Carotenoids
Volume 5: Nutrition and Health

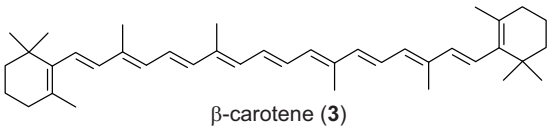
Chapter 18

Biological Activities of Carotenoid Metabolites

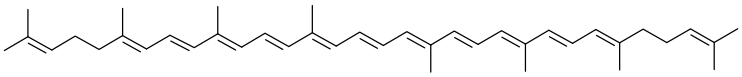
Xiang-Dong Wang

A. Introduction

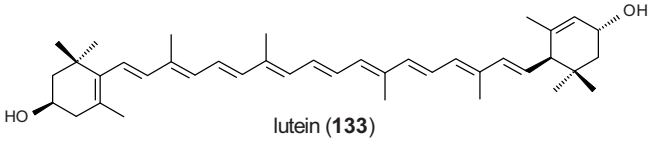
Considerable research effort has been expended in an attempt to substantiate and understand the potential roles of carotenoids in human health and disease, as described in previous *Chapters* in this *Volume*. Early studies dealt with β-carotene (**3**) and other provitamin A carotenoids, but more recent research efforts have focused on the potential roles in health and disease of the non-provitamin A carotenoids, such as lycopene (**31**) and lutein (**133**).



β-carotene (**3**)

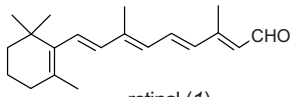


lycopene (**31**)

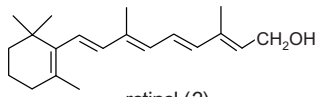


lutein (**133**)

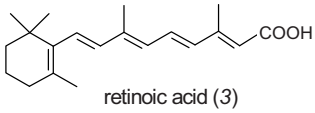
Carotenoids are lipophilic and the series of conjugated double bonds in the central chain of the molecule makes them susceptible to oxidative cleavage [1], to isomerization between the *trans* (*E*) and *cis* (*Z*) forms [2], and to the formation of potentially bioactive metabolites [3]. The best known metabolite of carotenoids is vitamin A, as retinal (*1*), retinol (*2*) and retinoic acid (*3*). In recent years, considerable efforts have been made to identify biological properties of carotenoid metabolites other than vitamin A and related retinoids. Better understanding of the molecular details behind the actions of these carotenoid oxidative metabolites may yield insights into both physiological and pathophysiological processes in human health and disease.



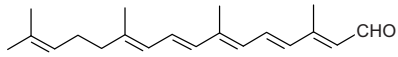
retinal (*1*)



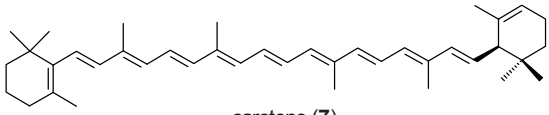
retinol (*2*)



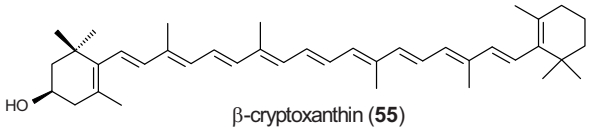
retinoic acid (*3*)



acycloretinal (*4*)



α-carotene (**7**)



β-cryptoxanthin (**55**)

For provitamin A carotenoids, such as β-carotene, α-carotene (**7**), and β-cryptoxanthin (**55**), central cleavage is a major pathway leading to vitamin A and its derivatives [4,5] (see *Chapter 8* and *Volume 4, Chapter 16*). This pathway has been substantiated by the cloning of a central cleavage enzyme, β-carotene 15,15'-oxygenase (BCO1), which can cleave carotenoids at their C(15,15′) double bond. It has been well demonstrated that retinoids, the most important oxidative products of provitamin A carotenoids, play an essential role in many critical biological processes, including vision, reproduction, metabolism, differentiation, haematopoiesis, bone development, and pattern formation during embryogenesis [6]. Considerable evidence demonstrates that the natural and synthetic retinoids may be effective in the prevention and treatment of a variety of human chronic diseases, including cancer [7]. Retinoids elicit these responses through their ability to regulate gene expression at specific target sites within the body [8,9].

An alternative pathway for carotenoid metabolism in mammals, the excentric cleavage pathway, was confirmed by the molecular identification of β-carotene 9,10-oxygenase (BCO2) in humans and animals. Recent biochemical characterization of BCO2 demonstrates that this enzyme catalyses the excentric cleavage not only of provitamin A carotenoids, but also of non-provitamin A carotenoids, such as lycopene. Recent experimental data suggest that carotenoid metabolites from the excentric cleavage pathway may have more important biological roles than their parent compounds. These metabolites may have specific actions on several important cellular signalling pathways and molecular targets, and may have both beneficial and detrimental effects in relation to cancer prevention [3,10,11]. The ability of carotenoids to modulate cell communication and signalling pathways, especially in relation to the cell cycle and apoptosis, is described in *Chapter 11*. This *Chapter* now discusses recent findings on the formation of metabolites of carotenoids, in particular β-carotene and lycopene, and addresses the question of whether the reported biological actions of carotenoids and their potential significance in chronic diseases such as cancer are in fact mediated by metabolites and not by the intact carotenoids themselves.

B. Carotenoid Metabolites

1. Enzymic central cleavage *in vitro*

a) β-Carotene 15,15′-oxygenase (BCO1)

As described in detail in *Chapter 8* and *Volume 4, Chapter 16*, carotenoids such as β-carotene, α-carotene, and β-cryptoxanthin are cleaved symmetrically at their central double bond by BCO1 [12,13]. This enzyme has been cloned in several species and its biochemical and enzymological characterization has been reported [14-18]. It has been detected in or isolated from several mouse and human tissues (*e.g.* liver, kidney, intestinal tract, and testis) which are important in carotenoid/retinoid metabolism. A purified recombinant BCO1, obtained *via* a human liver cDNA library, showed cleavage activity towards both β-carotene and β-cryptoxanthin, which has only one unsubstituted β ring [18], but with an approximately 4-fold lower affinity towards β-cryptoxanthin (K_m = 30.0 ± 3.8 μM) than towards β-carotene (K_m = 7.1 ± 1.8 μM) [18]. No cleavage of lycopene or zeaxanthin was detected. In other studies, no detectable activity of human retinal pigment epithelium BCO1 towards lycopene or lutein was observed [19]. No lycopene cleavage products were detected when lycopene was incubated with the *Drosophila* homologue of BCO1 [14] or with crude preparations of rat liver and intestine [20]. The presence of an unsubstituted β ring in the substrate appears to be a prerequisite for activity [21].

b) Central cleavage of lycopene

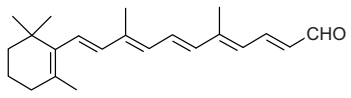
Indirect evidence for central cleavage of lycopene has been obtained, however. When a lycopene-accumulating strain of *Escherichia coli* was engineered to express also mouse BCO1, a distinct bleaching of colour was seen following induction, suggesting cleavage of lycopene [17]. In addition, purified recombinant mouse BCO1 was shown to display cleavage activity towards lycopene, but the expected central cleavage product acycloretinal (*4*) was only detected when the lycopene concentrations used were 2.5-3 times higher than the observed K_m (6 μM) for β-carotene. Taken together, these studies suggest that lycopene is, at best, a poor substrate for BCO1.

