Singlet molecular oxygen-quenching activity of carotenoids: relevance to protection of the skin from photoaging

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Carotenoids are known to be potent quenchers of singlet molecular oxygen [O₂ ($^{1}\Delta_{g}$)]. Solar light-induced photooxidative stress causes skin photoaging by accelerating the generation of reactive oxygen species via photodynamic actions in which O_2 ($^{1}\Delta_{g}$) can be generated by energy transfer from excited sensitizers. Thus, dietary carotenoids seem to participate in the prevention of photooxidative stress by accumulating as antioxidants in the skin. An in vivo study using hairless mice clarified that a O_2 (1 Δ_9) oxygenation-specific peroxidation product of cholesterol, cholesterol 5a-hydroperoxide, accumulates in skin lipids due to ultraviolet-A exposure. Matrix metalloproteinase-9, a metalloproteinase family enzyme responsible for the formation of wrinkles and sagging, was enhanced in the skin of ultraviolet-A -irradiated hairless mice. The activation of metalloproteinase-9 and the accumulation of 5α -hydroperoxide, as well as formation of wrinkles and sagging, were lowered in mice fed a β -carotene diet. These results strongly suggest that dietary β-carotene prevents the expression of metalloproteinase-9 (at least in part), by inhibiting the photodynamic action involving the formation of 5a-hydroperoxide in the skin. Intake of β-Carotene therefore appears to be helpful in slowing down ultraviolet-A -induced photoaging in human skin by acting as a O_2 ($^1\Delta_9$) quencher.

Key Words: singlet molecular oxygen, carotenoids, photoaging, cholesterol hydroperoxide, matrix metalloproteinase

real arotenoids are plant pigments containing long-chain hydro- \sim carbons with conjugated double bonds. More than 700 species are categorized as carotenoids. These hydrophobic compounds are often referred to as "biological antioxidants" because they possess potent singlet molecular oxygen $[O_2 (^{1}\Delta_g)]$ -quenching activity and unique free radical-scavenging activity.⁽¹⁾ To prevent radical chain reaction of lipid peroxidation occurring in biomembranes, chain-propagating lipid peroxyl radicals should be scavenged by hydrophobic antioxidants to yield stable non-radical products.⁽²⁾ In 1984, Burton and Ingold⁽³⁾ claimed that β -carotene exerts powerful free-radical scavenging activity at low partial pressures of oxygen (<150 Torr). Radicals trapped by β -carotene are stabilized by their delocalization at conjugated polyene structures, resulting in radical termination reaction. The partial pressure of oxygen in biological tissues under physiological conditions is maintained at such low levels, suggesting that β -carotene and other carotenoids can protect membranous lipids from free radical chain reaction-induced oxidative damages in vivo. It should be noted that α -tocopherol (vitamin E) takes a major part of hydrophobic antioxidants to prevent free radical chain reaction of phospholipid bilayers in biomembranes.(4,5)

Carotenoids are known to be powerful O₂ ($^{1}\Delta_{g}$) quenchers,⁽⁶⁾ and their activities are much higher than that of α -tocopherol

and other biological antioxidants.⁽⁷⁾ Their physical quenching rate constant is reported to be close to the diffusion-controlled rate constant ($\sim 10^{10} \text{ M}^{-1}\text{s}^{-1}$) and is 30–100 times higher than that of α -tocopherol (3 × 10⁸ M⁻¹s⁻¹).⁽⁸⁾ Thus, O₂ (¹ Δ _g)-quenching seems to be a unique characteristic for carotenoids. O₂ ($^{1}\Delta_{g}$) is a nonradical reactive oxygen species (ROS). The reaction of O_2 ($^{1}\Delta_{g}$) with olefinic and aromatic double bonds yields hydroperoxides and endoperoxides, respectively, through the ene reaction in which this electrophilic ROS binds directly to the two side carbons constituting double bonds. Three mechanisms have been proposed to be the source of O₂ ($^{1}\Delta_{g}$) in biological systems: (1) the myeloperoxidase reaction in the phagocytosis of neutrophils,⁽⁹⁾ (2) type-II photosensitized oxidation⁽¹⁰⁾ and (3) bimolecular decomposition of lipid hydroperoxides (Russell mechanism)⁽¹¹⁾ (Fig. 1). Recently, Miyamoto *et al.*⁽¹²⁾ also demonstrated that O₂ ($^{1}\Delta_{g}$) yields in the reaction of lipid hydroperoxides with biologically important oxidants such as metal ions, peroxynitrite and hypochlorous acid. However, there are few in vivo studies on the efficacy of dietary carotenoids as $O_2(^1\Delta_g)$ quenchers and their roles in the prevention of oxidative stress in biological systems.

