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APOCAROTENOIDS: EMERGING ROLES IN MAMMALS

Earl H. Harrison¹ and Loredana Quadro^{2,*}

¹Department of Human Sciences, The Ohio State University, Columbus, OH 43210, United States, harrison.304@osu.edu

²Department of Food Science; Rutgers Center for Lipid Research; and New Jersey Institute for Food, Nutrition, and Health, Rutgers University, New Brunswick, New Jersey 08901, United States

Abstract

Apocarotenoids are cleavage products of C40 isoprenoid pigments, named carotenoids, synthesized exclusively by plants and microorganisms. The colors of flowers and fruits or the photosynthetic process are examples of the biological properties conferred by carotenoids to these organisms. Mammals do not synthesize carotenoids, but obtain them from foods of plant origin. Apocarotenoids are generated upon enzymatic and non-enzymatic cleavage of the parent compounds both in plants and in the tissues of mammals that have ingested carotenoid-containing foods. The best-characterized apocarotenoids are retinoids (vitamin A and its derivatives) generated upon central oxidative cleavage of provitamin A carotenoids, mainly β -carotene. In addition to the well-known biological actions of vitamin A, it is becoming apparent that non-retinoid apocarotenoids also have the potential to regulate a broad spectrum of critical cellular functions, thus influencing mammalian health. This review will discuss the current knowledge about the generation and biological activities of non-retinoid apocarotenoids in mammals.

Keywords

carotenoid oxygenases; eccentric cleavage; non-enzymatic cleavage; metabolism; mammals

1. INTRODUCTION

Apocarotenoids are defined as cleavage products of parent carotenoids, C40 isoprenoid pigments synthesized exclusively by plants and microorganisms (21). Several hundred carotenoids exist in nature. The conjugated double bond system that characterizes their structure confers them efficient antioxidant properties, especially for reactive oxygen species such as singlet oxygen. Moreover, the double bond system enables carotenoids to absorb light in the visible spectrum providing the distinctive yellow and red colors to many plant flowers and fruits that accumulate carotenoids in their tissues. Photosynthetic organisms utilize carotenoids as integral components of the light-harvesting complex and as

*Corresponding author (L.Q.): Department of Food Science; Rutgers Center for Lipid Research; and New Jersey Institute for Food, Nutrition, and Health, Rutgers University, New Brunswick, New Jersey 08901, United States; phone: 848-9325491; lquadro@sebs.rutgers.edu.

photoprotectants (31). With the exception of a few arthropod species (61), animals cannot synthesize carotenoids. Nevertheless, they can obtain carotenoids from the diet and use them for pigmentation (as in the plumage of many birds), protection from light/oxidative stress (as in the macular pigment of the human fovea) and, in the case of carotenoids containing non-substituted β -ionone rings, as precursors of vitamin A upon conversion into retinoids (vitamin A and its derivatives). Of the roughly 50 different carotenoids present in the human diet, only about 10 have been detected in significant amounts in the human plasma, including provitamin A carotenoids (such as β -carotene, α -carotene and β -cryptoxanthin), and lycopene, lutein, zeaxanthin and β -cathaxanthin (83).

When the carbon skeleton of a carotenoid has been shortened by enzymatic or non-enzymatic removal of fragments, the nomenclature rules prescribe the prefix “apo” followed by its position to indicate that all of the carbon atoms beyond that carbon have been removed or replaced by hydrogen atoms (30) (Figure 1). Of course, retinal (vitamin A aldehyde) would represent a very important apocarotenoid derived from β -carotene and could be referred to as β -apo-15-carotenal (Figure 2). However, cleavage of a carotenoid such as β -carotene at other than the central double bond results in products without common names. The products are therefore named as apocarotenoids (30). For example, cleavage of β -carotene at the double bond adjacent to the central double bond results in formation of two possible apocarotenoid products, commonly referred to as β -apo-14'-carotenal and β -apo-13-carotenone (Figure 2). Naming of other apocarotenoids is similar (30). For example, cleavage of the asymmetric β -cryptoxanthin at the 9,10 and 9',10' double bonds yields two sets of products. Namely 3-hydroxy- β -apo-10'-carotenal and β -ionone for the 9,10 cleavage and β -apo-10'-carotenal and 3-hydroxy- β -ionone for the 9',10' cleavage [see Figure 3 and (18) for these structures and those of the cleavage products of other dietary carotenoids].

In addition to the biological functions of the intact carotenoid molecules, their enzymatic and non-enzymatic cleavage products perform a number of biological activities as well. Apocarotenoids are formed both in plants and in the tissues of animals that have ingested carotenoid-containing food. Plant apocarotenoids formation can be catalyzed by carotenoid cleavage dioxygenases (CCDs), a family of enzymes with different specificity for various carotenoid substrates (7, 34). Important regulators of plant growth and development, such as abscisic acid and carlactone, a precursor of stringolactones, are formed *via* these enzymatic cleavage pathways (59). However, the degradation of carotenoids through non-enzymatic processes produces even a larger number of potentially biologically active compounds in plants and, hence, in foods of plant origin (76). These non-enzymatic products can be generated since carotenoids act as antioxidants in response to abiotic factors, such as light and temperature, for example, or in response to changes in the intracellular oxidative status, such as those induced by the photosynthetic process. The formation and functions of apocarotenoids in plants has been recently reviewed in (7, 34), and it will not be discussed here.

