\textbf{\textbeta,\textbeta-Cardotene Decreases Peroxisome Proliferator Receptor \gamma Activity and Reduces Lipid Storage Capacity of Adipocytes in a \textbeta,\textbeta-Cardotene Oxygenase 1-dependent Manner*}\textsuperscript{1,2,3}

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Increasing evidence has been provided for a connection between retinoid metabolism and the activity of peroxisome proliferator receptors (Ppars) in the control of body fat reserves. Two different precursors for retinoids exist in the diet as preformed vitamin A (all-trans-retinol) and provitamin A (\textbeta,\textbeta-carotene). For retinoid production, \textbeta,\textbeta-carotene is converted to retinaldehyde by \textbeta,\textbeta-carotene monooxygenase 1 (Bcmo1). Previous analysis showed that Bcmo1 knock-out mice develop dyslipidemia and are more susceptible to diet-induced obesity. However, the role of Bcmo1 for adipocyte retinoid metabolism has yet not been well defined. Here, we showed that Bcmo1 mRNA and protein expression are induced during adipogenesis in NIH 3T3-L1 cells. In mature adipocytes, \textbeta,\textbeta-carotene but not all-trans-retinol was metabolized to retinoic acid (RA). RA decreased the expression of Ppar\gamma and CCAAT/enhancer-binding protein \alpha, key lipogenic transcription factors, and reduced the lipid content of mature adipocytes. This process was inhibited by the retinoic acid receptor antagonist LE450, showing that it involves canonical retinoid signaling. Accordingly, gavage of \textbeta,\textbeta-carotene but not all-trans-retinol induced retinoid signaling and decreased Ppar\gamma expression in white adipose tissue of vitamin A-deficient mice. Our study identifies \textbeta,\textbeta-carotene as a critical physiological precursor for RA production in adipocytes and implicates provitamin A as a dietary regulator of body fat reserves.

Among the many functions attributed to retinoids, its putative role in adipocyte biology and the regulation of body fat reserves have generated clinical and scientific interest (reviewed in Refs. 1, 2). Animal studies indicate that diets low in vitamin A favor adipose tissue formation (3, 4) and enhance formation of intramuscular fat (5). Regulation of fat reserves by dietary vitamin A can be explained by the metabolism of vitamin A to biologically active retinoid derivatives, which then impact the differentiation and function of adipose tissue. The vitamin A derivative all-trans-retinoic acid (RA)\textsuperscript{2} has been shown to inhibit adipocyte differentiation in cell culture (6, 7). In mature adipocytes, treatment with pharmacological doses of RA can induce lipolysis (4, 8), mitochondrial uncoupling (9, 10), and oxidative metabolism (11, 12) and influence the production of adipokines (13–16) both in cell culture and mouse models. Many of these effects are likely mediated by retinoic acid receptors (Rars). Rars are transcription factors that act in conjunction with retinoid X receptors (Rxrs) (17) to regulate the expression of numerous target genes in response to RA binding. Additionally, some effects of RA appear to arise from the activation of Ppar\beta and -\delta (8). Furthermore, the RA precursor retinaldehyde is present in fat \textit{in vivo} and can inhibit Ppar\gamma-induced adipogenesis (18).

A prerequisite for the initiation of vitamin A-dependent physiological processes is the production of these biologically active retinoids from dietary precursors. Surprisingly, high dose supplementation with preformed vitamin A does not affect body adiposity in mice (19). Hence, it is yet not clear whether dietary vitamin A can influence physiological energy balance and fat storage. Besides preformed vitamin A, \textbeta,\textbeta-carotene (BC) is a dietary source for retinoids. BC is oxidatively converted to RAL by the action of BC monooxygenase 1 (Bcmo1), an enzyme expressed in various tissues, including the intestine, liver, and adipocytes (20–22). Bcmo1 gene transcription is under the control of Ppar\gamma (23, 24), a key regulator of adipocyte differentiation and lipogenesis in mature adipocytes (reviewed in Ref. 25). However, the roles and relationships of Bcmo1 and Ppar in adipocyte biology and the regulation of fat reserves have yet to been further advanced.

Recent studies in knock-out mice indicate that Bcmo1 is the key enzyme for vitamin A production (26, 27). Aside from grossly impaired BC metabolism, this mouse mutant develops dyslipidemia, is more susceptible to diet-induced obesity, and shows increased expression of Ppar\gamma-induced genes in adipocytes (26). Importantly, these abnormalities were noted in Bcmo1-deficient mice well supplied with vitamin A, indicating

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\textsuperscript{4}The abbreviations used are: RA, all-trans-retinoic acid; BC, \textbeta,\textbeta-carotene; C/Ebp\alpha, CCAAT/enhancer-binding protein \alpha; FHR, fenretinide; iWAT, inguinal white adipose tissue; Ppar, peroxisome proliferator-activated receptor; RAL, all-trans-retinal; Rar, retinoic acid receptor; Rxr, retinoid X receptor; ROL, all-trans-retinol; RE, retinyl esters; TG, triacylglycerol; VAD, vitamin A-deficient diet; qRT-PCR, quantitative RT-PCR.
an important role of Bcmo1 for the regulation of lipid metabolism. Here, we here analyzed the role of Bcmo1 for retinoid metabolism of adipocytes by using both the NIH 3T3-L1 adipocyte cell culture system and mouse models.