It is unclear if the lycopene substrate used in the BCO1 studies *in vitro* described above [17-20] was the pure all-*E* form or contained *Z* isomers. As reported in the following Section, *Z* isomers of lycopene were better substrates than (all-*E*)-lycopene for BCO2 [22]. This raises the important question of whether BCO1 might also cleave *Z* isomers of lycopene to acycloretinoids. Reports on the use of (all-*E*)-lycopene as a supplement revealed dramatic increases in the 5*Z*, 9*Z* and 13*Z* isomers in blood and tissues [23-26].

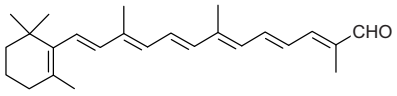
2. Excentric enzymic cleavage *in vitro*

a) β-Carotene 9,10-oxygenase (BCO2)

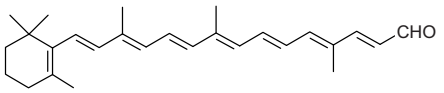
An alternative pathway for carotenoid metabolism in vertebrates is asymmetric cleavage at one of the other double bonds of the polyene chain, *i.e.* excentric cleavage [27-29]. The existence of this pathway was for a long time controversial [4,30,31], but has been substantiated by the identification of a series of homologous carbonyl cleavage products, including 14′-apo-β-caroten-14′-al (**513**), 12′-apo-β-caroten-12′-al (**507**), 10′-apo-β-caroten-10′-al (**499**), 8′-apo-β-caroten-8′-al (**482**), and 13-apo-β-caroten-13-one (*5*), along with retinoic acid, in tissue homogenates of humans, ferrets, and rats [32-35].



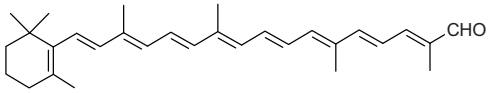
14'-apo-β-caroten-14'-al (**513**)



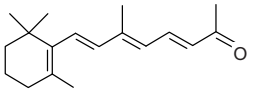
12'-apo-β-caroten-12'-al (**507**)



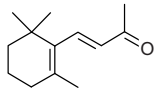
10'-apo-β-caroten-10'-al (**499**)



8'-apo-β-caroten-8'-al (**482**)



13-apo-β-caroten-13-one (*5*)



β-ionone (*6*)

A second cleavage enzyme, BCO2, has been cloned from mice, humans, and zebrafish [36]. BCO2 appears to be specific for the C(9,10) double bond; β-carotene, for example, gives rise to 10′-apo-β-caroten-10′-al (**499**) and β-ionone (*6*) [36]. Apo-β-carotenals can be precursors of vitamin A *in vitro* [28,37] and *in vivo* [38], by further cleavage. They can also be oxidized to their corresponding apo-β-carotenoic acids, which may then undergo a process similar to β-oxidation of fatty acids, to produce retinoic acid [35]. It is not known, however, whether other apo-β-carotenals with shorter carbon chain lengths are formed by further metabolism of the initial cleavage product, 10′-apo-β-caroten-10′-al, or are primary products of direct cleavage of other double bonds in the carotene molecule. Not much is known about the ability of BCO2 to cleave carotenoids other than β-carotene.

b) Excentric cleavage of lycopene

Ability to cleave lycopene was first demonstrated indirectly with strains of *Escherichia coli* engineered to synthesize and accumulate lycopene, and expressing the mouse BCO2 [36]. When BCO2 was induced, a distinct colour shift from red to white occurred, indicating cleavage. Following this, the ferret BCO2 gene has been cloned and characterized [22]; ferrets (*Mustela putorius furo*) and humans are similar in terms of carotenoid absorption, tissue distribution and concentrations, and metabolism [39,40]. The enzyme is expressed in the testis, liver, lung, prostate, intestine, stomach, and kidneys of ferrets, similar to the expression pattern of human BCO2 [41].

The recombinant ferret BCO2 catalysed the excentric cleavage of the C(9,10) double bond of (all-*E*)-β-carotene but not that of (all-*E*)-lycopene, though *Z* isomers of lycopene were cleaved effectively [22]. Based on the BCO2 expressed in Sf9 cells from the insect *Spodoptera frugiperda*, a K_m of 3.5 μM was estimated for (all-*E*)-β-carotene, but the kinetic

constants for lycopene could not be calculated because of the difficulty in controlling auto-isomerization, so that mixed isomers of lycopene had to be used as the substrate. Because the lycopene substrate mixture contained only ~20% as *Z* isomers, and the ferret BCO2 would not cleave (all-*E*)-lycopene, it can be speculated that the K_m for (*Z*)-lycopene is actually much lower than that of the lycopene isomer mixture. This indicates that (*Z*)-lycopene might be a better substrate than (all-*E*)-β-carotene for the ferret BCO2. It is not known why ferret BCO2 preferentially cleaves the 5*Z* and 13*Z* isomers of lycopene into 10′-apolycopenal. It has been suggested that the structure of the *Z* isomers of lycopene could mimic the ring structure of the β-carotene molecule and fit into the substrate-enzyme binding pocket. The different solubility properties may be a key factor, however; the *Z* isomers are more readily solubilized and much less prone to aggregation and crystallization than is (all-*E*)-lycopene (see *Volume 4, Chapter 5*). The observation that supplementation with (all-*E*)-lycopene results in a significant increase in the tissue concentration of (*Z*)-lycopene in animals and humans supports this [23-26].

3. Non-enzymic oxidative breakdown

The non-enzymic formation of carotenoid oxidation products *in vitro* is well known (see *Chapter 12* and *Volume 4, Chapter 7*). Because of the susceptibility of carotenoids to cleavage by auto-oxidation, radical-mediated oxidation, and singlet oxygen, such breakdown products may be formed *in vivo* by non-enzymic processes if the tissues are exposed to oxidative stress such as smoking and drinking. The possible biological importance of such processes and products is poorly understood.

