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

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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 monooxygenase 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) that converts all-*trans*-retinyl esters to 11-*cis*-retinol] 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

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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 monooxygenase 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 dietary supplement powder containing 40 to 50% 9-*cis*-BC from the alga *Dunaliella bardawil* (Ben-Amotz et al., 1988), also can improve vision in patients who have fundus albipunctatus 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 conversion to 9-*cis*-retinal through symmetric oxidative cleavage by the β,β-carotene-15,15′-monooxygenase (BCMO1). It is noteworthy that *Rpe65*(−/−) mice also possess some residual vision as a result of endogenous 9-*cis*-retinal, and levels of this compound also increase when mutant mice are kept in the dark (Fan et al., 2003). Thus, an enzymatic pathway for the production of this compound could exist in mammals. However, whether 9-*cis*-BC can promote 9-*cis*-retinal production is not confirmed by the literature, and the concept still lacks rigorous testing in animal models. As an alternative, a 9-*cis*-BC/all-*trans*-BC mixture could affect visual function because of its antioxidant and/or other effects (Demmig-Adams and Adams, 2002; Obulesu et al., 2011). 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.

In recent years, carotenoid oxygenases have been identified as one of several key participants in carotenoid metabolism (for review, see von Lintig, 2010). Thus, 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., 2005). Moreover, BCMO1 is abundant in the RPE of human eyes, and cell culture studies showed that human RPE cells can convert BC to retinoids (Yan et al., 2001; Chichili et al., 2005). In addition, β,β-carotene-9,10′-oxygenase (BCDO2), a mitochondrial dioxygenase expressed in various tissues, can convert BC and many other carotenoids by eccentric oxidative cleavage to apocarotenoid products (Kiefer et al., 2001).

By taking advantage of novel tools and reagents, including knockout mouse models for carotenoid oxygenases, we have analyzed and now report on certain biochemical features of 9-*cis*-BC metabolism. We also compared the relative efficacy of pharmacological interventions with 9-*cis*-R-Ac and 9-*cis*-β,β-carotene (9-*cis*-BC). We found that dietary 9-*cis*-BC fails to rescue vision in two mouse models of LCA.

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-Ac, 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_2Cl_2, and 0.1 ml of acetic acid anhydride was added. After 6 h at 10°C, the reaction was quenched with 0.1 ml of ethanol, CH_2Cl_2 was removed by flowing argon at 20°C, and 9-*cis*-R-Ac was purified by organic extraction and dried under argon (Batten et al., 2005). *Dunaliella bardawil* extracts were purchased from Nikken So Honsha Corp. (Gifu, Japan). All-*trans*-10′-β-apocartenal 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* supplement (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_2O and 4 ml of hexane and was capped and vortexed for 1 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 components 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., 2011b) 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_2OH 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 chromatographic 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 flow rate of 1.4 ml/min for β-10′-apocarotenal 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-apocarotenal oximes, respectively.

Mice and 9-*cis*-BC, All-*trans*-BC, and 9-*cis*-R-Ac 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*(−/−), *Bcdo2*(−/−), *Lrat*(−/−), 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;129Svj genetic background. Mice at 5 to 6 weeks of age were injected intraperitoneally with either dimethyl sulfoxide (DMSO), 9-*cis*-R-Ac, all-*trans*-BC, or 9-*cis*-BC. 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 DSMO, 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 NH_2OH, and 50% EtOH in H_2O, 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 Speed-Vac, 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 RNeasy system (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 and Assays on Demand probes (Applied Biosystems) for mouse *Bcdo2* (Mm00460051_m1) and for mouse *Bcmo1* (Mm00502437_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 C_t for 18s rRNA from the C_t for the gene of interest, thereby producing a ΔC_t value. The ΔC_t for each treatment sample was compared with the mean ΔC_t for control samples by using the relative quantification $2^{-(\Delta\Delta C_t)}$ 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. A contact lens electrode was placed on the eye and a reference electrode and ground electrode were positioned on the ear and tail, respectively. ERGs were recorded with a computerized system (UTAS E-3000; LKC Technologies, Inc., Gaithersburg, MD).

