Recycling of vitamin E in human low density lipoproteins

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Abstract Oxidative modification of low density lipoproteins (LDL) and their unrestricted scavenger receptor-dependent uptake is believed to account for cholesterol deposition in macrophage-derived foam cells. It has been suggested that vitamin E that is transported by LDL plays a critical role in protecting against LDL oxidation. We hypothesize that the maintenance of sufficiently high vitamin E concentrations in LDL can be achieved by reducing its chromanoxyl radicals, i.e., by vitamin E recycling. In this study we demonstrate that: *i*) chromanoxyl radicals of endogenous vitamin E and of exogenously added α-tocotrienol, α-tocopherol or its synthetic homologue with a 6-carbon side-chain, chromanol-α-C6, can be directly generated in human LDL by ultraviolet (UV) light, or by interaction with peroxyl radicals produced either by an enzymic oxidation system (lipoxygenase + linolenic acid) or by an azo-initiator, 2,2′-azo-bis(2,4-dimethylvaleronitrile) (AMVN); *ii*) ascorbate can recycle endogenous vitamin E and exogenously added chromanols by direct reduction of chromanoxyl radicals in LDL; *iii*) dihydrolipoic acid is not efficient in direct reduction of chromanoxyl radicals but recycles vitamin E by synergistically interacting with ascorbate (reduces dehydroascorbate thus maintaining the steady-state concentration of ascorbate); and *iv*) β-carotene is not active in vitamin E recycling but may itself be protected against oxidative destruction by the reductants of chromanoxyl radicals. We suggest that the recycling of vitamin E and other phenolic antioxidants by plasma reductants may be an important mechanism for the enhanced antioxidant protection of LDL.—**Kagan, V. E., E. A. Serbinova, T. Forte, G. Scita, and L. Packer.** Recycling of vitamin E in human low density lipoproteins. *J. Lipid. Res.* 1992. **33:** 385–397.

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Recent studies have shown that several different cultured cell types including endothelial cells, smooth muscle cells, monocyte-macrophages, and fibroblasts are capable of promoting the oxidative modification of low density lipoproteins (LDL) (1, 2). Oxidative modification of LDL results in several important changes in their properties: an increase in electrophoretic mobility due to an increase of the negative surface charge, increase of LDL density, hydrolysis of phosphatidylcholine and accumulation of free fatty acids and lysophosphatidylcholine, fragmentation of apoB, derivatization of lysine residues, and enhancement of lipofuscin-like fluorescence (3, 4).

The uptake of oxidatively modified LDL via a specific cell surface receptor, the scavenger receptor, is considered one of the possible mechanisms by which macrophages may accumulate cholesterol hence giving rise to foam cells (5). LDL oxidation and uptake by macrophages is suggested to be strongly dependent on the balance of pro- and antioxidants in the plasma (3, 6, 7). Rapid depletion of the endogenous antioxidants occurs during LDL oxidation (8–10). Consistent with this, exogenously added natural antioxidants (e.g., vitamin E) and antioxidant drugs (e.g., probucol) prevent the oxidative modification of LDL, suggesting possible retardation of atherogenesis (11–13).

Vitamin E has been shown to be crucially important in the antioxidant protection of LDL compared with other endogenous antioxidants (carotenoids, ubiquinols) (10, 12, 14, 15). LDL oxidation is substantially suppressed until vitamin E is depleted (10, 12, 16). Thus maintenance of sufficiently high vitamin E concentrations in LDL may be critical in protecting LDL against oxidation. The steady-state concentration of vitamin E in LDL is the outcome of: *i*) the flow of exogenous vitamin E to LDL, *ii*) its transfer from LDL to cellular membranes or other plasma lipoproteins, and *iii*) its metabolic redox reactions in LDL. Oxidation of vitamin E via intermediate formation of chromanoxyl radicals would reduce the vitamin E concentration. The replenishment of vitamin E might be possible if the reduction of chromanoxyl radical to chromanol by

Abbreviations: LDL, low density lipoproteins; AMVN, 2,2′-azo-bis(2,4-dimethylvaleronitrile); ESR, electron spin resonance; BHT, butylated hydroxytoluene; DHLA, dihydrolipoic acid.

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plasma or LDL reductants takes place, i.e., recycling of vitamin E.

Recently, the recycling of vitamin E from its phenoxyl radicals by enzymic electron transport and nonenzymic reductants (e.g., by ascorbate, thiols) had been demonstrated in membranes (17, 18). However, no evidence for vitamin E recycling in lipoproteins had been reported, although synergistic antioxidant effects of vitamin E and ascorbate were recently found to occur in LDL undergoing lipid peroxidation or exposed to oxidants (14, 19).

In this study we sought to: *1*) demonstrate whether chromanoxyl radicals of endogenous vitamin E and exogenously added α-tocotrienol, α-tocopherol, or its shorter hydrocarbon chain homologue, chromanol-α-C6, can be directly generated in human LDL by UVB-irradiation (290–400 nm) or by interaction with peroxyl radicals produced either by an enzymic oxidation system (lipoxygenase + linolenic acid) or by an azo-initiator, 2,2′-azo-bis(2,4-dimethyl-valeronitrile), (AMVN); *2*) determine whether reduction of vitamin E chromanoxyl radicals (recycling of vitamin E) by ascorbate occurs in LDL; and *3*) estimate whether reduced thiols (dihydrolipoic acid) interact with chromanoxyl radicals directly or via ascorbate-mediated mechanism in vitamin E recycling.

MATERIALS AND METHODS

Isolation of LDL

A pool of fresh plasma from normolipidemic subjects was used for isolation of LDL. EDTA (0.3 mM) was added to the plasma, the density was adjusted to 1.019 g/ml with solid NaBr, and the plasma was centrifuged at 40,000 rpm in a 50.3 Beckman rotor for 24 h at 4°C. The top 1 ml was harvested by aspiration and discarded. The infranatant was then adjusted to d 1.063 g/ml and centrifuged an additional 24 h after which the top 1 ml representing LDL (d 1.019–1.063 g/ml) was collected by aspiration. The LDL were dialyzed extensively against saline, Tris (10 mM), EDTA (0.3 mM), pH 7.4, and stored under nitrogen prior to further dialysis. For ESR measurements, LDL samples were dialized against phosphate buffer (50 mM, pH 7.4) prior to use.

Enrichment of LDL with exogenous chromanols

Two approaches were used to enrich LDL with exogenous chromanols. *1*) Chromanols were added to plasma before the isolation of LDL according to the procedure described by Esterbauer et al. (8). Freshly prepared plasma was supplemented with 0.8 mM chromanol dissolved in dimethylsulfoxide (DMSO) (10 μl DMSO/1 ml plasma) and then incubated at 37°C in a nitrogen atmosphere for a period of 6 h. Thereafter the LDL was isolated by ultracentrifugation. *2*) Chromanols were added to LDL suspension in ethanolic solution (5 μl of ethanol/ml) and preincubated for 10 min at 37°C. The concentration of exogenously added chromanols was 80 nmol/mg protein.

Electrophoresis

Electrophoretic mobility of LDL was assessed by agarose electrophoresis on Beckman Paragon gels used according to the manufacturers instructions. Gels were stained for lipid with fat 7B. To assess the integrity of LDL particles after exposure to various oxidants and antioxidants, aliquots of LDL were electrophoresed on nondenaturing 2–16% polyacrylamide gels according to the procedure of Krauss and Burke (20). Gels were stained with Coomassie G250 to visualize bands.

