Dietary 9-cis-β,β-Carotene Fails to Rescue Vision in Mouse Models of Leber Congenital Amaurosis

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ABSTRACT

Synthetic 9-cis-stereoisomers of vitamin A (all-trans-retinol) are especially promising agents for the fight against blinding diseases. Several studies suggested that 9-cis-β,β-carotene (9-cis-BC), a natural and abundant β-carotene isomer in the diet, could be the precursor of 9-cis-retinoids and thus could have therapeutic applications. Here we showed that 9-cis-BC is metabolized both in vitro and in vivo by two types of mouse carotenoid oxygenases, β,β-Carotene monoxygenase 1 (BCMO1), and β,β-carotene dioxygenase 2 (BCDO2). In the symmetric oxidative cleavage reaction at C15,C15’ position by BCMO1, part of the 9-cis-double bond was isomerized to the all-trans-stereoisomer, yielding all-trans-retinal and 9-cis-retinal in a molar ratio of 3:1. The asymmetric cleaving enzyme BCDO2 preferentially removed the 9-cis-ring site at the C9,C10 double bond from this substrate, providing an all-trans-β-10'-apocarotenal product that can be further metabolized to all-trans-retinal by BCMO1. Studies in knockout mouse models confirmed that each carotenoid oxygenase can metabolize 9-cis-BC. Therefore, treatment of mouse models of Leber congenital amaurosis with 9-cis-BC and 9-cis-retinyl-acetate, a well established 9-cis-retinal precursor, showed that the cis-carotenoid was far less effective than the cis-retinoid in rescuing vision. Thus, our in vitro and in vivo studies revealed that 9-cis-BC is not a major source for mouse 9-cis-retinoid production but is mainly converted to all-trans-retinoids to support canonical vitamin A action.

Introduction

An enzyme-based cyclic pathway for trans-to-cis isomerization of the visual pigment chromophore all-trans-retinal is intrinsic to mammalian retinal rod and cone vision. This pathway, called the visual or retinoid cycle, involves two cellular compartments, both rod and cone outer segments and closely associated retinal pigmented epithelium (RPE) (von Lintig et al., 2010). In addition, cones can be supported by a pathway involving Müller cells (Fleisch and Neuhauss, 2010; Wang and Kefalov, 2011). Genetic disruption of the visual cycle in mice results in rapid or slowly progressive death of rods and cones (Travis et al., 2007). For example, inactivating mutations in lecithin:retinol acyltransferase (LRAT) and retinoid isomerase [also known as RPE protein of 65 kDa (RPE65)] are associated with severe forms of retinitis pigmentosa (RP) including LCA in humans (Thompson and Gal, 2003). In contrast, perhaps because of redundancy in the redox system, mutations in retinol dehydrogenase 5, an enzyme responsible for oxidation of 11-cis-retinol to 11-cis-retinal, cause a mild form of retinal dysfunction called fundus albipunctatus and mild RP with slow dark adaptation (recovery of vision after illumination) and cone and rod degeneration (Travis et al., 2007; den Hollander et al., 2008).

Substantial efforts have been undertaken to establish therapies for patients who have blinding diseases affecting the retina (for review see Palczewski 2010; den Hollander et al. 2010). Among them, a pharmacological intervention with 9-cis-retinoids has been successfully established in animal models and is currently undergoing evaluation in patients

ABBREVIATIONS: RPE, retinal pigmented epithelium; LRAT, lecithin:retinol acyl transferase; RPE65, retinal pigmented epithelium protein of 65 kDa; RP, retinitis pigmentosa; 9-cis-BC, 9-cis-β,β-carotene; LCA, Leber congenital amaurosis; BCMO1, β,β-carotene monoxygenase 1; BCDO2, β,β-carotene dioxygenase 2; 9-cis-R-Ac, 9-cis-retinyl acetate; qRT, quantitative real-time; PCR, polymerase chain reaction; ETOH, ethanol; MeOH, methanol; HPLC, high-performance liquid chromatography; WT, wild-type; DMSO, dimethyl sulfoxide; MOPS, 4-morpholinepropanesulfonic acid; ERG, electroretinogram.
with different retinal diseases (Palczewski, 2010). This method relies on the fact that 9-cis-retinal can serve as a surrogate chromophore in 11-cis-retinal-deficient photoreceptors (Palczewski, 2006). 9-cis-Retinal binds to the opsin moieties of rod and cone visual pigments, thereby preserving vision and preventing retinal degeneration in homologous animal models for LCA (Van Hooser et al., 2000; Maeda et al., 2009).

