Low Density Lipoprotein Is Protected from Oxidation and the Progression of Atherosclerosis Is Slowed in Cholesterol-fed Rabbits by the Antioxidant *N,N'*-Diphenyl-Phenylenediamine

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Abstract

The oxidative modification of low density lipoprotein (LDL) may play an important role in atherosclerosis. We found that the antioxidant *N,N'*-diphenyl-1,4-phenylenediamine (DPPD) inhibits in vitro LDL oxidation at concentrations much lower than other reported antioxidants. To test whether DPPD could prevent atherosclerosis, New Zealand White rabbits were fed either a diet containing 0.5% cholesterol and 10% corn oil (control group) or the same diet also containing 1% DPPD (DPPD-fed group) for 10 wk. Plasma total cholesterol levels were not different between the two groups, but DPPD feeding increased the levels of triglyceride (73%, $P = 0.007$) and HDL cholesterol (26%, $P = 0.045$). Lipoproteins from DPPD-fed rabbits contained DPPD and were much more resistant to oxidation than control lipoproteins. After 10 wk, the DPPD-fed animals had less severe atherosclerosis than did the control animals: thoracic aorta lesion area was decreased by 71% ($P = 0.0007$), and aortic cholesterol content was decreased by 51% ($P = 0.007$). Although DPPD cannot be given to humans because it is a mutagen, our results indicate that orally active antioxidants can have antiatherosclerotic activity. This strongly supports the theory that oxidized LDL plays an important role in the pathogenesis of atherosclerosis. (*J. Clin. Invest.* 1992. 89:1885–1891.) Key words: arteriosclerosis • cholesterol • macrophage • probucol • scavenger receptors

Introduction

A prominent feature of atherosclerotic lesions is the cholesterol-loaded macrophage foam cell (1). During the progression of atherosclerosis, circulating monocytes adhere to the endothelium, penetrate the vessel wall, differentiate into macrophages, and become cholesterol-loaded (2). Low density lipoprotein (LDL) is believed to be the source of the cholesterol in foam cells, and LDL levels are positively correlated with risk of atherosclerosis and coronary artery disease (3). Conversely, HDL levels are negatively correlated with this disease (4), and HDL particles are believed to be able to accept excess cholesterol from foam cells and deliver this cholesterol to the liver via the reverse cholesterol transport pathway (5).

A model has been proposed for the biochemical mechanism by which LDL causes the appearance of foam cells (6). The "oxidized LDL hypothesis" states that atherosclerosis is caused not only by the native LDL particle itself, but by a modified LDL created by oxidative damage (6). LDL can be oxidatively modified in vitro by certain cultured cells (7–9) or by copper ions (7). Oxidized LDL is taken up via macrophage scavenger receptors (10), leading to cholesterol accumulation (6), whereas native LDL is not recognized by scavenger receptors and therefore does not cause cholesterol accumulation (11). Oxidized LDL is chemotactic for monocytes (12), and therefore oxidized LDL may recruit monocytes into the subendothelial space. Oxidized LDL is also cytotoxic (8), which may explain the endothelial damage that occurs during atherogenesis (13). Most studies of the in vitro effects of oxidized LDL have used LDL subjected to strong oxidizing conditions, but even "minimally modified" LDL has potent biological activity (14). These in vitro results support a role for oxidized LDL in the recruitment of monocytes into the artery and their conversion to cholesterol-loaded foam cells.

LDL oxidative modification probably occurs in vivo. Immunohistochemical studies suggest that atherosclerotic lesions contain oxidized LDL (15–17), and a modified form of LDL can be isolated from dissected lesions (18). Normal arterial tissue, however, contains no modified LDL (16), so it is conceivable that the modified LDL found in lesions is a consequence, rather than a cause, of atherosclerosis.

Among the most important in vivo evidence in support of the so-called "oxidized LDL hypothesis" comes from studies using probucol (4,4'-(isopropylidenedithio)bis[2,6-di-*t*-butylphenol]. Probucol is used clinically to lower plasma cholesterol levels (19), but probucol is also an antioxidant and was found to protect LDL from oxidative modification in vitro (20). Furthermore, lipoproteins isolated from humans or rabbits given probucol display increased resistance to oxidation ex vivo (20, 21). Most importantly, probucol slows the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit to an extent greater than that predicted from its cholesterol-lowering activity (22). Probucol has also been reported to be antiatherosclerotic in the cholesterol-fed rabbit (reviewed in reference 23).

