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Carotenoid-oxygenases: Key Players for Carotenoid Function and Homeostasis in Mammalian Biology

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Abstract

Humans depend on a dietary intake of lipids to maintain optimal health. Among various classes of dietary lipids, the physiological importance of carotenoids is still controversially discussed. On one hand, it is well established that carotenoids such as β,β -carotene are a major source for vitamin A that plays critical roles for vision and many aspects of cell physiology. On the other hand, large clinical trials have failed to show clear health benefits of carotenoids supplementation and even suggest adverse health effects in individuals at risk of disease. In recent years, key molecular players for carotenoid metabolism have been identified, including an evolutionarily well conserved family of carotenoid-oxygenases. Studies in knockout mouse models for these enzymes revealed that carotenoid metabolism is a highly regulated process and that this regulation already takes place at the level of intestinal absorption. These studies also provided evidence that of β,β -carotene conversion can influence retinoid-dependent processes in the mouse embryo and in adult tissues. Moreover, these analyses provide an explanation for adverse health effects of carotenoids by showing that a pathological accumulation of these compounds can induce oxidative stress in mitochondria and cell signaling pathways related to disease. Advancing knowledge about carotenoid metabolism will contribute to a better understanding of the biochemical and physiological roles of these important micronutrients in health and disease.

Keywords

Carotenoids; Retinoids; Carotenoid-oxygenases; Metabolism; Oxidative Stress

Carotenoids are tetraterpenoids (C_{40}) containing up to 15 conjugated double bonds. According to their chemical properties these isoprenoid pigments are divided into two classes; pure hydrocarbons such as β,β -carotene and lycopene are called carotenes, whereas their oxygenated derivatives such as zeaxanthin and lutein are called xanthophylls (Figure 1). Carotenogenic organisms include all photosynthetic plants, protists and bacteria, as well as some heterotrophic bacteria and some fungi. Carotenoids are accessory pigments in the antennae of chloroplasts, where they augment the light-harvesting capacity by absorbing light in the blue-green range of the visible spectrum (450–550 nm) and transferring the energy to chlorophylls. Carotenoids are also involved in photoprotection in plants and are

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known antioxidants [1]. Other carotenoid roles derive from the exploitation of their intense and attractive coloration. Hence, carotenoids are important in the pigmentation of flowers and fruits to attract animals for pollination and seed dispersal. In animals, carotenoids contribute in the pigmentation of invertebrates, birds and fishes. As a coloration of male birds, carotenoids have been shown to be an important trait for sexual attractiveness [2, 3]. Human tissues also retain considerable amounts of carotenoids that may play a role in preventing oxidative damage. Additionally, the central part of the primate retina, the macula lutea, owes its yellow color to high levels of the xanthophylls, lutein and zeaxanthin. These macular pigments have been suggested to filter short-wavelength visible light, to lessen chromatic aberration and to prevent the onset of age-related macular degeneration [4, 5].

Living organisms transform carotenoids to generate unique series of metabolites [6]. These cleavage products of carotenoids, apocarotenoids, are important signaling molecules and chromophores of photopigments in all kingdoms of living organisms. In higher plants, carotenoid-derived abscisic acid (ABA) and strigolactones influence processes, as diverse as seed dormancy, morphogenesis and environmental adaptation. Cleavage of carotenoids in plants also yields other derivatives (e.g., β -ionone, α -ionone and saffranal) which are important for coloration and chemoattraction (or repulsion) (for review see, [7]). Similarly, most animals metabolize carotenoids to diterpenoid molecules such as retinaldehyde, the visual chromophore [11], and all-*trans*-retinoic acid (RA), a hormone-like compound that regulates gene expression [12]. Retinaldehyde serves as a photosensory pigment of mammalian cone and rod visual pigments. A similar function is found in green algae [8] and it also is involved in light-dependent proton pumping in *Halobacterium* [9]. Additionally, asymmetric oxidative cleavage of carotenoids has been described in animals leading to the production of apocarotenoids different from retinoids [10]. Thus, carotenoids and their apocarotenoid derivatives exert critical physiological functions throughout many living organisms in the kingdoms of nature.

Out of the green wild yonder: Carotenoid cleaving enzymes

Vitamin A was recognized as an essential factor in food a century ago. Early research suggested that certain yellow plant pigments had the same activity as vitamin A. This phenomenon was explained in 1930 by Moore [11], who described a conversion of β,β -carotene into vitamin A in the small intestine of the rat, thus providing the first evidence that a plant-derived carotenoid is the direct precursor of retinoids. Karrer [12] elucidated the structure of β,β -carotene and proposed a central cleavage of the C (15,15') double bond of β,β -carotene to form two molecules of retinaldehyde. In cell free-extracts, cleavage of β,β -carotene by a 15,15'-oxygenase to produce two molecules of RAL has been reported [13, 14]. Enzymatic oxidative cleavage of carotenoids at a specific position of the polyene chain also has been proposed in bacteria and plants as a method for the synthesis of apocarotenoids. By analyzing the molecular basis of the ABA-deficient maize mutant, *vp14* (*viviparous* 14), the first carotenoid-cleaving enzyme was molecularly characterized [15]. These researchers proposed that enzymes related to VP14 catalyze oxidative cleavage of carotenoids in other organisms as well. Indeed, this breakthrough was followed by the molecular cloning and biochemical characterization of related carotenoid cleavage enzymes (CCEs) not only in plants but also in animals [16, 17], fungi [18], and bacteria [19]. CCEs depend on ferrous iron as a cofactor but do not contain a heme ring. Thus, CCEs belong to the family of non-heme iron oxygenases, but it is still controversial whether they act in a monooxygenase or dioxygenase fashion [20, 21]. Initially, evidence for a monooxygenase mechanism was provided for a vertebrate enzyme. However, exchange of the oxygen label of retinaldehyde with bulk water may have hampered this analysis. Studies with an eccentrically cleaving plant enzyme producing a ketone cleavage product (no exchange of the oxygen label) indicated a dioxygenase mechanism and thus incorporation of both oxygen

