ELSEVIER

Addition of lutein, lycopene, or β-carotene to LDL or serum in vitro: Effects on carotenoid distribution, LDL composition, and LDL oxidation

Joelle E. Romanchik,* Earl H. Harrison, and Diane W. Morel

Department of Biochemistry, MCP-Hahnemann School of Medicine, Allegheny University of the Health Sciences, Philadelphia, PA 19129 USA

Carotenoids are dietary antioxidants transported with plasma lipoproteins, primarily low-density lipoprotein (LDL). In this study in vitro methods were used to increase the amounts of specific, individual carotenoids in LDL. By addition of carotenoid to isolated LDL or to serum, followed by (re)isolation of the lipoproteins, samples of LDL were enriched 4- to 150-fold with lutein, 2- to 15-fold with lycopene, or 3- to 25-fold with β-carotene. Enrichment with specific carotenoids was achieved without affecting the electrophoretic mobility of the lipoprotein, its cholesterol to protein ratio, or the levels of other cartenoids or α-tocopherol. The distributions among lipoproteins of carotenoid added to serum were similar, but not identical, to the distributions of the endogenous carotenoids. In particular, for added lutein, a greater proportion was found in HDL, and for added β-carotene, more was found in very low-density lipoprotein (VLDL). We then studied the effect of enriching LDL with specific carotenoids on its susceptibility to oxidation by copper ions. Lutein, β-cryptoxanthin, lycopene, and β-carotene, the four major plasma carotenoids, and α-tocopherol were destroyed before the formation of lipid peroxidation products. The rates of destruction of the individual carotenoids differed; lycopene was destroyed most rapidly and lutein most slowly. Upon oxidation of β-carotene-enriched LDL, the rates of destruction of β-carotene, lycopene, and lutein were slowed and the lag times before the initiation of lipid peroxidation increased from 19 to 65 min. Neither effect was observed in LDL enriched with lutein or lycopene. Thus, β-carotene was unique among the carotenoids studied in having a small, but significant effect on LDL oxidation in vitro. (J. Nutr. Biochem. 8:681–688, 1997) © *Elsevier Science Inc. 1997*

Keywords: human; carotenoids; lipid peroxidation; LDL

Introduction

Carotenoids are a class of lipophilic pigments that are found in nature in a wide variety of fruits and vegetables. There is general interest in their metabolism and potential biologic and health-related function(s) because of well established correlations between ingestion of fruits and vegetables and decreased risk of chronic diseases such as cancer and atherosclerosis. Epidemiologic studies that have examined disease risk and intake of specific carotenoids have suggested an inverse correlation between plasma levels of α-tocopherol and carotenoids and the incidence of coronary heart disease.[1,2] However, in other studies supplementation of diets with β-carotene had no significant effect on cancer or cardiovascular disease (the Physician's Health Study, Ref. 3). Still other studies suggest that β-carotene, in conjunction with vitamin A, actually increased lung cancer (the CARET Study, Ref. 4).

To understand more about the function(s) of carotenoids, it is important to understand the transport and delivery of carotenoids to tissues. Work by us[5] and others[6–8] has shown that carotenoids are transported in plasma with lipoproteins, predominantly low density lipoprotein (LDL). Because carotenoids have the ability to function as antioxidants,[9] it is

Address correspondence and reprint requests to Dr. D.W. Morel, Allegheny University of the Health Sciences, MCP-Hahnemann School of Medicine, Department of Biochemistry, Philadelphia, PA 19129 USA.
*Current address: Department of Family and Consumer Sciences, P.O. Box 8034, Georgia Southern University, Statesboro, GA 30460 USA.
Supported by NIH R01 HL 49879.
Received March 27, 1997; accepted August 5, 1997.

655 Avenue of the Americas, New York, NY 10010

0955-2863/97/$17.00
PII S0955-2863(97)00119-8

possible they could function in part to protect LDL from oxidation. Such protection might be important in the development of atherosclerosis, a disease process hypothesized involve LDL oxidation.[10]

As a consequence of the possible antioxidant function for carotenoids in LDL, there is a need to be able to manipulate the levels of individual carotenoids in the lipoprotein. This can be done by increased ingestion of carotenoid-rich foods. However, as foods contain a mixture of carotenoids, it is difficult to examine the effect of an elevated concentration of a specific carotenoid. This is particularly true because there seems to be some competition for intestinal absorption among carotenoids.[11,12] An alternate approach has been to use dietary supplementation. Plasma (and hence LDL) levels of β-carotene have been successfully increased using β-carotene supplements, but such supplements of other pure carotenoids are not yet available. A number of investigators have attempted to increase the concentration of β-carotene in LDL by addition of the compound in solvent to LDL directly or to serum, followed by isolation of the LDL.[13–17] In some of these studies, β-carotene inhibited LDL oxidation,[13–15] whereas in others, LDL oxidation was unaffected or enhanced.[16,17] In many cases, however, the resulting lipoproteins were not extensively characterized.

In the present studies we addressed three issues. First, we sought to develop a method to increase the concentration of individual carotenoids in LDL without affecting its lipid composition, electrophoretic mobility, or content of endogenous antioxidants. As part of this we also wanted to determine whether addition directly to isolated LDL or addition to serum followed by isolation of LDL yielded better enrichment of the LDL. Second, we wanted to compare the distribution of added carotenoid with that of endogenous carotenoids. This has practical implications for using cartenoid-enriched serum to deliver carotenoids to cells in culture. Third, we wished to examine and compare the effect of enriching LDL with individual carotenoids on the susceptibility of that LDL to oxidation. A standard protocol using copper ions as initiators of lipid peroxidation was used in these studies.

