentiation medium, followed by 4 days of culture in maintenance medium (see **MATERIALS AND METHODS**). As shown in Figure 4, all-*trans*-retinoic acid treatment significantly diminished expression of all three genes, as expected. When added in combination with all-*trans*-retinoic acid, β -apo-13-carotenone failed to significantly diminish the observed effects of all-*trans*-retinoic acid inhibiting adipogenesis. Treatment with β -apo-13-carotenone alone had no effect on expression of these adipocyte genes.

In a second experiment with β -apo-13-carotenone, we harvested the cells at an earlier time point after induction of differentiation. We reasoned that antagonistic effects might be better detected as the 3T3-L1 preadipocytes begin to differentiate into adipocytes. Consequently, we altered our treatment protocol so that the 3T3-L1 cells were treated with β -apo-13-carotenone (5 μ M), all-*trans*-retinoic acid (0.1 μ M), or a combination of these for 1 day, starting the day before the cells were induced to differentiate (while still in growth medium). Cells were harvested immediately after the 2 day induction period for gene expression analysis. We once again examined *Lpl*, *Ap2*, and *Adipoq* expression, as well as expression of *Pparg*, a master regulator gene for adipocyte differentiation. Strikingly, β -apo-13-carotenone treatment alone significantly upregulated expression of *Lpl*, *Ap2*, and *Pparg* by 4.7-, 3.3-, and 4.2-fold, respectively (Figure 5). Again, all-*trans*-retinoic acid treatment blocked induction of 3T3-L1 adipocyte marker gene expression. When added in combination with all-*trans*-retinoic acid, β -apo-13-carotenone did not significantly diminish the effects of all-*trans*-retinoic acid on blocking 3T3-L1 differentiation, although data for all 4 genes trended in this direction. Considering the relatively low all-*trans*-retinoic acid concentrations that are present in unsupplemented growth or differentiation media containing 10% FBS, well under 1 nM (41), this marked stimulatory effect of β -apo-13-carotenone on several adipocyte marker genes may suggest that β -apo-13-carotenone has a modulatory effect on the 3T3-L1 cells that is independent from RAR antagonism, possibly through effects on RXR-mediated transcriptional activity (33, 42).

In order to better understand the possibility of a modulatory effect separate from antagonism of RARs, we examined the effects of β -apo-10'-carotenoic acid, as β -apo-10'-carotenoic acid was not reported to be a potent RAR antagonist (32, 33). For this experiment, 3T3-L1 cells were treated with β -apo-10'-carotenoic acid (5 μ M) or all-*trans*-retinoic acid (0.1 μ M) or a combination of both for 3 days starting 1 day before differentiation induction and throughout the 2 day period of culture in differentiation medium. β -Apo-10'-carotenoic acid treatment

significantly increased expression of *Lpl* and *Ap2* by 59% and 49%, respectively (Figure 6). However, expression levels of *Adipoq* and *Pparg* were not significantly different upon β -apo-10'-carotenoic acid treatment. Moreover, when added in combination with all-*trans*-retinoic acid, p-apo-10'-carotenoic acid also failed to significantly diminish all-*trans*-retinoic acid inhibition of adipogenesis (Figure 6). Thus, β -apo-10'-carotenoic acid alone also had a stimulatory effect on adipocyte differentiation, as indicated by increased levels of *Lpl* and *Ap2* marker gene expression.

Discussion

While our initial objective was to investigate the putative RAR antagonism of β -apo-13-carotenone and β -apo-14'-carotenal in living cells—through inhibition of all-*trans*-retinoic acid-induced P19 embryonal cell differentiation—we did not obtain strong evidence of RAR antagonism. P19 cells failed to respond to treatment with either β -apo-13-carotenone or β -apo-14'-carotenal. It should be noted that LE 540, which is reported in the literature to be a very potent RAR antagonist (34), including in cultured cells (43), also did not have a large effect on all-*trans*-retinoic acid-induced P19 cell differentiation either. We take our data collectively to indicate that the P19 cell model is not amenable for investigating β -apo-carotenoid antagonism of RAR signaling.

