Impact of LDL carotenoid and α-tocopherol content on LDL oxidation by endothelial cells in culture

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Abstract **Carotenoids and α-tocopherol are dietary, lipophilic antioxidants that may protect plasma lipoproteins from oxidation, a process believed to contribute to atherogenesis. Previous work demonstrated that after the Cu(II)-initiated oxidation of human low density lipoprotein (LDL) in vitro, carotenoids and α-tocopherol were destroyed before significant lipid peroxidation took place, and that α-tocopherol was destroyed at a much faster rate than were the carotenoids. Additionally, in vitro enrichment of LDL with β-carotene, but not with lutein or lycopene, inhibited LDL oxidation. In the present studies the impact of LDL carotenoid and α-tocopherol content on LDL oxidation by human endothelial cells (EaHy-1) in culture was assessed. LDL isolated from 11 individual donors was incubated at 0.25 mg protein/mL with EaHy-1 cells in Ham's F-10 medium for up to 48 h. Formation of lipid hydroperoxides was assessed by chemical analysis and the contents of lutein, β-cryptoxanthin, lycopene, β-carotene and α-tocopherol were determined by high performance liquid chromatography. The extent of lipid peroxidation correlated with the endogenous α-tocopherol content of the LDL but not with its content of carotenoids. As in the Cu(II)-initiated system, carotenoids and α-tocopherol were destroyed before significant peroxidation took place, but, in the cell-mediated system, α-tocopherol and the carotenoids were destroyed at comparable rates. Also, like the Cu(II)-initiated oxidation, enrichment of the LDL with β-carotene protected it from oxidation by the endothelial cells. However, enrichment with either lutein or lycopene actually enhanced the cell-mediated oxidation of the LDL. Thus, the specific content of carotenoids in low density lipoprotein (LDL) clearly modulates its susceptibility to oxidation, but individual carotenoids may either inhibit or promote LDL oxidation.—** Dugas, T. R., D. W. Morel, and E. H. Harrison. **Impact of LDL carotenoid and α-tocopherol content on LDL oxidation by endothelial cells in culture. *J. Lipid Res.* 1998.** 39: **999–1007.**

Supplementary key words lutein • β-cryptoxanthin • lycopene • β-carotene • antioxidant • atherosclerosis • free radical • lipids

The carotenoids are a group of plant pigments obtained in the diet from consumption of a variety of fruits and vegetables. These lipophilic compounds circulate in human plasma predominately in low density lipoprotein (LDL) (1–4). These micronutrients, some of which have pro-vitamin A activity, are not essential in the diets of humans. Since the finding that carotenoids efficiently quench singlet oxygen (5), however, much research has focussed on their possible antioxidant function in diseases such as cancer and atherosclerosis (6–8) whose etiologies are believed to involve free radical lipid oxidation (9).

Over 100 epidemiological studies have demonstrated a lower risk of both cancer and atherosclerosis associated with the intake of fruits and vegetables high in carotenoids and other antioxidant vitamins (10–13). One large intervention study in Linxian, China, showed that supplementation with β-carotene and α-tocopherol reduced the risk of cancer by 13% (14). Though these effects were positive, other intervention trials have shown different results. In one, supplementation with β-carotene had no effect on cancer or coronary heart disease (Physician's Health Study) (15), while in another, the α-Tocopherol, β-Carotene Cancer Prevention Study, supplementation with β-carotene in male Finnish smokers resulted in an 18% increase in lung cancer, but had no effect on coronary heart disease (16). In addition, in one intervention trial in the U.S., the Carotene and Retinol Efficacy Trials (CARET), supplementation of male and female smokers with both β-carotene and retinyl palmitate resulted in a 28% increase in lung cancer (17).

More recently, carotenoids other than β-carotene have gained attention. In particular, lycopene is being investigated for its possible activity in preventing prostate, stomach, and pancreatic cancer (18–20) and myocardial infarction (21). Before intervention trials are attempted, however, much more investigation of these carotenoids needs to be performed.

A major goal of the present investigation was to study

Abbreviations: LDL, low density lipoprotein; HPLC, high performance liquid chromatography; THF, tetrahydrofuran; BHT, butylated hydroxytoluene; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TBARS, thiobarbituric acid-reactive substances; FOX, ferrous oxidation/xylenol orange.

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the potential roles of all of the major carotenoids in human plasma on endothelial cell-mediated oxidation of LDL, which is implicated in atherogenesis (9). Previous investigations by our laboratory and others have focussed on chemically-initiated oxidation of LDL (9, 21–23). After Cu(II)-initiated oxidation of LDL, it was shown that α-tocopherol and the carotenoids were destroyed before significant oxidation took place, with α-tocopherol being destroyed at a faster rate than the carotenoids (21). Additionally, in vitro enrichment with β-carotene, but not with lutein or lycopene, inhibited LDL oxidation (21). Though much work has been reported for chemically initiated oxidation of LDL, little work has been reported for the presumably more pathophysiological initiation of oxidation by vascular cells.

We present here the results of studies where LDL oxidation was initiated using EaHy-1 cells, a human endothelial cell line. Results presented here show that as in the Cu(II)-initiated system, α-tocopherol and the carotenoids were destroyed before significant oxidation took place, but in this case, α-tocopherol and the carotenoids were destroyed at comparable rates. As with the Cu(II)-initiated system, β-carotene enrichment protected LDL from oxidation, but contrary to what was expected, enrichment with lutein and lycopene enhanced the oxidation. Finally, further analysis of the data shows that there was no correlation between the extent of oxidation and endogenous carotenoid levels, however, there was a strong correlation between oxidation and endogenous α-tocopherol.

