Chapter 28

Conversion of Carotenoids to Retinoids and Other Oxidation Products

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Dietary provitamin A carotenoids are cleaved to vitamin A at the central double bond by intestinal β-carotene-15,15'-dioxygenase, while eccentric cleavage at random position of conjugated double bonds is proposed for an additional pathway to vitamin A. Pig intestinal homogenate catalyzed exclusively cleavage reaction of the central double bond of β-carotene to produce two molecules of retinal, without any detectable formation of β-apocarotenals. High fat diets enhanced the enzyme activity and the level of cellular retinol binding protein type II in rat intestine. Dietary antioxidants and flavonols with a catechol structure in the B ring inhibited the enzyme activity *in vitro*. Thus, several food components would modulate metabolic conversion of provitamin A in intestinal cells and thereby the level of circulating provitamin A in the body. In contrast, carotenoids can be oxidized at random positions of conjugated double bonds in a non-enzymatic manner. Lycopene was oxidized *in vitro* to *acyclo*-retinal, *acyclo*-retinoic acid, and apolycopenals by autoxidation, and the oxidized lycopene induced apoptosis of HL-60 cells. Oxidation products formed *in vivo* might be involved in biological actions of carotenoids.

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Carotenoids are synthesized in microorganisms and plants, and work as antioxidants and antenna pigments in photosynthetic organs. Their oxidative metabolites play important roles as hormones in a wide variety of living organisms. The abscisic acid, which is synthesized through oxidative cleavage of neoxanthin, is a plant hormone related to water stress and senescence (*1*). Trisporic acid synthesized through cleavage of β-carotene in fungi is involved in the formation of zygote. Retinal works as a photo-sensor in vision of animals and retinoic acid regulates gene expression through activation of nuclear hormone receptors in vertebrates. They are synthesized through oxidative cleavage of β-carotene in animals. Thus, oxidative metabolites of carotenoids have been utilized as signal molecules by living organisms. Their formation are mediated and regulated by the enzymes, which catalyze the cleavage of specific double bonds in the respective carotenoids. In mammals, conversion of β-carotene to retinal is mediated by β-carotene-15, 15'-dioxygenase,which catalyzes cleavage reaction at the central double bond of provitamin A carotenoids (*2,3*). Although the dioxygenase plays an essential role for providing animals with vitamin A, the detailed characteristics and regulatory mechanism of the enzyme remain unknown. The enzyme is highly expressed in intestinal epithelium and liver, although the low activity is detected in other peripheral tissues. Thus, the intestinal epithelium is a primary site for conversion of dietary provitamin A carotenoids to vitamin A. Carotenoid absorption to intestinal cells is closely linked to digestion and absorption of dietary fat. The intestinal epithelium is exposed directly to various dietary components, which might influence the dioxygenase activity. The regulatory system of β-carotene dioxygenase in intestinal cells might be considered in this context as well as vitamin A status. In this contribution, effects of dietary fat and antioxidants on the intestinal dioxygenase activity are described.

Carotenoids have excellent quenching activity against singlet oxygen by physical energy transfer, and also have radical-scavenging activity by reaction with oxygen radicals. However, carotenoids are extremely vulnerable to oxidation and thereby work as prooxidants under certain conditions. In addition to the enzymatic cleavage, as described above, a number of compounds are formed from β-carotene by non-enzymatic oxidations, one of which cleaves conjugated double bonds at random positions under oxidative conditions. Retinal and β-apocarotenals are included among the oxidation products (*4*). Therefore, the oxidation products, which might be formed *in vivo* under oxidative conditions, could exert biological actions on human health. This contribution also describes the formation of cleavage products of lycopene, a typical dietary non-provitamin A carotenoid and effects of oxidized carotenoid preparations on proliferation of cancer cells.