4. Detection of central and excentric cleavage products *in vivo*

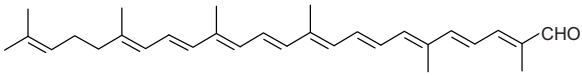
a) Metabolites of β-carotene

Retinol, retinal, retinoic acid and retinyl ester can be detected in both plasma and tissues of animals and humans. Although the conversion of β-carotene by BCO2 to other apocarotenoids remains to be determined directly, a recent study [42], suggests that excentric cleavage of ingested β-carotene does occur in humans *in vivo*. Application of the highly sensitive technique accelerator mass spectrometry, that can measure attomole amounts (1 in 10^{-18} parts) of ^{14}C, enabled the detection in human plasma of [^{14}C]-apo-β-caroten-8′-al and several other, unidentified [^{14}C]-labelled metabolites from a true tracer oral dose of (all-*E*)-[10,11,10′,11′-$^{14}C_4$]-β-carotene (1.01 nmol; 543 ng; 100 nCi) in human plasma. Although further study is needed to identify and characterize the additional metabolites, this observation is in agreement with the previous identification of excentric cleavage metabolites in animal models. Significant amounts of 8′-apo-β-caroten-8′-al (**482**), 10′-apo-β-caroten-10′-al (**499**) and 12′-apo-β-caroten-12′-al (**507**) were isolated from the intestines of chickens given dietary

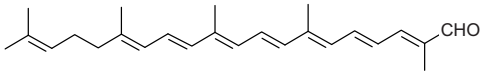
β-carotene [28,29]. Also, 12′-apo-β-caroten-12′-al and 10′-apo-β-caroten-10′-al, as well as retinoids, were isolated from ferret intestinal mucosa after perfusion of β-carotene *in vivo* [43,44].

b) Metabolites of lycopene

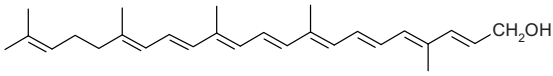
Labelled 8′-apolycopen-8′-al (**491**) and 12′-apolycopen-12′-al (*7*) were detected in rat liver 24 hours after dosing with [^{14}C]-lycopene [45]. A large quantity of unidentified polar short-chain compounds was also detected. 10′-Apolycopen-10′-ol (**504.3**) has been detected, together with several unidentified compounds, in the HPLC profiles of lung tissue from ferrets supplemented with lycopene for 9 weeks [22]; this compound is the reduction product of the predicted aldehyde cleavage product 10′-apolycopen-10′-al (*8*). Neither the latter nor 10′-apolycopen-10′-oic acid (**504.4**) was detected, so it is likely that 10′-apolycopen-10′-al is a short-lived intermediate compound which, as soon as it is formed, is rapidly reduced to its alcohol form *in vivo*.



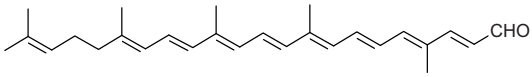
8'-apolycopen-8'-al (**491**)



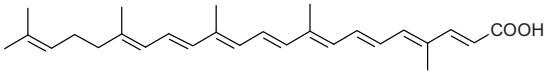
12'-apolycopen-12'-al (*7*)



10'-apolycopen-10'-ol (**504.3**)



10'-apolycopen-10'-al (*8*)



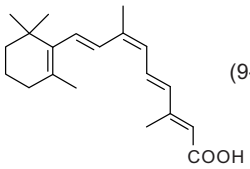
10'-apolycopen-10'-oic acid (**504.4**)

If 10′-apolycopen-10′-oic acid were present, its concentration was too low to be detected. It was demonstrated subsequently that incubation of 10′-apolycopen-10′-al with the post-nuclear fraction of hepatic tissue of ferrets resulted in the formation of either 10′-apolycopen-10′-ol or 10′-apolycopen-10′-oic acid, depending on the presence of either NAD^+ or NADH, respectively. Nonetheless, the presence of specific metabolites has not been consistent across different animal models.

C. Retinoids and the Retinoid Signalling Pathway

1. Retinoic acid and retinoic acid receptors

Provitamin A carotenoids, such as β-carotene and its excentric cleavage metabolites, can serve as direct precursors for (all-*trans*)-retinoic acid (*3*) and (9-*cis*)-retinoic acid (*9*) [35,46,47], which are ligands for retinoic acid receptors (RAR) and retinoid X receptors (RXR), respectively.



(9-*cis*)-retinoic acid (*9*)

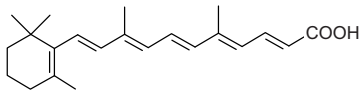
Retinoid receptors function as ligand-dependent transcription factors and regulate gene expression by binding as dimeric complexes to the retinoic acid response element (RARE) and the retinoid X response element (RXRE), which are located in the 5′ promoter region of responsive genes. RXR can form dimeric complexes not only with RAR but also with other members of the nuclear hormone receptor superfamily, such as thyroid hormone receptors (TR), the vitamin D receptor (VDR), peroxisome proliferator-activated receptors (PPAR), and possibly other receptors with unknown ligands, designated orphan receptors. Recent results have shown that decreased expression of all RAR and RXR receptor subtypes is a frequent event in non-small cell lung cancer [48]. Particularly, studies *in vivo* and *in vitro* indicate that RARβ expression, which can be induced by retinoic acid, is frequently reduced in various cancer cells and tissues [49]. Recent evidence also suggests that the RARβ subtypes, RARβ2 and RARβ4, have contrasting biological effects, as tumour suppressor and tumour promoter, respectively, in human carcinogenesis [50]. The down-regulation of all retinoid subclasses suggests a fundamental disruption of the regulation of the retinoid pathway in lung cancer [48]. Conversely, restoration of RARβ2 in an RARβ-negative lung cancer cell line has been reported to inhibit tumorigenicity in nude mice [51]. Retinoic acid can reverse the suppression of RARβ protein caused by benzo(a)pyrene diol epoxide by increasing transcription of RARβ,

in immortalized oesophageal epithelial cells [52] and lung cancer cells [53]. In a small-scale human trial, daily treatment with (9-*cis*)-retinoic acid for three months restored RARβ expression in the bronchial epithelium of former smokers [54]. Supplementing carcinogen-initiated AJ mice with (9-*cis*)-retinoic acid decreased lung tumour multiplicity and increased lung RARβ mRNA levels [55]. It has been shown that β-carotene supplementation prevents skin carcinoma formation by upregulating RARβ [56].

2. Effects of provitamin A carotenoids and their metabolites

a) β-Carotene and 14′-apo-β-caroten-14′-oic acid

Previously it was observed that the down-regulation of RARβ by smoke-borne carcinogens was completely reversed by treatment with either β-carotene or its oxidative metabolite, 14′-apo-β-caroten-14′-oic acid (*10*), in normal bronchial epithelium cells [57]. Further, transactivation of the RARβ2 promoter appeared to occur mainly as a result of the metabolism of 14′-apo-β-caroten-14′-oic acid to (all-*trans*)-retinoic acid [57]. Therefore, the molecular mode of action of provitamin A carotenoids can be mediated by retinoic acid, *via* transcriptional activation of a series of genes with distinct antiproliferative or proapoptotic activity, thereby eliminating cells with irreparable alterations in the genome, or killing neoplastic cells.



14'-apo-β-caroten-14'-oic acid (*10*)

It has been reported recently, however, that 14′-apo-β-caroten-14′-al, in contrast to 14′-apo-β-caroten-14′-oic acid, inhibited activation and responses of the nuclear receptors PPARγ, PPARα, or RXR, and promoted inflammation *in vivo* [58,59]. Although the question of whether this proinflammatory effect of 14′-apo-β-caroten-14′-al was related to dose was not addressed, this finding may help to explain the detrimental effect of β-carotene supplementation trials in smokers.