Our LC/MS/MS studies showed that P19 cells failed to accumulate in substantial quantities either β -apo-13-carotenone or β -apo-14'-carotenal, even though the cells readily accumulate all-*trans*-retinoic acid. Thus, mouse P19 cells, although highly responsive to all-*trans*-retinoic acid treatment, are refractory to β -apo-13-carotenone and β -apo-14'-carotenal because they cannot accumulate the compounds. These β -apo-carotenoids are either not taken up by P19 cells and/or are quickly metabolized by the cells. P19 cells express an impressive array of genes encoding proteins that are centrally involved in mediating retinoid uptake, metabolism and actions, including *Bco1*, *Bco2*, *Rbp1*, *Rbp4*, *Stra6*, *Rdh10*, *Aldh1a1*, *Aldh1a2*, *Aldh1a3*, *Crabp1*, *Crabp2*, *Rara*, *Rarb*, *Rarg*, *Cyp26a1*, *Cyp26b1*, and *Cyp26c1* (44). Moreover, many of these genes are induced when these cells are exposed to retinoids (Figures 1 and 2, and (44)). It is clear from this listing that P19 cells possess a very substantial metabolic capacity to take up and utilize all-*trans*-retinoic acid and its metabolic precursors. This is undoubtedly not the case for β -apo-carotenoids, which are either not taken up readily and/or rapidly metabolized within the P19 cells. Little is known about the cellular factors needed for the uptake, accumulation and metabolism of β -apo-carotenoids. Based on our studies, it seems likely to us that some cells and tissues may lack specific enzymes or other factors needed for acquiring and/or maintaining the many different β -apo-carotenoids, as we have observed to be true for P19 cells.

Interestingly, 3T3-L1 adipocyte differentiation, as assessed by marker gene expression is induced by β -apo-carotenoid treatment. β -Apo-13-carotenone and β -apo-10'-carotenoic acid both significantly elevated adipocyte marker genes in 3T3-L1 cells that had been induced to differentiate to adipocytes according to standard literature protocols. Specifically, β -apo-13-carotenone treatment significantly upregulated expression of *Lpl*, *Ap2*, and *Pparg*, while β -apo-10'-carotenoic acid upregulated expression of *Lpl* and *Ap2*. These effects for β -apo-13-carotenone were dependent on when the 3T3-L1 cells were treated, with larger effects being

observed early after induction of adipocyte differentiation (Figure 5 versus Figure 4). The observed stimulation of adipocyte marker gene induction by these β -carotene metabolites suggests that β -apo-carotenoids may have roles in modulating adipocyte development. These are clearly not strong dominating regulatory effects, since they can be overcome by all-*trans*-retinoic acid treatment, but rather, more subtle ones that fine tune adipogenesis. However, our data do support the conclusion that these two β -apo-carotenoids can influence the gene expression programs of differentiating adipocytes.

Based on transactivation assay data and its ability to block all-*trans*-retinoic acid-induced gene expression in HepG2 hepatocytes, β -apo-13-carotenone is proposed to be a very potent antagonist of all-*trans*-retinoic acid mediated transcription (32, 33). Why then did we not observe stronger effects of this β -apo-carotenoid in lessening the blockage of 3T3-L1 cell differentiation upon all-*trans*-retinoic treatment? One possibility is that β -apo-13-carotenone is not accumulated by differentiating 3T3-L1 cells to a significantly large extent to allow it to block the actions of the relatively large dose of all-*trans*-retinoic acid employed in our studies. But we did observe some β -apo-13-carotenone when treated 3T3-L1 preadipocytes were analyzed by LC/MS/MS (data not shown). However, as we observed for P19 cells, 3T3-L1 cells may much more avidly accumulate all-*trans*-retinoic acid than β -apo-13-carotenone, overwhelming β -apo-13-carotenone actions. We indeed observed effects of β -apo-13-carotenone on 3T3-L1 adipocyte marker gene expression when it was added alone to the culture medium. Possibly, these effects reflect that actions of β -apo-13-carotenone in blocking the actions of the endogenous all-*trans*-retinoic acid present in the adipocyte culture media that contain 10% FBS. Based on published work from Napoli and colleagues (41), the concentration of all-*trans*-retinoic acid in the culture media containing 10% FBS would be well under 1 nM, as opposed to 0.1 μ M in the supplemented media. This could explain why we observed an effect of β -apo-13-carotenone in stimulating 3T3-L1 adipocyte differentiation, but a lack of an effect when all-*trans*-retinoic acid was present in the media at a 100-fold greater concentration. Alternatively, since β -apo-13-carotenone can also affect RXR transcriptional activity (32, 42), it is possible that our observations arise due to effects on other nuclear hormone receptors important for 3T3-L1 adipocyte differentiation, ones that heterodimerize with RXR, including peroxisome proliferator-activated receptor- γ (30, 31, 45), liver X receptor- α (46), and the vitamin D receptor (47). Based on the data at hand, it is not possible to distinguish between these possibilities. However, we note that Prakash et al. (48) have reported that β -apo-14'-carotenoic acid, when added to the medium of cultured normal human bronchial epithelial cells, is converted to all-*trans*-retinoic acid and this affects RAR transcriptional activity. But this could not account for our observations since, when added alone to the culture medium, β -apo-13-carotenone had the opposite effect from all-*trans*-retinoic acid on 3T3-L1 gene expression profiles.