**EXPERIMENTAL PROCEDURES**

Materials—All chemicals, unless stated otherwise, were purchased from Sigma. All reagents for cDNA synthesis and quantitative real time PCR (qRT–PCR) were purchased from Applied BioSystems (Foster City, CA). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen. The M-PER mammalian protein isolation reagent, BCA, Bradford protein assay kits, and the ECL reagents were purchased from Pierce. The triacylglycerol assay kit (catalog no. TG-1-NC) was purchased from Zen Bio (Research Triangle Park, NC). Primary antibodies against PPARγ and Fabp4/aP2 were obtained from Cell Signaling Technologies (Beverly, MA). Primary antibody against Ras-regulated nuclear protein (Ran) was purchased from Abcam (Cambridge, MA). The anti-mouse and anti-rabbit horseradish peroxidase-conjugated secondary antibodies were purchased from Promega (Madison, WI).

Cell Culture—Mouse preadipocyte NIH 3T3-L1 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin sulfate and cultured at 37 °C in 5% CO2. To induce differentiation, 2 days after reaching confluence (day 0), cells were placed in differentiation media consisting of DMEM, 10% serum, 0.5 mM 3-isobutyl-1-methylxanthine, 10 μg/ml insulin, and 0.25 mM dexamethasone for 3 days. On day 3, cells were incubated with DMEM containing 10% serum and 10 μg/ml insulin for 2 more days. Medium was then replaced with fresh DMEM containing 10% FBS, and cells were allowed to differentiate further. Stock solutions of BC, all-trans-retinol (ROL), and citral were prepared in appropriate reagents as suggested by the manufacturer (Sigma). BC was from Wild (Germany), and fenretinide was from Toronto Research Chemicals (Ontario, Canada). With the exception of citral (10 μM), all other metabolites were prepared at a final concentration of 2 μM in tetrahydrofuran (THF) for delivery to cells. Final concentrations of the solvent in cell culture media were always 0.1% (v/v). Control cells were treated with complete medium containing 0.1% THF (v/v). For experimental treatments, the appropriate metabolite was added to mature NIH 3T3-L1 cells in THF on day 5. Treated cells then were treated with 1 mM of LE540 for 1 h prior to addition of BC (2 μM), and cells were incubated for an additional 24 h. After harvesting, cells were collected and quantified spectrophotometrically, as described previously (28). About 2 μg of total RNA was reverse-transcribed by using the High Capacity RNA-to-cDNA kit and following the manufacturer’s instructions (Applied BioSystems). q-PCR was carried out with TaqMan chemistry, namely TaqMan Gene Expression Master Mix and Assays on Demand probes (Applied BioSystems) for mouse Pparγ (Mm01184323_m1), mouse Bcmo1 (Mm00502437_m1), mouse Fabp4/aP2 (Mm0044588_m1), mouse C/Ebpα (Mm00514283_s1), mouse Cyp26a1 (Mm00514486_m1), mouse Raldh1 (Mm00657317_m1), and mouse Adh1 (Mm00507711_m1). The 18S rRNA (4319413E) probe set (Applied BioSystems) was used as the endogenous control. All real time experiments were done with the Applied BioSystems Step-One Plus qRT–PCR machine. To control for between-sample variability, mRNA levels were normalized to 18 S rRNA for each sample by subtracting the Ct for 18 S rRNA from the Ct for the gene of interest, thereby producing a ΔCt value. The ΔCt for each treatment sample was compared with the mean ΔCt for control samples by using the relative quantitation 2−ΔΔCt method to determine fold-changes (Applied BioSystems Technical Bulletin No. 2).

Semi-quantitative PCR—Total RNA was isolated from control or experimental NIH 3T3-L1 cells, and first-strand cDNA synthesis was achieved by using the High Capacity RNA-to-cDNA kit following the manufacturer’s instructions (Applied BioSystems). Semi-quantitative PCR was performed with primers as follows: mouse Fabp4/aP2 (adipocyte lipoprotein binding protein), 5′-GAACCTGGAGAAGCTTGTCCTCG-3′ and 5′-ACCAGTTGTCACCATCTCG-3′; mouse Raldh1, 5′-TGTCTTGGAGAAGCTTGGTCTC-3′ and 5′-CGTGGGAGCATTGACATGATGG-3′; mouse retinol saturase, 5′-TGCCAGATGTGAAGAAGCAG-3′ and 5′-GAAAGGGAATGTGTTCTTGG-3′; and mouse cyclophilin (Cyc) 5′-TCCAAAGCACGGAGAAAACTTTCG-3′ and 5′-CCTTCTTGGCTGGTCTGTCATTCC-3′. The PCR program included denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 60 s (for a total of 25 cycles). PCR products were separated by electrophoresis on 2% (w/v) agarose gels.