EXPERIMENTAL PROCEDURES

Materials

All carotenoids used in this study, either for HPLC quantitation or for in vitro enrichment, were selected for their high purity and quality. Lycopene and lutein were gifts from Dr. Gary Beecher of the USDA (Beltsville, MD). Lutein was also purchased from Kemin Industries (Des Moines, IA). β-Carotene was obtained from Fluka, and β-cryptoxanthin was a gift from Hoffman-LaRoche (Basel, Switzerland). A human endothelial cell line, EaHy-1, was a gift from Dr. Mahamad Navab at UCLA. Cell culture media, Dulbecco's modified Eagle's Medium (DMEM) and Ham's F-10 medium, trypsin·EDTA and heat-inactivated fetal bovine serum (FBS) were obtained from Bio-Whittaker (Walkersville, MD). HAT (5 mm hypoxanthine, 0.02 mm aminopterin, 0.8 mm thymidine) media supplement (Hybri-Max®, 50×) for hybridoma cells and gentamicin solution (10 mg/mL) were purchased from Sigma (St. Louis, MO). Reagents used for protein or oxidation assays, including Folin and Ciocalteu's Phenol Reagent, lauryl sulfate (sodium dodecyl sulfate), xylenol orange (3,3′-bis[N,N-di(carboxymethyl)-aminomethyl]-*o*-cresol-sulfonephthalein), butylated hydroxytoluene (2,6-di-*t*-butyl-*p*-cresol), and 2-thiobarbituric acid (4,6-dihydroxypyrimidine-2-thiol), were obtained from Sigma. Malonaldehyde bis(dimethyl acetal), or 1,1,3,3-tetramethoxypropane, 99%, was purchased from Aldrich. The tetrahydrofuran (THF) used in these experiments contained no BHT (butylated hydroxytoluene), so it was stored under N_2 and was pretested before each use with starch iodide test papers (Fisher) to insure that the THF was peroxide-free. All other incidental reagents or solvents were of high purity and were used as received.

Lipoprotein isolation

Plasma was collected from eleven different fasted donors (7 males and 4 females), denoted here as donors A–K. The donors were all healthy individuals between the ages of 22 and 45 years, who were not currently taking medication or vitamin supplements. After collection, plasma was either stored at −20°C or was used immediately. Lipoproteins were isolated by the sequential density gradient ultracentrifugation method described by Hatch and Lees (24). Low-density lipoprotein (LDL) was identified as the fraction with a density of 1.019–1.063 g/mL. Once the LDL was isolated, it was used immediately with no storage. Before incubation with cells, the LDL was first desalted by gel filtration (Pharmacia Biotech Sephadex® G-25 M (PD-10) gel filtration columns). Protein was determined by the modified Lowry protein assay (25).

In vitro enrichment

The LDL or plasma sample was first divided into two parts. One was enriched with carotenoid in vitro and the other, which served as an unenriched control, was stored untreated at 4°C. To achieve in vitro carotenoid enrichment, stock solutions of either 2 mm lutein in ethanol, 3 mm lycopene in THF, or 8 mm β-carotene in THF were first prepared. These stock solutions were checked for purity by HPLC before use and discarded if there was any evidence of degradation of the carotenoids. Plasma or LDL was then incubated at 4°C in the dark on a gentle rotary (blood) mixer with 2% v/v of the appropriate carotenoid stock solution for 16 h (overnight). The resulting concentrations of carotenoid incubated in the presence of plasma or LDL were thus either 40 μm lutein, 60 μm lycopene, or 160 μm β-carotene. The plasma or LDL was filtered using a 0.45-μm syringe filter to remove the majority of unincorporated carotenoid. The lipoproteins were then (re)isolated by the sequential density gradient ultracentrifugation method mentioned earlier, further reducing the amount of unincorporated carotenoid. Lipoprotein integrity was compared for the unenriched and enriched samples by measuring the electrophoretic mobility on an agarose gel (Beckman Paragon® lipoprotein (LIPO) agarose gel electrophoresis kit), as well as the cholesterol-to-protein and triglyceride-to-protein ratios for each of the two samples. Results of these measurements showed that there was no effect of in vitro enrichment on either LDL mobility on an agarose gel or on cholesterol- or triglyceride-to-protein ratios, suggesting that there was no change in physical properties or composition of the lipoprotein. We analyzed the lipoprotein carotenoid content before and after enrichment with each carotenoid. Importantly, the enrichment of LDL with specific carotenoids (i.e., lutein, lycopene, β-carotene) had no significant effect on the levels of the other endogenous carotenoids. Moreover, after enrichment there was no indication by HPLC analysis of any "new" carotenoid peaks or breakdown products. Thus, the enrichment of LDL with pure solution of a given carotenoid increased only that carotenoid in the lipoprotein.

Endothelial cell culture and incubation with LDL

The EaHy-1 cells used in these experiments, human aortic endothelial cells immortalized by hybridization to cells from a human adenoma, were shown previously to maintain their characteristic cobblestone morphology, presence of Factor VIII-related antigen, and ability to take up acetylated LDL (26–28). These cells were grown here in DMEM medium containing 10% FBS, 0.125 mg/mL gentamycin, 4.5 mg/mL glutamine, and HAT medium supplement (50×, dissolved in 10 mL deionized water and diluted 1:50 in the DMEM). Cells were plated into 6-well plates 3–4 days before the start of the oxidation experiments. Once cells were grown to confluence, the DMEM was removed and the cells were washed with sterile phosphate buffered saline (PBS).

