Concentration-dependent antioxidant activity of probucol in low density lipoproteins in vitro: probucol degradation precedes lipoprotein oxidation

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Abstract The ability of probucol, a lipid-lowering drug with antioxidant properties, to prevent the Cu^{2+}-induced oxidation of human plasma low density lipoproteins (LDL) was examined as a function of the concentration of probucol in LDL. In the absence of probucol, 3 μM Cu^{2+} induced half-maximal LDL lipid oxidation, as determined by the formation of thiobarbituric acid reactive substances (TBARS). Oxidation was associated with a loss of apolipoprotein B-100 and the appearance of higher molecular weight forms of the protein. In the presence of 0.6 mol% probucol (relative to phospholipid) and with 3 μM Cu^{2+}, the time required to obtain half-maximal LDL lipid oxidation increased from 130 to 270 min and was explained by an increase in the lag time prior to LDL lipid oxidation. Once rapid oxidation of LDL had begun, the rate of TBARS formation was similar to that for LDL containing no probucol. At a probucol concentration of 4.2 mol%, the antioxidant prevented the oxidation of LDL-lipids. The delay in Cu^{2+}-induced LDL oxidation with probucol corresponded to the time required for free radical-mediated processes to convert probucol to a spiroquinone and a diphenoquinone. These in vitro findings suggest that the potent antioxidant property of probucol is directly related to the amount of drug in the LDL particle and may have relevance to its antiatherosclerotic effects observed in vivo. —**Barnhart, R. L., S. J. Busch, and R. L. Jackson.** Concentration-dependent antioxidant activity of probucol in low density lipoproteins in vitro: probucol degradation precedes lipoprotein oxidation. *J. Lipid Res.* 1989. **30:** 1703–1710.

Supplementary key words malondialdehyde • Cu^{2+} oxidation • apolipoprotein B • 4-hydroxynonenal

During the past few years, a great deal of information has been published on the cellular mechanisms that contribute to the pathogenesis of atherosclerosis (1). It is now generally accepted that the accumulation of foam cells in the atherosclerotic lesion arises mainly from monocyte-derived macrophages (2). This cell type takes up limited amounts of normal low density lipoproteins (LDL), but avidly binds and internalizes modified LDL through a lipoprotein scavenger receptor. The exact LDL modifications that account for enhanced lipoprotein uptake are not known with certainty. It is known that modification of LDL by lipid oxidation products (3–5), monocytes/macrophages, neutrophils (6–8), endothelial cells (9, 10–14), and smooth muscle cells (9, 14–16) enhances LDL uptake by macrophages and renders LDL cytotoxic to cells in tissue culture. The mechanisms of free radical-mediated modification of LDL and the pathological consequences as it relates to human diseases have recently been reviewed (17–20).

In vivo, biologically modified LDL presumably results from free-radical formation in either the plasma or subendothelial space followed by nucleophilic attack of the radical on LDL lipid and protein. The antioxidant probucol, [4,4'-(isopropylidenedithio)bis(2,6-di-*tert*-butylphenol)], a drug used in the treatment of hypercholesterolemia, has recently been shown to inhibit oxidative modification of LDL (21, 22). Furthermore, probucol reduces atherosclerosis in the Watanabe heritable hyperlipidemic rabbit (23–25) and the cholesterol-fed rabbit (26). In the present study, we provide in vitro evidence that the concentration of probucol in LDL determines the extent of oxidative modification of the lipoprotein.

EXPERIMENTAL PROCEDURES

Isolation of LDL

Human plasma was obtained from normolipidemic fasting donors; sodium azide and EDTA were added immediately to final concentrations of 0.01% and 1 mM,

Abbreviations: LDL, low density lipoproteins; PDB, plasma density buffer; PBS, phosphate-buffered saline; MDA, malondialdehyde; TBARS, thiobarbituric reactive substances; HPLC, high performance liquid chromatography.

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respectively. LDL were isolated by ultracentrifugal flotation in KBr between densities 1.019–1.063 g/ml in a Beckman 45 Ti rotor at 6°C for 24 h at 44,000 rpm. LDL were stored at 4°C and dialyzed before use against a plasma density buffer (PDB) containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.01% NaN_3.