The effects of β -apo-10'-carotenoic acid on 3T3-L1 adipocyte marker gene expression that we observed (Figure 6) were surprising. β -Apo-10'-carotenoic acid does not show strong activity as an antagonist of all-*trans*-retinoic acid-mediated transcriptional activity (32). The most likely explanation explaining this observation would be that β -apo-10'-carotenoic acid may be acting in the cells through some novel mechanism that does involve direct disruption of RAR-mediated transcription. There is a growing body of evidence that all-*trans*-retinoic

acid acts non-genomically to affect signal transduction pathways within the cell (49, 50). Possibly, β -apo-10'-carotenoic acid acts in a similar/related manner within cells. This possibility merits further attention.

Our findings are intriguing in light of previous studies that found that long term supplementation with relatively large doses of β -carotene can reduce body adiposity in mice (51, 52) and with adipocyte β -carotene levels in humans (53). Like these other studies, we too have employed relatively large pharmacological concentrations of both β -apo-carotenoid and all-*trans*-retinoic acid in our treatments of P19 and 3T3-L1 cells, albeit ones that are often used in cell culture studies. Our data further raise the possibility that different β -apo-carotenoids can modulate adipogenesis in different ways. Data reported in this manuscript suggest that β -carotene consumption could have varying impacts on body adiposity between individuals depending on the particular combination of β -apo-carotenoids that are present within the body. Several genetic polymorphisms in the *Bco1* gene have been described that affect the enzyme's activity level, which may ultimately result in different species and concentrations of β -apo-carotenoids within different subjects. Such differences have been recently observed in mice totally lacking *Bco1* compared to wild type mice (54). Additional studies of other β -apo-carotenoids will need to be undertaken in order to further elucidate the modulatory role that β -apo-carotenoids may have in adipogenesis, as well as the specific mechanisms by which they may influence all-*trans*-retinoic acid action.

Conclusions

In summary, our studies in P19 cells indicate that the uptake and/or metabolism of β -apo-13-carotenone and β -apo-14'-carotenal does not allow for accumulation of these β -carotene metabolites in cells. This suggests that the RAR antagonist activities of each of these two β -apo-carotenoids will be limited by the intrinsic capacities of cells to obtain/retain sufficient quantities of each to allow for transcriptional regulatory actions to occur. Our data also suggest that β -apo-carotenoids, such as apo-13-carotenone and apo-10'-carotenoic acid, may have important modulatory effects on various cellular processes, and that some of these observed effects may be mediated through additional mechanisms other than RAR antagonism. Both of these conclusions will need to be explored in much more depth before the biological actions of the enzymatic and non-enzymatic β -apo-carotenoid by-products of β -carotene can be understood.

Acknowledgments

This work was supported by U.S. Public Health Services, National Institutes of Health grants R01 DK068437 (WSB), R01 HL049879 (EHH), and R21 AA021336 (WSB).

We wish to thank Dr. Hiroyuki Kagechika of the Institute of Biomaterials and Bioengineering at the Tokyo Medical and Dental University for providing the LE 540 used in some of our studies. The authors also wish to acknowledge Dr. Pierre-Jacques Brun for thoughtful discussions regarding our work.