Chronic exposure of human skin to solar light is known to

(1) Phagocytosis

$$H_2O_2 + CI^- \longrightarrow OCI^- + H_2O$$

 $H_2O_2 + OCI^- \longrightarrow {}^1O_2 + H_2O + CI^-$

(2) Type II Photosensitized oxidation



Fig. 1. O_2 (¹ Δ_g)-generating reactions in biological systems. ¹ O_2 : O_2 (¹ Δ_g).

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Fig. 2. Major pathways of cholesterol oxidation in biological systems. ¹O₂: O₂ (¹Δ₉).

induce photoaging by enhancing the oxidative stress in the dermis and epidermis. Dietary carotenoids seem to be helpful in the prevention of oxidative stress and resulting photoaging in the skin. There are many studies suggesting the effectiveness of carotenoid intake on the lowering of skin photoaging.^(13,14) In this review, we focus on the O₂ ($^{1}\Delta_{g}$)-quenching activity of carotenoids and its physiological significance from the viewpoint of protection of the skin from exposure to ultraviolet-A (UVA). We emphasize that the skin is the most plausible target for dietary carotenoids to exert their functions for human health.

UVA-Induced O₂ ($^{1}\Delta_{9}$) Generation and Lipid Peroxidation in the Skin of an Animal Model

Human skin is inevitably exposed to solar light during one's lifetime. Among solar-light exposure, the ultraviolet (UV) region often causes skin damage because of its high energy potential. UVC light (200-290 nm) is unlikely to reach the surface of the earth because of the barrier of the ozone layer. Thus, UVB (280-320 nm) and UVA (320-400 nm) regions are major sources inducing skin damage. UVB possesses higher energy to induce severe damage to the skin (e.g., DNA breaks). This UV light attacks the epidermis (<0.5 mm) but does not reach the dermis, whereas UVA can penetrate the dermis (1-4 mm).⁽¹⁵⁾ The initial step of UVA-induced skin damage is thought to be its interaction with skin chromophores.⁽¹⁶⁾ In general, chromophores act as photosensitizers to trigger photosensitized oxidation in skin tissue. This process accelerates oxidative stress in the skin by generating free radicals and/or O₂ ($^{1}\Delta_{g}$). Coproporphyrin produced from Propionibacterium acnes has been implicated as a photosensitizer in human skin tissue.⁽¹⁷⁾ Proteins of the extramatrix and 3hydroxypyridine chromophore have been suggested to be endogeneous photosensitizers present in human skin cells.^(18,19) Lipid hydroperoxides are produced as the primary oxidation products of skin lipids via photosensitized oxidation and after radical chain reactions.

