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Revisiting carotenoids as dietary antioxidants for human health and disease prevention

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Humans are unique indiscriminate carotenoid accumulators, so the human body accumulates a wide range of dietary carotenoids of different types and to varying concentrations. Carotenoids were once recognized as physiological antioxidants because of their ability to quench singlet molecular oxygen ($^1O_2$). In the 1990s, large-scale intervention studies failed to demonstrate that supplementary β-carotene intake reduces the incidence of lung cancer, although its antioxidant activity was supposed to contribute to the prevention of oxidative stress-induced carcinogenesis. Nevertheless, the antioxidant activity of carotenoids has attracted renewed attention as the pathophysiological role of $^1O_2$ has emerged, and as the ability of dietary carotenoids to induce antioxidant enzymes has been revealed. This review focuses on six major carotenoids from fruit and vegetables and revisits their physiological functions as biological antioxidants from the standpoint of health promotion and disease prevention. β-Carotene 9′,10′-oxygenase-derived oxidative metabolites trigger increases in the activities of antioxidant enzymes. Lutein and zeaxanthin selectively accumulate in human macular cells to protect against light-induced macular impairment by acting as antioxidants. Lycopene accumulates exclusively and to high concentrations in the testis, where its antioxidant activity may help to eliminate oxidative damage. Dietary carotenoids appear to exert their antioxidant activity in photo-irradiated skin after their persistent deposition in the skin. An acceptable level of dietary carotenoids for disease prevention should be established because they can have deleterious effects as prooxidants if they accumulate to excess levels. Finally, it is expected that the reason why humans are indiscriminate carotenoid accumulators will be understood soon.

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1. Introduction

"Carotenoids" is a general term for the yellow, red, and orange pigments containing a long-chain hydrocarbon with conjugated double bonds. More than 1100 carotenoids are found in nature and are typically present as C-40-based tetraterpenoid secondary metabolites in the plant kingdom, although C-45- and C-50-based carotenoids are also distributed in bacteria.[1] Carotenoids can be categorized as hydrocarbon carotenoids (carotenes and lycopene) and oxygenated carotenoids (xanthophylls) on the basis of the presence or absence of oxygen atoms in their structures.[2] They are exogeneous nutrients for animals because animals cannot synthesize them *de novo*. Birds and fishes are known to accumulate various carotenoids in their body by oxidative modification after ingestion. In contrast, dietary carotenoids have a range of different fates in the bodies of mammals, including humans. In 1984, Goodwin[3] proposed that mammals can be separated into three groups: indiscriminate accumulators, carotene accumulators, and carotene non-accumulators, on the basis of their patterns of absorption and accumulation of ingested carotenoids. Humans are unique indiscriminate accumulators and are believed to absorb more than 40 carotenoids as dietary components and accumulate them in the body indiscriminately. It was also confirmed that primates are uniquely distinguished by the wide range of carotenoids of different types and concentrations in their sera.[4] It is noteworthy that six major carotenoids accumulate in human plasma and tissues: α-carotene, β-carotene, lycopene, β-cryptoxanthin, lutein, and zeaxanthin (Fig. 1).[5,6]

The major dietary sources of each carotenoid are as follows. α-carotene: carrots, winter squash and pumpkin; β-carotene: dark orange and green fruits and vegetables; lycopene: tomatoes and tomato products; β-cryptoxanthin: tropical fruits and sweet red peppers; lutein: leafy greens, corn and green vegetables; zeaxanthin: egg yolk, corn, corn meal, and leafy greens.[7] The distribution of carotenoids in human plasma seems to reflect the composition of carotenoids ingested *via* the diet, especially fruit and vegetables.

In humans, the main biological function of dietary carotenoids is provitamin A activity. Among the six carotenoids mentioned above, α-carotene, β-carotene, and β-cryptoxanthin can act as provitamin A because they can release retinal by the central cleavage reaction with β-carotene 15,15′-oxygenase (BCO1) within epithelial cells during absorption in the small intestine. Nevertheless, a proportion of these three provitamin A carotenoids and the other three non-provitamin A carotenoids are absorbed from the small intestine without cleavage, and are transported into the blood circulation system in their original forms. Therefore, the biological functions of dietary carotenoids other than provitamin A activity have long attracted attention from the aspect of their health benefits. Such benefits include immunostimulatory function, promotion of intercellular gap junction formation, and adipocyte function, although the level of carotenoids in the blood may simply be an indicator of fruit and vegetable intake.

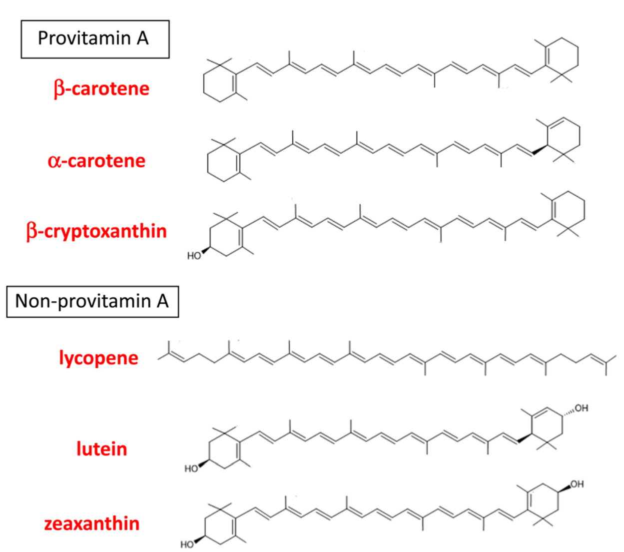

Fig. 1 Structures of major six carotenoids detected in human plasma and tissues.

In the 1980s, Sies[8] proposed the concept "oxidative stress"; that is, an imbalance of prooxidants that accelerate the production of reactive oxygen species (ROS) and antioxidants that attenuate ROS production. Oxidative stress was identified as a factor contributing to the etiology and progression of chronic diseases. In this context, the antioxidant activity of dietary carotenoids aroused much interest together with vitamin C and vitamin E because carotenoids were known to react readily with ROS. In the 2000s, the "oxidative stress" concept was revised to, "an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of the redox signaling and control, and/or molecular damage".[9] In addition, novel metabolites other than retinal, which may affect cellular redox signaling, were discovered as metabolic products of carotenoids produced during intestinal absorption and blood circulation. Thus, the antioxidant activity of carotenoids has attracted renewed attention in relation to the role of food-derived factors in human health.[10–12] This review article focuses on the six major dietary carotenoids and revisits their physiological functions as biological antioxidants from the viewpoint of health promotion and disease prevention.

2. Research background on carotenoids as dietary antioxidants

2.1 Progress of antioxidant research on carotenoids

Carotenoids are essential compounds for the survival of photosynthetic plants. These pigments coexist with chlorophylls in the thylakoid membranes of chloroplasts and protect plants from photodamage induced by the photodynamic action of chlorophylls.[13] This is the reason why chlorophyll-rich leafy vegetables contain remarkable amounts of carotenoids. In fruits, carotenoid pigments act as attractants for pollinators and seed-dispersing animals after fruit ripening, in addition to functioning as protectants from photodamage in irradiated regions. Carotenoids also serve as precursors of phytohormones such as abscisic acid, and aroma components such as β-ionone.[2,14]

In 1968, Foote *et al.*[15] discovered that β-carotene is capable of quenching highly reactive singlet molecular oxygen ($^1O_2$), a type of ROS that contributes to photooxidative damage in photosynthetic plants. Twenty years later, Di Mascio *et al.*[16] found that lycopene exhibited the highest quenching rate constant with $^1O_2$ among eight carotenoids including β-carotene, α-carotene, zeaxanthin, lutein, and β-cryptoxanthin. Thereafter, dietary carotenoids were frequently referred as powerful $^1O_2$ quenchers that participate in protection against oxidative stress in the human body.[17,18] Krinsky[19] raised the possibility that carotenoids inhibit the free radical reaction of lipid peroxidation by scavenging lipid radicals.

In the 1980s, the potential roles of ROS and ROS-scavenging natural antioxidants attracted much attention in relation to the etiology of carcinogenesis. Several studies evaluated the roles of carotenoids in the molecular mechanism of carcinogenesis, including oxidative modification of genomic DNA.[20,21]

The results of Peto *et al.*[22] implied that dietary β-carotene might exert its protective effect against human cancer by a mechanism other than its well-known provitamin A activity. In 1984, Burton and Ingold[23] proposed that β-carotene is a unique radical-trapping type lipid antioxidant because it exhibits good radical-trapping capacity at lower oxygen partial pressures under physiological conditions. They also found that, at higher oxygen pressures and relatively high concentrations, β-carotene can act as a prooxidant by promoting the radical chain reaction of lipid peroxidation. Much attention was then paid to the antioxidant effect of dietary carotenoids in free radical-mediated lipid peroxidation.[24,25] Terao *et al.*[26,27] demonstrated that xanthophylls, like β-carotene, can act as chain-breaking antioxidants against lipid peroxidation in solution and in liposomal membranes. Concerning the possibility that ingested carotenoids exert antioxidant activity in the human body, low-density lipoprotein (LDL) derived from the plasma of human subjects consuming a diet supplemented with lycopene-rich tomato juice showed increased resistance to $^1O_2$-induced LDL-cholesterol peroxidation, whereas this supplementation did not affect the free-radical chain reaction.[28] Increased resistance of plasma LDL to metal ion-induced lipid oxidation and the suppression of a lipid peroxidation biomarker in the plasma were detected after dietary intake of tomato products for 3 weeks.[29] The results of those studies implied that dietary carotenoids can improve the antioxidant defense of plasma LDL against lipid peroxidation. Because oxidized LDL is thought to participate in the incidence and progress of atherosclerosis leading to cardiovascular disease,[30] it was proposed that dietary carotenoids can help to prevent atherosclerosis by acting as antioxidants in LDL particles.

2.2 Expectations and confusion about the chemopreventive effect of carotenoid supplementation

In the 1980s, epidemiological evidence strongly suggested that carotenoid intake may reduce the risk of lung cancer and certain other cancers.[31,32] Several large-scale intervention studies were then carried out to determine whether supplementary intake of β-carotene exerted a preventive effect against the incidence of lung cancer (Table 1).

A major intervention trial involving approximately 30 000 people in Linxian County, China, was conducted from 1986 to 1991. The population in this region has one of the world's highest rates of esophageal/gastric cardia cancer and a persistently low intake of several micronutrients. Combined dietary supplementation with β-carotene, vitamin E, and selenium apparently reduced the risk of cancer in this population.[33] In contrast, the Physicians' study (1982–1995) targeting approximately 22 000 physicians in the U.S.A. showed that supplementation with β-carotene produced neither benefit nor harm in terms of the incidence of lung cancer and death from all causes.[34] The results of both the alpha-tocopherol, beta-carotene cancer prevention (ATBC) study involving nearly 30 000 male smokers in southwestern Finland[35] and the beta-Carotene and Retinol Efficacy Trial (CARET) involving approximately 18 000 men and women who were heavy cigarette smokers and men with occupational exposure to asbestos[36] strongly indicated that β-carotene supplementation increased the rate of death by lung cancer. The CARET intervention was stopped 21 months early because of the possible harmful effect of daily consumption of β-carotene combined with retinyl palmitate.