References

1. Bendich A, Olson JA. Biological actions of carotenoids. *FASEB J.* 1989; 3:1927–1932. [PubMed: 2656356]

2. Blaner, WS.; Olson, JA. Retinol and retinoic acid metabolism. In: Sporn, MB.; Roberts, AB.; Goodman, DS., editors. *The Retinoids: Biology, Chemistry, and Medicine*. Raven Press Ltd.; New York: 1994. p. 229-256.
3. Eroglu A, Harrison EH. Carotenoid metabolism in mammals, including man: formation, occurrence, and function of apocarotenoids. *J Lipid Res*. 2013; 54:1719–1730. [PubMed: 23667178]
4. von Lintig J. Colors with functions: elucidating the biochemical and molecular basis of carotenoid metabolism. *Annu Rev Nutr*. 2010; 30:35–56. [PubMed: 20415581]
5. von Lintig J, Vogt K. Filling the gap in vitamin A research. Molecular identification of an enzyme cleaving beta-carotene to retinal. *J Biol Chem*. 2000; 275:11915–11920. [PubMed: 10766819]
6. Redmond TM, Gentleman S, Duncan T, Yu S, Wiggert B, Gantt E, Cunningham FX Jr. Identification, expression, and substrate specificity of a mammalian beta-carotene 15, 15'-dioxygenase. *J Biol Chem*. 2001; 276:6560–6565. [PubMed: 11092891]
7. Paik J, During A, Harrison EH, Mendelsohn CL, Lai K, Blaner WS. Expression and characterization of a murine enzyme able to cleave β -carotene. The formation of retinoids. *J Bio Chem*. 2001; 276:32160–32168. [PubMed: 11418584]
8. Wyss A, Wirtz GM, Woggon WD, Brugger R, Wyss M, Friedlein A, Riss G, Bachmann H, Hunziker W. Expression pattern and localization of beta,beta-carotene 15,15'-dioxygenase in different tissues. *Biochem J*. 2001; 354:521–529. [PubMed: 11237856]
9. Yan W, Jang GF, Haeseleer F, Esumi N, Chang J, Kerrigan M, Compochiaro M, Compochiaro P, Palczewski K, Zack DJ. Cloning and characterization of a human beta,beta-carotene-15,15'-dioxygenase that is highly expressed in the retinal pigment epithelium. *Genomics*. 2001; 72:193–202. [PubMed: 11401432]
10. Kaulmann A, Bohn T. Carotenoids, inflammation, and oxidative stress--implications of cellular signaling pathways and relation to chronic disease prevention. *Nutr Res*. 2014; 34:907–929. [PubMed: 25134454]
11. Kiefer C, Hessel S, Lampert JM, Vogt K, Lederer MO, Breithaupt DE, von Lintig J. Identification and characterization of a mammalian enzyme catalyzing the asymmetric oxidative cleavage of provitamin A. *J Biol Chem*. 2001; 276:14110–14116. [PubMed: 11278918]
12. Hu KQ, Liu C, Ernst H, Krinsky NI, Russell RM, Wang XD. The biochemical characterization of ferret carotene-9'/10'-monooxygenase catalyzing cleavage of carotenoids in vitro and in vivo. *J Biol Chem*. 2006; 281:19327–19338. [PubMed: 16672231]
13. Harrison EH. Mechanisms involved in the intestinal absorption of dietary vitamin A and provitamin A carotenoids. *Biochim Biophys Acta*. 2012; 1821:70–77. [PubMed: 21718801]
14. Amengual J, Widjaja-Adhi AK, Rodriguez-Santiago S, Hessel S, Golczak M, Palczewski K, von Lintig J. Two carotenoid oxygenases contribute to mammalian provitamin A metabolism. *J Biol Chem*. 2013; 288:3408–34096.
15. Mangelsdorf, DJ.; Umesono, K.; Evans, RM. The retinoid receptors *The Retinoids: Biology Chemistry, and Medicine*. Sporn, MB.; Roberts, AB.; Goodman, DS., editors. Raven Press Ltd.; New York: 1994. p. 319-349.
16. Chambon P. The retinoid signaling pathway: molecular and genetic analyses. *Semin Cell Biol*. 1994; 5:115–125. [PubMed: 8068884]
17. Balmer JE, Blomhoff R. Gene expression regulation by retinoic acid. *J Lipid Res*. 2002; 43:1773–1808. [PubMed: 12401878]
18. Rhinn M, Dolle P. Retinoic acid signalling during development. *Development*. 2012; 139:843–858. [PubMed: 22318625]
19. Tang XH, Gudas LJ. Retinoids, retinoic acid receptors, and cancer. *Annu Rev Pathol*. 2011; 6:345–364. [PubMed: 21073338]
20. Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, Kotani K, Quadro L, Kahn BB. Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature*. 2005; 436:356–362. [PubMed: 16034410]
21. Graham TE, Yang Q, Blüher M, Hammarstedt A, Ciaraldi TP, Henry RR, Watson CJ, Oberbach A, Jansson PA, Smith U, Kahn BB. Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects. *N Engl J Med*. 2006; 354:2552–2563. [PubMed: 16775236]