Cholesterol is one of the major classes of skin lipids. The reactivity of cholesterol with $O_2(^1\Delta_g)$ is similar to that of unsaturated fatty acids (rate constant; $5.7 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ for cholesterol and 3×10^4 M⁻¹s⁻¹ for each non-conjugated *cis*-double bond for C18 unsaturated fatty acids⁽²⁰⁾). Figure 2 shows the different pathways of cholesterol oxidation between the reactions of cholesterol with O_2 ($^1\Delta_g$) and with free radicals. Cholesterol 5 α -hydroperoxide (Chol 5 α -OOH), a O₂ (¹ Δ_g) oxygenation-specific product of cholesterol,⁽²¹⁾ was detected in the skin when rats were administered pheophorbide, a type-II photosensitizer, and exposed to visible light.⁽²²⁾ Hairless mice have frequently been used as experimental models of photoaging of human skin.⁽²³⁾ We recently detected Chol 5 α -OOH, along with cholesterol 7 α / β -hydroperoxide (Chol $7\alpha/\beta$ -OOH), hydroperoxidized products of cholesterol derived from free radical attack or isomerization of Chol 5a-OOH, in the skin of hairless mice, and revealed that the level of Chol 5a-OOH increased significantly after UVA irradiation of these animals.⁽²⁴⁾ Thus, it is evident that $O_2(^{1}\Delta_g)$ participates in lipid peroxidation and the resulting oxidative stress in the UVAirradiated skin of hairless mice. Interestingly, Chol 5α -OOH was recently suggested to be a precursor of biologically active cholesterol aldehydes, i.e., cholesterol 5,6-secosterols.⁽²⁵⁾ Attention should be paid on the accumulation of Chol 5α -OOH and its decomposition to cholesterol aldehydes in skin tissue because these cholesterol aldehydes are reported to covalently modify proteins, resulting in various pathological conditions.(26-28)

In addition to cholesterol, unsaturated fatty acids are targets of O_2 (¹ Δ_g) oxygenation in UVA-exposed skin tissue. Unsaturated fatty acids such as oleic acid and linoleic acid in the skin of hairless mice are constituted as their esterified lipids such as phospholipids and alkyldiacyl glycerol. $^{(29,30)}$ O2 $(^{1}\Delta_{g})$ oxygenationspecific lipid hydroperoxide isomers appeared in the peroxidized phospholipids of mouse skin homogenate after irradiation with UVA light.⁽³¹⁾ We recently developed a new method for the gas chromatography-mass spectrometry (GC-MS) analysis of esterified fatty acid hydroperoxides, and demonstrated that $O_2(^{1}\Delta_g)$ oxygenation-specific hydroperoxides isomers accumulated in the skin lipids of hairless mice at higher levels due to the exposure of UVA.⁽³²⁾ These animal studies clearly indicated that O₂ ($^{1}\Delta_{g}$) originating from type-II photosensitized oxidation participates in the oxidative stress and injury to the skin seen after chronic exposure to UVA.

Accumulation of Dietary Carotenoids in Human Skin and Protection of UV-Induced Erythema

Carotenoids evidently accumulate in human skin through oral intake of carotenoid-rich foods and supplements. Carotenemia originates from excessive intake of carotene-containing vegetables, resulting in an increased plasma carotene level. It leads to their deposition in skin tissue ("carotenoderma"), indicating that dietary carotenes readily accumulate in human skin.⁽³³⁾ Interestingly, carotenemia and carotenoderma are recognized to be harmless and do not need to be treated. Stahl et al. (34) carried out repeat supplementation of β-carotene in 12 females for 12 weeks, and clarified that carotenoid levels increased in all areas of the skin, with the highest levels being in the forehead and palm of the hand. They also confirmed that the serum carotene level was positively correlated to the level in the skin. Compared with other mammals, humans absorb a wide variety of carotenoids into the body. Each carotenoid is transferred by simple diffusion and/or a transportermediated process in the epithelial cells of the small intestine, and then secreted into lymph as chylomicron.⁽³⁵⁾ A part of carotenoids can be metabolized into retinol and other oxidative chain-scission products by enzymatic and non-enzymatic cleavage during absorption into the small intestine.^(36,37)

An intervention study demonstrated that dietary carotene protects the human skin from the UV light-induced erythema.⁽³⁸⁾ Stahl *et al.*⁽³⁹⁾ found that combination of a relatively low dose of total carotenoids (25 mg/day) and vitamin E (*RRR*- α -tocopherol; 335 mg/day) significantly diminished the erythema on dorsal skin induced by illumination with UV light after 8 weeks. They also revealed that the intensity of erythema 24 h after the irradiation of UV light was diminished in a group receiving mixed carotenoids containing β -carotene, lutein and lycopene (8 mg each/day) for 12 weeks.⁽⁴⁰⁾ In a separate study, intake of tomato paste rich in lycopene (40 g/day) was also shown to be effective in the prevention of erythema formation induced by UV light after 10 weeks.⁽⁴¹⁾ These studies strongly suggest that dietary carotenoids accumulate preferentially in the skin and prevent it from UV-induced photooxidative damage by acting as antioxidants.

