Molecular aspects of β, β-carotene-9', 10'-oxygenase 2 in carotenoid metabolism and beyond

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Abstract

Carotenoids, the carotenes and xanthophyll, are essential components in human nutrition. β, β-carotene-9', 10'-oxygenase 2 (BCO2, also named as BCDO2) catalyzes the asymmetrical cleavage of carotenoids, whereas β, β-carotene-15, 15'-monooxygenase (BCMO1) conducts the symmetrical cleavage of pro-vitamin A carotenoids into retinoid. Unlike BCMO1, BCO2 has a broader substrate specificity and has been considered as an alternative way for vitamin A production. Different from BCMO1, a cytoplasmic protein, BCO2 is located in the inner mitochondrial membrane. The difference on the cellular compartmentation may reflect the different substrate specificity and physiological functions between BCMO1 and BCO2. The mutations of *BCO2* gene had been proved to be associated with yellow color of skin and fat tissue and milk in livestock. Mutation in intron 2 of *BCO2* gene is also supposed to be related to the expression of IL-18, a pro-inflammatory cytokine associated with obesity, cardiovascular diseases, and type 2 diabetes. Further, BCO2 is also associated with the development of macular degeneration, anemia, mitochondrial oxidative stress, and hepatic steatosis. This review of the literature will address most recent updates regarding the role of BCO2 in carotenoid metabolism, and discuss the potential impacts of BCO2 protein and the mutations in mammalian diseases.

Keywords: β, β-carotene-9', 10'-oxygenase 2, carotenoids, hepatic steatosis, interleukin 18, macular degeneration, mitochondrial stress

Introduction

Carotenoids are 40-carbons lipophilic pigments, which can be synthesized from plants, bacteria and fungi [1]. Carotenoids can be classified into two groups: the xanthophyll, which contains oxygenated groups and the carotenes, which are only hydrocarbons without oxygenated groups and less polar than the xanthophyll [2, 3]. Carotenoids present several important functions in human physiology. First, carotenoids, such as zeaxanthin and lutein, are functioned as antioxidants and the filters of blue light. These macula pigments decrease chromatic aberration to protect retina against light induced oxidative stress [4, 5]. Second, well-known provitamin A carotenoids such as β-carotene are the precursors of retinoid, which are important for eye function [1].

For most population of the world, provitamin A carotenoids are the major dietary source of vitamin A [6]. The vitamin A deficiency leads children blindness and increases children morbidity, especially in developing countries [7]. The amount of vitamin A produced from carotenoids is mainly based on the following two factors: the absorption and metabolism of intake carotenoid [8, 9]. The mode of vitamin A production was proposed as the symmetric oxidative cleavage of provitamin A carotenoids, such as β-carotene, α-carotene and β-cryptoxanthin. The gene encoding this symmetric cleavage enzyme β, β-carotene-15, 15'-monooxygenase (BCMO1) was cloned from several species, including human, mouse, chicken and *Drosophila* [10, 11]. However, recent biochemical studies showed that provitamin A carotenoid can also be catalyzed into apocarotenoids, which is different from retinoid. This eccentric cleavage, which was conducted by β, β-carotene-9', 10'-oxygenase 2 (BCO2, also named as BCDO2), was considered as an alternative way for vitamin A production [12, 13]. Different from BCMO1, BCO2 can also metabolize non-provitamin A carotenoids including

lutein, xanthophyll [14, 15]. BCMO1 and BCO2 have different intracellular localizations as BCMO1 located in the cytoplasm [11], but BCO2 located in the inner mitochondrial membrane [16]. The different cellular compartments may reflect the different substrate specificity and physiological functions between BCMO1 and BCO2. Therefore, in this review, we will focus on the biochemical characterization and protein function of BCO2 in nutrition.