Mayne *et al.*[37] reported that up to 5 years' daily supplementation with β-carotene (50 mg per day) produced an approximately 10-fold increase in the median plasma β-carotene concentration. In the ATBC study, the plasma β-carotene concentration was increased 15-fold (from 0.37 μM to 5.81 μM) by supplementation with β-carotene (20 mg per day) for 6.7 years.[38] These phenomena indicated that excess β-carotene accumulated in the plasma of smokers and men with occupational exposure to asbestos after β-carotene supplementation, and therefore acted as a potential prooxidant, resulting in harmful effects and the development of lung cancer.[39,40] In 2022, a systematic review on the role of β-carotene in primary chemoprevention of lung cancer reconfirmed that β-carotene supplementation may increase the risk of lung cancer.[41] Thus, it was concluded that nutritional prevention of cancer through β-carotene supplementation should not be recommended.[42]

The "oxidative stress" concept attracted much attention in the field of clinical medicine in the 1990s. Together with

Table 1 Large-scale intervention trials examining the relationship between β-carotene supplementation and incidence of lung cancer

Trial	Subject	Dose	Period	Result	Ref.
Linxian study	29 584 people (40–69 y.) living in Linxian, China	Daily β-carotene (15 mg), vitamin E (30 mg) and selenium (50 mg)	1986–1991	Significant difference in lowered total mortality (RR = 0.91), lower cancer rates (RR = 0.87) and lower stomach cancer rate (RR = 0.79)	33
Physicians' study	22 071 physicians (40–84 y.) living in US	β-Carotene (50 mg) on alternative days	1982–1995	No significant difference in malignant neoplasms, cardiovascular diseases, or death from all diseases	34
ATBC study	29 133 male smokers (50–69 y.) living in southwestern Finland	Daily β-carotene (20 mg)	1985–1993	Significantly higher incidence of lung cancer (+18%)	35
CARET trial	18 314 current smokers and asbestos-exposed workers	Daily β-carotene (30 mg) and retinyl palmitate (25 000 IU)	1988–1998	Significantly excess lung cancer incidence (RR = 1.36) and lung cancer mortality (RR = 1.59)	36

y.: years old and RR: relative risk.

vitamin E and vitamin C, carotenoids were expected to participate in the antioxidant network in the human body.[43] However, the 5-year Medical Research Council/British Heart Foundation Heart Protection Study in which daily antioxidant vitamin supplementation (600 mg vitamin E, 250 mg vitamin C, and 20 mg β-carotene) was provided to approximately 20 000 adults in the United Kingdom did not provide any evidence for significant effects of antioxidant supplementation on vascular events and cancer incidence.[44] Bjelakovic *et al.*[45,46] conducted a systematic review and meta-analysis of randomized trials using antioxidant supplements for disease prevention, and proposed that supplementary intake of β-carotene, vitamin A, and vitamin E may increase all-cause mortality. They concluded that antioxidant supplementation has no preventive effect and may be harmful to human health at doses higher than the recommended daily allowances. Thus, the optimal source of antioxidants is from the diet, not from antioxidant supplements in pill or tablet form.[47] In 2013, the Department of Health and Human Services (HHS) and the National Institutes of Health (NIH) in the U.S.A. warned of the deleterious effects of high-dose antioxidant supplements in some cases.[48]

Increasing the consumption of fresh fruits and vegetables is a favorable strategy to prevent non-communicable diseases including cancers, cardiovascular disease, diabetes, and chronic respiratory diseases.[49,50] The inverse association between fruit and vegetable intake and all-cause mortality was recently reconfirmed in a study involving nearly 100 000 Japanese people.[51] The HHS/NIH proposed three reasons for the discrepancy between beneficial and deleterious effects as follows: the beneficial health effect of fruits and vegetables may be caused by other substances or other dietary factors, the effects of the large doses used in supplements may differ from those of smaller amounts consumed in foods, and differences in the chemical composition of antioxidants in foods *versus* those in supplements may influence their effects.[48] In the case of carotenoids, excess β-carotene intake by ingesting supplements may induce its prooxidant effect, leading to enhanced oxidative stress in the human body. In all intervention studies, only β-carotene was provided as the carotenoid supplement, whereas foods contain a variety of carotenoids. In any case, the mechanism of action of the health benefits of dietary carotenoids remains unclear, except for their provitamin A activity.

Specific carotenoids derived from fruit and vegetables might be effective in some diseases. In this sense, observational studies suggested that lycopene may act as a preventive factor against prostate cancer.[52] The role of lutein and zeaxanthin in macular pigments attracted attention in connection with the prevention of age-related macular regeneration (AMD).[53] Dietary carotenoids have also been implicated in the protection of skin against photoaging because human skin is known to accumulate these pigments from foods.[54]

2.3 Discovery of novel carotenoid-metabolic pathways and interest in the function of novel metabolites

In 1965, Olson and Hayaishi[55] and Goodman and Huang[56] independently discovered the enzyme β-carotene 15,15′-oxygenase (BCO1) in the rat liver and intestine. This enzyme catalyzes the cleavage of the central region of β-carotene symmetrically to produce retinal. Although it was proposed that eccentric cleavage to produce apocarotenoids with different chain lengths may occur in the metabolic pathway of β-carotene, a series of reports refuted the participation of eccentric cleavage in the mechanism of vitamin A formation and emphasized that retinal is the sole product of β-carotene cleavage.[57] In 2001, Kiefer *et al.*[58] discovered a novel enzyme from mouse cDNA that catalyzes the asymmetric oxidative cleavage at the 9′,10′ double bond of β-carotene to yield β-apo-10′-carotenal and β-ionone. This enzyme, designated as β-carotene 9′,10′-oxygenase 2 (BCO2), was found to cleave lycopene to yield apo-10′-lycopenoids.[58,59] BCO2 prepared from ferret, an animal model of human carotenoid metabolism, was also found to cleave xanthophylls such as lutein, zeaxanthin, and β-cryptoxanthin, resulting in several isomers of 3-hydroxy-apo-10′-carotenal and 3-hydroxyionones.[60]

Khachik *et al.*[61] earlier showed that oxidation products, such as 3-hydroxy-β,ε-carotene-3′-one, accumulate in human plasma as oxidative metabolites of lutein and zeaxanthin. These ketocarotenoids, as well as apocarotenoids and apolycopenoids, may have specific biological activities in relation to human health, as they can act as powerful electrophilic compounds.[62,63] Apolycopenoids can activate the electrophile/antioxidant response element (EpRE/ARE) system by releasing NEF-E2-related factor 2 (Nrf2).[64] This event may promote biological antioxidant defenses by increasing the expression of antioxidant enzymes, as described later in this review. It is currently suggested that the mechanism of action of carotenoids involves an indirect effect of their oxidative products to promote antioxidant enzyme activity, as well as their inherent $^1O_2$-quenching and radical-trapping activities.

3. Absorption and metabolism of dietary carotenoids

3.1 Absorption of carotenoids in the intestine

Yonekura and Nagao[65] and von Lintig *et al.*[66] described a chain of processes from the ingestion of carotenoid-containing foods to the absorption of each carotenoid by intestinal epithelial cells. After ingestion of carotenoid-containing foods, carotenoids are released from the food matrix in the digestive tract. The lipophilic carotenoids incorporate into the lipid phase, which is emulsified in the stomach, and are then transferred to mixed micelles formed by the action of bile salts, biliary phospholipids, dietary lipids, and their lipase-hydrolysis products. The mixed micelles migrate to the brush border membranes of the lumen and then each carotenoid is absorbed by intestinal absorptive cells. In fruits and vegetables, xanthophylls are frequently present as their fatty acid esters, in which the hydroxy group at the β-ionone ring is esterified by various free fatty acids.[67] These esters are mostly hydrolyzed by pancreatic carboxyl lipase during the digestion process.[68,69] Thus, xanthophylls, that is, β-cryptoxanthin, lutein, and zeaxanthin, are present as free forms in human plasma. Fats and oils are good carriers for lipophilic carotenoids in the digestive tract, and they accelerate micelle formation by inducing the secretion of pancreatic lipase and bile acids. In fact, ingestion of fresh vegetables with larger proportions of oil in the dressing was shown to improve carotenoid absorption.[70]

Class B scavenger receptors, SR-B1 and CD36, contribute to the absorption of dietary carotenoids in the epithelium.[71–73] These proteins are expressed in the intestine and facilitate the uptake of carotenoids by enterocytes without energy expenditure. After intestinal absorption, carotenoids are incorporated into triacylglycerol-rich lipoproteins (chylomicrons) and secreted into the lymphatic system. Then, they are absorbed into the liver as chylomicron remnants and finally distributed around the whole body *via* the carotenoid-containing lipoproteins secreted from the liver.

3.2 Metabolism of carotenoids

Provitamin A carotenoids such as β-carotene are partly converted to vitamin A in the intestine.[66] That is, they are cleaved at the central position (between the 15- and 15′-position) to yield two molecules of retinal by the action of BCO1 located in the intestinal epithelial cells, followed by retinal dehydrogenase-dependent reduction to retinol (Fig. 2).

Retinol is finally circulated in the blood stream after binding with retinol binding protein-4 (PBP4) in the liver. Human BCO1 mRNA has also been detected in the liver, kidney, and several peripheral tissues, in which BCO1 may play a role in local vitamin A synthesis.[74] Dietary supply of provitamin A carotenoids at excess levels does not lead to symptoms of hypervitaminosis because vitamin A production is elegantly regulated by a negative feedback mechanism corresponding to dietary intake.[75]

BCO2, which catalyzes the cleavage between the 9′ and 10′ position of β-carotene resulting in β-apo-10′-carotenal, displays broad substrate specificity for carotenoids. Thus, this enzyme catalyzes the conversion of non-provitamin A carotenoids including acyclic lycopene as well as lutein and zeaxanthin as shown in Fig. 2. BCO2 is expressed in the intestine and several additional tissues, but its role in the metabolism of dietary carotenoids remains undefined. Interestingly, BCO2 is a mitochondrial enzyme associating with the inner membrane, in contrast to BCO1, which is a cytosolic enzyme.[76] Experiments on BCO2-deficient mice implied that BCO2 protects against carotenoid-induced oxidative damage in hepatic mitochondria.[77] In experiments using a human cell line, BCO2 was suggested to prevent carotenoid-caused oxidative stress and triggering of the apoptotic pathway in mitochondria by acting as a carotenoid scavenger and gatekeeper for the apoptotic pathway.[78] BCO2 systemic knock out-mice showed oxidative stress-induced perturbation of mitochondrial function in the liver[79] and the hypothalamus.[80] Therefore, BCO2 seems to be essential to regulate macronutrient mitochondrial metabolism in rodents.[81] Human BCO2 may also affect normal mitochondrial function, although its activity has not yet been fully clarified.