22. Klöting N, Graham TE, Berndt J, Kralish S, Kralisch S, Kovacs P, Watson CJ, Fasshauer M, Schön MR, Stumvoll M, Blüher M, Kahn BB. Serum retinol-binding protein is more highly expressed in visceral than in subcutaneous adipose tissue and is a marker of intra-abdominal fat mass. *Cell Metab.* 2007; 6:79–87. [PubMed: 17618858]
23. Maher JJ. Retinol binding protein 4 and fatty liver: A direct link? *Hepatology.* 2013; 58:477–479. [PubMed: 23703940]
24. Xia M, Liu Y, Guo H, Wang D, Want Y, Ling W. Retinol binding protein 4 stimulates hepatic sterol regulatory element-binding protein 1 and increases lipogenesis through the peroxisome proliferator-activated receptor-gamma coactivator 1 beta-dependent pathway. *Hepatology.* 2013; 58:564–575. [PubMed: 23300015]
25. Bobbert P, Weithäuser A, Anders J, Bobbert T, Kühl U, Schultheiss HP, Rauch U, Skurk C. Increased plasma retinol binding protein 4 levels in patients with inflammatory cardiomyopathy. *Eur J Heart Fail.* 2009; 11:1163–1168. [PubMed: 19926600]
26. Chavarria N, Kato TS, Khan R, Chokshi A, Collado E, Akashi H, Takayama H, Naka Y, Farr M, Mancini D, Schulze PC. Increased levels of retinol binding protein 4 in patients with advanced heart failure correct after hemodynamic improvement through ventricular assist device placement. *Circ J.* 2013; 76:2148–2152. [PubMed: 22785609]
27. Sharma RV, Mathur SN, Dmitrowskii AA, Das RC, Ganguly J. Studies on the metabolism of beta-carotene and apo-beta-carotenoids in rats and chickens. *Biochim Biophys Acta.* 1976; 486:183–194. [PubMed: 12834]
28. Wang XD, Tang GW, Fox G, Krinsky NI, Russell RM. Enzymatic conversion of beta-carotene into beta-apo-carotenals and retinoids by human, monkey, ferret and rat tissues. *Arch Biochem Biophys.* 1991; 285:8–16. [PubMed: 1899329]
29. Amengual J, Lobo GP, Golczak M, Li HNM, Klimova T, Hoppel CL, Wyss A, Palczewski K, von Lintig J. A mitochondrial enzyme degrades carotenoids and protects against oxidative stress. *FASEB J.* 2011; 25:948–959. [PubMed: 21106934]
30. Ziouzenkova O, Orasanu G, Sukhova G, Lau E, Berger JP, Tang G, Krinsky NI, Dolnikowski GG, Plutzky J. Asymmetric cleavage of β -carotene yields a transcriptional repressor of retinoid X receptor and peroxisome proliferator-activated receptor responses. *Mol Endocrinol.* 2007; 21:77–88. [PubMed: 17008383]
31. Ziouzenkova O, Plutzky J. Retinoid metabolism and nuclear receptor responses: New insights into coordinated regulation of the PPAR-RXR complex. *FEBS Letters.* 2008; 582:32–38. [PubMed: 18068127]
32. Eroglu A, Hruszkewycz DP, dela Sena C, Narayanasamy S, Riedl KM, Kopec RE, Schwartz SJ, Curley RW Jr, Harrison EH. Naturally occurring eccentric cleavage products of provitamin A β -carotene function as antagonists of retinoic acid receptors. *J Biol Chem.* 2012; 287:15886–15895. [PubMed: 22418437]
33. Eroglu A, Hruszkewycz DP, Curley RW Jr, Harrison EH. The eccentric cleavage product of β -carotene, β -apo-13-carotenone, functions as an antagonist of RXR α . *Arch Biochem Biophys.* 2010; 504:11–16.
34. Umemiya H, Fukasawa H, Ebisawa M, Eyrolles L, Kawachi E, Eisenmann G, Gronemeyer H, Hashimoto Y, Shudo K, Kagechika H. Regulation of retinoidal actions by diazepinylbenzoic acids. Retinoid synergists which activate the RXR-RAR heterodimers. *J Med Chem.* 1997; 40:4222–4234. [PubMed: 9435893]
35. Kim YK, Wassef L, Chung S, Jiang H, Wyss A, Blaner WS, Quadro L. β -Carotene and its cleavage enzyme β -carotene-15,15'-oxygenase (CMO1) affect retinoid metabolism in developing tissues. *FASEB J.* 2011; 25:1641–1652. [PubMed: 21285397]
36. Wongsiriroj N, Jiang H, Piantedosi R, Yang KJ, Kluwe J, Schwabe RF, Ginsberg H, Goldberg IJ, Blaner WS. Genetic dissection of retinoid esterification and accumulation in the liver and adipose tissue. *J Lipid Res.* 2014; 55:104–114. [PubMed: 24186946]
37. Clugston RD, Jiang H, Lee MX, Piantedosi R, Yuen JJ, Ramakrishnan R, Lewis MJ, Gottesman ME, Huang LS, Goldberg IJ, Berk PD, Blaner WS. Altered hepatic lipid metabolism in C57BL/6 mice fed alcohol: a targeted lipidomic and gene expression study. *J Lipid Res.* 2011; 52:2021–2031. [PubMed: 21856784]

