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GENERATION OF PROBUCOL RADICALS AND THEIR REDUCTION BY ASCORBATE AND DIHYDROLIPOIC ACID IN HUMAN LOW DENSITY LIPOPROTEINS

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Probucol, 4,4′-[(1-methylethylidene)bis(thio)]bis-[2,6-bis(1,1-dimethyl)phenol], is a lipid regulating drug whose therapeutic potential depends on its antioxidant properties. Probucol and α-tocopherol were quantitatively compared in their ability to scavenge peroxyl radicals generated by the thermal decomposition of the lipid-soluble azo-initiator 2,2′-azo-bis(2,4-dimethyl-valeronitrile), AMVN, in dioleoylphosphatidylcholine (DOPC) liposomes. Probucol showed 15-times lower peroxyl radical scavenging efficiency than α-tocopherol as measured by the effects on AMVN-induced luminol-dependent chemiluminescence. We suggest that probucol cannot protect α-tocopherol against its loss in the course of oxidation, although probucol is known to prevent lipid peroxidation in membranes and lipoproteins. In human low density lipoproteins (LDL) ESR signals of the probucol phenoxyl radical were detected upon incubation with lipoxygenase + linolenic acid or AMVN. Ascorbate was shown to reduce probucol radicals. Dihydrolipoic acid alone was not able to reduce the probucol radical but in the presence of both ascorbate and dihydrolipoic acid a synergistic effect of a stepwise reduction was observed. This resulted from ascorbate-dependent reduction of probucol radicals and dihydrolipoic acid-dependent reduction of ascorbyl radicals. The oxidized form of dihydrolipoic acid, thioctic acid, did not affect probucol radicals either in the presence or in the absence of ascorbate.

KEY WORDS: Antioxidants, probucol, α-tocopherol, ascorbate, dihydrolipoate, low density lipoproteins, free radicals.

INTRODUCTION

Increased levels of LDL and VLDL cholesterol are known risk factors for the development of atherosclerotic vascular disease. Oxidative modification of low density lipoproteins (LDL) and accelerated degradation of LDL via the scavenger receptor pathway in macrophages, has been implicated as a factor in the generation of macrophage-derived foam cells, the hallmark of atherosclerotic plaques.[1,2] It has been proposed that this process might lead to cholesterol accumulation in macrophages in the arterial wall *in vivo*.[3,4] LDL oxidation and uptake by macrophages is strongly dependent on the balance of pro- and antioxidants in the plasma.[3,5–7] Rapid depletion of the endogenous antioxidants (vitamin E, ubiquinols, β-carotene, vitamin C) occurs during LDL oxidation. Consistent with this, exogenously added natural

antioxidants and antioxidant drugs (e.g., probucol) prevent the oxidative modification of LDL.[8–12]

Probucol is a cholesterol-lowering agent effective in the treatment of type IIa and IIb hyperlipoproteinaemias, including polygenic (non-familial) hypercholesterolaemia and both heterozygous and homozygous forms of familial hypercholesterolaemia.[7,13,14] Probucol has also shown efficacy in hyperlipoproteinaemia secondary to nephrotic syndrome and diabetes mellitus.[13,14] The mechanism of the reduction in LDL cholesterol levels by probucol is yet to be fully elucidated, but recent evidence suggests that its antioxidant properties are involved.[13–15] Probucol was reported to prevent oxidation of LDL *in vitro* to an extent that directly related to its concentration in LDL.[16] The decrease in lipid oxidation was directly correlated with the inhibition of both oxidized-LDL-induced cholesteryl ester synthesis in cultured macrophages and to the inhibition of aortic atherosclerosis *in vivo*.[16]

Probucol has been claimed to act as a phenolic chain-breaking antioxidant presumably by donating a hydrogen atom to intermediate peroxyl and/or alkoxyl radicals.[17,18] This would result in formation of the probucol phenoxyl radical. The appearance of the ESR spectrum of the phenoxyl radical produced by one electron-oxidation of probucol with the half-time of the radical decay in ethanolic solution about 30 sec at a room temperature was reported.[13]