Conversion of Provitamin A Carotenoids to Vitamin A

One molecule of β-carotene is converted to two molecules of retinal through the cleavage at central double bond by β-carotene 15,15'-dioxygenase, which was discovered in the mid 1960s by two groups of Goodman and Olson (*2,3*). In the intestinal epithelium, retinal is further reduced to retinol, which is esterified and incorporated into chylomicron as retinyl ester (*5*). This is a central cleavage pathway in conversion of provitamin A carotenoid to vitamin A, and has been confirmed by several groups (*6-8*). In contrast to this symmetrical cleavage, an eccentric cleavage pathway was proposed as an additional pathway of vitamin A formation (*9*). β-Carotene is cleaved by enzyme at random position of conjugated double bonds to retinal, β-apocarotenals, and several short-chain carbonyl compounds. β-Apocarotenals are further cleaved to short-chain compounds or oxidized to retinoic acid through β-oxidation pathway. We have investigated the cleavage reaction with pig intestinal homogenate as the source of enzymes, in order to elucidate the extent to which the eccentric cleavage pathway contributes to vitamin A formation (*10*). The incubation of β-carotene with pig homogenate and subsequent extraction were carefully conducted under the presence of antioxidant to avoid oxidative degradation of β-carotene and retinal, which are extremely susceptible to chemical oxidation. Retinal, which was spontaneously isomerized immediately after formation from β-carotene, was quantified for each geometrical isomer separated by high-performance liquid chromatography on a normal phase column. The low recovery of retinal due to formation of an Schiff base with amino compounds present in tissue homogenate was overcome by formaldehyde treatment prior to extraction. We found more than 94% converson of β-carotene consumed to retinal and no formation of β-apocarotenals. These results clearly indicated that the homogenate of pig intestine converted β-carotene to retinal exclusively by central cleavage. Moreover, in the absence of antioxidant, rat intestinal homogenates was reported to cleave β-carotene randomly to β-apocarotenals (*11*). The results suggested the random cleavage by oxygen radical produced through enzyme reactions. Therefore, the chemical cleavage at random position should be taken into consideration carefully, although it is still uncertain whether the eccentric enzymatic cleavages play significant role in the conversion of β-carotene to vitamin A. Recently, cDNA encoding a carotene dioxygenase, which catalyzed exclusively the asymmetric cleavage at 9', 10' double bond of lycopene as well as β-carotene, was identified from mouse (*12*). In addition to the central cleavage enzyme, the asymmetric cleavage enzyme might work in metabolism of carotenoids in mammals.

Effects of Dietary Fat and Antioxidant on β-Carotene-15,15'-Dioxygenase Activity

β-Carotene-15,15'-dioxygenase was expressed exclusively in the intestinal mucosa and liver, as the brain, kidney and lung exhibited little activity (*13*). The dioxygenase converts dietary β-carotene absorbed into intestinal cells to vitamin A, while in the liver it might attack β-carotene circulated in blood or stored in liver. Therefore, the enzyme would affect the levels of provitamin A carotenoids and vitamin A in the tissues. However, the regulation of β-carotene dioxygenase activity has not been clarified well, although vitamin A status and dietary protein were known to affect the activity (*14-16*). In particular, the intestine is exposed directly to dietary components, which could affect the dioxygenase activity. Several dietary antioxidants were found that remarkably inhibited the enzyme activity *in vitro* (*17*). Flavonoids such as rhamnetin and quercetin with a catechol structure in this ring B inhibited at micromole concentrations. Synthetic antioxidants such as butylhyroxytoluene strongly inhibited the activity at 1 μM. These inhibitory effects were also confirmed in the conversion of β-carotene to retinol by human intestinal Caco-2 cells. These results suggest that some flavonoids, which are ingested with carotenoids, e.g. as green leafy vegetables, might repress the conversion of provitamin A carotenoids to vitamin A. Another factor, which potentially affects the conversion, would be dietary fat, because digestion and absorption of β-carotene are closely related to those of dietary fat. Fat is well known to enhance the absorption of lipophilic vitamins. The dietary fat enhances the formation of mixed micelles composed of bile acid, cholesterol, phospholipids and fat hydrolyzates. Solubilization of carotenoid into mixed micelles is prerequisite to transfer from intestinal tract to the intestinal cells (*18-20*). Moreover, carotenoids and retinyl ester are incorporated into chylomicron rich in triacylglycerol and secreted in the lymph. Dietary unsaturated fat has been reported to enhance the expression of cellular retinol binding protein type II (CRBP-II), which works as carrier protein for retinal and retinol for subsequent metabolism in intestine (*21*). Thus, the effect of dietary fats on the β-carotene dioxygenase activity in rats was investigated (*22*). The rats fed with high fat diets containing 15% of olive oil or soybean oil for 3 weeks showed significantly higher activity of β-carotene dioxygenase in intestinal mucosa than the control rats fed with a low fat diet containing 2.5 % soybean oil (Figure 1). The rats fed with 15% soybean oil showed a higher level of CRBP-II protein in intestinal mucosa than the control rats. The results suggest that the expression of β-carotene dioxygenase is modulated by dietary fat and that the dioxygenase and CRBP-II are closely linked in the conversion of β-carotene. The enhancement of the dioxygenase by dietary fat could be of biological relevance in terms of efficient conversion of β-carotene to vitamin A, when much of β-carotene is absorbed into the intestinal cells. The transcriptional