Incorporation of [^{14}C]probucol into LDL

[^{14}C]Probucol (11.0 mCi/mmol) was prepared as described previously for the unlabeled compound (27) from 2,6,di-*tert*-butylphenol (uniformly labeled with ^{14}C in the phenol ring) by Dr. E. R. Wagner (Merrell Dow Research Institute, Indianapolis, IN). The radiolabeled compound was 99.92% pure as determined by HPLC. [^{14}C]Probucol was solubilized in 100% ethanol to give a final stock solution of 3.1 mg/ml; 0–155 μg of probucol in 50 μl 100% ethanol was added in 10-μl aliquots to a 6.0 ml solution of LDL (0.50 mg protein/ml in PDB) contained in a 13 × 100 mm glass borosilicate tube. After each addition, samples were gently mixed by hand and after the final addition, were incubated for 45 min at 42°C. The samples were immediately cooled to 4°C, and unassociated [^{14}C]probucol was separated from LDL by ultracentrifugation; 2 ml of the incubation mixtures was layered onto 3 ml of a d 1.063 g/ml KBr solution in PDB. Samples were centrifuged at 6°C in a Beckman 50.3 Ti rotor for 18 h at 48,000 rpm. After centrifugation, unassociated probucol was removed in the top 2 ml of each tube. Then, LDL (determined visually by pigmentation) were recovered at the 1.063 g/ml interface and immediately dialyzed 48–72 h (to remove EDTA) against degassed 10 mM potassium phosphate, 0.15 M NaCl, pH 7.4, (PBS) in the dark at 4°C. No loss of radioactivity was observed during dialysis suggesting that the [^{14}C]probucol was incorporated into the LDL. After dialysis, samples were filtered through a Gelman Acrodisc (0.2 μm) filter. LDL-protein was then determined by the method of Lowry et al. (28) with bovine serum albumin as standard. Phospholipid-phosphorus was determined by the method of Bartlett (29). Mol% probucol in LDL was determined by ^{14}C radioactivity and calculated relative to LDL-phospholipid content. The LDL-protein to LDL-phospholipid weight ratio was 0.95.

Cu^{2+}oxidation of LDL

For all experiments, LDL were adjusted to a protein concentration of 0.2 mg/ml with PBS, and $CuSO_4$ was added to the final concentration as indicated in Results. Samples were then incubated in loosely capped, 16 × 100 mm borosilicate glass tubes at 37°C. At the appropriate times, oxidation was terminated by the addition of EDTA (1 mM final concentration). Thiobarbituric acid reactive substances (TBARS), a measure of lipid oxidation as malondialdehyde (MDA) generated upon acid hydrolysis, were determined as described by Kim and LaBelle (30). Briefly, to 0.5 ml of sample (100 μg LDL-protein) were added 2.0 ml of 50 μM potassium hydrogen phthalate, pH 3.5, and 1 ml of thiobarbituric acid (1% wt/vol in 0.05 M NaOH). The samples were vortexed and incubated in marble-covered glass tubes for 15 min at 100°C in a heating block. The samples were then cooled to room temperature and centrifuged 15 min at 3,000 rpm to clarify samples. The amount of MDA was determined as the MDA-TBA adduct by measuring the absorbance at 532 nm and calculating its concentration using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} (31); all assays were performed in duplicate.

Gel electrophoresis

LDL were examined by a denaturing gel system using 2–16% polyacrylamide gradient gels (PAA 2-16 Pharmacia Fine Chemicals Inc.) as described by the supplier. Samples (50 μg of LDL-protein in 0.25 ml) were delipidated with 1 ml of acetone–ethanol 1:1 (v/v) for 60 min at −20°C. The protein was pelleted by centrifugation at 3,000 rpm for 10 min, dried under a N_2 stream, and resolubilized in 50 μl of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.2% SDS, 0.05% bromophenol blue, 20% glycerol, and 5% β-mercaptoethanol. Samples were incubated at 60°C for 15 min, and 15 μg of each sample was loaded onto gels which were pre-electrophoresed at 10°C for 60 min at 70 V. The running buffer contained 40 mM Tris-HCl, pH 7.4, 20 mM sodium acetate, 2 mM EDTA, 0.2% SDS. Electrophoresis was performed at 125 V for 70 min. Gels were stained for 4 h in 25% methanol, 10% acetic acid and 0.1% Coomassie Brilliant Blue R-250, and then destained overnight in the same solution without dye.