**Immunoblotting Analyses**—Total protein from mouse adipose tissue or cultured cells was isolated as described previously (28) at indicated time points by using the M-PER mammalian protein extraction reagent with protease inhibitors (Roche Applied Science) according to the manufacturer’s instructions (Pierce). Mice were sacrificed by cervical dislocation. Appropriately adipose tissues were collected, rinsed in ice-cold phosphate-buffered saline (PBS) (pH 7.4), and snap-frozen in liquid nitrogen. Mouse adipose tissue (~100 mg) was then homogenized in liquid nitrogen with a mortar and pestle and transferred directly into M-PER buffer (500 μl) containing protease inhibitors and further processed as outlined by the manufacturer (Pierce). Samples were centrifuged at 12,000 rpm for 15 min at 4 °C, and protein content in the supernatant was determined by using either the bicinchoninic acid assay (BCA) reagent (Pierce) or the Bradford assay (Bio-Rad). Proteins (30–50 μg) were fractionated on 4–10% SDS-polyacrylamide gels with the Bio-Rad mini-gel system and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). Equal protein loading was
confirmed by Ponceau S staining and by immunoblotting analysis with anti-Ran. PVDF membranes were blocked by either 5% milk prepared in Tris/HCl (pH 7.4) and 100 mM NaCl, containing 0.05% Tween (TBS-T) or Super-block (Pierce) for 2–4 h and then immunoblotted with either anti-Pparγ, anti-FABP4/αP2, anti-Bcmo1 (1:1000 dilution), or anti-RAN antibody (1:5000 dilution) overnight at 4 °C. After multiple washes with TBS-T, membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature before being visualized with the ECL detection system (Pierce).

Quantitative Analysis of Immunoblotting Data—Measurements of signal intensity on PVDF membranes after immunoblotting with various antibodies were performed by using an AlphaImager densitometer with the AlphaEaseFC image processing and analysis software (Alpha Innotech). For statistical analyses, all data were expressed as integrated density values, which were calculated as the density values of specific protein bands/RAN density values and expressed as percentages of the control. All figures showing quantitative analyses include data obtained from at least three independent experiments.

Oil Red-O Staining—Cells were washed twice with PBS, fixed with 4% paraformaldehyde for a minimum of 1 h at room temperature, and then stained for 2 h with Oil Red-O solution (0.5% Oil Red-O in 100% isopropyl alcohol). After staining, cells were washed twice with PBS and visualized with an inverted microscope. Quantification of Oil Red-O stain from stained cells was performed by eluting the stain from cells with 1 ml of 100% isopropyl alcohol and then measuring the absorbance of the stain against a blank (100% isopropyl alcohol at 500 nm). This experiment was repeated three times and performed with at least four replicates per treatment.

Triacylglycerol Accumulation Assay—To determine the extent of lipid accumulation of experimental cells as compared with controls, we made use of the TG assay kit (Zen-Bio) and followed the manufacturer’s instructions. The reagent allows measurement of the concentration of glycerol released after lysing the cells and hydrolyzing the TG molecules. The TG concentration can then be determined from the glycerol values. For this experiment, mature adipocytes were treated with either 0.1% THF (vehicle control) or test compounds for 24 h, and the free glycerol released was assayed by using the TG assay kit for NIH 3T3-L1 adipocytes (Zen-Bio) and following the manufacturer’s instructions. The experiment was repeated twice with at least four replicates for each condition.