Carotenoids metabolism by BCMO1 and BCO2

Carotenoids are natural fat-soluble pigments synthesized in plants, bacteria and fungi, which play important role in animal and plant physiology. For carotenoid metabolism, the existence of carotenoid cleavage enzymes was discussed as early as 1965, however, the first cleavage enzyme VP14 was not identified until 1997 [17]. In animals, there are two carotenoids cleavage enzymes that have been identified. The first is BCMO1, which catalyzes the centric cleavage at 15, 15' double bond of β-carotene, forming two molecules of all-trans retinal. These all-trans retinal can be irreversibly oxidized into retinoic acid or reversibly reduced into retinol [10, 11] (Fig. 1.). The second enzyme is BCO2, which participates in eccentric cleavage of β-carotene at 9', 10' and/or 9, 10 double bond, forming apocarotenoid different from retinoid, including β-apo-10'-carotenal and β-ionone (Fig. 2.). Although BCMO1 functions as a major enzyme in vitamin A production, BCO2 is considered as an alternative pathway for forming vitamin A [18, 19]. Both BCMO1 and BCO2 are family members of nonheme iron oxygenase which are important in many physiological processes in animals and plants [20]. Beside BCMO1 and BCO2, the retinal pigment epithelium protein of 65 kDa (RPE65) was also included in this nonheme iron oxygenase family, which is crucial for vision [21, 22].

In recombinant human BCMO1 study, BCMO1 was found to cleave pro-vitamin A carotenoids, which contain at least one nonsubstituted β-ionone ring, such as α-carotene, β-carotene, or β-cryptoxanthin, but fails to catalyze the cleavage of non-pro-vitamin A carotenoids, such as zeaxanthin, lutein or lycopene [11]. BCMO1 was found to be present in the intestinal mucosa, where it facilitates the cleavage of majority of the carotenoids. BCMO1 was also presented in the classical steroidogenic cells, which are sensitive to vitamin A deficiency [23]. In this way, BCMO1 was proposed to be present in extra-intestinal tissues as a backup system for local synthesis of vitamin A when intake was decreased [24]. In mice, BCMO1 was found to play key roles in the retinoid production [25]. In humans, a heterozygotic mutation in *BCMO1* gene led to the increased plasma levels of β-carotene and decreased plasma concentrations of retinoid [26].

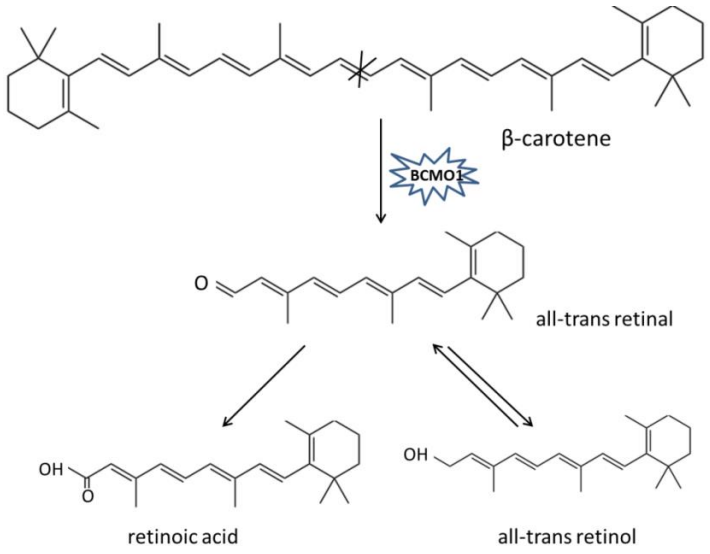

Fig. 1. Enzyme reaction mechanisms of BCMO1. BCMO1 catalyzes carotenoids by cleaving double bond at position 15, 15' of carotenoids. BCMO1, β-carotene-15, 15'-monooxygenase.