Protective Roles of Carotenoids on Skin Photoaging and Their Underlying Mechanism

Skin photoaging is characterized by the degradation of collagen and elastin, leading to an enhancement of the dermis with wrinkles and sagging.⁽⁴²⁾ The matrix metalloproteinase (MMP) family, in particular MMP-2 and MMP-9, are responsible for the collagenase activity on collagen type IV, which constitute the basement membrane of the skin.⁽⁴³⁾ Studies on cultured cell lines demonstrated that MMPs are activated by attack by ROS.⁽⁴⁴⁾ MMP-9 expression proceeds through the oxidative stress-induced mitogenactivated protein kinase (MAPK) pathway.⁽⁴⁵⁾ Activation of MMP- 9 induces the degradation of collagen in the dermis and basal membrane, resulting in the formation of deep and large wrinkles through disruption of the skin structure.⁽⁴⁶⁾ ROS-dependent MMP-9 expression induced by UVA exposure is therefore involved in the onset and progression of skin photoaging. We found that MMP-9 activity was enhanced by intracutaneous injection of a cholesterol hydroperoxide mixture (Chol-5aOOH, 45.6%; Chol 7α -OOH, 12.4%; Chol 7 β -OOH, 42.0%) into the skin of hairless mice.(47) This is the first report that lipid hydroperoxides can induce MMP activity in skin tissue. Although hydrogen peroxide (an inorganic hydroperoxide) and 4-hydroxynonenal (a reactive secondary product of lipid peroxidation) were shown to induce MMP expression in cultured cell lines,^(48,49) neither compounds exhibited any effect on MMP-9 activity in the skin of live rodents.⁽⁴⁷⁾ The precise mechanism through which MMP-9 is activated by cholesterol hydroperoxide mixture is incompletely understood. Cholesterol hydroperoxides may act as lipophilic redox signaling molecules by translocation between cell membranes.⁽⁵⁰⁾ Cholesterol hydroperoxides accumulate in the skin by type-II photosensitized oxidation through the generation of O₂ $(^{1}\Delta_{g})$.⁽²²⁾ Interestingly, an isoform of the glutathione peroxidase family, GPx-4, but not other isoforms, can convert cholesterol hydroperoxides to stable hydroxyl cholesterol through two-electron reduction.⁽⁵¹⁾ MMP-9 activation in the skin under UVA irradiation is at least partly mediated by the action of peroxidized cholesterol accumulated in the skin.

Supplementation of β -carotene in the diet was shown to increase β-carotene content in the skin tissue of hairless mice and our ex vivo study demonstrated that dietary β-carotene could inhibit O_2 (¹ Δ_g) oxygenation of unsaturated lipids in the skin of hairless mice exposed to UVA.⁽³¹⁾ Furthermore, we also found that dietary β -carotene suppresses MMP-9 expression together with accumulation of cholesterol hydroperoxides, in particular Chol 5 α -OOH, in the skin after UVA-irradiation.⁽⁴⁷⁾ The oxidation products of β-carotene accumulated in the skin of hairless mice were investigated to confirm the chemical reaction of β -carotene with ROS (Bando N, Minami Y, Terao J. unpublished data). It is known that β -carotene-5,8-endoperoxide is an oxidation product of β -carotene specific to O₂ ($^{1}\Delta_{g}$) oxygenation, whereas β -carotene 5,6-epoxide is produced by the free radical-mediated reaction of cholesterol and the isomerization of cholesterol 5,8-endoperoxide (Fig. 3).^(52,53) We found that β -carotene 5,8-endoperoxide and β carotene 5,6-epoxide were yielded as cholesterol oxidation products in the skin of hairless mice (Fig. 4A). A single dose of UVA to the dorsal skin of hairless mice enhanced the amount of these oxidation products as compared with skin without UVA irradiation (Fig. 4B). This indicated that β -carotene can react with O₂ ($^{1}\Delta_{g}$) together with free radicals to produce these oxidation products in the skin. This phenomenon is indirect evidence that β -carotene quenches O₂ ($^{1}\Delta_{g}$) to prevent skin photoaging through the physical quenching of $O_2(^1\Delta_g)$ simultaneously occurring with the chemical reaction.