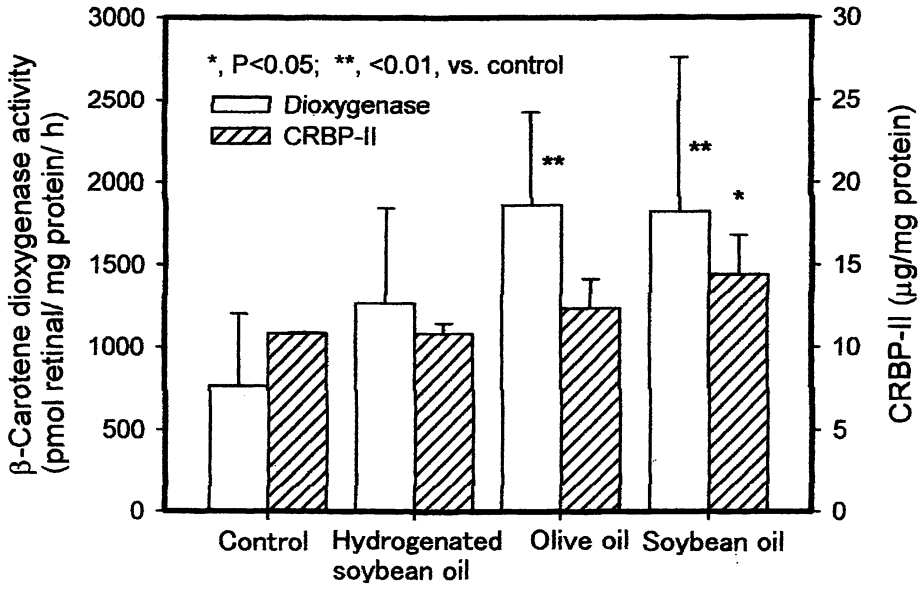


Figure 1. Effects of dietary fats on the levels of β-carotene dioxygenase activity and cellular retinol binding protein type II in rat intestinal mucosa. (Adapted with permission from reference 22. Copyright 1998 The American Society for Nutritional Science)

regulation of CRBP-II by dietary fatty acids has been intensively studied in relation to the peroxisome proliferator-activated receptors (*23*), while it remains unknown whether a similar mechanism for CRBP-II works in regulation of β-carotene dioxygenase. The recent success in the cloning of the dioxygenase (*24,25*) would facilitate the investigation on regulatory mechanism of the dioxygenase at the molecular level.

Non-enzymatic Cleavage of Carotenoids

The oxidation of provitamin A carotenoids to vitamin A is mediated through the specific cleavage at the central double bond by β-carotene dioxygenase. In contrast, non-enzymatic oxidation of carotenoids under *in vitro* oxidative conditions have been reported to produce a number of compounds, which include cleavage products at random positions of conjugated double bonds. 2,6-Cyclolycopene-1,5-diol was reported as an oxidation product of lycopene in human plasma (*26*), and 3-hydroxy-β,ε-carotene-3'-one as a major oxidation product of lutein in human retina (*27*). These oxidation products with a carbon skeleton of carotenoid might be formed *in vivo* by reaction with reactive oxygen species ROS. On the other hand, cleavage products of conjugated double bonds are particularly important, because retinoid-like substances might be formed from diverse carotenoids in addition to provitamin A carotenoids. Retinal and β-apocarotenals were produced under various conditions such as autoxidation in solvent, oxidation with peroxy radical initiators, singlet oxygen, cigarette smoke, and lipoxygenase (*4, 28-33*). A polar oxidation product of β-carotene, 5,8-endoperoxy-2,3-dihydro-β-apocarotene-13-one, which might be formed *in vitro* through cleavage at 13', 14' double bond of β-carotene, was reported to inhibit cell growth and cholesterol synthesis in MCF-7 mammary cancer cells (*34*). Furthermore, 4-oxo-retinoic acid, which was formed as an oxidation product at central double bond of canthaxanthin by incubation in cell culture medium, activated RARβ gene promoter and enhanced gap junctional communications (*35,36*). 3-Hydro-4-oxo-7,8-dihydro-β-ionone and 3-hydroxy-4-oxo-β-ionone were identified as the metabolites of canthaxanthin in rats and astaxanthin in rat hepatocytes, respectively (*37,38*). These two metabolites suggest that the cleavage at the 9', 10' double bond of the respective carotenoids occurs in biological tissues. Moreover, oxidized carotenoid preparations have recently been shown to have adverse effects on biological tissues (*39-43*). These observations strongly suggest that carotenoids are oxidized by reaction with ROS in biological tissues and that some products have potential biological effects, whether beneficial or harmful. Thus, it is worth evaluating the biological effects of oxidation products as well as intact carotenoids.