Fluorescence spectra

Fluorescence emission spectra of LDL suspensions in phosphate buffer (50 mM, pH 7.4, 0.2 mg protein/ml) were recorded in the spectral region 400–500 nm and excitation at 365 nm (slit widths were 6 nm), a maximum that is specific for oxidatively modified LDL, in accordance with the procedure described by Steinbrecher (21).

Generation of chromanoxyl radicals

Chromanoxyl radicals from α-tocotrienol, α-tocopherol, and its shorter hydrocarbon chain homologue were generated using: *1*) UV irradiation, *2*) an enzymic oxidation system (soybean 15-lipoxygenase + linolenic acid), and *3*) hydrophobic azo-initiator of peroxyl radicals, 2,2′-azo-bis(2,4-dimethylvaleronitrile) (AMVN). When the enzymic oxidation system was used the incubation medium (100 μl) contained LDL in 50 mM phosphate butter, pH 7.4, at 25°C. To avoid the interference of the endogenous lipoxygenase substrates in LDL (free polyunsaturated fatty acids), an excessive concentration of linolenic acid was used (1.4 mM). Linolenic acid was dissolved in ethanol (140 mM). Linolenic acid and lipoxygenase (3 U/μl) were subsequently added to the LDL suspension. With the azo-initiator the incubation medium was essentially the same but AMVN (5.0 mM) was added instead of (lipoxygenase + linolenic acid) and the reaction was carried out at 40°C.

Irradiation

Irradiation was by a solar simulator (Solar Light Co., model 14S), whose output closely matches the solar spectrum in the wavelengths 290–400 nm. The samples were illuminated directly in the ESR resonator

cavity; the distance between the light source and the sample was 30 cm. The intensity of the light at the sample surface in the spectral region 310–400 nm was 1.5 mW/cm² and was 10% of this value at 290 nm.

ESR measurements

ESR measurements were made on a Varian E 109E spectrometer in gas-permeable Teflon tubing (0.8 mm internal diameters, 0.013 mm thickness obtained from Zeus Industrial Products, Raritan, NJ). The gas-permeable tubing (approximately 8 cm in length) was filled with 60 µl of a mixed sample, folded into quarters, and placed in an open 3.0-mm internal diameter EPR quartz tube such that all of the sample was within the effective microwave irradiation area. ESR spectra were recorded either in the dark or under continuous UVAB irradiation by the solar simulator in the ESR cavity. Spectra were recorded at 100 mW power and 2.5 gauss modulation, and 25 gauss/min scan time. Spectra were recorded at room temperature (at 40°C with AMVN), under aerobic conditions by flowing oxygen through the ESR cavity. Chromanoxyl and ascorbyl radical ESR signals were recorded at 3245 gauss magnetic field strength, scan range 100 gauss, and time constant 0.064 sec.

HPLC measurement of α-tocopherol content

d-α-Tocopherol, d-α-tocotrienol, and chromanol-α-C6 was assayed by reverse phase HPLC using a C-18 column (Waters, Inc.) with an in-line electrochemical detector. The eluent was methanol–ethanol 1:9 (v/v), 20 mM lithium perchlorate. Tocopherol was extracted into hexane from sodium dodecyl sulfate-treated samples as described earlier (22).

HPLC measurement of β-carotene content

The extraction of the samples was carried out as follows: 190µl of phosphate buffer was added to 10 µl of LDL suspension, after which 200 µl of ethanol containing 0.025% butylated hydroxytoluene (BHT) was added. After saturating with sodium chloride and adding 10 µl of a solution of lycopene (2 mM) in tetrahydrofuran (as an internal standard), the samples were extracted twice with 6 volumes of hexane plus 0.025% BHT. The hexane was evaporated to dryness under nitrogen and the residue was reconstituted in 230 µl of a mixture of toluene–methanol 1:3 for HPLC analysis. The amount of lycopene used as an internal standard was 400 times higher than the content of endogenous lycopene in LDL sample (0.5 nmol/mg protein in our samples as measured in the absence of the exogenously added lycopene). Thus the calculation of the recovery of β-carotene after extraction could not be affected by endogenous lycopene. β-Carotene concentrations were determined by HPLC using a Beckman/Altex system with an Ultrasphere ODS, 5 mm, 4.6 × 150 mm reverse phase column (Beckman) and an elution solvent mixture of 82.5% methanol, 17.5% toluene, and 0.5% ammonium acetate at a flow rate of 1 ml/min. The effluent was monitored at 450 nm by a spectrophotometric detector.

Reagents used

Linolenic acid, soybean 15-lipoxygenase (101,000 U/mg protein), ascorbate, dihydrolipoic acid were from Sigma Chemical Company, St. Louis, MO; potassium phosphate dibasic, sodium phosphate monobasic were from Mallinckrodt, Inc., Paris, KY; 2,2′-azobis(2,4-dimethyl-valeronitrile) was from PolySciences, Inc., Warrington, PA; HPLC grade ethanol and methanol were from Fischer Scientific, Fair Lawn, NJ; 2R,4R′,8R′-α-tocopherol was a gift from Henkel Co.; thioctic acid was a gift from Asta MEDICA; and d-α-tocotrienol was a gift from Dr. Abdul Gapor and his colleagues at the Palm Oil Research Institute of Malaysia (PORIM). α-Tocopherol homologue with a 6-carbon side chain (chromanol-α-C6) was a gift from Prof. R. P. Evstigneeva (Institute for Fine Chemical Technology, Moscow, USSR).

RESULTS

Generation of chromanoxyl radicals from endogenous vitamin E in LDL

We used two different approaches to generate chromanoxyl radicals from endogenous vitamin E in LDL, *i*) exposure to irradiation by ultraviolet light absorbed by vitamin E:

$$\text{ChR-OH} + h\nu \rightarrow \text{ChR-O}^{\bullet} + e^- + H^+ \qquad \textit{Reaction 1})$$

or *ii*) oxidation by peroxyl radicals:

$$\text{ChR-OH} + \text{ROO}^{\bullet} \rightarrow \text{ChR-O}^{\bullet} + \text{R-OO-H} \qquad \textit{Reaction 2})$$

To produce the peroxyl radicals necessary in reaction 2, two different sources were chosen: *i*) the hydrophobic azo-initiator, AMVN, which thermally decomposes at a constant rate to generate radicals (23, 24), and *ii*) soybean 15-lipoxygenase, which produces peroxyl radicals of polyunsaturated fatty acids (linolenic acid in our experiments) (25).