The expression and activity of BCO2 has been verified in humans [23], cattle [27], sheep [9] and chicken [28]. BCO2 is highly expressed in hepatocytes, which are important for uptake and

processing of retinol [29]. Except β-carotene, lutein, lycopene, β-cryptoxanthin and zeaxanthin are all specific substrates for BCO2. For instance, BCO2 could cleave the acyclic carotenoids, lycopene. However, only the 13-cis- and 5-cis-isomers of lycopene could be cleaved by BCO2 [30]. BCO2 was also found to cleave lutein at both 9', 10' and 9, 10 double bond, leading to the formation of β-ionone, β-apo-10'-carotenal and apo-10, 10'- carotenedialdehyde [31]. Interestingly, the production of β-apo-10'-carotenal was significantly affected by the concentration of Fe^{2+}. When the concentration of Fe^{2+} decreased from 78% to 67%, the formation of β-apo-10'-carotenal was significantly decreased, showing that iron was a necessary cofactor for this enzymatic cleavage [19]. Therefore, these researches demonstrate that BCO2 displays much broader substrate specificity for carotenoids metabolism compared to BCMO1 [16, 32, 33].

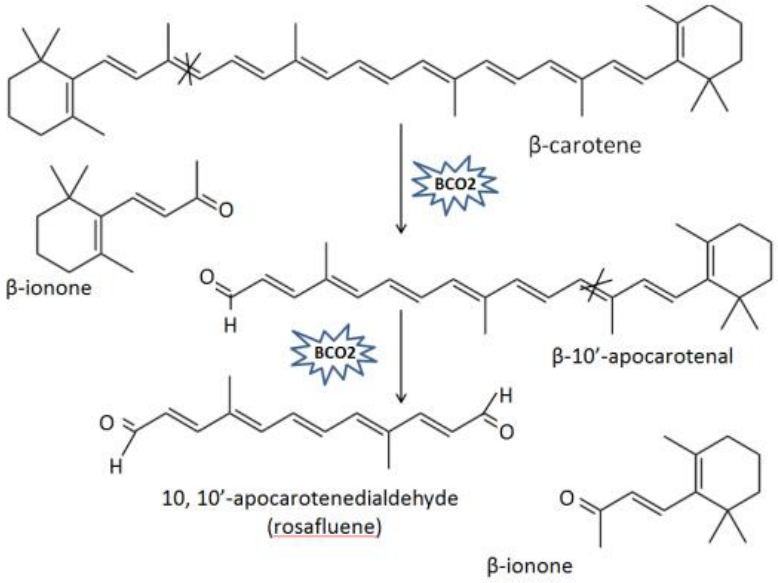

Fig. 2. Enzyme reaction mechanisms of BO2. BCO2, which is located on mitochondrial, catalyzes carotenoids by cleaving double bond at position 9, 10 and 9', 10' of carotenoids. BCO2, β,β-carotene-9', 10'-oxygenase 2.

***BCO2* genetics**

In the Entire genome of *Drosophila*, only *BCMO1* gene is found, which is encoded by *ninaB* gene. However, in vertebrates, two other genes besides *BCMO1*, *BCO2* and *RPE65*, are found. Based on the deduced amino acid sequence, *BCMO1*, *BCO2* and *RPE65* share approximately 40% overall sequence identity [34]. Additionally, there are 4 histidine residues within the amino acid sequence, conserved in their positions the same as several highly conserved regions which can be viewed as protein family motifs [35]. According to functional assays in vitro, two tyrosine residues, Y326 and Y235, are reported to be important for the activity of mouse *BCMO1*. Y235 is completely conserved in *BCMO1*, *BCO2* and *RPE65*. However, Y326 is well conserved in all *BCMO1* homologues and changed to tryptophan and glutamine in all *RPE65* and most *BCO2*, respectively [36]. In human, *BCO2* genomically map to chromosomal position 11q23.1. So far 19 different splice variants of human *BCO2* have been identified, 9 of which are protein coding (http: //www. ensemble.org). Among all of the 11 identified exonic SNPs in the open reading frame of human *BCO2*, only rs17113607 and rs10891338 have minor allele frequencies over 5% according to HapMap (http: //hapmap.ncbi.nlm.nih.gov). This indicates that splice variants could be important in introducing large inter-individual differences in BCO2 activity. For example, the intronic SNP rs2115753 at the locus of *BCO2* was correlated with IL-18 concentrations [37].

