Conversion of Dietary Carotenoids and Vitamin A into Bioactive Retinoids: Exploring trails blazed by Jim Olson

James Allen Olson Memorial Lecture

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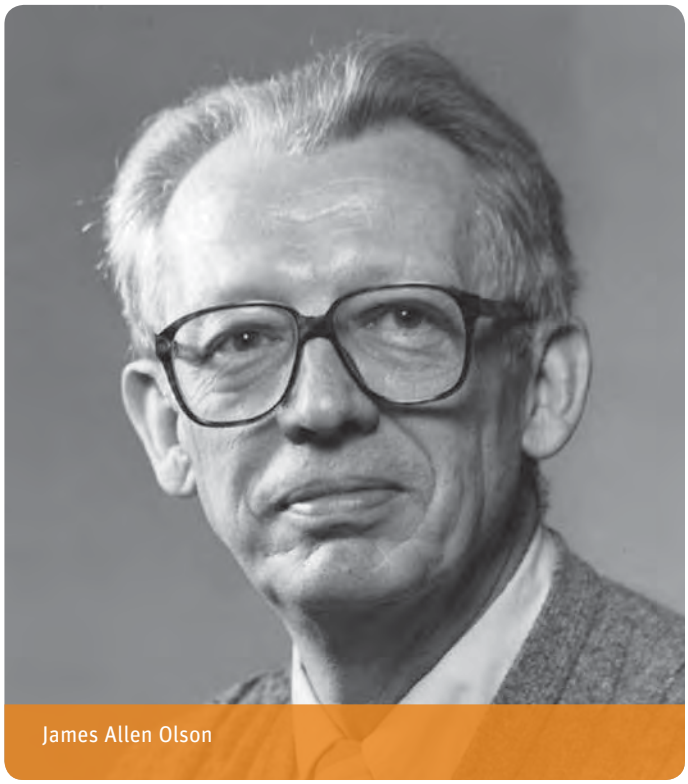