The basis of one explanation for this lies in the doses used and the free-radical-rich atmosphere in lungs of cigarette smokers [60-62]. This environment alters β-carotene metabolism and produces undesirable oxidative metabolites [62], which can affect many processes, *e.g.* they can facilitate the binding of metabolites of benzo(a)pyrene to DNA [63], down-regulate RARβ [61], up-regulate activator protein 1 (AP-1, c-Jun and c-Fos) activity [60], induce carcinogen-activating enzymes [64], enhance the induction of BALB/c 3T3 cell transformation by benzo(a)pyrene [65], inhibit gap junction communication in A549 lung

cancer cells [66] or impair mitochondrial functions [67]. The doses of β-carotene used in the ATBC (Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study) and CARET (Beta-Carotene and Retinol Efficacy Trial) studies were 20 to 30 mg per day, for 2-8 years, and these doses are 10-15 times the average daily dietary intake of β-carotene in the U. S. Such a pharmacological dose of β-carotene in humans could result in the accumulation of relatively high levels of β-carotene and its oxidative metabolites in the lung tissue, especially after long periods of supplementation. Potentially this could also lead to a decrease in lung retinoic acid concentration *via* induction of cytochrome P450 (CYP) enzymes [68]. It should be noted that excentric cleavage products, which may be formed in excess in cancerous lung tissue, have not been shown to bind competitively to RARβ at physiologically relevant levels [69]. It is possible, however, that the excentric cleavage products of carotenoids interfere with the binding of retinoic acid to its receptors when the retinoic acid level in tissues is low. This may be seen in the case of cigarette smoking and excessive alcohol drinking, which result in higher cytochrome P450 enzyme levels and breakdown of retinoic acid [68,70,71]. The loss of or low levels of retinoic acid, including both all-*trans* and 9-*cis* isomers, or the 'functional' down-regulation of retinoid receptors, because of the lack of retinoic acid, could interfere with retinoid signal transduction and result in enhanced cell proliferation and potentially malignant transformation. This is supported by previous studies with ferrets, showing that high dose β-carotene supplementation (equivalent to an intake of 30 mg of β-carotene/day/70 kg human, considered a pharmacological dose) and/or cigarette smoke exposure decreased levels of retinoic acid and RARβ protein, but increased levels of c-Jun and cyclin D1 proteins, and induced precancerous lesions in lung tissue [60,72].

Recently, further evidence was obtained to support the notion that the anti- or procarcinogenic response to β-carotene supplementation reported in human intervention trials and in animal studies may be related to the stability of β-carotene and its metabolites in different organ environments (such as high oxidative stress in the lung due to smoking or low antioxidants levels) as well as retinoic acid status in the lungs. A mixture of β-carotene (equivalent to 12 mg/day in human) together with the antioxidants α-tocopherol and ascorbic acid (which facilitates both recycling and stability of β-carotene and α-tocopherol, but was not used in the ATBC study and is expected to be low in this population of heavy smokers), provides protection against lung cancer risk by maintaining normal levels of retinoic acid [73]. This is in agreement with a previous study *in vitro* which showed that the addition of both ascorbic acid and α-tocopherol to an incubation mixture of β-carotene with ferret lung tissue can inhibit the smoke-enhanced production of excentric cleavage metabolites of β-carotene, increase the formation of retinal and retinoic acid [74] and decrease the smoke-induced catabolism of retinoic acid [68]. These studies and the known biochemical interactions of β-carotene, vitamin E and vitamin C (see *Chapter 12*) suggest that this combination of nutrients, rather than the individual agents, could be an effective chemopreventive strategy against lung cancer in smokers.

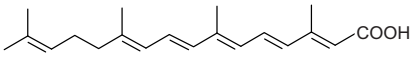
b) Other provitamin A carotenoids

Protective actions of other provitamin A carotenoids, namely β-cryptoxanthin and α-carotene, have been reported recently. Mechanisms for any effects of these carotenoids and their metabolites on the retinoid signalling pathway have not been elucidated, although their interactions with cleavage enzymes, depending on dose and the oxidative environment of the lungs, may be similar to those of β-carotene. In a recent cell culture study, it was observed that β-cryptoxanthin can inhibit lung cancer cell growth by increasing the expression of RARβ and transactivating RARE [75]. Another study [76] demonstrated that, in a yeast two-hybrid system, both β-cryptoxanthin and lutein exhibited RAR ligand activity but this was completely abolished by the RAR pan-antagonist LE540. Although their binding affinity was three orders of magnitude lower than that of (all-*trans*)-retinoic acid, β-cryptoxanthin and lutein were shown to bind to the RAR ligand-binding domain in the CoA-BAP system but not to the RXR ligand-binding domain, indicating that they can serve as ligands for RAR without being metabolized.

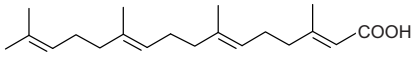
3. Effects of lycopene and its metabolites

Whereas up-regulation of retinoid receptor expression and function by provitamin A carotenoids may play a role in mediating the growth inhibitory effects of retinoids in cancer cells [57,75], it is not clear if non-provitamin A carotenoids and their metabolites may function in a similar fashion.

a) Acycloretinoic acid



acycloretinoic acid (*11*)



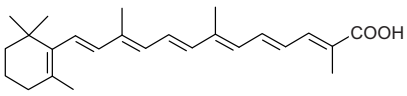
geranylgeranoic acid (*12*)

Several reports have evaluated the ability of the 'acycloretinoid' acycloretinoic acid (*11*), which would be a product of central cleavage of lycopene, to transactivate the RARE. It was demonstrated [77] that acycloretinoic acid can transactivate a RARE-reporter gene through an interaction with RARα. The potency of activation was approximately 100-fold lower than with retinoic acid, however. Binding affinity studies indicated that acycloretinoic acid had no appreciable binding affinity for RXRα, but bound RARα with an equilibrium dissociation constant in the range of 50-150 nM, two orders of magnitude lower than that of (all-*trans*)-retinoic acid. Intact lycopene did not show any significant binding to either receptor, but administration of lycopene led to a weak transactivation of the RARE-reporter gene [77]. Similar findings were reported for the RARβ2 promoter. Only when acycloretinoic acid was

provided at concentrations 500-fold higher than retinoic acid was an effect on luciferase and β-galactosidase reporter activity observed [78]. At the concentrations used in this study there was no effect of intact lycopene on reporter transactivation. In another study, acycloretinoic acid was found to have no significant effect on transactivation of RAR and RXR reporter systems [79]. Whereas no effect of acycloretinoic acid on retinoid signalling *in vivo* has been substantiated, a synthetic acyclic retinoid, E-5166 (geranylgeranoic acid, *12*), has been shown to transactivate retinoid reporter systems, and to have potential benefits in treatment of hepatocellular carcinoma [80-82].