**Extraction of BC and Retinoids for HPLC and LC/MS Analysis**—Retinoids and carotenoids were extracted from serum under a dim red safety light (600 nm) as described previously (26). Adipose tissue (~40 mg) was incubated with 100 μl of 12% pyrogallol in ethanol, 200 μl of 30% KOH in water, and 1 ml of ethanol for 2 h at 37 °C. After saponification, 1 ml of H2O was added. Retinoids were extracted twice with 3 ml of ether/hexane (2:1, stabilized with 1% ethanol) and diluted with 2 ml of H2O and 2 ml ethanol. After centrifugation for 5 min at 800 × g, the organic layer was collected. The extract was evaporated in a SpeedVac, and the residue was dissolved in HPLC solvent. For extraction of nonpolar and polar retinoids, 25 mg of NIH 3T3-L1 cells were homogenized in 200 μl of 2 M hydroyxylamine (pH 6.8) and 400 μl of methanol. After 10 min, 10 μl of 5 M KOH were added. Nonpolar retinoids were extracted with 750 μl of hexane, and the organic phase was collected. Then 600 μl of 4 N HCl and 30 μl of saturated NaCl solution were added to the aqueous phase. After acidification, polar retinoids were extracted with 750 μl of hexane. Organic phases were dried in a SpeedVac. Polar retinoids were dissolved in HPLC solvent 1 (81% hexane, 19% ethyl acetate, 12.5 μl of acetic acid per 100 ml), and nonpolar retinoids and BC were dissolved in HPLC solvent 2 (99.5% hexane and 0.5% ethanol). HPLC separation of BC and different retinoids and quantification of peak integrals were performed as described previously (29, 30). The identity of retinoic acid was further confirmed by LC/MS. The polar retinoid extract was injected onto a Zorbax Sil column (Agilent Technologies, Santa Clara, CA) equilibrated with 81% hexane, 19% ethyl acetate, and 12.5 μl of acetic acid per 100 ml. The eluant was directed into LQX mass spectrometer through an atmospheric pressure chemical ionization source (Thermo Scientific, Waltham, MA) working in a positive mode. Retinoic acid was identified based on its molecular mass (m/z = 301 [MH]+) and a fragmentation pattern of the parent ion identical with that of a synthetic standard (Sigma).

**Enzymatic Assays—**Recombinant Bcmo1 was expressed in Escherichia coli. Protein preparation and tests for enzymatic activity were conducted as described previously (31). Fenretinide (FHR) was purchased from Toronto Research Chemical (Toronto, Canada) and was dissolved in ethanol and delivered together with the BC substrate in n-octyl-β-d-thioglycosyranoside micelles (3% w/v). Animals and Diets—Animal experiments were performed in accordance with United States animal protection laws by the guidelines of the local veterinary authorities. The lecithin-retinol acyltransferase-deficient mice (Lrat−/−) were kept in a room with controlled conditions (24 °C, 12:12-h light/dark cycle) with free access to food and water. In the first experiment, 8-week-old Lrat−/− mice (n = 5 animals per group) were fed for 3 weeks with a vitamin A-sufficient diet, containing 15,000 IU/kg or vitamin A-deficient diet (VAD) (AIN-93G Growing Rodent Diet With or without Added vitamin A; Research Diets, New Brunswick, NJ). After this period, animals were sacrificed, and the liver and inguinal white adipose tissues (iWAT) were excised in their entirety, weighed, snap-frozen in liquid nitrogen, and stored at −80 °C until analysis. To analyze the effects of BC on iWAT, Lrat−/− mice were fed for 10 days with the VAD diet based on a AIN-93G formulation (Research Diets, New Brunswick, NJ). HPLC analysis revealed that BC was below detection levels in this diet (data not shown). After this period, animals (n = 3 per group) were gavaged one time with 0.5 mg of BC (DSM Nutritional Products, Sisseln, Switzerland) or 0.5 mg of ROL (Sigma) dissolved in 100 μl of corn oil. As control, animals were gavaged with vehicle only. After 24 h, animals were sacrificed, and the liver and iWAT were removed, snap-frozen in liquid nitrogen, and stored at −80 °C until further analyses. In an additional experiment, Lrat−/− mice were fed for 10 days with the VAD diet. The animals (n = 5 per group) were gavaged three times, 24 h apart, with 0.5 mg of BC (DSM Nutritional Products, Sisseln, Switzerland) or 0.5 mg of ROL (Sigma) dissolved in 100 μl of corn oil. After 24 h, animals
were sacrificed, and the liver and iWAT were removed, snap-frozen in liquid nitrogen, and stored at −80 °C until further analyses. In all experiments, animals had free access to water and food and were sacrificed in the fed state.

**H&E Staining of Mouse Adipose Tissue**—Mouse adipose tissues were fixed in neutral buffered formalin for 24 h and processed routinely into paraffin blocks, and representative histological sections (10 μm) of each specimen were stained with hematoxylin and eosin (Merck) according to the described standard staining protocol being used at the Histology Core Facility (Institute of Pathology, Visual Sciences Research Center, University Hospitals, Cleveland, OH). Images were collected through an Olympus BX 60 microscope equipped with a ×20 objective and a SPOT camera (Diagnostic Instruments, Sterling Heights, MI). Adipocyte images were then analyzed with Metamorph Imaging Software (Molecular Devices, Downingtown, PA). Approximately 600 individual adipocytes were sized per condition. Average areas of adipocytes are expressed in square microns.

**Statistical Analyses**—Results are presented as means ± S.D., and the number of experiments is indicated in the figure legends. For each experiment, all determinations were performed at least in triplicate. Statistical significance was assessed by using the two-tailed Student’s t test.