TABLE 1. Endogenous carotenoid composition of LDL samples from the various donors

Donor	Lutein	β-Cryptoxanthin	Lycopene	β-Carotene	α-Tocopherol
			nmol/mg protein		
A	0.07	0.16	0.24	0.15	6.87
B (I)	0.09	0.08	0.36	0.12	0.68
B (II)	0.05	0.17	1.23	0.67	8.14
C	0.10	0.10	7.60	0.28	9.88
D (I)	0.05	0.09	0.58	0.91	6.98
D (II)	0.06	0.03	0.16	0.11	3.60
E	0.06	0.10	2.65	0.82	3.28
F	0.26	0.05	0.29	0.18	3.65
G	0.02	0.03	0.29	0.59	2.88
H	0.14	0.09	5.85	0.82	10.4
I	0.08	0.04	0.58	0.10	5.35
J	0.04	0.03	0.56	0.03	6.90
K	0.08	0.03	0.51	0.04	4.52
Avg.	0.08 ± 0.06	0.08 ± 0.05	1.61 ± 2.39	0.37 ± 0.34	5.62 ± 2.87

The cells were then incubated at 37°C with 2 mL per well of 0.25 mg/mL LDL in Ham's F-10 medium for 0–48 h. As a control for the spontaneous oxidation of the LDL that might result from incubation at 37°C in the absence of cells, the 0.25 mg/mL LDL was also incubated in wells without cells at 37°C for 0–48 h. After various lengths of incubation, the LDL-containing medium was removed and aliquots of a stock solution of BHT in ethanol were added such that the final concentration was 25 μm BHT. This was done to prevent further oxidation of the samples both during storage and during the oxidation assays. Aliquots of medium containing BHT were taken for measurements of lipid oxidation and stored prior to assay at −20°C for less than 1 week. The cells were again washed with PBS, and the amount of remaining cell protein per well was measured using the modified Lowry protein assay (25). In brief, 1 mL reagent A (containing SDS and described in the modified Lowry assay) was placed in each well and was incubated for 1 h with gentle shaking of the plate. From each well, 100 μL was then taken for the assay.

Lipid oxidation determination

The amount of lipid oxidation that had taken place in each LDL sample was assessed using the TBARS (thiobarbituric acid-reactive substances) (29) and/or the FOX (ferrous oxidation/xylenol orange) (30) assays. Both the samples of LDL incubated in the presence of endothelial cells and the LDL incubated cell-free were assayed for lipid oxidation.

For the TBARS assay, 400 μL of each LDL sample was assayed as described previously (29). As a standard for the quantitation of TBARS, the assay was also performed using several dilutions of a known amount of malonaldehyde bis(dimethyl acetal) (Aldrich).

Before the FOX assay could be performed, the lipid hydroperoxides were first extracted from 100 μL of the LDL samples using the methanol extraction procedure described by Jiang, Hunt, and Wolff (30). The extracts were then dried under nitrogen and redissolved in 100 μL methanol–water 9:1 (v/v) solution. The FOX assay (30) was performed on the samples, as well as on dilutions of 30% hydrogen peroxide used as a standard.

Compositional analysis of the plasma or LDL

The cholesterol contents of the plasma or lipoproteins were determined using the Total Cholesterol 50 assay kit (enzymatic, colorimetric, from Sigma). Likewise, triglycerides were determined using the Triglyceride (GPO-Trinder) 50 assay kit (enzymatic, colorimetric, from Sigma).

To determine the carotenoid content of the LDL, the carotenoids were first extracted from 2-mL aliquots of the 0.25 mg/mL LDL samples using the method described by Barua et al. (31). The extracts were evaporated with a gentle stream of N_2 and were redissolved in 200 μL acetonitrile–isopropanol 1:1 solution. The carotenoid and α-tocopherol contents were then determined by the HPLC method reported originally by Barua and Furr (32). The analysis was performed using a Waters 600E HPLC pump, a 717 *plus* autosampler, and a 996 photodiode array detector. Conditions for the separation included a 25 cm, 4.6 ID Supercosil® (Supelco) C18 reverse-phase column, acetonitrile–dichloromethane–methanol–octanol 90:15:10:0.1 (v/v/v/v) containing 0.01% *t*-butylamine mobile phase, and a flow rate of 1 mL/min. Absorbance was monitored between 250 and 600 nm, and the UV spectrum was sampled at 2-sec intervals. Elution and absorption of the α-tocopherol and the carotenoids were determined on separate channels using Millenium software (Waters) version 2.10. For the α-tocopherol channel, the chromatogram was extracted at 290 nm, while for the carotenoid channel, at 450 nm. Quantitation and identification of the α-tocopherol or individual carotenoids were done by comparison to standards. The individual carotenoids and α-tocopherol were identified by comparison of both retention times and UV spectra. Carotenoid and α-tocopherol compositions of the various LDL samples were compiled and are listed in **Table 1**.