When the LDL suspension was irradiated in the ESR spectrometer cavity with simulated solar UV light, the chromanoxyl (tocopheroxyl) radical ESR signal appeared immediately (**Fig. 1**); this signal was not detected in the dark. Similarly, incubation of either of the peroxyl radical generators with LDL resulted in the appearance of a characteristic chromanoxyl radical

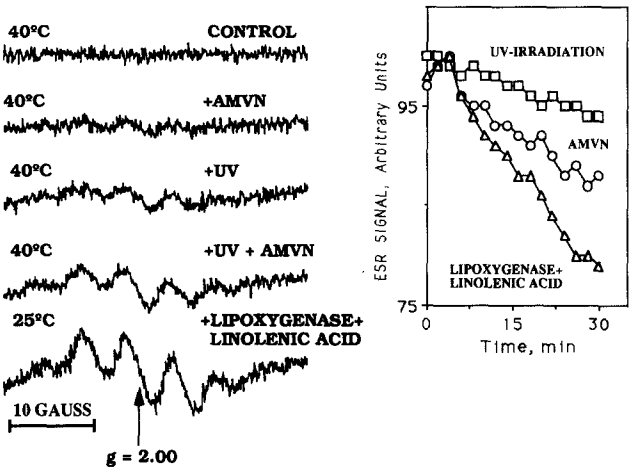

Fig. 1. ESR spectra of chromanoxyl radicals generated in LDL from endogenous vitamin E. Endogenous vitamin E chromanoxyl radicals in LDL were generated using an azo-initiator of peroxyl radicals, AMVN, or an enzymic oxidation system lipoxygenase + linolenic acid, or UV irradiation. The incubation medium (100 µl) contained LDL (14.0 mg protein/ml) in 50 mM phosphate buffer at pH 7.4. The endogenous vitamin E concentration in these LDL samples was 6.2 nmol/mg protein. AMVN (5 mM)-induced reaction was carried out at 40°C. Lipoxygenase (3 U/µl) + linolenic acid (1.4 mM)-induced reaction was carried out at 25°C. UVB irradiation alone or in combination with AMVN in these experiments was performed at 40°C. Other conditions are given in Methods. Insert: time course of vitamin E chromanoxyl radicals generated in LDL.

ESR signal of endogenous vitamin E with *g* values for the five resolved components of 2.0122, 2.0092, 2.0061, 2.0028, and 1.993 (Fig. 1). This signal was not observed in the absence of the oxidizing systems. Combination of UV-irradiation and AMVN additively enhanced the vitamin E radical signal indicating the involvement of independent radical generating mechanisms.

The magnitude of the vitamin E radical signal was a function of the LDL concentration in the suspensions and the vitamin E content in LDL. Vitamin E concentrations in LDL samples used in our experiments varied from 2.5 to 9.0 nmol/mg protein. Pronounced chromanoxyl radical ESR signals from endogenous vitamin E were obtained when the amount of vitamin E in the ESR samples was equal or higher than 30 nmol (normally under the conditions used when the concentration of vitamin E in the sample was higher than 3.0 nmol/mg protein and the LDL concentration in the suspension was higher than 10 mg protein/ml). The magnitude of the vitamin E radical ESR signal generated in LDL by UV-irradiation, AMVN, and lipoxygenase+linolenic acid decreased in time. The decay rate was very low for UV-induced radicals, high for lipoxygenase+linolenic acid-induced radicals, and intermediate for AMVN-induced radicals (Fig. 1).

Oxidative modification of LDL

To test whether oxidative modification of LDL (mediated by possible formation of peroxyl radicals) occurred during exposure to UV irradiation or during incubation with lipoxygenase+linolenic acid, we measured three parameters: *i*) the intensity of LDL fluorescence which is a marker of their oxidative modification (3, 21); *ii*) the electrophoretic mobility of LDL particles in agarose gel which is sensitive to an increase of the negative surface charge due to accumulation of lipid and protein oxidation products (26); and *iii*) nondenaturing gradient gel size distribution which reveals alterations in the physical properties of the particles.

Exposure of LDL to UV irradiation had no effect on the migration of LDL particles in agarose and polyacrylamide gels when compared to the control samples (**Fig. 2**). UV-irradiation did not increase the fluorescence intensity of LDL (**Table 1**). Thus, under UV exposure LDL were sufficiently protected against oxidative modification by endogenous antioxidants.

In contrast, incubation of LDL with lipoxygenase + linolenic acid over 50 min resulted in a significant increase of both fluorescence intensity and electrophoretic mobility, indicative of pronounced oxidative modification of LDL (Table 1, Fig. 2). Incubation with the enzymic oxidation system drastically altered LDL size distribution as noted in the nondenaturing gradient gel profile (Fig. 2). LDL particles in the 25.2 nm range shifted to very large particles in the range of 40.6 nm. This is consistent with aggregation of LDL which probably results from inter-particle cross-linking.

Reduction of UV-induced vitamin E radicals in LDL by ascorbate

To determine whether the vitamin E radicals induced in LDL by UV light could be reduced by ascorbate, we irradiated LDL suspension in the presence or absence of ascorbate. When ascorbate was added to the LDL suspension, chromanoxyl radical ESR signals generated by UV irradiation from endogenous vitamin E could not be observed. Instead, the characteristic ESR signal of ascorbyl radical was detected (**Fig. 3**). This signal decreased in time and was substituted by the progressive appearance of the chromanoxyl radical signal (Fig 3, and **Fig. 4A**). The ascorbate-induced delay in the reappearance of the vitamin E chromanoxyl signal was concentration-dependent (Fig. 4A). UV irradiation did not induce ascorbyl radical ESR signal from ascorbate in the absence of LDL. The oxidized form, dehydroascorbic acid, produced only a slight decrease (by 10–15%) of the magnitude of the vitamin E chromanoxyl radical ESR signal but did not

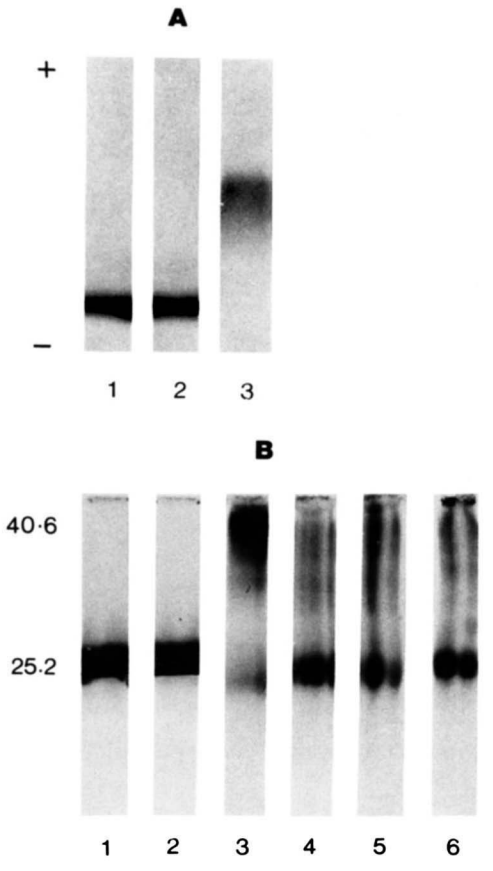

Fig. 2. Electrophoresis patterns of LDL exposed to UV irradiation or lipoxygenase + linolenic acid. A: Agarose gel electrophoretograms. B: Nondenaturing gradient gel profiles of LDL. 1, Control LDL (incubation without any additions); 2, LDL exposed to UV irradiation; 3, LDL exposed to lipoxygenase + linolenic acid; 4, lipoxygenase + linolenic acid plus exogenous α-tocotrienol; 5, lipoxygenase + linolenic acid plus exogenous α-tocopherol; 6, lipoxygenase + linolenic acid plus chromanol-α-C6. Incubation (irradiation) was for 50 min at 25°C; the concentration of exogenous chromanols was 80 nmol/mg protein. Other conditions as in Methods.

cause its disappearance from the ESR spectra which were continuously recorded for 90 min.