atoms into the substrate. Studies with mammalian β,β -carotene-15,15'-monooxygenase 1 (BCMO1) suggest that carotenoid cleavage involves the formation of a carbocation intermediate and cation- π stabilization by aromatic residues in the carotenoid-binding cleft [22]. Studies with animal CCEs revealed an additional enzymatic property of these enzymes; they can isomerize the C10,C11 double bonds of their substrates [23]. This reaction is critical for the formation of 11-*cis*-retinal or derivatives thereof such as 11-*cis*-3-OH-retinal. This geometric state of the chromophore can bind to the protein moiety (opsin) to form functional visual pigments. In insects, the oxidative cleavage of carotenoids and the all-*trans* to 11-*cis* isomerization of carotenoids is catalyzed by a single protein named NinaB [24]. In mammals, carotenoid cleavage and all-*trans* to 11-*cis* isomerization is catalyzed by two distinct family members, respectively named BCMO1 and retinal pigment epithelium 65 kDa protein (RPE65). RPE65 is not an actual carotenoid-oxygenase, but the long-sought retinoid isomerase in the vertebrate visual cycle and catalyst of the conversion of retinyl esters (RE) to 11-*cis*-retinol [25-28]. In contrast to carotenoid-oxygenases, RPE65 does not incorporate oxygen into its substrate, but the reaction also depends on ferrous iron [29]. Mutations in NinaB and RPE65 cause chromophore deficiency and blindness [30-32], thus emphasizing the importance of this class of proteins for animal vision.

The crystal structure has been resolved for three family members of carotenoid-oxygenase; a bacterial ACO (apocarotenoid-oxygenase) [33], plant VP14 [34], and vertebrate RPE65 protein [35]. The analyses of these structures revealed that the structural fold of CCEs is well conserved between family members of different kingdoms; the basic structural motif being a 7-bladed β -propeller. The iron cofactor is coordinated by four conserved histidine residues and three second shell glutamate residues. The iron is accessible through a long non-polar tunnel juxtaposing the substrate to the active center. This ground-breaking structural work will allow comparisons between CCEs for identification of functional site residues that participate in the isomerization and/or oxidative cleavage reaction at specific sites of the polyene carbon backbone of the substrates.

Mammalian genomes encode three different CCE family members

In mammals, three different members of the CCE family have been molecularly identified and biochemically characterized. RPE65 is expressed in the retinal pigment epithelium of the eyes and localizes to the endoplasmic reticulum. The critical role of RPE65 in visual chromophore production and regeneration is well established and has been extensively reviewed (e.g., [23]). The other two family members, BCMO1 and β,β -carotene-9,10-dioxygenase 2 (BCDO2), are true carotenoid-oxygenases and catalyze the oxidative cleavage of distinct double bonds of the polyene backbone of carotenoids (Figure 2). In this reaction carotenoid-oxygenases specifically interact with one of the two ionone rings of its carotenoid substrates in a specific manner [24]. For mammalian BCMO1 this interaction clearly requires a non-substituted β -ionone ring site, explaining its specificity for a limited number of proretinoid carotenoids such as β,β -carotene, α -carotene and β -cryptoxanthin [36]. BCDO2 catalyzes the oxidative cleavage of carotenoids at the 9,10 position [10]. Initially, BCDO2 was characterized as an enzyme that converts β,β -carotene to produce apocarotenal, β -10'-apocarotenal and β -ionone [10]. Recent research revealed that BCDO2 shows much broader substrate specificity and removes even hydroxylated ionone and ϵ -ionone ring sites from carotenoids such as zeaxanthin and lutein [37, 38]. Interestingly, BCDO2 can remove both ring sites from its substrates by oxidative cleavage at position C9,C10 and C9',C10', resulting in the formation of the C14-dialdehyde rosafluene and two ionone molecules [37]. Additionally, BCDO2 can also cleave acyclic carotenoids such as lycopene [39]. There is also a marked difference in the subcellular localization of the two mammalian carotenoid-oxygenases. BCMO1 is a cytoplasmic protein [36], whereas BCDO2 localizes to mitochondria [37]. The requirement for such compartmentalization of carotenoid

metabolism might be explained by the fact that BCMO1 and BCDO2 are expressed in same cell types (e.g. hepatocytes) and can metabolize the same substrate (β,β -carotene) [10]. Thus, expression in the same cellular compartment would lead to a competition between BCDO2 and BCMO1 for its common substrate β,β -carotene and may result in reduced production of retinoids. In summation, two different carotenoid metabolizing enzymes have been identified. BCMO1 is a cytosolic enzyme with narrow substrate specificity for proretinoid carotenoids and BCDO2 is a mitochondrial enzyme with broad substrate specificity for carotenoids. The identification of the genes encoding these enzymes allowed the generation of knockout mouse models by homologous recombination. These animals are versatile models to study the roles of different carotenoids in mammalian physiology.