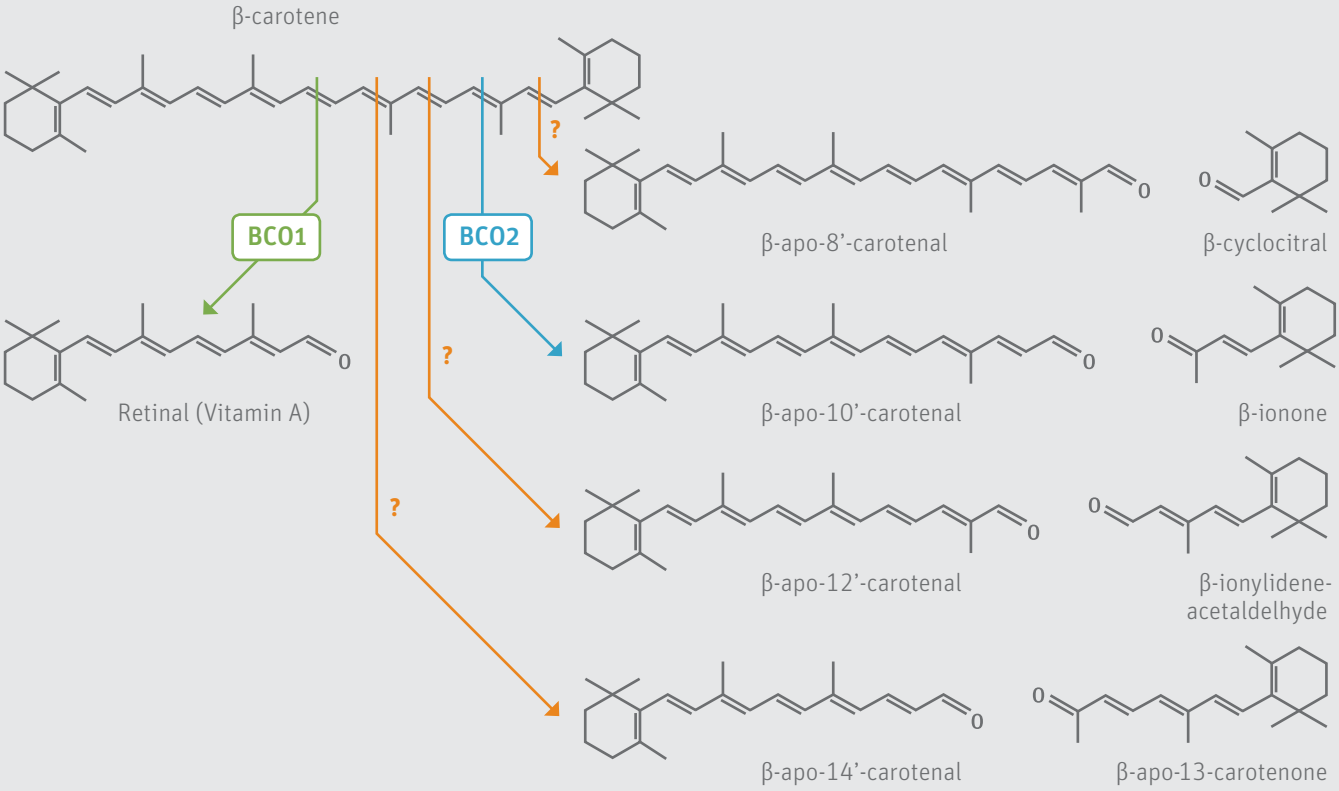
James Allen Olson

Vitamin A, carotenoids, and James Allen Olson

As readers of *Sight and Life* well know, vitamin A deficiency is one of the major micronutrient deficiencies worldwide and affects millions of children and women in the developing world. The widespread morbidity and mortality associated with the deficiency reflects the fact that the active forms of vitamin A (retinoids) are critical signaling molecules necessary in higher vertebrates for embryonic development, the regulation of gene transcription, visual transduction, immune function, and the control of metabolic processes. In spite of their importance in vertebrate development and physiology, the capability for the biosynthesis of molecules with retinoid activities is restricted to plants and microorganisms. Thus, animals, including humans, must obtain the essential vitamin A from the diet. Vitamin A activity in the diet comes from two sources: preformed vitamin A as retinyl esters in foods of animal origin, and provitamin A carotenoids, such as β-carotene, α-carotene, and β-cryptoxanthin, found in plant-derived foods. Indeed, in areas of the world with vitamin A deficiency, the major source of vitamin A is dietary carotenoids.

While the chemical and nutritional relationships of provitamin A carotenoids and vitamin A were appreciated by the 1930s it was in the last half of the 20th century that great advances in our understanding of metabolism, function, and public health significance of carotenoids and vitamin A led us to our current state of knowledge in these fields. While these advances were the results of the efforts of many basic scientists, clinicians, and public health experts, James Allen Olson stands out as one of the giants in the fields of vitamin A and carotenoids. What is particularly noteworthy about his work was that it involved a remarkable balance of basic research *and* the application of that research to the practical problem of vitamin A deficiency in hu-

FIGURE 1: Enzymatic cleavage of β-carotene. Oxidative cleavage of β-carotene at the central 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 retinal (β-apo-15-carotenal). Cleavage at the 9',10' double bond is catalyzed by β-carotene 9',10'-oxygenase 2 (**BCO2**) and yields β-apo-10'-carotenal and β-ionone. Eccentric cleavage at other double bonds may occur non-enzymatically or may be catalyzed by enzymes that are not yet fully characterized.



mans. In the latter area, his work on developing new ways to assess liver stores of vitamin A in humans continues to be critically important in determining the distribution and extent of vitamin A deficiency throughout the world.[1,2]

> "James Allen Olson stands out as one of the giants in the fields of vitamin A and carotenoids"