Recent intriguing results from a clinical trial indicated that supplementation with 9-cis-BC, readily available as a surrogate for 11-cis-retinal, can improve vision in patients who have fundus albinopunctatus caused by mutations in retinal dehydrogenase 5 (Rotenstreich et al., 2010). It has been further suggested that retinal dystrophies with similar mechanisms such as various types of RP would benefit from this pharmacological intervention. The authors proposed that this effect is probably mediated by 9-cis-BC as a precursor for 9-cis-retinoids (for review, see Parker, 1996). Thus, 9-cis-BC metabolism remains to be defined in detail.

In recent years, carotenoid oxygenases have been identified as one of several key participants in carotenoid metabolism (for review, see von Lintig, 2010). The critical importance of BCMO1 for retinoid production was demonstrated in a knockout mouse model (Hessel et al., 2007). BCMO1 is expressed in various human tissues, including the small intestine and parenchymal cells of liver (Lindqvist et al., 2009). Previous studies of 9-cis-BC metabolism were mainly conducted in the context of 9-cis-retinoic acid production. The latter compound specifically activates retinoid X receptors but also can activate nuclear hormone receptors such as the retinoic acid receptors (Heyman et al., 1992). However, the same studies reported conflicting results regarding the effectiveness of 9-cis-BC as a precursor for 9-cis-retinoids (for review, see Parker, 1996). Thus, 9-cis-BC metabolism remains to be defined in detail.

Materials and Methods

Materials. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Reagents for cDNA synthesis and quantitative real-time (qRT-)polymerase chain reaction (PCR) were obtained from Applied Biosystems (Foster City, CA). 9-cis-Retinal and all-trans-retinal were provided by Toronto Research Chemicals Inc. (North York, ON, Canada). In brief, to prepare 9-cis-R-As, 100 mg of 9-cis-retinal was reduced with 50 mg of sodium borohydride in 0.7 ml of EtOH at 0°C for 30 min. Solid 9-cis-retinol and 80 mg of 4-dimethylaminopyridine were dissolved in 0.4 ml of dry CH$_2$Cl$_2$ and 0.1 ml of acetic anhydride was added. After 6 h at 10°C, the reaction was quenched with 0.1 ml of ethanol, CH$_2$Cl$_2$ was removed by flowing argon at 20°C, and 9-cis-R-As was purified by organic extraction and dried under argon (Batten et al., 2005). Dunaliella bardawil extracts were purchased from Nikken So Honsa Corp. (Gifu, Japan). All-trans-10′-β-apo-8-carotenal was a gift from Dr. Hansgeorg Ernst (BASF, Ludwigshafen, Germany).

Preparation of All-trans-BC and 9-cis-BC from D. bardawil Extracts. All experimental procedures related to extraction, retinoid derivatization, and separation of retinoids were conducted under a dim red light provided by a safelight filter (transmittance >560 nm, No. 1; Eastman Kodak Co., Rochester, NY). Granules in one D. bardawil extract (1 capsule; 9 mg of carotenes) were homogenized in a Kontes glass-glass homogenizer with 2 ml of MeOH until the granules became fine and well mixed. The resulting suspension was placed in a Kimax glass tube along with 2 ml of H$_2$O and 4 ml of hexane and was capped and vortexed for 3 min before centrifugation for 5 min at 2000 rpm and 4°C. The upper layer was collected and dried in a SpeedVac (Eppendorf, Hamburg, Germany) and then dissolved in 4 ml of organic solvent composed of 75:25 MeOH-methyl tert-butyl ether. Sample solutions were separated on a ProntoSil 200-3-C30 3.0–μm column with 75:25 MeOH-methyl tert-butyl ether at a flow rate of 1.3 ml/min. For all purifications, 100 μl of the above solution was injected into the ProntoSil column. The peak fraction corresponding to 9-cis-BC (447 nm) was collected into a 5-ml glass tube and stored at −80°C.