RESULTS

Kinetics of endothelial cell-mediated oxidation of LDL and destruction of endogenous carotenoids and α-tocopherol

In agreement with previous findings (33, 34), EaHy-1 human endothelial cells, when incubated in Ham's F-10 medium, initiated significant lipid oxidation in the LDL (**Fig. 1**). Oxidation products measured using the FOX assay showed that a small amount of oxidation also took place in LDL incubated without cells, however, the amount of oxidation resulting from its incubation with the cells was considerably more pronounced. In addition, cell protein remained relatively constant over the incubation period (data not shown).

Figure 2 shows the results of oxidation of LDL samples from three donors (I, J, and K), containing very similar carotenoid and α-tocopherol profiles (Table 1). The kinetics of oxidation as measured by the TBARS and FOX assays were markedly different. Using the FOX assay, lipid

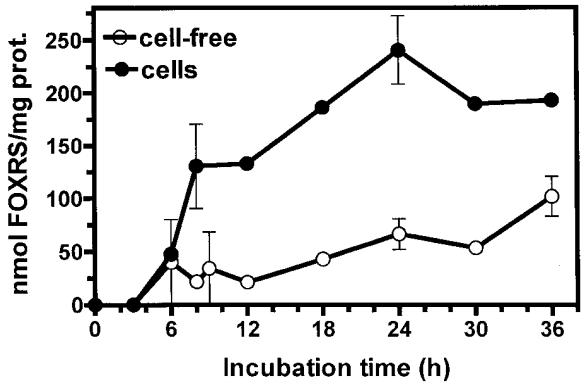

Fig. 1. Oxidation, measured as nmol/(mg protein) FOXRS (ferrous ion/xylenol orange-reacting substances), in 0.25 mg/mL human low-density lipoprotein (LDL) incubated in Ham's F-10 medium at 37°C with and without EaHy-1 endothelial cells. Results show the mean ± SD for three LDL samples (I, J, and K).

hydroperoxides, a direct product of lipid peroxidation, began to increase after a 4- to 6-h lag phase, reached a maximum at 24 h, and then decreased sharply. In the TBARS assay, short-chain aldehydes like malondialdehyde, breakdown products of lipid hydroperoxides, began to increase after a 10- to 12-hour lag phase, at least 2 h later than lipid hydroperoxides. They increased sharply for the first 20 h, then began leveling off through 36 h.

Both the endogenous carotenoids and α-tocopherol in the LDL were destroyed before significant oxidation took place (Fig. 2). In comparison to lipid hydroperoxides as measured by the FOX assay, about 50% of the carotenoids and α-tocopherol were destroyed before lipid oxidation began to increase by a measurable amount. Also note that the rates of destruction of α-tocopherol and the combined carotenoids (Fig. 2) were virtually indistinguishable.

In contrast to the relatively rapid destruction of LDL carotenoids in the presence of cells, LDL carotenoids were quite stable in culture medium when incubated in the absence of cells. Under these conditions, 90% of the total initial carotenoid was present at 18 h and thereafter declined to about 40% at 36 h (data not shown).

The averaged rates of destruction of the individual carotenoids and α-tocopherol from the same three LDL samples (from donors I–K) were also compared (**Fig. 3**). Results show that the rates of destruction of α-tocopherol, as compared to the individual carotenoids, were quite similar (Fig. 3). Likewise, there was little difference among the rates of destruction of individual carotenoids. While the results in Fig. 3 show that β-carotene was destroyed at a slightly lower rate than others, in additional experiments β-carotene was destroyed at the same rate as the other carotenoids.

Effect of in vitro enrichment on lipid oxidation

LDL collected from a given individual was divided into two samples. One sample served as the unenriched control and received no treatment beyond storage at 4°C. The other sample was enriched in vitro with a given carotenoid