Synergistic effects of ascorbate and dihydrolipoic acid in reduction UV-induced vitamin E radicals in LDL

To test whether thiols can affect the reduction of vitamin E radicals in LDL, we studied the time course of UV-induced chromanoxyl radicals in the presence of dihydrolipoic acid (DHLA) (Fig. 4B) and thioctic acid. DHLA alone decreased the vitamin E radical ESR signal by 30–35% but did not cause its transient disappearance as in the presence of ascorbate. When DHLA was added to the LDL suspension in combination with ascorbate, again only ascorbyl radical signals could be found in the ESR spectrum which decayed over time and was substituted by the reappearing vitamin E radical signal. However, the delay in the reappearance of the vitamin E chromanoxyl radical signal was much longer than in the presence of ascorbate only. At a given concentration of ascorbate, the duration of the delay was dependent on the concentration of DHLA added (Fig. 4B).

DHLA added to the LDL suspension in the presence of dehydroascorbate gave the same effect as in the presence of ascorbate: an immediate disappearance of the chromanoxyl radical ERS signal and appearance of the ascorbyl radical ESR signal (Fig. 4C).

Similarly, in LDL suspension in which ascorbate was depleted in the course of prolonged incubation (the ascorbyl radical ESR signal had disappeared and the chromanoxyl radical had reappeared), addition of DHLA caused a complete quenching of the chromanoxyl radical signal and the appearance of the ascorbyl radical signal (data not shown).

Thioctic acid did not affect vitamin E chromanoxyl radical ESR signal either alone or in combination with ascorbate. This suggests that DHLA (but not its oxidized form, thioctic acid) can synergistically enhance ascorbate-dependent reduction of vitamin E chromanoxyl radical in LDL. In a separate experiment we showed that DHLA did not inhibit the lipoxygenase reaction as measured by the formation of conjugated dienes in linolenic acid (data not shown).

Effect of ascorbate and dihydrolipoic acid on vitamin E radicals generated in LDL by lipoxygenase + linolenic acid

Lipoxygenase is considered to be a physiologically important LDL oxidant (27–29). Hence, in separate experiments we studied the oxidative modification of LDL by lipoxygenase + linolenic acid and the effects of ascorbate and dihydrolipoic acid on the chromanoxyl radicals generated in LDL form endogenous vitamin E.

When ascorbate was added to the LDL suspension, chromanoxyl radical ESR signals generated by lipoxygenase+linolenic acid from endogenous vitamin E could not be observed. Instead the characteristic ESR signal of ascorbyl radical was detected (**Fig. 5**). This signal decreased in time and was substituted by the progressive appearance of the chromanoxyl radical signal (Fig. 5). DHLA alone slightly suppressed the vitamin E ESR signal but did not produce its transient disappearance. In combination with ascorbate, DHLA had a synergistic effect: it substantially increased the duration of the lag period when vitamin E radical ESR signal could not be observed and ascorbyl radical signal was present (data not shown). In separate experiments with AMVN-induced chromanoxyl radicals, we obtained the results that were very similar to those with lipoxygenase+linolenic acid-induced chromanoxyl radicals (data not shown).

TABLE 1. Changes in the chromanol content and oxidation level (fluorescence intensity) of LDL by different oxidation systems

Samples	Chromanols, % of the control	Fluorescence Intensity % of the control
	Endogenous Vitamin E[a]	
Control (50 min incubation)	100	100
+ UV	84.0 ± 6.0	98.0 ± 6.0
+ Lipoxygenase + linolenic acid	<0.3	330.0 ± 32.0
+ Lipoxgenase + linolenic acid + ascorbate	40.0 ± 3.0	97.0 ± 6.5
+ Lipoxygenase + linolenic acid + DHLA	10.0 ± 1.0	120.0 ± 10.0
+ Lipoxygenase + linolenic acid + ascorbate + DHLA	70.0 ± 5.0	98.0 ± 8.0
	Exogenous Chromanols[b]	
Control (50 min incubation)	100	100
+ Lipoxygenase + linolenic acid + alpha-C6	20.0 ± 1.5	104.0 ± 9.0
+ Lipoxygenase + linolenic acid + alpha-C6 + ascorbate	73.6 ± 7.0	97.5 ± 5.0
+ Lipoxygenase + linolenic acid + alpha-C6 +DHLA	27.0 ± 2.0	105.0 ± 9.0
+ Lipoxygenase + linolenic acid + alpha-C6 + DHLA + ascorbate	88.0 ± 9.0	98.0 ± 8.0
+ UV + alpha-C6	74.0 ± 6.5	106.0 ± 9.0
+ UV + alpha-C6 + DHLA	82.5 ± 7.0	98.0 ± 7.0
+ UV + alpha-C6 + ascorbate	90.0 ± 8.5*	100.0 ± 5.0
+ UV + alpha-C6 + ascorbate + DHLA	95.0 ± 10.0**	99.0 ± 8.0
+ UV + alpha-tocotrienol	87.5 ± 8.0	100.0 ± 3.0
+ UV + alpha-tocopherol	78.5 ± 8.0	97.0 ± 8.0

[a]The concentration of endogenous vitamin E in these LDL samples was 6.2 nmol/mg protein. Incubation medium (100 µl) contained: LDL (14.0 mg protein/ml) in phosphate buffer, pH 7.4 at 25°C. Additions: lipoxygenase (3 U/µl)+linolenic acid (1.4 mM) ascorbate (1.5 mM), ascorbate (1.5 mM), DHLA (2 mM).

[b]The concentration of exogenously added chromanols was 80 nmol/mg protein (the concentration of endogenous vitamin E in these LDL samples was 2.5 nmol/mg protein). Incubation medium (100 µl) contained: LDL (10 mg protein/ml) in phosphate buffer, pH 7.4 at 25°C. Additions: d-α-tocopherol, d-α-tocotrienol or chromanol-α-C6 as indicated, ascorbate (1.5 mM), DHLA (2 mM). Incubation time was 50 min. Other conditions as in Methods. Data given as mean ± SD. when +UV+alpha-C6+ascorbate, +UV+alpha-C6+DHLA and +UV+alpha-C6+ascorbate+DHLA samples were compared with +UV+alpha-C6 significant differences as indicated by unpaired *t*-tests were: *$P<0.05$, **$P<0.01$.

Reduction of chromanoxyl radicals generated from exogenous chromanols

In samples where no detectable ESR signals of chromanoxyl radicals from endogenous vitamin E could be detected, they could be obtained by enrichment of LDL with exogenous chromanols. We used two procedures to obtain chromanol-enriched LDL as described in Methods.

First, addition of α-tocopherol dissolved in DMSO to plasma, 6 h incubation, and subsequent isolation of LDL as described by Esterbauer et al. (8). In our experiment this procedure resulted in almost 2.4-enrichment of LDL with α-tocopherol (from 4.4 nmol α-tocopherol/mg protein in control to 10.5 nmol α-tocopherol/mg protein in the enriched sample). The ESR spectrum of the vitamin E radical from the enriched samples was more resolved than that from non-enriched samples (**Fig. 6**). However, better resolved ESR spectra could be obtained by another procedure.

