Supplementation with Carotenoids Inhibits Singlet Oxygen-Mediated Oxidation of Human Plasma Low-Density Lipoprotein

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To study the role of carotenoids in the antioxidant defense against oxidative modification of low-density lipoprotein (LDL) particles, human LDL rich in β-carotene and lycopene was prepared from a healthy volunteer following long-term supplementation with tomato juice. This carotenoid-supplemented LDL accumulated cholesteryl ester hydroperoxides (CE-OOH) more slowly than the LDL prepared before supplementation when the suspensions containing these LDL were subjected to a singlet oxygen-generating system. However, there was no significant difference in the rate of CE-OOH accumulation between the two suspensions when they were exposed to a water-soluble radical generator. Therefore, it is strongly suggested that supplementation of LDL with carotenoids mainly improves the antioxidant defense against the attack of singlet oxygen.

Keywords: *Carotenoids; singlet oxygen; low-density lipoprotein; atherosclerosis*

INTRODUCTION

A wide variety of carotenoids are distributed in fruits and vegetables, and humans nonselectively absorb these compounds and accumulate them in plasma and tissues (Goodwin, 1984). The physiological function of carotenoids other than provitamin A activity is still obscure. Nevertheless, it is well-known that carotenoids can act as antioxidants by quenching singlet oxygen ($^1O_2$) (Foote and Denny, 1968; Dimasio et al., 1989) and scavenging free radicals (Burton and Ingold, 1984; Kennedy and Liebler, 1992; Palloza et al., 1992; Terao 1989; Boey et al., 1992). On the other hand, much attention has been given to the role of β-carotene in the prevention of cardiovascular disease (Gaziano and Hennekens, 1993). Oxidative modification of plasma low-density lipoprotein (LDL) is suggested to lead to the formation of lipid-laden foam cells in atherosclerotic lesions (Steinberg et al., 1989). Carotenoids within LDL particles may participate in the protection of LDL from the oxidative modification leading to the foam cell (Esterbauer, 1989). *In vitro* supplementation of LDL with β-carotene inhibited oxidation induced both by copper ion and by human monocyte macrophage (Jialal et al., 1991; Lavy et al., 1993). Dixon et al. (1994) reported that *in vivo* supplementation of LDL with β-carotene was also effective in the inhibition of copper-induced oxidation. However, Prinston et al. (1992) and Reaven et al. (1993) found that β-carotene supplementation did not increase the protection to copper-mediated oxidation of LDL.

We previously demonstrated that continuous ingestion of tomato juice by healthy volunteers significantly elevated the concentration of β-carotene and lycopene in their plasma (Sakamoto et al., 1994). This prompted us to investigate the effect of ingestion of tomato juice on the protection of LDL to oxidative modification. The present work was conducted to determine whether carotenoids within the LDL can serve as $^1O_2$ quenchers and/or free radical scavengers when LDL particles are exposed to oxidative attack.

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MATERIALS AND METHODS

Materials. *trans*-β-8′-Apocarotenal was obtained from the Sigma Chemical Co. (St. Louis, MO). Methylene blue and 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH) were purchased from Wako Pure Chemicals (Tokyo, Japan). Cholesteryl linoleate was obtained from the Sigma Chemical Co., and its hydroperoxy derivative was prepared by photosensitized oxidation using methylene blue (Ojima et al., 1993). Tomato juice was supplied by local distributors. Other reagents and solvents were of analytical or HPLC grade and were used without purification.

Preparation of LDL. Prior to the study, blood samples were collected from 76 healthy volunteers working at the same place (male and female; 20−51 years old), and the concentration of carotenoids in their plasma preparations was measured by using an HPLC technique (Ojima et al., 1993). One volunteer (26-year-old male) whose plasma was poor in carotenoid content was selected for this study. The plasma preparation was subjected to ultracentrifugation immediately in the density range 1.019−1.063 g/mL as previously described (Ojima et al., 1993). The LDL fraction obtained was dialyzed with phosphate-buffered saline (PBS; pH 7.4) containing 100 μM diethylenetriaminepentaacetic acid to prevent the prooxidant effect of contaminant metal ion. After dialysis, the LDL fraction was stored at −80 °C until use (20 days).