Recently the recycling of natural and synthetic phenolic antioxidants (α-tocopherol and its homologues, butylated hydroxytoluene, BHT and butylated hydroxyanisole, BHA) from their phenoxyl radicals by enzymic electron transport had been demonstrated in membranes.[19,20] Non-enzymic reductants (ascorbate, thiols) were shown to be active in the recycling of vitamin E and its homologues both in membranes and in human LDL.[21]

The present study was therefore designed to determine if (i) probucol radicals can be generated in human LDL by interaction with peroxyl radicals in concentrations sufficient for direct ESR measurements, and (ii) if reduction of probucol radicals occurs, how effective ascorbate and thiols are in this recycling of probucol. We also compared the radical scavenging efficiency of probucol with that of α-tocopherol in dioleoylphosphatidylcholine (DOPC) liposomes.

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 and it was adjusted to d 1.019 g/ml with solid NaBr and centrifuged 40,000 rpm, in a 50.3 Beckman rotor for 24 hr 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 hr 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 phosphate buffer (50 mM, pH 7.4) prior to use.

Fluorescence Spectra of LDL Suspensions

Fluorescence emission spectra of LDL suspensions in phosphate buffer (50 mM, pH 7.4, 0.2 mg protein/ml) wer 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.[22]

Generation of Probucol or α-Tocopherol Radicals

Probucol or α-tocopherol radicals were generated using: (1) an enzymic oxidation system (soybean 15-lipoxygenase + linolenic acid), or (2) a hydrophobic azoinitiator 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 (7.0 mg protein/ml) in 50 mM phosphate buffer, pH 7.4 at 25°C. The concentrations of exogenously added probucol or α-tocopherol were 80 nmoles/mg protein and 12 nmol/mg protein, respectively. Linolenic acid (1.4 mM) + lipoxygenase (3.0 U/μl) and probucol (or α-tocopherol) in ethanolic solution were subsequently added to 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.

Chemiluminescence Measurements

Generation of peroxyl radicals by AMVN and interactions of peroxyl radicals with probucol and α-tocopherol in dioleoylphosphatidylcholine (DOPC) liposomes were assayed by the chemiluminescence produced in the presence of luminol. The incubation medium contained: DOPC liposomal suspension (2.5 mM) in Tris-HCl buffer (pH 7.4 at 40°C), luminol (150 μM), AMVN (2.5 mM) and probucol (or α-tocopherol) in concentrations as indicated in the figure legends. The reaction was started by addition of AMVN. α-Tocopherol or probucol were incorporated into DOPC liposomes by sonication (60 sec at 4°C).

ESR Measurements

ESR measurements were made on a Varian E 109 E spectrometer in gas permeable Teflon tubings (0.8 mm internal diameter, 0.013 mm thickness obtained from Zeus Industrial Products, Raritan N.J. USA). The gas permeable tube (approximately 8 cm in length) was filled with 100 μl of a mixed sample, folded into quarters and placed in an open 3.0 mm internal diamter EPR quartz tube such that all of the sample was within the effective microwave irradiation area. Spectra were recorded at room temperature (or at 40°C with AMVN) under aerobic conditions by flowing oxygen through the ESR cavity at 100 mW power and 2.5 gauss modulation, and 25 gauss/minute scan time, 3245 gauss magnetic field strength, scan range 100 gauss, and time constant 0.0-64 sec.

Reagents

Probucol, linolenic acid, soybean 15-lipoxygenase (101 000 U/mg protein), ascorbate, dioleoylphosphatidylcholine were from Sigma Chemical Company, St. Louis MO, potassium phosphate dibasic, sodium phosphate monobasic from Mallinckrodt, Inc. Paris, KY, 2,2′-azo-bis(2,4-dimethyl-valeronitrile), AMVN, from PolySciences, Inc., Warrington, PA, HPLC grade ethanol and methanol from Fischer Scientific, Fair Lawn, N.J. Dihydrolipoic acid and lipoic (thiotic) acid were a gift from Asta Pharma, Frankfurt am Main. d-α-Tocopherol was a gift from Henkel Co. (La Grange, IL).