It has been widely assumed that the antiatherosclerotic activity of probucol is due to its antioxidant properties (6, 23). Very recent work by Mao et al. (24) however, has raised some doubts. These workers studied the compound MDL 29,311, which is an analogue of probucol that does not lower cholesterol but does have full antioxidant activity. MDL 29,311 was found to be much less effective than probucol at preventing

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atherosclerosis in the Watanabe rabbit (24). This implies that the antioxidant activity of probucol may not be the source of its antiatherosclerotic activity. It is possible that probucol is antiatherosclerotic because of its effects on high density lipoprotein (HDL) metabolism (19), especially the distribution of HDL subclasses (25, 26). These effects of probucol on HDL may lead to stimulation of reverse cholesterol transport (26, 27). In patients with familial hypercholesterolemia, probucol caused dramatic regression of tendon xanthomas (25). The extent of the regression correlated with the degree of HDL lowering, suggesting that the regression may be caused by probucol's influence on HDL metabolism (25). Finally, it has been suggested that probucol's antiatherosclerotic activity may be related to inhibition of interleukin 1 (IL-1) secretion by macrophages (28, 29).

The oxidized LDL hypothesis could be further tested in animal models of atherosclerosis by using potent, orally active antioxidants that do not alter plasma cholesterol levels. The compound *N,N'*-diphenyl-1,4-phenylenediamine (DPPD)[1] is an orally active antioxidant (30–33). Diets containing DPPD can prevent or alleviate symptoms of vitamin E deficiency in rabbits (31) and rats (32, 33). In the present work, we show that DPPD very effectively prevents oxidation of LDL, and also that feeding DPPD slows the progression of atherosclerosis in cholesterol-fed rabbits without affecting plasma cholesterol levels.

Methods

Animals and treatments. Pasteurella-free male New Zealand White rabbits (2.5 kg) were obtained from Hare Marland, Hewitt, NJ, and housed and cared for as set forth in the Animal Welfare Act. 14 control rabbits were fed a chow containing 10% corn oil and 0.5% cholesterol, and 12 DPPD-fed rabbits were given the same diet containing 1% DPPD (Aldrich Chemical Co., Milwaukee, WI). This dose is the same as the dose of probucol typically given to rabbits (21–23). The diets were prepared by milling Purina high-fiber rabbit pellets with corn oil and cholesterol, with or without DPPD, to produce a homogeneous coarse powder. Rabbits were fed ad libitum. The animals were bled periodically for measurements of plasma cholesterol levels. After 27 d of feeding, fasting plasmas were obtained and analyzed for triglyceride and for the distribution of cholesterol in lipoprotein fractions. Lipoproteins isolated from these plasma samples and from samples taken after 62 d of feeding were subjected to oxidation assays as described below.

Analysis of extent of atherosclerosis. After 71 d of feeding, the rabbits were killed and the aortas were removed, rinsed in saline, cleaned of adhering tissue, and trimmed to include the region from the aortic root to 1 cm below the superior mesenteric artery. The aortas were sliced open longitudinally, photographed, and then finely minced. Lesion area in the thoracic region (from the distal end of the aortic arch to the mesenteric artery) was determined from the photographs by quantitative morphometric analysis using a Joyce-Lobel Magiscan image analyzer (Compix Inc., Mars, PA). The minced aortic tissue was weighed, then extracted as described by Folch et al. (34). Aliquots of the extracts were subjected to silica gel thin-layer chromatography in the solvent system hexane/diethyl ether/acetic acid 80:20:1. Regions of free cholesterol and esterified cholesterol were scraped and lipids were extracted from the silica gel using chloroform:methanol 1:1. These extracts were subjected to the cholesterol assay described by Rudell and Morris (35). This procedure yields values for free cholesterol and for esterified cholesterol; these values were added together to obtain a value for total cholesterol.

Lipoprotein isolation and assays of lipoprotein oxidation. Blood was collected using EDTA as the anticoagulant, and plasma was obtained by centrifugation. To measure the distribution of cholesterol in lipoprotein fractions, 200-μl aliquots of plasma were diluted to 1.0 ml, adjusted to appropriate densities and centrifuged in a model TL-100 ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA). The tube was cut in half, and cholesterol was measured in the upper and lower fractions.