The cleavage reaction of lycopene, one of typical non-provitamin A carotenoids was investigated (*44*). Lycopene was solubilized in three different media, toluene, Tween 40 aqueous solution, and liposomal suspension, which was used as a model for biological tissues. The lycopene was autoxidized by incubating at 37°C and gave a number of oxidation products with absorption in the UV-VIS region. Among them, a series of carbonyl compounds with different chain length were formed by cleavage of conjugated double bonds of lycopene. Eight cleavage products, namely of 3,7,11-trimethyl-2,4,6,10-dodecatetraen-1-al, 6,10,14-trimethyl-3,5,7,9,13-pentadecapentaen-2-one, *acyclo*-retinal (3,7,11,15,-tetramethyl-2,4,6,8,10,14-hexadecahexaen-1-al), apo-14'-lycopenal, apo-12'-lycopenal, apo-10'-lycopenal, apo-8'-lycopenal, and apo-6'-lycopenal were identified (Figure 2). This result was consistent with the autoxidation of β-carotene in organic solvents (*29,30*). Furthermore, *acyclo*-retinoic acid (3,7,11,15,-tetramethyl-2,4,6,8,10,14-hexadecahexaenoic acid), an acyclic analogue of retinoic acid, was also detected as an oxidation product of lycopene. The incubation of 50 μM lycopene solubilized in liposome for 24 h produced 64 nM *acyclo*-retinal and 1-2 nM *acyclo*-retinoic acid. Although the amount of *acyclo*-retinoic acid formed was far less than that of *acyclo*-retinal in aqueous media, pig liver homogenate showed a remarkable ability to convert *acyclo*-retinal to *acyclo*-retinoic acid. Thus, *acyclo*-retinoic acid is potentially formed from *acyclo*-retinal, when lycopene is oxidized in biological tissues. The results in this study and in previous reports suggest that any carotenoid with long conjugated double bonds is cleaved at random positions by reacting with ROS in biological tissues. Some of the oxidation products and their metabolites might have biological activity, such as retinoids.

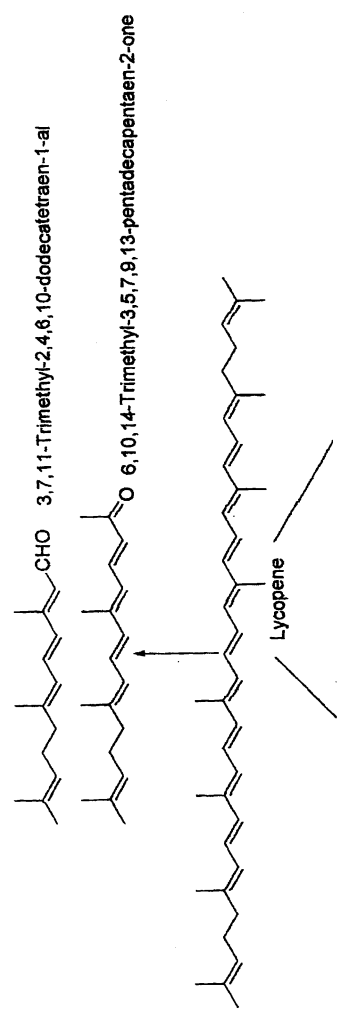
Effects of Oxidized Carotenoids on Proliferation of Cancer Cells