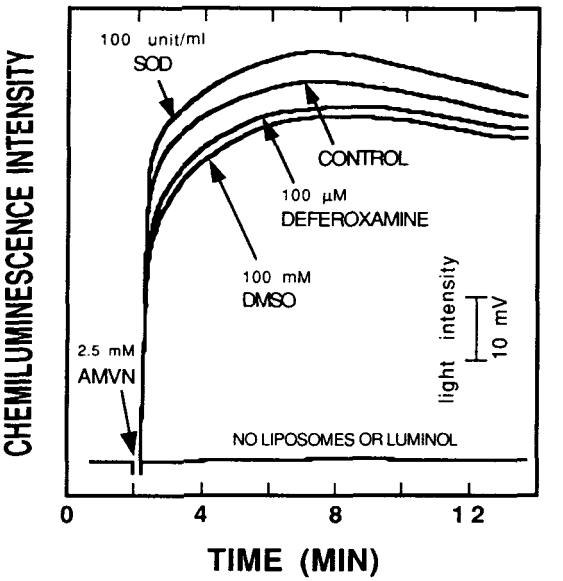

FIGURE 1 Luminol-dependent chemiluminescence induced by AMVN-derived radicals in DOPC liposomes. Effects of SOD, DMSO and Deferoxamine. Incubation conditions: 2.5 mM DOPC liposomes in 20 mM Tris-HCl buffer (pH 7.4 at 40°C), 150 μM luminol, 2.5 mM AMVN. Other concentrations as indicated.

RESULTS

Interaction of phenolic antioxidants with peroxyl radicals results in the formation of the corresponding phenoxyl radicals and hydroperoxides:

$$\text{Ph-OH} + \text{ROO}\cdot \rightarrow \text{Ph-O}\cdot + \text{R-OO-H} \qquad (1)$$

We have used two different approaches to follow the reactions of probucol with peroxyl radicals in which its phenoxyl radicals could be formed: chemiluminescence measurements of AMVN-derived peroxyl radicals interacting with luminol, or direct ESR measurements of probucol radicals.

Chemiluminescence Measurements of Probucol Radicals Generation and Recycling in Liposomes

Interaction of peroxyl radicals generated in AMVN-containing DOPC liposomes by their incubation at 40°C with luminol gave a characteristic chemiluminescent response (Figure 1). This response was not observed when either AMVN, luminol or DOPC liposomes were omitted. AMVN-induced chemiluminescence of luminol was not sensitive to oxygen-radical quenchers (SOD, DMSO) and the iron-chelator, deferoxamine. However, α-tocopherol, the lipid-soluble scavenger of peroxyl radicals, incorporated into DOPC liposomes, produced inhibition of the chemiluminescent response (Figure 2A). Half-maximal effect of α-tocopherol was achieved at 7.5 μM.

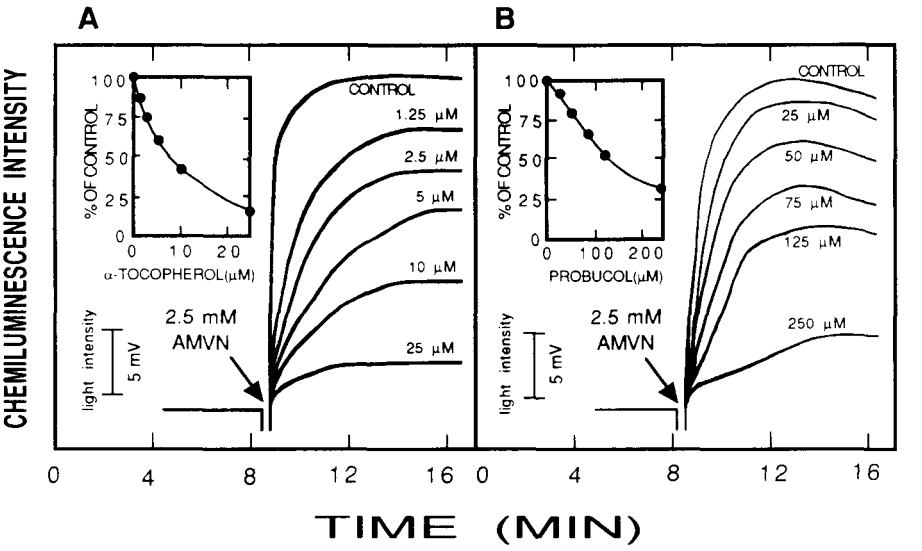

FIGURE 2 Luminol-dependent chemiluminescence induced by AMVN-derived radicals in DOPC liposomes. A – Effect of α-tocopherol. Insert: Concentration dependence of chemiluminescence quenching by α-tocopherol. B – Effect of probucol. Insert: Concentration dependence of chemiluminescence quenching by probucol. Conditions as in Figure 1.