Apocarotenoids are also formed in mammalian tissues. Among these compounds, the best-characterized are retinoids generated upon oxidative cleavage of provitamin A carotenoids, mainly β -carotene. In mammalian tissues, retinoids are predominantly generated *via* central cleavage of β -carotene, mediated by the cytosolic β -carotene 15,15' dioxygenase enzyme

(BCO1) yielding to two molecules of retinaldehyde (84). This latter can then be directly oxidized to retinoic acid, or reduced to retinol, which upon esterification generates retinyl esters, the storage form of vitamin A in tissues (9). Vitamin A is critical to sustain human health exerting its functions predominantly through the transcriptional regulatory activity of retinoic acid, the ligand of the nuclear receptors retinoic acid receptors (RARs)/retinoic X receptors (RXRs) that modulate the expression of hundreds of genes (39). Moreover, the role of retinaldehyde as the chromophore of rhodopsin constitutes the basis for the essential role of vitamin A in vision (43). The functions of retinoids (vitamin A apocarotenoids) will not be discussed in this review. We will focus exclusively on the generation and potential biological functions of non-retinoid apocarotenoids in mammalian tissues.

2. NON-RETINOID APOCAROTENOIDS: ENZYMATIC VS. NON-ENZYMATIC GENERATION

2.1. Central and eccentric enzymatic cleavage of dietary carotenoids

Early studies on carotenoid metabolism in animals focused on provitamin A carotenoids and their enzymatic conversion to vitamin A. The first in vitro studies published in 1965 used extracts from rat liver and intestinal mucosa to demonstrate enzymatic oxidative cleavage of β -carotene at the central double bond to yield retinal (26, 64). In 2000, the enzyme responsible for the conversion of β -carotene to retinal was identified in *Drosophila* (85) and chicken (93) and was named β -carotene-15,15'-oxygenase 1 (BCO1, also known as CMOI, CCE, BCMO1 and CMO1). BCO1 has also been identified in humans (50, 51, 96), mice (66, 71), rat (80), cows (60), zebrafish (46) and *C. elegans* (15). In 2001, another carotenoid cleavage enzyme that catalyzes eccentric cleavage, β -carotene-9',10'-oxygenase (BCO2, also known as BCDO2, CMOII, and CMO2) was identified in human, mouse and zebrafish (41, 42). Figure 2 shows the cleavages of β -carotene catalyzed by BCO1 and BCO2 and highlights that other cleavages occur non-enzymatically, as discussed in the next section.

The enzymatic properties and substrate specificity of BCO1 and BCO2 have been characterized using purified recombinant proteins from human (BCO1) and chicken (BCO2). For BCO1, the purified enzyme catalyzed the oxidative cleavage of β -carotene with a $V_{\max}$ = 197.2 nmol retinal/mg BCO1/hr, K_m = 17.2 μ M and catalytic efficiency k_{cat}/K_m = 6098 $M^{-1}min^{-1}$. The enzyme also catalyzed the oxidative cleavage of α -carotene, β -cryptoxanthin and β -apo-8'-carotenal, β -apo-10'-carotenal, β -apo-12'-carotenal, β -apo-14'-carotenal to yield retinal. The catalytic efficiency values of these substrates are lower than that of β -carotene (16). Surprisingly, BCO1 catalyzed the oxidative cleavage of lycopene to yield acycloretinal with a catalytic efficiency similar to that of β -carotene. The enzyme did not cleave lutein, zeaxanthin, and 9-cis β -carotene (16). These results show that BCO1 favors full length provitamin A carotenoids as substrates, with the notable exception of lycopene. Subsequent study of purified BCO1 revealed that the enzyme mechanism was a dioxygenase with molecular oxygen as the other substrate (17). Like BCO1, purified recombinant BCO2 reacts with the provitamin A carotenoids β -carotene, α -carotene, and β -cryptoxanthin. However, its catalytic activity with β -carotene as substrate is at least 10-fold lower than that of BCO1, suggesting that it plays a minor role in generating vitamin A from provitamin A carotenoids (18). In further contrast to BCO1, purified recombinant BCO2

also reacts with 9-cis- β -carotene and the non-provitamin A carotenoids zeaxanthin and lutein, and does not react with all-trans-lycopene and β -apocarotenoids (18). Apo-10'-carotenoids were detected as enzymatic products (18). Thus, BCO2 cleaves specifically at the 9'-10' bond to produce apo-10'-carotenoids (18, 42). BCO2 has been shown to function in preventing the excessive accumulation of carotenoids (see below), and its broad substrate specificity is consistent with these findings. The substrate specificity of the two enzymes is summarized in Figure 3. As shown in the figure, the asymmetric cleavage at the 9',10' bond also generates β -ionone in the case of β -carotene and similar volatiles in the case of other substrates. Although β -ionone has been shown to inhibit cell proliferation and induce apoptosis both in vitro and in vivo (5, 52–54), little is known about the biological activity of the “shorter” apocarotenoids in mammals and these molecules are not discussed further here.

2.2. Non-enzymatic oxidative cleavage of carotenoids

The study of carotenoid oxygenase enzymes is complicated by the fact that carotenoids are also prone to non-enzymatic oxidation during incubation, extraction and processing. Maret and Hansen incubated β -carotene under conditions similar to the early in vitro studies of Goodman and Olson in the absence of enzyme, and detected eccentric cleavage products or β -apocarotenoids (29). Further studies reported the production of β -apocarotenoids and retinal from the incubation of β -carotene with intestine homogenates (82, 90). However, the same group later reported that retinal is the sole oxidative cleavage product if the incubations are done with α -tocopherol (97), suggesting that the β -apocarotenoids were formed by chemical oxidation. It is very likely that non-enzymatic cleavages of carotenoids other than β -carotene (i.e., α -carotene, β -cryptoxanthin, lutein, zeaxanthin, and lycopene) also produce the corresponding apocarotenoids. Thus, apocarotenoids are endogenously present in fresh plant tissues and can be formed during food processing and storage. An excellent recent report comprehensively analyzed the β -carotene and β -apocarotenoids of a variety of plant foods, including those biofortified with β -carotene (76). While different types of fresh foods had different amounts of β -apocarotenoids relative to their β -carotene content, the levels of β -apocarotenoids were low (0–4 ng/g) in all cases. While storage led to degradation of β -carotene over time and the appearance of β -apocarotenoids, the quantities of β -apocarotenoids generated (up to about 500ng/g) were far less than that of β -carotene lost. Given that all the other carotenoids are also subject to non-enzymatic cleavages of their C=C bonds the potential exists for the generation of a very large number of apocarotenoids. Most of these molecules have not been studied in mammalian systems and these remain to be the subjects of future research.

