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Inhibition of Oxidation of Low Density Lipoprotein by Vitamin E and Related Compounds

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The oxidation of low density lipoprotein (LDL) was carried out aiming specifically at elucidating the antioxidant action of α-tocopherol. Lipophilic and hydrophilic azo compounds and copper induced the oxidation of LDL similarly to give cholesterol ester and phosphatidylcholine hydroperoxides as major products. The antioxidant potency of α-tocopherol in LDL was much poorer than in homogeneous solution. Doxyl stearic acids were used as spin probe and incorporated in LDL. The rate of reduction of doxyl nitroxide in LDL by ascorbate decreased with increasing distance from the LDL surface. From the competition between the spin probe and α-tocopherol in scavenging radical, it was found that the efficacy of radical scavenging by α-tocopherol became smaller as the radical went deeper into the interior of LDL. On the other hand, 2,2,5,7,8-pentamethyl-6-chromal spared the spin label regardless of the position of nitroxide. The antioxidant activity of chromanols against LDL oxidation increased with decreasing length of isoprenoid side chain at the 2-position. All these results were interpreted by location and low mobility of α-tocopherol in LDL. The tocopherol mediated propagation was observed notably at low rate of radical flux, but this was suppressed by reductant such as ascorbic acid and ubiquinol.

Key words: Lipid peroxidation, low density lipoprotein, vitamin E, antioxidants, free radicals

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); CE, cholesterol ester; CEOOH, cholesterol ester hydroperoxide; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance; HPLC, high pressure liquid chromatography; LDL, low density lipoprotein; NS, N-oxyl-4,4′-dimethyloxazolidine derivatives of stearic acid; NMS, methyl ester of NS; 5-NS, 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolidinyloxyl; 7-NS, 2-(5-caboxylpentyl)-2-undecyl-4,4-dimethyl-3-oxazolidynyloxyl; 10-NS, 2-(8-carboxyloctyl)-2-octyl-4,4-dimethyl-3-oxazolidinyloxyl; 12-NS, 2-(10-carboxyldecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl; 16-NS, 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl; PBS, phosphate buffered saline; PC, phosphatidylcholine; PCOOH, phosphatidylcholine hydroperoxide; PMC, 2,2,5,7,8-pentamethyl-6-chromanol; UQ_1H_2, ubiquinol-1; probucol, 4,4′-(isopropylidenedithio)bis(2,6-di-*tert*-butylphenol).

INTRODUCTION

There is now an increasing evidence which suggests that oxidative modification of low density

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lipoprotein (LDL) increases its atherogenic properties[1–4]: that is, the oxidatively modified LDL is recognized by a scavenger receptor of macrophage[5,6] rather than normal LDL receptor,[7] which results in its unregulated uptake and eventual formation of cholsteryl ester-loaded cells. In fact, polyclonal and monoclonal antibodies directed against LDL modified by oxidation, malonaldehyde or 4-hydroxynonenal were found in atherosclerotic plaques of hyperlipidemic rabbits.[8–11] Furthermore, the oxidatively modified LDL is thought to play a role in recruitment and retention of monocytes/macrophages,[12] and has the potential to damage endothelial cells.[13,14] Accordingly, the protection of LDL from oxidation by antioxidant received much attention.[15,16]

LDL has several antioxidants in itself such as vitamin E (α- and γ-tocopherol), ubiquinol, β-carotene and lycopene.[17] On a molar base, α-tocopherol is the most abundant, lipophilic radical-scavenging antioxidant contained in LDL.[18] The action of vitamin E as an antioxidant in homogeneous solution has been studied extensively and is now well understood,[19,20] but the role of vitamin E against the oxidative modification of LDL is not well elucidated and has been the subject of controversy.[18,21–43] Esterbauer and his coworkers found that the oxidation of polyunsaturated lipids occurred only after a significant drop in vitamin E,[25] and Jessup *et al.*[24] showed that oxidative modification of LDL by cultured macrophages or Cu(II) ions did not occur unless LDL was depleted of vitamin E. It has been found that oral supplementation with α-tocopherol increased both vitamin E content of LDL and its resistance to oxidation[29] and also that the supplementation of culture media with vitamin E prevents oxidative modification of LDL by cells.[36] Similar protective effects of vitamin E have been reported recently.[33,43] Epidemiological studies also show that the incidence of ischemic heart disease mortality is inversely correlated with the level of plasma vitamin E.[44,45] More recent, large-scale prospective studies show that the use of large doses of vitamin E supplements is associated with a significantly decreased risk of coronary heart disease.[46,47]

On the other hand, it has been also found that the content of vitamin E did not fully correlate with the oxidizability or resistence of the LDL to oxidation.[18] Gebicki *et al.*[22] studied the oxidation of LDL induced by γ-irradiation and observed no correlation between the initial levels of vitamin E in LDL and its oxidizability. Stocker and Bowry[32,37] proposed recently that the peroxidation is propagated within lipoprotein particles by reaction of the vitamin E radical with polyunsaturated fatty acid moieties in the lipid and that vitamin E acts as a prooxidant.