To obtain carotenoid-supplemented LDL, the volunteer supplemented his daily diet with 160 g of tomato juice for 19 days. The amount of tomato juice per day was equivalent to a single lycopene dose of 0.44 μmol/kg of body weight. At the end of the supplementation period, blood was again collected and the plasma prepared immediately by centrifugation. The LDL fraction was obtained by the same method as described above and stored at −80 °C for 1 day.

Oxidation of LDL. The LDL suspension was diluted by PBS to adjust the final concentration of cholesteryl ester (1.57 μmol/mL), and 990 μL of the resulting suspension was mixed with 10 μL of a methylene blue solution. The final concentration of methylene blue was 1.0 mM. The mixed suspension was then photoirradiated by fluorescent lamp (light intensity at the sample, 10 000 lx) at 37 °C with continuous shaking.

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Table 1. Carotenoids and α-Tocopherol Contents in LDL Fractions before and after Supplementation

	contents (nmol/mg of protein)					
	zeaxanthin/lutein	cryptoxanthin	lycopene	α-carotene	β-carotene	α-tocopherol
before supplementation	0.20	0.26	0.11	0.12	0.15	28.1
after supplementation	0.29	0.14	0.73	0.21	0.87	35.6

At specific intervals, an aliquot of the suspension (100 μL) was withdrawn and an ethanol solution (100 μL) of *trans*-β-8′-apocarotenal was added to the mixture as an internal standard. A solution of *n*-hexane and dichloromethane (4:1, v/v, 1.0 mL) was added to the mixture, and the resultant mixture was centrifuged (3000 rpm for 3 min). The supernatant (800 μL) was removed and evaporated under nitrogen gas. The residue was dissolved in a mixture of methanol, acetonitrile, dichloromethane, and water (7:7:2:0.16, v/v/v/v) for HPLC analysis. In the case of radical generator-induced oxidation of LDL, the concentration of cholesteryl ester was adjusted in the same way and mixed with the solution of AAPH (final concentration, 1.0 mM) instead of the methylene blue solution. The mixture was incubated in the dark at 37 °C. The following procedures were the same as those for phooxidized LDL.

Determination of Cholesteryl Ester Hydroperoxides (CE-OOH). CE-OOH were determined by reversed phase HPLC with UV detection at 235 nm as described previously (Ojima et al., 1993). The concentration of CE-OOH was tentatively calculated from the standard curve of the hydroperoxy derivative of cholesteryl linoleate.

Determination of Carotenoids and α-Tocopherol. Carotenoids were quantified by HPLC using a column of Lichrospher RP 18-5 (E. Merck, Darmstadt, Germany) with an eluting solvent of methanol, acetonitrile, dichloromethane, and water (7:7:2:0.16, v/v/v/v) at a flow rate of 1.0 mL/min. The effluent was monitored at 450 nm using a Shimadzu SPD-10AV spectrophotometric detector (Shimadzu, Kyoto, Japan). A column of Shim-Pack CLC-ODS (Shimadzu) was used for the determination of α-tocopherol and eluted with methanol and acetonitrile (1:9, v/v). The flow rate was set at 3.5 mL/min and the effluent was monitored with a spectrofluorophotometer (Shimadzu RF-10A, Shimadzu) at an extinction wavelength of 298 nm and an emission wavelength of 325 nm.

Determination of Other Components. The concentration of protein in the preparation was determined using the Protein Assay Kit II (Bio-Rad Laboratories Inc., Hercules, CA). The concentrations of total cholesterol and free cholesterol were determined enzymatically using Cholesterol E Test-Wako and Free Cholesterol-E Test-Wako (Wako Pure Chemicals., Tokyo, Japan). The concentration of cholesteryl ester was calculated by subtraction of the concentration of free cholesterol from that of total cholesterol.