**RESULTS**

**Bcmo1 Expression Is Induced during Adipocyte Differentiation**—The murine Bcmo1 promoter contains a DR1-type peroxisome proliferator-responder element (supplemental Fig. S1A) that binds Ppar/ΔR heterodimers (23), prompting us to examine expression patterns of Bcmo1 during adipocyte differentiation in the well established mouse NIH 3T3-L1 adipocyte cell culture model. Adipocyte differentiation was monitored by using Oil Red-O staining to assess lipid accumulation (supplemental Fig. S1B) and by following the mRNA expression of adipose marker genes, i.e. Ppar, fatty acid-binding protein 4 (Fabp4/aP2), and retinol saturase (RetSat). Semi-quantitative PCR analysis revealed that Bcmo1 mRNA expression increased during adipocyte differentiation and achieved maximal levels in mature NIH 3T3-L1 adipocytes (Fig. 1A). qRT-PCR analysis confirmed that Bcmo1 mRNA levels were 5-fold elevated in mature adipocytes as compared with preadipocytes (supplemental Fig. S1C). Immunoblotting revealed a 7-fold increase of Bcmo1 at the protein level upon adipocyte differentiation (supplemental Fig. S1D) and by following the mRNA expression of adipose marker genes, i.e. Ppar, fatty acid-binding protein 4 (Fabp4/aP2), and retinol saturase (RetSat). Semi-quantitative PCR analysis revealed that Bcmo1 mRNA expression increased during adipocyte differentiation and achieved maximal levels in mature NIH 3T3-L1 adipocytes (Fig. 1A). qRT-PCR analysis confirmed that Bcmo1 mRNA levels were 5-fold elevated in mature adipocytes as compared with preadipocytes (supplemental Fig. S1C). Immunoblotting revealed a 7-fold increase of Bcmo1 at the protein level upon adipocyte differentiation (supplemental Fig. S1D). Thus, as expected for a Ppar-γ-inducible target gene, Bcmo1 expression increased during adipocyte differentiation at both the mRNA and protein levels.

**BC but Not ROL Decreases Lipid Content of Mature Adipocytes**—We next tested whether the Bcmo1 substrate BC can impact adipocyte biology by treating mature NIH 3T3-L1 adipocytes with either 2.0 μM BC or vehicle alone (control cells). Additionally, cells were treated with 2 μM ROL, the other circulating precursor of biologically active retinoids. After 24 h, cells were fixed, and Oil Red-O staining for lipids was performed. BC-treated cells showed less staining for lipids compared with ROL-treated and control cells (Fig. 1C). Microscopic inspection revealed that lipid droplets were reduced in size and number in BC-treated compared with control or ROL-treated cells (Fig. 1C, bottom panel). Spectrophotometric quantification indicated that BC-treated cells displayed a 2.8-fold reduced staining with Oil Red-O compared with nontreated and ROL-treated cells (supplemental Fig. S1E). Likewise, the triacylglycerol (TG) content of adipocytes was 2.7-fold reduced upon BC but not ROL treatment of these differentiated cells (Fig. 1D).

**Fenretidine Selectively Inhibits BC Effects on Adipocytes**—Our study showed that BC can reduce TG content in mature NIH 3T3-L1 adipocytes. To discriminate between the effects of parent BC and its retinoid derivatives, we needed a highly selective inhibitor of Bcmo1 enzymatic activity. Positively charged retinoids such as retinylamine selectively block the activity of related plant carotenoid oxygenases (34). By taking into consideration the chemical properties of retinylamine and aromatic amide conjugates have recently been shown to inhibit the activity of related plant carotenoid oxygenases (34). A compound that combines the chemical properties of retinylamine and aromatic amides is FHR. To test whether FHR inhibits Bcmo1 activity, we expressed murine Bcmo1 in E. coli and delivered BC in n-octyl-β-D-thioglucopyranosside micelles. Recombinant Bcmo1 metabolized BC to RAL, and the reaction was linear for 10 min (Fig. 2A). We then delivered BC in n-octyl-β-D-thioglucopyranoside micelles in the presence of increasing amounts of FHR. As shown in Fig. 2B, FHR inhibited the activity of recombinant Bcmo1 in a dose-dependent manner. So we then treated mature NIH 3T3-L1 adipocytes with BC in the absence and presence of FHR. After 24 h, we stained cells with
Oil Red-O for lipids (Fig. 2C) and measured cellular TG content (Fig. 2D). Although BC treatment alone reduced TG content, this effect was largely prevented by FHR. Treatment with FHR only did not result in a reduction of TG content (Fig. 2D). Thus, FHR selectively inhibited the effects of BC on adipocyte lipid content, indicating that they are dependent on Bcmo1 activity.