LDL has a heterogeneous structure, phosphatidylcholine (PC) and free cholesterol compose an outer monolayer, while cholesteryl ester (CE) and triglyceride form core. A large protein termed apolipoprotein B-100 is embedded in the outer layer. It is generally thought that vitamin E is incorporated in the outer monolayer. However, there has been no clear experimental evidence to prove it. In a heterogeneous medium like LDL, the antioxidant efficiency will be determined not only by its chemical reactivity toward oxygen radicals but also by physical factors such as location and mobility. For example, probucol acts as a more potent antioxidant than α-tocopherol in LDL, although the chemical reactivity of probucol toward oxygen radicals is much smaller than that of α-tocopherol.[27] The present study has been undertaken to elucidate the dynamics of action of vitamin E and related compounds as an antioxidant against oxidation of LDL.

MATERIALS AND METHODS

Materials

LDL was separated from human plasma of healthy donors by ultracentrifugation as described previously[27,48] within a density cut-off of 1.019 to 1.063 g/ml and then dialyzed with cellulose membrane in phosphate-buffered saline (PBS, pH 7.4) containing 100 μM EDTA. EDTA

was removed by dialysis prior to oxidation with copper. It was sterilized with Mille-GV filter after dialysis. The protein concentration of LDL was measured by Lowry's method.[49]

The spin probe, N-oxyl-4,4'-dimethyloxazolidine derivatives of stearic acid (NS) were purchased from Aldrich (Milwaukee, WI) and used as received. The azo radical initiators, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN), were provided by Wako Pure Chemical Industries (Osaka, Japan) and used as received. 2*R*,4'*R*,8'*R*-α-Tocopherol and chromanols with different side chain length were kindly supplied from Eisai Company (Tokyo, Japan). The structure of spin probe and antioxidants used in this study is shown in Figure 1.

Oxidation of LDL and its lipids

The oxidation of LDL (0.25 mg protein/ml unless otherwise noted) was carried out at 37°C under air in PBS (pH 7.4). The oxidation was initiated by the addition of cupric chloride or AAPH dissolved in PBS or by AMVN which was added into the LDL suspensions as an ethanol solution. The hydrophilic antioxidant was added as its aqueous solution, while lipophilic antioxidant was added as an ethanol solution. The spin probe was also added into LDL suspensions as an ethanol solution, followed by incubation. The final concentration of ethanol was always kept below 2%. The aliquots of the reaction mixture were taken out at appropriate time intervals and subjected to analyses for antioxidants and lipid hydroperoxides, after stopping the oxidation by the addition of 100 μM EDTA and freezing.

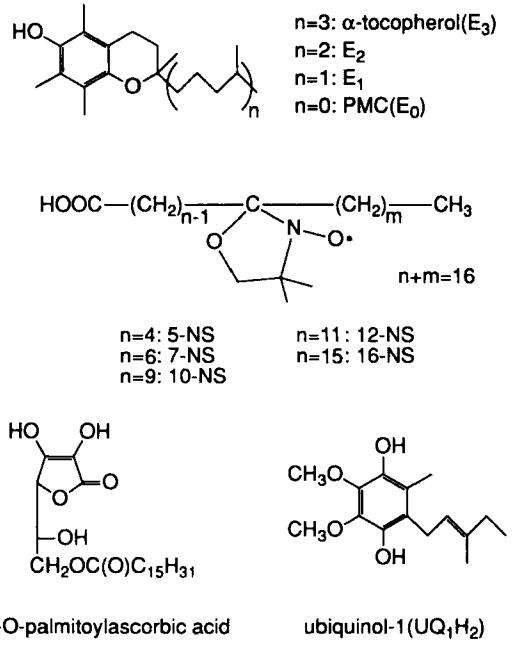

FIGURE 1 Structure of antioxidants and spin probes used in this study and their abbreviations.

The lipids and lipophilic antioxidants were extracted from LDL with twice as much chloroform and methanol mixture (2:1 by vol) and dissolved in hexane after removal of chloroform. The oxidation of this hexane solution was initiated by the addition of AMVN at 37°C under air.

Analysis of oxidation products and antioxidants

Lipid hydroperoxides and α-tocopherol were extracted from reaction mixture with twice as much chloroform/methanol (2/1 by vol) by mixing with vortex mixer for 1 min followed by centrifuging for 3 min at 12,000 rpm. The aliquots of chloroform phase containing small amount of methanol were injected to HPLC. The lipid hydroperoxides were detected by an absorption at 234 nm and α-tocopherol by electrochemical detector (Model ECP-1, Kotaki, Chiba, Japan) set at + 1,050 mV. The analytical conditions for HPLC are summarized in Table 1.

Electron spin resonance (ESR) study

The ESR spectra were recorded on an X-band JEOL-FE1X spectrometer. The spin probe was incorporated into LDL by incubation with 13.1 mg LDL/ml containing 1% ethanol. The consumption of spin probe was followed by measuring its ESR signal intensity. The conditions were as follows: magnetic field, 327 ± 1 mT; sweep time, 8 mT/min; microwave power, 1 mW; modulation

TABLE 1 Typical analytical conditions for PC and CE hydroperoxides, α-tocopherol and its analogues.