Single-Flash Recording. The duration of white light flash stimuli (from 20 μs to 1 ms) was adjusted to provide a range of illumination intensities (from −3.7 to 1.6 log candela · s/m^2). Three to five recordings were made at sufficient intervals (from 3 s to 1 min) between flash stimuli to allow recovery from any photo-bleaching effects.

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

Isolation and Purification of 9-*cis*-BC. For 9-*cis*-BC 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*-BC and 9-*cis*-BC, 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*-BC at intervals up to 10 days and found it was stable under these storage conditions (Supplemental Fig. 1B).

BCMO1 and 9-*cis*-BC Metabolism. To determine whether 9-*cis*-BC can be converted by BCMO1, we first expressed recombinant murine BCMO1 in *E. coli* and then assayed this reaction by a previously established procedure (Oberhauser et al., 2008). Recombinant murine BCMO1 was incubated with 20 μM all-*trans*-BC for 5 min, and lipids were isolated by HPLC. As expected, this analysis revealed that all-*trans*-BC 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*-BC 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*-BC and 9-*cis*-BC increased with prolonged incubation (Fig. 2, A and B). We next determined BCMO1 conversion rates for 9-*cis*-BC and all-*trans*-BC by incubating the same BCMO1 enzyme preparation with 20 μM concentrations of each substrate. These analyses showed that BCMO1 metabolized all-*trans*-BC 5-fold faster than 9-*cis*-BC (Fig. 2, A and B). Again, all-*trans*-BC was mainly converted to all-*trans*-retinal. With 9-*cis*-BC 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, K_m and V_{max} 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*-BC, the K_m value was estimated to be 53.6 μM and V_{max} was estimated to be 188.6 pmol retinal/min × mg. With 9-*cis*-BC, the K_m value was approximately 14.3 μM, and V_{max} was 11.8 pmol retinal/min × mg.

Thus, although BCMO1 demonstrated a lower K_m value for the 9-*cis*-BC stereoisomer, it metabolized 9-*cis*-BC at a much lower rate than the all-*trans*-BC stereoisomer. It is noteworthy that 9-*cis*-BC was not converted to equimolar amounts of the 9-*cis*-retinal and the all-*trans*-retinal stereoisomer by cleavage at the C15,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.