Thus, AMVN-induced chemiluminescence in the presence of DOPC liposomes results from the interaction of AMVN-derived peroxyl radicals with luminol.

Probucol added to DOPC liposomes in ethanolic solution also inhibited AMVN-induced chemiluminescence of luminol in a concentration-dependent manner (Figure 2B). Half-maximal inhibition was attained at 120 μM of probucol in the incubation system. This agrees well with the reported efficiency of probucol in inhibiting lipid peroxidation in membranes and in LDL.[15–18] Thus probucol appears to be an efficient scavenger of peroxyl radicals but its activity is about 15-times weaker than α-tocopherol.

Addition of ascorbic acid to the AMVN-containing DOPC liposomes incubated in the presence of luminol totally quenched the chemiluminescent response (Figure 3). This quenching effect was transient and the chemiluminescence curve followed its typical time course after ascorbate was consumed. When probucol was present in the incubation medium the ascorbate-induced suppression of the chemiluminescent response was significantly stronger: the chemiluminescence curve did not return to its control level. This difference in the chemiluminescence intensity in the presence or in the absence of probucol may be due to probucol recycling from its phenoxyl radical in the presence of ascorbate:

$$\text{Ph-O}\cdot + \text{Ascorbate} \rightarrow \text{Ph-OH} + \text{Ascorbyl}\cdot \qquad (2)$$

We suggest that in the presence of ascorbate, probucol was regenerated in part which would give chemiluminescence quenching corresponding to a higher probucol concentration. These data, however, can be considered only as an indirect evidence for ascorbate-dependent probucol regeneration. Therefore, in the next set of experiments we directly followed the reactions of probucol phenoxyl radicals by ESR technique.

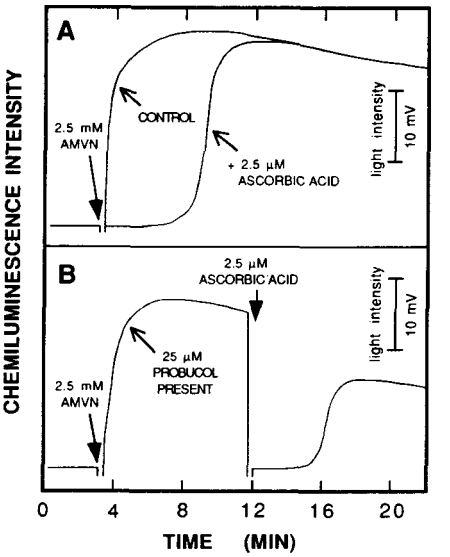

FIGURE 3 Effect of ascorbate and probucol on luminol-dependent chemiluminescence induced by AMVN-derived radicals in DOPC liposomes. A – Effect of ascorbic acid in the absence of probucol. B – Effect of ascorbic acid in the presence of probucol. Conditions as in Figure 1.

ESR Measurements of Probucol Radical Generation and Recycling in LDL

To test whether any oxidative modification of LDL (mediated by possible formation of peroxyl radicals occurred during incubation with (lipoxygenase + linolenic acid) or with AMVN we measured the intensity of LDL fluorescence which is a marker of their oxidative modification.[22] Incubation of LDL with (lipoxygenase + linolenic acid) over 60 minutes at 25°C or with AMVN at 40°C resulted in a significant increase in fluorescence intensity, indicative of pronounced oxidative modification of LDL (Table 1). Under conditions used, (lipoxygenase + linolenic acid)-system was a

TABLE I
Oxidative modification of human LDL by AMVN or lipoxygenase + linolenic acid and protective effects of α-tocopherol and probucol