	PCOOH	*CEOOH*	*α-tocopherol*	*α-tocopherol analogues*
Column	LC-Si (5 cm) + LC-Si (15 cm) (Supeclo, Tokyo)	LC-8 (5 cm) + LC-8 (25 cm) (Supelco, Tokyo)	LC-18 (25 cm) (Supelco, Tokyo)	LC-18 (5 cm) + LC-8 (25 cm) (Supelco, Tokyo)
Eluent	MeOH/*t*-BuOH/PB (6:3:1) 1.0 ml/min	MeOH/*t*-BuOH (95:5) 1.0 ml/min	MeOH/*t*-BuOH (9:1) containing 50 mM $NaClO_4$ 1.0 ml/min	MeOH/H_2O (96/4) containing 50 mM $NaClO_4$ 1.0 ml/min

MeOH: methanol; *t*-BuOH: tert-butyl alcohol; PB: phosphate buffer

frequency, 100 kHz; and modulation amplitude, 0.02 mT.

Reproducibility and error in oxidations and analyses

The oxidation run was carried out repeatedly several times. Although the content of α-tocopherol was different and accordingly lag time and rate of oxidation varied with LDL samples from different donors, they showed the similar pattern of oxidation. The experimental error in oxidations and analyses was within ± 15%.

RESULTS

The oxidation of LDL was induced by either AAPH, AMVN or copper. The formations of PC and CE hydroperoxides and consumption of α-tocopherol were not observed appreciably in the absence of initiator, but the addition of any one of them immediately induced the lipid hydroperoxide formation and α-tocopherol consumption. The examples are illustrated in Figure 2, which shows that, as observed and reported previously,[27,30,48] LDL was oxidized similarly by these initiators to give CE and PC hydroperoxides as major products. One of the interesting points which is relevant to the aim of this study is that considerable lipid hydroperoxides were formed even during the presence of α-tocopherol.

In order to estimate the antioxidant potency of α-tocopherol in LDL, the antioxidant activities of α-tocopherol were compared in solution and in LDL (Figure 3). The lipids and lipophilic antioxidants were extracted from LDL with chloroform-methanol and they were oxidized in hexane in the presence of AMVN. As observed previously,[32,48] PC was oxidized exclusively in hexane and the formation of CE hydroperoxide was small. Interestingly, α-tocopherol suppressed the oxidation almost completely in hexane but considerable formation of CE and PC hydroperoxides was observed in LDL, suggesting that the antioxidant activity of α-tocopherol in hexane solution is far better than that in LDL.

In order to clarify the reason for such a marked difference in antioxidant potency of α-tocopherol in homogeneous solution and in LDL, the efficacy of radical scavenging by α-tocopherol in LDL was estimated by using a spin label. It has been found previously[50] that the spin label, N-oxyl-4,4'-dimethyloxazolidine derivatives of stearic acid, acts as an antioxidant against lipid peroxidation and that it is consumed with an extent of oxidation. α-Tocopherol competes with the spin label and, when α-tocopherol scavenges radicals faster than the spin label, the spin label is spared. Thus, the efficacy of radical scavenging by α-tocopherol can be estimated from the relative effectiveness for sparing the spin label. Figure 4 shows the disappearance of two kinds of spin labels, 5-NS and 16-NS, incorporated into LDL during the oxidation induced by AAPH in the absence and presence of either α-tocopherol or PMC incorporated exogenously into LDL. In the absence of any added antioxidant, 5-NS was consumed slowly at first and then at a faster rate. Apparently, the