Methods and materials

Materials

β-carotene was purchased from Sigma Chemical Co. (St. Louis, MO USA) and lutein was purchased from Kemin Industries (Des Moines, IA USA). Lycopene was a gift from Dr. Gary Beecher of the USDA (Beltsville, MD USA), and β-cryptoxanthin was a gift from Hoffmann-La Roche (Nutley, NJ USA).

Chemical analyses

Carotenoids and α-tocopherol were extracted from 0.25 to 0.50 mL LDL (0.4 to 1.0 mg protein/mL) by the method of Barua et al.[18] and were separated by reverse-phase high-performance liquid chromatography (HPLC) on a 25-cm Supelcosil LC-18 column (5 μm, 4.6 mm) (Supelco, Bellefonte, PA USA) using a mobile phase of acetonitrile:dichloromethane:methanol:1-octanol (90:15:10:0.1 v/v/v/v) at a flow rate of 1 mL/min.[19] Analytes were detected by monitoring their absorbance at 436 nm (carotenoids) or 290 nm (α-tocopherol) using a variable wavelength detector (Hewlett Packard Series 1050). Amounts were calculated based on standard curves constructed with pure compounds.

Total cholesterol was measured enzymatically using a commercial kit (Sigma Catalog No. 352-50 Sigma Chemical). Protein was assayed by a modified Lowry procedure.[20]

Lipid peroxidation was assessed in three ways. Thiobarbituric acid-reacting substances (TBARS), a widely used, indirect measure of lipid hydroperoxides, was assayed as described by Morel and Chisolm.[21] A direct measure of lipid hydroperoxides was made using the ferrous ion oxidation-xylenol orange (FOX) assay developed by Jiang et al.[22] In addition, for some experiments, the formation of conjugated dienes, an intermediate in the formation of lipid hydroperoxides, was monitored continously in a spectrophotometer (Beckman ACTA II) as the increase in absorbance at 234 nm.[23]

Isolation of LDL

Serum from fasted human subjects was collected by centrifugation of clotted blood (500 × g, 30 min, 4°C). A broad cut LDL fraction (1.006 to 1.063 g/mL) was isolated from serum by sequential ultracentrifugation,[24] followed by dialysis against phosphate buffered saline (PBS) containing 1 mM EDTA. For direct enrichment of LDL, freshly isolated plasma LDL (d = 1.019 to 1.063 g/mL) was obtained from the departmental lipoprotein core laboratory and dialyzed against PBS with 1 mM EDTA. After the enrichment incubations, LDL was reisolated by a single ultracentrifugation after adjusting the density to 1.063 g/mL, then again dialyzed against PBS containing 1 mM EDTA. The purity of all LDL samples was confirmed by agarose gel electrophoresis (Beckman Lipogels) with lipid specific staining (Sudan Black B). In all cases, only a single band was observed. Samples of LDL (in PBS with 1 mM EDTA) were stored at 4°C, under nitrogen, in the dark, for less than 1 week before compositional analysis and use in oxidation experiments.

In vitro enrichment of LDL with carotenoids

Aliquots from stock solutions of 2 mM lutein in ethanol, 6 mM lycopene in tetrahydrofuran (THF), or 8 mM β-carotene in THF, checked for purity by UV spectroscopy and HPLC, were added to isolated LDL (0.5 to 1.0 mg protein/mL) or to whole serum to final concentrations of 40 μM lutein, 120 μM lycopene, or 160 μM β-carotene, and 2.0% (v/v) solvent. Samples were incubated for 8 hr at 37°C, in the dark, under nitrogen, in a water bath sonicator. In a number of pilot experiments, the conditions of these incubations were varied to arrive at this procedure for optimal incorporation of the carotenoids. Samples of LDL or serum serving as controls were incubated as described above with either ethanol or THF (2.0% v/v) or held at 4°C with no addition of solvent. After the incubation, LDL was isolated or reisolated from these samples as described above and then passed through a 5-μm filter. Samples of LDL were then dialyzed against PBS containing 1 mM EDTA. The electrophoretic mobility on agarose gels of these enriched LDL and their cholesterol to protein ratios were determined and compared with those of the 4°C controls.

Oxidation of LDL

LDL was diluted to 0.25 mg protein/mL with PBS and oxidized at 37°C for 3 hr in the dark, with a concentration of $CuSO_4$ that was 5 μM above the concentration of EDTA present in each LDL. Aliquots collected at various times during the oxidation were protected from further oxidation by addition of butylated hydroxytoluene (BHT) to a final concentration of 20 μM. A portion was frozen at −20°C for subsequent analysis of thiobarbituric acid reacting substances. Ethanol and ethyl acetate (2:5 v/v) were added

Table 1 Concentrations of endogenous carotenoids in control LDL and fold enrichment of specific LDL-associated carotenoids after addition of carotenoid to either LDL or serum