For preparative isolation of rabbit lipoproteins, aliquots of plasma were pooled to create three independent plasma samples from each group of rabbits (control and DPPD-fed). These samples were used to isolate lipoproteins in the density ranges $d < 1.019$ g/ml and $1.019 < d < 1.063$ (LDL) by standard procedures (36). The $d < 1.019$ fraction includes all of the β-VLDL, which is a major component of lipoproteins in the cholesterol-fed rabbit (37). All ultracentrifugation buffers included 1 mM EDTA to prevent oxidation. After isolation, the lipoproteins were dialyzed against phosphate-buffered saline containing no EDTA, then sterile filtered, and analyzed for cholesterol and DPPD content (see below). Rabbit lipoproteins (400 μg of cholesterol/ml) were subjected to oxidation by incubation at 37°C in F-10 medium containing 10 μM $CuSO_4$ (7). At various times, samples were assayed for the presence of thiobarbituric acid-reactive substances (TBARS) as described (38) except the color was quantitated by measuring fluoresence at an excitation wavelength of 515 nm and emmission wavelength of 553 nm (39). Results are expressed as nanomoles of malondialdehyde equivalents per milligram of lipoprotein cholesterol. In some experiments, the ability of oxidized lipoproteins to bind to macrophage scavenger receptors was assessed by measuring inhibition of macrophage uptake of radioiodinated oxidized human LDL. Human LDL was radioiodinated using the trapped ligand tyramine cellobiose (40), then oxidized with 10 μM $CuSO_4$. This oxidized ^{125}I-LDL (5 μg cholesterol/ml) was incubated with mouse peritoneal macrophages for 5 h at 37°C in the presence or absence of rabbit lipoproteins (250 μg of cholesterol/ml) previously subjected to oxidation. Uptake was measured by quantitating cell-associated ^{125}I as previously described (10).

Human LDL was isolated from plasma of normal volunteers by standard procedures (36). Cellular modification of LDL was performed using the RECB4 line of rabbit aortic endothelial cells (obtained from D. Steinberg, San Diego) as previously described (10). The extent of oxidation was measured by the TBARS assay, as described above. In some cases, inhibitors were added at various concentrations, and the concentration required for 50% inhibition (EC_{50}) was estimated from the dose-response curve. Although duplicates within a given experiment usually varied by < 10%, there was some day-to-day variability in the estimate of EC_{50} values.

Analytical methods. Plasma levels of total cholesterol and triglycerides were measured using kits obtained from Boehringer-Mannheim Diagnostics, Indianapolis, IN. DPPD was quantitated in plasma and in isolated lipoproteins by measuring the intrinsic fluoresence of DPPD. Samples were extracted as described by Bligh and Dyer (41), and the chloroform phase was dried under argon. The residue was redissolved in 2-propanol and its fluoresence intensity was measured at an excitation wavelength of 310 nm and emmission wavelength of 405 nm. A standard curve was produced by dissolving DPPD in 2-propanol. The fluoresence intensity of extracts of control plasma served as a blank in the assay.

Statistical analyses. All values are reported as mean±SEM. The *P* values reported were calculated from Student's unpaired two-tailed *t* test, except were noted. Calculations were performed using the InStat program from GraphPAD Software, San Diego, CA.

Results

DPPD inhibits the in vitro oxidative modification of LDL. DPPD was a very effective inhibitor of the oxidative modifica-

1. *Abbreviations used in this paper:* BHT, butylated hydroxytoluene; DPPD, *N,N'*-diphenyl-1,4-phenylenediamine; β-VLDL, β-migrating very low density lipoprotein; $d < 1.019$, the lipoprotein fraction with a density < 1.019 g/ml; TBARS, thiobarbituric acid-reactive substances.

tion of LDL by endothelial cells (EC_{50} of 0.04 μM) and by $CuSO_4$ (EC_{50} of 0.3 μM) (Table I). These EC_{50} values for DPPD are 10–100 times lower than the EC_{50} values for butylated hydroxytoluene (BHT) or probucol (Table I; also compare with references 7, 20, 42–44). DPPD is one of the most effective inhibitors of LDL oxidation published to date.

Structure–activity relationship for the antioxidant activity of DPPD. Antioxidants, in general, are compounds that can lose one hydrogen atom but not become a reactive radical (45). The hydrogen atom quenches other, more reactive radicals, and thereby terminates free radical chain reactions (45). We tested a number of compounds related to DPPD to elucidate the structural requirements for antioxidant activity (Fig. 1). The terminal phenyl groups of DPPD (compound I) are important: insertion of methylene groups between the nitrogens and the phenyl rings (compound II) slightly increased EC_{50}, and replacing the phenyl groups with methyl groups (compound III) increased EC_{50} about fivefold. Simplified versions of DPPD were not nearly as effective: phenylenediamine (IV) and diphenylamine (V) have EC_{50}'s > 1 μM.