b) Other lycopene metabolites

The question arises of whether 10′-apolycopen-10′-oic acid could also be an activator of RARs. Three cell lines, which represent different stages of lung carcinogenesis, namely NHBE, a normal human bronchial epithelial cell line, BEAS-2B, an immortalized human bronchial epithelial cell line, and A549 cells, a non-small cell lung cancer cell, were incubated with increasing concentrations of 10′-apolycopen-10′-oic acid (3-5 μmol/L) [83]. After 48 hours, a dose-dependent increase in RARβ mRNA expression was observed in both NHBE and BEAS-2B cell lines. The effect of 10′-apolycopen-10′-oic acid was similar to that of (all-*trans*)-retinoic acid. Was the increased RARβ mRNA expression due to increased transactivation of the RARβ promoter region? To investigate the involvement of the RARE in the promoter, located between -53 and -37 bp, site-directed mutagenesis was utilized to abolish the RAR binding site. This mutation completely abolished induction of promoter activity by both retinoic acid and 10′-apolycopen-10′-oic acid. These results suggest that the growth inhibitory actions of 10′-apolycopen-10′-oic acid may be mediated through retinoid signalling. On the other hand, other studies show that 12′-apo-β-caroten-12′-oic acid (**510**) can inhibit the growth of HL-60 cells [84] and 14′-apo-β-caroten-14′-oic acid (*10*) can stimulate the differentiation of U937 leukaemia cells [85] and inhibit the growth of breast cancer cells [69].

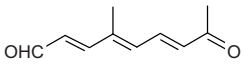


12'-apo-β-caroten-12'-oic acid (**510**)

These effects appear not to be due to cellular conversion of the apo-β-carotenoid to retinoic acid because no retinoids were detected in the cells after treatment with the apocarotenoids. It is possible, therefore, that breakdown products of β-carotene may play a role in regulating cell function which does not depend on their ability to be metabolized to retinoic acid. This is also supported by the finding that apocarotenoids have very low binding affinity to RAR [69]. Although 14′-apo-β-caroten-14′-oic acid can induce transcriptional activity of the RARβ2

promoter *via* its conversion into retinoic acid in the normal bronchial epithelial cells [57], it is possible that the conversion of β-carotene into retinoic acid is impaired in transformed cells.

This retinoid-independent activity of provitamin A carotenoid metabolites may be similar to the biological activity of non-provitamin A carotenoid metabolites. It has been shown that acycloretinoic acid, which is not a ligand for RAR and RXR [78], inhibited the growth of HL-60 human promyelocytic leukaemia cells [86], human mammary cancer cells [77], and human prostate cancer cells and this effect was significantly greater than those of (9-*cis*)-retinoic acid and (all-*trans*)-retinoic acid [87]. In addition, it has been shown that lycopene oxidation products enhance gap junctional communication [88] (see also Section **D**.4). A retinoic acid receptor antagonist did not suppress reporter activity induced by lycopene, indicating that gene activation by retinoids and by non-provitamin A carotenoids occurs by different mechanisms [89].



MON (*13*)

An oxidative product of lycopene, (*E*,*E*,*E*)-4-methyl-8-oxo-2,4,6-nonatrienal (MON, *13*), that induced apoptosis in HL-60 cells, was identified [90]. A dose-dependent decrease of cell viability was observed, with a concomitant increase in chromatin condensation and nuclear fragmentation, characteristic of apoptosis.

In spite of these observations with cell cultures, however, the physiological significance of these lycopene products remains unknown because none of them has been detected in biological systems.

c) Retinoid-dependent and retinoid-independent roles of carotenoid metabolites

Beyond participating in known retinoid signalling pathways, carotenoid metabolites appear to have retinoid-independent roles, signalling through other nuclear receptors (*e.g.* currently characterized orphan receptors with unknown ligands) or interacting with signalling pathways through transcriptional 'cross-talk'. Since RXRs function not only as heterodimeric partners of other nuclear receptors (*e.g.* VDR, PPAR), but also as active transducers of tumour suppressive signals [7], it will be interesting to investigate whether the biological activity of carotenoids or their metabolites is mediated through interaction with RARs, RXRs, PPAR, VDR or other orphan receptors. Recently, supplementation with (9-*cis*)-retinoic acid in combination with 1α,25-dihydroxyvitamin D3 was shown to reduce vitamin D-induced toxicity symptoms compared to those in mice that were supplemented with vitamin D alone, thereby suggesting an interaction between the two compounds [91]. In addition, carotenoids may be beneficial for bone formation by up-regulating vitamin D receptor levels [92]. It has been shown that both the PPARγ ligand ciglitazone and an RXR ligand cooperatively

promoted transcriptional activity of RAREβ and induced RARβ expression in human lung cancer cells [57].

D. Effects of Carotenoid Metabolites on Other Signalling and Communication Pathways

1. Nuclear factor-E2 related factor 2 (Nrf2) signalling pathway

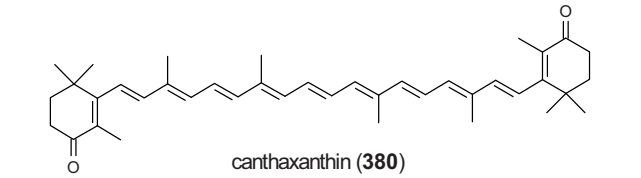
a) Phase II enzymes and antioxidant-response elements

In recent years, evidence has begun to accumulate indicating that some beneficial effects of carotenoids may be due to induction of the phase II enzymes that have important detoxifing and antioxidant properties in combating foreign substances (xenobiotics) including potential carcinogens [93]. Induction of phase II enzymes is mediated through *cis*-regulatory DNA sequences known as antioxidant response elements (ARE) that are located in the promoter or enhancer region of the gene [94]. The major ARE transcription factor Nrf2 (nuclear factor E2-related factor 2) is a primary agent in induction of antioxidant and detoxifying enzymes [95] and is essential for the induction of several phase II enzymes, including glutathione S-transferases (GSTs) and NAD(P)H:quinone oxidoreductase (NQO1) [96]. The induction of these and other phase II detoxifying/antioxidant enzymes, such as haem oxygenase-1 (HO-1), glutathione reductase (GR), glutamate-cysteine ligase (catalytic subunit, GCLC, and modifier subunit, GCLM), microsomal epoxide hydrolase 1 (mEH), and the UDP glucuronosyl-transferase 1 family polypeptide A6 (UGT1A6), results in the detoxication of carcinogens and the inactivation of reactive oxygen species (ROS), thus contributing to the protective effect of chemopreventive agents [95]. Under normal conditions, most of the Nrf2 is sequestered in the cytoplasm by 'Kelch-like erythroid Cap'n'Collar homologue-associated protein 1' (Keap 1) and only residual nuclear Nrf2 binds to the ARE to drive basal activities. Exposure to some chemopreventive agents leads to the dissociation of the Nrf2-Keap1 complex in the cytoplasm and the translocation of Nrf2 into the nucleus. The nuclear accumulation of Nrf2 subsequently activates target genes of phase II enzymes [95]. Because of its critical roles in detoxication and antioxidant processes in carcinogenesis, Nrf2 has been recognized as a potential molecular target for cancer prevention [95]. It has been shown that various dietary and synthetic compounds, *e.g*. sulforaphane [97], curcumin [98], and (-)-epigallocatechin 3-gallate [99], can induce gene expression mediated by Nrf2-ARE; this could be one mechanism for their reported chemopreventive effects.

