Inhibition by β -Carotene and Astaxanthin of NADPH-Dependent Microsomal Phospholipid Peroxidation

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Summary To evaluate the antioxidant effects of β -carotene and astaxanthin, rat liver microsomes were exposed to a mixture of chelated iron ($\text{Fe}^{3+}/\text{ADP}$) and NADPH. The carotenoids (190 pmol/mg protein) were incorporated into some of these microsomal membranes, and phospholipid hydroperoxides (PLOOH), thiobarbituric acid reactive substances (TBARS) and endogenous α -tocopherol content were measured over time after the initiation of oxidant stress. In control microsomes, oxidant stress led to accumulation of 1,865 ($\pm$ 371) pmol PLOOH/mg protein during the initial 10-min peroxidation reaction, followed by a more gradual increase during the subsequent 20-min of reaction. PLOOH accumulation during the initial 10-min reaction period was reduced to 588 ($\pm$ 169) pmol/mg protein with β -carotene present and 800 ($\pm$ 288) pmol/mg protein with astaxanthin present. During the following 20-min of incubation, PLOOH levels declined in the carotenoid-supplemented microsomes but continued to increase at a slower rate in control preparations. TBARS did not show such large accumulation as observed in PLOOH during the initial 10-min incubation in any microsomal sample. The presence of carotenoids in the microsomal membrane partially inhibited the loss of α -tocopherol, especially during the later phase of oxidant stress. When lipid peroxidation is generated by membrane-bound cyt-P450, the specific measurement of PLOOH clearly demonstrates that the presence of carotenoids provides antioxidant protection.

Key Words antioxidant, astaxanthin, β -carotene, lipid peroxidation, microsomes

The peroxidation of membrane phospholipids has potential importance in relation to the oxidative damage that occurs during atherogenesis (1–4), aging

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(5–9) and ischemia-reperfusion injury (10,11). Cellular phospholipids consist primarily of phosphatidylethanolamine and phosphatidylcholine, which preferentially distribute respectively in the inner and outer membrane leaflet of the membranous phospholipid bilayers (12–14). During oxidant stress to the membrane, these phospholipids form phosphatidylethanolamine hydroperoxide (PEOOH) and phosphatidylcholine hydroperoxide (PCOOH) (15). These phospholipid hydroperoxides (PLOOH) can lead to abnormality of membrane functions, and further perturbation of cellular signaling pathways. The increase in PLOOH in biological membrane is regarded as one of the most direct evidences of oxygen radical-mediated injury since PLOOH is the primary oxidation product of membrane lipids (4,6–9). The PLOOH accumulation reflects directly a balance between the peroxidation of the membrane lipids and the reduction of peroxidized lipids (15).

Carotenoids have been expected as potential membrane antioxidants due to reactivity with singlet molecular oxygen and peroxy radical (16–19). Several studies on the effects of carotenoids on radical-initiated phospholipid peroxidation in microsomes and model membrane have been reported (19,20). However, the effects of different carotenoids on the accumulation of PEOOH and PCOOH have not been extensively investigated, in spite of the importance of measuring lipid hydroperoxide as the most direct evidence for membrane lipid peroxidation. Since the previous studies (16) have been carried out using much higher concentrations (25 μM –1 mM) of carotenoids, we thought it important to investigate further the antioxidant effect at lower concentrations (0.5–1 μM), as in intact organella membranes (i.e., liver, microsomes and erythrocytes). On the other hand, a simultaneous comparison of antioxidant effect of non-polar carotenoid and polar carotenoid has never been performed for the microsome system.

Therefore, we selected β -carotene (structure [1] in Fig. 1) as the non-polar carotenoid and astaxanthin (structure [2] in Fig. 1) as the polar carotenoid and evaluated their antioxidant activities at a lower concentration (0.4 μM) in NADPH-dependent liver microsomal peroxidation. The specific determination of both PEOOH and PCOOH formed during this reaction was accomplished by the use of high-performance liquid chromatography combined with chemiluminescence detection (CL-HPLC), in which a mixture of cytochrome *c* and luminol was utilized as the post-column chemiluminescence cocktail (21–23).

Fig. 1. Chemical structures of β -carotene [1] and astaxanthin [2].

MATERIALS AND METHODS

Chemicals. β -Carotene was purchased from Sigma Chemical (St. Louis, MO, USA). Astaxanthin was kindly provided by Nihon Roche (Tokyo, Japan). *d*- α -Tocopherol was obtained from Eisai (Tokyo, Japan). All other chemicals used were at least of reagent grade.