The para arrangement of the nitrogens in DPPD is essential; the *meta* analogue of DPPD (compound VII) is inactive. The nitrogens of DPPD are also essential; replacing them with carbon (VIII) or sulphur (IX) destroys antioxidant activity. These results lead us to conclude that DPPD is an effective antioxidant because it can lose one hydrogen atom from each nitrogen, and become *N,N'*-diphenyl-1,4-quinoneimide. This chemistry is analogous to reactions of hydroquinone (compound VI, Fig. 1), which itself is an antioxidant, although a much weaker antioxidant than DPPD.

Analyses of plasma of rabbits fed cholesterol or cholesterol plus DPPD. To test the oxidized LDL hypothesis of atherosclerosis, we tested the ability of DPPD to prevent atherosclerosis in the cholesterol-fed rabbit model. 14 New Zealand White rabbits were fed a diet containing 0.5% cholesterol and 10% corn oil ("control rabbits") and 12 rabbits were fed the same diet also containing 1% DPPD ("DPPD-fed"). Both groups of rabbits gained weight during the study; the control rabbits started at 2.6±0.06 kg and their weight increased by 33±3%, and the DPPD-fed rabbits started at 2.5±0.11 kg and their weight increased by 24±4% (these differences were not statistically significant). Plasma cholesterol levels increased during the study for both groups of rabbits (Fig. 2). Cholesterol exposure for each rabbit was calculated as the area under the curve of plasma cholesterol versus time. Cholesterol exposure for the two groups were (milligrams of cholesterol per deciliter × days × 10^{-3}): control rabbits, 99±7; DPPD-fed, 105±8 (P = 0.53; not significant).

After 27 d of feeding, fasting plasma was obtained and analyzed (Table II). The DPPD-fed group had higher levels of plasma triglyeride (73%; P = 0.007) and of HDL cholesterol (26%; P = 0.045); other parameters were not significantly different between the two groups.

Plasma levels of DPPD were measured at 27 and 62 d. When expressed in micromolar, there is an apparent increase from 35±3 μM at 27 d to 59±5 μM at 62 d (P = 0.0005; paired t test). If the levels are expressed as nanomoles DPPD per milligram cholesterol, however, then there is no significant difference: 1.7±0.1 nmol at 27 d and 1.8±0.1 nmol at 62 d (P = 0.5). Lipoproteins isolated after 62 d of feeding were analyzed for DPPD content (see Methods). The d < 1.019 fraction had 1.0±0.2 nmol DPPD/mg cholesterol, and the LDL fraction had 0.88±0.2 nmol DPPD/mg cholesterol. The d > 1.21 fraction accounted for 41±8% of the DPPD in plasma.

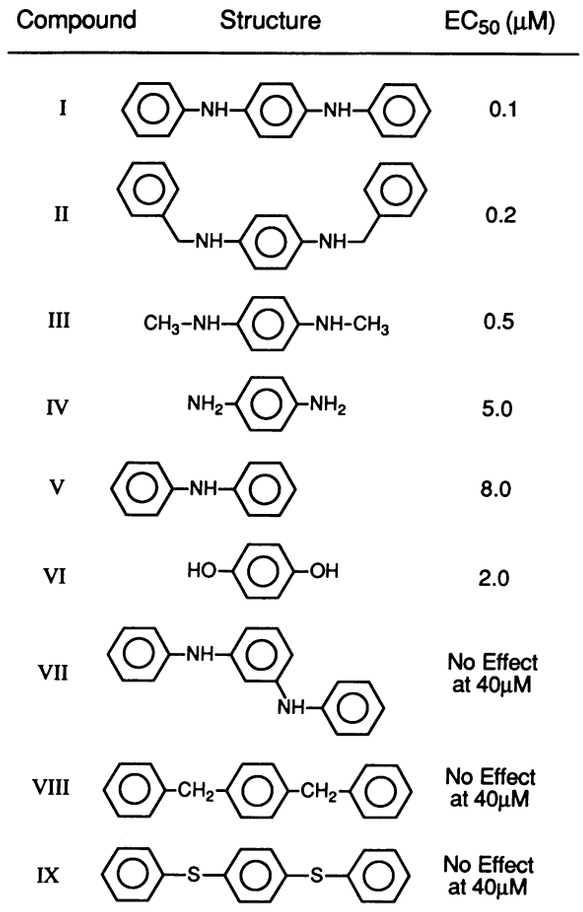

Figure 1. Structural requirements for antioxidant activity. Various compounds were tested for the ability to inhibit $CuSO_4$-mediated oxidation of LDL. EC_{50} values were estimated graphically from dose-response curves. Compound I is DPPD. In the experiments performed to determine the EC_{50} values given here, the EC_{50} for DPPD was found to be 0.1 μM. This is slightly lower than the mean value of 0.3 μM reported in Table I (see Methods). Compounds were either obtained from Aldrich Chemical Co. or synthesized at Merck Sharp & Dohme Research Laboratories, Rahway, NJ.