Other methods

Probucol and its oxidation products were separated by a modification of a previously described method (32) by HPLC (Waters) following their extraction from LDL. Briefly, 100 μl of [^{14}C]probucol LDL-protein (0.2 mg/ml) was extracted with 2 ml of ether–ethanol 3:1 (v/v), and the supernatant fraction was removed and dried with a stream of N_2 to concentrate the sample. The pellet was resolubilized with 200 μl acetonitrile–hexane–0.1 M ammonium acetate 90:6.5:3.5, and 160 μl was injected by a Water's Wisp^{TM} onto a Water's Delta Pak C18 reverse phase column (300 Å, 3.9 mm × 15 cm). The mobile phase buffer was acetonitrile–water 85:15. Samples were eluted over 30 min at a flow rate of 1.5 ml/min at ambient temperature; the eluent was monitored at 240, 255, and 420 nm. The elution times were: probucol (10 min), 3,3',5,5'-tetra-*tert*-butyl-diphenoquinone (24 min), 3,3'5,-5'-tetra-*tert*-butyl bisphenol (4.5 min), and 3,3',5,5'-tetra-*tert*-butyl spiroquinone (22 min). [See Fig. 7 for the structure of these compounds.] The spiroquinone standard was produced directly from probucol by oxidation with a four-

fold excess of lead dioxide in hexane (W. H. Braun and R. R. Fike, unpublished results). The diphenoquinone standard was purchased from Lancaster Synthesis Ltd., Mindham, NH. The bisphenol standard was prepared by zinc reduction of the diphenoquinone. Mass spectra were determined in the negative ion mode using a Finnigan MAT, model 4600 mass spectrophotometer.

Fluorescence spectra were recorded at 25°C on an SLM 4800S fluorometer as described previously (22). The formation of aldehydic lipid oxidation products was monitored by excitation at 360 nm and emission spectra were recorded from 380 to 600 nm (33). The fluorescence intensity at wavelengths of 410 and 470 nm was used to determine the relative concentrations of 4-hydroxynonenal and MDA, respectively.

RESULTS

The Cu^{2+} concentration-dependent oxidation of LDL is illustrated in **Fig. 1**. For this experiment, a pool of LDL was prepared from normolipidemic subjects and oxidation was initiated with 0–50 μM $CuSO_4$ for 14 h at 37°C. The extent of lipid oxidation was determined by a standard TBARS assay (30), a measure of MDA formation. In the absence of added copper, LDL contained approximately 4 nmol MDA/mg LDL-protein, a value consistent with that reported by Yagi (19). The maximum amount of lipid peroxides generated in 14 h was 27 nmol MDA/mg LDL-protein with 10 μM Cu^{2+}. The Cu^{2+} concentration required for half-maximal oxidation was 3 μM.

In the next experiment, 3 μM Cu^{2+} was then used to examine the kinetics of oxidation of LDL in the presence and absence of probucol (**Fig. 2**). In the absence of probucol, the rate of TBARS formation increased up to 9 h and then leveled off or was slightly decreased at 24 h. With these conditions, Cu^{2+}-induced lipid oxidation generated 16 nmol MDA/mg LDL-protein. In the presence of probucol (incorporated into LDL at 0.3, 0.6, 1.0, and 4.2 mol%, relative to phospholipid), the time ($t_{1/2}$) required to reach half-maximum oxidation increased with increasing concentrations of probucol (Fig. 2), suggesting that probucol contained in the LDL particle is responsible for the inhibition of lipid oxidation. From the data in Fig. 2 inset, $t_{1/2}$ increased from 130 min to 270 min in the presence of 0.6 mol% probucol. At an initial concentration of 4.2 mol% probucol in LDL, limited lipid oxidation occurred even after 24 h incubation.