BCMO1 is the key enzyme for vitamin A production

In mammals, the molecular and biochemical basis of vitamin A function has been well established. The vitamin A-derivative 11-*cis*-retinal serves as chromophore of cone and rod visual pigments [40]. Moreover, vitamin A is the precursor for all-*trans*-retinoic acid (RA), which is required for a wide range of biological processes, including embryonic and fetal development, cell differentiation and metabolic control. This hormone-like compound is the ligand of retinoic acid receptors (RARs) that require retinoid X receptors (RXRs) as partners [41]. The active receptor complex is a RAR/RXR heterodimer that binds specific DNA regulatory sequences and regulates gene transcription in response to RA binding.

β,β -Carotene is the major source for retinoids in the human diet. The formal first step in retinoid metabolism is the conversion of β,β -carotene to retinaldehyde, from which all other retinoids can be synthesized by endogenous enzymes. Studies in a BCMO1 knockout mouse model demonstrated the critical role of this enzyme for this process. On diets providing β,β -carotene as the sole source for vitamin A, these mice become retinoid-deficient and instead accumulate large amounts of β,β -carotene [25]. Recently, in human studies, a patient with a heterozygotic mutation in *BCMO1* was described with both elevated plasma β,β -carotene levels and low plasma retinol levels [42]. At the protein level of BCMO1, this mutation resulted in replacement of a highly conserved threonine by a methionine residue (T170M). Biochemical characterization of the recombinant T170M BCMO1 protein variant showed that its activity was $\approx 90\%$ lower than that of the wild-type protein. Moreover, single base pair polymorphisms in the human *BCMO1* gene have been identified that decreased intestinal β,β -carotene conversion efficiency [43]. Studies in BCMO1-deficient mice showed that the expression of the second carotenoid-oxygenase BCDO2 is upregulated in the liver and that some β -10'-apocarotenoids are produced [44]. Increased expression of BCDO2 in BCMO1-deficient mice has been also reported in other tissues such as the lung [45]. Apocarotenoid formation indicates that β,β -carotene can be at least, in part, metabolized by BCDO2 in BCMO1-deficiency.

Regulation of intestinal vitamin A production

The small intestine is responsible for absorbing dietary lipids such as carotenoids and delivering them to the organism as triglyceride-rich lipoproteins. Intestinal lipid absorption is a complex process that evidently depends on membrane receptors/transporters [46]. For carotenoids, it is now clear that scavenger receptors such as SR-B1 and CD36 facilitate their absorption [47]. Additionally, SR-B1 also facilitates the intestinal absorption of tocopherols (vitamin E) [48]. Studies in a *Drosophila* mutant showed that SR-B1/ NinaD is critical for the absorption of both fat and soluble vitamins [49, 50].

Upon absorption, proretinoid carotenoids are readily converted to vitamin A by the action of BCMO1 in enterocytes of the intestinal mucosa [16, 51]. The primary cleavage product,

retinaldehyde, is then converted to retinol and REs. These REs, along with REs produced from preformed dietary vitamin A, are packed into chylomicrons that are secreted into the blood. In humans, substantial amounts of absorbed carotenoids are not cleaved in the intestine (up to 40% of dietary intake) [52] so they, along with other lipids, become incorporated and associate with circulating lipoproteins [53].

Intervention studies in healthy volunteers have led to the description of a low-responder phenotype and a low-converter phenotype [54]. Low or poor responders are defined as individuals who show little variation in plasma β,β -carotene levels after acute or chronic supplementation. This huge inter-individual variability in conversion efficiency of β,β -carotene has been reported in several studies [54-57]. Furthermore, pharmacokinetic studies with labeled β,β -carotene indicate that low responders display a lower conversion efficiency compared to normal responders [58]. Genetic variation in the human *BCMO1* gene may very well be contributing to these different phenotypes [59, 60]. Additionally, studies in animal models reported that intestinal *BCMO1* activity is influenced by dietary retinoids (for the current model, see Figure 3). This regulation involves retinoic acid signaling via RARs and evidently takes place at the transcriptional level [61]. Interestingly, studies in *BCMO1*-deficient mice revealed that not only carotenoid conversion by *BCMO1* but also carotenoid absorption is repressed by retinoids [62]. Recently, a gut-specific homeodomain transcription factor *ISX* was identified that controls intestinal *SR-BI* and *BCMO1* mRNA expression [63, 64]. *SR-BI* is normally found on the apical surfaces of absorptive epithelial cells, and its levels decrease from the duodenum to ileum [65-67] in contrast to the increasing duodenum-ileum gradient for *ISX* [63, 64]. In *ISX*-deficient mice, *SR-BI* expression is significantly enhanced and its expression extends to more distal parts of the intestine [64]. Interestingly, *ISX* expression is reduced in vitamin A-deficiency [64], further implicating this transcription factor as a key regulatory factor for intestinal carotenoid metabolism. The crosstalk between β,β -carotene/retinoid metabolism and *ISX* is mediated via a retinoic acid responsive element in the *ISX* promoter to which RARs can bind [62]. These findings suggest that retinoic acid produced from dietary precursors regulates the intestinal expression of *SR-BI* and *BCMO1* by inducing *ISX*. Indeed, treatment of vitamin A — deficient animals with RA induced *ISX* expression and decreased *SR-BI* and *BCMO1* expression. Thus, *ISX* acts as a retinoic acid-sensitive ‘gatekeeper’ that controls vitamin A production. This negative feedback regulation of vitamin A production may contribute to the low and high responder phenotypes in humans. The low responder phenotype may represent the vitamin A sufficient state; whereas the high responder phenotype may represent a demand for vitamin A. The intestinal regulation of vitamin A production also explains why β,β -carotene supplementation does not cause hypervitaminosis A. In contrast, preformed dietary retinoids can cause this problem because retinol is absorbed in a non-regulated manner [68]. Further studies are required to fully elucidate the role of this diet responsive regulatory network on vitamin A homeostasis. Considering the involvement of *SR-BI* for the absorption of other dietary isoprenoid compounds such as tocopherols (vitamin E) and xanthophylls (macula pigments), dietary preformed retinoids may more generally impact homeostasis of these important dietary lipids.