Microsome preparation. Liver microsomes were prepared from male Sprague-Dawley rats (6 wk old; Funabashi Farm, Chiba, Japan). Liver was homogenized in 5 volumes of ice-cold 0.25 M sucrose containing 50 mM KCl, 2 mM MgCl_2 and 20 mM Tris-HCl buffer (pH 7.4) (24). The liver homogenate was centrifuged for 15 min at $12,000 \times g$ at 4°C , and the supernatant was collected. After ultracentrifugation of the supernatant at $105,000 \times g$ for 60 min, microsomes were collected from the pellet. The microsomes were washed with recentrifugation at $105,000 \times g$ for 60 min in 0.15 M KCl. The washed microsomes were suspended in 50 mM Tris-HCl buffer (pH 7.4) and stored at -30°C until used. Microsomal protein was determined by the method of Lowry et al. (25).

Incorporation of carotenoid into microsomes. Ten microliters of carotenoid in ethanol (either $115 \mu\text{M}$ β -carotene or $115 \mu\text{M}$ astaxanthin) was added to 1 mL of microsomal suspension (protein concentration, 4.6 mg/mL) and gently vortexed for 1 min in the dark to incorporate the carotenoid into the microsomal membrane. The same amount of ethanol, free of carotenoid, was added to the control microsomes.

The carotenoid-incorporated microsomes were centrifuged at $105,000 \times g$ for 60 min, and the carotenoid content of the pellet was assayed. Approximately 75% of the total carotenoid (190 pmol/mg protein) added to the preparation was found in the microsome pellet.

Peroxidation of microsomes with $\text{NADPH}/\text{Fe}^{3+}/\text{ADP}$. A solution containing both ADP (22 mM) and FeCl_3 (1.3 mM) was prepared in Tris-HCl buffer (50 mM, pH 7.4). A solution of NADPH (1 mM) was prepared in the same buffer. These solutions were mixed with the microsomes to initiate lipid peroxidation (26).

The reaction mixture contained 2.1 mg microsome protein/mL along with 1.0 mM ADP, 0.06 mM Fe^{3+} and 0.05 mM NADPH in a total reaction volume of 2.2 mL. The incubation mixture was shaken in the dark at 37°C under air atmosphere.

Lipid extraction from microsomes. To 1 mL of microsomal suspension, 4 mL of chloroform/methanol (2:1, v/v; containing 90 nM butylated hydroxytoluene (BHT) as antioxidant) was added and mixed vigorously for 1 min. The mixture was centrifuged at $1,000 \times g$ for 15 min. The lower chloroform layer was collected and evaporated to dryness under a nitrogen stream. The microsomal total lipids were redissolved in an appropriate amount of chloroform/methanol (2:1, v/v) and analyzed by CL-HPLC for phospholipid hydroperoxides.

Phospholipid hydroperoxide determination. Both PEOOH and PCOOH in microsomal total lipids were simultaneously determined with CL-HPLC as de-

scribed in method papers by Miyazawa et al. (21–23). CL detection was carried out with a CLD-100 detector (Tohoku Electronic Industries, Sendai, Japan), which employed a cooled photomultiplier tube (PMT) to suppress PMT dark current and improve the S/N ratio of the HPLC analysis. For the hydroperoxide-specific post-column chemiluminescence reagent, a mixture of luminol and cytochrome *c* in 50 mM borate buffer (pH 10.0) was utilized (21–23). Calibration was made with authentic PEOOH and PCOOH standards, as described previously (22).

TBARS assay. Microsomal TBARS, in the presence of 0.04% (w/v) BHT as antioxidant, were measured at 535 nm by the method of Buege and Aust (27), and TBARS was expressed as malondialdehyde.

Carotenoid and α -tocopherol assay. Microsomes were submitted to extraction by the methods of Stahl et al. (28) and Barua and Furr (29) with slight modifications.

To each 1 mL of microsome suspension, 1 mL of 2-propanol and 0.5 mL of 100 mM sodium dodecyl sulfate were added, and the sample was vortexed for 1 min. Then 6 mL of *n*-hexane/dichloromethane (5:1, v/v; containing 1.2 mM BHT) was added, and the sample was sonicated for 3 min (NS80-1.50 sonicator, Nihonseiki, Tokyo, Japan). After an additional 1 min vortexing, the sample was centrifuged at $1,000\times g$ for 15 min and the supernatant was collected. The solvent of the supernatant fraction was evaporated under nitrogen, and the residual fraction was redissolved in 50 μ L of a HPLC column eluant for HPLC analysis.