Enzyme Kinetics. Previously described plasmids used for the expression of recombinant murine BCMO1 and BCDO2 (Kiefer et al., 2001; Amengual et al., 2011) were transfected into Escherichia coli BL21. Protein expression and tests for enzymatic activity were performed according to a published protocol (Oberhauser et al., 2008). In brief, appropriate amounts of all-trans-BC and 9-cis-BC dissolved in hexane were mixed with 25 μl of 12% (w/v) n-octyl β-D-thioglucopyranoside dissolved in EtOH and dried in a SpeedVac. Then, enzyme solution (100 μl) was added, and the solution was vortexed for 20 s. Enzymatic assays were incubated at 28°C for the indicated time periods and then stopped by the addition of 100 μl of NH$_4$OH and 200 μl of MeOH. Lipophilic compounds were extracted with 400 μl of acetone and 500 μl of hexane. Extraction with hexane was repeated twice, and the resulting organic phases were combined, dried in a SpeedVac, and redissolved in 200 μl of HPLC solvent. HPLC analysis was performed on a normal-phase Zorbax Sil (5-μm, 4.6 × 150 mm) column (Agilent Technologies, Santa Clara, CA) with chromato-

graphic separation achieved by isocratic flow of 10% ethyl acetate-hexane at a flow rate of 1.4 ml/min for retinal oximes and 30% ethyl acetate-hexane at a flow rate of 1.4 ml/min for 10′-β-apo-8-carotenal oximes. For quantification, HPLC systems were scaled with known amounts of different all-trans-stereoisomer and 9-cis-stereoisomers of retinal oximes or all-trans-10′-β-apo-8-carotenol oximes, respectively.

Mice and 9-cis-BC, All-trans-BC, and 9-cis-R-As Administration. Animal experiments were conducted according to accepted standards of humane care and use of laboratory animals and were approved by the Case Western Reserve University Animal Use and Care Committee (protocol number 2008-0074). The generation and genotype of Bcmo1($−/−$), Bcd2($−/−$), Lrux($−/−$), and Rpe65($−/−$) mice have been described previously (Redmond et al., 1998; Batten et
al., 2004; Hessel et al., 2007; Amengual et al., 2011b). All knockout mouse strains and wild-type (WT) control mice had a mixed C57/BL6;129Sv genetic background. Mice at 5 to 6 weeks of age were injected intraperitoneally with either dimethyl sulfoxide (DMSO), 9-cis-R-∆c, all-trans-∆c, or 9-cis-∆c. All injections were delivered in a total volume of 60 µl through a 28 1/2-gauge needle. Each injection contained 0.16 mg of a test compound dissolved in DMSO, and a total of five daily injections were given. Mice were maintained in the dark during this injection period. Animals subsequently were sacrificed under dim red safety light, and their livers and eyes were removed, immediately placed on dry ice, and stored in a −80°C freezer before carotenoid and retinoid analyses.

Carotenoid and Retinoid Analyses. Eyes were homogenized in 1 ml of retinoid analysis buffer (50 mM MOPS, 10 mM NH4OH, and 50% EtOH in H2O, pH 7.0). Samples were allowed to incubate at room temperature for 20 min, and then 1 ml of EtOH was added to stop the reaction. Next 5 ml of hexane was added to the homogenate and vortexed for 1 min before centrifugation for 5 min at 2000 rpm and 4°C. The collected hexane phase was evaporated in the SpeedVac, and 300 µl of fresh hexane was added. Samples were eluted on a normal-phase HPLC system using an Agilent silicon column at 1.4 ml/min with a gradient of hexanes and ethyl acetate (0–15 min: 0.5% ethyl acetate; 15–60 min: 6% ethyl acetate) as described previously (Amengual et al., 2011b). Extraction of carotenoids and retinoids and retinyl ester saponification of the liver were performed as described previously (Amengual et al., 2011a).

RNA isolation and qRT-PCR. RNA was isolated from mouse adipose tissue or cultured adipocytes (± indicated treatments) with the TRIzol reagent (Invitrogen, Carlsbad, CA), purified by using the RNaseasy kit (Qiagen, Valencia, CA) and then quantified spectrophotometrically, as described previously (Lobo et al., 2010). Approximately 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). qRT-PCR was performed with TaqMan chemistry, namely TaqMan Gene Expression Master Mix Assays on Demand probes (Applied Biosystems) for mouse Bedo2 (Mm00460051_m1) and for mouse Bcmol (Mm00520437_m1), respectively. The 18S rRNA probe set (Applied Biosystems) was used as the endogenous control. All real-time experiments were performed with the Applied Biosystems Step-One Plus qRT-PCR machine. To control for between-sample variability, mRNA levels were normalized to 18S rRNA for each sample by subtracting the Ct from 18s rRNA from the Ct for the gene of interest, thereby producing a ΔCt value. ΔCt for each treatment sample was compared with the mean ΔCt for control samples by using the relative quantification formula 2−ΔΔCt method to determine fold-changes (Applied Biosystems Technical Bulletin 2).