Additions to LDL Suspension*	Fluorescence Intensity, Arbitrary Units
None (control)	100
Lipoxygenase + Linolenic Acid	360 ± 30
Lipoxygenase + Linolenic Acid + α-Tocopherol (12.0 nmol/mg protein)	98 ± 10
Lipoxygenase + Linolenic Acid + Probucol (80 nmol/mg protein)	102 ± 12
AMVN	240 ± 18
AMVN + α-Tocopherol (12.0 nmol/mg protein)	105 ± 14
AMVN + Probucol (80 nmol/mg protein)	101 ± 10

*Incubation medium contained: LDL (7.0 mg protein/ml) in 50 mM Trsi-HCl buffer (pH 7.4 at 25°C). The enzymic oxidation system (Lipoxygenase + Linolenic Acid) or the azo-initiator, AMVN, were added to the LDL suspension and the sample was transferred to a gas permeable tubing saturated with oxygen.

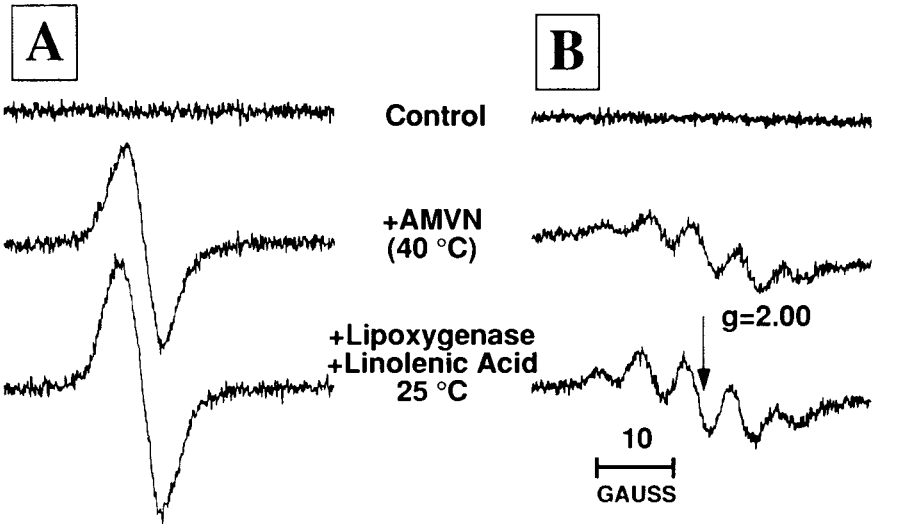

FIGURE 4 ESR spectra of radicals generated by AMVN or Lipoxygenase + Linolenic Acid in human LDL from exogenously added probucol or α-tocopherol. Incubation conditions: LDL suspension (7.0 mg protein/ml, 2.8 nmol of endogenous vitamin E/mg protein) in 50 mM Tris-HCl buffer (pH 7.4), α-tocopherol (12.0 nmol/mg protein) or probucol (80 nmol/mg protein). Other conditions as indicated in Methods. A – in the presence of probucol; B – in the presence of α-tocopherol.

stronger oxidative modifier of LDL than AMVN. Exogenously added α-tocopherol or probucol completely protected LDL against oxidative modification by both (lipoxygenase + linolenic acid) or AMVN (Table 1) which was indicative of incorporation of the antioxidants into LDL particles.

Human LDL suspensions with a vitamin E (α-tocopherol) content higher than 3 nmoles/mg protein and at concentrations higher than 10 mg protein/ml gave pronounced ESR signals of tocopheroxyl radical from endogenous vitamin E upon addition of lipoxygenase + linolenate or AMVN, as was reported earlier.[20] When these two conditions were not fulfilled the signals of endogenous vitamin E radicals were not detectable in the ESR spectra (Figure 4, control). Addition of α-tocopherol or probucol to such suspensions of human LDL in the presence of AMVN at 40°C resulted in an appearance of their radical signals (Figure 4 A, B). The unresolved ESR signal of probucol radical made it impossible to identify whether one or both of its -OH groups were converted into phenoxyl radicals. These signals were not observed in the absence of AMVN. Similar but more pronounced ESR signals were obtained by addition of α-tocopherol or probucol to LDL suspensions incubated at 25°C in the presence of lipoxygenase + linolenic acid. No ESR detectable signals of probucol or α-tocopherol were obtained in the absence of LDL both by incubation with AMVN or with lipoxygenase + linolenic acid. This suggests that only LDL-associated antioxidants appear in the ESR spectra.