HPLC separation was carried out with a C18 column (TSK gel ODS-80TS, 4.6×250 mm, Tosoh, Tokyo, Japan): the mobile phase was acetonitrile/methanol/dichloromethane/water/propionic acid (39/39/19/2/1, v/v) at 1.0 mL/min flow rate. The determination of β -carotene, astaxanthin and α -tocopherol was carried out by two Jasco 970-UV detectors (Japan Spectroscopic, Tokyo, Japan) connected in series: the first detector was set at 450 nm for carotenoid analysis, and the second detector was set at 300 nm for determination of α -tocopherol (30).

Statistical analysis. The data were expressed as mean $\pm$ standard deviation (SD). Statistical comparisons were made with ANOVA analysis. Statistical significance was accepted at a *p* value of <0.05 .

RESULTS

Inhibition of microsomal phospholipid hydroperoxide accumulation by carotenoids

Figure 2 shows the typical chemiluminescence chromatograms recorded when the microsomal total lipids extracted from the control and carotenoid-treated microsomes were injected into the CL-HPLC. Both PEOOH and PCOOH were clearly detected as major PLOOH of the microsomes. Rapid accumulation of both PEOOH and PCOOH was observed in control microsomes incubated with NADPH/ Fe^{3+} /ADP, as shown in Fig. 3. The formation of PCOOH consistently occurred at a rate 4.5-fold higher than PEOOH. The addition of either β -carotene or astaxanthin to the microsomes led to a 50–75% inhibition of the accumulation

Fig. 2. Typical CL-HPLC chromatograms of PEOOH and PCOOH in the NADPH-dependent liver microsomal peroxidation system. The microsomal total lipids extracted from the control (A), β -carotene-incorporated (B) and astaxanthin-incorporated (C) microsomes during the initial 10-min peroxidation were analyzed.

of both kinds of PLOOH (Fig. 3). During the later course of incubations, the presence of carotenoids resulted in a decrease in microsomal PLOOH levels, while the PLOOH levels were maintained at high levels in the non-carotenoid treated control microsomes.

Effects of carotenoids on microsomal TBARS accumulation

For all samples, no significant change in TBARS was observed during the first 10-min incubation (Fig. 4). During the following incubation, there was some increase in all samples, to about 50% above the background. However, there was no statistical difference in this rate of TBARS accumulation between the control and carotenoid-supplemented microsome samples.

Loss of microsomal carotenoids

Figure 5 shows the loss of β -carotene and astaxanthin in microsomes during peroxidation initiated by a combination of NADPH/ Fe^{3+} /ADP. Both β -carotene and astaxanthin were consumed at a constant rate during the 30-min incubation. A substantial amount of each carotenoid remained in the microsomes at the end of 30-min of incubation (i.e., 82 pmol β -carotene/mg protein, 43% of the initial value and 55 pmol astaxanthin/mg protein, 30% of the initial value). There were no significant differences in the rate of degradation of either carotenoid.

Fig. 3. Effect of carotenoid on the increase of PEOOH (A) and PCOOH (B) in the NADPH-dependent liver microsomal peroxidation system. The complete microsome system (total volume of 2.2 mL; 2.1 mg microsomal protein/mL, 1.0 mM ADP, 0.06 mM Fe^{3+} , 0.05 mM NADPH in 50 mM Tris-HCl buffer at pH 7.4) was incubated at 37°C in the absence (control microsomes) and presence of β -carotene (190 pmol/mg microsomal protein) or astaxanthin (182 pmol/mg microsomal protein). Each point represents the mean $\pm$ SD of three experiments. *Significantly different from the control microsomes at $p < 0.01$.

Effect of carotenoids on endogenous microsomal α -tocopherol level

Figure 6 shows the effect of β -carotene and astaxanthin on the endogenous α -tocopherol contents of microsomes during peroxidation initiated by a combination of NADPH/ Fe^{3+} /ADP. In the control microsomes, approximately 95% of the endogenous α -tocopherol was degraded during 10-min incubation, and α -tocopherol could not be detected at the end of 30-min incubation. The presence of carotenoids slightly but significantly delayed the loss of α -tocopherol, and some endogenous α -tocopherol was still present in the microsomes at the end of 30-min

Fig. 4. Effect of carotenoid on TBARS formation in the NADPH-dependent liver microsomal peroxidation system. The reaction conditions were the same as those given in Fig. 3. Each point represents the mean $\pm$ SD of three experiments.

Fig. 5. Loss of microsome carotenoid during NADPH-dependent lipid peroxidation. The reaction conditions were the same as in Fig. 3. Each point represents the mean $\pm$ SD of three experiments. *Significantly different from the microsomes at 0-min incubation at $p < 0.01$.

peroxidation.