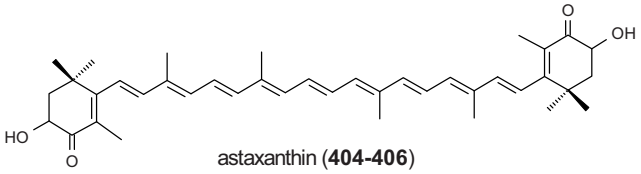
b) Effects of carotenoids and their metabolites

Not only β-carotene but some non-provitamin A carotenoids, including lycopene, have been shown to induce several phase II enzymes both *in vivo* and *in vitro* [92,100,101]. An

induction of UDP-glucuronosyltransferase and NQO1 was observed in rats fed various carotenoids [100]. Whilst canthaxanthin (**380**) and astaxanthin (**404-406**) induced phase II activity, lycopene and lutein had no effect after 15 days of feeding.



canthaxanthin (**380**)



astaxanthin (**404-406**)

In another study, a dose-dependent induction of several phase I and II enzymes was demonstrated in female Wistar rats supplemented with lycopene at doses ranging from 0.001 to 0.1 g/kg body weight for 2 weeks [101]. Hepatic ethoxyresorufin O-dealkylase (EROD) and benzyloxyresorufin O-dealkylase (BROD) activity increased approximately 2-fold and 50%, respectively, suggesting activation of the cytochrome P450 enzyme CYP1A. In addition, several liver and red blood cell phase II enzyme activities, such as GR, GST and NQO1, were significantly increased by feeding lycopene. The induction of phase II enzymes by lycopene has been reported in other animal studies [102], but it was not determined whether this induction was due to the intact carotenoid or its metabolites. This has been addressed in other studies.

c) Lycopene metabolites

An ethanolic extract containing lycopene and unidentified hydrophilic oxidative derivatives was shown to induce phase II enzymes and activate ARE-driven reporter gene activity with a similar potency to lycopene [92], but those chemically produced oxidative derivatives have not been found in mammalian tissues. Evidence has been obtained recently to show that 10′-apolycopen-10′-oic acid, derived from cleavage of lycopene, induces phase II enzyme expression *in vitro* [103]. Work with BEAS-2B human bronchial epithelial cells has shown a dose-dependent and time-dependent increase in the accumulation of nuclear Nrf2 protein, following 10′-apolycopen-10′-oic acid treatment [103]. In addition, 10′-apolycopen-10′-oic acid significantly induced mRNA expression of several phase II enzymes, including HO-1, NQO1, GST, GR,GCLC and GCLM, mEH and UGT1A6, compared to treatment with THF alone [103]. Additionally, 10′-apolycopen-10′-al, 10′-apolycopen-10′-ol and 10′-apolycopen-10′-oic acid were all effective in activating the Nrf2-mediated induction of HO-1 [103],

although the mechanisms of this remain unknown. The activation of Nrf2 is complex and is controlled through multiple regulatory mechanisms, including Keap1-mediated ubiquitination and degradation, subcellular distribution, and phosphorylation. 10′-Apolycopen-10′-al showed stronger induction of HO-1 than did 10′-apolycopen-10′-oic acid and 10′-apolycopen-10′-ol. Its aldehyde group is highly reactive, and is capable of forming Schiff bases with the amino groups of protein and of reacting with other cellular macromolecules, *e.g*. directly modifying the reactive cysteine residues in Keap1 and interrupting Keap1-mediated Nrf2 ubiquitination and degradation. It is also possible that these lycopenoids affect upstream signalling pathways, such as mitogen activated protein kinases (MAPKs), phosphoinositol 3-kinase (PI3K), epidermal growth factor receptor (EGFR) and protein kinase C (PKC), which all have been shown to play a role in the regulation of Nrf2-ARE in lung epithelial cells. Clearly, further investigation is needed.

It is known that intact lycopene can function as an antioxidant *in vitro* (see *Chapter 12*) and there is evidence that lycopene metabolites could also have antioxidant functions. Pre-treatment of BEAS-2B cells with 10′-apolycopen-10′-oic acid (3-10 μM) for 24 hours resulted in a dose-dependent inhibition of both endogenous ROS production and H_2O_2-induced oxidative damage, as measured by release of lactate dehydrogenase [103]. This decrease in ROS was comparable to that in control cells treated with the antioxidant *t*-butylhydroquinone.

Thus lycopene metabolites in general, and 10′-apolycopen-10′-oic acid in particular, may possess antioxidant activity by inducing antioxidant enzymes. It will be interesting to investigate whether metabolites of other carotenoids can induce phase II detoxifying/antioxidant enzymes.

2. Carotenoid metabolites and the mitogen-activated protein kinase pathway

Among the members of the mitogen-activated protein kinase (MAPK) family are Jun N-terminal kinase (JNK), extracellular-signal-regulated protein kinase (ERK) and p38 mitogen-activated protein kinase. They are activated by phosphorylation in response to extracellular stimuli and environmental stress and may play an important role in carcinogenesis [104,105]. JNK was shown to phosphorylate the protein c-Jun on sites Ser-63 and Ser-73 and to increase activator protein 1 (AP-1) transcription activity, thereby mediating cell proliferation and apoptosis [104,105] (see *Chapter 11*). ERK also induces c-Jun through phosphorylation and activation of the AP-1 component ATF1 at Ser-63 [106]. On the other hand, MAPK phosphatases (MKPs), a family of dual-specificity protein phosphatases, can dephosphorylate both phosphothreonine and phosphotyrosine residues to inactivate JNK, ERK and p38 both *in vitro* and *in vivo* [107,108]. It has been shown that phosphorylated-JNK, phosphorylated-ERK, and phosphorylated-p38 are preferred substrates for the isomer MKP-1 *in vivo* [107,108].

a) β-Carotene and metabolites

Previously, expression of AP-1, c-Jun and c-Fos was shown to be up-regulated in the lungs of smoke-exposed ferrets supplemented with β-carotene [61], compared to the control animals. This overexpression of AP-1 was positively associated with increased levels of cyclin D1 protein and with squamous metaplasia in the lungs of animals exposed to smoke [61]. It is conceivable that chronic excess β-carotene intake may modulate MAPK signalling and cause abnormal cell cycle regulation, and promote carcinogenesis. This hypothesis is supported by the observation that smoke exposure and/or high dose β-carotene activated the phosphorylation of JNK and p38, but significantly reduced lung MKP-1 protein levels [109]. In contrast, low dose β-carotene attenuated smoke-induced JNK phosphorylation by preventing down-regulation of MKP-1 [109]. This inhibitory effect of low dose β-carotene supplementation could be due to increased lung retinoic acid levels in smoke-exposed animals; it is known that retinoic acid can inhibit phosphorylation of MAPKs, such as JNK and ERK, by upregulation of MKP-1 [110-112].