	Endogenous carotenoids* nmol/mg protein				Fold enrichment (enriched/control)		
Sample	Lutein	Lycopene	β-Carotene	Added Carotenoid	Lutein	Lycopene	β-Carotene
LDL-1	0.28	2.0	0.29	None	1	1	1
				Lutein	**32**	1.0	1.0
				Lycopene	1.3	**3.2**	1.4
				β-Carotene	1.3	0.9	**5.1**
LDL-2	0.16	0.70	0.29	None	1	1	1
				Lutein	**150**	0.8	0.9
				Lycopene	1.7	**8.3**	1.0
				β-Carotene	1.9	0.8	**18**
LDL-3	0.25	0.37	0.22	None	1	1	1
				Lutein	**21**	1.1	1.3
				Lycopene	1.2	**2.5**	0.9
				β-Carotene	1.0	1.1	**3.4**
Serum-A	0.25	0.22	0.63	None	1	1	1
				Lutein	**5.8**	1.0	0.7
				Lycopene	1.5	**13**	1.1
				β-Carotene	1.2	1.0	**9.0**
Serum-B	0.41	0.52	0.51	None	1	1	1
				Lutein	**3.6**	1.1	1.3
				Lycopene	0.9	**15**	1.4
				β-Carotene	0.8	0.7	**8.4**
Serum-C	0.24	0.61	0.12	None	1	1	1
				Lutein	**4.2**	0.8	5.3
				Lycopene	0.8	**1.6**	4.5
				β-Carotene	0.9	1.1	**25**

*No significant differences in concentrations were seen when LDL or serum were held at 4°C or incubated with solvent (ethanol or THF) alone; thus, the values shown here represent an average of values obtained for the 4°C, 37°C + ETOH, and 37°C + THF controls.

to the remainder to begin extraction of lipids, including carotenoids, α-tocopherol, and lipid hydroperoxides, and then frozen at −20°C. After thawing, extraction of carotenoids and α-tocopherol and analysis by HPLC was completed as described above.[18,19] A portion of the lipid extract was also used to assay for FOX-reactive lipid hydroperoxides. In some experiments, the time course of conjugated diene formation was monitored by the increase in absorbance at 234 nm during oxidation of a portion of the LDL in a spectrophotometer cuvette.

Calculations and statistical analysis

To estimate the relative rates of destruction of individual carotenoids in each oxidation, data shown in *Figures 3* and *5* were replotted as the natural logarithm (ln) of the fraction of carotenoid remaining versus time. In general, these plots were nearly linear, suggesting approximately first-order kinetics. Extrapolating data from two points along this line, rates of destruction for individual carotenoids were then calculated by

$$R_d = \frac{-(ln_a - ln_b)}{t_a - t_b} \times C_i$$

where

R_d = rate of destruction of carotenoid (pmols/min/mg protein)
ln_a and ln_b = ln fraction of carotenoid remaining at time a and b
t_a and t_b = two different time points during the oxidation
C_i = initial concentration of carotenoid (pmols/mg protein)

In some cases, these data were not multiplied by the initial concentration and are expressed as a percent of initial concentration destroyed per minute. To compare the effects of carotenoid enrichment on the rates of carotenoid destruction, a half time ($t_{1/2}$) for carotenoid destruction, based on the approximate first-order kinetics, was calculated as $0.693 \times C_i/R_d$.

Results are expressed as means ± SEM. The statistical significance of differences between pairs of measurements was assessed by the Wilcoxen signed-rank test. Differences were considered statistically significant at $P < 0.05$.

Results

Enrichment of LDL in vitro with carotenoids and characterization of the resulting lipoproteins

Table 1 shows the concentrations of lutein, lycopene, and β-carotene in control LDL and the fold enrichment of each carotenoid after addition of a specific carotenoid either to three individual, isolated LDL samples or to three distinct serum samples (followed by isolation of the LDL) as described in the methods. The overall degree of enrichment of LDL that was obtained for each carotenoid varied considerably among individual samples. Concentrations of lycopene and β-carotene in LDL were increased 2- to 15-fold and 3- to 25-fold, respectively. For these carotenoids, there was little difference in the range of enrichments of LDL achieved by addition of the carotenoid to isolated

lipoprotein or to whole serum. In contrast, the degree of enrichment of LDL with lutein was substantially higher when the carotenoid was added to isolated LDL (21- to 150-fold) as compared to when it was added to serum (4- to 6-fold). In general, the concentrations of the other endogenous carotenoids did not change under any of the treatment conditions. All enriched LDL samples exhibited similar electrophoretic mobility on agarose gels and negligible baseline levels of oxidized lipids. Moreover, no consistent changes in α-tocopherol content or cholesterol:protein ratio were detected in LDLs enriched with specific carotenoids as compared with their respective controls (data not shown). Thus, the enrichment procedures, whether via addition to LDL directly or to serum with LDL isolation, led to enhanced quantities of a specific carotenoid in LDL without generation of oxidized lipids, changes in endogenous lipids, or alteration in electrophoretic mobility.

To determine how tightly the added carotenoid associated with LDL, four of the six samples of LDL enriched with carotenoids (shown in *Table 1*) were subjected to agarose gel electrophoresis after reisolation by ultracentrifugation. Bands corresponding to LDL and the origin were scraped and lipid extracts were analyzed for carotenoids. Most (>75%) of the carotenoid that floated with LDL on ultracentrifugation also migrated with LDL on electrophoresis, demonstrating that added carotenoids were tightly associated with LDL.