The overall span of human *BCO2* gene spans 11 exons across 42.5 kilobases (kb) on chromosomal 11q23.1 (Fig. 3.). The coding sequence is 1424 bases (Accession Number is NM_001256400). According to the genetic analysis of the 5' UTR domain of BCO2, several regulatory transcription factor binding sites are found in the BCO2 gene promoter section, including TATA-binding protein (TBP), activator protein family (AP), and signal transducer and activator of transcription family (STAT) family (Fig. 4.). AP is a family of transcription factors,

composed of proteins including c-Jun, c-Fos, JDP and ATF. Regulation of the human *BCO2* gene expression by AP in response to a variety of stimuli, including growth factors, cytokines, stress, and viral and bacterial infections. AP also participates in several cellular processes, such as proliferation, differentiation, and apoptosis [38, 39]. These characteristics in human *BCO2* gene promotor region indicate that the human *BCO2* gene may be a stress responsive gene and participate in several important cellular processes.

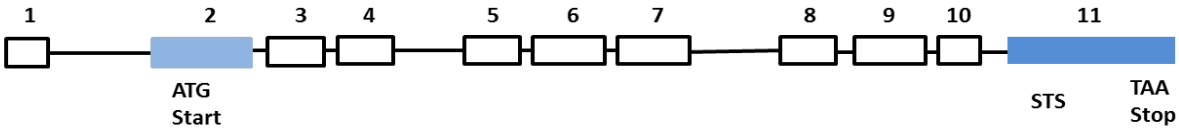

Fig. 3. Human *BCO2* gene (NM_001256400) structure. Exon-intron structure drawn based on approximate scale. Exon 2 (light blue) contains the translation start codon. Exon 11 (dark blue) contains the stop code and sequence tagged site (STS).

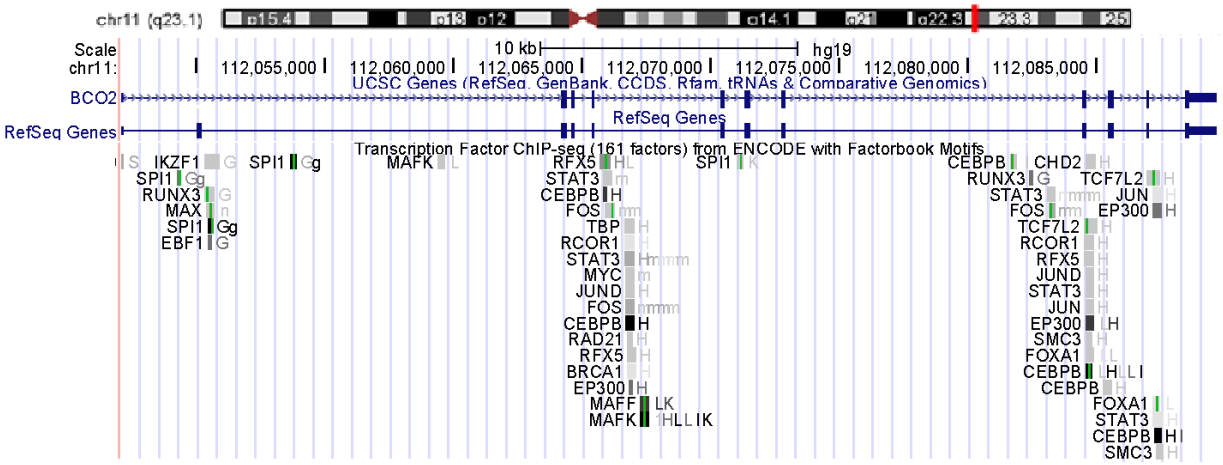

Fig. 4. Regulatory transcription factor binding sites in the human *BCO2* gene promoter. The human BCO2 gene is on chromosomal 11q23.1. Scale bar at top indicates 10 kb. Assessed from http://genome.ucsc.edu/.