It can be concluded that dietary carotenoids suppress photodynamic action involving O₂ ($^{1}\Delta_{\text{g}}$) oxygenation in UVA-exposed skin, and that this phenomenon is a promising strategy for the prevention of skin photoaging. An alternative mechanism from O₂ ($^{1}\Delta_{\text{g}}$) quenching may be due to their physical properties affecting cellular and subcellular membranes. A recent study suggested that carotenoids affect the lipid raft structure of cellular membranes in which physiologically essential reactions occur in relation to various signal transduction pathways.⁽⁵⁴⁾ Further studies are warranted on the mechanism of the protective role of carotenoids in skin photoaging.

Conclusion

Carotenoids have long been known to be the most effective O_2 ($^{1}\Delta_{g}$) quenchers and that they readily accumulate in human skin



Fig. 3. Structures of primary oxidation n products of β -carotene with reactive oxygen species. ¹O₂: O₂ (¹ Δ ₉).



Fig. 4. Effect of ultraviolet-A (UVA) irradiation on the accumulation of primary oxidation products of β -carotene in the skin of hairless mice. A: HPLC chromatogram of β -carotene oxidation product standards and extracts from skin tissue. B: Comparison of the concentrations of primary oxidation products from mouse skin with and without UVA irradiation. Hos: HR-1 hairless mice (male, 8 weeks of age) were fed a diet containing β -carotene (50 mg/100 g diet) and α -tocopherol (5 mg/100 g diet) for 3 weeks. The mice covered with leather on half of their back received UVA light at 47 J/cm² on one occasion. Irradiated and non-irradiated areas were obtained after 12 h from the irradiation period, extracted with ethanol/hexane (1/1 vol) and analyzed using HPLC equipped with an ODS-80Ts column (4.6 × 250 mm) (Tosoh Company, Tokyo, Japan) and a mobile phase consisting of methanol/ acetonitrile/dichloromethane/water (7/7/2/0.16 by vol.) at 450 nm.



Fig. 5. Proposed relationship between dietary β-carotene and peroxidation of cholesterol in ultraviolet-A (UVA)-induced skin photoaging. ¹O₂: O₂ (¹Δ₉), Chol 5α-OOH: Cholesterol 5α-hydroperoxide, Chol 7α/β-OOH: 7α/β-hydroperoxide, MMP: matrix metalloproteinase.

due to the intake of carotenoid-rich foods and supplements. The results of animal model experiments indicate the accumulation of cholesterol hydroperoxides in the skin are accelerated by UVA irradiation and that these peroxidized skin lipids mediate the enhancement of MMP expression, resulting in the formation of skin wrinkles and sagging. Dietary carotenoids can suppress MMP expression (at least in part) by attenuating the accumulation of peroxidized skin lipids, including cholesterol hydroperoxides (Fig. 5). In this context, the antioxidant activity of dietary carotenoids principally targets the skin. Carotenoids play a major part in the quenching of O_2 ($^{1}\Delta_{g}$) generated by UVA-dependent photodynamic action among a wide variety of dietary antioxidants. The intake of carotenoid-rich foods and supplements therefore appear to be helpful for protecting the skin from photoaging.

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