**BC but Not ROL Is Converted to RA in Mature Adipocytes—**To determine the biologically active retinoid derivative that mediates this effect, we extracted nonpolar and polar retinoids and performed HPLC analyses. In BC-treated adipocytes, no increase of ROL and/or retinyl ester (RE) levels was found (Fig. 3A), but these cells produced RA (9.6 ± 2 pmol/mg) (Fig. 3A), the identity of which was confirmed by tandem mass spectrometry [MH]+ = 301 and [MH – H2O]+ = 283 (Fig. 3, C and D). Treatment of adipocytes with BC in the presence of the Bcmo1 inhibitor FHR reduced the production of RA by 8-fold (1.2 ± 0.42 pmol/mg) (Fig. 3A). In ROL-treated adipocytes, a strong increase in RE and ROL levels was detected, but RA was below the detection limit (Fig. 3B). Thus, BC and ROL have different metabolic fates in NIH 3T3-L1 adipocytes, being metabolized to RA and RE, respectively.

**BC Represses Ppar/CCAAT/Enhancer-binding Protein α Expression in Mature Adipocytes—**RA treatment inhibits adipocyte differentiation (18). Treatment of cells with BC in the presence of citral, an inhibitor of retinal dehydrogenases (Raldhs), did not reproduce the inhibitory effects of treatment with BC alone on Ppar mRNA and protein levels and C/Ebpα mRNA levels (Fig. 4, A and D). Additionally, we analyzed the effect of the different treatments on adipocyte expression of Faby4/aP2, a gene well known to be regulated by Ppar. BC treatment reduced Faby4/aP2 mRNA and protein expression 2.8- and 4.6-fold, respectively. Again, this effect was not observed in cells treated with BC in the presence of FHR or citral or upon treatment with either ROL or RA (Fig. 4, D and E). Thus, we conclude that endogenous RA derived from BC cleavage can decrease the expression of key lipogenic transcription factors in mature adipocytes.

**Effects of BC in Mature Adipocytes Are Mediated by Ras—**Our study showed that Bcmo1-dependent RA production can
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FIGURE 3. ββ-Carotene but not all-trans-retinol is converted to all-trans-retinoic acid in mature adipocytes. A, HPLC traces at 360 nm of polar retinoid extracts from 12.5 mg of untreated (a), ROL-treated (b), RA-treated (c), BC-treated and FHR-pretreated (d), and BC-treated (e) mature NIH 3T3-L1 adipocytes. Inset shows the spectrum of RA peak of BC-treated mature NIH 3T3-L1 adipocytes. B, HPLC traces at 325 nm of nonpolar retinoid extracts from 12.5 mg of untreated (a), BC-treated (b), and ROL-treated (c) mature NIH 3T3-L1 adipocytes. The inset shows the spectrum of RE peak of ROL-treated mature NIH 3T3-L1 adipocytes. C, averaged MS spectrum collected for retinoic acid extracted from the peak indicated with RA in A. A prominent peak at m/z = 301 corresponds to molecular mass of retinoic acid ([MH]+). Full mass spectrum showed ions that can be attributed to its dehydration product (m/z = 283). D, fragmentation pattern of a parent ion selected at m/z = 301 with characteristic peaks corresponding to loss of H₂O or COOH group (m/z = 283 and 255, respectively).

down-regulate C/Ebpa and Pparγ expression in mature NIH 3T3-L1 adipocytes. Inhibitory effects of RA on adipogenesis are largely mediated by RARs (7). To investigate whether the effects of BC-derived RA in mature adipocytes are RAR-dependent, we first analyzed Cyp26a1 mRNA levels. Cyp26a1 is an RA hydroxylase, and its mRNA expression is induced by RA in a strictly RAR-dependent manner (38, 39). In mature adipocytes treated with BC alone, Cyp26a1 mRNA levels were 2.8-fold induced compared with control cells (supplemental Fig. S3A). This induction was not seen in mature adipocytes treated with either BC and FHR or BC and citral together. Additionally, ROL-treated cells failed to show induction of Cyp26a1 mRNA expression (supplemental Fig. S3A). Thus, in agreement with RA production, BC treatment induced the expression of this RAR-responsive gene. To provide direct evidence that the effects of BC on adipocyte gene expression are mediated by Rars, we took advantage of the Rar pan-antagonist, LE450 (40).

NIH 3T3-L1 adipocytes were pretreated with LE450 for 1 h prior to the addition of BC. After 24 h, we harvested cells and determined mRNA expression levels of Cyp26a1, Pparγ, and Fabp4/aP2. Although BC treatment alone induced Cyp26a1 and reduced Pparγ and Fabp4/aP2 mRNA levels, these effects were largely prevented by pretreatment with LE450 (supplemental Fig. S3, B–D). Additionally, treatment with LE450 prevented the effects of BC on the TG content of adipocytes (supplemental Fig. S3E). Thus, we conclude that the effects of BC on mature adipocytes are RAR-dependent.