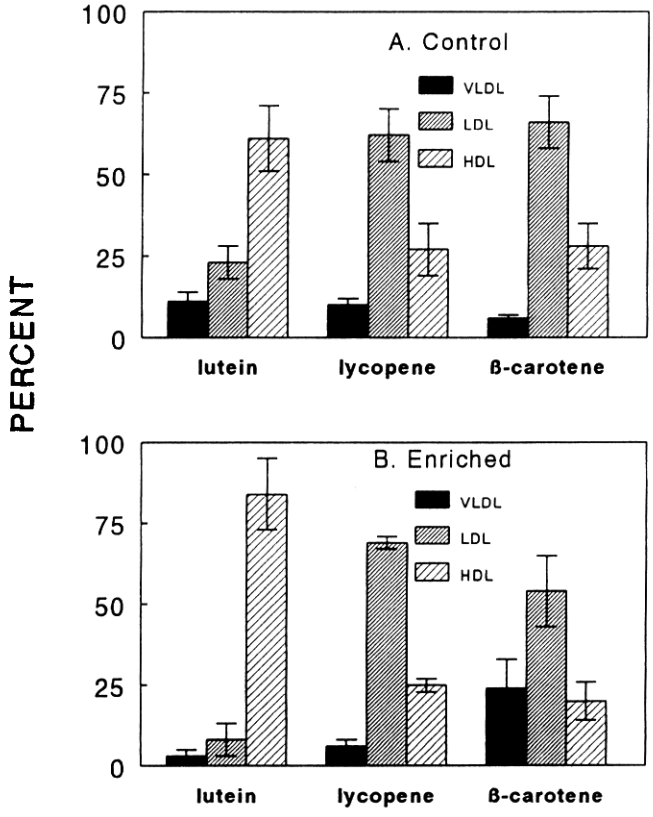

Figure 1 Percent distribution of lutein, lycopene, and β-carotene among lipoproteins in control serum (Panel A) or in serum enriched with specific carotenoids (Panel B) by addition of carotenoid in solvent. The distributions of other carotenoids, as well as the distributions of α-tocopherol and cholesterol were not altered by enrichment with a specific carotenoid. Data shown represent $n = 3$ serum samples (mean ± SE), the same as those presented in *Table 1*.

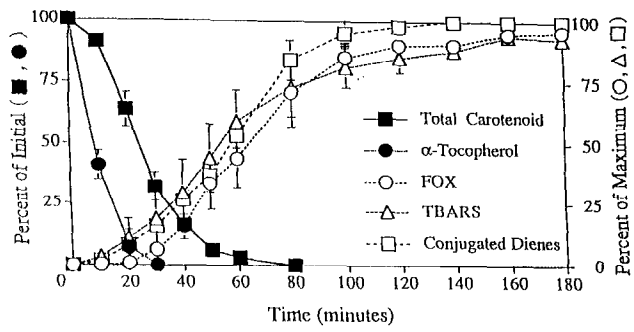

Figure 2 Disappearance of total carotenoid and α-tocopherol and formation of lipid peroxidation products, assessed as FOX-reactive lipid hydroperoxides, TBARS, and conjugated dienes, during oxidation of human LDL (0.25 mg protein/mL, 5 μM $CuSO_4$, 37°C) from five fasted individuals. Initial concentrations, expressed as mean ± SEM, were 0.872 ± 0.092 μM for total carotenoid (representing the sum of the masses of lutein, β-cryptoxanthin, lycopene, and β-carotene) and 8.56 ± 0.23 μM for α-tocopherol.

We also investigated the distributions among the lipoprotein classes of the individual carotenoids added to the three serum samples (*Table 1*) and compared them to the distributions observed for the endogenous carotenoids. The results are shown in *Figure 1*. For lycopene, the distributions of endogenous and added carotenoid were nearly identical. However, for lutein and β-carotene, whereas the distributions of endogenous and added compound were qualitatively similar, there were modest, quantitative differences. For all three samples of lutein-enriched serum, lutein associated to a greater extent with HDL (and less with LDL) than in control serum, whereas for all three samples of β-carotene-enriched serum, the major difference was a greater association of β-carotene with VLDL. In all cases, the amounts of carotenoids recovered in the three lipoprotein fractions accounted well for the amounts found in whole serum.

Oxidation of LDL containing endogenous carotenoids

Our first experiments on oxidation utilized LDL isolated from the serum of five fasting individuals. The kinetics of destruction of endogenous carotenoids and α-tocopherol and the formation of oxidized lipids during the Cu^{+2}-mediated oxidation of LDL are shown in *Figure 2*. The assays measuring FOX-reactive lipid hydroperoxides, TBARS, and conjugated dienes gave similar results when plotted as percent of maximal oxidation over the 3-hr time course. In general, only minimal oxidation was observed before 30 min. By that time, α-tocopherol was completely destroyed, and approximately 75% of total carotenoid was also destroyed. The remaining 25% of carotenoid was destroyed by 60 to 80 min, a time period during which increasing, but not complete, oxidation of the LDL was observed. The finding that individual carotenoids are completely destroyed before substantial oxidation of LDL is consistent with the hypothesis that carotenoids can act as antioxidants to protect LDL from oxidation.