Babino *et al.*[82] demonstrated that oxidative stress induces the expression of the gene encoding human BCO2, and proposed that the preventive function of BCO2 against oxidative stress is conserved in primates. The induction of BCO2 in human tissues may prevent excessive accumulation of carotenoids and explain the observation that carotenoid levels decrease in oxidative stress-related chronic disease states.[66] However, Li *et al.*[83] found that human BCO2 in the retina is inactive as a xanthophyll-cleavage enzyme, in contrast to the high activity of mouse BCO2. In contrast, the products of the reaction between lycopene and BCO2 showed antioxidant properties, in that they activated Nrf2 to induce antioxidant enzymes including heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase-1 (NQO1), glutathione S-transferase (GST), and glutamate–cysteine ligases in human bronchial

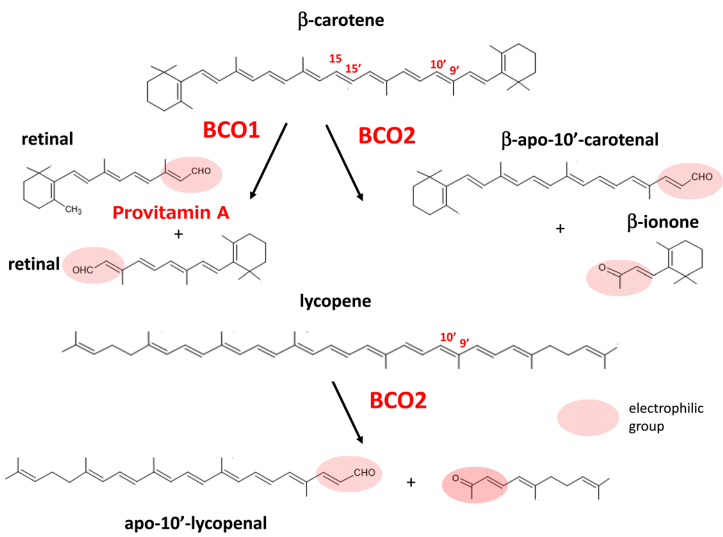

Fig. 2 Oxidative cleavage of double bonds in carotenoids by BCO1 and BCO2.

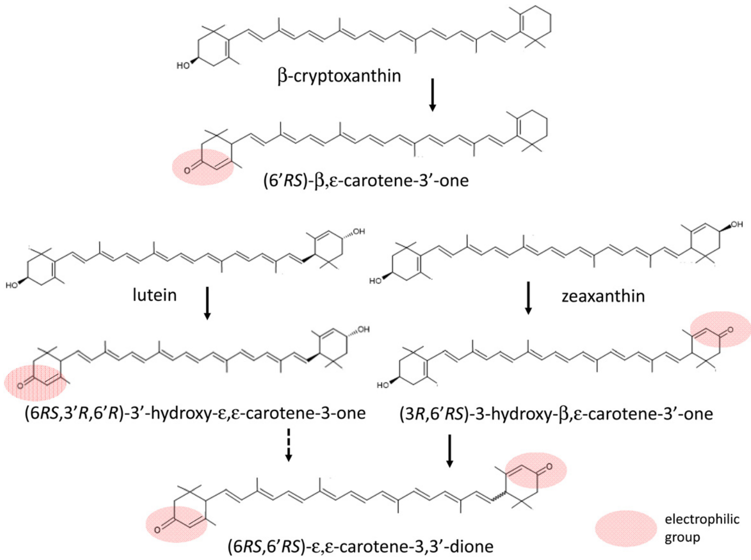

Fig. 3 Proposed mechanism for oxidative modification to yield ketocarotenoids from xanthophylls (modified from ref. 85).

cells.[84] At present, the levels of BCO2 activity and its physiological functions in humans with respect to the metabolism of dietary carotenoids are still unclear.

In 1992, Khachik *et al.*[61] detected 3′-hydroxy-ε,ε-caroten-3-one, 3-hydroxy-β,ε-caroten-3′-one, and ε,ε-carotene-3,3′-dione, which were formed by the oxidation of lutein and zeaxanthin in human plasma. This implied that oxidative metabolism of xanthophylls occurs at their hydroxy group of the β-ionone ring in human tissues. Nagao *et al.*[85] confirmed this oxidative modification of xanthophylls in the mouse liver. Their results suggested that this oxidative activity to convert the 3-hydroxy β-end group to a keto group also exists in humans because the same ketocarotenoids produced by oxidation of lutein, zeaxanthin, and β-cryptoxanthin in the mouse liver were detected in human plasma after ingestion of mandarin orange juice (Fig. 3).

Microsomal NAD^+-dependent dehydrogenase is likely to be responsible for this oxidative modification, although the enzyme itself has not yet been characterized.

3.3 Accumulation of dietary carotenoids in the human body

Böhm *et al.*[86] estimated that a total plasma carotenoid concentration lower than 1000 nmol L^{-1} is associated with a higher risk of chronic diseases. They also clarified from the data on the average daily intake of carotenoids that plasma concentrations reflecting normal carotenoid intake in a healthy varied diet are ∼1725 nmol L^{-1} (total carotenoids), comprising 100 nmol L^{-1} α-carotene, 500 nmol L^{-1} β-carotene, 600 nmol L^{-1} lycopene, 230 nmol L^{-1} β-cryptoxanthin, and 330 nmol L^{-1} lutein and zeaxanthin. A variety of factors including diet, sex, age, and body mass index affect the plasma concentration of each carotenoid.[87] Habitual alcohol drinking and cigarette smoking adversely affect plasma carotenoid concentrations.[90] The carotenoid concentrations were found to be significantly higher in the liver (∼16 500 nmol kg^{-1}), adrenal gland (9400 ± 7800 nmol kg^{-1}), and testis (∼87 550 nmol kg^{-1}) than in the kidney (3050 ± 4210 nmol kg^{-1}) and lung (1905 ± 2820 nmol kg^{-1}).[86] Lycopene accounts for the majority of carotenoids in the testis, although β-carotene is the major carotenoid in other tissues.[88] Lutein and zeaxanthin uniquely accumulate at the macula, where they are collectively called macular pigment.[89] Once they accumulate in each tissue, carotenoids are speculated to be excreted from the body with and without metabolic conversion. The details of the excretion process are not yet known. The half-lives of carotenoids in human plasma were reported to be 76 days for lutein, 45 days for α-carotene, 39 days for β-cryptoxanthin, 38 days for zeaxanthin, 37 days for β-carotene, and 26 days for lycopene in healthy adult women,[90] and 33–61 days for zeaxanthin and lutein, less than 12 days for β-carotene, α-carotene, and β-cryptoxanthin, 12–33 days for lycopene in healthy men.[91]

4. Mechanism of antioxidant activity of carotenoids

4.1 General comments

The antioxidant activity of carotenoids is explained by two mechanisms, one of which involves direct scavenging or quenching of ROS and lipid radicals. The other is indirect promotion of the antioxidant defense system *via* their involvement in cellular redox signaling pathways. $^1O_2$ quenching is well characterized as a representative mechanism of carotenoids that function as biological antioxidants to regulate oxidative stress. It should be noted that carotenoids may also act as prooxidants by accelerating the radical chain reaction of lipid peroxidation when they react with oxygen radicals or lipid radicals under specified conditions. In the indirect action mechanism, electrophilic apocarotenoids, generated by the reaction of BCO2 with parent carotenoids, participate in a signal transduction pathway inducing Nrf2 activation. Ketocarotenoids derived from the oxidative metabolism of xanthophylls may also contribute to this pathway because ketocarotenoids possess electrophilic properties owing to their α,β-unsaturated carbonyl structure. The relationship between the site of oxidative damage and the location of carotenoids within the cells and extracellular fluids seems to affect the efficacy of carotenoids as biological antioxidants.

4.2 Generation of singlet oxygen in biological systems

The presence of $^1O_2$ means that an oxygen molecule has been excited to the singlet state (spin quantum number S = 0) from the ground state triplet oxygen molecule ($^3O_2$: $O_2(^3\Sigma_g^-)$) by energy transfer. There are two species of $^1O_2$: $O_2(^1\Sigma_g^+)$ and $O_2(^1\Delta_g)$. Long-lived $O_2(^1\Delta_g)$ is probably responsible for the oxygenation reaction in biological systems because $O_2(^1\Sigma_g^+)$ is short-lived owing to its rapid transition to $O_2(^1\Delta_g)$.[92] $^1O_2$ attacks the electron-rich π-bond of organic compounds because this molecule possesses an empty anti-binding molecular orbital π_y^*. The reaction with olefines containing allylic hydrogen yields allylic hydroperoxide by an ene reaction, whereas endoperoxide is produced by [4 + 2] cycloaddition of *cis* 1,3-diene (Fig. 4).[93,94]

$^1O_2$ can be produced by either light-dependent or light-independent (dark reaction) processes in biological systems.[95] Type II photosensitized oxidation is responsible for the production of $^1O_2$ in the light-dependent process. That is, $^1O_2$ is generated from ground-state $^3O_2$ by ultraviolet-A (UV-A) or visible light energy transfer through the photodynamic action of the sensitizer, as shown in Fig. 5.[96]

An excited sensitizer reacts with organic compounds in type I photosensitized oxidation, while it reacts with $^3O_2$ to yield $^1O_2$ by energy transfer or superoxide anion ($O_2^{\bullet-}$) by electron transfer in the type II reaction.[97,98] Riboflavin,[99] *N*-formylkynurenine,[100,101] which is a metabolic intermediate of tryptophan, and chlorophylls and their related porphyrin compounds such as pheophytin and pheophorbide[102–104] are frequently referred to as natural photosensitizers inducing photooxidation in biological systems. Psoralene and its related furocoumarins

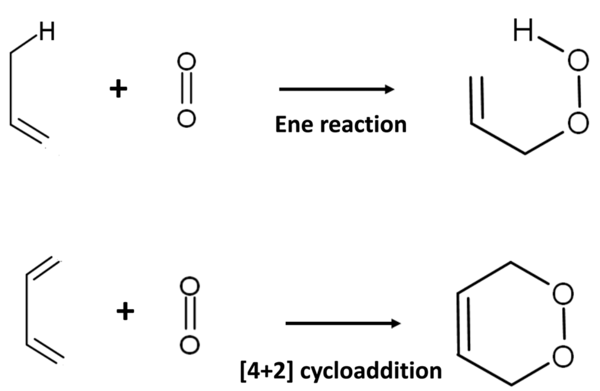

Fig. 4 Oxygenation reaction of $^1O_2$ with olefines and *cis* 1,3-diene.