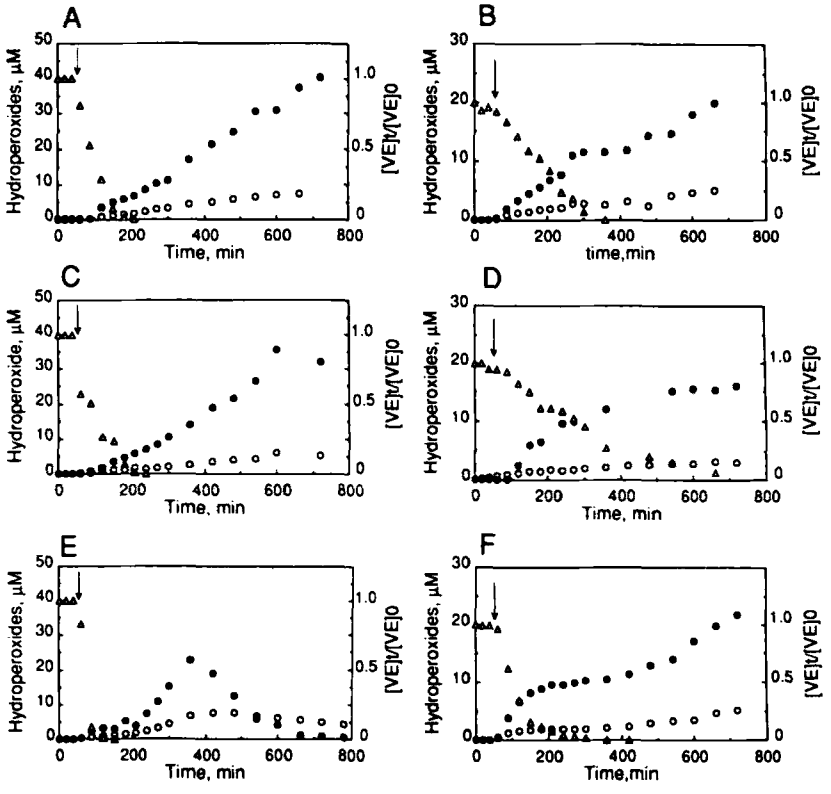

FIGURE 2 Oxidations of human LDL (0.25 mg protein/ml) in 10 mM phosphate buffered saline, pH 7.4, induced by either AAPH, AMVN or copper (II) chloride at 37°C in air. The initiators were added 50 min after incubation at the point indicated by an arrow and the consumption of endogenous α-tocopherol (VE, △) and formations of PCOOH (○) and CEOOH (●) were followed as described in Materials and Methods. The endogenous α-tocopherol concentration was 1.87 μM. A: 3.0 mM AAPH; B: 0.40 mM AAPH; C: 2.0 mM AMVN; D: 0.40 mM AMVN; E: 5.0 μM $CuCl_2$; F:0.50 μM $CuCl_2$.

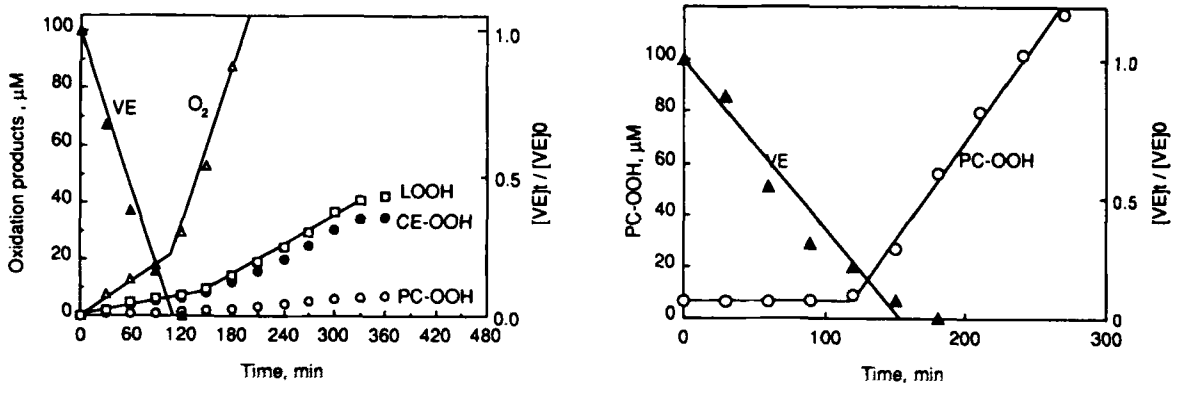

FIGURE 3 Inhibition by α-tocopherol of oxidations of LDL (left) and lipids extracted from it (right) at 37°C in air. Left: LDL (0.25 mg protein/ml) was oxidized with AAPH (0.50 mM) in phosphate buffer saline, pH 7.4. The initial endogenous α-tocopherol was 1.26 μM. Right: Lipids and α-tocopherol were extracted from 1 ml LDL suspensions containing 2 mg protein with 2 ml chloroform/methanol (2/1 by vol). Chloroform layer (1 ml) was collected into a flask, from which chloroform was removed under reduced pressure and then 1 ml hexane was added. The hexane solution was oxidized by adding 3 mM (final concentration) AMVN. △: oxygen uptake; ▲: α-tocopherol (VE); ●: CEOOH; ○: PCOOH; □: CEOOH + PCOOH

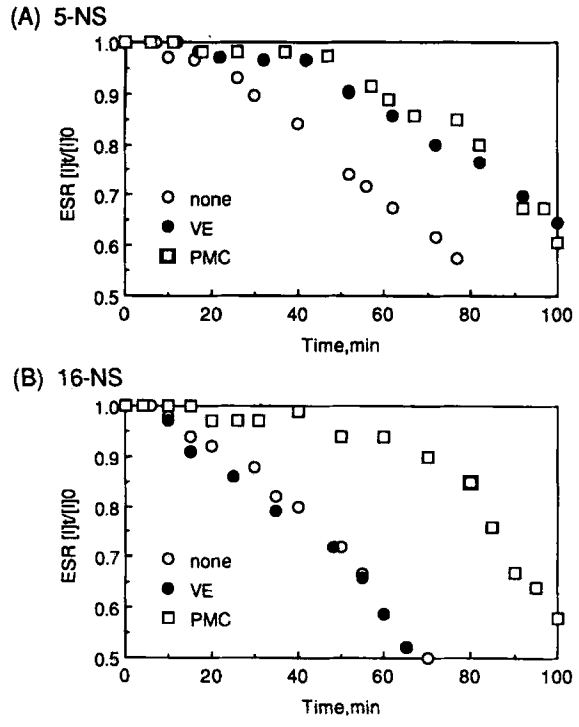

FIGURE 4 Effects of α-tocopherol (VE) and PMC on the consumption of 5-NS and 16-NS during the oxidation of LDL. The spin probe, α-tocopherol and PMC were incorporated into LDL as described in Materials and Methods and then LDL (1.69 mg protein/ml) was oxidized in the presence of 20 mM AAPH at 37°C under air. The consumption of (A) 5-NS or (B) 16-NS was followed with ESR signal intensity. ○: without antioxidant addition; ●: with 10 μM α-tocopherol added exogenously; □: with 10 μM PMC