BCMO1 expression in peripheral tissues and the embryo

In humans, substantial amounts of absorbed β -carotene are not cleaved in the intestine by *BCMO1* (up to 40% of dietary intake) [52] and along with other lipids become incorporated in chylomicrons and found associated with circulating lipoproteins [53]. Circulating carotenoids in association with lipoproteins can be then taken up by the lipoprotein specific receptors. In mice, *BCMO1* is expressed in both the intestine and liver but also in peripheral tissues, including the mammalian embryo [10, 69-71]. In humans, analyses of *BCMO1* mRNA expression levels revealed a comparable picture [72]. Immunohistology showed that

BCMO1 is expressed in the mucosal and glandular cells of stomach, small intestine, and colon, in liver, cells comprising the exocrine glands in pancreas, glandular cells in prostate, endometrium, and mammary tissue, kidney tubular cells, and keratinocytes of skin squamous epithelium [73]. Additionally, steroidogenic cells in testis, ovary, and the adrenal gland, as well as skeletal muscle cells express BCMO1 [73]. Moreover, BCMO1 is abundantly expressed in the retinal pigmented epithelium of the eyes [72, 74]. Together, these studies suggest that a local tissue specific-conversion of β,β -carotene can contribute to retinoid metabolism in peripheral tissues.

BCMO1 deficiency affects embryonic retinoid metabolism

Loredana Quadro and coworkers provided evidence that BCMO1 can maintain retinoid homeostasis in embryonic tissues of VAD mice [71]. In an elegant genetic approach, they generated *RBP*^{-/-} *BCMO1*^{-/-} double mutant female mice. In *RBP*-deficiency, mice depend on a continuous dietary vitamin A supply (RE in chylomicrons) [75]. These double mutant mice were crossed with *RBP*^{-/-} male mice so that dams were deficient both for *RBP* and BCMO1 whereas their offspring carried a functional *BCMO1* allele. Pregnant dams were then subjected to dietary vitamin A restriction and received β -carotene or vehicle by IP injection. The offspring developed severe embryonic malformations when supplemented with vehicle only. In contrast, β,β -carotene could maintain retinoid homeostasis and promoted normal development of the offspring (with a functional *BCMO1* allele). Thus, β,β -carotene can serve as retinoid precursor in embryonic tissue. A developmental role of BCMO1 also has been reported in lower vertebrates such as the zebrafish [76].

Additionally, the study by Quadro and coworkers provided evidence that the BCMO1 protein influences embryonic retinoid homeostasis. These researchers reported that dietary vitamin A restriction induced more severe developmental malformations in *RBP*^{-/-} *BCMO1*^{-/-} double mutant offspring than in *RBP*^{-/-} single mutant offspring [71]. This observation was unexpected because diets essentially provided no β,β -carotene in these experiments. The authors found that embryonic retinoid metabolism was impaired in BCMO1-deficient embryos also in the absence of β,β -carotene. LRAT expression was significantly reduced as compared to embryos carrying a wild-type BCMO1 allele. Accordingly, BCMO1-deficient embryos had much lower levels of RE and RA, the developmentally active form of vitamin A. Thus, the authors established a critical role of BCMO1 protein for embryonic retinoid metabolism.

A role of BCMO1 in the regulation of body fat reserves

Among the many functions attributed to retinoids, its putative role in adipocyte biology and the regulation of body fat reserves has generated clinical and scientific interest. The vitamin A derivative RA acid has been shown to influence adipocyte differentiation [77, 78] and fat deposition [79], mitochondrial uncoupling [80, 81], oxidative metabolism [82, 83] and adipokine expression [84-87] in adipose tissues. These effects are mediated in part via the classical retinoic acid receptors (RARs), which, upon retinoid binding, regulate the expression of direct target genes [88] and interfere with the activity of other transcription factors, including early adipogenic transcription factors [77, 78]. Additionally, retinoic acid can influence peroxisome proliferator-activated receptor (PPAR)-mediated responses by activating the retinoid X receptor (RXR) moiety of permissive PPAR:RXR heterodimers [89, 90] and, possibly, by serving as an agonist, activating ligand of the PPAR β/δ isoform [91, 92]. PPARs are lipid-activated transcription factors involved in the regulating the expression of genes related to lipid and glucose metabolism [93, 94]. PPAR γ , in particular, is pivotal for adipocyte differentiation and hypertrophy [95]. Additionally, the retinoic acid

precursor retinaldehyde has been shown to inhibit PPAR γ -induced adipogenesis both in adipocyte cell cultures and mouse models [96].