Compared with mouse *BCO2* gene, in exon 3, there is an insertion of GGT AAA GCT GCA in the human *BCO2* gene. This insertion DNA sequence is coding sequence, which codes amino acid sequence in human BCO2 protein. In this way, the major difference between human BCO2

enzyme and mouse BCO2 enzyme is the existence of 4 amino acid residues GKAA in human BCO2. This specific insertion of GKAA is unique to primates and is absent in sheep, rats, mice, cows and ferrets according to the NCBI protein database search. This GKAA sequence is also absent in mouse and human RPE65, which can cleave all-trans-retinyl esters [40].

The effects of *BCO2* gene mutation in animals

The deficiency of BCO2 was found to be associated with carotenoids accumulating in the adipose tissues, such as subcutaneous adipose tissue [41], which leads to occurrence of yellow fat in sheep [42], cow [27] and chicken [28]. In the yellow fat experimental sheep, the *BCO2* coding region was sequenced and the nonsense mutation of *BCO2* was found in nucleotide position 196 (c.196C>T). This nonsense mutation introduced a stop codon in position 66 of amino acid, while the full-length protein BCO2 in sheep contains 575 amino acids. It was hypothesized that the mutant in *BCO2* gene forms a non-functional enzyme [43].

In cows, BCO2 was associated with the yellow color of the adipose tissue and milk, which was mainly caused by deposition of carotenoids [27]. Association analysis showed that there were significant differences among cattle with different *BCO2* genotypes in concentration of β-carotene and color of subcutaneous fat. Compared with genotypes of *BCO2* AA, GA and GG, the animals with AA had a higher concentration of β-carotene and more yellow fat than those animals with GG or GA genotype. The A allele which is in bovine *BCO2* exon 3 is a nonsense mutation. As the full length of BCO2 protein in cattle is 530 amino acids, the change in this allele results in a different polypeptide with presumably a lost in BCO2 enzyme function presumably [44]. The data showed that there is a strong link between this *BCO2* SNP and fat color in cattle.

IL-18, found in Kupffer cells and macrophages, is an important pro-inflammatory cytokine which pays key roles in innate and acquired immunities. It has been shown that the plasma concentration of IL-18 is associated with different diseases, including atherosclerosis, cardiovascular diseases and type 2 diabetes [37, 45, 46]. In humans, variants at the *BCO2* locus were associated with IL-18 levels, but not carotenoid levels in either plasma or macula [37]. A 2-stage genome-wide association study among women of Women's Genome Health Study (WGHS) and European ancestry from the Nurses' Health Study (NHS) was performed to test this association between *BCO2* and *IL-18*. In the stage of discovery, 7 SNPs located at the *IL18-BCO2* locus were significantly related with concentrations of IL-18. According to these combined analyses, SNPs rs2115763, rs7106524 and rs1834481 were found to show significant association between the *BCO2* and *IL-18* gene. SNP rs2115763 was found to be the strongest among these three SNPs. Further selection analysis showed that SNPs rs1834481 and rs2115763 were associated with IL-18 levels independently. Variation of 2.9% plasma levels of IL-18 could be explained together by these 2 SNPs. SNP rs2115763, which presents the strongest association, is located in intron 2 of the BCO2 gene and also in the same location of the IL-18 gene [37]. It is reported that IL-18 level was significantly lower in the BCO2 knockout mice [30]. However, the underlying mechanism between the IL-18 level and SNP rs2115763 in the *BCO2* gene is unknown. SNP rs2115763 might regulate transcription of *IL-18* gene directly, as the *BCO2* gene is upstream of the IL18 gene. Another possibility is that SNP rs2115763 is an indicator of *IL-18* gene functional mutation, affecting plasma levels of IL-18 [45, 46].