Autoxidation of lycopene produced acyclic analogues of retinal and retinoic acid *in vitro*. It is worth evaluating whether the oxidized lycopene preparation works as a retinoid-like substance, in order to elucidate the potential biological effects of oxidized carotenoids. All-*trans*-retinoic acid is known to induce differentiation of human promyelocytic leukemia HL-60 cells to granulocyte. Moreover, lutein (*45*), lycopene (*46*), saffron carotenoids (*47*) and β-carotene (*45, 46, 48*) inhibit the growth of HL-60 cells by inducing differentiation. However, it is still obscure whether the growth inhibition is caused by the intact carotenoids or by their oxidation products. Therefore, the effects of a series of acyclic carotenoids including lycopene and their oxidized preparations on growth and differentiation of HL-60 cells was evaluated (*49*). Oxidation mixture of each carotenoid was prepared by incubating 1 mM carotenoid in toluene at

37°C for 24 h. The carotenoid preparations were incubated at 6 μM as intact carotenoid with HL-60 cells for 5 days, and thereafter the cell growth and differentiation were evaluated. As shown in Figure 3, ζ-carotene and phytofluene significantly inhibited the cell growth to 45.2 and 68.4 % of the control, respectively. Lycopene tended to inhibit cell growth, whereas phytoene and β-carotene did not inhibit the cell growth. ζ-Carotene and phytofluene were unstable in the medium under the culture condition, although they showed a significant growth inhibition against HL-60 cells. On the other hand, β-carotene was stable during incubation in the medium, followed by lycopene. Surprisingly, the oxidation mixture of phytofluene, ζ-carotene, and lycopene more strongly inhibited the growth of HL-60 cells than the respective intact carotenoids. In particular, lycopene drastically increased the inhibitory effect on the cell growth by oxidation prior to supplementation. The oxidation mixture of β-carotene was far less inhibitory than that of lycopene, although the degree of oxidation was at the same level in both preparations. These results suggest that oxidation products formed from specific carotenoids inhibit cell growth rather than intact carotenoids themselves.

Cell cycle-arrest (*50,51*), enhancement of gap junctional communication (*52*) and induction of apoptosis (*53-55*) and differentiation have been reported as possible mechanisms for growth inhibition of cancer cells by carotenoids. The acyclic carotenoid preparations, which inhibited the growth of HL-60 cells, did not induce differentiation in our study, although some carotenoids were reported to induce differentiation in HL-60 cells. Exposure of HL-60 cells to phytofluene, ζ-carotene and oxidation mixture of lycopene at 10 μM for 24 h induced the morphological changes characterized by reduction of cell volume, chromatin condensation, nuclear fragmentation and production of the apoptotic bodies. An apoptotic DNA ladder was observed in HL-60 cells treated with phytofluene, ζ-carotene and oxidation mixture of lycopene at 10 μM for 24 h, but not in those treated with the purified lycopene (data not shown). Thus, phytofluene, ζ-carotene and oxidation mixture of lycopene induced apoptosis in HL-60 cells. These results indicate that acyclic carotenoids and their oxidation products show an apparent growth inhibition by inducing apoptosis.

These carotenoid preparations were also evaluated to examine if they could affect proliferation of human prostate cancer cells. Phytofluene, ζ-carotene, and lycopene significantly reduced the viability of three prostate cancer cells (*56*). Moreover, each oxidation mixture of these carotenoids also reduced the viability as effectively as the intact carotenoids. These results suggest that oxidation products formed from phytofluene, ζ-carotene and lycopene caused reduction of the cell viability of the human prostate cancer cells as well as HL-60 cells. Isolation and identification of oxidation products, which cause apoptosis induction against HL-60 cells, are currently underway. These results strongly