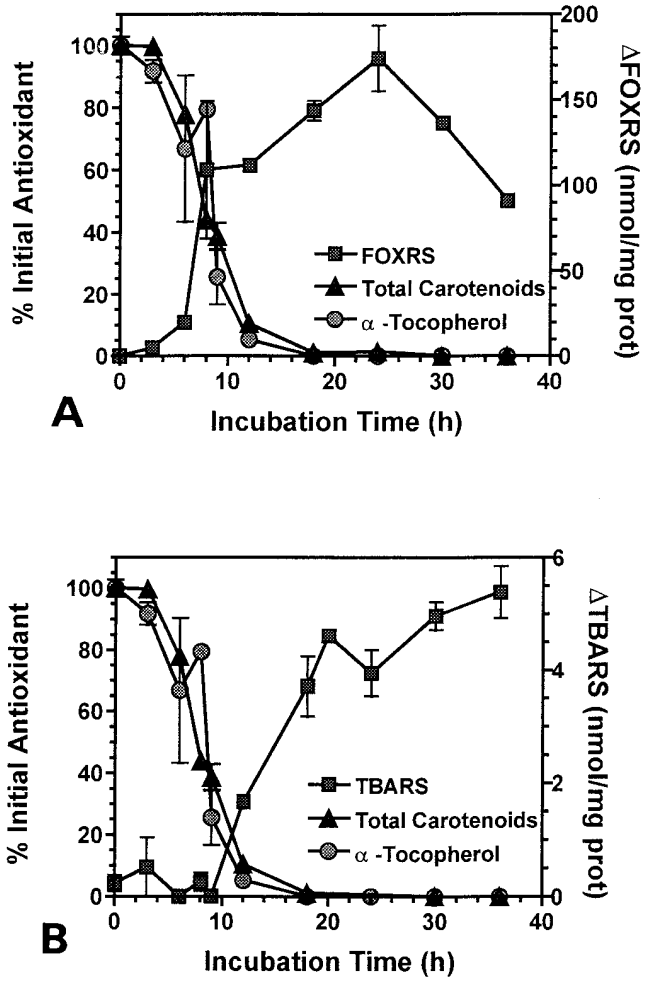

Fig. 2. Destruction of total carotenoid (the sum of lycopene, lutein, β-cryptoxanthin, and β-carotene) and α-tocopherol and increase in lipid oxidation products, measured as A) FOXRS and B) TBARS after endothelial cell-mediated oxidation of 0.25 mg/mL human LDL in Ham's F-10 medium. Initial antioxidant concentrations of the LDL samples (n = 3, donors I, J, and K) were 0.66–0.80 nmol(mg protein) total carotenoids and 4.52–6.90 nmol/(mg protein) α-tocopherol. The background amount of FOXRS and TBARS detected in cell-free samples of LDL was subtracted from the amount observed in samples incubated with cells. The remaining amount of oxidation products thus represents only the oxidation initiated by the cells and is denoted here as Δnmol/(mg protein) TBARS or FOXRS.

by the method described above in *Experimental Procedures*. Both samples were incubated with the same endothelial cells (subcultures of the same flask) at the same time.

Five LDL samples from four different donors (donors B, C, D, and H) were enriched in vitro with lutein. LDL from donor B was sampled twice, about 6 months apart. Treatment with lutein resulted in a 5- to 18-fold increase in LDL lutein. Representative results from two donors, donors B and C, are shown in **Fig. 4**. Fold enrichments in LDL lutein in these two samples were 13 and 11, respectively. In four out of five experiments, there was a dramatic enhancement of lipid oxidation in the sample of enriched LDL as compared to its unenriched control. In the LDL sample with the highest enrichment (18-fold, donor

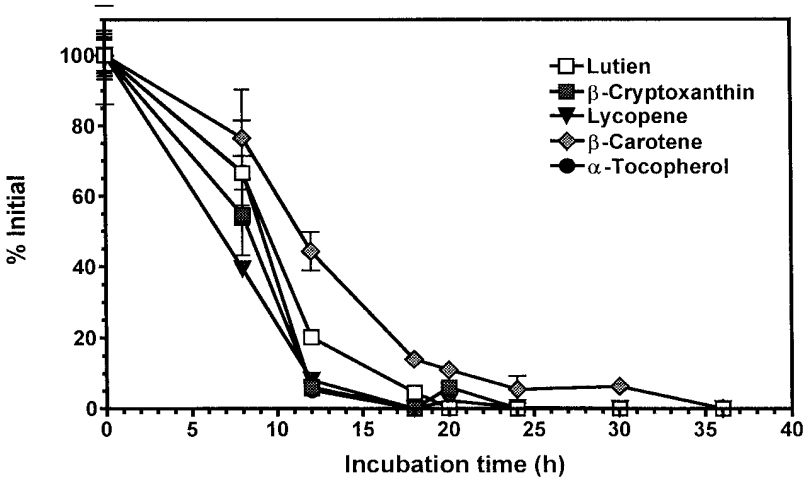

Fig. 3. Destruction of the individual carotenoids and α-tocopherol after endothelial cell-mediated oxidation of 0.25 mg/mL LDL (n = 3, donors I, J, K). Initial concentrations of LDL carotenoids and α-tocopherol, in nmol/(mg protein), were 0.067 ± 0.023 lutein, 0.033 ± 0.006 β-cryptoxanthin, 0.550 ± 0.360 lycopene, 0.056 ± 0.038 β-carotene, and 5.59 ± 1.21 α-tocopherol.

H), there was a very slight decrease in oxidation as compared to the unenriched LDL (data not shown).

Three samples of LDL from three different donors (donors A, F, and H) were enriched in vitro with lycopene and were incubated with EaHy-1 cells. Enrichment resulted in a 2- to 8-fold increase in lycopene. Results from two representative samples (donors A, H) are shown in **Fig. 5**. In these two samples, LDL lycopene was enriched 3- and 8-fold, respectively. In all experiments, lycopene enrichment resulted in enhancement of the oxidation observed in the unenriched control. An 8-fold enrichment did produce a more pronounced increase in oxidation than did a 2-fold enrichment.

Finally, six LDL samples collected from four donors (donors B, D, E, and G) were enriched with β-carotene. In this case, plasma from donors B and D was collected twice, each blood sample drawn approximately 6 months apart. Enrichments achieved for β-carotene ranged from 2- to 12-fold over the unenriched sample. Results from oxidation of two of the LDL samples (donors D, G) are shown in **Fig. 6**. Enrichments in LDL β-carotene in these two samples were 5- and 7-fold, respectively. In each and every experiment, the amount of oxidation observed in the in vitro enriched sample was less than in the unenriched control. In the case of β-carotene enrichment, there seemed to be no correlation between the amount of enrichment and effectiveness of inhibition of the oxidation.