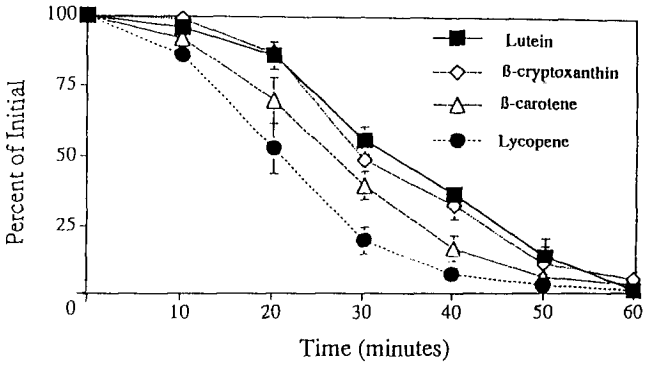

Figure 3 Disappearance of individual carotenoids during oxidation of human LDL (0.25 mg protein/mL, 5 μM $CuSO_4$, 37°C) from five fasted individuals. Initial concentrations, expressed as mean ± SEM, were 0.160 ± 0.036 μM lutein, 0.068 ± 0.008 μM β-cryptoxanthin, 0.424 ± 0.048 μM lycopene, and 0.220 ± 0.048 μM β-carotene.

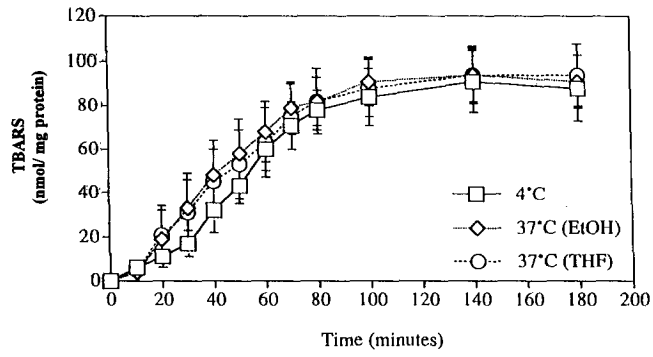

Figure 4 Formation of TBARS during oxidation of control LDL (0.25 mg protein/mL, 5 μM $CuSO_4$, 37°C). The LDL from five individuals, obtained from human serum or isolated LDL, were incubated with ethanol or tetrahydrofuran (2.0% v/v) as solvent controls for the enrichment protocol or held at 4°C as described in Methods and materials.

Figure 3 shows the kinetics of destruction of individual carotenoids in these same LDL during the first 60 min of oxidation. Lycopene, the carotenoid found in highest concentration in all samples of LDL, was destroyed most quickly, at approximately 17 pmol/min. Lutein and β-cryptoxanthin, found in much lower concentrations, were destroyed at considerably slower rates of approximately 5 pmols/min. The rate of destruction of β-carotene was intermediate at about 11 pmols/min. These rates of destruction were determined as described in Experimental Procedures. Differences in the rates of destruction of individual carotenoids are consistent with the hypothesis that individual carotenoids may vary in their effectiveness at protecting LDL from oxidation.

Oxidation of LDL enriched with carotenoids

Having developed methods for the in vitro enrichment of LDL with specific carotenoids, we next compared the kinetics of oxidation for LDL enriched with specific carotenoids to that of the same LDL without enrichment (control LDL). These experiments were performed with the LDL samples whose carotenoid contents are presented in *Table 1*. *Figure 4* shows the formation of TBARS in control LDL samples that were incubated at 37°C with either ethanol or THF (2.0% v/v), or held at 4°C during the enrichment protocol (see Methods and materials). The lag times before oxidation as well as rates and extent of TBARS formation were similar under all three incubation conditions, thereby showing that the conditions of the enrichment protocol did not alter the susceptibility of LDL to oxidation. This was also seen when oxidation was monitored using the FOX assay (data not shown).

The effects of enrichment of these LDL with specific carotenoids on the kinetics of destruction of the major carotenoids during oxidation were also determined. To compare the effects of the enrichment conditions on the disappearance of each carotenoid, half times of destruction were calculated from the rates of destruction as described in Experimental Procedures. As shown in *Table 2*, enrichment of the LDL with lutein prolonged the half time of lutein destruction but had no effects on the rates of destruction of the other carotenoids. Enrichment with lycopene had no effect on the rates of destruction of any of the carotenoids. In contrast, enrichment with β-carotene inhibited the destruction of lutein and lycopene as well as β-carotene itself. The protective effect of enrichment of the LDL with β-carotene on the rates of destruction of carotenoids is shown more clearly in *Figure 5*. The rates of destruction of lutein, lycopene, and β-carotene were significantly slower in LDL enriched with β-carotene (2.6%, 4.3%, and 3.0% per min, respectively) compared with control LDL (4.0%,

Table 2 Half times (minutes) for destruction of LDL-associated carotenoids during oxidation of human LDL: comparison between control LDL samples and those enriched with specific carotenoids

Enrichment condition	Carotenoid							
	Lutein		β-Cryptoxanthin		Lycopene		β-Carotene	
	Control	Enriched	Control	Enriched	Control	Enriched	Control	Enriched
Lutein	16 ± 6	52 ± 28	9 ± 1	11 ± 2	9 ± 2	9 ± 2	9 ± 1	14 ± 5
enriched vs control*	**P = 0.03**		NS		NS		NS	
Lycopene	20 ± 5	52 ± 24	10 ± 1	10 ± 1	9 ± 2	14 ± 5	10 ± 2	9 ± 2
enriched vs control*	NS		NS		NS		NS	
β-Carotene	20 ± 5	85 ± 59	10 ± 1	14 ± 4	9 ± 2	21 ± 8	10 ± 2	19 ± 3
enriched vs control*	**P = 0.03**		NS		**P = 0.03**		**P = 0.03**	

*Statistical comparisons of half times for carotenoid destruction in five individual LDL samples enriched with a specific carotenoid and control LDL were made using Wilcoxen signed-rank test ($P < 0.05$ considered significant and shown in bold type).