Second, chromanols (α-tocopherol, α-tocotrienol, chromanol-α-C6) dissolved in ethanol were added to LDL suspension and shaken for 10 min. This procedure resulted in the appearance of well-resolved ESR spectra of the chromanoxyl radicals when sufficiently

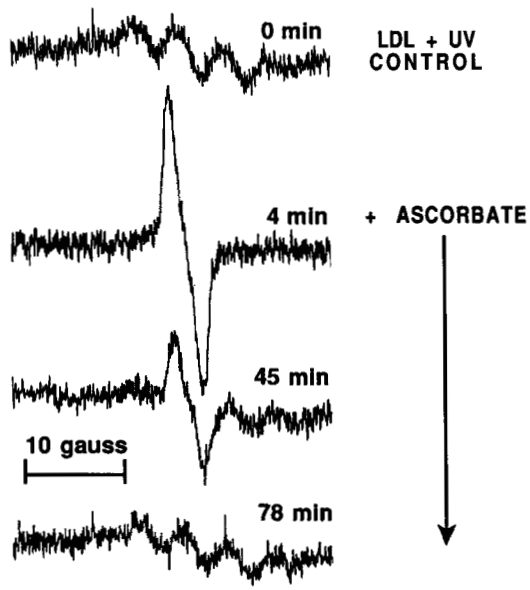

Fig. 3. ESR spectra of vitamin E chromanoxyl and ascorbyl radicals generated by UV irradiation in LDL suspensions in the presence of ascorbate. Incubation medium (100 µl) contained LDL (14.0 mg protein/ml) in 50 mM phosphate buffer, pH 7.4. The concentration of endogenous vitamin E in LDL samples was 6.2 nmol/mg protein. Ascorbate (500 µM) was added after the first recording of the chromanoxyl ESR signal was completed.

high concentrations of chromanols were used. Signals from exogenous chromanols had the same characteristic parameters in the ESR spectra. The magnitude of the ESR signal in LDL followed the order: chromanol-α-C6> α-tocotrienol > α-tocopherol (Fig. 6). In the absence of LDL, α-tocopherol, α-tocotrienol, and chromanol-α-C6 added to a buffer in ethanolic solution gave faint and poorly resolved ESR signals of their radicals in the presence of lipoxygenase + linolenic acid (Fig. 6) or under UV irradiation.

It should be noted that exogenously added chromanols strongly protected LDL against oxidative modification by lipoxygenase + linolenic acid. No significant increase in lipoxygenase + linolenic acid-induced LDL fluorescence was detected in the presence of exogenously added chromanols. The lipoxygenase + linolenic acid-induced formation of large particles (40.6 nm) detected by nondenaturing gradient gel electrophoresis was at least partially prevented by the addition of chromanols (Table 1 and **Table 2**, Fig. 2).

Essentially the same results were obtained when the effects of ascorbate and dihydrolipoic acid on the chromanoxyl radicals generated by UV irradiation or lipoxygenase + linolenic acid were studied in LDL suspensions enriched with exogenous chromanols by either of the enrichment procedures used. Ascorbate delayed the appearance of the chromanoxyl radical during which time the ascorbyl radical ESR signal was observed, and was further replaced by the reappearing chromanoxyl radical signal (**Fig. 7**). In the presence of the same ascorbate concentration, the delay in the reappearance of chromanoxyl radical signals decreased in the order: chromanol-α-C6 > α-tocotrienol > α-tocopherol (Fig. 7). In LDL with no chromanoxyl radical ESR signal from endogenous vitamin E, lipoxygenase + linolenic acid generated the ascorbyl radical ESR signal upon addition of ascorbate. However, the magnitude of the ascorbyl radical signal was 30–40% lower than that obtained from LDL enriched with exogenous chromanols.

DHLA (but not its oxidized form, thioctic acid) synergistically enhanced ascorbate-dependent reduction of chromanoxyl radicals from exogenous chromanols, although it was inefficient in direct interaction with chromanoxyl radicals (data not shown).

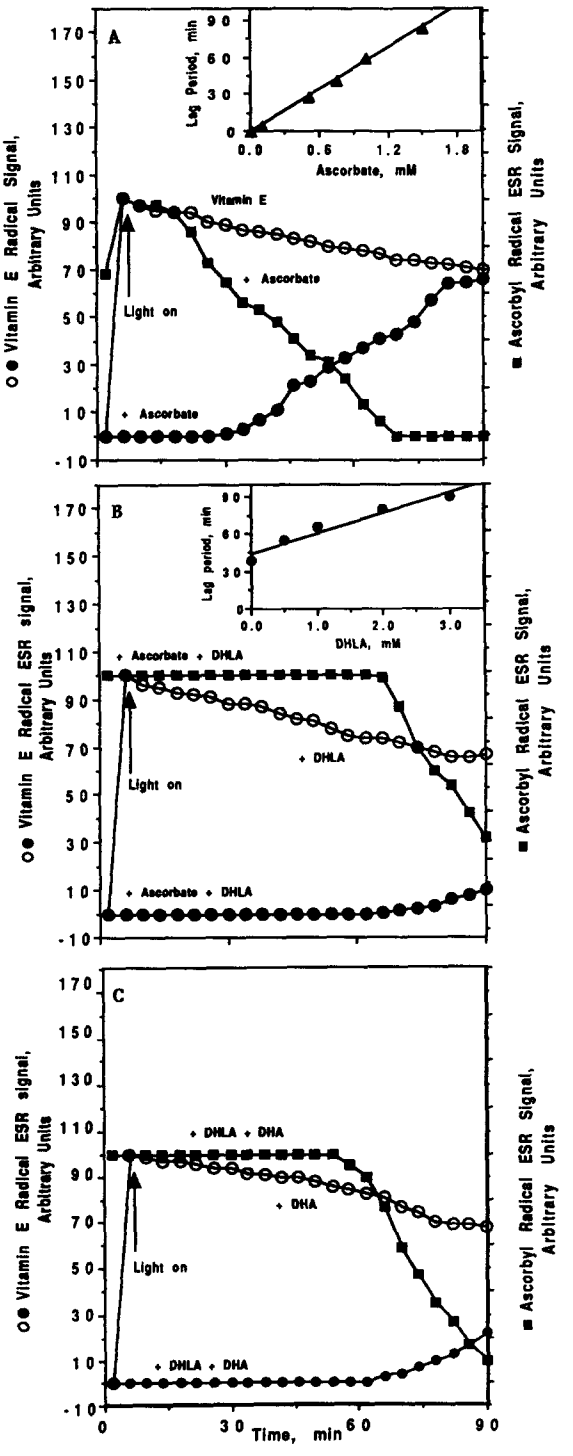

Fig. 4. Time-course of UV-induced vitamin E chromanoxyl and ascorbyl ESR signals in LDL suspensions. A: Effect of ascorbate: ascorbate, 500 μM. Insert: dependence of the lag period (during which vitamin E chromanoxyl radical ESR signal was not observed) on the concentration of ascorbate. B: Effect of dihydrolipoic acid plus ascorbate, ascorbate, 500 μM; DHLA, 2 mM. Insert: dependence of the lag period (during which vitamin E chromanoxyl radical ESR signal was not observed) on the concentration of dihydrolipoic acid. (The ascorbate concentration was 500 μM.) C: Effect of dehydroascorbic acid and dihydrolipoic acid, dehydroascorbate (DHA), 500 μM; DHLA, 2mM. Incubation medium (100 μl) contained: LDL samples with endogenous vitamin E (6.2 nmol/mg protein, 14 mg protein/ml), ascorbate, 500μM in phosphate buffer, pH 7.4, at 25°C. All values are given as a percentage of the maximal magnitude obtained. Irradiation as described in Methods.