3. THE CHALLENGE OF DETECTING APOCAROTENOIDS

Today, almost all quantitative analyses of retinoids, carotenoids, their metabolites and derivatives use high-performance liquid chromatography (HPLC) coupled with various modes of detection (57, 72). The HPLC columns most often used are so-called “reverse-phase” columns packed with hydrophobic (C18) matrices. Compounds elute in order of polarity with more water-soluble compounds eluting first and more non-polar compounds eluting later. Separations are optimized by using gradient elution with mixtures of various

organic solvents. In the case of carotenoids, a special “C30” column has been developed to facilitate resolution of the cis-isomers of carotenoids. For more polar compounds (i.e., RA and its derivatives and apocarotenoids acids) “normal-phase” silica-based matrices are often used, and compounds elute in reverse order of polarity. Quantitation of compounds present in sufficient amounts is commonly achieved by using UV absorption detection, often with a diode array detector that allows recording of the complete UV absorption spectrum of each peak. Thus, peak purity, identity and amount of analyte can all be assessed, provided that appropriate purified standards are available. Because the sensitivity of UV detection precludes unambiguous quantitation of analytes, including retinoic acid, apocarotenoids, and minor isomers that are present in only very small amounts (40), more sensitive methods of analysis and detection have been developed. Today, the most powerful analytical techniques available for the unambiguous quantitative analysis of retinoids and carotenoids and their metabolites are those involving HPLC coupled with various modes of mass spectral detection, broadly called LC-MS. A number of mass spectrometer configurations can be used with differences in mode of ionization and configurations of single or multiple mass analyzers. In general, the application of LC/MS-MS techniques with Multiple Reaction Monitoring (MRM) gives the greatest reliability in terms of sensitivity and specificity of analyses. By selecting a major initial “parent” ion of the molecule of choice and then simultaneously detecting one or more specifically produced “daughter ions”, the interference by other isobaric molecules in the mixture is greatly minimized. These methods still depend critically on the availability of purified standards. Indeed, the most rigorous quantitation is best achieved by having standards of the compound of interest that are labeled with stable isotopes such as ^{13}C or ^2H . The biggest drawback to LC/MS-MS analyses is that the instrumentation is very expensive and complex and requires a high degree of skill for its maintenance and operation. Also, it requires rigorous and time-consuming assay validation. Given these issues, it is to be expected that there may be discrepancies in the levels of apocarotenoids reported in various foods and biological matrixes. In general, detection and quantitation of apocarotenoids in foods is somewhat easier than their analysis in animal and human plasma and tissues. This is because the endogenous levels are likely higher in foods and the amount of source is usually not limiting. Nonetheless, since the levels of apocarotenoids are very low, relative to the parent carotenoid, it is crucial to control for the possible generation of the compounds during sample preparation and analysis. An example of a recent LC-MS/MS method developed to detect and quantitate the β -apocarotenals and the apolycopenals in foods and human plasma is shown in Figure 4 (13). In this method the limit of quantitation (LOQ) for each of these compounds in plasma was about 50 pM.

4. APOCAROTENOIDS FROM FOODS

4.1. β -Apocarotenoids in foods

β -Apocarotenoids can also be formed via autoxidation, thermal degradation of β -carotene, or during food processing. The spontaneous autoxidation of toluene solutions of β -carotene with 100% oxygen at 60°C for 120 min yielded a homologous series of carbonyl cleavage products of β -carotene including retinaldehyde, β -apo-13-carotenone, β -apo-14'-carotenal, β -apo-12'-carotenal, and β -apo-10'-carotenal which were separated by reverse-phase HPLC, and individual peaks were characterized with an on-line diode array detector (28). In a

simulated deodorization using purified palm oil and β -carotene in which the individual compounds in fractions were isolated using a silicic acid column, β -apo-13-carotenone, β -apo-14'-carotenal, and retinaldehyde were identified by infrared spectroscopy and mass spectrometry (65). In another food related study, thermal degradation (various heat treatments) of β -carotene yielded β -apo-8'-carotenal, β -apo-10'-carotenal, β -apo-12'-carotenal, β -apo-14'-carotenal and these oxidation products were found to be 5% of initial β -carotene (56). β -apocarotenoids are also present in the diet (24, 74). Processed mango juice, acerola juice and dried apricots were analyzed by thin layer chromatography, high-performance liquid chromatography, and visible absorption spectra obtained by a photodiode array detector, and mass spectra. These processed foods were tested for the presence of β -apocarotenals including β -apo-8', 10', 12', and 14'- carotenal derived from β -carotene. None of these oxidation products were detected in acerola juice, or dried apricots and only β -apo-12'-carotenal was found in mango juice (74). Harrison and colleagues (24) reported the existence of β -apocarotenoids in orange-fleshed melons, i.e., an unprocessed food. The levels of β -apo-13-carotenone and β -apocarotenals including β -apo-8', 10', 12', and 14'-carotenal in melons (a cantaloupe and a greenhouse grown Orange Dew) were measured by liquid chromatography mass spectrometry (LC/MS). In both melons the 5 β -apocarotenoids were each present at approximately 30 pmol/g of wet weight, and in total represented about 1.5% the level of β -carotene in the melons.