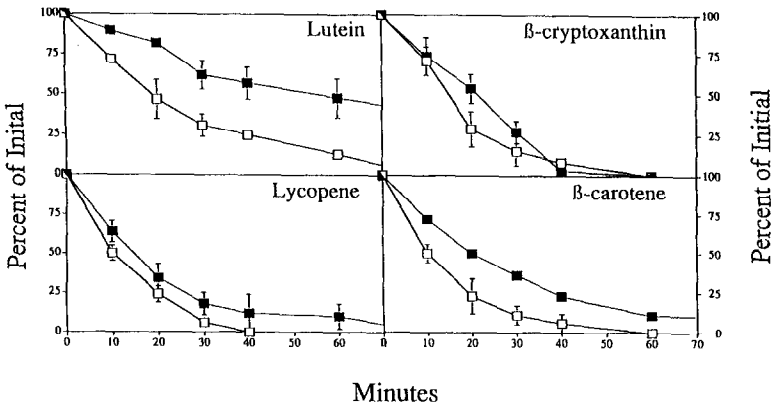

Figure 5 Disappearance of individual carotenoids during oxidation of LDL (0.25 mg protein/mL, 5 μM $CuSO_4$, 37°C) for five individual LDLs enriched with β-carotene (■) and their respective controls (□).

8.9%, and 5.8% per min, respectively), resulting in the prolonged half times for destruction shown in *Table 2*.

The time course for oxidation of the LDL enriched with lutein, lycopene, or β-carotene was assessed as TBARS and compared to that for control LDL. These data are shown in *Figure 6*. No differences in the kinetics of oxidation of control LDL and LDL enriched with lutein (4- to 150-fold) or lycopene (2- to 15-fold) were observed. However, for samples of LDL enriched with β-carotene (3- to 25-fold), lag times before initiation of oxidation (65 ± 29 min) were significantly longer than those of control LDL (19 ± 7 min) ($P = 0.03$). After initiation of oxidation, the rate of TBARS formation and the maximal extent of oxidation of these LDL did not differ. Measures of the formation of FOX-reactive lipid hydroperoxides paralleled those observed using the TBARS assay (data not shown). Thus, the rates of carotenoid destruction and the time course of oxidation show that only β-carotene protects LDL from Cu^{+2}-mediated oxidation in vitro.

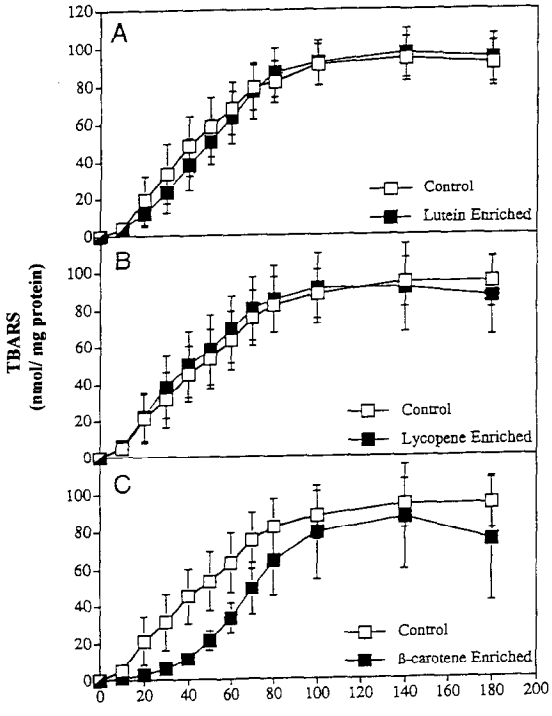

Figure 6 Formation of TBARS during oxidation of LDL (0.25 mg protein/mL, 5 μM $CuSO_4$, 37°C). Samples of LDL were obtained from human serum or isolated LDL incubated (8 hr, 37°C) with lutein in ethanol (Panel A, ■), lycopene in THF (Panel B, ■), or β-carotene in THF (Panel C, ■) or appropriate solvent controls (□). For each treatment, $n = 5$.

Discussion

These studies provide new information on the effects of increasing the concentration of a specific carotenoid in serum or in LDL on its distribution among lipoproteins, the composition of LDL and the susceptibility of LDL to an in vitro oxidation. The protocol used to increase the concentration of specific carotenoids within the LDL particle provides a powerful tool to examine the potential function(s) of carotenoids, in this case as antioxidants. It is particularly noteworthy that the chemical composition and properties of the LDL as well as concentrations of other endogenous antioxidants are unchanged by this enrichment procedure. Hence, this method allows us to evaluate specifically the potential biological effects of individual LDL-associated carotenoids. It is of interest to note that the concentrations of β-carotene in LDL achieved with this in vitro enrichment procedure are fairly comparable to those seen with oral supplementation.[17,25] Moreover, since oral supplements are not available for lutein and lycopene, this in vitro method allows direct comparison of the effects of enriching with individual carotenoids against a background of the same endogenous carotenoids.