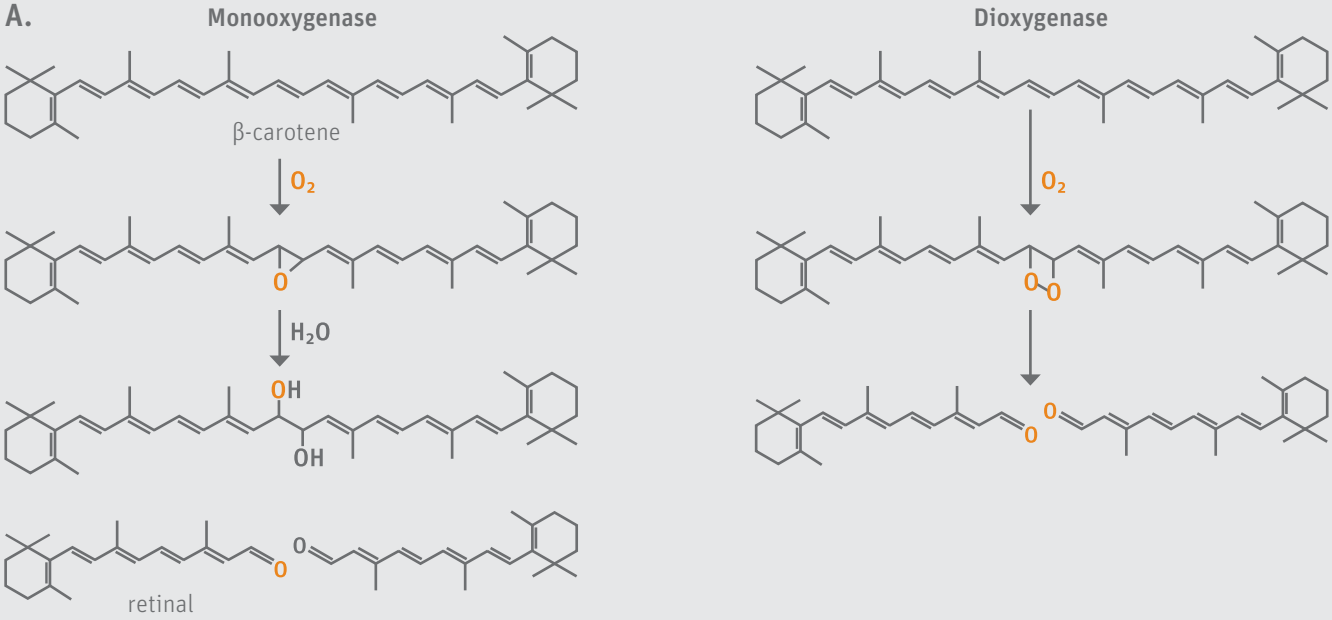
In addition to his important work on human vitamin A deficiency, Jim Olson always maintained an active research program that yielded important advances in understanding the basic biochemistry and metabolism of vitamin A. Indeed, his first publications after establishing his own laboratory focused on the enzymatic conversion of β-carotene to vitamin A.[3,4,5,6] He also had a long-standing interest in the so-called "eccentric" cleavage of β-carotene to the β-apocarotenoids.[7,8,9] This brief review attempts to highlight the current state of knowledge in this area as developed by many investigators who have followed these trails blazed by Jim Olson.

Central vs. eccentric cleavage of β-carotene and the enzymes involved

In 1965, Olson (and independently Goodman) published the first *in-vitro* studies using extracts from rat liver and intestinal mucosa to demonstrate enzymatic oxidative cleavage of β-carotene at the central double bond to yield retinal.[10,11] However, it would be 35 years before the enzyme was purified and characterized at the molecular level. In 2000, the enzyme responsible for the conversion of β-carotene to retinal was identified in Drosophila[12] and chicken,[13] and was named β-carotene-15,15'-monooxygenase 1 (BCO1). BCO1 has also been identified in humans[14,15,16] and mice,[17,18] and subsequently in a number of other taxa. In 2001, another carotenoid cleavage enzyme that catalyzes eccentric cleavage, β-carotene-9',10'-oxygenase (BCO2), was identified in humans, mice and zebrafish by von Lintig and colleagues.[19]

FIGURE 2: BCO1 reaction mechanism study. Human BCO1 is a dioxygenase. **A**. The putative reaction mechanisms of BCO1: A monooxygenase incorporates an oxygen atom from O_2 in one retinal molecule, and an oxygen atom from water into the other. A dioxygenase incorporates only atoms from O_2 into the cleavage products. **B**. Theoretical percentages of ^{18}O-retinal that will be obtained for oxygen labeling experiments with BCO1 as a monooxygenase and as a dioxygenase. **C**. Summary of results of oxygen labeling experiments with purified recombinant BCO1. The numbers separated by commas are the % ^{18}O enrichment of the retinal product from individual experiments done on different days. Retinal obtained from the BCO1-β-carotene reaction contains predominantly the same oxygen isotope as O_2. Control incubation of active BCO1 with ^{18}O-retinal in $H_2{}^{16}O$ and ^{16}O-retinal in $H_2{}^{18}O$ account for the oxygen exchange that occurred in the corresponding BCO1-β-carotene reactions. Thus, BCO1 incorporates solely oxygen from O_2 during the oxidative cleavage of β-carotene, and is therefore a dioxygenase.