If the suggestion on the ascorbate dependent recycling of probucol according to a reaction (2) is correct we may expect that addition of ascorbate would result in a transient disappearance of probucol phenoxyl radical ESR signal and a concomittant transient appearance of ascorbyl radical signal. The ESR spectra of radicals generated

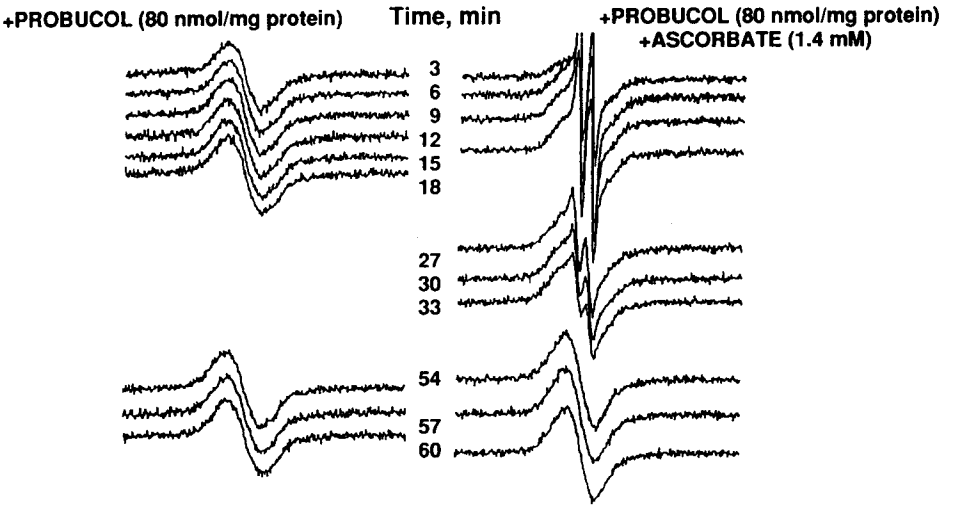

FIGURE 5 Serial ESR spectra of probucol radicals generated by Lipoxygenase + Linolenic Acid in human LDL in the absence or presence of 1.4 mM ascorbate. Condition as in Figure 4.

by lipoxygenase + linolenic acid oxidation of probucol-containing LDL in the presence or absence of ascorbate demonstrated that ascorbate caused a transient disappearance of the probucol radical signal and its substitution by an ascorbyl radical signal (Figure 5). Consumption of ascorbate in the course of incubation results in the disappearance of ascorbyl radical signal and reappearance of probucol signals (Figures 5, 6).

When another reductant, dihydrolipoic acid, was added to probucol-containing LDL, only a small decrease in the magnitude of the probucol radical ESR signal occurred. In the presence of both ascorbate and dihydrolipoic acid a synergistic effect on probucol recycling was observed. The probucol radical ESR signal disappeared and the delay in its reappearance was 2.5-times longer than in the presence of only ascorbate (Figure 7). At the same time the ascorbyl radical ESR signal was persistent and was substituted by the reappearing probucol radical signals, but only after 60–70 minutes of incubation.

DISCUSSION

Evidence for the importance of the antioxidant function of probucol in its therapeutic potential, particularly in its antiatherogenic effects is accumulating.[4,7,13] It is suggested that probucol is transported in lipoproteins, including LDL, where it acts as an effective antioxidant to prevent oxidative modification of LDL thus blocking the "scavenger" receptor-mediated uptake of LDL by macrophages with subsequent formation of fatty-streak lesion of atherosclerosis.[13–15]

It is known that vitamin E (α-tocopherol) is the major endogenous chain-breaking lipid-soluble antioxidant in membranes and LDL.[10,12,23–25] Thus it is important to quantitatively compare the radical scavenging efficiency of probucol with that of α-tocopherol. Our data shows that probucol is an efficient scavenger of peroxyl