When carotenoid in solvent was added to serum or to isolated LDL, the extent of carotenoid enrichment achieved varied considerably from one LDL sample to another and for different carotenoids. The basis of this variability is not known but may be related to the poor solubility of the carotenoids in the largely aqueous environments in suspensions of lipoproteins or serum. We did find that the range of enrichments of LDL achieved by addition of either lycopene or β-carotene to serum with subsequent isolation of LDL or directly to pre-isolated LDL were similar. In contrast, the enrichment of LDL with lutein was much less when lutein was added to serum (with subsequent LDL isolation) than when lutein was added directly to LDL. This result is likely related to the presence of phospholipid-rich HDL in whole serum. Both the current and previous studies[5] showed that

the distribution of endogenous lutein differed from those of lycopene or β-carotene in its greater association with HDL. For added β-carotene, its distribution was shifted toward a greater association with VLDL than that for endogenous β-carotene. Thus, for studies such as tissue culture experiments in which serum enriched in vitro or lipoproteins from that serum are used as delivery vehicles for carotenoids, it is important to realize that the distributions of carotenoids among lipoproteins may be different than those of the endogenous carotenoids.

Once we had carefully characterized LDL following enrichment with specific carotenoids, we examined the ability of these individual carotenoids to inhibit LDL oxidation. Our findings that enrichment of LDL with β-carotene modestly inhibits the oxidation of LDL under the conditions used here are consistent with those of Jialal et al.,[13] Lavy et al.,[15] and Naruszewicz et al.[14] In these studies, β-carotene added to LDL or serum in a small volume of solvent inhibited the oxidation of LDL or Lp (a) by Cu^{2+}-mediated oxidation in a cell-free system as well as by cultures of monocyte-macrophages. In contrast, Reaven et al.[17] found that β-carotene enrichment of LDL by oral supplementation had no effect on copper-mediated oxidation of the lipoprotein, whereas Gaziano et al.[16] found such enrichment to promote copper-mediated oxidation. Certainly, when taken together, all of the investigations on the effects of increasing β-carotene in LDL suggest that, at best, it can slightly delay the onset of lipid peroxidation. Thus, any potential beneficial effect of dietary β-carotene supplementation on atherosclerosis might be more related to possible effects of β-carotene on cells and not to its action as an LDL-associated antioxidant.

We have also provided new information on the effects of carotenoid enrichment of LDL on the kinetics of destruction of individual carotenoids during copper-mediated oxidation. Each carotenoid studied had different effects on the process, and these effects were largely unrelated to lipid peroxidation in LDL. While β-carotene enrichment prolonged the destruction halflife of lutein, lycopene, and β-carotene itself, the effect of the first two carotenoids cannot be relevant to lipid peroxidation since enrichment of the LDL with these two carotenoids had no effect. Furthermore, lutein enrichment markedly increased its own halflife for oxidative destruction but had no effect on lipid peroxidation.

To our knowledge, the studies reported here are the first to examine the effects of enrichment of LDL with either lycopene or lutein on the oxidation of LDL and to compare their effects to that of β-carotene. Both lycopene and lutein differed from β-carotene in that neither had any effect on the oxidation of LDL. This is seen despite (1) enrichments comparable to (or greater than) those obtained with β-carotene, and (2) evidence that all three of these carotenoids exhibit antioxidant properties in solution as deactivators of peroxyl radicals and/or singlet oxygen quenchers.[26,27] Thus, while the different carotenoids may not have intrinsically different properties as antioxidants, in a supermolecular complex such as LDL they may be differentially exposed to the oxidant or exhibit differential reactivity. It remains to be determined if these specific carotenoids in LDL are more or less effective when the lipoprotein is exposed to different oxidative conditions.

In conclusion, in vitro enrichment is an excellent means by which to enhance the concentration of an individual carotenoid in its physiological plasma transporter, LDL, without altering other aspects of LDL's composition. The limited effect of β-carotene, and the absence of effect of either lutein or lycopene, on copper mediated oxidation of LDL suggest that further studies are needed to more fully understand the potential physiological and/or prophylactic function(s) of β-carotene and other carotenoids in cardiovascular disease.

References

1 Gey, K.F., Puska, P., Jordan, P., and Moser, U.K. (1991). Inverse correlation between plasma vitamin E and mortality from ischemic heart disease in cross-cultural epidemiology. *Am. J. Clin. Nutr.* **53,** 326S–334S

2 Kardinaal, A.F.M., van't Veer, P. Brants, H.A.M., van den Berg, H., van Schoonhoven, J., and Hermus, R.J.J. (1995). Relation between antioxidant vitamins in adipose tissue, plasma, and diet. *Am. J. Epidemiol.* **141,** 440–450

3 Hennekens, C.H., Buring, J.E., Manson, J.E., Stamfer, M., Rosner, B., Cook, N.R., Belanger, C., LaMotte, F., Gaziano, J.M., Ridker, P.M., Willett, W., and Peto, R. (1996). Lack of effect of long-term supplementation with beta-carotene on the incidence of malignant neoplasms and cardiovascular disease. *New Engl. J. Med.* **334,** 1145–1149