RESULTS

Effect of Tomato Juice Supplementation on the Concentration of Carotenoids in Plasma LDL. The average concentration of the plasma carotenoids of 76 volunteers was 1.93 μM, whereas that of the selected one for this study was 0.43 μM. This value was increased to 1.88 μM after supplementation with tomato juice for 19 days. In particular, the concentrations of lycopene and β-carotene were elevated from 0.05 and 0.02 μM to 0.60 and 0.49 μM, respectively. Table 1 shows the concentration of each carotenoid and α-tocopherol in LDL fractions obtained from these plasma preparations. Similarly to the plasma preparation, the contents of lycopene and β-carotene within LDL were increased about 6.0-fold by supplementation with tomato juice. The contents of α-tocopherol, α-carotene, and zeaxanthin or lutein also increased slightly although that of cryptoxanthin decreased considerably. Total carotenoid content increased from 0.84 protein to 2.23 nmol/mg of protein by the supplementation. Therefore, the LDL fraction from the plasma after supplementation and that from the plasma before supplementation were used in the following experiments as carotenoid-supplemented LDL and carotenoid-poor LDL, respectively.

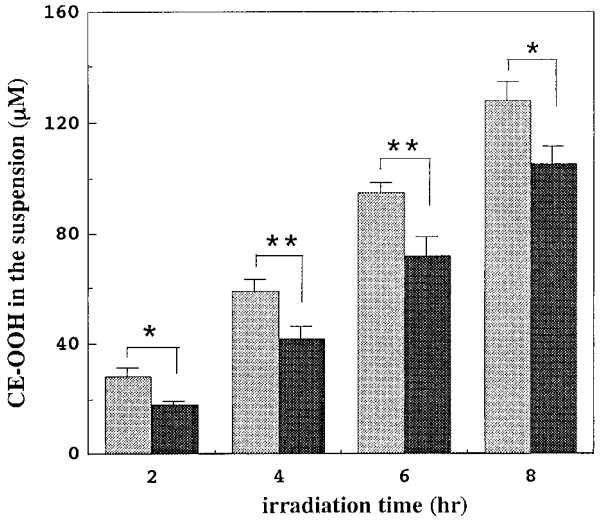

Figure 1. Accumulation of CE-OOH by photoirradiation of human plasma LDL in the presence of methylene blue: dark columns, carotenoid-supplemented LDL; light columns, carotenoid-poor LDL. The concentration of cholesteryl ester in the two suspensions was adjusted to 1.57 μmol/mL. The protein concentration of the preparations were 0.50 and 0.32 mg/mL for LDL before supplementation and LDL after supplementation, respectively. The concentration of total carotenoids, lycopene, β-carotene, and α-tocopherol were 0.417, 0.054, 0.072, and 12.04 μmol/L for carotenoid-poor LDL and 0.714, 0.233, 0.280, and 11.39 μmol/L for carotenoid-supplemented LDL, respectively. CE-OOH were not detected in the preparations before incubation. Results were expressed as the mean ± SD of three experiments. Asterisks indicate significant differences between carotenoid-supplemented LDL and carotenoid-poor LDL: *, $p < 0.05$; **, $p < 0.01$.

Comparison of the Methylene Blue-Sensitized Oxidation and AAPH-Induced Oxidation between Carotenoid-Supplemented LDL and Cartoenoid-Poor LDL. Methylene blue-sensitized oxidation of the two LDL suspensions was compared by adjusting the concentration of cholesteryl ester of the two suspensions. The concentration of total carotenoids was 0.71 μM for the suspension of carotenoid-supplemented LDL and 0.42 μM for the suspension of carotenoid-poor LDL. In contrast, the two suspensions contained almost the same level of α-tocopherol, that is, 11.39 μM for carotenoid-supplemented LDL and 12.04 μM for carotenoid-poor LDL, respectively. In the both suspensions, CE-OOH accumulated linearly with the elapse of photoirradiation time as shown in Figure 1. The amount of CE-OOH in the carotenoid-supplemented LDL suspension was lower than that in the carotenoid-poor LDL in each incubation period, indicating that carotenoid-supplemented LDL was more resistant to photosensitized oxidation than carotenoid-poor LDL. In each LDL suspension, β-carotene and lycopene were consumed slower than α-tocopherol, although these three compounds decreased simultaneously (Figure 2). In addition, consumption rates of the two carotenoids were slightly slower in carotenoid-supplemented LDL than in the carotenoid-poor LDL.