38. McBurney MW. P19 embryonal carcinoma cells. *Int J Dev Biol.* 1993; 37:135–140. [PubMed: 8507558]
39. Schwarz EJ, Reginato MJ, Shao D, Krakow SL, Lazar MA. Retinoic acid blocks adipogenesis by inhibiting C/EBP β -mediated transcription. *Mol Cell Biol.* 1997; 17:1552–1561. [PubMed: 9032283]
40. Berry DC, Noy N. All-*trans*-retinoic acid represses obesity and insulin resistance by activating both peroxisome proliferation-activated receptor β/δ and retinoic acid receptor. *Mol Cell Biol.* 2009; 29:3286–3296. [PubMed: 19364826]
41. Horst RL, Reinhardt TA, Goff JP, Koszewski NJ, Napoli JL. 9, 13-Di-*cis*-retinoic acid is the major circulating geometric isomer of retinoic acid in the periparturient period. *Arch Biochem Biophys.* 1995; 322:235–239. [PubMed: 7574681]
42. Sun J, Narayanasamy S, Curley RW Jr, Harrison EH. β -Apo-13-carotenone regulates retinoid X receptor transcriptional activity through tetramerization of the receptor. *J Biol Chem.* Epub ahead of print.
43. Brun PJ, Grijalva A, Rausch R, Yuen JJ, Watson E, Das BC, Shudo K, Kagechika H, Leibel RL, Blaner WS. Retinoic acid receptor signaling is required to maintain glucose stimulated insulin secretion and β -cell mass. *FASEB J.* Epub ahead of print.
44. Chen Y, Reese DH. The retinol signaling pathway in mouse pluripotent P19 cells. *J Cell Biochem.* 2011; 112:2865–2872. [PubMed: 21618588]
45. Hamza MS, Pott S, Vega VB, Thomsen JS, Kandhadayar GS, Ng PW, Chiu KP, Pettersson S, Wei CL, Ruan Y, Liu ET. De-novo identification of PPARGgamma/RXR binding sites and direct targets during adipogenesis. *PLoS ONE.* 2009; 4:e4907. [PubMed: 19300518]
46. Juvet LK, Andersen SM, Schuster GU, Kalen KT, Tobin KA, Hollung K, Haugen F, Jacinto S, Ulven SM, Bamberg K, Nebb HI. On the role of liver X receptors in lipid accumulation in adipocytes. *Mol Endocrinol.* 2003; 17:172–182. [PubMed: 12554745]
47. Wood RJ. Vitamin D and adipogenesis: new molecular insights. *Nutr Rev.* 2008; 66:40–46. [PubMed: 18254883]
48. Prakash P, Liu C, Hu KQ, Krinsky NI, Russell RM, Wang XD. β -Carotene and β -apo-14'-carotenoic acid prevent the reduction of retinoic acid receptor pin benzo[a]pyrene-treated normal human bronchial epithelial cells. *J Nutr.* 2004; 134:667–673. [PubMed: 14988465]
49. Al Tanoury Z, Piskunov A, Rochette-Egly C. Vitamin A and retinoid signaling: genomic and nongenomic effects. *J Lipid Res.* 2013; 54:1761–1775. [PubMed: 23440512]
50. Wei LN. Non-canonical activity of retinoic acid in epigenetic control of embryonic stem cell. *Transcription.* 2013; 4:158–161. [PubMed: 23863198]
51. Lobo GP, Amengual J, Li HNM, Golczak M, Bonet ML, Palczewski K, von Lintig J. β , β -Carotene decreases peroxisome proliferator receptor γ activity and reduces lipid storage capacity of adipocytes in a β , β -carotene oxygenase 1-dependent manner. *J Biol Chem.* 2010; 285:27891–27899. [PubMed: 20573961]
52. Amengual J, Gouranton E, van Helden YGJ, Hessel S, Ribot J, Kramer E, Klec-Wilk B, Razny U, Lietz G, Wyss A, Dembinska-Klec A, Palou A, Keijer J, Landrier JF, Bonet ML, von Lintig J. Beta-carotene reduces body adiposity of mice via BCMO1. *PLoS ONE.* 2011; 6:e20644. [PubMed: 21673813]
53. Östh M, Öst A, Kjolhede P, Strålfors P. The concentration of β -carotene in human adipocytes, but not the whole-body adipocyte stores, is reduced in obesity. *PLoS ONE.* 2014; 9:e85610. [PubMed: 24416432]
54. Lee SA, Jiang H, Trent CM, Yuen JJ, Narayanasamy S, Curley RW Jr, Harrison EH, Goldberg IJ, Maurer MS, Blaner WS. Cardiac dysfunction in β -carotene-15,15' dioxygenase-deficient mice is associated with altered retinoid and lipid metabolism. *Am J Physiol Heart Circ Physiol.* 2014; 307:H1675–H1684. [PubMed: 25260612]