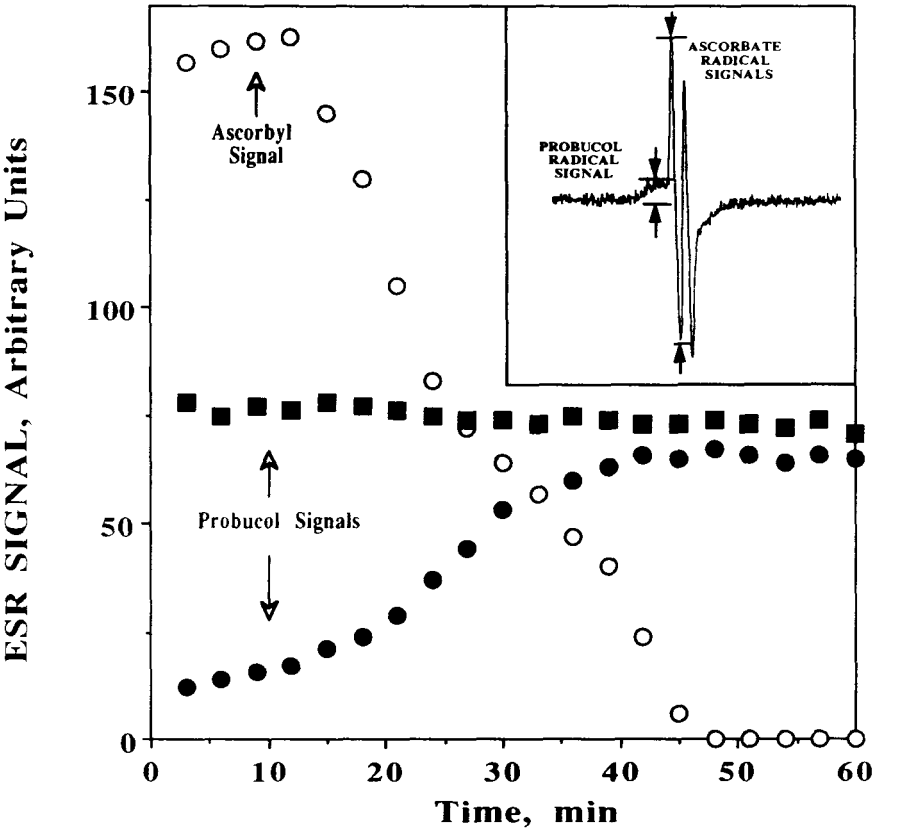

FIGURE 6 Effect of ascorbate on the time-course of probucol radical ESR signals generated by Lipoxygenase + Linolenic Acid in LDL. Conditions as in Figure 4. ○ – ascorbyl radical signal; ■ – probucol radical signal in the absence of ascorbate (control); ● – probucol radical signal in the presence of ascorbate. Insert: a representative ESR spectrum showing the superimpositioning of probucol and ascorbyl radicals. Magnitude of probucol radical signal was defined as the distance from the baseline to the maximum of the shoulder as indicated in the insert. Magnitude of ascorbyl radical signal was defined as the distance from the peak to the trough of the high-field component of the signal as indicated in the insert.

radicals. However, its radical scavenging activity in the lipid bilayer of DOPC liposomes is 15 times lower than that of α-tocopherol. This means that probucol can not efficiently compete with α-tocopherol in the reaction with peroxyl radicals and can hardly be consumed before α-tocopherol in the course of peroxidation. Thus it is not surprizing that probucol does not prevent α-tocopherol loss in LDL induced by oxidative stress, as was suggested by Jailal *et al.*[27] However, the maintenance of sufficiently high probucol concentration in LDL is very important, since probucol itself protects LDL against oxidative modification: probucol degradation precedes LDL peroxidation.[15]

Our results show that the maintenance of probucol in LDL may be due to its regeneration from its phenoxyl radicals by ascorbate, a physiologically important