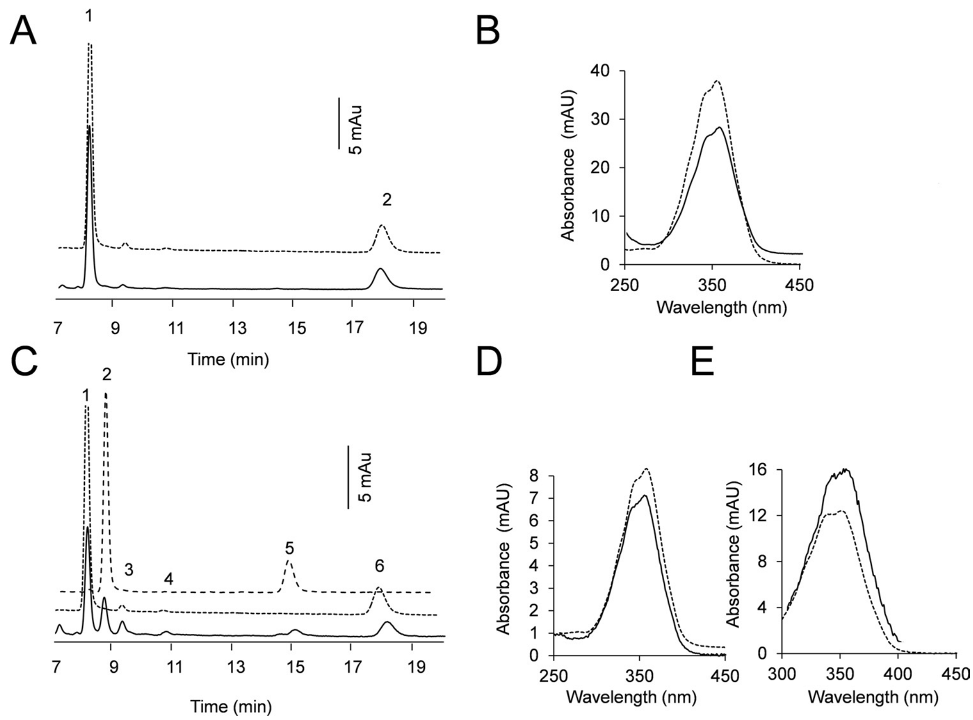

Fig. 1. Products of recombinant murine BCMO1 enzymatic activity. Protein extracts of recombinant murine BCMO1 were incubated in the presence of 20 μM all-*trans*-BC or 9-*cis*-BC. After 5 min, lipids were extracted and separated by normal-phase HPLC. A, HPLC monitored at 360 nm. Upon incubation of BCMO1 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.

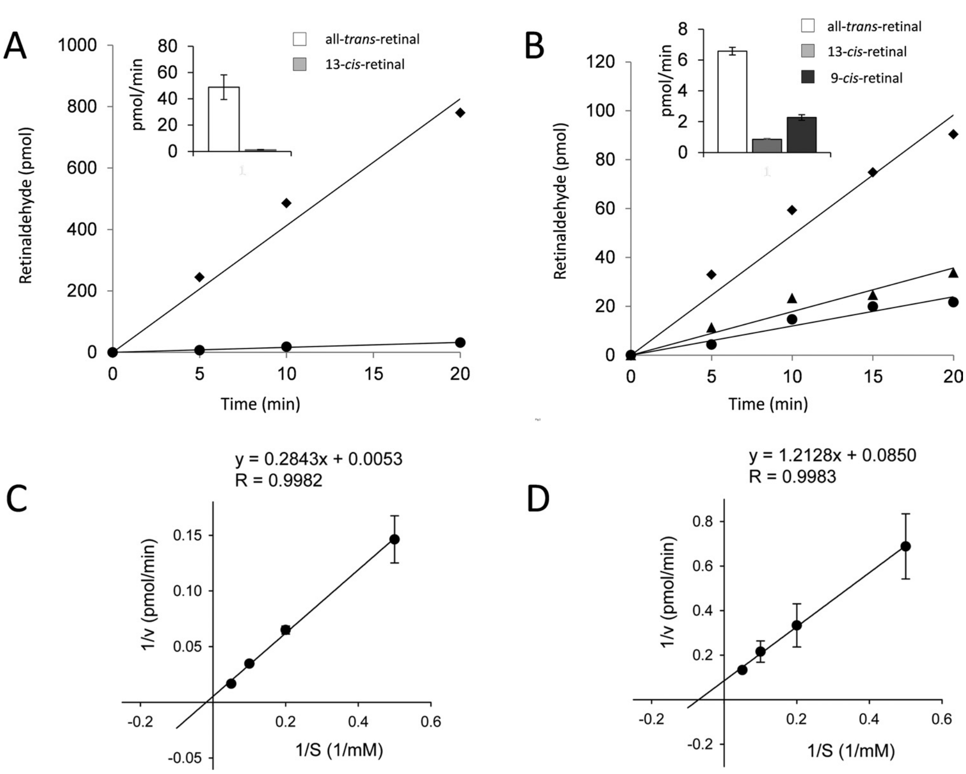

Fig. 2. Kinetics of the conversion of all-*trans*-BC and 9-*cis*-BC by murine BCMO1. Protein extract with recombinant murine BCMO1 was incubated in the presence of 20 μM all-*trans*-BC or 9-*cis*-BC. Reactions were stopped after different time intervals; lipids were extracted and subjected to HPLC analysis. 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 means $\pm$ 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 $\pm$ 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′-apocartenal 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). BCDO2-catalyzed turnover rates for 9-*cis*-BC and all-*trans*-BC were comparable (Fig. 3B). 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 *Bcdo2*(−/−) 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 *Bcdo2*-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 *Bcmo2*(−/−) 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 *Bcdo2*(−/−) mice, 9-*cis*-retinol levels were slightly higher