A.



Adapted from dela Sena et al[22]

B. Theoretical results

% ^{18}O-retinal			
$^{16}O_2$-$H_2{}^{18}O$		$^{18}O_2$-$H_2{}^{16}O$	
Monooxygenase	Dioxygenase	Monooxygenase	Dioxygenase
50	0	50	100

C. Experimental results

% ^{18}O-retinal	
$^{16}O_2$-$H_2{}^{18}O$	$^{18}O_2$-$H_2{}^{16}O$
BCO1 + β-carotene	BCO1 + β-carotene
3, 6, 10	79, 85
BCO1 + ^{16}O-retinal (>99% atom)	BCO1 + ^{18}O-retinal (91% atom)
5, 7, 13	67, 84

The study of carotenoid oxygenase enzymes is complicated by the fact that carotenoids are also prone to nonenzymatic 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.[20] Intestinal homogenates were used in most of the early *in-vitro* studies of BCO1. However, the intestine also contains peroxidases that can potentially oxidize carotenoids.[20] **Figure 1** shows the cleavages of β-carotene catalyzed by BCO1, BCO2, and as yet undefined mechanisms.

BCO1 only reacts with carotenoids with at least one unmodified ionone ring and at least 30 carbons. Among these, β-carotene has been shown to be the best substrate for BCO1.[14,21] The human enzyme also catalyzes cleavage of the β-apocarotenals to yield retinal.[22]

BCO1 is a soluble (cytosolic) enzyme[14,23] and exists as a monomer.[24,25] *In vivo* it is expressed in a variety of tissues. Early experiments have shown high BCO1 activity in tissue homogenates from intestines,[10,11,26] and it is no surprise that BCO1 expression is particularly high in intestine and liver.

Both Olson & Hayasi[10] and Goodman & Huang[11] suggested that the central cleavage enzyme was a dioxygenase based on substrate and cofactor requirements, but did not rule out a monooxygenase mechanism. In 2001, shortly after purified enzyme was available, a monooxygenase mechanism was indeed proposed for BCO1 by Leuenberger et al.[27] In this study, α-carotene was incubated with purified chicken BCO1 and horse liver aldehyde dehydrogenase in an 85% $^{17}O_2$-95% $H_2{}^{18}O$ environment. The resulting products (retinol and α-retinol) were purified by HPLC and silylated. Using gas chromatography (GC)-MS, the authors found virtually equal enrichment of ^{17}O and ^{18}O in both silylated retinols, suggesting a monooxygenase mechanism for BCO1. However, it is possible that the long reaction time (7.5 hours) and extensive processing (reduction of the aldehydes, purification, silylation) favored oxygen exchange between the initial aldehyde products and the medium. We have recently reinvestigated the reaction mechanism of human BCO1 using highly purified recombinant human enzyme, short reaction times and using both ^{18}O water and ^{18}O gas.[28] These new results described in **Figure 2** show clearly that the enzyme is a dioxygenase.

"The enzyme BCO1 is a dioxygenase"

The most obvious function of BCO1 is to generate retinal (vitamin A aldehyde) from dietary carotenoids. Indeed, BCO1 knockout mice tend to have high levels of stored carotenoids and low levels of retinol and retinyl esters.[29] A loss-of-function mutation in BCO1 has been identified in a patient with hypercarotenemia and hypovitaminosis A.[24] However, BCO1 knockout mice have been shown to develop liver steatosis, elevated free fatty acids and obesity, even on a vitamin A sufficient diet.[29,30] This suggests a greater metabolic role for BCO1 than just generating retinal from provitamin A carotenoids.

Unlike BCO1, BCO2 is able to catalyze the oxidative cleavage of xanthophylls such as zeaxanthin and lutein.[31,32] The kinetic parameters obtained by Mein and colleagues also suggest that zeaxanthin and lutein are better substrates than β-cryptoxanthin. Kinetic data for different carotenoids with BCO2 are lacking, but the limited information available suggests that BCO2 has broader substrate specificity with respect to substrate molecule shape. In contrast to BCO1, BCO2 is a mitochondrial enzyme, and has been suggested to function primarily in preventing oxidative stress due to carotenoid accumulation by breaking down excess carotenoids.[23,31] There are also differences in expression among tissues which suggest that BCO2 may play roles that are quite distinct from those of BCO1.