Electroretinogram. All electroretinogram (ERG) procedures were performed by previously published methods (Maeda et al., 2009). In brief, mice under a safety light were anesthetized by intraperitoneal injection of 20 µl/g b.wt. of 6 mg/ml ketamine and 0.44 mg/ml xylazine diluted with 10 mM sodium phosphate, pH 7.2, containing 100 mM NaCl. Pupils were dilated with 1% tropicamide. Mice were maintained in the dark during this injection period. Animals subsequently were sacrificed under dim red safety light, and their livers and eyes were removed, immediately placed on dry ice, and stored in a −80°C freezer before carotenoid and retinoid analyses.

Results

Isolation and Purification of 9-cis-∆c. For 9-cis-∆c purification, we used commercially available preparations of the unicellular algae D. bardawil (Ben-Amotz et al., 1988). Lipids were extracted and separated by HPLC with an Agilent C30 silicon column, which revealed that the extract contained two major compounds with absorption maxima and retention times described for all-trans-∆c and 9-cis-∆c, respectively (Supplemental Fig. 1A). We then established a quantitative HPLC method for collecting each BC stereoisomer (Supplemental Fig. 1B). Separated BC stereoisomers were promptly stored in DMSO at −80°C in light-tight glass vials until further use. To determine the stability of these preparations, we reanalyzed the geometric composition of isolated 9-cis-∆c at intervals up to 10 days and found it was stable under these storage conditions (Supplemental Fig. 1B).

Bcmol and 9-cis-∆c Metabolism. To determine whether 9-cis-∆c can be converted by Bcmol, we first expressed recombinant murine Bcmol in E. coli and then assayed this reaction by a previously established procedure (Oberhauser et al., 2008). Recombinant murine Bcmol was incubated with 20 µM all-trans-∆c for 5 min, and lipids were isolated by HPLC. As expected, this analysis revealed that all-trans-∆c was converted to all-trans-retinal (Fig. 1, A and B). Aside from the all-trans-retinal product, trace amounts of the 13-cis-retinal became detectable, but other retinal stereoisomers were absent. When similar assays were performed with 20 µM 9-cis-∆c as the substrate, HPLC analysis detected that the retinal cleavage products were the all-trans-retinal, 9-cis-retinal, and 13-cis-retinal stereoisomers as confirmed by cochromatography with authentic standards (Fig. 1, C and D). Moreover, production of different retinal stereoisomers from all-trans-∆c and 9-cis-∆c increased with prolonged incubation (Fig. 2, A and B). We next determined Bcmol conversion rates for 9-cis-∆c and all-trans-∆c by incubating the same Bcmol enzyme preparation with 20 µM concentrations of each substrate. These analyses showed that Bcmol metabolized all-trans-∆c 5-fold faster than 9-cis-∆c (Fig. 2, A and B). Again, all-trans-∆c was mainly converted to all-trans-retinal. With 9-cis-∆c as substrate, all-trans-retinal, 9-cis-retinal, and 13-cis-retinal stereoisomers were formed at a molar ratio of approximately 9:3:1 (Fig. 2B). Finally, Km and Vmax values were determined by incubating the same enzyme preparation with increasing amounts of the two BC substrates for 5 min (Fig. 2, C and D). For all-trans-∆c, the Km value was estimated to be 53.6 µM and Vmax was estimated to be 188.6 pmol retinal/min × mg. With 9-cis-∆c, the Km value was approximately 14.3 µM, and Vmax was 11.8 pmol retinal/min × mg.