The function of BCO2 in Nutrition

Genetic studies in sheep, cows and chickens revealed that BCO2 gene mutations could alter homeostasis of carotenoid metabolism. This was also confirmed in a mouse model. In

accordance with the subcellular location of BCO2 (in the inner mitochondrial membrane), carotenoids were found to be accumulated in mitochondria and impair respiration [16]. This process leads to cellular oxidative stress and alteration of signaling pathways related to cell proliferation and survival. Therefore, BCO2 functions as a key regulator of preventing the adverse effect caused by excess carotenoids. However, we are still at the early stage of understanding the importance of BCO2 in health and disease. The advanced knowledge of BCO2 function might help us to better understand the biochemical, developmental, and physiological roles of carotenoids.

Function of BCO2 on eye

Carotenoids, such as lutein and zeaxanthin, accumulated in the retina and circulating system, were supposed to be associated with common eye diseases, such as diabetic retinopathy, age-related macular degeneration (AMD) and so on [9, 47, 48]. The researchers tested in various genes related to zeaxanthin and lutein status for association with AMD in Age-Related Eye Disease Study (CAREDS). 424 SNPs from 24 candidate genes were genotyped in 1663 of 2005 CAREDS participants. After adjusting for ancestry and age, 24 variants from five genes (*BCMO1*, *BCO2*, *NPCL1L1*, *ABCG8* and *FADS2*) were related to AMD independently. SNP rs2250417 in BCO2 is one of the strongest statistical significance for carotenoid-candidate genes association with AMD. The two minor alleles for SNP rs2250417 in *BCO2* were accounted for almost increasing 50% risk for AMD. Carotenoids with hydroxylated β-ionone ring including cryptoxanthin and lutein and zeaxanthin are substrates for BCO2, which is located in mitochondrial. As discussed above, mutations of BCO2 might cause accumulation of zeaxanthin and lutein in skin of livestock to form yellow fat. However, in this present study, the variants of BCO2 were not related to concentrations of lutein and zeaxanthin in the macular pigment and

blood. This finding suggested that BCO2 mutations had tissue specific influences. What's more, the demonstrated function of BCO2 in inhibiting oxidative stress indicated that BCO2 could be associated with the lower damage and oxidative stress to mitochondrial in retinal pigment epithelium [49]. Variants of BCO2 might also be associated with AMD through mechanisms involving an impact on the activity of the enzymes involving in inflammation and lipid homeostasis [50, 51]. One of these common variants, rs2115763 in *BCO2*, is weak linked with rs2250417, which was also reported to relate with AMD in this study [34, 52]. However, these researches were still at their early stage.

Knock Down of BCO2 Causes Anemia and Apoptosis of Blood Cells

To further understand the importance of BCO2 in development, mRNA was used to knock down BCO2 gene in zebrafish [53]. No defects in growth pattern or obvious malformations of organ systems, such as eyes and brain, were observed in BCO2 knock down (BCO2 -/-) morphants [14]. However, the red blood cells in BCO2 -/- morphants were no longer red and the blood filling rate of heart was also reduced in BCO2 -/- morphants compared with wild type groups. All these results showed that knock down of BCO2 caused anemia. Although the primitive erythropoiesis was not affected in BCO2 -/- morphants, the expression of a later erythrocyte differentiation marker, embryonic α-globin (hbae3), was increased. During the same time, fragmented nuclei were also presented in erythrocytes of BCO2 -/- morphants, indicating that occurrence of apoptosis of red blood cells [54, 55]. It was also reported that the differentiating blood cells were more sensitive to apoptosis when BCO2 was deficient. Thus, BCO2 is associated with the development of red blood cells [14].