4.2. Apolycoplenoids in foods

The occurrence of apolycoplenoids such as apo-6'-lycopenal and apo-8'-lycopenal in tomato extracts and tomato paste (91) and in raw tomatoes (8) was reported some time ago. Recently, in addition to apo-6'- and 8'-lycopenal, apo-10', 12', and 14'- lycoplenals were found in both raw and processed food products (44). The sum of the lycoplenals was 6.5 $\mu\text{g}/100\text{ g}$ of Roma tomato and 73.4 $\mu\text{g}/100\text{g}$ of tomato paste. These amounts are about 0.2% of the lycopene content of these foods. Also, in the same study all of these apolycoplenals (apo-6', 8', 10', 12', and 14'-) were detected in human plasma at a total concentration of about 2 nM, also about 0.2% of the plasma lycopene concentration. Whether the plasma apolycoplenals originated from enzymatic cleavage of lycopene or from consumption of apolycoplenal containing fruits and vegetables remain still unclear, though.

4.3 Intestinal absorption of apocarotenoids

Only limited information is available on whether the apocarotenoids found in foods can be absorbed intact in the intestine. This important area warrants future investigation. In one controlled human study, subjects were fed high β -carotene tomato juice for four weeks (35 $\mu\text{g}/\text{day}$) or high lycopene tomato juice for four weeks (120 $\mu\text{g}/\text{day}$) (13). Only β -apo-13-carotenone was detected above the limit of quantitation (50 pM) at a concentration of 0.5 nM. These data suggest that apocarotenoids are either not absorbed or are rapidly metabolized by the intestine. This same conclusion is supported by preliminary in vitro studies using human intestinal CACO-2 cells (20). In these experiments the cells were given 1–5 μM β -apo-8'-carotenal, β -apo-10'-carotenal, or β -apo-13-carotenone. There was rapid uptake but extensive metabolism of these compounds by the cells.

5. APOCAROTENOID FUNCTIONS

5.1. β -apocarotenoids functions

A handful of evidence of a potentially critical biological activity of β -apocarotenoids dates back to the 1960s and early 70s (1, 2, 77). These reports indicated that apocarotenoids form β -carotene, such as β -apo-8'-carotenal, β -apo-10'-carotenal and β -apo-12'-carotenal could restore growth in vitamin A-deprived quails, rats and chickens. At the time of these earlier investigations, however, it was not unequivocally established whether β -apocarotenoids could perform this action exclusively as retinoid precursors or *per se*. The cloning and characterization of the mammalian enzyme that cleaves β -carotene eccentrically, β -carotene-9',10'-oxygenase (BCO2) (42), spearheaded a number of seminal discoveries on the functions of this enzyme and its cleavage product. In this first report, von Lintig and colleagues (42) demonstrated that BCO2 cleaves β -carotene specifically at the C9C10 double bond, thus generating exclusively β -apo-10'-carotenal and β -ionone, as later confirmed by Dela sena et al (18). Thus, it was inferred that β -apocarotenals of other chain lengths were likely to be formed *via* non-enzymatic pathway, as previously proposed by Wang and colleagues (89). These authors suggested that β -apocarotenoids generated from β -carotene could play an important role in the biosynthesis of retinoic acid from β -carotene. Nevertheless, mice lacking *Bco1* became vitamin A deficient when β -carotene was the only source of vitamin A in their diet, despite the expression of *Bco2*, thus indicating potential different physiological function for this enzyme and its cleavage products (32). Subsequently, the von Lintig's group investigated the biochemical properties of this enzyme and analyzed the consequences of BCO2 deficiency in a mouse model (3). First, they confirmed the mitochondrial localization of the protein and then showed that BCO2 displayed broad substrate specificity and catalyzed oxidative cleavage both at position 9,10 and 9',10' of the carbon backbone of carotenes and xanthophylls. They also generated *Bco2*^{-/-} mice that were fed with diet supplemented with xanthophylls because these carotenoids could not be metabolized by BCO1, still expressed in *Bco2*^{-/-} animals. Remarkably, they found that the tissues of the supplemented mice accumulated lutein and zeaxanthin metabolites (i.e., 3-dehydro derivatives). Moreover, such accumulation induced mitochondrial dysfunction, as assessed by increase in manganese superoxide dismutase and reduced rates of ADP-dependent respiration. The authors provided evidence that carotenoid accumulation directly interfered with the electron transport chain, despite the mitochondria were structurally intact; and showed that increased ROS production and depolarization of mitochondria membranes was induced in HepG2 cells upon treatment with zeaxanthin and lutein or their 3-dehydro derivatives, as well as with β -carotene. This impairment of mitochondria function was associated with the induction of cell signaling pathways related to oxidative stress and disease. Clearly, β -carotene elicited ROS production and decreased depolarization of mitochondria membranes in a BCO2-dependent manner leading to the activation of apoptotic pathways (55). Later, Amengual and colleagues (4) conducted *in vivo* studies with single and double knockouts mice for *Bco1* and *Bco2*, to establish more specifically the contributions of these enzymes to β -carotene metabolism. They demonstrated that BCO1 is the major β -carotene-metabolizing enzyme in adult mouse tissues and that BCO2 alone does not significantly contribute to β -carotene homeostasis, even though it was once again confirmed that a β -apo-10'-carotenoid (β -apo-10'-carotenol)