Dietary BC but Not ROL Decreases Pparγ Expression—Our studies in cell culture indicated that BC plays an important role for retinoid signaling in mature adipocytes. Hence, one would expect that dietary BC but not preformed vitamin A exerts a similar role in animals. Therefore, we analyzed the effects of vitamin A deficiency as well as ROL and BC supplementation on adipose tissues of mice. Lrat−/− mice cannot convert ROL to RE and lack retinoid stores in the liver (41). Therefore, these mice are highly susceptible to dietary vitamin A deficiency (41, 42). To induce vitamin A deficiency in these mice, we fed 8-week-old Lrat−/− animals a diet either lacking any source of vitamin A (n = 5) or a vitamin A-sufficient diet (n = 5). After 3 weeks, we sacrificed the animals and isolated iWAT. As expected, HPLC analysis showed that retinoid levels were significantly reduced in iWAT of animals subjected to vitamin A deprivation (supplemental Fig. S4A). We next determined Pparγ and F fab4/aP2 mRNA expression levels in the iWAT of these animals by qRT-PCR. Despite vitamin A deprivation, these mice exhibited no changes as compared with siblings maintained on a vitamin A-sufficient diet (Fig. 5A). Additionally, histology showed that adipocytes were comparable in size between the vitamin A-deficient and -sufficient animals (Fig. 5B and supplemental Fig. S4B). Thus, dietary restriction of preformed vitamin A had no effect on Pparγ mRNA expression and activity in iWAT of mice.

We next gavaged vitamin A-deprived Lrat−/− mice with the same amount of BC and ROL, respectively, or vehicle only. We then measured retinoid levels in iWAT of mice. Upon ROL treatment, retinoid levels significantly increased over levels in vehicle only-treated controls. In contrast, in BC-treated animals, Pparγ mRNA levels were 3-fold decreased, and Cyp26a1 mRNA expression levels in the iWAT of these animals by qRT-PCR. Despite vitamin A deprivation, these mice exhibited no changes as compared with siblings maintained on a vitamin A-sufficient diet (Fig. 5A). Additionally, histology showed that adipocytes were comparable in size between the vitamin A-deficient and -sufficient animals (Fig. 5B and supplemental Fig. S4B). Thus, dietary restriction of preformed vitamin A had no effect on Pparγ mRNA expression and activity in iWAT of mice.
mRNA levels were 2.1-fold increased. Similar results were obtained after three consecutive treatments (24 h apart) with ROL and BC (Fig. 5E), indicating that BC but not ROL increased RA signaling and reduced Pparγ in iWAT.

We also measured effects of different treatments on mRNA expression of key enzymes of retinoid metabolism in iWAT (Fig. 5, D and E). Bcmo1 and retinal dehydrogenase 1 (Raldh1) mRNA levels were 4- and 2.1-fold increased, respectively, after a single treatment with BC as compared with vehicle only-treated animals. Raldh1 acts downstream of Bcmo1 and converts the BC cleavage product RAL to RA. In contrast, Adh1 mRNA expression was 4-fold decreased under this condition. This alcohol dehydrogenase catalyzes RAL to ROL conversion. No changes in the mRNA expression of these genes were observed in ROL-treated animals. Thus, BC induced mRNA expression of genes encoding key enzymes for RA production in iWAT, including Bcmo1 and Raldh1, and repressed expression of the enzyme for RAL to ROL conversion.

DISCUSSION

Here, we showed in cell culture and mouse models that BC is the physiological precursor for RA production in adipocytes. RA production from BC strictly depended on the activity of Bcmo1 that was expressed as a Pparγ-induced gene during adipogenesis. BC but not ROL reduced Pparγ expression and lipid content of mature adipocytes. These findings provide evidence that Bcmo1 plays a critical role for the cross-talk between Pparγ and Rar signaling pathways and implicates BC as an important dietary regulator of fat storage capacities of adipocytes.

FIGURE 4. β,β-Carotene but not all-trans-retinol represses expression of key adipogenic markers. A–C, quantitative RT-PCR analysis of Pparγ, C/EBPα, and Fabp4/aP2 mRNA levels in mature NIH 3T3-L1 adipocytes, respectively, with and without treatments as indicated. D, representative Pparγ and Fabp4/aP2 immunoblots of mature NIH 3T3-L1 adipocytes with treatments as indicated. Ras-related nuclear protein (Ran) was used as a protein loading control. Error bars represent ± S.D., n = 3 per condition. Two-tailed Student’s t test; *p < 0.001. Differentiated adipocytes (D, day 5). C, citral.