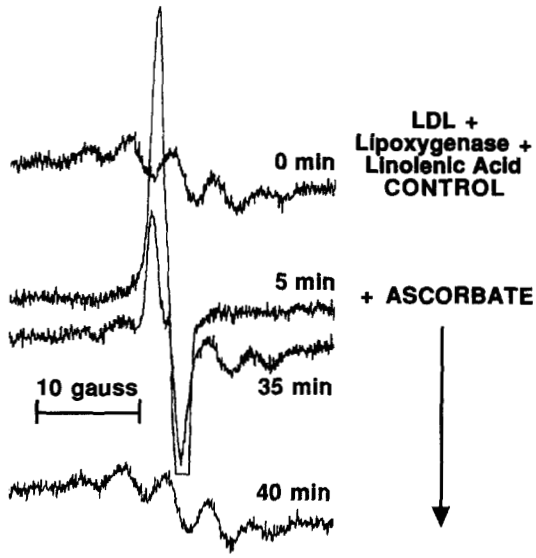

Fig. 5. ESR spectra of radicals generated by the lipoxygenase + linolenic acid oxidation system in LDL in the presence of ascorbate. Incubation medium (100 μl) contained LDL (14.0 mg protein/ml) in 50 mM phosphate buffer, pH 7.4. The concentration of endogenous vitamin E was 6.2 nmol/mg protein. Lipoxygenase (3 U/μl) + linolenic acid (1.4 mM)-induced reaction was carried out at 25°C. Ascorbate (1.5 mM) was added after the first recording of the chromanoxyl ESR signal was completed.

Consumption of chromanols in LDL and their protection by reductants interacting with chromanoxyl radicals

Generation of chromanoxyl radicals from endogenous LDL vitamin E or from exogenously added chromanols was accompanied by their consumption in the course of incubation. Under the conditions used, lipoxygenase + linolenic acid caused a dramatic decrease, and UV irradiation caused only a slight decrease in the concentrations of both endogenous vitamin E and exogenously added chromanols (Table 2 and **Table 3**). In accordance with this, the ESR signals of chromanoxyl radicals in LDL were observed for up to 90 min with only a slow decrease in their magnitude when UV irradiation was used for their generation and a much faster decay when lipoxygenase + linolenic acid oxidation was used (Fig. 1).

Ascorbate preserved both endogenous vitamin E and exogenously added chromanols against oxidative destruction (Table 2). With exogenously added chromanols the sparing effect of ascorbate decreased in the order: chromanol-α-C6>α-tocotrienol>α-tocopherol (Table 2). Combination of ascorbate + DHLA gave better protection against oxidative destruction than ascorbate alone, although the effect of DHLA alone was very weak (Table 1).

Protection of β-carotene against oxidative destruction

Another lipophilic antioxidant present in LDL, β-carotene, was destroyed both by lipoxygenase + linolenic acid and by UV irradiation (Table 3). Under the conditions used, the UV-induced loss of endogenous β-carotene was much less than its lipoxygenase-induced consumption. Exogenously added chromanols only partially protected endogenous β-carotene against oxidative destruction by lipoxygenase. Chromanol-α-C6 exerted significant protection, while α-tocopherol and α-tocotrienol were much less effective (data not shown).

Reductants interacting with chromanoxyl radicals enhanced protection of endogenous β-carotene against destruction. Chromanol-α-C6 and ascorbate alone or in combination with DHLA were efficient in prevention of β-carotene loss upon LDL UV irradiation. DHLA was much less efficient alone than in combination with ascorbate (**Fig. 8**).

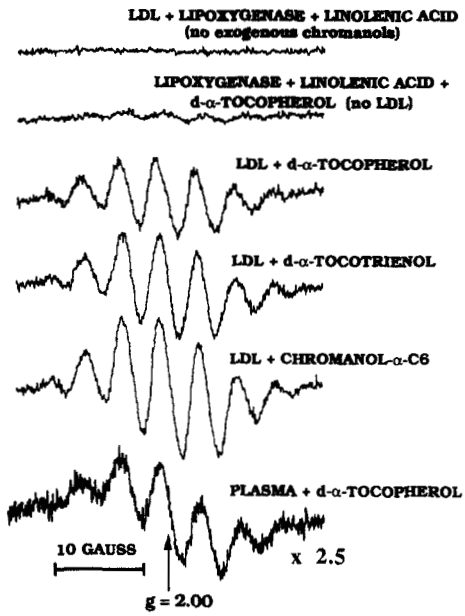

Fig. 6. ESR spectra of chromanoxyl radicals generated by an enzymic oxidation system lipoxygenase + linolenic acid in LDL enriched with exogenous chromanols. α-Tocopherol, α-tocotrienol or chromanol-α-C6 in ethanolic solution were added to LDL suspension. The concentration of exogenously added chromanols was 80 nmol/mg protein. The concentration of endogenous vitamin E in these LDL samples was 2.5 nmol/mg protein. LDL concentration was 10 mg protein/ml. In the absence of LDL α-tocotrienol and chromanol-α-C6 also gave poorly resolved ESR spectra. α-Tocopherol was added to plasma after which LDL were isolated (PLASMA + d-α-TOCOPHEROL). The concentration of endogenous α-tocopherol in this sample of nonenriched LDL was 4.4 nmol/mg protein; after enrichment the total concentration of α-tocopherol was 10.5 nmol/mg protein. LDL concentration was 9.4 mg protein/ml. Incubation medium (100 μl) contained: 50 mM phosphate buffer, pH 7.4, at 25°C, lipoxygenase (3 U/μl), linolenic acid (1.4 mM).

TABLE 2. Changes in the tocopherol, tocotrienol or chromanol-alpha-C6 content and in oxidation level (fluorescence intensity) of LDL induced by lipoxygenase+linolenic acid in the presence of ascorbate

Additions to LDL Suspension	Loss of Chromanols	Fluorescence Intensity
	nmol/mg protein	*% of control*
Control (no additions)	0	100
+ d-Alpha-tocopherol + (lipoxygenase + linolenic acid)	23.0 ± 2.0	98.0 ± 4.5
+ d-Alpha-tocotrienol + (lipoxygenase + linolenic acid)	44.0 ± 4.5	100.0 ± 6.0
+ Chromanol-alpha-C6 + (lipoxygenase + linolenic acid)	64.0 ± 5.0	97.0 ± 5.5
+ d-Alpha-tocopherol + ascorbate + (lipoxygenase + linolenic acid)	10.0 ± 1.5	98.0 ± 5.0
+ d-Alpha-tocotrienol + ascorbate + (lipoxygenase + linolenic acid)	15.0 ± 1.0	99.0 ± 8.0
+ Chromanol-alpha-C6 + ascorbate + (lipoxygenase + linolenic acid)	21.0 ± 2.5	100.0 ± 9.0

The concentration of exogenously added chromanols was 80 nmol/mg protein (the concentration of endogenous vitamin E in these LDL samples was 2.5 nmol/mg protein). Incubation medium (100 μl) contained: LDL (10 mg protein/ml) in phosphate buffer, pH 7.4 at 25°C. Additions: lipoxygenase (3 U/μl)+linolenic acid (1.4 mM), d-α-tocopherol, d-α-tocotrienol or chromanol-α-C6 (80 nmol/mg protein), ascorbate (1.5 mM), ascorbate (1.5 mM), DHLA (2 mM). Data given as mean ± SD. Incubation time was 50 min. Other conditions as in Methods.