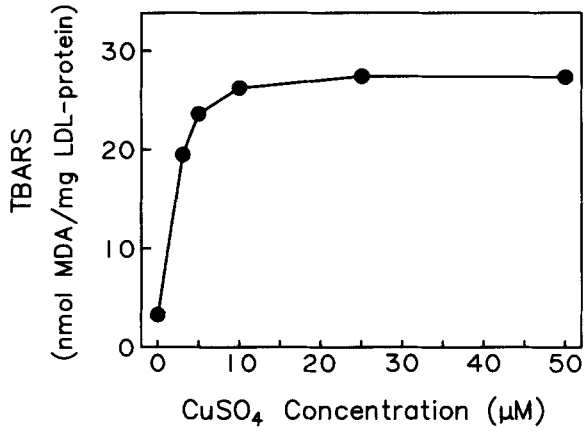

Fig. 1. Effect of $CuSO_4$ concentration on the oxidation of LDL as measured by TBARS. LDL (0.2 mg protein/ml) were incubated with increasing concentrations of $CuSO_4$ for 14 h at 37°C. After the addition of EDTA (final concentration 1 mM), TBARS were determined as described in Experimental Procedures. Values represent the mean of duplicate analysis.

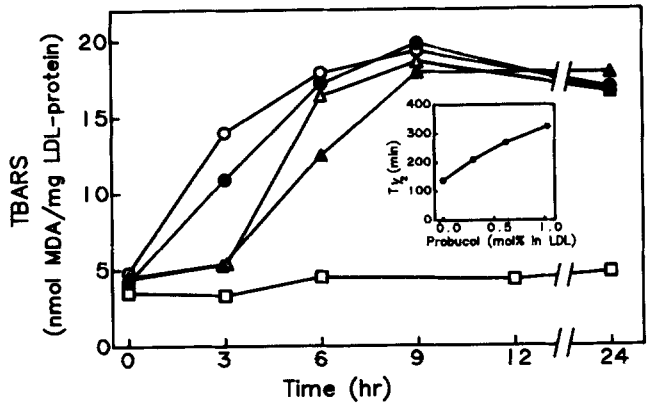

Fig. 2. Antioxidant activity of probucol on Cu^{2+}-modified LDL. LDL (0.2 mg protein/ml) containing increasing mol% probucol (—○—, 0.0; —●— 0.3; —△— 0.6; —▲— 1.0; —□— 4.2 mol%) were incubated at 37°C in the presence of 3 μM $CuSO_4$. At the indicated times aliquots were removed, EDTA (final concentration 1 mM) was added, and TBARS were determined as described in Experimental Procedures. Values represent the mean of duplicate analysis. The inset is a replot of the time at which one-half of maximal oxidation occurred as a function of probucol concentration in LDL.

Recently, Fong et al. (34) showed that Cu^{2+} oxidation of LDL results in the degradation of apoB-100. Therefore, we examined the ability of probucol to prevent this degradation. Probucol was incorporated into LDL at 0.3, 0.62, and 1.0 mol%. After Cu^{2+} oxidation the samples were then subjected to SDS-polyacrylamide gel electrophoresis. As is shown in **Fig. 3**, the protein staining band corresponding to apoB-100 (MW ~550 kDa) was eliminated by 3 h in the absence of probucol, and the gel showed a heterogeneous mixture of higher molecular weight forms of the protein suggesting that the protein had either aggregated or had become cross-linked; with these conditions of oxidation no degradation of apoB-100 to lower molecular weight forms of the protein was observed even after silver staining the gel (data not shown). With 0.6 mol% probucol, most of the 550 kDa band of apoB-100 had disappeared at 3 h. However, with 1.0 and 4.2 mol% probucol only a slight decrease in apoB-