BCO2 Is Related to Carotenoid-induced Oxidative Stress

Lobo GP and his colleagues showed that the protection of cells against apoptosis induced by carotenoid is strictly dependent on BCO2 enzymatic function [56]. After transfecting with murine BCO2 gene, transfected cells could transiently express BCO2. Compared to control cells, the mitochondrial membrane was more polarized in transfected cells. Intrinsic apoptotic markers were assessed in these transfected cells after treatment with carotenoid. Activated caspase-3, which is a mediator of intrinsic apoptosis, was not changed in the transfected cells compared to control cells. Conversely, knock down of BCO2 in cells caused increased ROS production when cells were treated with carotenoids. More importantly, these BCO2 -/- cells showed initiation of apoptosis after incubation with carotenoid for 2 hours [56, 57]. Thus, BCO2 expression is associated with cellular oxidative stress (Fig. 5.).

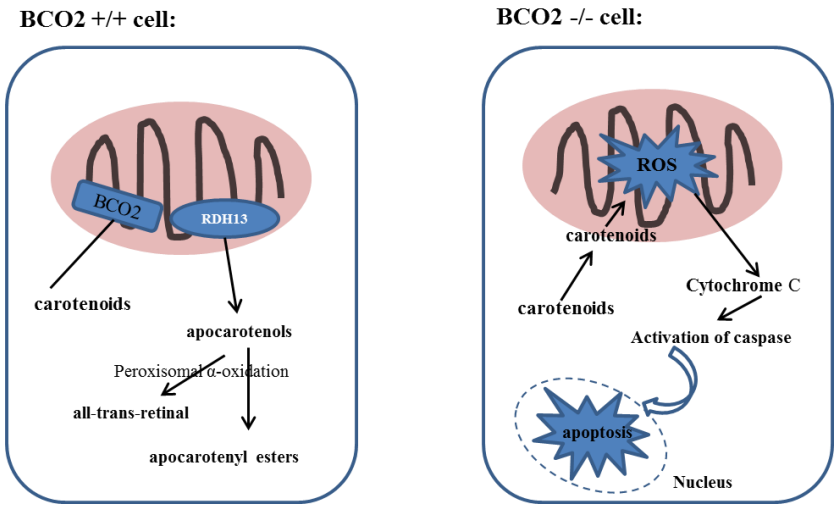

Fig. 5. The Role of Mitochondrial BCO2 in Cells. In cells that express endogenous BCO2, carotenoids can be degraded into apocarotenoids by BCO2. The apocarotenoids can then be metabolized by RDH13 into corresponding apocarotenols. These apocarotenols might then be metabolized by peroxisomal α-oxidation into all-trans-retinal or apocarotenyl esters. In cells without expression of BCO2, carotenoids could accumulate in the mitochondria. This kind of accumulation would interfere with respiration and produce ROS. These changes could cause cytochrome C release and then activate the caspase and eventually lead to cell apoptosis.

BCO2 Is Important in Prevention of Hepatic Steatosis

A high fat diet induces hepatic steatosis in mice through a possible mechanism of increasing ROS levels and suppressing mitophagy and mitochondrial biogenesis. Hepatic concentration of zeaxanthin and lutein and expression of BCO2 in hepatic mitochondria were lowered in mice fed with a high fat diet. These results suggested that BCO2 might be a stress responsive gene in mice [58]. BCO2 knockout mice also showed lipid accumulation in liver [14]. However, elevated expression of BCO2 attenuated the hepatic steatosis. All these results indicated that BCO2 protein is important in prevention of hepatic steatosis. Knockout of BCO2 might cause mitochondrial dysfunction in the liver and increased hepatic cell susceptibility to oxidative stress [59-61].