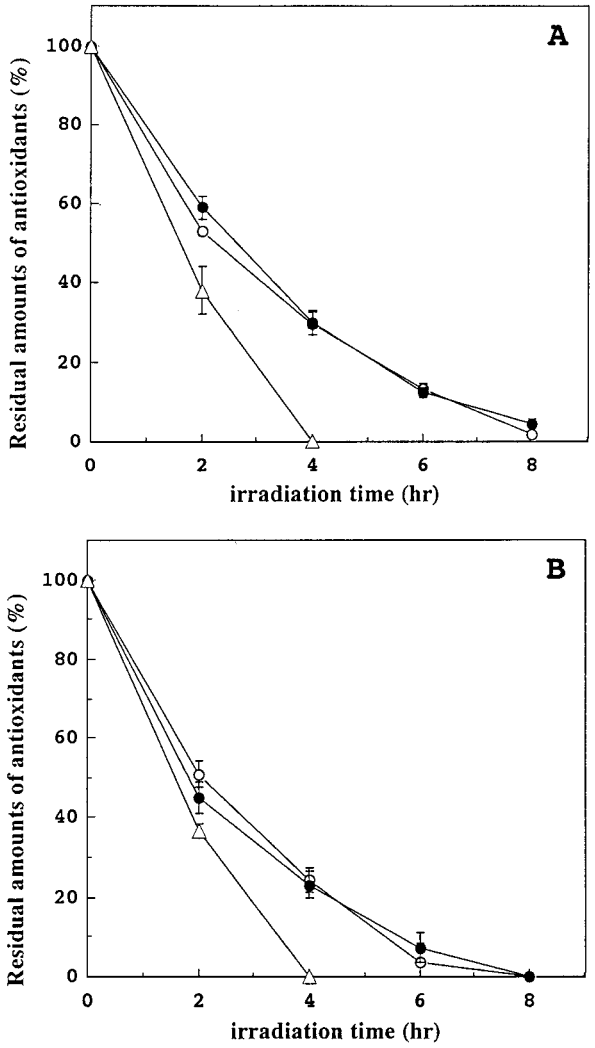

Figure 2. Loss of lycopene, β-carotene, and α-tocopherol by photoirradiation of the suspension of human plasma LDL in the presence of methylene blue: (A) carotenoid-supplemented LDL; (B) carotenoid-poor LDL. ○, lycopene; ●, β-carotene; △, α-tocopherol. Results were expressed as the mean ± SD of the three experiments.

Figure 3 shows the accumulation of CE-OOH in AAPH-induced oxidation of the two suspensions. Contrary to the results of photosensitized oxidation, there was no significant difference in the amounts of CE-OOH at each incubation period between the two suspensions. This indicates that the oxidizability against AAPH-derived radicals is indistinguishable between carotenoid-supplemented LDL and carotenoid-poor LDL in this reaction system.

DISCUSSION

Stahl et al. (1992) reported that the lycopene concentration in human serum did not increase when unprocessed tomato juice was consumed, although the consumption of heat-processed tomato juice increased its serum concentration. Rupture of cell walls during the process may be accompanied by the release of lycopene from the cells. Thus, processes related to heating seem to improve the availability of lycopene. However, we succeeded in the preparation of carotenoid-rich LDL from a healthy volunteer by repeated dosing with unprocessed tomato juice. Interestingly, β-carotene, as well as lycopene, accumulated in the LDL at a higher level, although the amount of β-carotene corresponded