DISCUSSION

This study shows that both β -carotene and astaxanthin provide antioxidant protection against NADPH-dependent microsomal lipid peroxidation, as measured by the formation of PLOOH. During NADPH-dependent peroxidation of micro-

Fig. 6. Loss of microosomal endogenous α -tocopherol during NADPH-dependent lipid peroxidation. The reaction conditions were the same as those given in Fig. 3. Each point represents the mean $\pm$ SD of three experiments. *Significantly different from the control microsomes at $p < 0.01$.

somes, both PEOOH and PCOOH accumulated over a 30-min incubation (Figs. 2 and 3). In addition, we found that the accumulation of PCOOH was 4.5-fold greater in microsomes than PEOOH. In rat liver microsomes, the molar ratio of phosphatidylethanolamine and phosphatidylcholine is 1:2 (24). This difference in peroxide accumulation between PCOOH and PEOOH reflects, in part, their relative content in the microsomal membrane, together with the difference in unsaturation of their constituent fatty acids.

Both β -carotene and astaxanthin significantly prevented the accumulation of PEOOH and PCOOH in the microsomes (Figs. 2 and 3). From studies in model membranes, it has been concluded that the lengthy hydrocarbon structure of β -carotene lies fully within the hydrophobic acyl region of the bilayer (31). By contrast, astaxanthin has two polar oxygen groups at each end of the molecule, so that this polar carotenoid spans the membrane and each polar end associates with the polar phospholipid head groups (32). Since the reactive polyene chain of each carotenoid is well within the bilayer and provides effective protection against radical reactions propagated inside the membrane, no big difference in antioxidant effectiveness may be observed. This explanation coincides with the finding that the decay of β -carotene and astaxanthin during peroxidation is almost equivalent as revealed in Fig. 5. These observations indicate that there is no meaningful difference in the antioxidant activity between non-polar carotenoid and polar carotenoid in the microsome membrane system.

The TBA reaction was not able to demonstrate a protective effect of added carotenoid against microosomal lipid peroxidation as shown in Fig. 4. In part, this may be due to the high background value (1.5 nmol TBARS/mg protein) of the classic TBA assay (27). In contrast, the background value of PLOOH in native

microsomes collected from animals consuming normal amounts of dietary tocopherol was very low (22 pmol PLOOH/mg protein). The TBA method is generally not appropriate to determine the change in lipid hydroperoxide, especially at the pmol level.

Endogenous α -tocopherol in microsomes was rapidly consumed in all samples during NADPH-dependent lipid peroxidation (Fig. 6). However, in the presence of carotenoids, the tocopherol was not fully eliminated, consistent with the hypothesis that a significant portion of the free radicals is quenched by the carotenoids, thus sparing part of the α -tocopherol. This may be reflected in the decay of carotenoids during peroxidation (Fig. 5). The retardation of the radical-induced loss of endogenous α -tocopherol by carotenoid has also been proposed by Palozza and Krinsky (33).

We observed that carotenoid protective effects were seen even though the amount of carotenoid present was less than the amount of tocopherol in the membranes. The protective effects of carotenoids at this lower level (190 pmol/mg protein; 0.4 μ M) have not been reported previously, except when singlet oxygen is the source of oxidant stress. However, previous work (33) relied primarily on TBARS as the indicator of oxidant stress; in this study, TBARS assay failed to demonstrate protection by carotenoids, but a specific assay of PLOOH showed protection. This may reflect our hypothesis that the antioxidative function of carotenoids is essentially involved in the very initial stage of microsomal membrane phospholipid peroxidation (30).

In studies of chicks, Mayne and Parker (34) could not obtain reliable protection of the hepatic microsomal membrane after high-dose carotenoid supplementation which increased the membrane carotenoid content, but their study also relied on the TBA assay. The application of PLOOH analysis by CL-HPLC will be of interest in animal models of dietary carotenoid supplementation. Most recently, we explained that dietary β -carotene prevents *in vivo* PLOOH formation in red blood cells of mice using CL-HPLC (30). On the other hand, the prevention of skin lipid peroxidation with dietary palm oil carotene has been demonstrated using CL-HPLC (35).

In this study, both β -carotene and astaxanthin revealed effective antioxidant activity against PLOOH formation in microsomal membrane. The antioxidant efficacies of non-polar β -carotene and polar astaxanthin were almost the same in the microsomal membrane. Such antioxidant function in the microsomal membrane was evidently confirmed even under much lower concentrations of carotenoids, at 0.4 μ M (190 pmol/mg protein), than previous investigations (33). Additionally in this study, it was first confirmed that PCOOH is formed in the liver microsome membrane at a rate 4.5-fold higher than PEOOH.

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