Table I. EC_{50} Values of BHT, Probucol, and DPPD against Oxidation of LDL

Compound	EC_{50} against oxidation by:	
	$CuSO_4$	Endothelial cells
	μM	
BHT	8.8±1.5 (8)	4.7±1.6 (5)
Probucol	3.8±0.4 (2)	3.6±1.0 (2)
DPPD	0.32±0.12 (6)	0.044±0.02 (4)

Values are mean±SEM, except for probucol where the mean and range of duplicates is given. Numbers in parentheses are number of independent measurements.

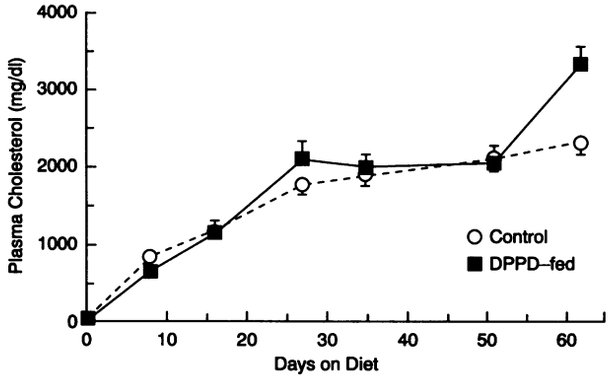

Figure 2. Rabbit plasma cholesterol levels versus time on cholesterol/corn oil diet. The values are means±SEM. Control rabbits (○) and DPPD-fed rabbits (■) were significantly different only at 8 d ($P = 0.01$) and at 62 d ($P = 0.003$). One rabbit that was not in the atherosclerosis study was fed the DPPD-containing diet for 135 d, at which time it had a cholesterol level of 2,620 mg/dl. The areas under the curves were not significantly different between the two groups (see text).

Lipoproteins from rabbits fed DPPD are resistant to oxidation ex vivo. Lipoproteins were isolated from rabbits in each treatment group and subjected to oxidizing conditions as described in Methods. Lipoproteins from DPPD-fed rabbits were much more resistant to oxidation than lipoproteins from control rabbits, both at 27 d (Table III) and at 62 d (Fig. 3). The time course of oxidation in Fig. 3 shows that the lipoproteins from DPPD-fed rabbits eventually are oxidized, i.e., the DPPD in the lipoproteins dramatically increases the lag time of oxidation. Previous work has shown that antioxidants such as vitamin E also increase lag time for oxidation of LDL (46, 47).

The absolute value of TBARS produced during 24-h $CuSO_4$ oxidation of control rabbit lipoproteins was variable (data not shown; also compare Table III and Fig. 3). This variability may arise because of variability in the time course of oxidation: maximum TBARS may not always have been achieved after 24 h of incubation with $CuSO_4$. We did find, however, that oxidation of LDL from control rabbits led to recognition by the macrophage scavenger receptor (Fig. 3 *B*), whereas LDL from DPPD-fed rabbits was not readily converted to a scavenger receptor ligand (Fig. 3 *B*).

LDL from DPPD-fed rabbits prevents oxidation of human LDL. We found that the DPPD present in LDL from DPPD-fed rabbits could prevent the oxidation of human LDL, i.e., the DPPD present in one LDL particle prevented the oxidation of another LDL particle. Human LDL was subjected to copper oxidation in the presence of various concentrations of (*a*) authentic DPPD, (*b*) control rabbit LDL, or (*c*) LDL from DPPD-

Table II. Plasma and Lipoprotein Analyses at 27 d

Variable	Control rabbits	DPPD-fed rabbits	*P* value
		mg/dl	
Plasma cholesterol	1,753±150	2,077±194	0.19
Plasma triglyceride	67±9	116±15	0.007
$d < 1.019$ cholesterol	1,612±144	1,946±187	0.16
LDL cholesterol	103±15	83±12	0.31
HDL cholesterol	38±3	48±4	0.045

All values are mean±SEM.