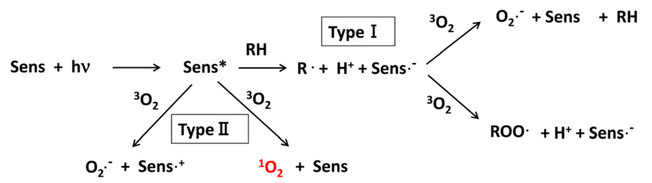

Fig. 5 Mechanism of reactive oxygen species (ROS) generation in photosensitized oxidation reaction. Sens: photosensitizer, Sens*: excited photosensitizer, $h\nu$: light energy, $^3O_2$: triplet state oxygen molecule, $^1O_2$: singlet state excited oxygen molecule, RH: organic compound, $ROO^\bullet$: peroxyl radical, $O_2^{\bullet-}$: superoxide anion radical.

derived from citrus fruits such as oranges and grapefruits are potential photosensitizers leading to photo-carcinogenesis[105] and are believed to act as type II sensitizers because they can produce $^1O_2$ under UV-A irradiation.[106,107] Further research is required to understand the effect of citrus consumption on the risk of photo-carcinogenesis,[108] although the daily intake of psoralene seems to be below the safety threshold.[109]

Squalene hydroperoxides, which are the products of $^1O_2$ oxygenation of squalene, were detected in the human skin surface after exposure to sunlight[110] or UV-A radiation.[111] It was suggested that squalene functions to prevent light-induced lipid peroxidation in human skin surface by acting as the first target of $^1O_2$.[112] Nevertheless, squalene hydroperoxides may cause oxidative damage in the skin.[113] Extracellular matrix proteins[114] and 3-hydroxypyridine derivatives[115] are proposed to act as endogenous photosensitizers in photo-oxidative damage in human skin cells. Coproporphyrin produced from cutaneous *Propionibacterium acnes* was found to generate $^1O_2$ by acting as a type II photosensitizer on the skin surface under exposure to UV radiation.[116]

Two major processes are proposed for light-independent $^1O_2$ generation in biological systems.[117,118] Firstly, the so-called Russell mechanism is the autocatalytic decomposition of peroxyl radicals ($RCOO^\bullet$) involving cyclization *via* a tetraoxide (RCOO–OOCR) intermediate (eqn (1)).[119]

$$\begin{aligned} R_1COO^\bullet + R_2COO^\bullet &\rightarrow R_1COO\text{--}OOCR_2 \\ &\rightarrow R_1C = O + R_2\text{--}COH + {}^1O_2 \end{aligned} \quad (1)$$

Direct evidence for the generation of $^1O_2$ from lipid peroxyl radicals was provided using ^{18}O-labeled linoleic acid hydroperoxide[120] or cholesterol hydroperoxide[121] and near-infrared emission analysis. Generation of $^1O_2$ from lipid peroxyl radicals by the Russell mechanism implies that radical chain lipid peroxidation progresses together with the generation of both $^1O_2$ and chain-propagating lipid peroxyl radicals. Miyamoto *et al.*[122] indicated that the reaction of lipid hydroperoxides with peroxynitrite leads to $^1O_2$ generation through the formation of lipid peroxyl radicals and the tetraoxide intermediate according to the Russell mechanism. They also suggested that cytochrome *c*-promoted phospholipid oxidation yields $^1O_2$ from an excited triplet carbonyl intermediate, as well as from lipid peroxyl radicals *via* the Russell mechanism.[123] Interestingly, Mano *et al.*[124] recently demonstrated that chemically and enzymatically nascent excited carbonyls generate $^1O_2$ *via* energy transfer to $^3O_2$. The alternative mechanism is a reaction of hypochlorous acid (HOCl) with hydrogen peroxide (H_2O_2) (eqn (2)).[125]

$$\begin{aligned} H_2O_2 + Cl^- &\rightarrow OCl^- + H_2O \\ H_2O_2 + OCl^- &\rightarrow {}^1O_2 + H_2O + Cl^- \end{aligned} \tag{2}$$

Thus, excited $^1O_2$ is partly generated by the myeloperoxidase reaction in phagocytosing leukocytes. It was also reported that linoleic acid hydroperoxide can generate $^1O_2$ by a reaction with HOCl.[126] Notably, haloamines of amino acids and polyamines, especially bromoamines, were found to generate $^1O_2$ in a reaction with H_2O_2.[127] $^1O_2$ may be readily generated during the inflammation process because inflammation is tightly linked with phagocytosis by leucocytes. The $^1O_2$ generated during inflammation can lead to serious biological defects.

4.3 Reactions of $^1O_2$ with biomolecules and their consequences

The reaction of $^1O_2$ with unsaturated fatty acids produces isomeric hydroperoxides *via* an ene reaction mechanism.[128] The radical chain reaction of lipid peroxidation also produces isomeric hydroperoxides from unsaturated fatty acids. However, the isomeric composition of hydroperoxides derived from $^1O_2$ oxygenation is different from that of those produced by the radical chain reaction because $^1O_2$ oxygenation is a non-radical reaction.[129] In the case of $^1O_2$ oxygenation with linoleic acid (octadeca-9*Z*,12*Z*-dienoic acid), $^1O_2$ attacks the double bond-constituting carbon at either the 9- and 10-position or the 12- and 13-position to yield four hydroperoxyoctadecadienoic acid (HpODE) isomers, 9-HpODE, 10-HpODE, 12-HpODE, and 13-HpODE, by the transfer of allylic hydrogen and the shift of the double bond to the adjacent position (Fig. 6).

The rate constant of $^1O_2$ oxygenation with unsaturated fatty acids ($k_r \simeq 10^5\ M^{-1}\ s^{-1}$) is much larger than that of the radical chain reaction ($k_r \simeq 10^2\ M^{-1}\ s^{-1}$), whereas $^1O_2$ oxygenation terminates without continuation in a chain.[130] Umeno *et al.*[131,132] reported that 10-hydroxyoctadedienoic acid (10-HODE) and 12-HODE, which are specifically derived from $^1O_2$ oxygenation of linoleic acid, were detected in human plasma after reduction and saponification treatments. They suggested that these HODEs are potential biomarkers for the early detection of type 2 diabetes because monocytes seem to be recruited to adipose cells where myeloperoxidase is activated to yield $^1O_2$ during hyperglycemia.[132]

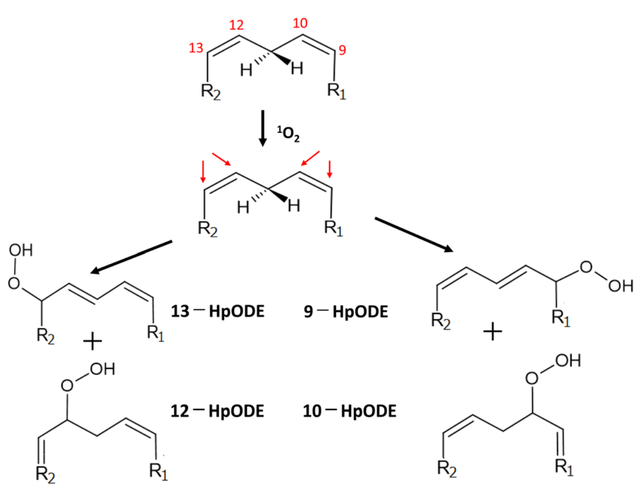

Fig. 6 Production of isomeric hydroperoxides by $^1O_2$ oxygenation of linoleic acid. $R_1 = (CH_2)_7{-}COOH$, $R_2 = (CH_3)_4{-}CH_3$. HpODE: hydroperoxyoctadecadienoic acid.

Cholesterol is an essential constituent of cellular and subcellular membrane lipids. The radical chain reaction scarcely occurs with cholesterol because cholesterol lacks double allyl hydrogens to be eliminated. Nevertheless, this molecule can be a target of $^1O_2$ oxygenation owing to the presence of a double bond between the 5-position and 6-position carbons.[130] The rate constant for the reaction of $^1O_2$ with the double bond of cholesterol does not substantially differ from that for the reaction of $^1O_2$ with the *cis*-double bond of unsaturated fatty acids.[133] This means that $^1O_2$ can attack cholesterol and unsaturated fatty acids equally, resulting in isomeric hydroperoxycholesterols (Chol-OOHs). Fig. 7 shows the pathway of $^1O_2$ oxygenation of cholesterol.

Cholesterol 5α-hydroperoxide (Chol 5α-OOH), which is produced by an ene reaction at the 5,6-double bond position[134] was found to serve as a precursor of cholesterol 5,6-secosterol A and B.[135] These aldehydic cholesterols are called "atheronals" because they are frequently detected in atherosclerotic lesions, and are suggested to participate in the etiology of cardiovascular diseases.[136] Because of their high electrophilicity, aldehydic cholesterols and ketocholesterols can exert potential biological activity by irreversible covalent modification of proteins.[137] Therefore, cholesterol may function as a target molecule in $^1O_2$-dependent lipid peroxidation occurring in biological systems.

Ferroptosis is a nonapoptotic form of cell death and characterized as the occurrence of iron ion- and ROS-dependent lipid peroxidation.[138] Glutathione peroxidase-4 (GPX-4) occupies an essential position in the mitigative cascade of ferroptotic cell death by reducing phospholipid hydroperoxides.[139] Among all the GPX isoforms, only GPX-4 reduces Chol-OOH to hydroxycholesterol (Chol-OH).[140] Chol-OOH is inferior to unsaturated fatty acid hydroperoxides and their esterified phospholipid hydroperoxides as the substrate for GPX-4.[141] The cellular level of lipophilic antioxidants including vitamin E may regulate this type of programmed cell death.[142] Homma *et al.*[143] showed that $^1O_2$ can induce ferroptosis in mouse hepatoma cells exposed to a chemical $^1O_2$ generator. Therefore, the role of dietary carotenoids and vitamin E in $^1O_2$ quenching during ferroptotic cell death will be of much interest.