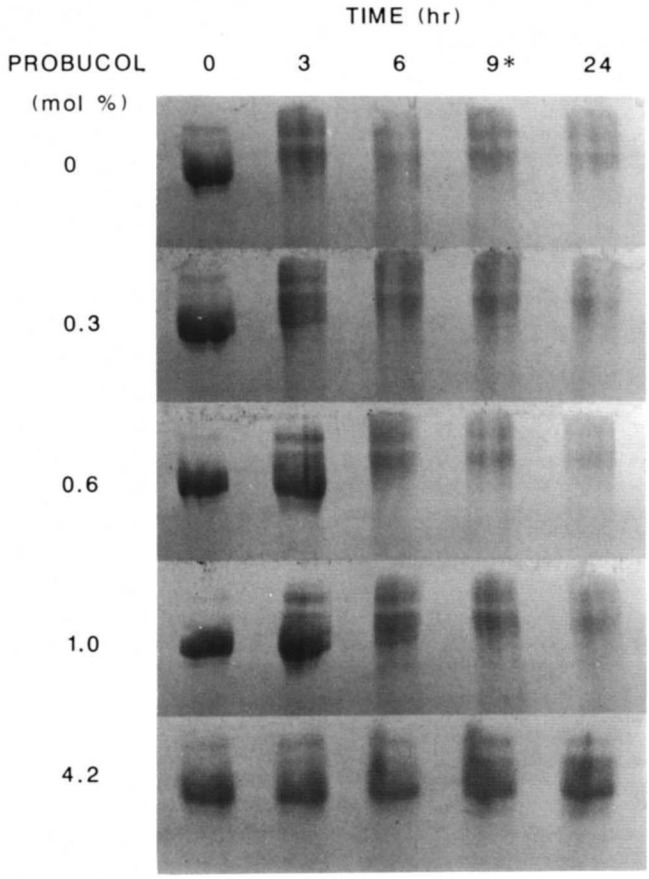

Fig. 3. Polyacrylamide gel electrophoresis of Cu^{2+}-oxidized LDL. SDS-PAGE was performed on the samples from Fig. 2. Fifteen μg LDL-protein was applied to each lane. *At 4.2 mol% probucol, a 12-h incubation was used instead of 9-h incubation.

100 was evident at 3 h. After 9 h of oxidation, apoB-100 disappeared, except in samples containing 4.2 mol% probucol.

To determine whether the Cu^{2+}-mediated oxidation of LDL resulted in the breakdown of probucol and formation of oxidation products, LDL (0.2 mg protein/ml) containing 4.6 mol% probucol was incubated with 40 μM Cu^{2+} for 40 h. Two ml of the incubation mixture was then extracted with 40 ml of ether–ethanol and the lipid-soluble fraction was subjected to HPLC. **Fig. 4** shows that Cu^{2+} oxidation was associated with the formation of spiroquinone and diphenoquinone; the bisphenol derivative of probucol was not detected. The identification of the spiroquinone and diphenoquinone was based on identical elution times on HPLC and UV/visible spectra as compared to the authentic compounds. In addition, the identity of the spiroquinone was confirmed by mass spectrum analysis. The molecular ions observed for the spiroquinone were identical to the standard compound: 514 (parent ion), 440, 408, 236, and 106 mass units.

The results described above suggested that the antioxidant activity of probucol in LDL is related to the concentration of the drug in the lipoprotein particle. Therefore, we examined the relationship between the concentration of probucol in LDL, the onset of peroxidation of LDL-lipids, and the formation of the spiroquinone and diphenoquinone. [^{14}C]Probucol was incorporated into LDL at either 0.6 or 1.2 mol% and then the lipoproteins were incubated with 3 μM Cu^{2+}. At various times after the addition of Cu^{2+}, LDL were extracted with ether–ethanol and the lipid soluble fraction was separated by HPLC in order to determine the concentration of probucol. The formation of protein adducts, as a result of lipid oxidation, was also monitored by changes in fluorescence emission spectra (22, 33) and compared to the onset of oxidation as measured by the TBARS assay. **Fig. 5** shows the kinetics of lipid oxidation of LDL following the addition of 3 μM Cu^{2+}. In the absence of probucol an initial slow oxidation rate of 2.0 nmol MDA/h per mg LDL-protein was observed up to 2 h. At 0.6 and 1.2 mol% probucol, initial