Regulation of fat reserves by dietary vitamin A could be explained by the metabolism of vitamin A to biologically active retinoid derivatives, which can then impact the differentiation and function of adipose tissue. Surprisingly, obesity and obesity associated diseases are prevalent in western societies, with adequate dietary vitamin A supply. Additionally, high dose supplementation with preformed vitamin A does not affect body adiposity in mice [97]. Besides preformed vitamin A, β,β -carotene is found in the circulation and BCMO1 expression has been reported in several tissues, including the intestine, liver and adipocytes (see above). It was recently determined that, BCMO1 gene transcription is under the control of PPAR γ , a key regulator of adipocyte differentiation and lipogenesis in mature adipocytes [98]. The finding of responsiveness of BCMO1 to PPAR provides evidence for a possible link between the regulation of fatty acid and β,β -carotene metabolism. Moreover, studies in BCMO1 knockout mice showed that this mouse mutant develops dyslipidemia, and is more susceptible to diet-induced obesity, and shows increased expression of PPAR γ -induced genes in adipocytes, even when supplemented well with vitamin A, further indicating an important role for BCMO1 in the regulation of lipid metabolism [25].

Recently, it was demonstrated that like PPAR γ , BCMO1 is induced (5-7 fold) both at the mRNA and protein levels, during differentiation of NIH 3T3-L1 pre-adipocyte cells to mature adipocytes [99]. Treatment of mature adipocytes with the BCMO1 substrate β,β -carotene resulted in a reduced staining for lipids (Oil Red O stain) and triglyceride (TG) content, while microscopic inspection showed that lipid droplets were reduced in size and number (Figure 4). Correspondingly, mRNA and protein expression levels of PPAR γ and its downstream target fatty acid binding protein 4 (FABP4/aP2) were also significantly reduced in β,β -carotene treated mature adipocytes. No such observations were apparent in vitamin A treated mature adipocytes.

To further discriminate between the effects of parent β,β -carotene and its retinoid derivatives, this study used (4-hydroxy-phenyl) retinamide ((fenritinide, (FHR)), a highly selective inhibitor of BCMO1 enzymatic activity. Although β,β -carotene treatment alone reduced TG content and decreased expression of the lipogenic marker PPAR γ , this effect was largely prevented by FHR. Thus treatment of mature adipocytes with FHR selectively inhibited the anti-adipogenic effects of β,β -carotene, indicating that they are dependent on BCMO1 activity. To determine the biologically active retinoid derivative, polar and non-polar retinoids from both β,β -carotene and all-*trans*-retinol treated mature adipocytes were isolated and analyzed by HPLC. This analysis showed that β,β -carotene is metabolized to all-*trans*-retinoic acid, while all-*trans*-retinol was metabolized to RE, thus β,β -carotene and all-*trans*-retinol have different metabolic fates in mature adipocytes. In fact, endogenous all-*trans*-retinoic acid derived from β,β -carotene cleavage decreased the expression of key lipogenic transcription factors, namely CCAAT/enhancer-binding protein α (C/EBP α) and PPAR γ . It has been shown that the inhibitory effects of all-*trans*-retinoic acid on adipogenesis are mediated by RARs. In line with this concept, a 2.8 fold induction of *Cyp26a1* at the transcriptional level (mRNA) was observed. *Cyp26a1* is an RA hydroxylase, whose mRNA expression is induced by RA in a strictly RAR-dependent manner. Thus in agreement with RA production, β,β -carotene treatment induced the expression of this RAR-responsive gene. Taken together *in vitro* studies in cell culture indicated that β,β -carotene plays an important role for retinoid signaling in mature adipocytes.

To expand these observations to animals, the effects of vitamin A deficiency as well as β,β -carotene and all-*trans*-retinol supplementation on adipose tissues of mice was analyzed in *LRAT*^{-/-} mice (which lack retinoid stores in the liver as they cannot convert all-*trans*-retinol

to RE in most tissues) [100]. Examination of inguinal white adipose tissue from these mice showed no changes in the mRNA expression of either PPAR γ or its downstream target *aP2* as compared to *LRAT*^{-/-} mice on vitamin A sufficient (VAS) diets. Additionally, morphometric analysis showed that adipocytes were comparable in size between the VAS and vitamin A deficient (VAD) animals. However, gavage of β,β -carotene resulted in a 3-fold decrease in PPAR γ and *aP2* mRNA expression and 2.1 fold increase in *Cyp26a1* mRNA expression, indicating that these mice produce RA which in turn influences adipogenesis. No such results were observed in all-*trans*-retinol gavaged vitamin A deficient *LRAT*^{-/-} mice, which further strengthens the importance of BCMO1 in influencing adipogenicity.