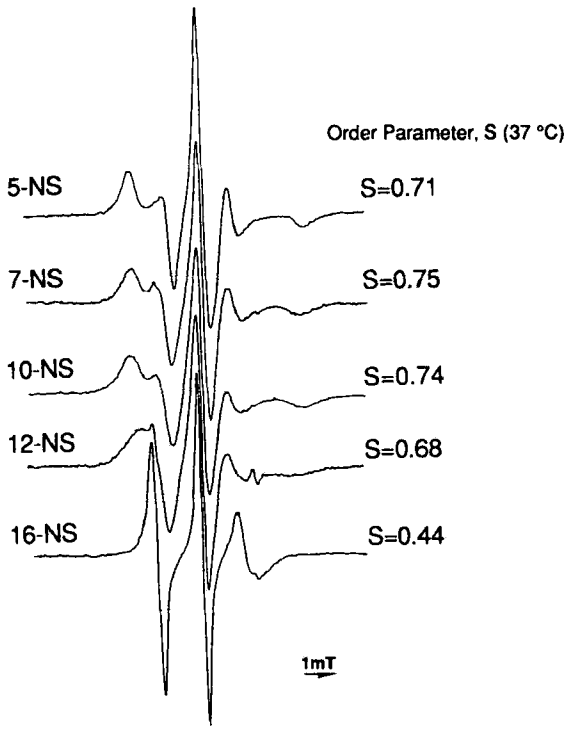

FIGURE 5 The ESR spectra of 5-NS, 7-NS, 10-NS, 12-NS and 16-NS incorporated into LDL and measured as described in Materials and Methods.

endogenous α-tocopherol spared 5-NS at the initial stage. When either α-tocopherol or PMC was incorporated exogenously into LDL, 5-NS was spared quite markedly and then it was consumed at a similar rate as that in the absence of either α-tocopherol or PMC. The spin probe 16-NS was also consumed similarly, but, interestingly, α-tocopherol did not spare 16-NS, while PMC did. Substantially the same results were observed with only endogenous α-tocopherol without exogenously added antioxidant (data not shown). Furthermore, AMVN gave the same results as AAPH (data not shown).

In order to prove that the spin probe NS was incorporated into LDL in such a way that the free carboxyl group was placed at the surface of LDL particle and the stearic acid chain was embedded perpendicularly into the interior of LDL outer monolayer, the ESR spectra of the spin labels having nitroxide group at different positions of stearic acid chain and the interaction of these nitroxide groups of NS and ascorbate were studied. The ESR spectra of 5-NS, 7-NS, 10-NS, 12-NS and 16-NS are shown in Figure 5. The ESR parameters for the paramagnetic nitroxide moiety situated at different positions of the carbon chain give us information on fatty acid mobility and membrane fluidity. The ESR order parameters for 5-NS, 7-NS, 10-NS, 12-NS, and 16-NS incorporated into LDL were obtained, respectively, as 0.71, 0.75, 0.74, 0.68, 0.44. The doxyl stearic acid methyl ester (NMS) was also used. The ESR order parameters for both 5-NMS and 12-NMS were 0.65. The rotational correlation time obtained by 16-NS in hexane,

benzene, decane, methyl linoleate aqueous emulsions, soybean PC liposomes and human LDL was, respectively, 0.20×10^{-10}, 0.32×10^{-10}, 0.41×10^{-10}, 2.7×10^{-10}, 4.3×10^{-10}, and 12.9×10^{-10} s. These numbers suggest that the spin probe was incorporated into LDL as assumed and that the surface of LDL particle is quite rigid while the inner monolayer becomes more fluid.

The nitroxide group of NS has been shown to interact quite rapidly with ascorbate in homogeneous solution.[50] However, as shown in Figure 6, when ascorbate was added into the aqueous suspensions of LDL into which the spin probe had been incorporated beforehand, the spin probe was consumed only slowly, the rate getting smaller as the nitroxide group went deeper into the interior of LDL. The spin probes 10-NS, 12-NS, and 16-NS were reduced by ascorbate at the similar rate. The spin probe 5-NMS was reduced slower than 5-NS. It may be noteworthy that cholesteryl 16-doxylstearate incorporated into LDL core was not reduced by ascorbate but it was reduced by 6-O-palmitoylascorbic acid (data not shown).

The above results suggest that the efficiency of radical trapping by α-tocopherol decreases as the radicals go deeper into the interior of LDL, as observed in liposomal membranes.[50] The results in Figure 4 suggest that PMC may act as a stronger antioxidant than α-tocopherol in LDL. To test this, the effects of side chain length of chromanols on the antioxidant activity against LDL oxidation were studied. The data summarized in Table 2 show clearly that the rates of formation of PC and CE hydroperoxides decreased as the length of side chain of chromanols decreased and that PMC inhibited the oxidations of lipids almost completely.