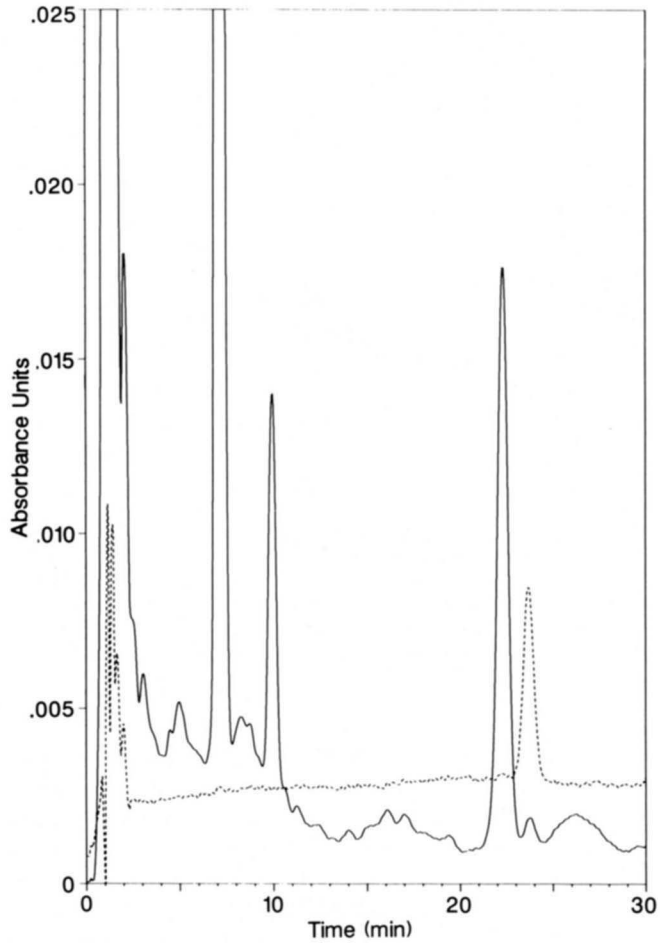

Fig. 4. HPLC separation of the lipid-soluble fraction of Cu^{2+}-oxidized LDL containing 4.6 mol% probucol. LDL were oxidized with 40 μM Cu^{2+} for 40 h. The LDL were extracted with ether–ethanol and chromatographed on HPLC as described in Experimental Procedures. Probucol (10 min) and spiroquinone (22 min) were monitored at 240 nm (solid line) and diphenoquinone (24 min) was monitored at 420 nm (dashed line).

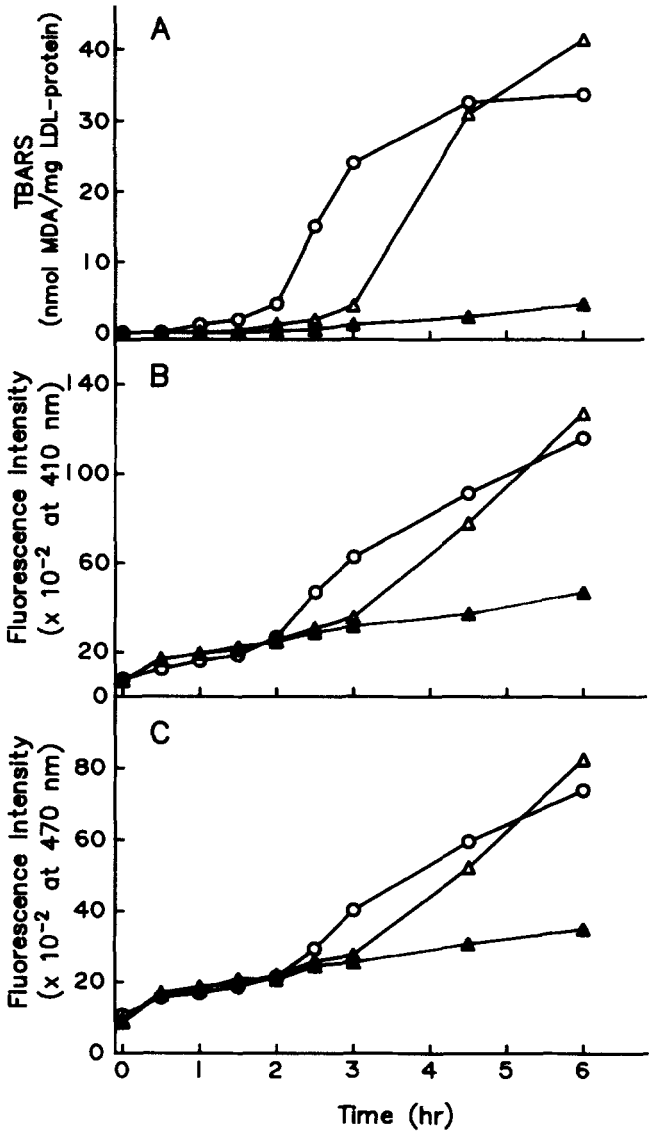

Fig. 5. Antioxidant activity of probucol on Cu^{2+}-induced oxidation of LDL. LDL containing 0, —○—; 0.6, —△—; and 1.2, —▲— mol% probucol were oxidized at 37°C with 3 μM $CuSO_4$. At the indicated times, samples were removed and TBARS were determined (panel A). Oxidatively modified protein adducts in LDL were also monitored by fluorescence changes at 410 nm (panel B) and 470 nm (panel C).