Relatively high β-carotene supplementation (equivalent to 12 mg/day in humans) in the presence of ascorbic acid and α-tocopherol blocked smoke-induced phosphorylation of JNK and ERK completely by preventing smoke-induced reductions in retinoic acid levels in the lungs of ferrets [113]. The combined antioxidants also inhibited smoke-induced oxidative stress, assessed by Comet analysis [113]. These data may help to explain the conflicting results of the negative human β-carotene intervention trials, which used high doses of β-carotene, *versus* the positive observational epidemiological studies which showed that diets high in fruits and vegetables containing β-carotene (but at much lower concentrations than in the intervention studies and with other antioxidants present) are associated with a decreased risk for lung cancer.

b) Lycopene and metabolites

Lycopene has also been shown to inhibit JNK, p38 and ERK, and the transcription factor, nuclear factor-κB (NF-κB) [114]. 10′-Apolycopen-10′-oic acid showed dose-dependent inhibition of cell growth and induced apoptosis in human THLE-2 liver cells by stimulating the cyclin-dependent kinase inhibitor p21 and by reducing activation of JNK and cyclin D1 gene expression [115]. It is possible, therefore, that the inhibition of JNK activation by combined antioxidants, including both provitamin A and non-provitamin A carotenoids, may help to ‘rescue’ the functions of RARs; it has been reported recently that activation of JNK contributes to RAR dysfunction by phosphorylation of RARα and by inducing its degradation through the ubiquitin-proteasomal pathway [116]. It has been shown that RARα can activate the RARE of RARβ, suggesting a possible accessory role for RARα in RARβ expression [117]. Further examination of effects of provitamin A and non-provitamin A carotenoids on the stability and degradation of RARs through JNK-mediated pathways should be considered.

3. Carotenoid metabolites and the insulin-like growth factor-1 (IGF-1) pathway

It has been suggested that the signalling system involving insulin-like growth factors (IGF) may play a role in the biological action of lycopene [118,119]. IGF-1 and IGF-2 are mitogens (mitosis inducers) that play a central role in regulation of cellular proliferation, differentiation, and apoptosis [120]. By binding to membrane IGF-1 receptors, IGFs activate intracellular phosphatidylinositol 3′-kinase (PI3K)/Akt/protein kinase B and Ras/Raf/MAPK pathways, which regulate various biological processes such as cell cycle progression, survival, and transformation [121]. IGFs are sequestered in circulation by a family of binding proteins (IGFBP1 – IGFBP6), which regulate the availability of IGFs to bind to IGF receptors [121]. Disruption of normal IGF signalling, leading to hyperproliferation and survival signal expression, has been implicated in the development of several tumour types [122]. Indeed, strong positive associations have been found between plasma IGF-1 levels and risk of prostate cancer [123], breast cancer [124], and colorectal cancer [125]. Recent epidemiological studies provide supportive evidence that lycopene may have a chemopreventive effect against a broad range of epithelial cancers, particularly prostate, breast, colorectal, and lung cancer [126-129].

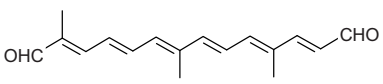
A possible mechanism was indicated when it was shown [118-120] that IGF-1-stimulated cell growth and DNA-binding activity of the AP-1 transcription factor were reduced by physiological concentrations of lycopene in endometrial, mammary (MCF-7), and lung (NCI-H226) cancer cell lines. Lycopene has been shown to inhibit IGF-1-stimulated insulin receptor substrate 1 phosphorylation and cyclin D1 expression, block IGF-1-stimulated cell-cycle progression [118,130], and increase membrane-associated IGFBPs [118,131]. Consistent with previous findings from studies *in vitro*, recent epidemiological studies demonstrated that higher dietary intake of lycopene is associated with lower circulating levels of IGF-1 [132] and higher levels of IGFBPs [133,134].

The effect of lycopene on prevention of IGF signalling in cigarette smoke-related lung carcinogenesis has been examined in the ferret model [24]. Plasma IGF-1 levels were not affected by cigarette smoke exposure or lycopene supplementation, but IGFBP-3 levels were raised by lycopene supplementation and decreased by smoke exposure. Lycopene increased plasma IGFBP-3 regardless of smoke exposure status. Increased plasma IGFBP-3 was associated with inhibition of cigarette smoke-induced lung squamous metaplasia, and with decreased levels of proliferating cell nuclear antigen (PCNA), phosphorylated Bad, and cleaved caspase 3, suggesting inhibition of cell proliferation and induction of apoptosis [24]. These results, along with others, suggest that interference with IGF-1 signalling could be an important mechanism by which lycopene may exert an anticancer action. There is recent evidence that lycopene metabolites may be partly responsible for this effect. Treatment with 10′-apolycopen-10′-oic acid (5-20 μM) resulted in a dose-dependent increase in IGFBP-3 mRNA levels in THLE-2 human liver cells, whereas similar concentrations of retinoic acid, lycopene, and acycloretinoic acid showed no significant effect [135].

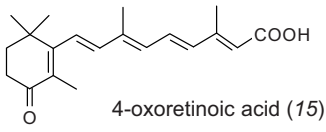
4. Carotenoid metabolites and gap-junction communication

Gap-junction communication has been implicated in the control of cell growth *via* differentiation, proliferation and apoptosis [136]. A large body of evidence now indicates loss of gap-junctional communication (GJC) to be a hallmark of carcinogenesis [137] and the targeting of the gap-junction proteins, connexins, has been suggested as a possible strategy for chemoprevention. Retinoids and carotenoids increase gap-junction communication between normal and transformed cells [90,138]. Both provitamin A and non-provitamin A carotenoids were shown to inhibit carcinogen-induced neoplastic transformation [139] and to upregulate connexin 43 (Cx43) mRNA expression [90,138]. Treatment with retinoic acid increased Cx43 expression within 6 hours, but carotenoid treatment required approximately three times longer to produce the same response [140,141]; this lag in activity is often attributed to the formation of active metabolites.

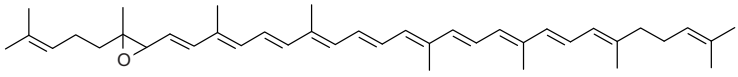
Several lines of evidence from experiments *in vitro* indicate that carotenoid oxidation products/metabolites may be responsible for increased GJC, especially in the case of lycopene. After oxidation of lycopene with hydrogen peroxide and osmium tetroxide, a product, 2,7,11-trimethyltetradecahexaene-1,14-dial (*14*), was isolated and this induced gap-junction communication as effectively as did retinoic acid. The oxidation product lycopene 5,6-epoxide (**222**), which is found in tomatoes, was shown to increase Cx43 expression in human keratinocytes [142].