was generated in vivo from β -carotene by its activity. However, β -carotene emerged as a poor substrate for BCO2 in vivo. Rather, the enzyme seemed to prefer carotenoids with 3-OH- β -ionone ring sites as a substrate. Most importantly, they showed that BCO1 can use long chain β -apocarotenoids as a substrate ensuring that they could be utilized for vitamin A production, at the same time preventing the tissue accumulation of these compounds that could potentially compete with vitamin A. The authors proposed that this “stepwise cleavage” could be critical for the conversion of asymmetric provitamin A carotenoids, such as β -cryptoxanthin, into retinoids. From these and other data, von Lintig and colleagues proposed that the “eccentric enzymatic cleavage of β -carotene can occur in the test tube but is largely absent in living organisms” (67). This remarkable body of work from von Lintig and colleagues established the notion that the asymmetric cleavage by BCO2 contributes to protect mitochondria from toxic effects of excessive carotenoids (including β -carotene) which otherwise would lead to oxidative stress. This notion was later confirmed by others in the context of studies aimed at defining the link between carotenoid metabolism and chronic diseases, such as metabolic disorders, age-related macular degeneration (AMD) and cancer (6, 27, 37, 47, 81, 92). Nevertheless, these findings did not address the mechanism(s) of the β -carotene toxicity. Is this due to the intact molecule *per se* or to its derivatives? How do carotenoids exactly impair mitochondria functions? Moreover, the question as to whether β -apo-10'-carotenoids may have a potential biological role remained unanswered not only in adult tissues but also in the developing ones.

Evidence has emerged in support of the notion that β -apocarotenoids act indeed as nuclear receptor antagonists. Harrison *et al.* (23) showed that β -apocarotenoids of various chain lengths and forms (aldehyde, acid, ketone) antagonized all-*trans* retinoic acid-induced transactivation of all three RAR isoforms at nM concentrations, by directly competing with retinoic acid for receptor binding or by modulating the RXR oligomeric state, as in the case of β -apo-13-carotenone (79). Notably, endogenous levels of β -apo-13-carotenone in human plasma range from 3 to 5 nM, near to the concentration of the natural RAR agonist, retinoic acid (23). β -apocarotenoids also inhibited the all-*trans* retinoic acid-induced expression of retinoid-responsive genes in HepG2 cells (23) and antagonized the activation of RXR α by 9-*cis* retinoic acid (22). Also, β -apo-14'-carotenol effectively inhibited agonist-induced RXR α , PPAR α and PPAR γ activation, decreasing adipogenesis (98). The finding that β -carotene can generate RAR agonists (all-*trans* retinoic acid), through symmetric cleavage, as well as potent RAR antagonists (β -apocarotenoids) has been proposed to have implications for the unexpected and negative effects of high doses of β -carotene in human clinical trials of cancer prevention (23, 75, 88). Further in vitro study from Blaner and colleagues (86) confirmed that the β -apo-13-carotenone or β -apo-10'-carotenoid stimulated adipocyte marker gene expression in 3T3-L1 cells. However, in this study β -apo-carotenoid failed to attenuate the blockage of 3T3-L1 cell differentiation upon all-*trans*-retinoic treatment. Therefore, the authors suggested that in addition to acting as transcriptional antagonists, some β -apocarotenoids may function through other mechanisms, for example through non-genomic activities. Recently, Costabile *et al.* (14) provided the first in vivo evidence of the transcriptional regulatory activity of β -apocarotenoid(s) by showing that the β -carotene metabolites generate a feed-forward regulatory mechanism to facilitate placental-fetal transfer of β -carotene *via* modulation of placental lipoprotein biosynthesis. Specifically, the

authors proposed that when β -carotene is available, placental *Bco2* transcription increases, thus enhancing the generation of β -apo-10'-carotenal from β -carotene *via* asymmetric cleavage. This metabolite increases the transcription of *Hnf4a*, which in turn increases transcription and activity of microsomal triglyceride transfer protein (MTP) - the master regulator of intracellular assembly and secretion of apoB-lipoproteins (35) - to enhance the transfer of intact β -carotene towards the embryo. Their finding suggested that neither *Mttp* nor *Hnf4a* are likely direct targets of the β -apo-10'-carotenoid(s) action. Thus, a direct target of the β -apocarotenoid action upstream of *Hnf4a* still remains to be identified. Also, it remains to be established which form (alcohol, acid, aldehyde) represent the actual active β -apocarotenoid in this process.

5.2. Apolycopenoids functions

Apolycopenoids are lycopene derivatives of various chain length that can be formed by autooxidation, radical-mediated oxidation, and singlet oxygen as well as by enzymatic cleavage mediated by BCO2. In this latter instance, apo-10'-lycopenal, apo-10'-lycopenol, and apo-10'-lycopenoic acid are predominantly generated from *cis*-lycopene isomers (87). Noteworthy, apo-6'-, apo-8'-, apo-10'-, apo-12'-, and apo-14'-lycopenals are present endogenously in lycopene-containing foods and have been detected in human plasma in concentrations of 0.12–0.73 nM upon consumption of tomato juice (44). Chemopreventive effects have been attributed to lycopene metabolites, such as apo-10'-lycopenoic acid, against carcinogen-induced cancer development in animal models *in vivo* (48). This activity was later linked to the induction of antioxidant/phase II detoxifying enzymes (49). In BEAS-2B human bronchial epithelial cells apo-10'-lycopenoic acid induced accumulation of nuclear factor E2-related factor 2 (Nrf2) protein, as well as mRNA expression of heme oxygenase-1 (HO-1) in a dose- and time-dependent manner. Moreover, pretreatment of BEAS-2B cells with apo-10'-lycopenoic acid resulted in a dose-dependent inhibition of both endogenous reactive oxygen species production and H₂O₂-induced oxidative damage, as measured by release of lactate dehydrogenase (49). Furthermore, as retinoic acid, apo-10'-lycopenoic acid was shown to transactivate RARs, albeit at higher concentrations (48). This activity was proposed to account for the growth inhibitory effect of apo-10'-lycopenoic acid. As in the case of the parent compound lycopene, apo-10'-lycopenoic acid has also been proposed to inhibit cell proliferation by modulating the IGF signaling. Indeed, treatment with apo-10'-lycopenoic acid increased IGFBP-3 expression in lung BEAS-2B cells, liver THLE-2 cells, and prostate PC-3 cells (87). Chemopreventive activity was also shown by apo-12'-lycopenal in androgen-dependent DU145 prostate cancer cells (although at supra-physiological levels). This compound reduced proliferation in part by inhibition of cell cycle progression (25). Supplementation of apo-10'-lycopenoic acid in mice was also effective at inhibiting hepatic steatosis in obese *ob/ob* mice (12) and at attenuating high saturated fat diet (HSFD)-induced liver inflammation as well as tumor number and volume in *C57Bl/6J* mice (36). Most importantly, Wang and colleagues (38) demonstrated that the biological activity of lycopene is different from that of its metabolite apo-10'-lycopenoic acid in the absence of BCO2 expression. Both lycopene and apo-10'-lycopenoic acid supplementation were effective at reducing HSFD-induced liver steatosis in *Bco2*^{-/-} mice. However, whereas the apo-10'-lycopenoic acid mediated ability to prevent hepatic steatosis and inflammation in