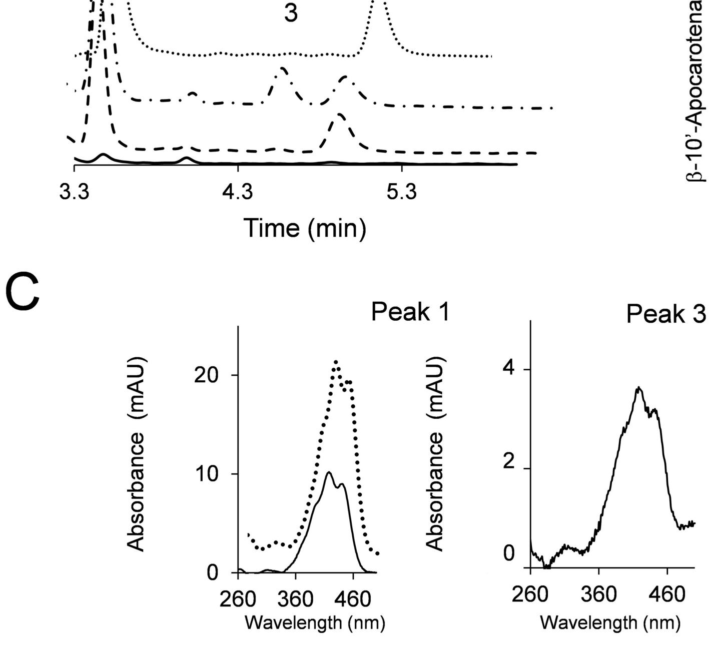

Fig. 3. Reactions of BCDO2 with all-*trans*-BC and 9-*cis*-BC. The same BCDO2 enzyme preparation was incubated in the presence of 20 μM all-*trans*-BC or 9-*cis*-BC. Reactions were stopped after 5 min, and lipids were extracted and separated by HPLC. Note that the apocarotenoids were converted to the corresponding oximes during extraction. A, HPLC at 420 nm. Lower trace, 9-*cis*-BC without incubation; dashed trace, all-*trans*-BC; dashed-dotted trace, 9-*cis*-BC; dotted trace; all-*trans*-β-10′-apocarotenal standard. Peak 1, 10′-β-apocarotenal oxime (*syn*); peak 2, 10′-β-apocarotenal oxime (*anti*); peak 3, 9-*cis*-10′-β-apocarotenal. B, conversion rates of all-*trans*-BC and 9-*cis*-BC. Values represent the mean ± S.D. of three independent enzymatic assays. C, spectral characteristics of peak 1, 10′-β-apocarotenal oxime (*syn*) (left) produced by the enzymatic reaction (solid line) and from the authentic standard (dotted line), and peak 3, 9-*cis*-10′-β-apocarotenal. mAU, milliabsorbance units.