Highlights

- β -apo-13-carotenone and β -apo-14'-carotenal are not taken up and/or are rapidly metabolized by P19 cells
- 3T3-L1 adipocyte marker gene expression is induced by β -apo-carotenoid treatment
- Effects on 3T3-L1 gene expression may involve nuclear receptors that heterodimerize with RXR

Figure 1. β -Apo-13-carotenone treatment of mouse P19 cells does not markedly disrupt P19 cell differentiation induced upon all-*trans*-retinoic acid exposure

The effects of vehicle (Ctrl), all-*trans*-retinoic acid (RA), provided in the medium at 0.1 μ M, β -apo-13-carotenone (Apo), provided in the medium at 1 μ M, or a combination of the two (Apo + RA) on mRNA expression levels of three genes important to P19 cell differentiation (*Hoxa1*, *Hoxa2*, and *Hoxa3*) and two canonical retinoic acid-dependent genes (*Cyp26a1* and *Rarb*), 2 and 4 hours after initiation of the treatments. All data are normalized to *Gapdh* mRNA levels. Values are presented as means $\pm$ SEM ($n = 3$). Expression levels that are denoted with different letters are significantly different ($P < 0.05$).

Figure 2. The retinoic acid receptor pan-antagonists β -apo-14'-carotenal and LE 540 do not significantly disrupt all-*trans*-retinoic acid induction of P19 cell differentiation

The effects of vehicle (Ctrl), all-*trans*-retinoic acid (RA), provided in the medium at 0.1 μ M, β -apo-14'-carotenal (Apo), provided in the medium at 1 μ M, or a combination of all-*trans*-retinoic acid and β -apo-14'-carotenal (Apo + RA), LE 540 (LE), provided at a medium concentration 1 μ M, or a combination of both all-*trans*-retinoic acid and LE 540 on mRNA expression levels of three genes important to P19 cell differentiation (*Hoxa1*, *Hoxa2*, and *Hoxa3*) and two canonical retinoic acid-dependent genes (*Cyp26a1* and *Rarb*), 2 and 4 hours after initiation of the treatments. All data are normalized to *Gapdh* mRNA levels. Values are presented as means $\pm$ SEM (n = 3). Expression levels that are denoted with different letters are significantly different (P < 0.05).