BC Is a Physiological Precursor for RA Production in Adipocytes—Upon cloning Bcmo1, a surprising result was that steady-state mRNA levels of the vitamin A-forming enzyme were relatively high in peripheral nondigestive tissues, including adipocytes (20–22). These findings indicate that besides intestinal conversion of BC and transport of vitamin A, a tissue-specific conversion of the provitamin may influence retinoid-dependent physiological processes. Bcmo1-deficient mice accumulate BC in various tissues, including adipocytes, indicating that the BC substrate is well distributed within the body (26). Promoter analysis revealed that Bcmo1 is a Ppar-induced gene that contains a peroxisome proliferator-binding responsive element that binds Ppar/RXr heterodimers (23). In fact, we showed here that Bcmo1 expression increased during adipogenesis of NIH 3T3-L1 cells both at the mRNA and protein levels. Treatment of mature NIH 3T3-L1 adipocytes with BC resulted in RA production. RA production was significantly reduced in the presence of FHR that inhibited enzymatic activity of recombinant Bcmo1. In contrast, treatment of mature NIH 3T3-L1 adipocytes with performed vitamin A resulted in RE but not RA production. In conformity with the cell culture study, gavage of BC but not ROL induced expression of the classical RA-induced target gene Cyp26a1 in the iWAT of vitamin A-deficient mice.

This critical role of BC for RA metabolism might be explained by specific biochemical characteristics of adipocyte. Previous studies have revealed that enzymatic activity of Bcmo1 is stimulated in the presence of cellular retinol-binding protein 1 (Crbp1) (27). Crbp1 can bind RAL, the BC cleavage product, and is expressed in various tissues, including adipocytes (43). In the liver, Crbp1 stimulates RE formation, the storage form of vitamin A, from Crbp1-bound retinoids in a LRAT-dependent manner (44). Adipocytes express different acyltransferases than Lrat for RE formation (41). Additionally, the expression of the RAL to ROL-converting enzyme Adh1 is decreased during adipogenesis (18). Additionally, our study showed that BC treatment decreased Adh1 mRNA expression, whereas it induced Bcmo1 and Raldh1 mRNA expression in iWAT. Thus, reduced expression of Adh1 and, on the other hand, induced expression of Bcmo1 and Raldh1 (this study and see Ref. 18) likely explain why the primary BC cleavage product, RAL, is predominantly metabolized into RA in adipocytes.

BC Decreases Pparγ Signaling and Lipid Storage Capacity of Adipocytes—Previous analyses of Bcmo1 knock-out mice have already indicated a specific role of Bcmo1 for retinoid metabolism in adipose tissues. Bcmo1−/− mice show increased expres-
sion of Ppary-induced genes in iWAT and are more susceptible to diet-induced obesity (26). Our current analysis provided a molecular and mechanistic explanation for this phenotype by showing that Bcmo1 plays an important role for RA production in adipocytes. RA is the ligand of Rars that act in conjunction with Rxrs. During preadipocyte to adipocyte conversion, liganded Rars in mature 3T3-L1 adipocytes RA derived from BC conversion may have a role in mediating this process toward energy expenditure, including reproduction, a process in which BC-derived retinoids play a pivotal role (47). In the absence of BC, adipocytes increasingly store energy in the form of fat. In humans, circulating BC levels are inversely correlated with risk of type 2 diabetes, a pathology associated with obesity (36, 48–50). Additionally, reduced plasma levels of carotenoids, including BC, are commonly found in obese children (46). Thus, the complex role of BC in adipocyte biology in health and disease deserves further research.

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FIGURE 5. Effects of β,β-carotene and vitamin A on adipose tissue of Lrat<sup>−/−</sup> mice. A and B, Lrat<sup>−/−</sup> mice (n = 5 per group) were maintained either on a VAD or a vitamin A-sufficient diet (VAS). A, relative mRNA expression of key adipogenic genes in iWAT of Lrat<sup>−/−</sup> mice provided with diets indicated. B, representative images of H&E-stained sections of iWAT from Lrat<sup>−/−</sup> mice provided with diets as indicated. The scale bar represents 200 μm. C and D, Lrat<sup>−/−</sup> mice were maintained for 10 days on a vitamin A-deficient diet. The animals (n = 3 per condition) were then gavaged with 0.5 mg of BC or ROL dissolved in 100 μl of corn oil or vehicle only. 24 h after gavage, animals were sacrificed. Retinoid levels (retinyl esters and all-trans-retinol) (C) and mRNA levels of Bcmo1, Ppary, Cyp26a1, Adh1, and Raldh1 (D) were quantified in iWAT of animals. In the experiments, error bars represent ± S.D., n = 3 per group. Two-tailed Student’s t test; *, p < 0.05 as compared with vehicle only-treated animals. E, Lrat<sup>−/−</sup> mice were maintained for 10 days on a vitamin A-deficient diet. The animals (n = 5 per condition) were then gavaged with 0.5 mg of BC or ROL dissolved in 100 μl of corn oil. Gavage was performed three times in 24-h intervals. 24 h after the last gavage, animals were sacrificed, and mRNA levels of Bcmo1, Ppary, Fasp4/Ap2, Cyp26a1, Adh1, and Raldh1 were determined in iWAT. In the experiments, error bars represent ± S.D.; n = 5 per group. Two-tailed Student’s t test; #, p < 0.05 as compared with all-trans-retinol-treated animals. VAS, vitamin A-sufficient diet.