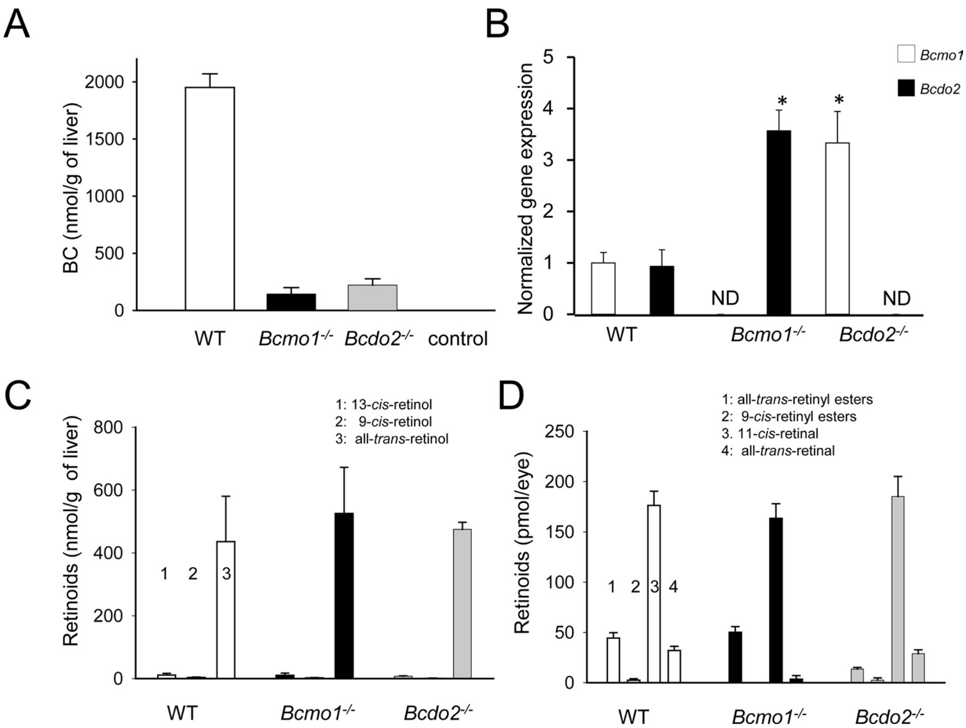

Fig. 4. Retinoid profiles in mouse eyes and liver after administration of β,β-carotene isomers. *Bcmo1*(−/−), *Bcdo2*(−/−), and WT mice were treated with 9-*cis*-BC at 1.0 mg/mouse via intraperitoneal injection over the course of 5 consecutive days. Retinoid profiles in the eyes and liver were evaluated by normal-phase silica column chromatography. Trace levels of 9-*cis*-retinyl esters detected in WT and *Bcdo2*(−/−) mouse eyes were not observed in *Bcmo1*(−/−) or WT mice treated with DMSO. A, significant amounts of β,β-carotenes were detected in WT liver, whereas their levels were lower in *Bcmo1*(−/−) and *Bcdo2*(−/−) mice ($< 5\%$ compared with WT) after 9-*cis*-BC treatment. B, qRT-PCR analysis of BCMO1 and BCDO2 expression in livers of indicated mouse strains values are represented as fold changes compared with WT and are normalized to levels of 18S rRNA. Error bars represent ± S.D.; $n = 5$/genotype. Two-tailed Student's *t* test, *, $p \leq 0.01$. ND, not detectable. C, retinoid composition of the livers of different mouse strains upon 9-*cis*-BC injections. D, retinoid composition of the eyes with five daily 9-*cis*-BC injections. Three mice representing each genotype were treated.

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 (Rotenstreich 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*(−/−) and *Rpe65*(−/−) mice treated with 9-*cis*-R-Ac. 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*(−/−) and *Rpe65*(−/−) 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.