male mice was associated with SIRT1-signaling activation in the liver, the lycopene-mediated activity was linked to modulation of lipid metabolism in adipose tissue (mesenteric).

Recently, a novel synthetic process for apo-11, apo-13, and apo-15 lycopeneoids has been reported. Some of these acyclic apolycopeneoids can function as antagonists of retinoic acid, like some of their cyclized analogs β -apocarotenoids (63). Whereas currently there is no information on the levels of apo-13-lycopeneone or the apo-15-lycopeneoids in human plasma or tissues, the generation of these important reagents may be critical to expand this area of research.

5.3. Functions of other apocarotenoids

Wang and colleagues (58) first reported that eccentric enzymatic cleavage by BCO2 occurs in vitro at the 9,10 and 9',10' double bond of hydroxy carotenoids, such as the non-provitamin A zeaxanthin and lutein, and provitamin A β -cryptoxanthin producing both volatile and non-volatile apo-carotenoid cleavage products, including 3-OH- β -ionone, 3-OH- α -ionone, β -ionone, 3-OH- α -apo-10'-carotenal, 3-OH- β -apo-10'-carotenal, and β -apo-10'-carotenal. These findings were later confirmed by Dela Sena and colleagues (18) who also reported a small amount 3-hydroxy- β -apo-8'-carotenal generated from BCO2-mediated cleavage of β -cryptoxanthin. Overall, whether these hydroxyl compounds are formed in mammalian tissues and whether their generation may be linked to the potential biological actions of their parent compounds remain to be established and certainly warrant future studies. Interestingly, keto-carotenoids derived from dietary xanthophylls were detected in the liver of mice fed lutein as well as in plasma of normal human subjects. Furthermore, β , ϵ -caroten-3'-one was significantly increased upon ingestion of β -cryptoxanthin suggesting that humans and mice have oxidative activity to convert the 3-hydroxy β -end group of xanthophylls to a 3-oxo ϵ -end group (62).

Apocarotenoids are abundant in plants where they play major roles as signaling molecules in regulating development, as well as pigments, repellents, chemoattractants, and as determinants of their aroma (7, 34). Consequently, many foods or food-derivatives of plant origin (such as spices, for example) are highly enriched in these metabolites. Very little is known in regards to how apocarotenoids from foods are absorbed and metabolized within different tissues in the body. Uptake studies with two different cell lines (PC19 and NIH3T3) have led Blaner and colleagues to hypothesize that acquisition and/or metabolism of different β -apo-carotenoids is controlled by tissues-specific mechanisms (86). Yet, further investigations are needed to elucidate these processes. Limited information pertains also to the potential physiological role of food-derived apocarotenoids in mammals. However, a larger literature has emerged on the potential therapeutic applications of apocarotenoids. In this respect, the two most studied ones to date are bixin and crocin, mainly due to their commercial value and broad use in the food industry (94).

Bixin is an apocarotenoid of 25 carbon atoms with 9 double bonds highly concentrated in the seeds of *Bixa orellana* L. (73). Lycopene is described as a bixin precursor, although it appears that it can also be generated from other not yet identified biosynthetic pathways (11). For centuries, in different parts of the world, a plethora of health-beneficial properties

has been attributed to various mixtures of compounds extracted from different parts of the *Bixa orellana* (73). Among the components of these extracts, bixin (from the seeds) has been shown to function as a powerful antioxidant, specifically due to its capability to scavenge ROS (10). Given that increased ROS and free radicals promote a number of pathological conditions in humans, the function of bixin to reduce oxidative damage in various mammalian tissues has been linked to its protective roles against the side effects of chemotherapeutic drugs, and the development of diabetes and its complications, including recently cardiac injury (95). In this report, it was shown that bixin reduced fibrosis, inflammation and ROS production in the heart of high fat-diet fed mice and in cardiac muscle cells (95).