It has been known that vitamin E acts as an antioxidant not only by itself but also cooperatively or even synergistically with other antioxidants. In the present study, the effects of ubiquinol and fatty acid ester of ascorbic acid were examined. It was found that ubiquinol-10 was not readily incorporated into LDL *in vitro* and hence ubiquinol-1 having much shorter side chain was used. When LDL was oxidized in the presence of 10 μM (final concentration) ubiquinol-1 with either AAPH or AMVN, ubiquinol-1 was consumed faster than endogenous α-tocopherol and neither CE hydroperoxide nor PC hydroperoxide was formed appreciably during its presence (Figure 7). 6-O-Palmitoylascorbic acid also spared α-tocopherol and suppressed the oxidation of LDL induced by either AAPH or AMVN efficiently (Figure 8). Ascorbic acid added into aqueous phase also suppressed α-tocopherol consumption and lipid hydroperoxide formation efficiently (data not shown) as observed previously.[30]

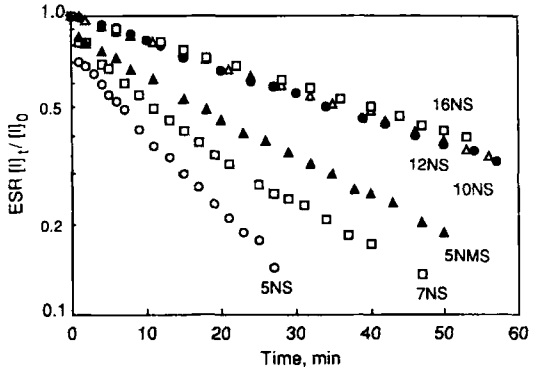

FIGURE 6 Reduction of spin probes NS incorporated into LDL by ascorbate. The spin probe, 5-NS (○), 7-NS (□), 10-NS (△), 12-NS (●) or 16-NS (□), (final concentration 30 μM) was incorporated into LDL (13.1 30 mg/ml) by adding its ethanol solution, followed by incubation. The 5-doxyl stearic acid methyl ester (5-NMS, ▲) was also incorporated into LDL similarly. Ascorbate (5.2 mM) was then added to the LDL suspensions and the disappearance of NS was followed with ESR at 37°C. $[I]_0$ and $[I]_t$ denote ESR signal intensity of the spin probe at time 0 and t respectively.

DISCUSSION

The above results obtained by using spin probe enable us to draw two important conclusions; firstly vitamin E is located predominantly in the outer monolayer placing active phenolic group at or near the surface of LDL particle and secondly, the efficiency of radical scavenging by vitamin E decreases as the radical goes deeper into the interior of LDL.

TABLE 2 Rates of formation of phosphatidylcholine and cholesterol ester hydroperoxides (PC-OOH and CE-OOH) in the oxidation of 0.25 mg protein/ml LDL induced by either 3 mM AAPH, 2 mM AMVN or 5 μM $CuCl_2$ in the absence and presence of 10 μM α-tocopherol analogues at 37°C under air.

	AAPH		*AMVN*		*$CuCl_2$*	
	PCOOH	*CEOOH*	*PCOOH*	*CEOOH*	*PCOOH*	*CEOOH*
none	4.0	5.5	1.8	5.6	3.1	9.6
E_2	1.5	3.8	3.9	5.9	0.36	1.5
E_1	0.77	2.7	0.60	2.8	0.17	1.5
E_0	0	0	0.67	3.0	0	0

α-Tocopherol analogues were incorporated into LDL as described in Materials and Methods and then the LDL was subjected to oxidation by adding an initiator.