Thus, although Bcmol1 demonstrated a lower Km value for the 9-cis-∆c stereoisomer, it metabolized 9-cis-∆c at a much lower rate than the all-trans-∆c stereoisomer. It is noteworthy that 9-cis-∆c was not converted to equimolar amounts of the 9-cis-retinal and the all-trans-retinal stereoisomers by cleavage at the C15′ position. Production of the all-trans-stereoisomer was 3-fold higher than that of the 9-cis-stereoisomer, indicating that the 9-cis-double bond was partially isomerized to the all-trans configuration during this reaction.
Fig. 1. Products of recombinant murine BCMO1 enzymatic activity. Protein extracts of recombinant murine BCMO1 were incubated in the presence of 20 μM all-trans-BC or 9-cis-BC. After 5 min, lipids were extracted and separated by normal-phase HPLC. A, HPLC monitored at 360 nm. Upon incubation of BCMO1 with all-trans-BC significant amounts of all-trans-retinal were produced (solid line). The identity of this compound was verified by cochromatography with an authentic standard (dotted line). Note that all-trans-retinal was converted to the corresponding syn- and anti-oxime forms during extraction. Peak 1, all-trans-retinal oxime (syn); peak 2, all-trans-retinal oxime (anti). B, spectra of all-trans-retinal oxime (syn) from the enzymatic reaction mixture (solid line) and the authentic standard (dotted line). C, HPLC monitored at 360 nm. Upon incubation of BCMO1 with 9-cis-BC, different retinal stereoisomers became detectable (solid line). The identity of these compounds was verified by cochromatography with authentic standards for all-trans-retinal (dotted line) and 9-cis-retinal (dashed line). Note that different retinal stereoisomers were converted to the corresponding syn- and anti-oxime forms during extraction. Peak 1, all-trans-retinal oxime (syn); peak 2, 9-cis-retinal oxime (syn); peak 3, 13-cis-retinal oxime (syn); peak 4, 13-cis-retinal oxime (anti); peak 5, 9-cis-retinal oxime (anti); and peak 6, all-trans-retinal oxime (anti). D, spectra of all-trans-retinal oxime (syn) from the enzymatic reaction mixture (solid line) and the authentic standard (dotted line). E, spectral characteristics of 9-cis-retinal oxime (syn) from the enzymatic reaction mixture (solid line) and the authentic standard (dotted line). mAU, milliabsorbance units.

Fig. 2. Kinetics of the conversion of all-trans-BC and 9-cis-BC by murine BCMO1. Protein extract with recombinant murine BCMO1 was incubated in the presence of 20 μM all-trans-BC or 9-cis-BC. Reactions were stopped after different time intervals; lipids were extracted and subjected to HPLC analysis. Each value shown represents the average of three independent experiments. A, all-trans-BC. B, 9-cis-BC. Insets show the amounts of retinal stereoisomers produced per minute by the same enzyme preparation. Values represent the mean ± S.D. from three independent enzymatic assays. C and D, Lineweaver-Burk plots of reactions with (C) all-trans-BC and (D) 9-cis-BC. Each value represents the mean ± S.D. of three independent enzymatic assays.
BCDO2 Also Catalyzes the Conversion of 9-cis-BC. Besides BCMO1, BCDO2 can contribute to 9-cis-BC metabolism. To analyze the putative role of BCDO2 in 9-cis-BC metabolism, we performed enzyme assays with recombinant murine BCDO2 by incubating the same enzyme preparation with either 20 μM all-trans-BC or 9-cis-BC (Fig. 3A). After a 5-min incubation, putative cleavage products were isolated and subjected to HPLC analysis. With all-trans-BC, significant amounts of all-trans-β-10'-apocarotenal were produced as verified by cochromatography with an authentic standard (Fig. 3, A and C). With 9-cis-BC as substrate, all-trans-β-10'-apocarotenal was also the major cleavage product (Fig. 3, A and C). Aside from this stereoisomer, another likely 9-cis-β-apocarotenal cleavage product with a comparable spectrum but different retention time was produced (Fig. 3, A and C). With 9-cis-BC as substrate, the all-trans-stereoisomer product exceeded the amount of the 9-cis-stereoisomer by approximately 7-fold, indicating that BCDO2 preferentially removed the β-ionone ring from the 9-cis-configured site of BC.

Metabolism of 9-cis-BC in WT and Carotenoid Oxygenase-Deficient Mouse Models. We next analyzed the contribution of each carotenoid oxygenase to 9-cis-BC metabolism by taking advantage of Bcmo1−/− and Bcd2−/− mouse lines. In a previous study, we reported that intestinal BC absorption is repressed with consumption of vitamin A-sufficient diets (Lobo et al., 2010). In addition, several studies indicate that intestinal absorption of 9-cis-BC is significantly lower than that of all-trans-BC. Therefore, to avoid problems with intestinal absorption, we intraperitoneally injected 9-cis-BC dissolved in DMSO. This route of BC administration has been shown to result in efficient retinoid production in mice (Kim et al., 2011). Here we injected animals with 0.2 mg of 9-cis-BC dissolved in 60 μl of DMSO five times at daily intervals and maintained them continuously in the dark. After 5 days, we analyzed BC and retinoids in the liver. Whereas WT mice accumulated 9-cis-BC in this organ, accumulation of 9-cis-BC was significantly lower in Bcmo1−/− and Bcd2−/− deficient animals (Fig. 4A). This result was surprising because previous studies indicated that BC accumulates in large quantities in Bcmo1 knockout mice but not in WT animals (Hessel et al., 2007). A possible explanation for such accumulation was revealed when we measured mRNA expression levels of the two carotenoid oxygenases in different mouse strains. Thus, in knockout mice, expression of each remaining carotenoid oxygenase were significantly increased over WT levels (Fig. 4B). Because both carotenoid oxygenases can metabolize 9-cis-BC as shown above, this increased expression probably explains the lower 9-cis-BC levels in knockout compared with WT mice.