Table III. Oxidation of Rabbit Lipoproteins after 27 d of Feeding

Lipoprotein fraction	Group	TBARS	
		Unoxidized	Oxidized
		pmol/mg cholesterol	
$d < 1.019$	Control	150±91	9877±237
$d < 1.019$	DPPD-fed	193±81	380±10
LDL	Control	94±24	1650±305
LDL	DPPD-fed	105±18	248±27

Lipoproteins were isolated as described in Methods, and then oxidized by incubation at 37°C for 24 h in F-10 medium containing 10 μM $CuSO_4$. Oxidation was measured by the TBARS assay, as described in Methods. Unoxidized lipoproteins were mixed with F-10 and then subjected to the TBARS assay. Values are means±SEM.

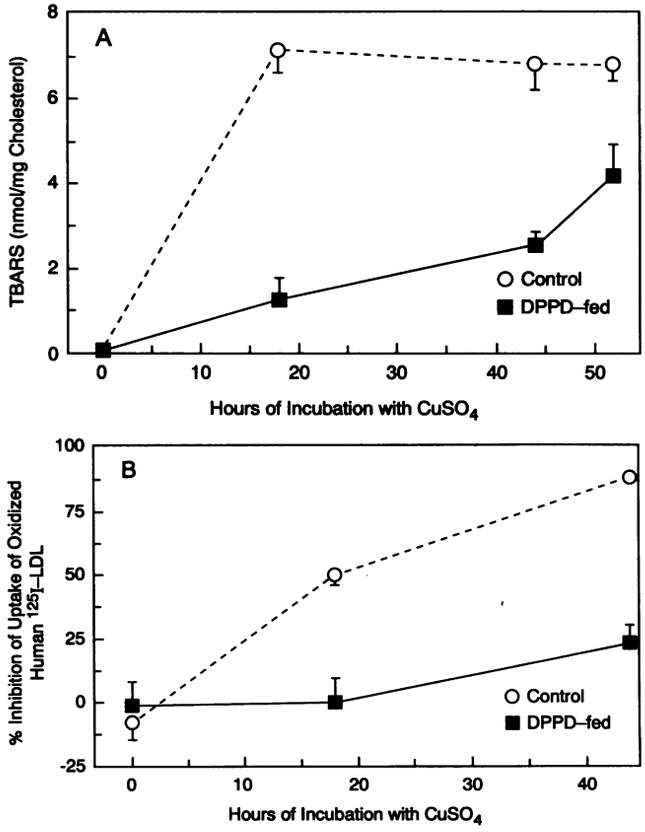

Figure 3. Time course of oxidation of rabbit LDL. LDL was isolated from control and DPPD-fed rabbits after 62 d of feeding. LDL preparations were incubated in F-10 medium containing 10 μM $CuSO_4$ and at various times samples were removed and subjected to two assays. (*A*) TBARS results are expressed as nanomoles of malondialdehyde equivalents per milligram of LDL cholesterol. (*B*) Scavenger receptor recognition was measured by the ability of the rabbit LDL to inhibit macrophage uptake of copper-oxidized ^{125}I-labeled human LDL; results are expressed as percent inhibition of uptake. All values are means±SEM. For assay details see Methods. (○) LDL from control rabbits; (■) LDL from DPPD-fed rabbits.

fed rabbits. The data are presented in Fig. 4. Increasing amounts of control rabbit LDL led to increasing amounts of TBARS per milliliter, because the rabbit LDL is oxidized along with the human LDL. DPPD itself, and LDL from DPPD-fed rabbits, both block oxidation of human LDL, at similar total concentrations of DPPD. These results show that either DPPD is rapidly exchanged between lipoproteins, or that DPPD in lipoproteins can terminate lipid peroxidation events occurring outside the particle itself.

Effect of DPPD feeding on atherosclerosis. DPPD feeding decreased lesion area in the thoracic aorta by 71% ($P = 0.0007$) and decreased total cholesterol content of the aorta by 51% ($P = 0.007$) (Table IV). Both cholesteryl ester and free cholesterol content were lower in the DPPD-fed rabbits (Table IV). Fig. 5 shows a scatter plot of lesion area versus cholesterol exposure (area under the curve of plasma cholesterol with time) for all the rabbits. DPPD-fed rabbits were clearly protected from atherosclerosis compared to controls. There was a large range in severity of atherosclerosis within the control group; this phenomenon has been discussed by Henry (48).