BCO1 knockout mice have elevated expression of BCO2, and vice versa.[33,34] Upon β-carotene supplementation, BCO1 knockout mice accumulate β-apo-10'-carotenol, the alcohol form of the β-carotene-BCO2 cleavage product.[30] BCO1 knockout mice accumulate 3,3'-didehydrozeaxanthin and 3-dehydrolutein upon supplementation with zeaxanthin and lutein, respectively.[31] This is consistent with the findings of other groups that these xanthophylls are not substrates for BCO1.

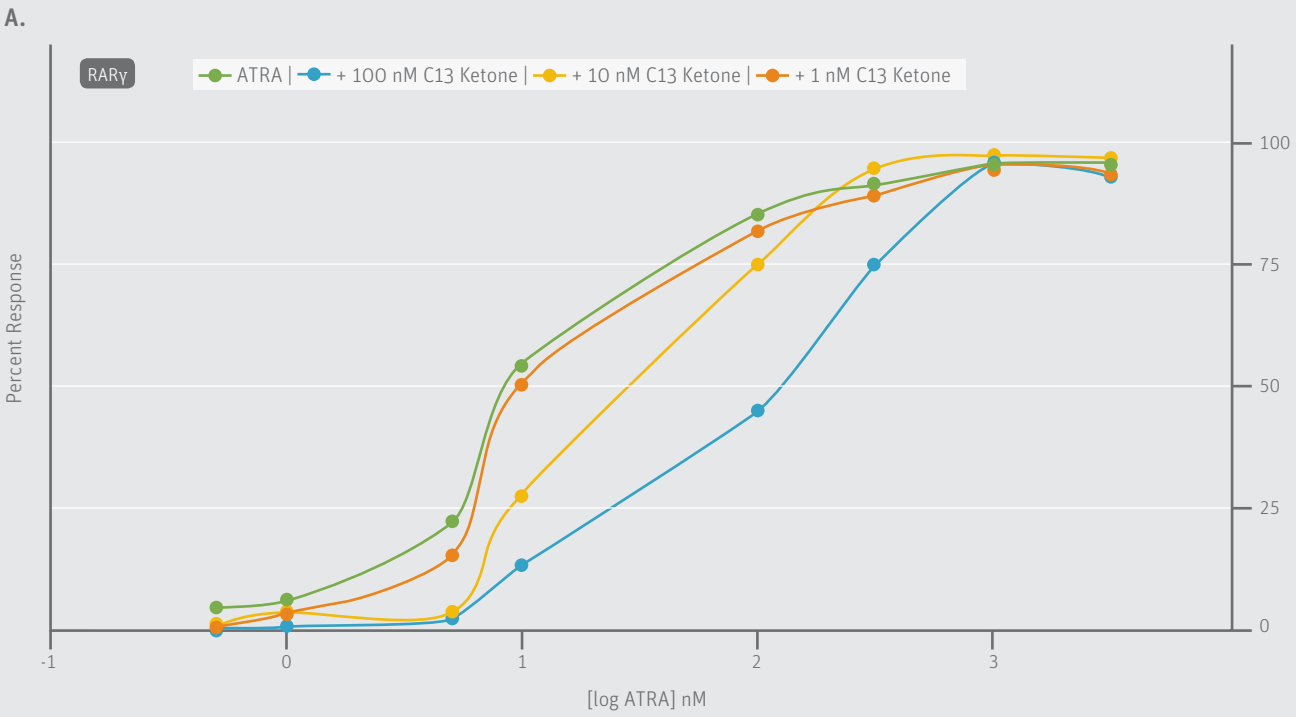
Occurrence, metabolism, and function of β-apocarotenoids in vertebrates