rates were 1.3 and 0.7 nmol MDA/h per mg LDL-protein, respectively, suggesting that these initial rates were decreased in the presence of probucol. A rapid oxidation phase followed this initial slow phase. In the absence of probucol, the onset of rapid lipid oxidation did not occur for 2 h as determined by the TBARS assay (Fig. 5A) or by the increase in fluorescence due to the formation of aldehyde-protein adducts of 4-hydroxynonenal (Fig. 5B) or MDA (Fig. 5C). This delay in lipid oxidation is probably related to α-tocopherol and other natural antioxidants present in the LDL particle (33). In the presence of 0.6 mol% probucol, rapid oxidation was not apparent until 3 h, a time at which greater than 80% of probucol had been oxidized and converted into the spiroquinone and diphenoquinone (**Fig. 6**). In LDL containing 1.2 mol% probucol, the rate of formation of lipid oxidation products remained at an initial slow rate up to 6 h (Fig. 5) as determined by all three assays. The rapid phase of lipid oxidation with 0 and 0.6 mol% probucol occurred at rates of 18.6 and 22.1 nmol MDA formed/h per mg LDL-protein, respectively.

The rate of probucol degradation was calculated from Fig. 6 and compared to the rate of lipid oxidation (Fig. 5). LDL containing 0.6 mol% probucol corresponds to 5.3 nmol of probucol/mg of LDL-protein or approximately 10 molecules of probucol per LDL particle (mol wt 2×10^6). With 0.6 mol% probucol, the rate of oxidative degradation of probucol was 2.1 nmol probucol degraded/h per mg LDL-protein. If probucol were consumed at this rate, we calculate that it would take 2.5 h to degrade all of the probucol and initiate lipoprotein oxidation. As is shown in Fig. 5, the onset of rapid MDA formation did not occur until 2.5–3 h after the addition of Cu^{2+} and is in close agreement with the calculation.

DISCUSSION

Oxidative modification of LDL produces lipid oxidation products (18, 33), loss of reactive amino groups on the surface of LDL (11, 35), decreased heparin binding (22), and a breakdown of LDL phospholipids and apoB-100 (12, 13). The presence of the antioxidant probucol in LDL prevents these oxidative modifications (21, 22). The present study focused on the fate of probucol itself during Cu^{2+}-induced oxidative modification of LDL. With half-maximal concentrations of Cu^{2+} (3 μM), we showed that

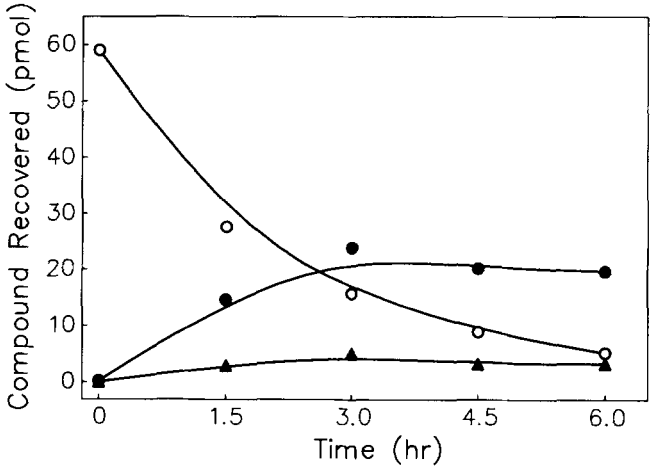

Fig. 6. Cu^{2+}-induced modification of probucol. LDL containing 0.6 mol% [^{14}C]probucol were incubated with 3 μM $CuSO_4$. At the indicated times, the amount of probucol (—○—), spiroquinone (—●—), and diphenoquinone (—▲—) was determined by radioactivity after separation by HPLC (see Experimental Procedures).