BCO2 Modulates the Impact of Dietary Lycopene on Hepatic Nuclear Receptor-, Stress- and Metabolism-related Gene Expression

Lycopene, mainly found in dietary tomato, is one of the most abundant carotenoids in human plasma. Recently, lycopene has attracted more attention because of its impact on a decreased risk of certain chronic diseases, including cancers and cardiovascular diseases [62]. Therefore, large amounts of work have been expended to study the biological properties of lycopene. Within the past few years, apo-lycopenoids, which are the major derivatives of lycopene cleaved by BCO2, were found to present several biological benefits [63]. The lycopene induced effects on nuclear receptor expression was affected by the BCO2 presence. For instance, the tomato diet-induced up-regulation of androgen receptor (Ar) was dependent on the presence of BCO2. Increased hepatic expression of Ar has been linked to lower risk of hepatocellular carcinoma [64], indicating a role of BCO2-mediated expression of hepatic Ar by dietary tomato. Lycopene-

induced down-regulation of 7 nuclear receptors was also dependent on BCO2 status. The impacted nuclear receptors and coactivators play diverse roles, including mitochondrial metabolism (Ppargc1b) [65], carcinogenesis (Nr3c1, Esrra) [66], liver metabolic regulation (Esrra) [67], inflammation and immunity (Nr3c1) [68], gene transcription silencing by histone deacetylation (Hdac3), heme-mediated transcription repression (Nr1d2) [69] and mediated of steroid receptor–driven gene transcription (Ncoa2 and Ncoa4). The effect of lycopene diet on metabolism- and stress-related genes was also related to BCO2. However, the pattern is not in consistent with the nuclear receptor-related genes [30, 70].

Discussion

Carotenoids, such as zeaxanthin, lutein and their metabolites, *meso*-zeaxanthin, are macular pigments of human retina, which are known to function as antioxidant and light-screening players in nature [71-73]. The chemically and spatially accumulation of high levels of xanthophyll in the retina is a specific feature of the primates compared with other mammals, but the biochemical mechanism for this unique feature is unclear. [5, 74, 75]. To better understand the underlay mechanism, it is hypothesized that none BCO2 expression, or enzyme activity of BCO2 is inactive in primates' retina. However, studies had already shown that BCO2 is expressed in both human and mouse retina. Therefore, enzyme activity of human BCO2 was investigated by cell-based carotenoid cleavage assay. According to this study, BCO2 was shown as an inactive carotenoid cleavage enzyme. Another study showed that xanthophyll carotenoids highly uptake by human ARPE-19 cells, however, no cleavage products of xanthophyll were found in these cells [76]. Moreover, no eccentric cleavage products were found when human subjects were given labeled supplements of β-carotene [77, 78]. No association between human *BCO2* SNPs and optical density of macular pigment was reported, which also imply that human

BCO2 cannot catalyze macular pigments. Moreover, human BCO2 was demonstrated with the inability to bind with appropriate carotenoids substrates in the active site. This might be another reason that human BCO2 is inactive in human retina. Therefore, consistent expression of BCO2 in the human retina together with carotenoid cleavage enzyme inactivity indicates that BCO2 may serve as unknown enzymatic or regulatory function [40].

We are still at the early start of understanding importance of BCO2 in health and disease. As BCO2 located in the inner mitochondrial membrane [16], BCO2 might impact on normal mitochondrial function through target on the ETC/OXPHOS, including inhibit the activity of different complexes, increase the proton leak or decrease the ATP production efficiency. As discussed above, knockout of BCO2 in liver could increase the production of ROS [14]. Recently, it has been proposed that mitochondria could prevent the ROS production through forming supercomplexes [79]. There is accumulated evidence demonstrating that mitochondrial respiratory chain complexes are associated to form different supermoleculars, known as supercomplexes [80, 81]. Recent evidence supports the function of these supercomplexes, including (1) the formation of mitochondrial respiratory chain supercomplexes could minimize the production of ROS which is generated from complex I [79], (2) the mitochondrial respiratory chain supercomplexes may intergrate the cytochrome c and coenzyme Q to induce kinetic advantage via facilitating efficient transportation of electrons through substrate channeling [82], (3) mammalian complex I is assembled in mitochondrial respiratory chain supercomplexes, which help to stable the complex I [83, 84]. Thus, it is interested to investigate the association between BCO2 and formation of mitochondrial respiratory chain supercomplexes. This might give us the reason that BCO2 knockout could cause increasing ROS production.

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