$^1O_2$ and the hydroxyl radical ($^\bullet$OH) are major oxidants of cellular DNA that lead to deleterious effects.[144,145] $^1O_2$ is a key player contributing to oxidative DNA damage in light-irradiated human skin, as described by Di Mascio *et al.*[146] Among the four nucleobases, guanine shows remarkedly high activity toward $^1O_2$, followed by cytosine, adenine, and then

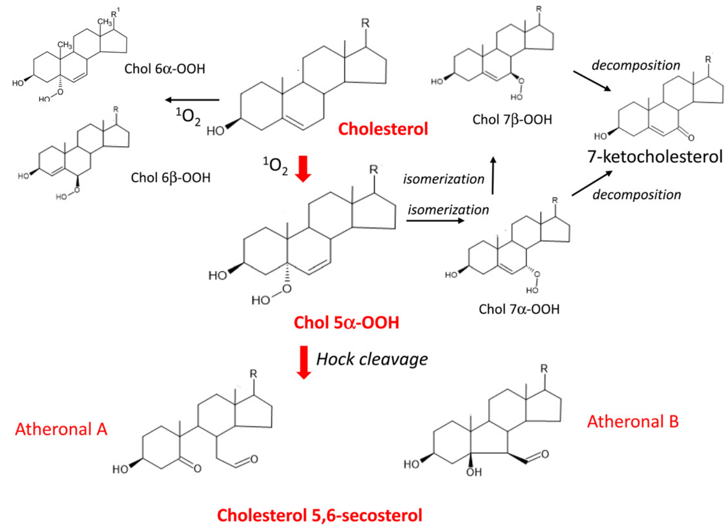

Fig. 7 Pathway of $^1O_2$ oxygenation of cholesterol leading to the production of atheronal A and B. R = C_8H_{17}.

thymine.[147] The rate constants for guanosine and deoxyguanosine are ~10^6 M^{-1} s^{-1}, whereas those of other nucleosides are 10^3–10^4 M^{-1} s^{-1}, indicating that guanine is the sole target in $^1O_2$ oxygenation of DNA and other nucleic acids.[147] Guanine contains electron-rich conjugated double bonds between the 4- and 5-carbons and the 7- and 8-carbons, so it readily reacts with $^1O_2$ *via* a [4 + 2] Diels–Alder-type cycloaddition reaction. 4,8-Endoperoxide is formed as an intermediate of $^1O_2$ oxygenation of the imidazole ring, resulting in 8-hydroperoxydeoxyguanosine (8-OOHGuo). Finally, 8-oxodeoxyguanosine (8-oxoGuo) appears after the reduction of hydroperoxy group of 8-OOHGuo (Fig. 8).[148,149]

Among the amino acids constituting functional proteins, cysteine, histidine, methionine, tryptophan, and tyrosine are known to react with $^1O_2$ specifically (Fig. 9).[150]

The rate constants of $^1O_2$ oxygenation of these amino acids are reported to be approximately 10^6–10^7 M^{-1} s^{-1}.[146,151] In solution, $^1O_2$ oxygenation of cysteine is proposed to produce a persulfoxide intermediate and then a disulfide *via* a reaction

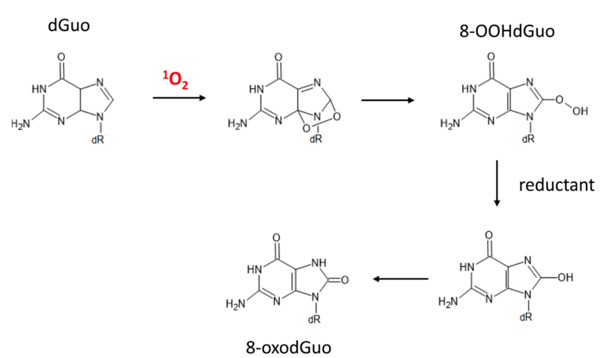

Fig. 8 Degradation pathway of deoxyguanosine induced by $^1O_2$ oxygenation. dR = 2-deoxyribose. dGuo: deoxyguanosine, 8-OOHdGuo: 8-hydroperoxy-deoxyguanosine, 8-oxodGuo: 8-oxo-7,8-dihydroguanosine.

with another unprotonated thiol.[151,152] In the case of methionine, persulfoxide is the first intermediate, and a free amino group attacks this intermediate to generate dehydromethionine and H_2O_2 at neutral pH.[153] Histidine reacts with $^1O_2$, yielding endoperoxides by cycloaddition of $^1O_2$ across the 2,4- and 2,5-carbons of the imidazole ring.[154] Rearrangements of these endoperoxides give rise to three isomeric hydroperoxides. Their subsequent decomposition followed by the nucleophilic attack of the α-amino group produce bicyclic products.[155,156] $^1O_2$ reacts with tyrosine *via* Diels–Alder [4 + 2] cycloaddition to the phenolic ring, producing 1,4-endoperoxide, and subsequent opening of the endoperoxide structure yields labile hydroperoxide.[157,158] Then, bicyclic hydroperoxide is formed by Michael-type addition of the free amino group to the phenolic ring. This cyclization reaction does not occur in the case of tyrosine-containing peptides. The $^1O_2$ oxygenation products of tryptophan are *N*-formylkynurenine and 3-hydroxypyrroloindole.[159] *N*-Formylkynurenine is a key metabolite of tryptophan metabolism in biological systems, and it is generated by the enzyme indoleamine 2,3-dioxygenase 1 (Ido1). This enzyme is expressed in arterial endothelial cells, where it contributes to the regulation of blood pressure during inflammation. It was recently demonstrated that Ido1 generates $^1O_2$ in the presence of H_2O_2 to yield 3-hydroperoxypyrroloindole, which decreases blood pressure *via* oxidation of a specific cysteine residue of endothelial PKG1a (protein kinase G1a).[160] This means that $^1O_2$ derived from the enzymatic reaction serves as a redox signal molecule in the artery under inflammation conditions.[161] It is expected that the roles of $^1O_2$ in pathophysiological conditions will be resolved in the near future.

In terms of the reaction of proteins with $^1O_2$, *N*-formylkinurenine was detected among the reaction products in UV-A-irradiated human lens epithelial cells in the presence of porphyrin.[162] It was confirmed that methionine, histidine, and tryptophan residues are selectively oxidized by the reaction of $^1O_2$ with lysozymes.[163] However, methionine, histidine, and

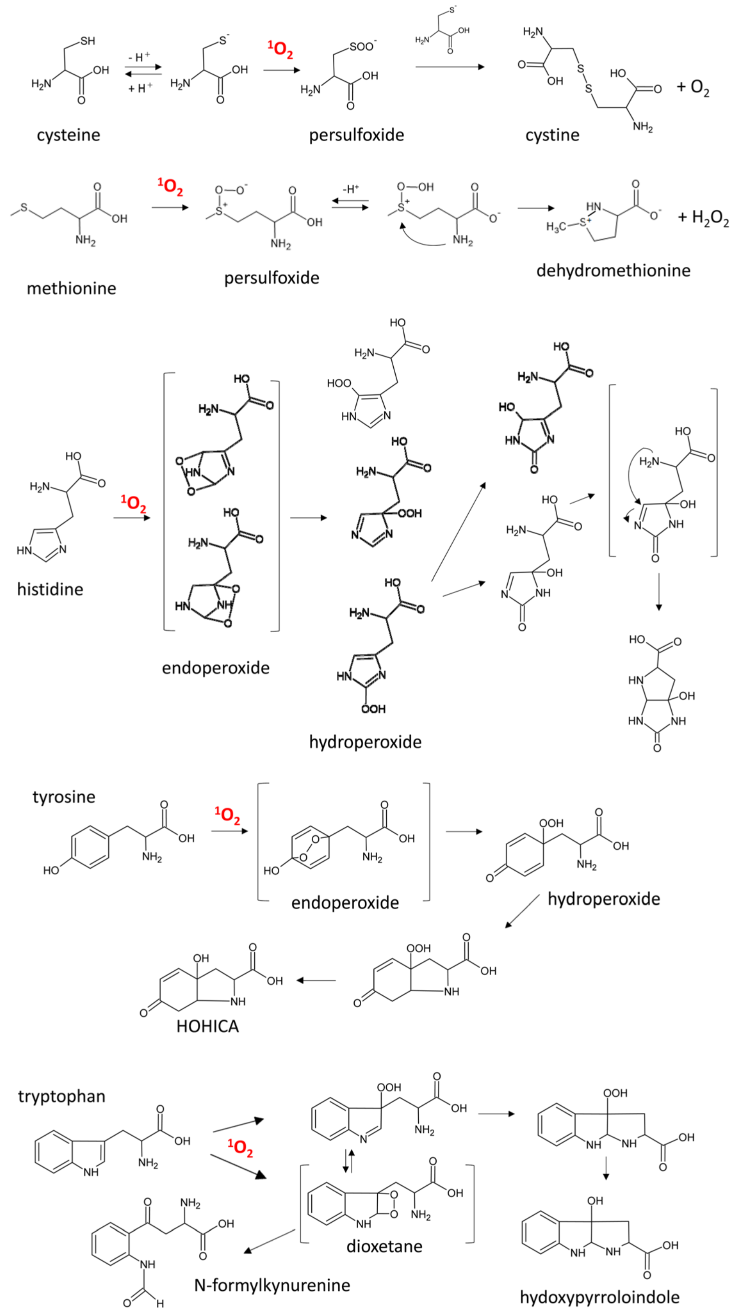

Fig. 9 Main pathways for $^1O_2$ oxygenation of amino acids. HOHICA: 3-a-hydroxy-6-oxo-2,3,3a,6,7,7a-hexahydro-1*H*-indol-2-carboxylic acid.

tryptophan, but not tyrosine, are oxidized in $^1O_2$ oxygenation of cytochrome *c*.[164] Crosslinking between certain residues including histidine and lysine occurs during the reaction of $^1O_2$ with proteins.[164,165] The $^1O_2$ oxygenation of proteins is more complex than that of other biomolecules, and thus, many aspects of these reactions remain obscure.

4.4 Singlet oxygen quenching mechanism

Carotenoids seem to exert a potential role in human health and disease by acting as powerful antioxidants against $^1O_2$.[166] The antioxidant effect of carotenoids against $^1O_2$ is based on the physical quenching of $^1O_2$ (eqn (3)) and a chemical reaction with $^1O_2$ (eqn (4)).

Physical quenching:

$$\begin{aligned} {}^1O_2 + {}^1\mathrm{CAR} &\rightarrow {}^3O_2 + {}^3\mathrm{CAR}^* \\ {}^3\mathrm{CAR}^* &\rightarrow {}^1\mathrm{CAR} + \mathrm{heat} \end{aligned} \tag{3}$$

Chemical reaction:

$$ {}^1O_2 + {}^1\mathrm{CAR} \rightarrow \text{reaction products.} \tag{4}$$

In physical quenching, $^1O_2$ is deactivated to the ground state oxygen molecule ($^3O_2$) by energy transfer from $^1O_2$ to the ground state carotenoid molecule (^{1}CAR). The resulting excited triplet state carotenoid molecule (^{3}CAR*) returns to the original ^{1}CAR by releasing its excited energy to the exogeneous environment as heat energy.[167] In the chemical reaction, $^1O_2$ reacts with carotenoids to yield oxygenated products. For example, the addition of $^1O_2$ to the 5- and 8-positions of β-carotene generates β-carotene 5,8-endoperoxide.[168,169] The rate constant of the physical quenching reaction (k_q) is about 1000 times larger than that of the chemical reaction (k_r), indicating that one carotenoid molecule can quench about 1000 molecules of $^1O_2$ efficiently prior to its consumption by the chemical reaction with $^1O_2$. In solution, the rate constant of the physical quenching reaction is close to that of the diffusion-controlled reaction (~10^{10} M^{-1} s^{-1}) and 30–100 times larger than that of a representative lipophilic antioxidant, α-tocopherol.[170] Using a competitive reaction method, Aizawa *et al.*[171] confirmed the superiority of dietary carotenoids to α-tocopherol in terms of their ability to quench $^1O_2$.