4 Omenn, G.S., Goodman, G.E., Thornquist, M.D., Balmes, J., Cullen, M.R., Glass, A., Keogh, J.P., Valanis, B., Williams, J.H., Barnhart, S., and Hammar, S. (1996). Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *New Engl. J. Med.* **334,** 1150–1155

5 Romanchik, J.E., Morel, D.W., and Harrison, E.H. (1995). Distribution of carotenoids and α-tocopherol among lipoproteins do not change when human plasma is incubated in vitro. *J. Nutr.* **125,** 2610–2617

6 Krinsky, N.I., Cornwell, D.G., and Oncley, J.L. (1958). The transport of vitamin A and carotenoids in human plasma. *Arch. Biochem. Biophys.* **73,** 233–246

7 Mathews-Roth, M.M. and Gulbrandsen, C.L. (1974). Transport of β-carotene in serum of individuals with carotenemia. *Clin. Chem.* **20,** 1578–1579

8 Bjornson, L.K., Kayden, H.J., Miller, E., and Moshell, A.N. (1976). The transport of α-tocopherol and β-carotene in human blood. *J. Lipid Res.* **17,** 343–352

9 Sies, H., Stahl, W., and Sundquist, A. (1992). Antioxidant functions of vitamins. *Ann. N.Y. Acad. Sci.* **669,** 7–20

10 Berliner, J.A. and Heinecke, J.W. (1996). The role of oxidized lipoproteins in atherogenesis. *Free Rad. Biol. Med.* **20,** 707–727

11 White, W.S., Stacewicz-Sapuntakis, M., Erdman, Jr., J.W., and Bowen, P.E. (1994). Pharmacokinetics of β-carotene and canthaxanthin after ingestion of individual and combined doses *J. Am. Coll. Nutr.* **13,** 665–671

12 Kostic, D., White, W.S., and Olsen, J.A. (1995). Intestinal absorption, serum clearance and interaction between lutein and β-carotene when administered to human adults in separate or combined doses. *Am. J. Clin. Nutr.* **62,** 604–610

13 Jialal, I., Norkus, E.P., Cristol, L., and Grundy, S.M. (1991). β-Carotene inhibits the oxidative modification of low-density lipoprotein. *Biochim. Biophys. Acta* **1086,** 134–138

14 Naruszewicz, M., Selinger, E., and Davignon, J. (1992). Oxidation of lipoprotein (a) and the effect of beta-carotene. Metabolism **41,** 1215–1224

15 Lavy, A., Ben Amotz, A., and Aviram, M. (1993). Preferential inhibition of LDL oxidation by the all-trans isomer of beta-carotene in comparison with 9-cis beta-carotene. *Eur. J. Clin. Chem. Clin. Biochem.* **31,** 83–90

16 Gaziano, J.M., Hatta, A., Flynn, M., Johnson, E.J., Krinsky, N.I., Ridker, P.M., Hennekens, C.H., and Frei, B. (1995). Supplementation with beta-carotene in vivo and in vitro does not inhibit low density lipoprotein oxidation. *Atherosclerosis* **112,** 187–195

17 Reaven, P.D., Khouw, A., Beltz, W.F., Parthasarathy, S., and Witztum, J.L. (1993). Effect of dietary antioxidant combinations in

humans: protection of LDL by vitamin E but not by β-carotene. *Arterioscler. Thromb.* **13,** 590–600

18 Barua, A.B., Batres, R.O., Furr, H.C., and Olson, J.A. (1989). Analysis of carotenoids in human serum. *J. Micronutr. Anal.* **5,** 291–302

19 Barua, A.B. and Furr, H.C. (1992). Extraction and analysis by high-performance liquid chromatography of carotenoids in human serum. *Methods Enzymol.* **213,** 273–281

20 Markwell, M.K., Haas, S.M., Bieber, L.L., and Tolbert, N.E. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **87,** 206–210

21 Morel, D. and Chisolm, G. (1989). Antioxidant treatment of diabetic rats inhibits lipoprotein oxidation and cytotoxicity. *J. Lipid Res.* **30,** 1827–1834

22 Jiang, Z.Y., Hunt, J.V., and Wolff, S.P. (1992). Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. *Anal. Biochem.* **202,** 384–389

23 Esterbauer, H., Gebicki, J., Puhl, H., and Jurgens, G. (1992). The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Rad. Biol. Med.* **13,** 341–390

24 Hatch, F.T. and Lees, R.S. (1968). Practical methods for plasma lipoprotein analysis. *Adv. Lipid Res.* **6,** 1–68

25 Micozzi, M.S., Brown, E.D., Edwards, B.K., Bieri, J.G., Taylor, P.R., Khachik, R., Beecher, G.R., and Smith Jr., J.C. (1992). Plasma carotenoid response to chronic intake of selected foods and β-carotene supplements in men. *Am. J. Clin. Nutr.* **55,** 1120–1125

26 DiMascio, P., Murphy, M., and Sies, H. (1991). Antioxidant defense systems: the role of carotenoids, tocopherols, and thiols. *Am. J. Clin. Nutr.* **53,** 194S–200S

27 Liebler, D. (1993). Antioxidant reactions of carotenoids. *Ann. N.Y. Acad. Sci.* **691,** 20–31