Correlations between LDL oxidation and endogenous antioxidants

Examination of the data from individual donors suggested that there was a relationship between the total content of lipophilic antioxidants and the extent of lipid peroxidation. In order to explore this possibility, correlation plots were constructed to show the relationship between the formation of lipid hydroperoxides (as ΔFOXRS) and total endogenous carotenoids and α-tocopherol (data not shown). There was a significant correlation between the two, with $r = 0.54$ and $P = 0.0030$. There was no significant correlation between lipid hydroperoxides and total carotenoid alone, as $r = 0.25$ and $P = 0.20$ (**Fig. 7**). Furthermore, correlation plots for the relationship between lipid hydroperoxides and α-tocopherol showed a significant correlation, even greater than when comparing lipid

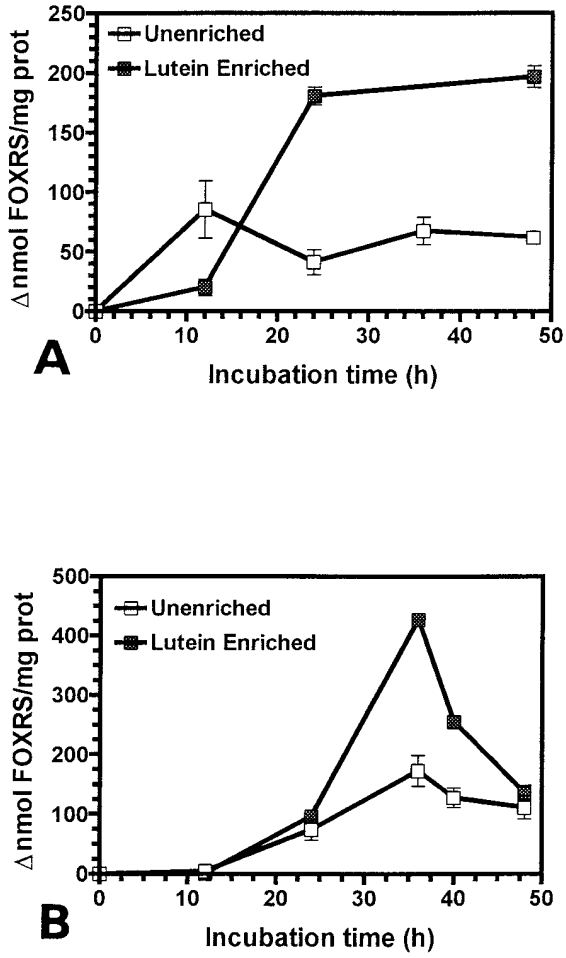

Fig. 4. Effect of in vitro lutein enrichment on the extent of lipid oxidation. Data represent lipid hydroperoxides (measured using the FOX assay) after endothelial cell-mediated oxidation of 0.25 mg/mL LDL from A) donor B(II) and B) donor C. Fold enrichments in LDL lutein were 13 and 11, respectively, over the endogenous levels given in Table 1. Enrichment of the LDL with lutein had no effect on the endogenous levels of other carotenoids or α-tocopherol.

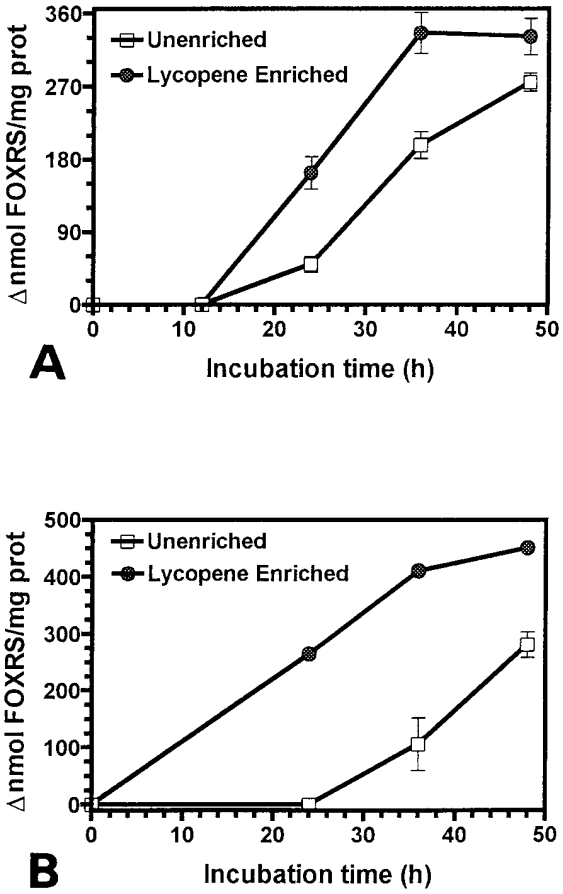

Fig. 5. Effect of in vitro lycopene enrichment on the extent of endothelial cell-mediated LDL oxidation. FOXRS were measured after incubation of 0.25 mg/mL LDL from A) donor A and B) donor H, at 37°C with EaHy-1 human endothelial cells. Enrichment of LDL lycopene were 3- and 8-fold, respectively, over the endogenous levels given in Table 1. Enrichment of LDL with lycopene had no effect on the endogenous levels of other carotenoids or α-tocopherol.