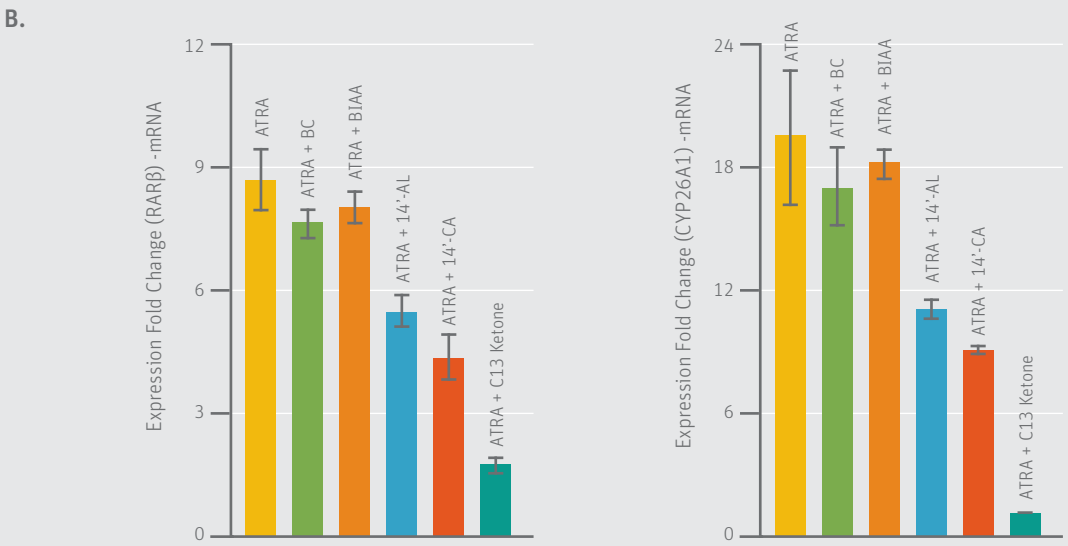
β-apocarotenoids are found endogenously in both natural foods[35] and diets prepared for experimental animals with β-carotene beadlets.[36] Thus, it is likely that these compounds can be absorbed in the intestine directly from the diet, but there are no studies on this point.

Additionally, β-apocarotenoids can arise in humans and rats from β-carotene metabolism. In one study, β-apo-8'-carotenal was detected in the plasma of a healthy man three days following ingestion of a small oral tracer dose of [^{14}C]-β-carotene.[37] Our group has conducted studies in which the concentrations of β-carotene, β-apo-8'-, 10'-, 12'- and 14'-carotenal and β-apo-13-carotenone were measured in serum and tissues (liver and heart) of wild type and BCO1 knockout mice that were on a β-carotene-containing diet.[36,38] In general, these compounds are found in serum and tissues at nanomolar concentrations. The levels of β-apo-13-carotenone have been measured in human plasma by liquid chromatography (LC)-MS and found to be 3–5 nM.[39]

The possible activation or inhibition of nuclear receptors, including retinoic acid receptors (RARs) and retinoid X receptors (RXRs), by β-apocarotenals has been studied by several groups. In general, the β-apocarotenoids are only very weak

FIGURE 3: β-apo-13-carotenone is a potent antagonist of retinoic acid receptor-mediated induction of reporter gene expression and blocks all-*trans* retinoic acid (ATRA) induction of endogenous gene expression. A. Dose response curves for transactivation of RARγ (**upper panel**) by ATRA in the absence (**green**) or presence of 1 nm (**orange**), 10 nm (**yellow**), or 100 nm (**blue**) β-apo-13-carotenone (C13 ketone). **B**. Induction of expression of mRNAs for RARβ (**left lower panel**) or cytochrome P450, 26A1 (CYP26A1) (right lower panel) by 10 nm ATRA treatment alone or by co-treatment with ATRA and the test compounds at 10 nm, including β-carotene (BC), β-ionylideneacetic acid (BIAA), β-apo-14'-carotenal (14'-AL), β-apo-14'-carotenoic acid (14'-CA), and β-apo-13-carotenone (C13 ketone). mRNA levels were quantified by RT-PCR and are shown as the fold induction compared with vehicle-treated cells (n = 3); mean ± SD.





Adapted from Eroglu et al[39]

agonists. However, there is accumulating evidence that some of the β-apocarotenoids are retinoid receptor antagonists. Thus, Ziouzenkova et al[40] describe RXRα activation in the presence of a synthetic agonist of RXR and β-apo-8'-, 12'- and 14'- carotenals in human bovine cells. Only β-apo-14'-carotenal effectively inhibited agonist-induced RXRα activation with an inhibition constant (Ki) of 500 nM. In addition, RXR partner nuclear receptors were tested including PPARα, PPARβ/δ, and PPARγ. It was found that β-apo-14'-carotenal decreased agonist-induced PPARα and PPARγ activation very effectively, and PPARβ/δ modestly. Most of the experiments in this study were conducted with 1–10 μM β-apo-14'-carotenal.