Discussion

We have shown that the antioxidant DPPD effectively inhibits the oxidative modification of LDL, with an EC_{50} value 10–100 times lower than that of probucol or BHT. Lipoproteins from rabbits fed DPPD were much more resistant to oxidation than lipoproteins from control rabbits. DPPD feeding also resulted in reduced severity of atherosclerosis in cholesterol-fed rabbits, without affecting plasma cholesterol levels.

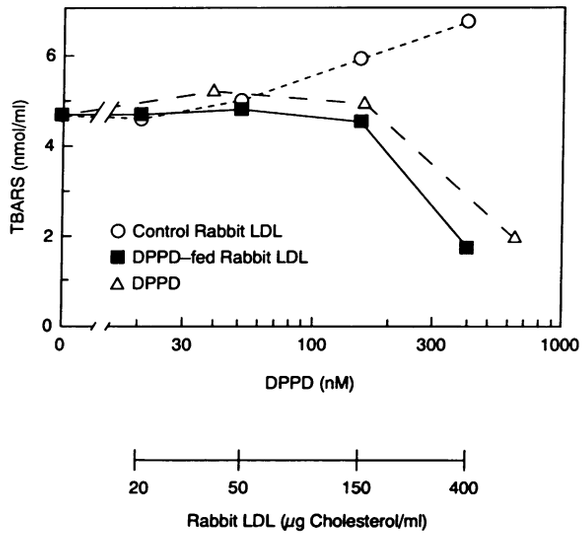

Figure 4. LDL from DPPD-fed rabbits inhibits the oxidation of human LDL. Human LDL (100 μg protein/ml) was incubated with 10 μM $CuSO_4$ in F-10 medium in the presence of various amounts of: DPPD (△), control rabbit LDL (○) or DPPD-fed rabbit LDL (■). After 24 h, TBARS were measured and are expressed as nanomoles of malondialdehyde equivalents per milliliter. Concentration of DPPD is expressed in nanomolar and concentration of rabbit LDL is expressed in micrograms cholesterol per milliliter. The LDL from DPPD-fed rabbits had 1.03 nmol DPPD/mg cholesterol, and both scales are applicable to that material. Data shown is from one of three similar experiments performed with two different batches of DPPD-fed rabbit LDL.

Table IV. Feeding DPPD Decreased Lesion Area and Cholesterol Content in Aortas from Cholesterol-fed Rabbits

	Control	DPPD	*P* value
Number of rabbits	14	12	
Lesion area (%)	42±7	12±3	0.0007
Cholesterol content (*mg/g wet weight*)			
Cholesteryl esters	9.9±1.5	5.2±1.2	0.025
Free cholesterol	4.1±0.5	1.6±0.2	0.0002
Total cholesterol	14.0±2.0	6.8±1.3	0.0067

Values are means±SEM. Aortas from three age-matched rabbits that were never fed the cholesterol/corn oil diet contained 0.30±0.03 mg of total cholesterol per g wet weight of tissue.

We chose to test DPPD as a potential antiatherosclerotic agent purely on the basis of its antioxidant activity. DPPD feeding did not have dramatic effects on lipid metabolism, but it significantly decreased the extent of atherosclerosis. Our observations strongly support the oxidized LDL hypothesis of atherosclerosis as proposed by Steinberg and colleagues (6). Recently Björkhem et al. (49) reported that feeding the antioxidant BHT decreased atherosclerotic lesion area in cholesterol-fed rabbits. In that study, lipoproteins isolated from the BHT-fed rabbits were not more resistant to oxidation than lipoproteins from control animals (49), so it is difficult to directly correlate the anti-atherosclerotic effect of BHT with protection of LDL from oxidation.

It has been widely suggested that the antiatherosclerotic activity of probucol is related to its antioxidant activity (6, 21–23). Very recently, however, the probucol analogue MDL 29,311 was shown to be comparable to probucol with respect to antioxidant activity, but had much less antiatherosclerotic activity than probucol (24). This implies that some portion of the antiatherosclerotic activity of probucol is due to other pharmacologic actions. DPPD is a much more potent antioxidant than probucol (Table I), and therefore presumably much more po-