We next investigated whether 9-cis-BC injection can result in 9-cis-retinoid production in Bcmo1−/− and Bcd2−/− mice by analyzing their liver retinoid levels. Because retinoids mainly exist in the form of retinyl esters in the liver, we saponified liver samples and separated different retinol stereoisomers by HPLC (Fig. 4C). The major retinoid found was the all-trans-retinol stereoisomer with only trace amounts of 9-cis-retinol and 13-cis-retinol detected. In WT and Bcd2−/− mice, 9-cis-retinol levels were slightly higher...
than in Bcmo1(−/−) mice, but this difference did not achieve statistical significance. We also analyzed the retinoid composition in the eyes of these two knockout mouse strains (Fig. 4D). This analysis revealed that retinoid composition and levels in dark adapted eyes were comparable in the WT and knockout mice, with 11-cis-retinal and all-trans-retinyl esters identified as the major retinoids. Trace amounts of 9-cis-retinyl esters were present in the eyes of WT and Bcdo2(−/−) mice but were absent in Bcmo1(−/−) mice. These experiments provide in vivo evidence that both carotenoid oxygenases can contribute to 9-cis-BC metabolism in mice. However, intraperitoneal injection of 9-cis-BC did not result in significant production of 9-cis-retinoids in the eyes and livers of these animals.

**Metabolism of 9-cis-BC in Lrat(−/−) and Rpe65(−/−) Mice.** In a recent study, it was proposed that 9-cis-BC can improve vision in patients who have RP (Rotensteinrech et al., 2010). To test this proposal in animal models of this condition, we injected 6-week-old Lrat(−/−) and Rpe65(−/−) mice intraperitoneally with all-trans-BC, 9-cis-BC, or vehicle (DMSO) control. At this early stage, mutant mice still display structurally intact photoreceptors (Wenzel et al., 2007). On importance, we also included 9-cis-R-Ac-injected mice as positive controls in this study. In this procedure, animals were given daily intraperitoneal injections of 0.2 mg of each compound dissolved in 60 μl of DMSO. After 5 days of treatment, improvement of visual function was evaluated by ERG recording and 9-cis-retinal level in the eyes were quantified by normal-phase HPLC (Fig. 5). In Lrat(−/−) mice, this analysis revealed that significant amounts of 9-cis-retinal were present in the eyes after the 9-cis-R-Ac injections. In contrast, 9-cis-BC administration did not increase this compound in the eyes. As expected, all-trans-BC- and vehicle control-injected mice showed the same negative result. In contrast, Rpe65(−/−) mice showed an increase in 9-cis-retinal after injections with either 9-cis-BC or 9-cis-R-Ac over levels found in vehicle only controls. No such increases were observed in mice that had undergone the all-trans-BC regimen. 9-cis-Retinal levels in the eye after the 9-cis-R-Ac injection schedule was 25-fold higher than those of 9-cis-BC-injected RPE65-deficient animals (Fig. 5, A and B). ERG recording showed significant improvement of retinal function only in Lrat(−/−) mice. In contrast, administration with 9-cis-BC did not improve light sensitivity of the eyes (Fig. 5, C and D). This result was consistent with the 9-cis-retinal level in Lrat(−/−) mice.

Thus, 9-cis-BC was not effective in promoting 9-cis-retinal production and restoring vision in the eyes of two mouse models of LCA. In contrast, 9-cis-R-Ac was efficiently used for 9-cis-retinal production and restored vision in the eyes of these mouse models.