The possibility that the β-apocarotenoids might function as antagonists rather than agonists of nuclear receptors has been supported by the results of recent studies in the author's laboratory, some of which have been previously reviewed in *Sight and Life* magazine.[41] In one study, we investigated the effects of β-apocarotenoids on RXRα signaling.[42] Transactivation assays were performed to test whether β-apocarotenoids activate or antagonize RXRα. None of the β-apocarotenoids tested activated RXRα. Among the compounds tested, β-apo-13-carotenone was found to antagonize the activation of RXRα by 9-*cis*-RA and was effective at concentrations as low as 1 nM. β-Apo-14'-carotenal and β-apo-14'-carotenoic acid were also found to be antagonists of RXRα, but with less potency than β-apo-13-carotenone. Molecular modeling studies revealed that β-apo-13-carotenone makes molecular interactions like an antagonist of RXRα.

In another study,[39] we found that β-apo-14'-carotenal, β-apo-14'-carotenoic acid, and β-apo-13-carotenone antagonized retinoic acid induced transactivation of all three of the retinoic acid receptors (RARs) and were effective at nanomolar concentrations. These compounds compete directly with retinoic acid for high affinity binding to purified receptors. The binding affinity for β-apo-13-carotenone is 4–5 nM, close to that of retinoic acid itself, while that of β-apo-14'-carotenal and β-apo-14'carotenoic acid is 5–10 times lower. Molecular modeling studies confirmed that β-apo-13-carotenone can directly interact with the ligand binding site of the retinoid receptors. As shown in **Figure 3** β-apo-13-carotenone and the β-apo-14'-carotenoids inhibited both RARγ-mediated reporter gene activity and retinoic acid-induced expression of retinoid responsive genes in HepG2 cells. These findings have important implications, as they suggest that β-apocarotenoids may function as naturally occurring retinoid antagonists.

"β-Apocarotenoids may function as naturally occurring retinoid antagonists"

Concluding remarks

The enzymatic conversion of dietary β-carotene to biologically active forms of vitamin A has been studied since the early 20th century. Starting with the pioneering work of Jim Olson and DeWitt Goodman in 1965, we now appreciate that the major pathway for the conversion of dietary β-carotene to vitamin A involves BCO1. The enzyme catalyzes the central cleavage of provitamin A carotenoids at the 15,15' double bond to yield two molecules of retinal (vitamin A aldehyde). It also cleaves each of the β-apo-carotenals (viz. β-apo-8', -10', -12', and -14'-carotenal) to generate retinal directly. This explains the vitamin A activity observed on feeding these β-apocarotenals. The enzyme functions as a dioxygenase, as speculated early on by both Olson and Goodman, but this mechanism was only recently rigorously established.

Although work on the basic enzymology of BCO1 may seem esoteric, understanding the structure, mechanism, and substrate specificity of the enzyme actually has relevance to public health. Thus, it is now appreciated that various SNPs (single nucleotide polymorphisms) in the BCO1 gene lead to different efficiencies in the conversion of provitamin A carotenoids to vitamin A. Indeed, genetic variation in the enzyme may partly explain the differences among humans in the efficiency with which they can convert provitamin A carotenoids to vitamin A and thus their susceptibility to vitamin A deficiency.

"Understanding BCO1 has relevance to public health"

We now also have a much fuller appreciation of the pathways of eccentric cleavage of β-carotene to the other β-apocarotenals (other than retinal) than when Olson and others tackled this problem in the last half of the 20th century. The seminal work of Johannes von Lintig and colleagues in identifying and characterizing BCO2 has led to a much greater, but more complex, picture of the role of metabolites of dietary carotenoids in metabolism and physiology. Thus it is now clear that, while BCO2 is not a major player in the generation of vitamin A, the enzyme plays important roles in the metabolism of non-provitamin-A carotenoids, such as the xanthophylls, lutein and zeaxanthin. It also appears to play even wider roles in lipid metabolism and mitochondrial function.

Finally, the demonstration of the occurrence of β-apocarotenoids in the diet and the fact that some of these metabolites have potent biological activities on their own leads us to a greater appreciation of the richness and complexity of the many roles that dietary carotenoids play in human health and disease. Jim Olson would be very pleased, because he always knew this.

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