Table 2 shows the rate constants of $^1O_2$ quenching by major carotenoids in solution and liposomal membranes.[16,167,172] Because nine or more conjugated double bonds are required for $^1O_2$ quenching, neither retinol nor retinoic acid serve as $^1O_2$ quenchers.[173] Di Mascio *et al.*[16] clarified that lycopene has the highest $^1O_2$-quenching ability in solution. However, the $^1O_2$-quenching activity of each carotenoid is significantly decreased in the liposomal model membrane system because the mobility of carotenoids is restricted when located in the lipid bilayer of the liposome structure.[174] This means that the $^1O_2$-quenching effect of carotenoids is unexpectedly low in biological systems as compared with that in solution because lipophilic carotenoids are presumed to be concentrated in the phospholipid bilayer of cellular and subcellular biomembranes. Carotenoids contain a long-chain hydrocarbon that stretches in lipid bilayer membranes,[175] and polar carotenoids such as xanthophylls have their polar group anchored in the opposite polar zone of the membrane.[176] The difference in orientation in a model membrane between β-carotene and zeaxanthin was pointed out by Cerezo *et al.*[177] Bosio *et al.*[178] emphasized that intracellular $^1O_2$ is not efficiently quenched by β-carotene in β-carotene-incorporated mammalian cells. Thus, the efficiency of carotenoids as $^1O_2$ quenchers *in vivo* remains a subject of argument. Nevertheless, carotenoids present in biomembranes together with α-tocopherol are likely to inhibit $^1O_2$-dependent oxidative damage *in vivo.*[179]

4.5 Reaction with free radicals and antioxidant/prooxidant effects

It has been suggested that carotenoids can act as antioxidants by scavenging free radicals, which induce the free radical-mediated radical chain reaction of lipid peroxidation.[180] Carotenoids are proposed to inhibit this chain reaction efficiently at low-oxygen pressure in biological environments.[23,181] In the lipid phase, the radical chain reaction occurs *via* chain-initiating radicals ($X^•$) and propagates until chain-propagating lipid peroxyl radicals ($LOO^•$) disappear from this phase. Chain-breaking antioxidants can stop the chain reaction by scavenging $LOO^•$. Carotenoids react with $LOO^•$ at the position of conjugated double bonds to yield the radical adduct of the carotenoid and $LOO^•$. This carbon radical ($LOO\text{-}CAR^•$) is subject to resonance stabilization at low oxygen pressure, so that the chain-termination reaction prevails over the chain-propagation reaction (Fig. 10).

Carotenoids were earlier reported to inhibit liposomal lipid peroxidation significantly by scavenging chain-propagating $LOO^•$.[27] However, the antioxidant activity of carotenoids as free radical scavengers was found to be much lower than that of α-tocopherol.[182,183] Furthermore, assuming that the concentration of α-tocopherol is higher than that of carotenoids in cellular and subcellular membranes, it is reasonable to assume that carotenoids are significantly inferior to α-tocopherol as free radical scavengers against the radical chain reaction of lipid peroxidation occurring in biomembranes.

Carotenoids may change from antioxidants to prooxidants under increased oxygen partial pressure. Under higher oxygen

Table 2 Rate constants for $^1O_2$ quenching reaction by carotenoids

	k_q ($\times10^8$ M^{-1} s^{-1})				
	Homogenous solvent			DPPC[b] liposomes	
Carotenoids	EtOH/CHCl$_3$/H$_2$O[a]	benzene	Micelle solution	RB[c]	PBA[d]
Lycopene	310	170	20	24	23
α-Carotene	190	120	—	—	—
β-Carotene	140	130	24	23	25
Zeaxanthin	100	120	25	2.3	1.7
Lutein	80	66	33	1.1	0.82
β-Cryptoxanthin	60	—	—	—	—

"—": not determined. [a] EtOH/CHCl$_3$/H$_2$O (50/50/1, v/v/v). [b] DPPC: dipalmitoylphosphatidylcholine. [c] Water-soluble rose bengal (RB) was used as type II photosensitizer. [d] Lipid soluble 1-pyrenebutyric acid (RBA) was used as type II photosensitizer.

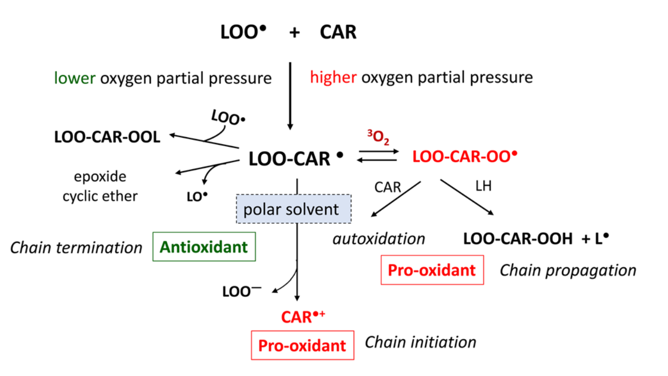

Fig. 10 Antioxidant and prooxidant mechanisms of carotenoids in their radical trapping reaction. CAR: carotenoid, LH: unsaturated lipids, $LO^•$: lipid alkoxyl radical, $LOO^•$: lipid peroxyl radical, $LOO\text{-}CAR^•$: lipid peroxyl-carotene radical, $LOO\text{-}CAR\text{-}OO^•$: lipid peroxyl-carotene peroxyl radical, LOO^-: lipid peroxyl anion, $CAR^{•+}$: carotenoid cation radical.

partial pressure, LOO-CAR$^•$, which is generated by the addition reaction of a carotenoid with LOO$^•$, reacts with the ground state oxygen molecule ($^3O_2$) to yield a peroxyl radical (LOO-CAR-OO$^•$). These radicals abstract a hydrogen molecule from unsaturated lipids to yield a lipid radical (L$^•$), thereby accelerating the chain-propagation reaction of lipid peroxidation. El-Agamey and McGarvey[184] showed that the addition reaction of a carotenoid with LOO$^•$ progresses differently depending on the properties of the reaction solvent. In polar solvents, a carotenoid radical cation (CAR$^{•+}$) and a peroxyl anion (LOO$^-$) are produced from LOO-CAR$^•$, while in non-polar solvents the S_Hi reaction proceeds to yield epoxides or cyclic ethers from LOO-CAR$^•$.

Switching from an antioxidant to a prooxidant is a critical factor when estimating the role of carotenoids in human health and disease prevention.[185] Boehm *et al.*[186] demonstrated that lycopene switched from an antioxidant to a prooxidant in human lymphoid cell membranes when the oxygen concentration increased. This switch can also depend on the carotenoid concentration, whereby carotenoids at higher concentrations can act as prooxidants.[187] Furthermore, the reaction products of carotenoids with free radicals can also determine whether carotenoids act as antioxidants or prooxidants.[188] The carotenoid radical cation (CAR$^{•+}$) serves as a strong oxidant of biological components, leading to oxidative damage.[188] For example, these radicals participate in the oxidative modification of amino acids such as tyrosine, tryptophan, and cysteine.[189] However, as shown in eqn (5) and (6), vitamin E (T-OH) and vitamin C (AsA) may have protective roles against the prooxidant effect of carotenoids by converting CAR$^{•+}$ to the original CAR form.[190,191]

$$CAR^{•+} + T\text{–}OH \rightarrow CAR + T\text{–}O^• \quad (5)$$

$$CAR^{•+} + AsA \rightarrow CAR + AsA^• \quad (6)$$

Palozza *et al.*[39] noted that carotenoids may act as prooxidants at relatively high concentrations and under chronic oxidative stress. In large-scale β-carotene intervention studies, namely the CARET study and the ATBC trial, it was speculated that the reason why β-carotene supplements were deleterious to smokers was the combined effect of excess β-carotene accumulation and vitamin C deficiency.[189]

Peroxynitrite (ONOO$^-$), generated by the reaction of nitric oxide (NO) with the superoxide anion radical ($O_2^{•-}$), is a powerful oxidant in biological systems.[192] Carotenoids are suggested to scavenge ONOO$^-$ by producing nitro compounds.[193–195] Therefore, carotenoids may suppress oxidative damage derived from the combination of NO and O2$^{•-}$ in biological systems.

4.6 Activation of the Nrf2 signaling pathway and indirect antioxidant activity

Dietary antioxidants may exert their physiological functions during oxidative stress by modulating intracellular signaling pathways that affect the translocation of transcription factors.[196] It is known that carotenoids control the expression of genes encoding antioxidant enzymes by enhancing the transcription of the *Nrf2* gene.[197,198] In this sense, carotenoids act as indirect antioxidants, by enhancing other antioxidant defenses that protect against the oxidative stress that causes chronic diseases.

An elaborate mechanism underlies the activation of the Nrf2 transcription factor, as shown in Fig. 11.[199,200]

In a steady state, Nrf2 is bound to Kelch-like ECH-associated protein-1 (Keap1) resulting in ubiquitinated Nrf2, which is successively decomposed by the ubiquitin–proteasome system. When intracellular oxidative stress is elevated owing to the imbalance between oxidants and antioxidants, a cysteine residue in Keap1 is oxidized to create an intramolecular disulfide bond. This leads to a structural transition, and ultimately the release of Nrf2 from the Nrf2–Keap1 complex without ubiquitination. Free Nrf2 is easily translocated into the nucleus where this transcription factor induces the expression of genes encoding phase II enzymes, antioxidant enzymes, and other enzymes that protect against cellular stress. Nrf2 induces gene expression by forming a heterodimer with the small Maf transcription factor, and then binding to the electrophile response element (EpRE) in the promoter region of its target genes. Electrophilic compounds can also react with the SH group of Keap1 to activate Nrf2 by the same mechanism. Thus, Keap1 regulates the activity of the Nrf2 signaling pathway and acts as a sensor for oxidative and electrophilic stresses. Antioxidant enzymes induced by the Nrf2–Keap1 system include the glutamate–cysteine ligase regulatory subunit (GCLm) for the synthesis of glutathione, as well as heme oxygenase-1 (HO-1) and NAD(P):quinone oxidoreductase-1 (NQO-1).