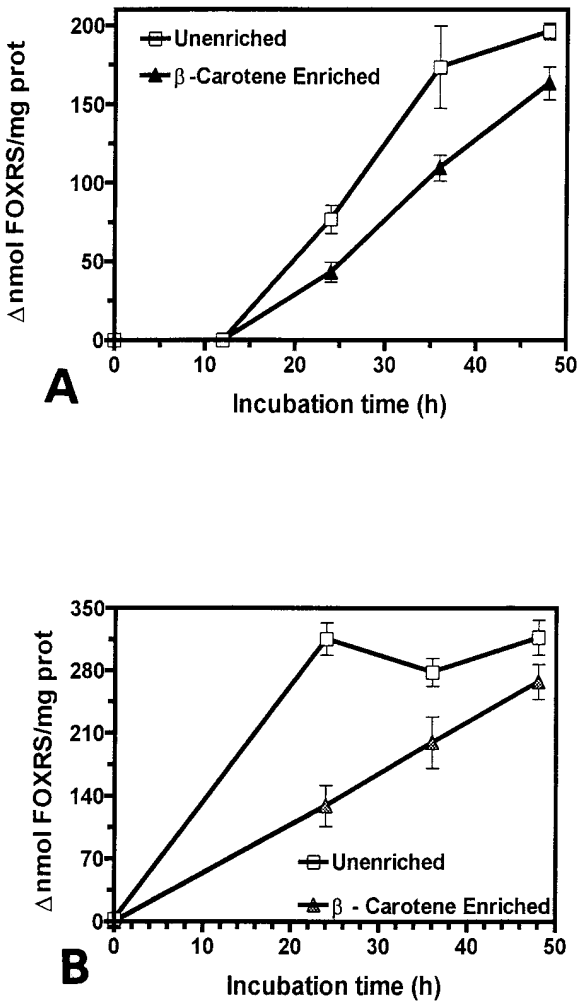

Fig. 6. Effect of in vitro β-carotene enrichment on the extent of LDL oxidation. These data represent lipid hydroperoxides (FOXRS) after endothelial cell-mediated lipid oxidation of 0.25 mg/mL LDL from A) donor E and B) donor G. Enrichments in LDL β-carotene were 11- and 7-fold, respectively, over the endogenous levels given in Table 1. Enrichment of LDL with β-carotene had no effect on the endogenous levels of other carotenoids or α-tocopherol.

hydroperoxides and total carotenoid and α-tocopherol combined, with $r = 0.72$ and $P < 0.0001$ (Fig. 7).

DISCUSSION

Much work has been reported examining the role of α-tocopherol and β-carotene in preventing oxidative damage to LDL, as might occur during atherogenesis. To date, however, little work has been done to examine the antioxidant effectiveness of carotenoids other than β-carotene in LDL. The primary aim of this work, therefore, was to determine whether or not the most prominent carotenoids in LDL prevent its oxidation by endothelial cells. This was done by assessing the kinetics of carotenoid destruction as the oxidation took place and the impact of enrichment of the LDL in a given carotenoid on the extent of oxidation.

One complication with the study of carotenoids other than β-carotene is that no pure supplements of these compounds are approved for human use, making in vivo enrichment experiments difficult. Furthermore, in vivo supplementation of a single carotenoid by ingestion of a large quantity of a given vegetable(s) is problematic, as many fruits and vegetables contain a combination of carotenoids. We have therefore introduced here a method similar to what we have used before for enriching LDL in vitro with a particular carotenoid.

In the past, investigators often initiated oxidation in LDL by chemical means, using, for example, Cu(II) or a variety of azo initiators. For example, we recently studied the kinetics of destruction of β-carotene and α-tocopherol after Cu(II)-initiated LDL oxidation and showed that α-tocopherol was destroyed at a faster rate than β-carotene (22). Both antioxidants, however, were destroyed before significant lipid oxidation took place. Here, we have instead used a more physiological method of initiating oxidation using EaHy-1 cells, a human endothelial cell line, in culture. Because of the current dogma that oxidation

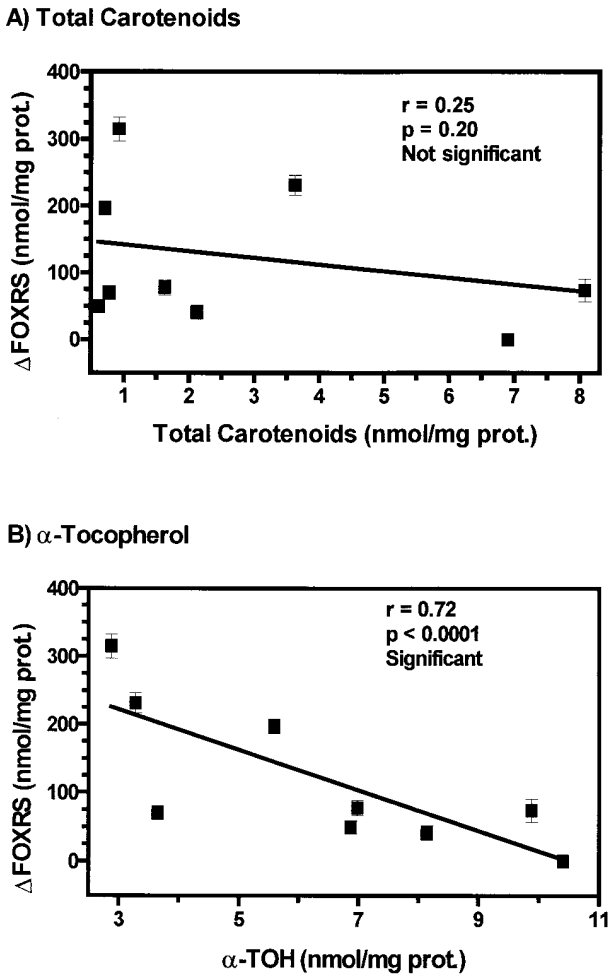

Fig. 7. Correlation between lipid hydroperoxides and endogenous LDL antioxidants after 24 h of cell-mediated oxidation. Eleven samples of LDL were incubated independently with EaHy-1 human endothelial cells at 37°C for 24 h. Lipid hydroperoxides were measured using the FOX assay. Correlation data were obtained using GraphPad Prizm software.

involved in atherogenesis is cell-mediated, we believe that this form of initiation is much more relevant to pathophysiology. Using endothelial cell-mediated oxidation, the carotenoids and tocopherol were destroyed before a measurable amount of oxidation took place; however, in this case, the rates of destruction of α-tocopherol and the carotenoids were identical (Fig. 2). This could suggest that in systems of cell-mediated LDL oxidation the carotenoids are comparable antioxidants to α-tocopherol.