The role of β,β -carotene for RA metabolism might be explained by specific biochemical characteristics of adipocyte. Studies has revealed that enzymatic activity of BCMO1 is stimulated in the presence of cellular retinol-binding protein 1 (CRBP1) [101]. CRBP1 can bind RAL, the primary β,β -carotene cleavage product, and is expressed in various tissues including adipocytes [102]. In the liver, CRBP1 stimulates RE formation, the storage form of vitamin A, from CRBP1-bound retinoids in a LRAT-dependent manner [103]. Adipocytes express different acyl-transferases than LRAT for RE formation [100]. Additionally, the expression of the RAL to retinol converting enzyme ADH1 is decreased during adipogenesis [96]. Moreover, β,β -carotene treatment decreased *ADH1* mRNA expression, whereas it induced mRNA expression of the retinal dehydrogenase *RALDH1* in iWAT of mice [99]. Thus, these biochemical characteristics may explain that β,β -carotene is predominantly metabolized into RA in adipocytes.

Cumulatively, these studies provided evidence that BCMO1 plays a role in the cross-talk between PPAR γ and RAR signaling pathways and implicates β,β -carotene as an important dietary regulator of fat storage capacities of mice adipocytes. Many observational studies indicate that the consumption of food rich in plant-derived micronutrients such as carotenoids is inversely related to the incidence of obesity and associated disease. However, there are limited molecular explanations for this effect, though this assumption is widely accepted but not a strictly proven fact. Thus, the putative role of β,β -carotene for this process requires further research.

BCDO2 and apocarotenoid signaling molecules

Though the role of BCMO1 for retinoid metabolism has been well established, less is known about the second carotenoid-oxygenase, BCDO2. As described above, biochemical studies indicate that this enzyme displays broad substrate specificity and converts both carotenes and xanthophylls by oxidative cleavage at the 9',10' and 9,10 double bonds. Apocarotenoids such as β -14'-apocarotenal and β -13'-apocarotenal have shown to influence the activities of nuclear receptors such RXR and PPAR α and γ [104, 105]. In ferrets, 10'-lycopenals are produced upon lycopene supplementation [39]. Studies in *BCMO1* knockout mice, with elevated *BCDO2* mRNA expression levels, show that lycopene tissue distribution is significantly altered compared to wild-type mice [106]. BCDO2-deficient mice accumulated significantly more lycopene than wild-type mice, implicating BCDO2 in the metabolism of this carotenoid [107]. In rats, lycopene supplementation results in altered expression of PPAR γ in several tissues [108]. Additionally, lycopene derivative apo-10'-lycopenoic acid has been shown to transactivate RARs with relative high potency [109]. This compound also was shown to inhibit lung tumor growth in cell culture and mouse models [109]. Furthermore, lycopene supplementation inhibited hepatic carcinogenesis in rats with induced fatty livers [110]. All these studies clearly show that apo-carotenoids can influence physiological processes. However, pathways for the biochemical modifications of the primary apocarotenal cleavage product await molecular and biochemical description. It must

also be considered that these compounds exist in relatively high amounts in plant food. Thus, diet-derived apocarotenoids may influence nuclear receptor activities as well. More research is needed to understand the role of BCDO2 for the production of the numerous apocarotenoid molecules that could be formed from dietary carotenoids and their putative impact on mammalian biology.

BCDO2, carotenoid homeostasis, and mitochondria

The broad substrate specificity of BCDO2 implicates this enzyme in the metabolism of both carotene and xanthophylls. A critical role of BCDO2 for xanthophyll metabolism was substantiated by findings in chickens. The yellow skin color of chickens (xanthophylls) is determined by *cis*-acting and tissue-specific regulatory mutation(s) that inhibit expression of *BCDO2* in skin [111]. Additionally, it was shown that mutations in *BCDO2* gene cause the yellow fat phenotype (xanthophyll accumulation) of sheep [112]. Moreover, the carotenoid content of cow milk and serum was demonstrated to be significantly altered by a mutation that abolished BCDO2 function [113].

To further analyze the physiological role for BCDO2 in a model organism, a *BCDO2*^{-/-} knockout mouse model was established [37]. Ablation of BCDO2 expression was confirmed by protein analysis of isolated hepatic mitochondria, using a BCDO2 specific antibody. *BCDO2*^{-/-} mice develop normally and were fertile on standard chow-diet. Dietary supplementation of *BCDO2*^{-/-} mice with either 50 mg/kg zeaxanthin or lutein revealed impairment in carotenoid metabolism. Gross abdominal sectioning showed an accumulation of these carotenoids, evident by strong yellow coloring of adipose tissue in only *BCDO2*^{-/-} mice and not WT mice supplemented with the same diets. HPLC analysis of lipid extracts of livers revealed that only small amounts of xanthophylls from these diets were present in *BCDO2*^{-/-} mice, but these mice accumulated large amounts of metabolites of these carotenoids with different retention times and absorption spectra as compared to the parent carotenoids. LC-MS/MS identified these accumulated compounds as 3-didehydro-carotenoids [37]. These compounds have been recently described as major xanthophyll derivatives in wild-type mice with very short half-life times [114]. Notably, no accumulation of apocarotenoid cleavage products was identified in WT mice, indicating that the cleavage products are rapidly metabolized/ degraded.