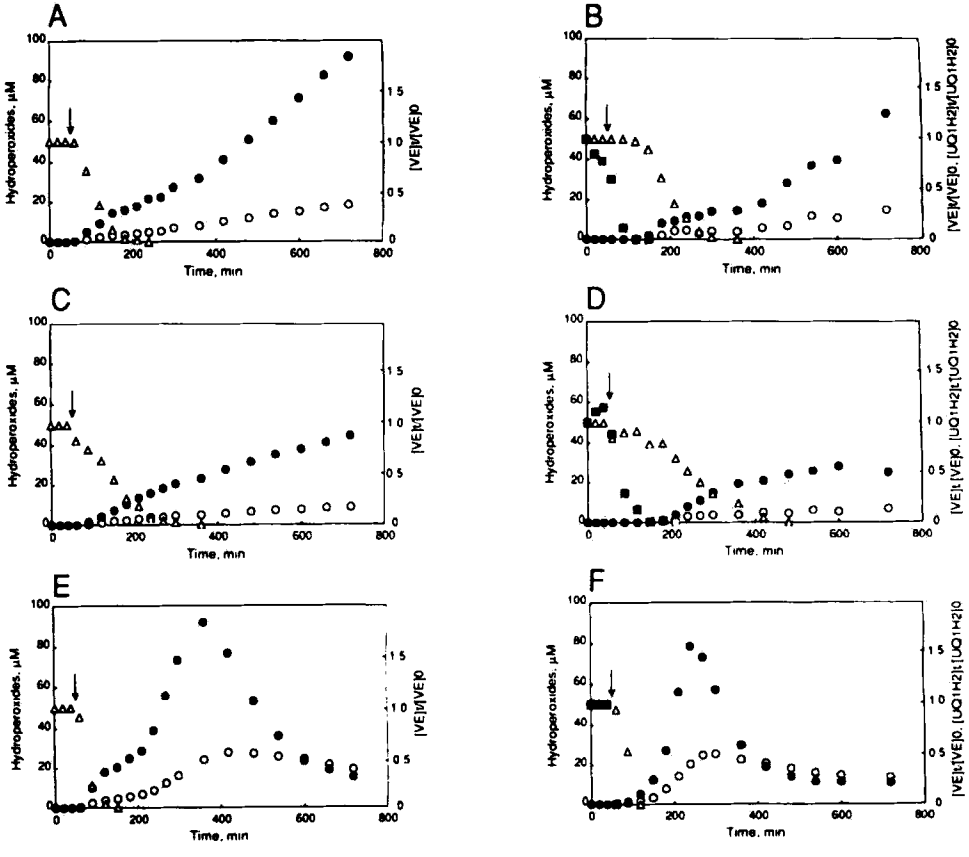

FIGURE 7 Effects of ubiquinol-1 (UQ_1H_2) on the oxidation of human LDL at 37°C in air. Ubiquinol-1 was prepared and incorporated into LDL by incubation as described in Materials and Methods. The oxidation of LDL (0.25 mg protein/ml) was induced by either AAPH, AMVN or Cu which was added at the time indicated by an arrow (50 min). The formations of PC (○) and CE (●) hydroperoxides and consumptions of α-tocopherol (VE) (△) and ubiquinol-1 (UQ_1H_2) (■) were followed. A: 3 mM AAPH, no UQ_1H_2; B: 3 mM AAPH, 10 μM UQ_1H_2; C: 2 mM AMVN, no UQ_1H_2; D: 2 mM AMVN, 10 μM UQ_1H_2; E: 5 μM $CuCl_2$, no UQ_1H_2; F: 5 μM $CuCl_2$, 10 μM UQ_1H_2.

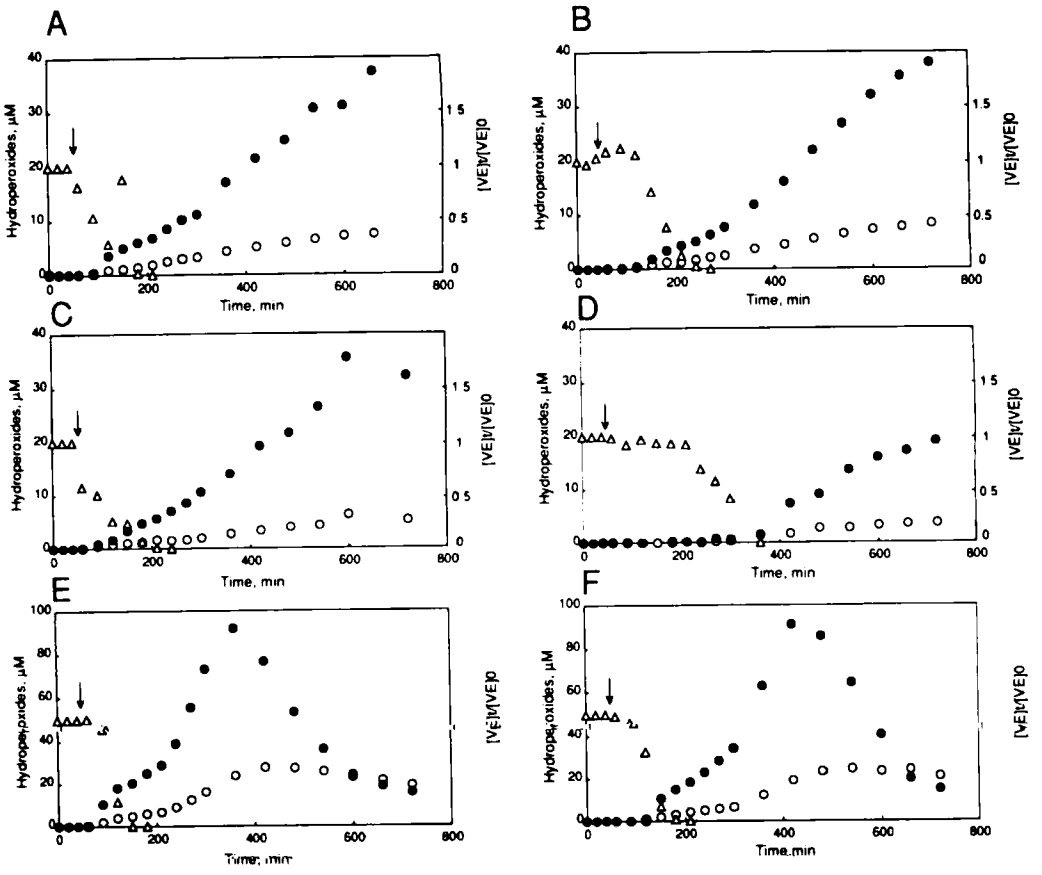

FIGURE 8 Effect of 6-O-palmitoylascorbic acid on the oxidation of LDL (0.25 mg protein /ml) initiated by either (A) (B) 3 mM AAPH, (C) (D) 2 mM AMVN, or (E) (F) 5 μM $CuCl_2$ at 37°C in air. The formation of PC (○) and CE (●) hydroperoxides and consumption of α-tocopherol (VE, △) were followed as described in Materials and Methods. (A), (C), (E): no 6-O-palmitoylascorbic acid; (B), (D), (F): 10 μM 6-O-palmitoylascorbic acid.