Previous experiments using Cu(II) as an initiating species showed that oxidation as measured by various assays, including the TBARS and FOX assays, showed similar kinetics (22). Oxidation measured here after cell-mediated oxidation using these same two assays, however, showed different kinetics. In these studies, oxidation takes place over a 36–48 h period rather than 2–3 h. This more protracted (and likely more physiologic) time course allows for discernment between lipid hydroperoxide generation and formation of breakdown products. As one might expect, malondialdehyde, a breakdown product of lipid oxidation (measured using the TBARS assay), began to increase at a later timepoint than lipid hydroperoxides, a direct product of lipid oxidation (measured using the FOX assay). Thus, in these experiments we have focussed on the direct assay of lipid hydroperoxide formation (i.e., FOXRS), in order to monitor what is likely more physiologically relevant.

The effect of enriching LDL with a specific carotenoid on the amount of lipid oxidation taking place in the LDL particle over a 48 h incubation with cells was then explored. As observed previously, the extent of enrichment with carotenoids varied considerably from preparation to preparation. The factors responsible for this variation are not known, however, enrichment of LDL with carotenoids does not affect the content of other lipids or the electrophoretic mobility of the lipoprotein.

In three of four experiments, lutein enrichment enhanced oxidation (Fig. 4). Similarly, enrichment of LDL with lycopene resulted in enhancement of oxidation in each of three experiments (Fig. 5). The results observed here for LDL enrichment with lutein and lycopene and cell-mediated oxidation are different from what we observed for Cu(II)-initiated oxidation of LDL, where lutein and lycopene enrichment showed no effect (22). As the enhancement of oxidation was observed in a pathophysiological system, these results suggest that dietary enrichment with these two carotenoids could actually accelerate atherogenesis in vivo. It is possible, however, that LDL enriched with ingested carotenoids might differ from that enriched with the in vitro methods used here. In any case, these results thus warrant further investigation.

Finally, the only carotenoid studied here found to inhibit the amount of lipid oxidation was β-carotene. In each of six experiments, enrichment with β-carotene somewhat protected the LDL from oxidation (Fig. 6). It was noted, however, that the largest effect was observed in LDL from donor G, which, before enrichment, contained only modest carotenoid and tocopherol levels.

Thus, as with chemical oxidation, the behavior of LDL enriched with lutein and lycopene differed qualitatively from those enriched with β-carotene. This could indicate a qualitative difference between the compounds. If, however, in vitro enrichment results in incorporation of a carotenoid in a manner that is not physically the same as in a normal LDL particle, perhaps the true effect of the carotenoid on LDL oxidation is masked. The location of the carotenoid in the lipoprotein particle could indeed affect its ability to act as an antioxidant. Prooxidant effects of enrichment on LDL oxidation, such as those shown here for lycopene and lutein, have also been observed for α-tocopherol (35, 36). In these experiments, LDL oxidation was initiated using a water-soluble azo initiator. It was argued that because the phenolic moiety of α-tocopherol was likely oriented toward aqueous solution, it could react with a water-soluble initiating radical to form the stable phenoxyl radical, normally characteristic of antioxidation by α-tocopherol (37, 38). Because the water-soluble initiating radical would have difficulty entering the lipid layer to initiate lipid oxidation, few lipid peroxyl radicals would at

that point exist to react with the tocopherol-derived phenoxyl radical for termination. As the phenoxyl radical must also terminate itself, it could instead react with a molecule of unoxidized lipid to propagate the chain of lipid oxidation. Perhaps in enriching the LDL in vitro we have incorporated carotenoid only near the surface and not necessarily where they might normally be located (39). Similar to α-tocopherol, the carotenoid radical intermediate formed from reaction with an initiating species in the aqueous phase might also react to propagate the chain (pro-oxidation) rather than break the chain (anti-oxidation). In light of these questions, it would therefore be worthwhile to extend these studies to in vivo enrichments of carotenoids (however difficult that may be), α-tocopherol, and a combination of the two, to confirm the results presented here.

While enrichment results indicate that β-carotene is somewhat effective in inhibiting LDL oxidation, there was no correlation between the levels of endogenous total carotenoids (Fig. 7) or β-carotene alone (data not shown) and lipid peroxidation in LDL. Furthermore, correlations between α-tocopherol and LOOH did show a marked and significant correlation. This suggests that, as predicted by others (10, 23), α-tocopherol may be the major antioxidant in the LDL particle during cell-mediated oxidation. On the other hand, enrichment of a donor low in endogenous antioxidant (donor G) with β-carotene did inhibit LDL oxidation markedly. It may be that the antioxidant effectiveness of β-carotene is not observed because the normal endogenous levels, small by comparison to α-tocopherol, are too small to elicit an effect. It may only become an important antioxidant after enrichment or supplementation, at which point the levels of β-carotene become more comparable to the levels of endogenous α-tocopherol.

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