2,7,11-trimethyltetradecahexaene-1,14-dial (*14*)



4-oxoretinoic acid (*15*)



lycopene 5,6-epoxide (**222**)

Acycloretinoic acid was also shown to increase GJC [78], but this effect was achieved only at high concentrations, indicating that the contribution of acycloretinoic acid to the activity of lycopene on GJC appears to be minimal. While the Cx43 promoter does not contain a RARE, it has been reported that RAR antagonists inhibited upregulation by retinoids and had no influence on the effect of carotenoids [143]. The modulating effect of oxidation products and enzymic cleavage metabolites of lycopene on GJC could, therefore, provide two separate pathways for increasing GJC. Whether 10′-apolycopenoids contribute to the activity of lycopene on GJC warrants further study, however. In addition, two decomposition products of the non-provitamin A carotenoid canthaxanthin, namely the (all-*trans*) and (13-*cis*) isomers of 4-oxoretinoic acid (*15*) have the same activity as canthaxanthin on enhancing cell-cell gap-

junctional communication in murine fibroblasts [144,145]. 4-Oxoretinoic acid has been shown to serve as a ligand of the nuclear receptor, RARβ [146]. Whether canthaxanthin can regulate gene expression *via* this metabolite remains to be determined.

E. Overview and Conclusions

An understanding of the impact of carotenoid oxidation products and bioactive metabolites is important in understanding the health effects of carotenoids. It appears that while small quantities of carotenoid metabolites can offer protection against chronic diseases and certain cancers, larger amounts may actually be harmful, especially when coupled with a highly oxidative environment (*e.g.* the lungs of a cigarette smoker or liver of an excessive alcohol drinker). The potential effects, beneficial and harmful, attributed to carotenoids and their metabolites are summarized in Fig. 1.

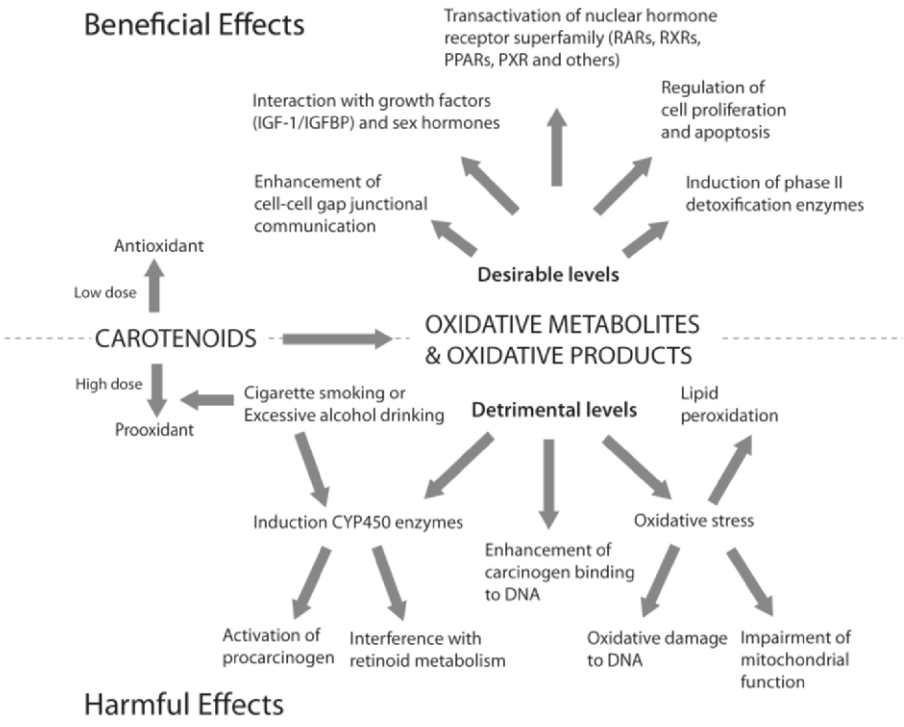


Fig. 1. Summary of biological effects of carotenoids and their metabolites and oxidation products. With low-dose treatment, carotenoids are likely to have antioxidant properties and produce small, desirable levels of metabolites, leading to beneficial effects. With high-dose treatment, carotenoids may have pro-oxidant properties, especially in smokers. The higher levels of oxidative products may be detrimental and lead to harmful effects. (Adapted from [4]).

Various effects of carotenoids on cellular functions and signalling pathways have been reported, as summarized in Fig. 2. An important question that remains unanswered is whether these effects are a result of the direct actions of intact carotenoids or of their derivatives, for example products of central or excentric cleavage of provitamin A and non-provitamin A carotenoids. Whilst evidence is presented in this *Chapter* to support the latter, more research is needed to identify and characterize additional carotenoid metabolites and breakdown products, and their biological activities; this could provide invaluable insights into the mechanisms underlying the actions of carotenoids.

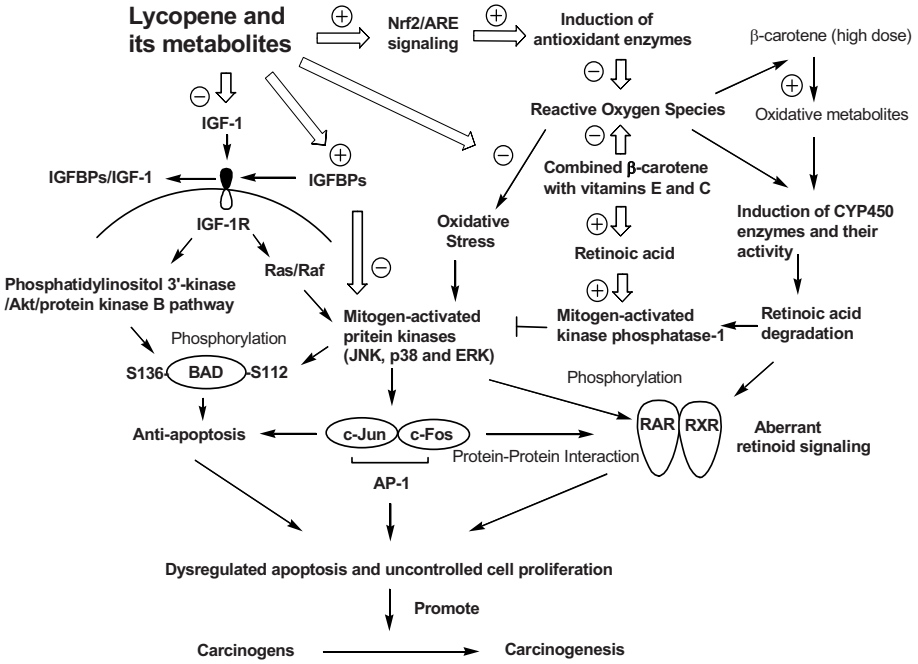


Fig. 2. Diagram illustrating the complex interactions between signalling pathways, especially those that result in impaired regulation of apoptosis and uncontrolled cell proliferation, leading to carcinogenesis. Interactions of carotenoids and their metabolites with these processes are indicated here and discussed in the text.

Finally, in considering the efficacy and complex biological functions of carotenoids in human chronic disease prevention, it appears that combining provitamin A carotenoids (*e.g.*, β-cryptoxanthin) with other antioxidants would be a particularly useful approach for chemoprevention. Antioxidants such as ascorbic acid and α-tocopherol limit the formation of excessive oxidative cleavage products of carotenoids in an oxidative environment. In addition, provitamin A carotenoids combined with non-provitamin A carotenoids (such as lycopene and lutein), which target different signalling pathways, could provide complementary or synergistic protective effects against chronic diseases including certain kind of cancers.

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