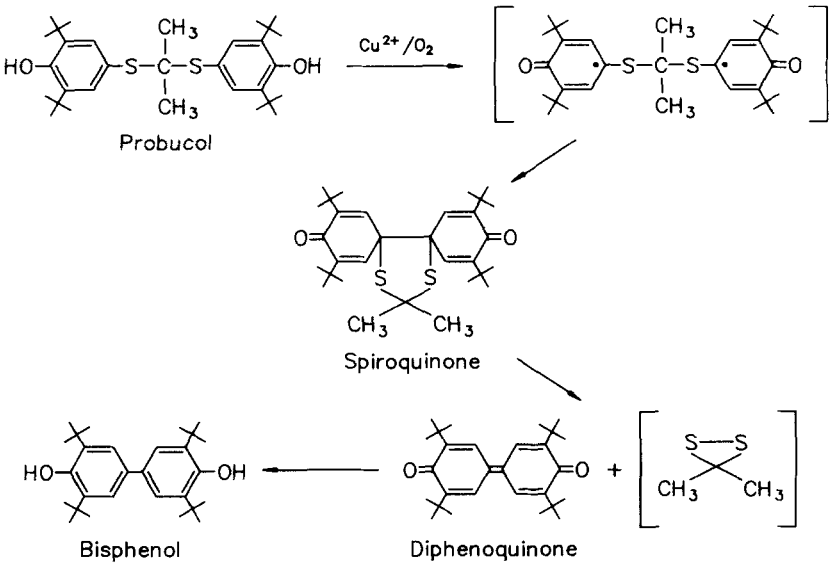

Fig. 7. Proposed pathway for the Cu^{2+}-induced degradation of probucol.

the onset of LDL oxidation was dependent on the amount of probucol in the lipoprotein particle. The increase in fluorescence of LDL with excitation at 360 nm and emission at 410 and 470 nm provided a method to monitor the kinetics of formation of protein adducts of 4-hydroxynonenal and MDA, respectively, after the addition of Cu^{2+}. By this method we found, even in the absence of probucol, a delay in fluorescence increase and TBARS formation; this is probably due to the presence of natural antioxidants, α-tocopherol and carotenoids, in LDL. Esterbauer et al. (33) have shown that α-tocopherol contained in LDL is highly sensitive towards oxidative degradation and only when the antioxidant is depleted from the LDL particle does lipid oxidation occur. The presence of probucol in LDL further delayed the onset of lipid oxidation; the time required to reach 50% oxidation was directly proportional to the amount of probucol in LDL up to 1.0 mol%. At higher probucol concentrations, the presence of probucol prevented oxidative damage to LDL under these experimental conditions. As is shown in Fig. 6, probucol is destroyed by the Cu^{2+}-mediated oxidation of LDL. Only after there is a >90% depletion (corresponding to an average of less than 1 molecule of probucol per LDL particle of 2×10^6 Da) of probucol from LDL is there extensive LDL oxidation and loss of apoB-100.

The time course for formation of products from both lipid oxidation and loss of apoB-100 suggests that probucol must be quantitatively destroyed before modification of LDL occurs. This implies that probucol is a potent free-radical terminator; its efficacy in preventing LDL oxidation may be explained, in part, by its presence in LDL and possibly by a less rigid localization compared to the lipids. Localization of the oxidation products of probucol in the lipoprotein core is suggested by preliminary experiments in which a variety of reducing agents with varying degrees of hydrophilicity failed to convert the spiroquinone and diphenoquinone to the bisphenol (**Fig. 7**). However, in organic solvent and in the presence of 1 mM 2-mercaptoethanol, they were rapidly converted to the bisphenol.

The specificity of the radical scavenged by probucol to produce degradation of the drug is not known and is under investigation. However, Bedwell and Jessup (36) used a ^{60}Co source to generate various oxygen-centered free radicals and showed that the hydroxyl radical is the most potent agent in oxidizing LDL-lipids. If this is the free radical, then it presumably extracts the two phenolic hydrogen atoms from probucol to form the unstable intermediate shown in Fig. 7. This intermediate may then form the stable spiroquinone which, as suggested previously (37), can undergo homo- or heterolytic scission to form the diphenoquinone. In the present in vitro system with Cu^{2+} oxidation, approximately 50%, and possibly (transiently) all, of the oxidized probucol in LDL was converted first to the spiroquinone and then to the diphenoquinone. Experiments are currently in progress to determine the in vivo antioxidant properties of the probucol degradation products shown in Fig. 7. If these metabolites prove to be potent antioxidants, then probucol may generate an oxidation product that can cycle between the diphenoquinone and bisphenol, thus providing a regenerating free radical scavenger cycle.

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