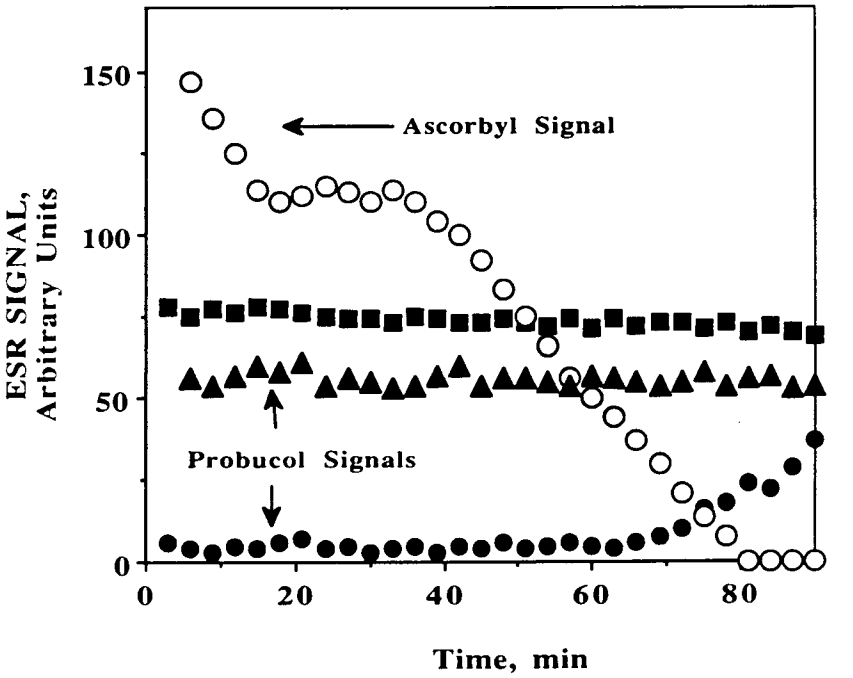

FIGURE 7 Effect of ascorbate + dihydrolipoic acid (DHLA) on the time-course of probucol radical ESR signals generated by Lipoxygenase + Linolenic Acid in LDL. Conditions as in Figure 4. Ascorbate and DHLA concentrations were 1.4 mM and 0.8 mM, respectively. ○ – ascorbyl radical signal in the presence of DHLA; ■ – probucol signal in the absence of either ascorbate or DHLA (control); ▲ – probucol radical signals in the presence of DHLA (no ascorbate); ● – probucol radical signal in the presence of both ascorbate + DHLA.

reductant found in plasma in concentrations as high as 50 μM.[27] Dihydrolipoic acid, a stronger reductant than ascorbate (redox potential of dihydrolipoate/lipoate is −0.32 v compared with 0.08 v for ascorbate/dehydroascorbate),[28] can synergistically enhance ascorbate-dependent regeneration of probucol by continuously recycling ascorbate consumed in the course of its interaction with the phenoxyl radical of probucol. The time course of ESR radical signals suggests that a stepwise recycling of probucol may occur: probucol phenoxyl radical is reduced by ascorbate, which in turn is being regenerated by dihydrolipoic acid (Figure 8).

Earlier we have shown that this mechanism can operate efficiently in LDL with phenoxyl radicals of vitamin E.[21] Thus recycling of endogenous antioxidants and antioxidant drugs in LDL may provide a novel mechanism for decreasing *in vivo* oxidation of LDL and hence the risk of premature atherosclerosis. Future studies will be aimed at a search for the optimal combinations of phenolic antioxidants and their "recyclers".

Acknowledgements

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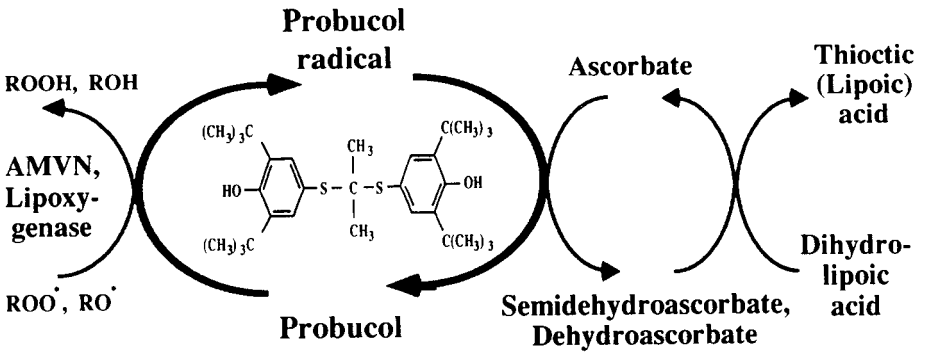

FIGURE 8 A scheme showing possible interactions of ascorbate and dihydrolipoic acid with probucol radicals generated by AMVN or Lipoxygenase + Linolenic Acid.

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