DISCUSSION

There is a consensus that antioxidants are essential in protecting LDL against chemical modification by reactive intermediates formed and released during oxidative stress (9–16). However, the relative importance of different types of antioxidants, either water-soluble or lipid-soluble, in LDL protection is only now becoming appreciated. Previous reports suggest that ascorbate, vitamin E, or ubiquinols each may play a key role in LDL protection (9–16, 30, 31). Reports of synergistic effects of antioxidants in inhibiting lipid peroxidation suggest that the concerted action of different antioxidants may give the highest efficiency of LDL antioxidation (19, 32, 33). The synergistic mechanism(s) of antioxidant action in LDL has not yet been elucidated.

It was recently shown that the enhancement of phenolic antioxidants in membranes may be achieved by their recycling from phenoxyl radicals (34). However, the possible role of antioxidant recycling, and in particular of vitamin E recycling, in the maintenance of their sufficiently high concentrations in LDL has not been studied thus far, although generation of tocopheroxyl radicals by copper- or lipoxygenase-initiated oxidation was recently demonstrated (35).

Generation of vitamin E chromanoxyl radicals in LDL

We have used three different approaches that simulate possible sources of LDL oxidative modification in vivo to generate chromanoxyl radicals from vitamin E. *1*) UVB irradiation which is known to be absorbed over 70 μm through the stratum corneum and the

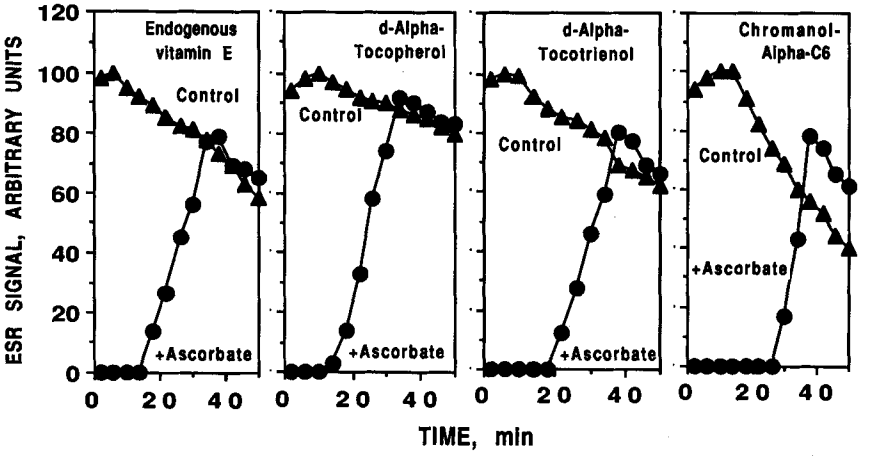

Fig. 7. Time-course of chromanoxyl radical ESR signals generated by the lipoxygenase + linolenic acid oxidation system in LDL from endogenous vitamin E or exogenously added α-tocopherol, α-tocotrienol, or chromanol-α-C6. Effect of ascorbate. In each case ascorbate (1.5 mM) was added before the recording of the first ESR spectrum. The concentrations of endogenous vitamin E were 6.2 nmol/mg protein in the sample to which no exogenous chromanols was added and 2.5 nmol/mg protein in the samples to which chromanols (80 nmol/mg protein) were added. All values are given as a percentage of the maximal magnitude obtained. Other conditions as in Fig. 6.

epidermal cell layer of the skin, may potentially modify LDL in the interstitial fluid bathing the epidermal cells (36, 37). UVB was recently shown to oxidatively modify both lipid and protein components of LDL and to deplete endogenous antioxidants (vitamin E, β-carotene) (37, 38). *2*) Leukocyte 15-lipoxygenase is believed to participate in oxidative modification of LDL in vivo (28, 29). It was recently reported that soybean 15-lipoxygenase can oxidatively modify LDL, rendering them cytotoxic (39). *3*) AMVN models toxic effects of hydrophobic xenobiotic molecules capable of producing reactive radicals that can induce LDL peroxidation (23, 24). AMVN can initiate chain free radical oxidation of LDL lipids in vitro (19).

Our results show that all three methods are able to generate chromanoxyl radicals from endogenous vitamin E or from exogenously added chromanols which had identical parameters of their ESR spectra and differed only by their magnitudes.

When chromanols were added to LDL suspensions in ethanolic solution, a difference in the magnitude of chromanoxyl radical signals generated from α-tocotrienol, α-tocopherol, or it synthetic homologue was observed. This may be accounted for by different mobility and uniformity of distribution of these chromanol molecules in the LDL lipid phase. Such difference in chromanol distribution has been previously reported for other biological systems including liver mitochondrial and microsomal membranes (18, 40). Both natural chromanols (α-tocopherol and α-tocotrienol) and the synthetic homologue with the shorter side-chain, chromanol α-C6, were substantially incorporated into LDL as evidenced by: *i*) the lack of their ESR signal in the absence of LDL, and *ii*) complete protection of LDL against (lipoxygenase + linolenic acid)-induced oxidation by exogenous chromanols.

Direct evidence for the reduction of chromanoxyl radicals (recycling of chromanols) in LDL by ascorbate

The transient disappearance of the chromanoxyl radical ESR signal and appearance of the ascorbyl radical signal after addition of ascorbate to UV-irradiated LDL can be only due to the reduction of chromanoxyl radical:

$$\text{ChR-O}^{\bullet} + \text{Asc} \rightarrow \text{ChR-OH} + \text{Asc}^{\bullet} \qquad \textit{Reaction 3})$$

since no other radicals were present to interact with the reductants. It is worth mentioning that LDL did not undergo oxidative modification under UV irradiation. Since UV irradiation did not induce the ascorbyl radical ESR signal in the absence of vitamin E, its effect in LDL might be only due to the reduction of chromanoxyl radicals to form chromanols. These data for the first time directly demonstrate that ascorbate can recycle endogenous vitamin E in LDL as was suggested earlier to explain a synergistic antioxidant interaction of vitamin E with ascorbate (17, 41, 42).

TABLE 3. Content of endogenous α-tocopherol and β-carotene in LDL suspensions incubated with different oxidation systems

Samples	d-α-Tocopherol	β-Carotene
	nmol/mg protein	*pmol/mg protein*
Control (50 min incubation)	6.2 ± 0.3	323.0
+ AMVN	4.1 ± 0.2*	
+ UV	5.2 ± 0.3*	81.2
+ Lipoxygenase + linolenic acid	< 0.1	5.0

Incubation medium (100 μl) contained: LDL (14.0 mg protein/ml) in phosphate buffer, pH 7.4, at 25°C. Additions: AMVN (5 mM) or lipoxygenase (3 U/μl) + linolenic acid (1.4 mM). Incubation time was 50 min. Irradiation and other conditions as in Methods. Data given as means ± SD. Significant differences from the control sample as indicated by unpaired *t*-tests were: $^{*}P < 0.01$.