**Discussion**

Double bonds in the carbon backbone of carotenoids and their retinoid derivatives can exist in cis and trans configurations. In animals, 11-cis-retinal (or derivatives thereof) constitutes the chromophore of animal visual pigments (Wald, 1968). In addition, 9-cis-retinoic acid can activate nuclear hormone receptors such as retinoic acid receptors and specifically the retinoid X receptors (Heyman et al., 1992). These are transcription factors that play important roles in processes as diverse as embryonic development, immunity, and metabolic control. Moreover, 9-cis-retinal can bind the opsin moiety of visual pigments to form iso-rhodopsin. However, whether 9-cis-retinoids play a physiological role and how these compounds are synthesized from dietary precursors is controversial. Therefore, in this study we investigated 9-cis-BC metabolism and evaluated the effectiveness of 9-cis-BC support of 9-cis-retinal production in the eyes of mouse models of RP.
9-cis-BC Metabolism in Mammals. In gerbils, 9-cis-BC can be used as a dietary source of vitamin A but with only approximately 38% efficiency compared with all-trans-BC (Deming et al., 2002). In addition, this study showed that there was no increase in 9-cis-retinoids in the livers of mice upon 9-cis-BC supplementation. Our biochemical analyses and studies in animal models provide a mechanistic explanation for these observations. In the symmetrical cleavage reaction, BCMO1 converted 9-cis-BC into the all-trans-retinal, 9-cis-retinal, and 13-cis-retinal stereoisomer in a molar ratio of 9:3:1 (Fig. 6). This finding contrasts with reports that 9-cis-BC is converted in a 1:1 molar ratio to 9-cis-retinoids and all-trans-retinoids (Wang et al., 1994; Hébuterne et al., 1995). However, it does agree with observations in cell-free intestinal and liver extracts from rats, indicating a geometric composition of the cleavage products comparable with that we report with the recombinant enzyme (Nagao and Olson, 1994). These findings suggest that BCMO1 has intrinsic isomerase activity similar to that recently demonstrated for structurally related enzymes in insects (Oberhauser et al., 2008; Voolstra et al., 2010). This enzyme converts all-trans-carotenoids to 11-cis-retinoids and all-trans-retinoids to support visual pigment production. This intrinsic isomerase activity is also in agreement with recent biochemical and structural analyses of this class of nonheme iron oxygenases, revealing that the ferrous iron in the active site is accessible by a hydrophobic tunnel and a specific interaction with one half-site of the carotenoid substrate (Kloer et al., 2005; Kiser et al., 2009). Measurements of the $K_m$ value showed that BCMO1 has even a higher affinity for 9-cis-BC than all-
Thus, interaction of BCMO1 with the 9-
trans-BC; b, 9-
retinal; d, all-
-9-retinal; c, all-
trans-BC in different mouse models. a, cis-retinoids from 9-
these findings provide an explanation for the lack of production of significant amounts of 9-
someric site of BC resulting in the formation of all-
trans would lead to the production of 9-
retinal and all-
ring sites of this substrate. Exclusion of the 9-
trans-BC, indicating that the enzyme can interact with both 
ring sites of this substrate. Exclusion of the 9-cis-ring site would result in a doubling of the $K_m$ value as recently shown for the insect enzyme with asymmetric carotenoid substrates (Oberhauser et al., 2008). Several lines of evidence indicate that a carboxylation intermediate is formed during reactions with these enzymes (Kiser et al., 2009; Poliakov et al., 2009). In this carboxylation, intermediate C–C bonds can undergo trans-to-cis and probably cis-to-trans isomerization as well. Thus, interaction of BCMO1 with the 9-cis ring site should lead to an isomerization and the production of all-trans-retinal (Fig. 6). In contrast, interaction with the all-trans site would lead to the production of 9-cis-retinal and all-trans-retinal. The latter mechanism could explain our observation that the all-trans-stereoisomer exists in a 3-fold molar excess over the 9-cis-stereoisomer after enzymatic cleavage by BCMO1. Then there is an additional mechanism that reduces formation of 9-cis-retinoids from 9-cis-BC. BCD02 preferentially removed the 10'-ionone ring site from the 9-cis-stereoisomic site of BC resulting in the formation of all-trans-10'-apocarotenal, which can then undergo a second cleavage reaction catalyzed by BCMO1 to form all-trans-retinal (Fig. 6). Indeed, we found a small increase of 9-cis-retinoids in the eyes and liver of Bcd02-deficient mice that overexpress BCMO1, thus indicating that some 9-cis-retinoids are produced in the absence of BCD02. Subsequent cleavage of 9-cis-BC by both carotenoid oxygenases also contributes to the lower vitamin A-producing efficiency of 9-cis-BC compared with that of all-trans-BC. Furthermore, it provides an explanation for the finding that the stereoisomeric composition of liver retinoids was comparable after 9-cis-BC or all-trans-BC supplementation in WT animals (Deming et al., 2002). Conversion of 9-cis-BC by BCD02 is also favored by the kinetics of 9-cis-BC conversion by BCMO1. BCMO1 showed a much lower conversion rate with the cis-stereoisomer, which also explains the observation that 9-cis-BC accumulates in tissues of WT mice. In knockout mice, this accumulation was less pronounced because of the compensatory increased expression of each remaining carotenoid oxygenase.