The apocarotenoid crocin and safranal are the main components of saffron, the dehydrated red stigma of the flower of *Crocus sativus* L., a widely cultivated perennial plant (78). Saffron is a spice used for flavor enhancement and as a preservative in food (45) and its quality is determined by the concentration of its bioactive compounds. The apocarotenoids in saffron are proposed to derive from zeaxanthin (7). Crocetin and its glycosylated form, crocin, give the characteristic red pigmentation of the saffron stigmas, whereas safranal gives the aroma. Having been reported to function as an anti-inflammatory, antioxidant, anti-proliferative and pro-apoptotic molecule, the list of beneficial effects of crocin in humans is almost omni-comprehensive, showing protective effect against diseases of the heart, central and peripheral nervous system, and liver, to name a few (reviewed in (68)). Interestingly, these effects described in animal models and cell culture systems, were reported at relatively low concentrations as those attainable upon intake of crocin-containing foods (78). Saffron is also known for its chemopreventive activity through apoptosis, anti-oxidant activity, immune modulation, enhancement of cell differentiation, inhibition of cell proliferation, modulation of carcinogen metabolism, regulation of cell growth and cell cycle progression and stimulation of cell-to-cell gap junction communication. These actions have been predominantly attributed to crocin, although the exact molecular mechanisms have not been fully understood. Interestingly, it has been proposed a direct interaction of the apocarotenoid with different critical molecules within the cell, such as a) the inhibitory effect on DNA and RNA synthesis by interacting with DNA and causing conformational changes; b) the inhibitory effect on histone DNA complex formation; c) cell cycle arrest through p53 dependent and independent mechanisms causing apoptosis; d) and the interaction of carotenoids with topoisomerase II, an enzyme responsible for DNA-protein interaction (reviewed in (33, 68)).

6. CONCLUSIONS AND PERSPECTIVES

It is becoming increasingly clear that non-retinoid apocarotenoids have the potential to regulate a broad spectrum of critical cellular functions, thus influencing health in humans and, more generally, in mammals. However, the research in this area is also clearly in its infancy posing a number of fundamental questions that warrant further investigations. Since apocarotenoids can be generated in the tissues of mammals that have ingested foods containing the parent carotenoid compound or obtained directly from food sources highly enriched in apocarotenoids (generated in the plant or during the processing of these foods), it is imperative to understand more about the bioavailability of these molecules. What is the

contribution of apocarotenoids obtained directly from foods vs. those generated in the tissues from their parent compound to the total tissue apocarotenoids content? Accurate measurements of the most common apocarotenoids of dietary origin - yet, still a rather large variety of molecules - needs to be achieved in the foods as well as in plasma and tissues of humans and experimental models. This knowledge is critical to correlate their intake and tissues levels with the biological effects. The development of novel, efficient protocols to synthesize pure apocarotenoids to be used as standards in analytical techniques as well as the improvement in sensitivity of such methodologies is desirable to attain these goals.

Different forms of apocarotenoids (acid, alcohol, aldehyde, ketone) have been detected in human tissues and animal models. However, it has not unequivocally established which one(s) may be the active form(s). If conversion from one apocarotenoid form (inactive) to another (active) is required for some of these carotenoid derivatives to exert their biological function, which are the mammalian enzymes (if they exist) that perform these conversions? Is it reasonable to assume that apocarotenoids may be substrates for the well-known enzymes of the retinoid metabolic pathways, for example? Interestingly, von Lintig and colleagues (4) showed that β -apo-10'-carotenol can be esterified by LRAT, bind to RBP, and taken up by target cells from its RBP-bound form by STRA6, implying that, at least at high concentrations, this apocarotenoid could compete with the vitamin A metabolic pathways. Addressing these questions is crucial to understand the mechanisms of action of apocarotenoids and, potentially, their tissue-specificity. In the same vein, it would be important to investigate how the intracellular trafficking of these compounds is carried out. If (at least some) apocarotenoids are generated by the action of BCO2 in the mitochondria, they must be able to efflux from these organelles by crossing the mitochondrial membrane(s) to exert their functions in the cytoplasm, for instance. Moreover, if they act as transcriptional regulators, they must traverse the nuclear membrane to interact with specific nuclear receptors. Whether this trafficking is achieved by means of specific transporters or channels located in the above-mentioned biological membranes remains to be established.

The list of beneficial health effects ascribed to the actions of apocarotenoids is already quite long and it is growing even faster. The development of cancer, cardiovascular diseases, metabolic syndrome, neurodegenerative pathologies - to name a few - has been shown to be influenced by these dietary-derived compounds. They seem to act by regulating key cellular metabolic and signaling pathways, including lipid metabolism, IGF and SIRT-1 signaling, oxidative stress, phase I and II xenobiotic-metabolizing enzymes. However, the molecular mechanisms underlying these activities are still not fully understood. In vitro experiments have shown that β -apocarotenoids and lycopeneoids can interact directly with nuclear receptors to regulate transcription, and in vivo targets of this activity are beginning to be identified. Can such broad range of biological affects of apocarotenoids be ascribed exclusively to their role as transcriptional regulators? Some literature begins to provide insights into potential direct interaction of the apocarotenoids with different critical molecules within the cell, such as DNA, DNA-protein complexes, or specific kinases. Thus, it seems plausible that, like in the case of their "sibling compounds" retinoids (69), extra-nuclear and non-transcriptional effects (non-genomic) may account for their biological functions.

In addition to the most studied β -carotene, lycopene, lutein and zeaxanthin, other carotenoids are emerging as beneficial for mammalian health, such as β -cryptoxanthin and astaxanthin, for example (19, 70). To date, these actions have been mainly attributed to the anti-inflammatory and antioxidant effects of the parent compounds. Nevertheless, it cannot be excluded that their potential promoting role may be driven by their derivatives. This issue remains to be ascertained. Lastly, many other apocarotenoids are generated in plants that are then ingested as a food. Is there a biological function(s) for these less common apocarotenoids?

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TERMS AND DEFINITION LIST

Apocarotenoids

Apocarotenoids are carotenoids derivatives originated upon enzymatic and non-enzymatic cleavage of the C40 parent compound.

Non-retinoid apocarotenoids

Cleavage products of carotenoids that do not generate vitamin A derivatives (retinoids).