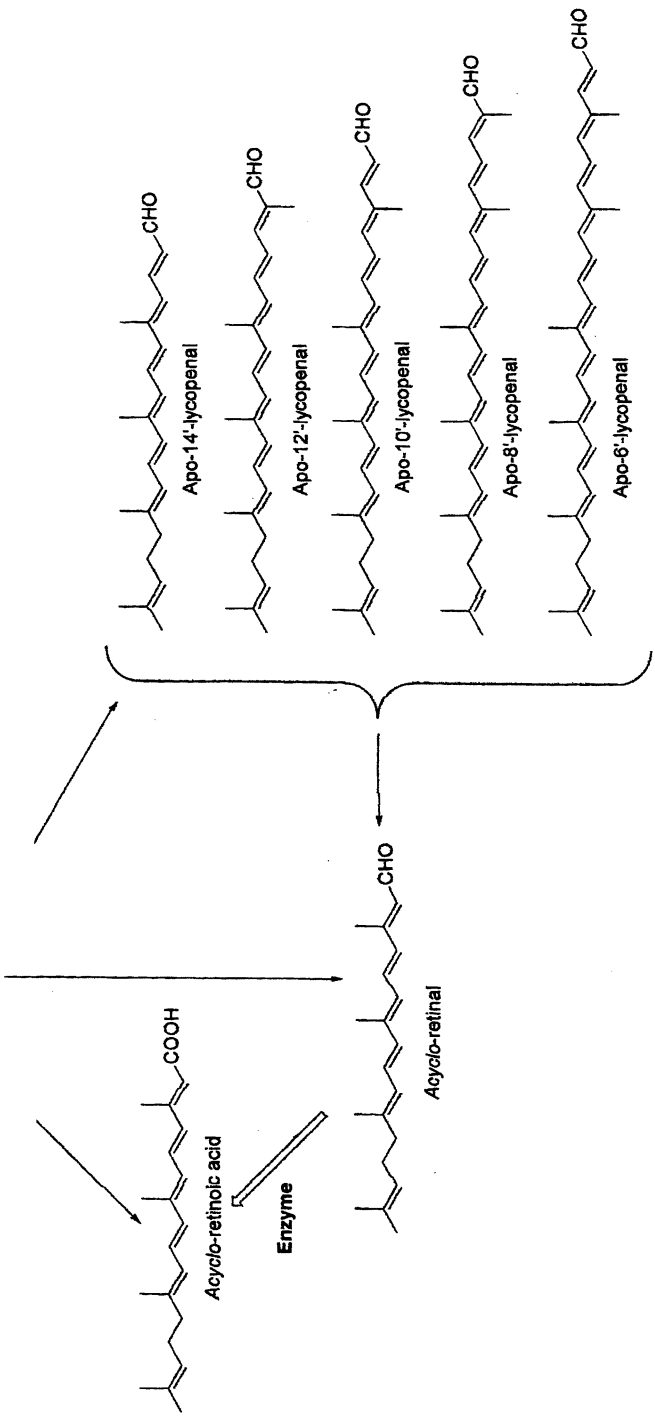


Figure 2. Cleavage products formed from lycopene by autoxidation.

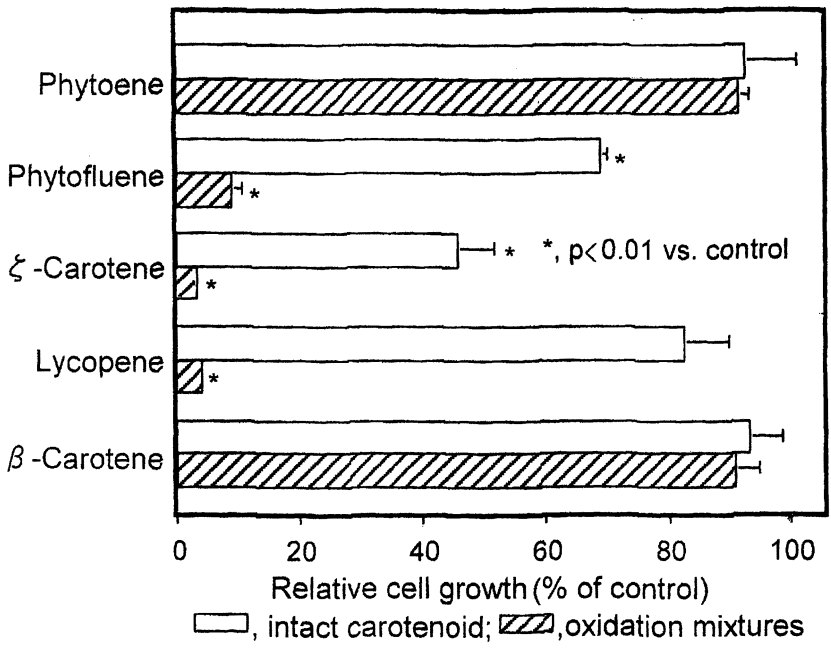


Figure 3. Effects of carotenoid preparations on the growth of HL-60 cells.

suggest that oxidation products as well as intact carotenoids affect proliferation of cancer cells.

Thus, several dietary components were found to affect oxidative metabolism of provitamin A carotenoids. More detailed characterization of regulatory mechanisms is still needed to clarify the bioavailability of provitamin A carotenoids. The non-enzymatic oxidation products of carotenoids have been shown to have the potential to exert biological actions by affecting proliferation of cancer cells. *In vivo* oxidation of carotenoids and its relation to biological actions deserves further studies.

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