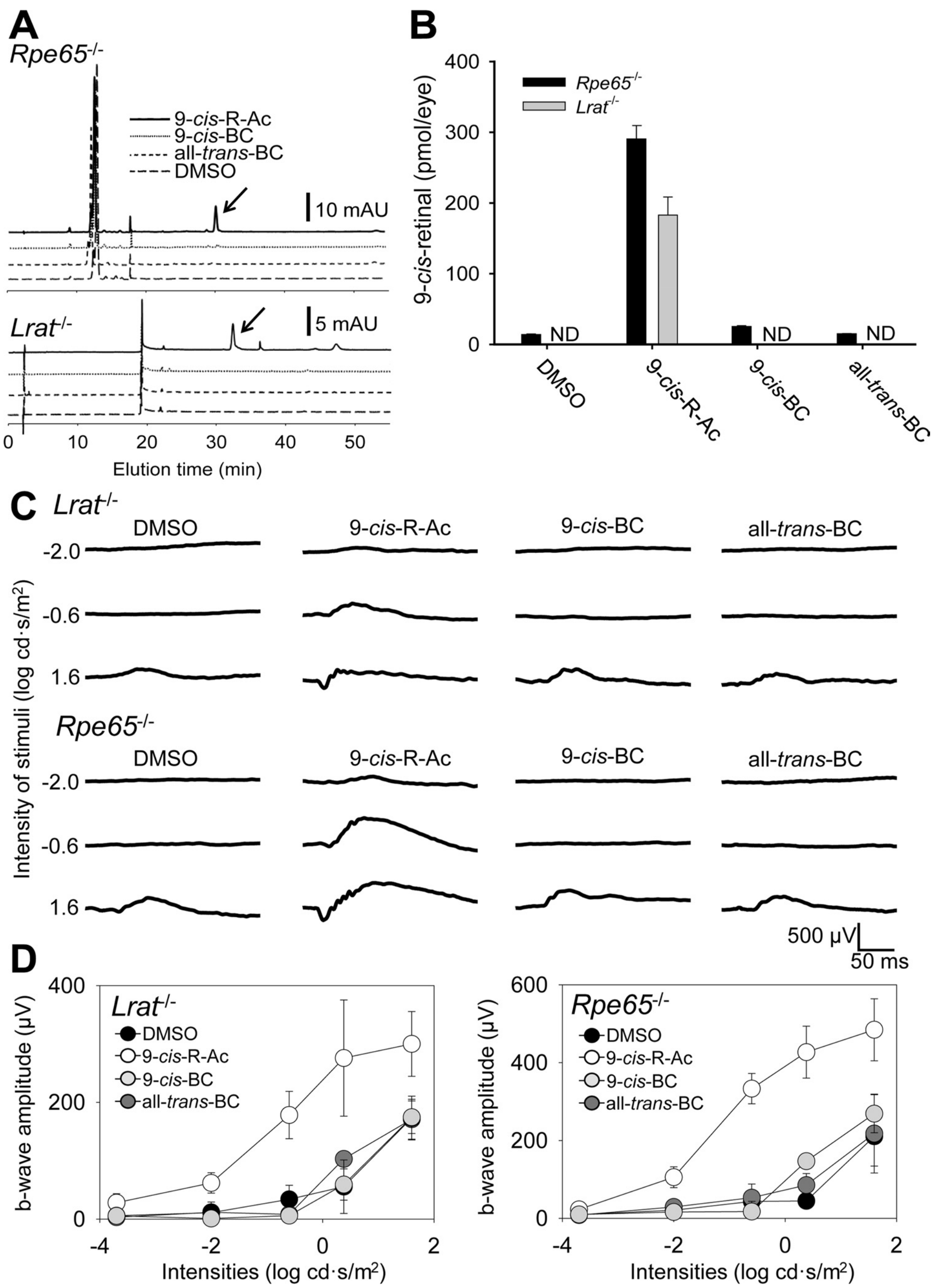

Fig. 5. Eye retinoid profile and retinal function after administration of BC isomers and 9-*cis*-R-Ac. Purified BC isomers and 9-*cis*-R-Ac at 1.0 mg/mouse (total) in DMSO were administrated over the course of 5 consecutive days via intraperitoneal injection to *Rpe65*(−/−) and *Lrat*(−/−) mice maintained on a low vitamin A diet. After 5 days without treatment or light exposure, eye retinoid profiles were determined by normal-phase silica column chromatography and improvement of retinal function was evaluated by ERG. A, representative chromatograms from each group of mice are shown. Visual pigment regeneration was evaluated by determining levels of 9-*cis*-retinal in the eye, which reflect isorhodopsin levels in the retina. 9-*cis*-Retinal was detected in eyes of *Lrat*-deficient and *Rpe65*-deficient mice treated with 9-*cis*-R-Ac (A, black arrows), whereas significantly lower levels of 9-*cis*-retinal were found in eyes of 9-*cis*-BC-treated *Rpe65*-deficient animals but not of *Lrat*-deficient animals (A and B, less than 4.0% compared with 9-*cis*-R-Ac treatment). mAU, milliabsorbance units. A and B, trace level amounts of 9-*cis*-retinal were detected in the eyes of either mutant strain treated with all-*trans*-BC or DMSO. C, serial ERG responses in RPE65-deficient and LRAT-deficient mice after treatment with each compound were compared. D, amplitudes of functional b waves versus intensity of stimuli were plotted. Apparent improvement of ERG responses were only observed in 9-*cis*-R-Ac-treated mice. ND, not detectable. For RPE65-deficient mice, six, seven, seven, and two mice were treated with DMSO, all-*trans*-BC, 9-*cis*-BC, and 9-*cis*-R-Ac, respectively. Likewise three, three, four, and four LRAT-deficient mice were treated with all-*trans*-BC, 9-*cis*-BC, and 9-*cis*-R-Ac, respectively. ERG responses were obtained from two mice (four eyes) of the RPE65-deficient and LRAT-deficient mice that were treated with each compound and prepared separately from retinoid analyses.