Central and eccentric cleavage

Central cleavage refers to the cleavage at the 15,15' double bond of the C40 carotenoid parent molecule. This is enzymatically mediated by the action of β -carotene-15,15'-oxygenase (BCO1). Eccentric cleavage refers to the enzymatic cleavage at the 9,10 or 9',10' double bond of the parent carotenoid compound. It is mediated by β -carotene-9',10'-oxygenase (BCO2). Eccentric cleavages at other position of the C40 isoprenoid chain can also occur non-enzymatically by autooxidation.

Transcriptional activity

It is performed by hormone-like molecules that by binding to specific nuclear receptors can modify their ability to control the transcription of their target genes.

Non-genomic activity

It is performed by hormone-like molecules that act outside the nucleus activating signaling cascade upon binding to specific proteins. The cellular response induced by non-genomic activities is generally extremely rapid.

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SUMMARY POINTS

1. Most of the current knowledge regarding the biological functions of non-retinoid apocarotenoids pertains to derivatives of β -carotene, lycopene and zeaxanthin.
2. Apocarotenoids can be formed *via* enzymatic cleavage mediated by β -carotene-9',10'-oxygenase (BCO2) from parent carotenoids accumulated in the tissues of mammals consuming plant-based foods. Alternatively they can be obtained from foods of plant origin naturally enriched in apocarotenoids or containing apocarotenoids generated from the parent compounds during food processing.
3. A plethora of cellular metabolic and signaling pathways have been reported to be modulated by apocarotenoids *in vitro* and *in vivo*, resulting in beneficial effects for almost all organs in the body. Nevertheless, their molecular mechanisms of actions have not been fully elucidated.
4. β -apocarotenoids and apolycopeneoids can function as nuclear receptor antagonists regulating the transcription of genes involved in such pathways. Apolycopeneoids have also been shown to function as agonist for nuclear receptors, albeit at higher concentrations. Whether some of their biological functions can be ascribed also to non-genomic activities remain to be unequivocally established.
5. Bixin (derived from lycopene) has been proposed to function predominantly as a powerful antioxidant, specifically due to its capability to scavenge ROS. Crocin (derived from zeaxanthin) has been proposed to directly interact with critical molecules within the cell, such as DNA, histone DNA complex, and topoisomerase II, for example.
6. Very limited information is available in regards to the potential of other apocarotenoids to modulate biological functions in mammals.

FUTURE ISSUES

1. To understand if and how apocarotenoids can function as dietary modulators of health in humans and, more generally, in mammals, further studies are needed to define the bioavailability of these compounds. This knowledge is critical to correlate their intake and tissues levels with the biological effects.
2. Currently, inconsistent data exist in the literature in regards to the doses of apocarotenoids used in both *in vitro* and *in vivo* experiments, and often such doses could be considered supra-physiological. It is desirable that future studies will be performed at more physiological concentrations of these compounds.
3. So far, only a few indirect targets of the transcriptional regulatory activity of apocarotenoids (β -apocarotenoids) have been recognized *in vivo*. To understand the full spectrum of their biological activity more of such targets,

especially the direct ones, need to be identified. In vivo direct targets of the transcriptional regulatory activity of apolycopenoids are currently unknown.

4. A clear mapping of the cellular metabolic and signaling pathways that can be regulated by apocarotenoids is needed. Given the extremely variable experimental settings described in the literature, the data currently available are often conflicting and not fully comprehensive.
5. The molecular mechanisms of intracellular trafficking and metabolism of apocarotenoids remain to be elucidated.
6. Apocarotenoids from β -carotene and lycopene, as well as a handful of apocarotenoids from other parent carotenoids (such as zeaxanthin) are currently being studied. However, many more apocarotenoids are generated in plants that are then ingested as a food or likely in the tissues of the animals that consumed such foods. Is there a biological function(s) for these less common apocarotenoids?
7. The majority of the apocarotenoid functions studied so far pertain to adult tissues. With the exception of their activity as regulators of specific placental metabolic pathways, their potential role in the developing tissues remains to be established.

Figure 1. The numbering of the carbons of a carotenoid structure.
The structure of β -carotene is shown.

Figure 2. Products of the central and eccentric cleavages of β -carotene.

Oxidative cleavage of β -carotene at the 15,15' double bond is catalyzed by the enzyme β -carotene 15,15'-oxygenase 1 (BCO1) and leads to the generation of two molecules of retinaldehyde (β -apo-15-carotenal). Cleavage at other double bonds leads to the formation of β -apocarotenals and β -apocarotenones. For example, the cleavage at the 9',10' double bond is catalyzed by β -carotene 9',10'-oxygenase 2 (BCO2) and leads to the formation of β -apo-10'-carotenal and β -ionone. Eccentric cleavage at other double bonds may occur non-enzymatically or may be enzyme catalyzed. Figure adapted with permission from (21).

Substrate	Chemical Structure
β -carotene
α -carotene
β -cryptoxanthin
Lycopene
β -apo-8'-carotenal
β -apo-10'-carotenal
β -apo-12'-carotenal
β -apo-14'-carotenal
Zeaxanthin
Lutein
9-cis β -carotene

Figure 3. Sites of enzymatic cleavages of dietary carotenoids and apocarotenoids catalyzed by purified vertebrate BCO1 and BCO2.

BCO1 cleaves exclusively at the 15,15' double bond, as shown in blue. BCO2 cleaves almost exclusively at the 9',10' double bond, as shown in red. Figure adapted with permission from (16).

Figure 4. LC-MS/MS profile of β -apocarotenals and apolycopenals in human plasma. LC/MS mass chromatograms of β -apocarotenoids and apolycopenoids. Compounds were separated on a reverse phase C30 column using a gradient of methanol, methyl-t-butyl ether and water. Colored tracings represent different mass transitions that are used to quantitate isobaric compounds.