A consequence of carotenoid accumulation in the *BCDO2*^{-/-} mice was apparent in liver histology. Histological analysis revealed that *BCDO2*^{+/-} and *BCDO2*^{-/-} mice developed liver steatosis with large lipid droplets in hepatocytes and a significantly increased TG content. This phenotype was not evident in age matched *BCDO2*^{-/-} mice raised on a chow diet, indicating that this pathology was induced by carotenoid accumulation. Since, carotenoids also accumulated in *BCDO2*^{+/-} mice, it was indicative that loss of a single allele can cause halpoin sufficiency and impairs carotenoid homeostasis. Furthermore, *BCDO2* mRNA expression analysis by qRT-PCR revealed that *BCDO2* expression was induced 7-fold in WT mice as compared to *BCDO2*^{+/-} mice, thereby preventing accumulation of these carotenoids.

In agreement with the enzyme's intracellular localization, carotenoids accumulated in isolated hepatic mitochondria of *BCDO2*^{-/-} mice. Carotenoids are lipophilic molecules with an extended polyene chromophore that can act as an electron sink. Additionally, these rigid lipids also may disturb membrane topology. Immunoblot analysis of manganese superoxide dismutase (MnSOD), an enzyme indicative of general mitochondrial stress and dysfunction, showed a 9-fold increase in hepatic mitochondria isolated from carotenoid-supplemented *BCDO2*^{-/-} mice as compared to WT mice. Detailed analysis of the mitochondrial respiration chain (ETC) revealed that ADP-dependent respiration, respiration with high ADP, and

uncoupled respiration rates of mitochondria isolated from *BCDO2*^{-/-} on the carotenoid diets were all significantly reduced, as compared to siblings on the similar but carotenoid-free diet.

Disturbances in the mitochondrial ETC generally result in the production of excessive reactive oxygen species (ROS). Indeed, *in vitro* studies in human liver HepG2 cells showed that zeaxanthin, lutein or their 3-dehydro derivatives, the latter isolated from livers of *BCDO2*^{-/-} mice, all could induce ROS production, in a dose dependent manner. Additionally, treatment of these cells with β,β -carotene evidently resulted in ROS. Conversely, overexpressing recombinant murine BCDO2 in HepG2 cells significantly reduced ROS production, further demonstrating the importance of this carotenoid-oxygenase in preventing oxidative stress. Accordingly, determination of the mitochondrial membrane potential using the well established JC-1 dye technique yielded similar outcomes. Again, overexpressing recombinant murine BCDO2 in HepG2 cells significantly ameliorated membrane depolarization in carotenoid-treated cells.

Additionally, various components of the oxidative-stress regulated signaling pathways were analyzed by immunoblot analysis of hepatic and cardiac tissues of *BCDO2*^{-/-} and WT animals maintained on supplemented carotenoid diets. Analysis of the hypoxia inducible factor 1 α (HIF1 α), phospho-AKT and phospho-MAPK kinase pathways all showed a 5-7 fold induction at the protein levels in hepatocytes and cardiac tissues isolated from *BCDO2* mice. Similar observations were also noted in *BCDO2* heterozygous mice, but no such induction was observed in WT mice on similar carotenoid diets. The induction of these cell signaling pathways can at least in part counteract carotenoid-induced impairment in *BCDO2*-deficient mice. However, a overstimulation of these pathways also is associated with disease, including cardiovascular and neurodegenerative disease, type 2 diabetes and cancer.

For *BCDO2*, a single base pair polymorphism in intron 2 has been identified and correlates with altered blood levels of interleukin 18, a pro-inflammatory cytokine associated with type 2 diabetes and cardiovascular disease [115]. A relationship between *BCDO2* and cardiovascular disease has also been observed in mice [116]. An induction of cell signaling pathways related to oxidative stress and cell proliferation has been previously described in rats and ferrets supplemented with supraphysiological doses of β,β -carotene [117, 118]. Large clinical trials revealed that high dose β,β -carotene supplementation can harm individuals at risk of disease, such as smokers and asbestos workers (for review, see [119, 120]). Studies in *BCDO2* knockout mice implicate mutation in the *BCDO2* gene as a genetic risk factor and may provide a mechanistic explanation for adverse health effects of carotenoids.

Concluding remarks

A ubiquitous family of non-heme iron oxygenases has been identified that modify double bonds of carotenoids and their apocarotenoid derivatives by *trans*-to-*cis* isomerization and oxidative cleavage. Mammalian genomes encode three distinct family members. RPE65 is the isomerase in the visual cycle, whereas BCMO1 and BCDO2 catalyze the oxidative cleavage of carotenoids. BCMO1 cleaves at the C15,C15' double bond and has a limited substrate specificity for proretinoid carotenoids such as β,β -carotene. The second enzyme cleaves carotenoids at the C9,C10 double bond and displays broad substrate specificity. Studies in knockout mouse models for these enzymes identified BCMO1 as the key enzyme for retinoid production. Analysis in this mouse model also helped to unravel a diet-responsive regulatory network that controls intestinal vitamin A production. In this process, intestine-specific homeodomain transcription factor ISX acts as a RA-sensitive gatekeeper