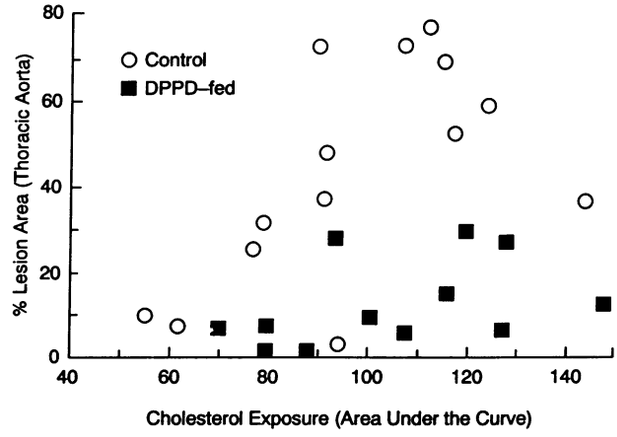

Figure 5. Dependence of lesion area on cholesterol exposure, plotted for each rabbit in the study. Cholesterol exposure was calculated as the area under the curve of plasma cholesterol level versus time. Area under the curve is given in units of (milligrams of cholesterol per deciliter) (days) (10^{-3}). (○) Control rabbits; (■) DPPD-fed rabbits.

tent than MDL 29,311. This may explain the greater anti-atherosclerotic activity of DPPD as compared to MDL 29,311. The oxidized LDL hypothesis would be further strengthened if other potent orally active antioxidants are shown to protect LDL from oxidation ex vivo and to prevent atherosclerosis in vivo.

DPPD feeding increased plasma triglycerides by 73% and HDL cholesterol by 26% (Table II). Elevated HDL cholesterol levels are widely believed to be antiatherosclerotic (4). In cholesterol-fed diabetic rabbits, dramatically elevated triglyceride levels correlate with decreased severity of atherosclerosis (50). The small differences in HDL and triglyceride between the groups in our study are probably insufficient to explain the large differences in severity of atherosclerosis. Furthermore, within each of the two treatment groups, neither HDL cholesterol level nor triglyceride levels correlated with extent of atherosclerosis. Despite these observations, we cannot completely exclude the possibilities that DPPD is antiatherosclerotic because it alters lipid metabolism in some subtle fashion or because it influences cellular processing of lipoproteins or cholesterol.

A substantial portion of the plasma cholesterol in the cholesterol-fed rabbit is in β-migrating very low density lipoprotein (β-VLDL) (37). This abnormal lipoprotein has been suggested to be intrinsically atherogenic because cultured macrophages accumulate cholesterol when incubated with β-VLDL (51). This has led to the suggestion that lipoprotein oxidation does not play a role in atherosclerosis in cholesterol-fed rabbits. At least three lines of evidence, however, imply that lipoprotein oxidation is important in this disease model: (*a*) Previous studies have shown that rabbit β-VLDL is susceptible to oxidation and the oxidized form is recognized by the macrophage scavenger receptor (52). We also found that the $d < 1.019$ lipoprotein fraction, which would be predominantly β-VLDL, is susceptible to oxidation (Table III). Oxidized LDL can cause the production of cytokines (14, 53) and is chemotactic for monocytes (11, 52), and these effects might also be mediated by oxidized β-VLDL. (*b*) Rosenfeld et al. (54) have recently shown that foam cells isolated from atherosclerotic lesions of cholesterol-fed rabbits contain oxidation specific lipid-protein adducts. (*c*) The recent study using BHT (49), and the present work with DPPD, show that antioxidants can decrease atherosclerosis in the cholesterol-fed rabbit. Taken together, the evidence suggests that lipoprotein oxidation is an important part of atherogenesis in cholesterol-fed rabbits.

The growing evidence in support of the oxidized LDL hypothesis has led to discussions of possible clinical trials of antioxidants (55). We have now shown that DPPD is a potent antioxidant and is anti-atherosclerotic in rabbits. Unfortunately, DPPD could never be given to patients because it is a mutagen: DPPD caused chromosome rearrangements in cultured cells (56), and it was positive in the Ames test for mutagens (57). Therapeutic use of potent antioxidants to treat vascular disease must await the discovery of nontoxic antioxidants.

Acknowledgments

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Note added in proof. We have further analyzed our data by plotting lesion area versus aortic cholesteryl ester content for each rabbit. Least-squares lines were drawn for each group, and we found that the slope for the control group was twice the slope for the DPPD-fed group ($P < 0.05$). Thus in the DPPD-fed group there was significantly less lesion area per cholesteryl ester content. This suggests the following conclusion: β-VLDL may be sufficient for aortic cholesteryl ester accumulation, whereas oxidized lipoproteins may be necessary for the creation of visible lesions. We thank Dr. Ira Tabas, Columbia University, for suggesting this analysis.

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