Figure 3. P19 cells do not accumulate β -apo-13-carotenone from the culture medium but do accumulate substantial levels of medium all-*trans*-retinoic acid

LC/MS/MS separation of synthetic purified β -apo-carotenoids (**Panel A**). This method allows for the separation of all β -apo-carotenal (β -apo-8'- (8-AL), β -apo-10'- (10-AL), β -apo-12'- (12-AL), and β -apo-14'-carotenal (14-AL)) and β -apo-carotenoic acid (β -apo-8'- (8-CA), β -apo-10'- (10-CA), β -apo-12'- (12-CA) and β -apo-14'-carotenoic acid (14-CA) metabolites of β -carotene, as well as β -apo-13-carotenone (13-One), β -ionone, β -carotene, and the internal standard retinyl acetate. For P19 cells incubated for 4 h in culture medium containing 1 μ M β -apo-13-carotenone, no β -apo-13-carotenone (13-One) could be detected in P19 cell extracts (**Panel B**). All-*trans*-retinyl acetate (Retinyl Acetate) was employed as an internal standard for assessing β -apo-carotenoid recovery from P19 cell extracts. Unlike β -apo-13-carotenone, P19 cells readily accumulated all-*trans*-retinoic acid from culture medium supplemented with 0.1 μ M all-*trans*-retinoic acid (**Panel C**). D₅-All-*trans*-retinoic acid was employed as an internal standard for assessing retinoid recovery from P19 cell extracts.

Figure 4. β -Apo-13-carotenone treatment of differentiating 3T3-L1 adipocytes for 7 days does not stimulate adipocyte-specific gene expression or arrest the inhibitory effects of all-*trans*-retinoic acid on differentiation

As described in the text, the culture medium of differentiating 3T3-L1 adipocytes was supplemented for 7 days with either vehicle alone (Ctrl), all-*trans*-retinoic acid (0.1 μ M final concentration), β -apo-13-carotenone (5 μ M), or a combination of all-*trans*-retinoic acid (0.1 μ M) and μ -apo-13-carotenone (5 μ M). Subsequently, expression levels of 3 adipocyte-specific genes (lipoprotein lipase (Lpl), Ap2 and adiponectin) were assessed by qPCR. All data are normalized to *Gapdh* mRNA levels. Values are presented as means $\pm$ SEM (n = 3). Expression levels that are denoted with different letters are significantly different (P < 0.05).

Figure 5. Short-term (1 day) treatment of differentiating 3T3-L1 adipocytes early in the differentiation program with β -apo-13-carotenone stimulates 3T3-L1 adipocyte differentiation as assessed by adipocyte-specific gene expression

As described in the text, the culture medium of differentiating 3T3-L1 adipocytes was supplemented for 1 day with either vehicle alone (Ctrl), all-*trans*-retinoic acid (0.1 μ M final concentration), β -apo-13-carotenone (5 μ M), or a combination of all-*trans*-retinoic acid (0.1 μ M) and β -apo-13-carotenone (5 μ M). Subsequently, expression levels of 4 adipocyte-specific genes (lipoprotein lipase (Lpl), Ap2, adiponectin, and Pparg) were assessed by qPCR. All data are normalized to *Gapdh* mRNA levels. Values are presented as means $\pm$ SEM (n = 3). Expression levels that are denoted with different letters are significantly different (P < 0.05).

Figure 6. Supplementation of 3T3-L1 adipocyte culture medium early in the differentiation program with β -apo-10'-carotenoic acid stimulates 3T3-L1 adipocyte gene expression

As described in the text, the culture medium of differentiating 3T3-L1 adipocytes was supplemented for 3 days with either vehicle alone (Ctrl), all-*trans*-retinoic acid (0.1 μ M final concentration), β -apo-10'-carotenoic acid (5 μ M), or a combination of all-*trans*-retinoic acid (0.1 μ M) and β -apo-10'-carotenoic acid (5 μ M). Subsequently, expression levels of 4 adipocyte-specific genes (lipoprotein lipase (Lpl), Ap2, adiponectin, and Pparg) were assessed by qPCR. All data are normalized to *Gapdh* mRNA levels. Values are presented as means $\pm$ SEM (n = 3). Expression levels that are denoted with different letters are significantly different (P < 0.05).