that controls carotenoid absorption and conversion to vitamin A. Increasing evidence has been acquired that a tissue-specific BCMO1 also plays an important role in a tissue-specific context. BCMO1-deficient embryo show altered vitamin A homeostasis. Furthermore, β -carotene conversion to vitamin A can maintain normal development in the embryo under conditions of limited maternal vitamin A supply. A tissue-specific conversion of β -carotene to retinoids can also influence retinoid-dependent processes in adult mammals. In adipocytes, a reciprocal influence between the β , β -carotene metabolite retinoic acid and PPAR γ activity has been demonstrated. Importantly, preformed dietary vitamin A does not show this activity. These findings suggest that a tissue-specific conversion of β , β -carotene influence nuclear receptor activities that help regulate energy balance. Genetic studies in chickens, cows, and sheep showed that mutations in the *BCDO2* gene can indeed alter carotenoid metabolism. A critical role of *BCDO2* for carotenoid homeostasis has been confirmed in a mouse model. In accordance with the mitochondrial localization of this enzyme, carotenoids accumulated in these organelles and impaired respiration. This impairment caused oxidative stress and induced cell signaling pathways related to cell survival and proliferation. This action of carotenoids may explain adverse health effects of these compounds reported in clinical studies and identify *BCDO2* as a key player for their prevention. It also shows that carotenoid homeostasis – as it is already a well known fact for other isoprenoid lipids such as cholesterol – must be tightly regulated to avoid disease. In humans, genetic polymorphisms exist that alter carotenoid and retinoid homeostasis. In the future, this genetic variability as well as regulatory aspects must be considered when effects of carotenoids on health and disease are evaluated. Advancing knowledge about carotenoid metabolism will contribute to the understanding of the biochemical, physiological, developmental, and medical roles of carotenoids and their numerous derivatives.

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Abbreviations

C/EBPα	CCAAT/enhancer-binding protein α
BCMO1	β,β -carotene-15,15'-monooxygenase 1
BCDO2	β,β -carotene-9,10-dioxygenase 2
CCE	carotenoid cleaving enzyme
CD36 (SCARB3)	Cluster of Differentiation 36
FABP4 (aP2)	fatty acid-binding protein 4
FHR	fenretinide
HPLC	high-performance liquid chromatography
iWAT	inguinal white adipose tissue
PPAR	peroxisome proliferator-activated receptor
SR-BI	scavenger receptor class B type I
RAL	all- <i>trans</i> -retinal
RA	all- <i>trans</i> -retinoic acid
RAR	retinoic acid receptor
RXR	retinoid X receptor
RetSat	retinol saturase
RPE65	retinal pigment epithelium 65 kDa protein
RE	retinyl esters
ROS	reactive oxygen species
TG	triacylglycerol
VAD	vitamin A-deficient diet
VAS	vitamin A-sufficient diet
WT	wild type

Figure 1.
Chemical structure of major carotenoids in the human blood.

Figure 2.

β,β -carotene metabolism in a cell that express both types of carotenoid-oxygenases. Insets at the top show images immunostained expressing recombinant V5-tagged murine BCMO1 and BCDO2. Nuclei (blue) are stained with DAPI. The scale bar gives 10 μ M. Note that BCMO1 is distributed in the cytosol, whereas BCDO2 localizes to mitochondria. Enzymes and proteins involved β,β -carotene metabolism: 1, Cellular uptake of β,β -carotene is mediated by scavenger receptors such as SR-BI and CD36; 2, BCMO1 converts β,β -carotene by symmetric oxidative cleavage to all-*trans*-retinal; 3, all-*trans*-retinal can be oxidized to retinoic acid by retinal dehydrogenases; 4, all-*trans*-retinal also can be reduced to all-*trans*-retinol by alcoholdehydrogenases and short chain dehydrogenases; all-*trans*-retinol can be esterified by the lecithin: retinol acyl transferase and by acylCoA: retinol acyl transferases; 6, BCDO2 removes β -ionone rings from β,β -carotene by successive oxidative cleavage at 9,10 and 9',10' position.

Figure 3.

Negative feedback regulation of intestinal β,β -carotene absorption and conversion. **A.** In vitamin A deficiency, SR-BI and BCMO1 expression are highly elevated and distally extended throughout the intestine. In contrast, ISX expression is highly reduced. Note that a similar pattern of expression of SR-BI and BCMO1 is found in ISX-deficient mice [64]. **B.** In vitamin A sufficiency, ISX expression is increased and proximally extended. In contrast, SR-BI and BCMO1 expression are highly reduced in a distal gradient throughout the intestine [64]. **C.** Schematic overview of intestinal β,β -carotene metabolism. SR-BI facilitates the absorption of dietary β,β -carotene from the intestinal lumen. BCMO1 converts absorbed β,β -carotene by symmetric oxidative cleavage to all-*trans*-retinal. all-*trans*-Retinal is reduced to all-*trans*-retinol and then esterified to retinyl RE. RE together with intact β,β -carotene is packed in chylomicrons and secreted into the circulation. all-*trans*-Retinal can be also converted to all-*trans*-retinoic acid. This hormone like compound activates via retinoic acid receptors (RAR) and retinoid X receptors (RXR) gene transcription of ISX. ISX in turn inhibits the expression of SR-BI and BCMO1 genes and reduces intestinal β,β -carotene absorption and conversion to retinoids.

Figure 4.

β,β -Carotene but not all-*trans*-retinol reduces triacylglycerol content in mature NIH 3T3 L1 adipocytes. **A.** Photographs of cell culture plates stained with oil-red for lipids. Non-differentiated NIH 3T3 L1 cells show weak staining, whereas mature NIH 3T3 L1 adipocytes show strong red staining for lipids. Oil red staining of mature NIH 3T3 L1 adipocytes treated with one μM β,β -carotene (BC) or all-*trans*-retinol (ROL). BC but not retinol reduces triacylglycerol content of these cells. For further details see text and reference [99].