However, this interpretation is not unequivocal when peroxyl radicals are involved in the generation of chromanoxyl radicals (reaction 1). The interaction of the reductant with peroxyl radicals:

$$\text{ROO}^{\bullet} + \text{Asc} \rightarrow \text{ROO-H} + \text{Asc}^{\bullet} \qquad \textit{Reaction 4})$$

would give the same effect, i.e., formation of ascorbyl radicals and a delay in the appearance of chromanoxyl radicals. In fact the results obtained with UV-induced and with lipoxygenase + linolenic acid-induced chromanoxyl radicals of vitamin E were essentially the same: transient disappearance of the chromanoxyl radical ESR signal and appearance of the ascorbyl radical signal. The contribution of each of the two possible mechanisms (interaction with peroxyl radicals or with chromanoxyl radicals) in the overall sparing of vitamin E against lipoxygenase + linolenic acid-induced oxidation remains to be elucidated.

Synergistic interaction of ascorbate with dihydrolipoic acid in chromanoxyl radicals reduction

Thiols are known to synergistically enhance antioxidant potency of vitamin E to prevent or inhibit membrane lipid peroxidation (29, 30, 43). This effect was attributed to the ability of thiols to directly reduce tocopheroxyl radicals thus providing for vitamin E regeneration (43–45). Recently we have shown that reduced thiols have a very low efficiency in direct vitamin E recycling but can recycle vitamin E in liposomes, membranes, and tissue homogenates by an ascorbate-mediated reaction (46).

We used dihydrolipoic acid as a model of a strong thiol reductant (redox potential of the couple dihydrolipoic acid/thioctic acid is –0.32V (47). We tested the effect of DHLA on the recycling of vitamin E in LDL in the presence or absence of ascorbate. We

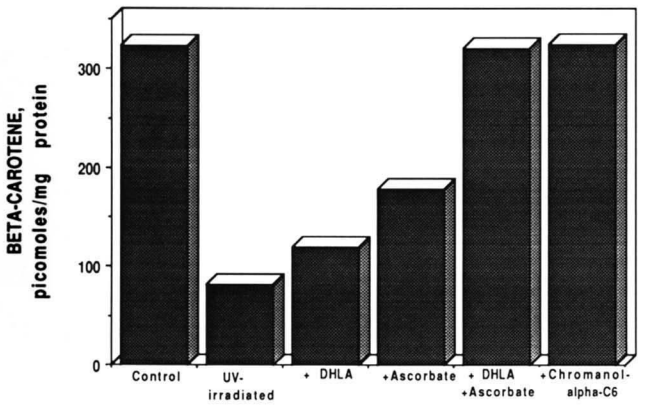

Fig. 8. Endogenous β-carotene content in LDL exposed to UVB irradiation: effects of exogenously added chromanol-α-C6, ascorbate or DHLA. Incubation medium (100 μl) contained: LDL (10 mg protein/ml) in phosphate buffer, pH 7.4, at 25°C. Ascorbate (in the buffer), DHLA (in methanol) and chromanol-α-C6 (in ethanol) were added to give the final concentrations 1.5 mM, 2.0 mM, and 80 nmol/mg protein, respectively. Irradiation was performed as described in Methods.

showed that DHLA (but not its oxidized form thioctic acid) synergistically interacted with ascorbate to reduce chromanoxyl radicals of endogenous vitamin E and exogenous chromanols. This synergistic effect of DHLA is due to its ability to maintain the steady-state concentration of ascorbate by reducing dehydroascorbate. This agrees with the previous reports that show much higher reaction rates of ascorbate in the reduction of phenoxyl or nitroxyl radicals as compared to the rates of their reduction by thiols (48, 49).

It is known that intracellular concentrations of thioctic (lipoic)/dihydrolipoic acid are within the range 5–30 nmol/g (50). Normal human urine contains 200 nmol/ml lipoic acid in a conjugate form (50). Since dihydrolipoic acid is not expected to be present in plasma (but low levels of other thiols are present), it is unlikely that dihydrolipoic acid participates in LDL vitamin E recycling under normal conditions. However, thioctic/dihydrolipoic acid antioxidant couple is presently used for the treatment in a variety of diseases, including liver and neurological disorders (51). In this case elevated levels of dihydrolipoic acid may occur in plasma.

Our results suggest that water-soluble reducing antioxidants present in plasma (i.e., ascorbate and thiols) may prevent oxidative modification of LDL not only in their own right, but also by recycling the natural lipophilic antioxidant, vitamin E, in LDL. Such agents may act independently (ascorbate) or synergistically (ascorbate + DHLA).

There may be one rather unexpected consequence of the UV-induced generation of vitamin E chromanoxyl radicals in LDL. The vitamin E concentration in LDL is higher than the concentration of any other lipid-soluble antioxidant (4, 8). Moreover, the vitamin E absorbance spectrum (maximum at 295 nm) extends well into the solar spectrum. When vitamin E absorbs solar UV light and becomes a free radical, it may deplete other water-soluble reductive antioxidants (ascorbate, thiols) by the mechanism of its own regeneration. Thus, under conditions of high intensity UV irradiation, vitamin E in addition to its peroxyl radical scavenging activity may act as a UV-sensitizer, enhancing UV-induced consumption of antioxidants (52).

Role of vitamin E in protecting β-carotene against oxidation in LDL

Vitamin E, ubiquinols, and carotenoids (β-carotene, lycopene) are the lipid-soluble antioxidants of LDL (4, 8). While vitamin E and unbiquinols are typical chain-breaking phenolic antioxidants, carotenoids do not owe their antioxidant potency to their redox properties. As an example of the interaction of vitamin E with a non-redox active lipid-soluble antioxidant, we have chosen β-carotene.

We have recently reported that β-carotene was not able to reduce chromanoxyl radicals, i.e., recycle vitamin E (52). The results of this study showed that both vitamin E and β-carotene were depleted by UV irradiation and by lipoxygenase. UV irradiation resulted in a pronounced consumption of endogenous β-carotene but only a slight decrease in endogenous vitamin E. This may be explained by the differences in the UV-absorption bands of β-carotene and α-tocopherol and by the spectral characteristic of our light source. The solar simulator used emits light mainly within the 300–400 nm range with less than 10% of the energy in the 290–300 nm range. Thus the emitted light was heavily absorbed by β-carotene and was only slightly absorbed by α-tocopherol. Both exogenously added chromanols and the reductants of chromanoxyl radicals (ascorbate or ascorbate + DHLA) protected endogenous β-carotene against oxidation induced by UV irradiation. This suggests that vitamin E may be important in protecting not only LDL phospholipids and proteins against peroxyl radicals, but also β-carotene in LDL. Soybean 15-lipoxygenase with linolenic acid caused pronounced depletion of both vitamin E and β-carotene. This suggests that the peroxyl radicals of linolenic acid readily interact with both α-tocopherol and β-carotene in LDL.

In conclusion, our results on UV-induced generation of vitamin E radicals in LDL and their reduction by water-soluble antioxidants for the first time directly demonstrate the recycling of endogenous vitamin E in human LDL. It is quite possible that other phenolic antioxidants (vitamin E homologues, probucol, buty-

lated hydrotoluene (BHT), and its homologues) may undergo quite similar recycling from their corresponding phenoxyl radicals thus substantially increasing the antioxidant protection of LDL.

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