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-

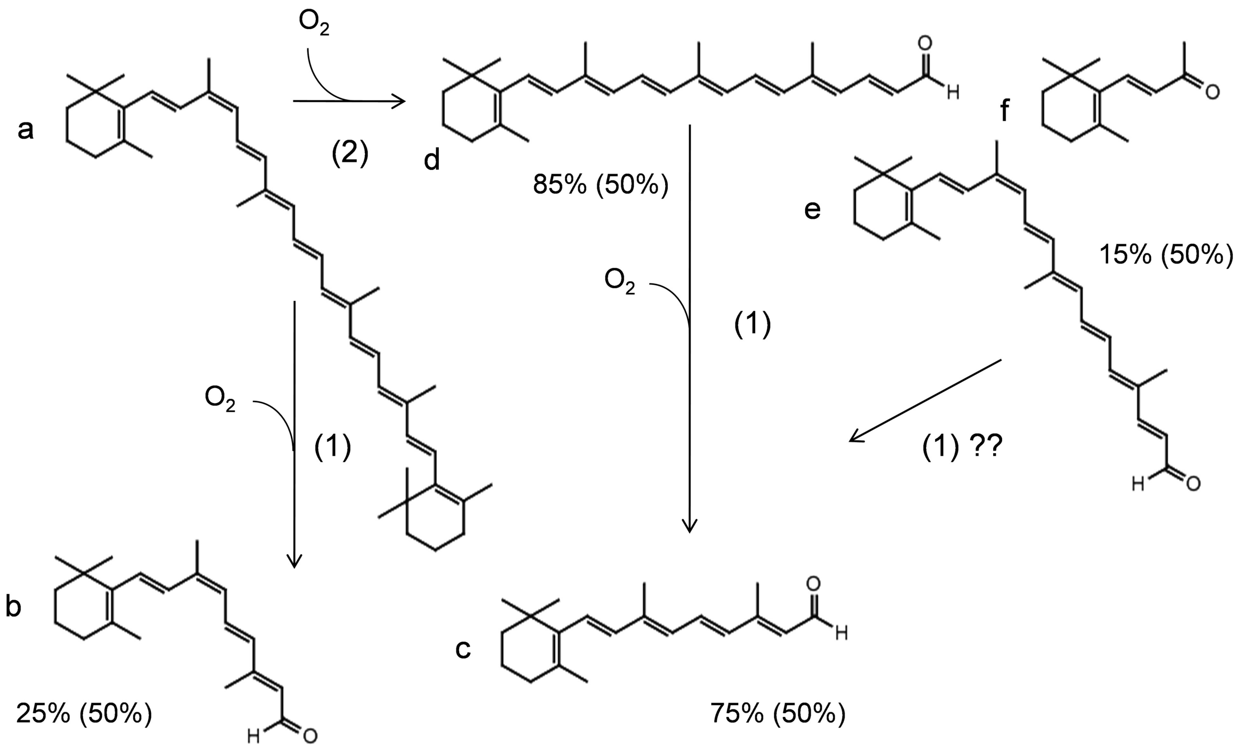

Fig. 6. Model of 9-*cis*-β,β-carotene metabolism in mice. 9-*cis*-BC can be metabolized by the two different carotenoid oxygenases, BCMO1 (1) and BCDO2 (2). The percentage of occurrence of the different cleavage product stereoisomers was determined by enzymatic assays with recombinant proteins. For BCMO1, the discrepancy of these values from the theoretical value (50%) indicates that the enzyme can interact with both β-ionone ring sites and that the enzyme possesses intrinsic isomerase activity when interacting with the 9-*cis*-ring site. For BCDO2, 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*-β-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*-β-10′-apocarotenal; e, 9-*cis*- β-10′-apocarotenal, f, β-ionone; ??, not experimentally tested.