A critical question is whether our findings in mouse models can be translated to the human situation. In contrast to rodents, humans absorb a significant portion of dietary BC intact and display relatively high blood levels of this compound. In addition, mechanisms exist to transport carotenoids to the eyes as demonstrated by the accumulation of macular pigments (zeaxanthin and lutein) in the fovea lutea. Thus, 9-cis-BC might be absorbed intact and transported to the eyes to be locally converted to 9-cis-retinoids. Molecular players for carotenoid metabolism are well conserved between rodents and humans (for review, see von Lintig, 2010). In one study, an individual with a heterozygotic mutation in the $BCMO1$ gene is expressed in various human tissues (Lindqvist et al., 2005). For intestinal absorption, studies in humans showed that the all-trans-isomer is much better absorbed from the diet than the 9-cis

**Fig. 6.** Model of 9-cis,β-β-carotene metabolism in mice. 9-cis-BC can be metabolized by the two different carotenoid oxygenases, BCMO1 (1) and BCD02 (2). The percentage of occurrence of the different cleavage product stereoisomers was determined by enzymatic assays with recombinant proteins. For BCMO1, the discrepancy of these values from the theoretical value (50%) indicates that the enzyme can interact with both 10'-ionone ring sites and that the enzyme possesses intrinsic isomerase activity when interacting with the 9-cis-ring site. For BCD02, the discrepancy of these values from the theoretical value (50%) indicates that the enzyme preferentially removes the 9-cis ring site. The cleavage product all-trans-10'-apocarotenal can be further metabolized by BCMO1. The second cleavage product 9-cis-β-10'-apocarotenal also is probably metabolized by BCMO1. In conclusion, these findings provide an explanation for the lack of production of significant amounts of 9-cis-retinoids from 9-cis-BC in different mouse models. a, 9-cis-BC; b, 9-cis-retinal; c, all-trans-retinal; d, all-trans-10'-apocarotenal; e, 9-cis-β-10'-apocarotenal; f, 10'-ionone; ??, not experimentally tested.
geometric states of BC (Stahl et al., 1993; Gaziano et al., 1995). Studies in humans also brought up the proposal that the 9-cis double bond of BC is isomerized to the all-trans double bond during absorption (Stahl et al., 1993). After 13C-labeled 9-cis-BC supplementation, the resulting [13C]retinoids existed mainly in the all-trans configuration (You et al., 1996). Thus, 9-cis-BC is not well absorbed in humans, and absorbed 9-cis-BC is mainly converted to all-trans-retinoids. The latter finding is probably explained by the same enzymatic properties of human BCMO1 and BCDO2 as here described for their murine counterparts.

Taken together, our studies provide a mechanistic explanation for the observation that 9-cis-BC is mainly converted to all-trans-retinoids, which can be further metabolized to all-trans-retinoic acid and 11-cis-retinol to support canonical retinoid action. These findings are in agreement with recent studies that evaluated the physiological role of 9-cis-retinoids in mammalian biology. Mouse studies indicate that only the all-trans-retinoic acid stereoisomer is required for normal retinoid production from dietary precursors under physiological conditions.

Implications for the Pharmacological Use of 9-cis-BC and 9-cis-R-Ac.

In pharmacological settings, agonists for 9-cis-R-Ac have been successfully used to restore retinoid X receptors, so-called rexinoids, have been developed and 9-cis-R-Ac has been shown to be effective in several animal models of RP (Palczewski, 2010). In a recent study, supplementation of 9-cis-BC in animal models of RP has been shown to restore retinoid X receptors in the eyes of mice (You et al., 1996). Thus, 9-cis-BC is not well absorbed in humans, and absorbed 9-cis-BC is mainly converted to all-trans-retinoids. The latter finding is probably explained by the same enzymatic properties of human BCMO1 and BCDO2 as here described for their murine counterparts.

References


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