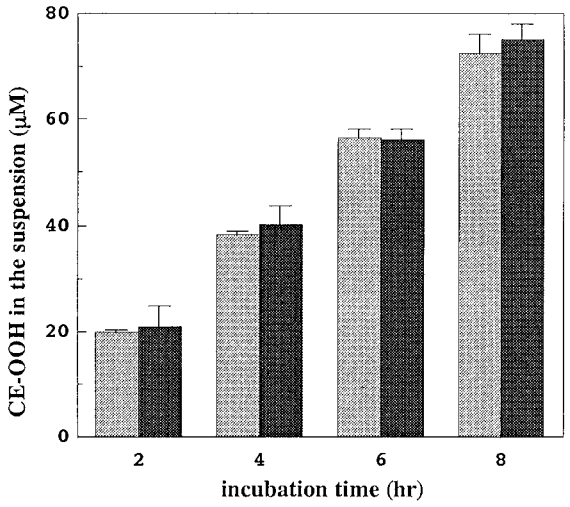

Figure 3. Accumulation of CE-OOH by AAPH-induced oxidation of the suspension of human plasma LDL: dark columns, carotenoid-supplemented LDL; light columns, carotenoid-poor LDL. The suspensions of the two LDL preparations were the same as those used in the experiments in Figure 2. Results were expressed as the mean ± SD of three experiments.

to only 3% of that of lycopene in tomato juice. Our previous study using 65 volunteers also demonstrated that continuous supplementation of tomato juice increased the concentrations of both β-carotene and lycopene (Sakamoto et al., 1994).

Recently, Wagner et al. (1993) showed that lycopene and β-carotene are included in the principal $^1O_2$ quenchers within human LDL. They suggested that beneficial effects of these compounds in health may in part be due to the elimination of $^1O_2$ occurring *in vivo*. The present study selected methylene blue-sensitized photooxidation (type II) as an $^1O_2$-generating system (Ojima et al., 1993) and has clearly shown that carotenoid-supplemented LDL is more resistant to $^1O_2$-mediated oxidation than carotenoid-poor LDL (Figure 1). Although α-tocopherol is also included in the principal $^1O_2$ quenchers in LDL (Wagner et al., 1993), no difference in the content of α-tocopherol was found between carotenoid-supplemented LDL and carotenoid-poor LDL. It has been demonstrated that phagocytosing neutrophils (Steinbeck et al., 1992) and stimulated macrophages (Steinbeck et al., 1993) can generate this oxygen species. Therefore, it seems plausible that LDL in the blood stream or within the arterial wall can be exposed to $^1O_2$. As far as we know, the present study is the first to demonstrate that the supplementation of LDL with carotenoids increases the resistance against the $^1O_2$ oxidation of LDL.

Many studies have shown that β-carotene can serve as a chain-breaking antioxidant against free radical-mediated lipid peroxidation by trapping chain-propagating peroxyl radicals (Burton and Ingold, 1984; Kennedy and Liebler, 1992; Palloza et al., 1992). We previously clarified that carotenoids other than β-carotene also possess activity as chain-breaking antioxidants in solution (Terao, 1989) and model membranes (Boey et al., 1992). However, we could not find inhibition of the free-radical attack of LDL by the supplementation of LDL with carotenoid (Figure 3). Gaziano et al. (1995) also clarified that supplementation with β-carotene *in vivo* does not enhance the protection of LDL against free-radical oxidation. In general, the free-radical-scavenging activity of carotenoids is much lower than that of α-tocopherol (Krinsky, 1989). Thus, it is unlikely that the supplementation of carotenoids increases the antioxidant activity of LDL against free-radical attack.

It is suggested that a trace level of lipid hydroperoxides is required for the continuous radical chain oxidation of LDL induced by copper ion (Thomas et al., 1994) and myoglobin (Hogg et al., 1994). It can be therefore concluded that $^1O_2$ quenching by carotenoids in LDL particles at least partly has a physiological role in the prevention of atherosclerotic lesions, because lipid hydroperoxides formed by the reaction of LDL lipids with $^1O_2$ can trigger the continuous chain oxidation.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; CE-OOH, cholesteryl ester hydroperoxides; LDL, low-density lipoprotein; $^1O_2$, singlet oxygen.

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