Ascorbic acid reduces the spin probe quite rapidly in homogeneous solution.[50] Figure 6 shows that the rate of reduction of NS by ascorbate decreased as the nitroxide radical went deeper into the interior of LDL, as observed with liposomal membranes.[51] The ESR spectra and parameters show that the spin probes are incorporated into LDL as assumed, that is, the free carboxyl group is placed at the LDL-particle surface and the stearic acid chain is embedded perpendicularly into the LDL interior. These results are in agreement with the reported ones.[52] It may be noteworthy that ascorbate alone did not suppress the oxidation of LDL efficiently when it was induced by lipophilic AMVN but that ascorbate acted as a synergist in the presence of α-tocopherol in LDL.[30] These results and the fact that ascorbic acid spares α-tocopherol efficiently in LDL suggest that α-tocopherol is incorporated in the outer monolayer of LDL. It appears that such a location of α-tocopherol in LDL has been accepted considering the hydrogen bonding between phenolic hydroxyl group and water, but no experimental evidence has been presented so far.

The efficiency of α-tocopherol in sparing NS renders additional experimental proof for the above point. It was found previously[50] that the spin label NS acted as an antioxidant probably by scavenging radicals and it was consumed as the oxidation proceeded. α-Tocopherol when present

simultaneously competes with the spin label in scavenging radicals and spares it. The efficiency of radical scavenging by α-tocopherol can be estimated from the effectiveness for sparing NS by α-tocopherol. As shown in Figure 4, α-tocopherol spared 5-NS markedly but it could not spare 16-NS efficiently, implying that the active site of α-tocopherol is closer to the surface of LDL particles and that the vertical mobility of α-tocopherol is restricted.

Another interesting feature of the results in Figure 4 is that PMC can spare 16-NS as well as 5-NS quite efficiently in LDL as observed in liposomal membranes.[50] Furthermore, the antioxidant activity of chromanols against LDL oxidation increased with decreasing length of side chain (Table 2), and PMC suppressed AAPH and copper-induced oxidation completely. This must be ascribed to the higher mobility of chromanols with shorter side chain length. In support of this, it was found previously that the mobility of PMC was quite high within and between the membranes.[53,54]

The overall potency of α-tocopherol as an antioxidant is determined not only by the rate of radical scavenging but also by a relative importance of several competing reactions of α-tocopheroxyl radical.[55,56] It may attack substrate, scavenge another peroxyl radical to give an adduct, react with another vitamin E radical to give a dimer, or be reduced by a reductant such as vitamin C and ubiquinol to regenerate vitamin E. If the concentrations of the peroxyl and/or vitamin E radicals and reductant are low, α-tocopheroxyl radical might attack lipid and/or lipid hydroperoxide to initiate the chain oxidation of lipids.

Figure 2 shows that the rate of formation of lipid hydroperoxides was fast right after the addition of any initiator and that it then declined as α-tocopherol was consumed. This is especially evident at lower concentration of the initiator, that is, when the fluxes of radicals are low. Our earlier report (Figure 2 of ref. 30) also showed the similar trend but its significance was overlooked. Bowry, Stocker, and Ingold[32,36,37] have also observed these phenomena and from the detailed and extensive discussion they proposed that α-tocopheroxyl radical acted as a chain-transfer agent: that is, α-tocopherol reacts with aqueous radicals more readily than the lipids in LDL to give α-tocopheroxyl radical, which attacks lipids and abstracts hydrogen to give lipid radical. In other words, α-tocopherol enhances the fraction of aqueous radicals that react with lipids in LDL. In agreement with this proposal named tocopherol mediated propagation, they found that the rate of AAPH-initiated peroxidation of LDL enriched with α-tocopherol either *in vitro* or by oral supplementation was larger than that of non-enriched control.[32,37] Bowry *et al.*[32] also found that the efficiency of chain initiation measured from the rate of α-tocopherol consumption was increased by α-tocopherol supplementation. Such a prooxidant effect of α-tocopherol is convincing when the oxidation of LDL is induced by aqueous radicals, that is, by assuming that α-tocopherol reacts with the radical at the LDL surface much more readily than the lipids in LDL particles and that the resulting α-tocopheroxyl radical attacks lipids. Such an attack of α-tocopheroxyl radical on polyunsaturated lipids has been observed in solution,[57,58] although the rate should be much smaller in LDL than in homogeneous solution.

The effects of ascorbic acid, ubiquinol and related compounds also support the above conclusion. Bowry, Ingold and Stocker[32,36] explained how the reagents such as ascorbic acid and ubiquinol which reduce α-tocopheroxyl radical strongly inhibit lipid peroxidation in vitamin E-containing LDL. Stocker *et al.*[23] found that the LDL oxidation was reduced significantly by the addition of either ascorbic or ubiquinol-10. The data of the present study (Figures 7 and 8) also show that ascorbic acid ester and ubiquinol-1 inhibited the oxidation quite efficiently.

In conclusion, it may well be stated that the efficiency of radical scavenging by vitamin E is small in LDL compared in homogeneous solution and becomes smaller as the radical goes deeper

into the interior of LDL, probably due to its low mobility within LDL and low fluidity of LDL. It may be assumed that the scavenging of radicals within LDL core by vitamin E is difficult. It may be also said that, as reported by Bowry *et al.*,[32,36,37] α-tocopherol might act as a prooxidant under certain conditions, but such an effect may not be important *in vivo* where the reductants such as ascorbic acid reduce α-tocopheroxyl radical.

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