Experiments using cultured cells have demonstrated that carotenoids induce the expression of genes encoding antioxidant enzymes by activating Nrf2 signaling pathways. For example, lycopene protected mouse photoreceptor cells against light-induced oxidative damage through Nrf2 activation.[201] Lutein significantly enhanced Nrf2 translocation to

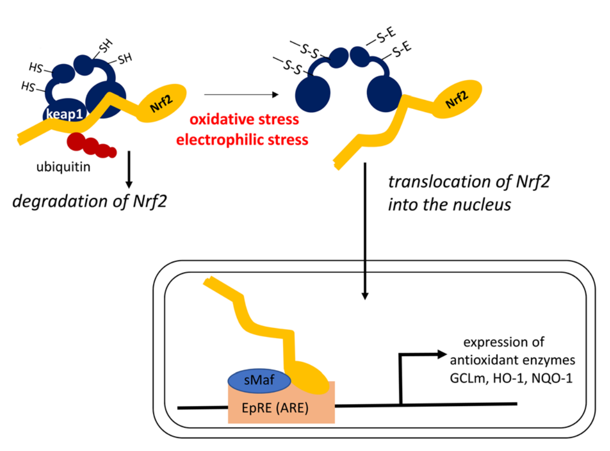

Fig. 11 Oxidative/electrophilic stress-induced activation of the Nrf-2/Keap1 pathway, which induces the expression of genes encoding antioxidant enzymes. EpRE (ARE): electrophile response element (antioxidant response element).

the nucleus and increased the abundance of NQO1, GCLm, and HO-1 in human retinal pigment epithelial cells,[202] and protected cerebrovascular endothelial cells against β-amyloid peptide-induced oxidative stress by upregulating Nrf2 expression.[203] Zeaxanthin promoted the nuclear translocation of Nrf2 in human retinal pigment epithelium cells exposed to *tert*-butyl hydroperoxide-induced oxidative stress.[204] Similarly, β-cryptoxanthin attenuated H_2O_2-induced oxidative stress in human renal tubular epithelial cells by promoting Nrf2 nuclear translocation.[205]

In vivo studies on the indirect antioxidant effects of carotenoids have generally used rodent models. In a cortical impact model mimicking traumatic brain injury, administration of β-carotene to mice reduced ROS levels in the brain, accompanied by nuclear accumulation of Nrf2 and decreased Keap1 expression, indicating that β-carotene alleviated oxidative stress by modulating the Nrf2/Keap1-mediated pathway.[206] Rats fed with a high-fat diet supplemented with lycopene and tomato extract showed increased abundance of nuclear Nrf2 and HO-1 proteins and inhibition of nonalcoholic steatohepatitis-promoted hepatocarcinogenesis.[207] Supplementation with lycopene was found to alleviate oxidative stress-induced neuroinflammation and cognitive impairment of D-galactose-loaded mice by modulating the Nrf2/nuclear factor kappa B (NF-κB) transcriptional pathway.[208] Lycopene also ameliorated renal injury by activating the Nrf2 antioxidant signaling pathway in the aflatoxin B1-induced nephrotoxicity mouse model[209] and attenuated oxidative stress and inflammation *via* the interaction of NF-κB, mitogen-activated protein kinases (MAPKs), and Nrf2 signaling pathways in the chronic prostatitis/chronic pelvic pain syndrome rat model.[210] Lycopene alleviated hepatic injury in ischemia reperfusion-stressed mice *via* Nrf2 activation[211] and suppressed phthalate-induced oxidative stress in phthalate-treated mice *via* mediating the Nrf2 signaling pathway in Leydig cells.[212] Lutein showed a protective effect against hepatotoxicity by enhancing Nrf2 signaling in the arsenic-induced oxidative stress mouse model,[213] and suppressed oxidative stress and inflammation in ovariectomized rats by Nrf2 activation.[214] Zeaxanthin supplementation to rats increased the level of GSH, which contributed to the reduced lipid and protein oxidation in the retina.[204] β-Cryptoxanthin ameliorated metabolic risk factors by regulating Nrf2 pathways and NF-κB pathways in rats with insulin resistance induced by a high-fat diet.[215]

Wang[216] explored whether the physiological effect of lycopene results from its metabolites generated by the activity of BCO2, that is, apo-10′-lycopenoids. In fact, it was confirmed that the BCO2-generated lycopene metabolites apo-10′-lycopenoic acid and apo-10′-lycopenal induced the nuclear accumulation of Nrf2, leading to the induction of antioxidant enzymes such as HO-1 in human bronchial cells.[217] Bohn *et al.*[218,219] suggested that certain carotenoid metabolites act as suitable electrophiles to react with Nrf2. Linnewiel *et al.*[220] demonstrated that carotenoid oxidation products including apocarotenals, but not hydrophobic carotenoids lacking an electrophilic group, actively mediate Nrf2 during stimulation of the Nrf2 signaling pathway by carotenoids. The proposed reaction of apocarotenals with the SH group of Keap1 is shown in eqn (7).

$$\mathrm{R{-}SH + R'{-}CH = CH{-}CH = O \rightarrow R'{-}\underset{\displaystyle \mathrm{R{-}S}}{\underset{|}{CH}}{-}CH_2{-}CH{=}O} \tag{7}$$

Ketocarotenoids with the 3-oxo β-end group derived from oxidative modification of xanthophylls may also participate in antioxidant defenses. It has been suggested that their electrophilic α,β-unsaturated carbonyl group may contribute to the activation of Nrf2 signaling, leading to the regulation of oxidative stress and inflammation.[88] However, the exact mechanism by which dietary carotenoids activate Nrf2 and enhance cellular antioxidant defenses has not been fully demonstrated yet. Many questions remain about the specificity of each enzymatic and/or non-enzymatic oxidation product as the electrophiles inducing nuclear Nrf2 translocation and expression of antioxidant enzymes. Other mechanisms may also contribute to the release of Keap1 from the Nrf2–Keap1 complex by carotenoids. Research to date suggests that the direct promotion of *Nrf2* gene expression may be involved in the indirect antioxidant activity of carotenoids. In addition, it should be noted that the metabolism of dietary carotenoids differs between rodents and humans. The rodents used for *in vivo* experiments are carotene non-accumulators, whereas humans are indiscriminate accumulators.[3] Wu *et al.*[221] pointed out the differences in the properties and distribution of BCO2 between mice and humans. These differences may make it difficult to interpret the results of rodent studies in terms of their relevance to humans. Nevertheless, it is expected that these difficulties will be overcome and that the efficacy of dietary carotenoids as indirect antioxidants for human health will be evaluated accurately.

5. Contribution of antioxidative carotenoids to the prevention of human diseases

5.1 Age-related macular degeneration

AMD is one of the major age-related eye diseases in elderly people suffering from severe visual impairment.[222] The macula is an oval-shaped pigmented area in the center of the human retina with a diameter of around 5.5 mm. It is responsible for central, high-resolution, and color vision. Oxidative stress and inflammation induced by chronic light exposure are believed to be closely related to the onset of AMD, depending on the wavelength, the irradiation time, and the intensity of the light source.[223,224] Blue light with a wavelength of around 450 nm in sunlight causes severe macular damage because the energy of blue light is around 100 times stronger than that of red light. Xanthophylls including lutein, zeaxanthin, and *meso*-zeaxanthin ((3*R*,3′*S*;*meso*)-zeaxanthin), in which the double bond of the lutein ionone ring is shifted to the neighboring site, are specifically distributed in the human retina.[225]

Compared with other human cell types, macular cells accumulate these xanthophylls exclusively and to high levels.[226,227] Lutein supplementation was known to increase macular pigment optical density in a dose-dependent manner.[228] Landrum *et al.*[229] compared macular lutein/zeaxanthin concentrations between control and AMD-affected eyes, and proposed that lower levels of macular pigmentation are a causative factor in AMD development. Overall, dietary lutein and zeaxanthin are thought to help to prevent AMD by acting as macular pigments.[227,230] There are two mechanisms by which macular pigments prevent AMD.[231,232] One is a shielding effect against phototoxic blue light, as the maximum absorption wavelength of lutein/zeaxanthin is nearly 440 nm in accordance with that of blue light. The other mechanism is an antioxidant effect to scavenge or quench ROS generated under exposure to short-wavelength visible light or UV radiation. Several oxidation products of lutein and zeaxanthin have been detected in the human retina, indicating that lutein and zeaxanthin may act as direct antioxidants to protect the macula against short-wave visible light or UV radiation.[233]

Several pilot-scale intervention trials have explored the effects of lutein supplementation on vision impairment. Visual function was found to be improved when lutein was administered to AMD patients.[234–236] It was suggested that lutein supplementation can improve the visual field and visual acuity in patients with retinitis pigmentosa or Usher syndrome.[237,238] Zeaxanthin supplementation provided distinct visual benefits to elderly patients with early atrophic macular degeneration by improving foveal cone-based visual parameters.[239] McGill *et al.*[240] demonstrated that rhesus macaques fed with a diet lacking lutein and zeaxanthin for their whole lifetime displayed significantly higher fundus autofluorescence, indicating that a long-term deficiency of dietary lutein and zeaxanthin induces the progression of macular disease in primates. A large-scale intervention study, the Age-Related Eye Disease Study (AREDS), was conducted with 3640 elderly AMD patients for 6.3 years, and demonstrated that daily supplementation with antioxidants (vitamin C 500 mg, vitamin E 400 IU, and β-carotene 15 mg) and zinc (80 mg as zinc oxide) significantly reduced the development of advanced AMD.[241] A second study, the Age-Related Eye Disease Study 2 (AREDS2), was conducted in 2006–2012. AREDS2 enrolled 4203 elderly people with a high risk of advanced AMD and explored the effect of adding lutein (10 mg per day) + zeaxanthin (2 mg per day) to the formulation used in the initial AREDS.[242] The results implied that lutein + zeaxanthin play a role in reducing the risk of progression to advanced AMD when given without β-carotene, although the addition of lutein + zeaxanthin to the AREDS formulation did not further reduce the risk. An epidemiological 5-year follow-up study of AREDS2 suggested that lutein + zeaxanthin was an appropriate replacement for β-carotene in AREDS2 supplements, as lutein + zeaxanthin supplementation did not increase the risk of lung cancer, unlike β-carotene supplementation.[243] Thus, it is conclusive that lutein and zeaxanthin are essential dietary factors for preventing and treating AMD through selective accumulation in the macula and protection against light-induced macular impairment by acting as antioxidants.