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 carbocation intermediate is formed during reactions with these enzymes (Kiser et al., 2009; Poliakov et al., 2009). In this carbocation, 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. BCDO2 preferentially removed the β-ionone ring site from the 9-*cis*-stereoisomeric site of BC resulting in the formation of all-*trans*-β-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 *Bcdo2*-deficient mice that overexpress BCMO1, thus indicating that some 9-*cis*-retinoids are produced in the absence of BCDO2. 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 BCDO2 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 *BCMO1* who evidenced both elevated plasma BC levels and low plasma retinol levels was described previously (Lindqvist et al., 2007). In addition, the *BCDO2* 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* 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 ^{13}C-labeled 9-*cis*-BC supplementation, the resulting [^{13}C]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*-retinal 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 development (Mic et al., 2003). Moreover, a recent analysis showed that 9-*cis*-retinoic acid is largely absent in mouse blood and tissues, except for the pancreas (Kane et al., 2010). Thus, there is no requirement for extensive 9-*cis*-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 retinoid X receptors, so-called rexinoids, have been developed to fight cancer and metabolic diseases (de Lera et al., 2007). In addition, 9-*cis*-R-Ac has been successfully used to restore vision and prevent retinal degeneration in animal models of RP (Palczewski, 2010). In a recent study, supplementation with *D. bardawil* extracts (containing 40–50% 9-*cis*-BC) has reportedly been beneficial for patients who have fundus albipunctatus, a form of RP (Rotenstreich et al., 2010). Because unliganded opsin in chromophore-deficient photoreceptors provides a sink for 9-*cis*-retinal, we compared the relative potency of 9-*cis*-R-Ac and 9-*cis*-BC for this process. After a 5-day intervention with similar amounts of 9-*cis*-BC, all-*trans*-BC, and 9-*cis*-R-Ac, we analyzed ocular retinoid composition of *Rpe65*(−/−) and *Lrat*(−/−) mice. In *Lrat*(−/−) mice, both BC stereoisomers failed to promote 9-*cis*-retinal production. However, as expected, 9-*cis*-R-Ac proved successful in this process. In *Rpe65*(−/−) mice, a slight increase in 9-*cis*-retinal was observed with 9-*cis*-BC injection that was absent in all-*trans*-BC-supplemented animals. However, this increase was 25-fold lower than observed after 9-*cis*-R-Ac injections and improvement of retinal function was not obvious in ERG responses obtained from *Lrat*(−/−) and *Rpe65*(−/−) mice after 9-*cis*-BC administration. This pronounced difference is probably explained by our finding that only a portion of 9-*cis*-BC is metabolized to 9-*cis*-retinoids because of *cis*-to-*trans* isomerization by BCMO1 and subsequent removal of the 9-*cis*-β-ionone ring site by BCDO2. Nevertheless, this finding can explain why the eyes of *Rpe65*(−/−) mice can contain some 9-*cis*-retinal. The absence of 9-*cis*-retinal in photoreceptors of LRAT mice indicates that retinyl ester formation is required to accumulate 9-*cis*-retinoids produced from 9-*cis*-BC in the eyes. We previously have shown that LRAT acts downstream of the retinoid transporter STRA6 to enhance the uptake of retinoids bound to the serum retinol-binding protein (Isken et al., 2008). This dependence can obviously be bypassed by 9-*cis*-R-Ac. However, there also is less 9-*cis*-retinal accumulation in the eyes of *Lrat*(−/−) mice compared with *Rpe65*(−/−) mice after 9-*cis*-R-Ac injections. Thus, compared with 9-*cis*-R-Ac, 9-*cis*-BC is a poor source for 9-*cis*-retinal in mouse models of LCA, and because of poor absorption and conversion to all-*trans*-retinoids, probably in humans as well.

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Authorship Contributions

Participated in research design: Maeda, Palczewski, and von Lintig.

Conducted experiments: Maeda, Perusek, Amangual, and Babino.

Performed data analysis: Maeda, Palczewski, and von Lintig.

Wrote or contributed to the writing of the manuscript: Palczewski and von Lintig.

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