5.2 Skin photodamage and photoaging

Human skin is inevitably exposed to solar light during a person's lifetime. During exposure to sunlight, wavelengths in the UV region with higher energy potential often induce skin damage. Although UV-C radiation (200–290 nm) does not reach the earth's surface because it is absorbed by the ozone layer, UV-B (280–320 nm) and UV-A (320–400 nm) radiation in sunlight are capable of irradiating human skin. UV-B attacks the epidermis to induce severe skin damage, *e.g.*, direct DNA strand breakage and generation of ROS.[244] UV-A can penetrate the dermis to interact with skin chromophores, which then act as photosensitizers to trigger photosensitized oxidation and generate ROS in skin tissue.[245,246] Visible light (400–700 nm) was also reported to contribute to skin damage by producing ROS.[247]

Sunburn is acute skin damage after overexposure to UV radiation and characteristically yields erythema in the skin tissue.[248] Photoaging is chronic skin damage after long-term exposure to sunlight, and is observed as winkles and sagging. Carotenoids are expected to prevent sunlight-induced skin damage because they are powerful antioxidants in skin tissue.[249] Stahl *et al.*[250] demonstrated that the carotenoid level in skin was increased after 12 week's daily ingestion of 24 mg β-carotene in 12 women. The highest accumulation of β-carotene was in forehead skin (1.36 ± 0.23 nmol g^{-1}), followed by skin on the palm of the hand (1.03 ± 0.12 nmol g^{-1}), and then skin on the back of the hand (0.54 ± 0.52 nmol g^{-1}). Several intervention studies with supplements or a carotenoid-rich diet documented the efficiency of carotenoids in photoprotection in human skin, as measured by decreased sensitivity to UV-induced erythema.[251] Stahl *et al.*[252] demonstrated that the combined intake of mixed carotenoids (25 mg per day) and α-tocopherol (500 IU per day) for 8 weeks suppressed erythema formation after 24 h of blue light irradiation. They also clarified that supplementation with β-carotene (24 mg per day) and supplementation with mixed carotenoids (β-carotene, lutein, lycopene, each 8 mg per day) for 12 weeks reduced the intensity of erythema after 24 h of UV irradiation.[253] Daily intake of tomato paste was shown to improve the photoprotection of skin from UV irradiation. In intervention studies where tomato paste (16 mg lycopene per day) was supplied to healthy adults for 10 weeks[254] and to healthy women for 12 weeks,[255] dorsal erythema formation at 24 h after UV irradiation was lower in those that consumed supplements than in those in the control group (with no tomato paste supplementation). In 2013, Meinke *et al.*[256] conducted a study on 24 healthy volunteers ingesting mixed carotenoid-rich capsules for 8 weeks, and found that increased cutaneous carotenoid levels increased the radical scavenging activity of the skin and provided significant protection against visible and near-infrared light.

Photoaging occurs in the dermis where UV-A radiation may induce ROS-dependent breakdown of extracellular matrix proteins through upregulation of matrix metalloproteinases (MMPs).[257] Interestingly, $^1O_2$ was already thought to mediate UV-A radiation-induced synthesis of an interstitial collagenase, MMP-1, in dermis cells.[258] Then, Polte and Tyrrel[259] reported that peroxidation of membrane lipids is involved in MMP-1 expression in skin fibroblast cells in response to UV-A radiation. The author's group developed a photoaging skin model using hairless mouse with chronic exposure to UV-A radiation. The results of studies using this model suggested that cholesterol hydroperoxides (Ch-OOH) derived from $^1O_2$ oxygenation of membrane lipids mediate the activation of MMP-9, leading to the formation of wrinkles and sagging.[260,261] It was also found that dietary β-carotene can prevent both the expression of MMP-9 and the formation of Chol-OOH in the skin. The results of these animal studies support the idea that dietary carotenoids accumulated in the dermis help to prevent photoaging by acting as direct antioxidants against ROS.[262]

5.3 Antioxidant activity of lycopene in prostate and testis

Epidemiological studies have reported that the plasma lycopene concentration is inversely correlated with the risk of prostate cancer, suggesting that high consumption of lycopene-rich tomatoes is helpful to prevent the incidence of this disease.[263,264] However, a prospective study of lycopene and tomato intake in approximately 29 000 men with a follow-up period of 4.2 years did not support the hypothesis that greater lycopene/tomato product consumption protects against prostate cancer.[265] A systematic review of intervention trials on lycopene supplementation concluded that there is insufficient evidence to support or refute the use of lycopene to prevent prostate cancer.[266] Further studies are required to assess the impact of dietary lycopene consumption upon the incidence of prostate cancer, as a population that may benefit from lycopene supplementation is likely to be predicted.[267,268]

Lycopene accumulates to higher levels in the testis and seminal plasma than in other organs and fluids.[269] The administration of 2 mg lycopene twice a day for 3 months to 30 patients with idiopathic infertility improved their sperm concentration and mobility.[270] Daily ingestion of tomato juice containing 30 mg lycopene for 12 weeks by 44 male infertility patients increased their sperm motility.[271] Ribeiro *et al.*[272] reported that an imbalance between ROS production and antioxidants leads to impairment of sperm function, and semen ROS levels are strongly related to infertility in men. It is therefore rational to conclude that dietary lycopene acts as an essential antioxidant in the testis to prevent ROS-induced sperm dysfunction.

5.4 Antioxidant effect of carotenoids in cardiovascular disease and other chronic diseases

A systematic review of cohort and case-control studies by Bahonar *et al.*[273] in 2000–2017 showed that higher intake of the six main carotenoids (lycopene, α/β carotene, lutein, zeaxanthin, and astaxanthin) was associated with reduced risks of stroke and other cardiovascular events, with multiple mechanisms other than direct antioxidant activity involved in their beneficial effects. Upregulation of paraoxonase 1 (PON1), an antioxidant enzyme attached to high-density lipoprotein with anti-atherogenic potential, was recently proposed to be the antioxidant mechanism by which β-carotene and lycopene protect LDL from lipid peroxidation.[274] A systematic review on diabetic retinopathy therapies concluded that carotenoids can potentially delay the initiation and progression of diabetic retinopathy.[275] In liver diseases including non-alcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD), carotenoids exert a hepatoprotective effect by acting as antioxidants, provitamin A, and regulators of lipid metabolism in hepatocytes.[276,277] Interestingly, a meta-analysis of 12 observational studies showed that total carotenoid intake is inversely associated with depression symptoms, suggesting that dietary intake of carotenoids may help in reducing the risk of depression.[278] At present, the antioxidant activity of dietary carotenoids cannot be definitively identified as the key factor in the prevention of these chronic diseases. However, it is reasonable to conclude that dietary carotenoids elevate the body's own antioxidant defense system through Nrf2 signaling pathways, as well as acting as direct scavengers or quenchers of ROS.[279]

6. Conclusion

Sies[280] recently proposed that oxidative stress can be defined as reductive stress (a deviation to the opposite side of the redox balance), oxidative eustress (physiological deviation within the redox control), and oxidative distress (supraphysiological deviation exceeding redox control), depending on the type of deviation from the steady-state cellular redox balance. In humans, the pleiotropic regulation of oxidative stress can help to preserve lifelong health and prevent a variety of chronic diseases. Carotenoids are among the exogeneous antioxidants that contribute to this regulation. Fig. 12 summarizes the function of dietary carotenoids as exogeneous antioxidants working in the human body.

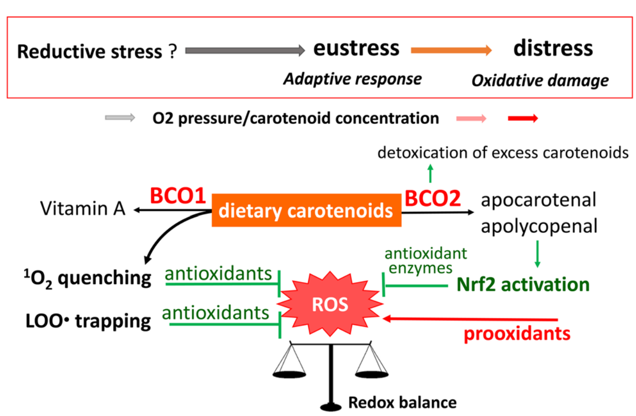

Fig. 12 Proposed scheme for the functions of dietary carotenoids as exogeneous antioxidants in the human body.

Although dietary carotenoids of fruit and vegetable origin are partly converted to vitamin A by the enzyme BCO1, a large proportion is translocated to several tissues and biological fluids. In those tissues and fluids, carotenoids exert a direct antioxidant function based on their inherent structures, and/or an indirect antioxidant function after oxidative modification through the actions of BCO2 and other enzymes or non-enzymatic processes. Physical quenching of $^1O_2$ is an essential part of the direct antioxidant function of carotenoids. Research in recent decades has revealed how $^1O_2$ is generated *in vivo* and how it contributes to the development of biological disorders.[146,281,282] Therefore, $^1O_2$ quenching is recognized as a key role of carotenoids in the prevention of oxidative stress-related diseases. However, the relationship between the site and target of $^1O_2$ generation and the location of hydrophobic carotenoids in microenvironments such as phospholipid bilayers of biomembranes is yet to be elucidated, even though the reaction mechanism of carotenoids with $^1O_2$ is well established. In-depth research is still required to assess the efficacy of carotenoids as $^1O_2$ quenchers *in vivo*.

The antioxidant activity of carotenoids derived from their radical-trapping activity is greatly affected by their concentration and the partial pressure of oxygen at the site of action. Notably, carotenoids are liable to switch from antioxidants to prooxidants in the lipid peroxidation process. Among mammals, humans eccentrically and indiscriminately accumulate carotenoids. As such, humans are predicted to accumulate supplementarily ingested carotenoids in their body to high concentrations, which may induce a switch to prooxidant activity. In addition, each carotenoid is distributed differently in the human body. The mechanisms of, and the reasons for, the high accumulation and different distributions of various carotenoids in the body are still unknown. However, it is known that the dietary lutein and zeaxanthin selectively distributed in the macula contribute to the preservation of vision by protecting the macula from light-induced oxidative stress. The characteristic accumulation of lycopene in the testis may also reflect a pertinent function to protect against oxidative stress, although experimental evidence for this is still insufficient. Skin is an important target for dietary carotenoids to exert their antioxidant activity because human skin is inevitably exposed to light-induced oxidative stress and dietary carotenoids readily accumulate in the skin.

Recently, the induction of antioxidant enzymes *via* activation of the Nrf2 signaling pathway has attracted much attention as an alternative antioxidant function of dietary carotenoids. This induction relates to cellular redox regulation, which leads to oxidative eustress. The electrophilic property is key for the activation of Nrf2, but carotenoids do not have this property *per se*. Instead, their oxidative metabolites that are produced by the activity of BCO2 can induce Nrf2 activation, leading to the promotion of antioxidant defenses. At present, it is difficult to present an overview of the roles of BCO2 in carotenoid metabolism because the distribution and activity of BCO2 in human tissues are yet to be clarified. Further research is urgently required to clarify the role of BCO2 in the antioxidant effect of carotenoids.

Intervention studies verified that β-carotene supplementation had harmful effects on smokers and people exposed to asbestos. An acceptable level of carotenoids other than β-carotene should be assessed for clinical applications. At present, it is recommended to obtain a variety of carotenoids by eating a wide range of fruits and vegetables. It can be hypothesized that, during the process of evolution, the human body has used the antioxidant activity of dietary carotenoids instead of eliminating their potential toxicity. It is expected that carotenoid research will progress and will soon be able to explain why humans are indiscriminate carotenoid accumulators.

Conflicts of interest

The author declares no conflict of interest.

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