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Low density lipoprotein rich in oleic acid is protected against oxidative modification: Implications for dietary prevention of atherosclerosis

(macrophage/lipid peroxidation/linoleic acid/monounsaturated fat/polyunsaturated fat)

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ABSTRACT **Oxidative modification of low density lipoprotein (LDL) enhances its potential atherogenicity in several ways, notably by enhancing its uptake into macrophages. *In vivo* studies in the rabbit show that inhibition of LDL oxidation slows the progression of atherosclerotic lesions. In the present studies, rabbits were fed either a newly developed variant sunflower oil (Trisun 80), containing more than 80% oleic acid and only 8% linoleic acid, or conventional sunflower oil, containing only 20% oleic acid and 67% linoleic acid. LDL isolated from the plasma of animals fed the variant sunflower oil was highly enriched in oleic acid and very low in linoleic acid. These oleate-rich LDL particles were remarkably resistant to oxidative modification. Even after 16-hr exposure to copper-induced oxidation or 24-hr incubation with cultured endothelial cells, macrophage uptake of the LDL was only marginally enhanced. The results suggest that diets sufficiently enriched in oleic acid, in addition to their LDL-lowering effect, may slow the progression of atherosclerosis by generating LDL that is highly resistant to oxidative modification.**

A number of lines of evidence suggest that oxidative modification of low density lipoprotein (LDL) converts it to a more atherogenic form (reviewed in ref. 1). It has been shown that oxidative modification of LDL does indeed occur *in vivo* (2–5) and that inhibition of such oxidation slows the progress of atherosclerotic lesions, at least in the LDL receptor-deficient rabbit model (6, 7). These latter studies were done utilizing probucol as the antioxidant, but other antioxidants in principle would exert similar protective effects.

Oxidative modification of LDL, whether induced by incubation with cells or as a result of autooxidation in the presence of transition metal ions, is linked to oxidation of its polyunsaturated fatty acids. These fatty acids undergo extensive breakdown during LDL oxidation, yielding an array of lower molecular weight fragments (ketones, aldehydes, alcohols, and alkanes) (8, 9), some of which form covalent bonds with LDL apolipoprotein B (3, 4, 10). This lipid–protein conjugation is probably crucial in generating a form recognized by the acetyl LDL receptor of the macrophage because we know that the delipidated apoprotein of oxidized LDL is recognized by the acetyl LDL receptor (11). Actually, it now appears that more than one receptor is involved in the specific, saturable uptake of oxidatively modified LDL (12, 13).

LDL particles rich in polyunsaturated fatty acids, presenting more double bonds accessible for oxidative attack, should be more readily oxidatively modified and, in principle, more atherogenic. Yet diets in which polyunsaturated fatty acids are substituted for saturated fatty acids are considered to be beneficial in that they lower plasma cholesterol levels (14). In fact, dietary intervention studies have shown that such diets appear to reduce the risk of clinical coronary heart disease (15), presumably by decreasing plasma LDL levels. However, it is quite possible that the LDL in such patients *is* more readily oxidized and *is* therefore more atherogenic but that the decrease in LDL concentration more than compensates for any such increased susceptibility of LDL to oxidative modification. In other words, a polyunsaturated fatty acid-rich diet may have two opposing effects but with an overall balance that is favorable—i.e., antiatherogenic. Now, diets enriched in monounsaturated fatty acids have been shown to be just as effective as diets enriched in polyunsaturated fatty acids in lowering plasma LDL levels (16, 17). Thus, the use of monounsaturated fats in the diet, rather than polyunsaturated fats, might confer additional protection by generating LDL particles relatively resistant to oxidative modification without sacrificing the beneficial effect of lowering LDL levels.

In the present studies, we have taken advantage of the recent development of a variant sunflower oil containing more than 80% oleate (Trisun 80). The plasma LDL from rabbits fed this oil was greatly enriched with oleate and depleted of linoleate. It was strikingly resistant to oxidative modification.

MATERIALS AND METHODS

Materials. An oleate-enriched variant of sunflower oil (Trisun 80) and conventional sunflower oil were provided by SVO Enterprises (Eastlake, OH) (18). Rabbit diets containing 10% (wt/wt) oil (oleate-rich or conventional sunflower oil) were prepared for us by ICN. Standard Rabbit Chow was from O. H. Kruse Feed Company (El Monte, CA). New Zealand White (NZW) rabbits (females; 2.5-kg body weight) were purchased from Holberts Rabbitry (Spring Valley, CA). The sources of other chemicals are described elsewhere (11, 12).

Experimental Design and Lipoprotein Purification. Each of three diet groups contained eight NZW rabbits. Four animals from each group were sacrificed at 6 weeks and the remaining four at 10 weeks. Blood was collected in 0.1% EDTA/1 μM D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone, and the plasmas from each group were pooled for isolation of LDL (3). A mixture of gentamicin (100 μg/ml), chloramphenicol (50 μg/ml), and phenylmethylsulfonyl fluoride (0.5 mM) was added to the plasmas and to all solutions used during purification. LDL samples were dialyzed extensively against phosphate-buffered saline containing 0.01% EDTA, adjusted

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Abbreviations: LDL, low density lipoprotein; EC, endothelial cell(s); TBARS, thiobarbituric acid-reactive substances; NZW, New Zealand White.

to similar protein concentrations using dialysis buffer (to equalize the total EDTA per sample), stored at 4°C in the dark, and used within 2 weeks of isolation. Protein was determined by the method of Lowry *et al.* (19).

LDL protein (5 mg) from each plasma pool was radioiodinated by the method of Salacinski *et al.* (20), and the specific activity of each was adjusted to 60,000 cpm per μg of protein. Acetylation of LDL was carried out as described by Basu *et al.* (21).

Fatty Acid Composition. Lipids from LDL and from diets were extracted by a modification of the method of Folch *et al.* (22). The fatty acids were transmethylated (23) and analyzed in a Varian gas chromatograph model 3700, equipped with a column of 10% Silar 5CP on 100/120 Gas Chrom Q2.

Oxidative Modification of LDL. ^{125}I-labeled LDL (^{125}I-LDL; 100 μg of protein per ml) was incubated for 8, 16, or 24 hr at 37°C with either 5 μM copper(II) acetate (no cells) or with monolayers of rabbit aortic endothelial cells (EC) in Ham's F-10 medium. Lipid peroxidation was assessed in terms of thiobarbituric acid-reactive substances (TBARS) in the medium (24). Results are expressed as malondialdehyde equivalents per mg of LDL protein.

Conjugated Dienes. The formation of conjugated dienes was measured by incubating 200 μg of the LDL protein in a cuvette with 5 μM Cu^{2+} in 2 ml of Ham's F-10 medium. Absorbance at 234 nm was measured continuously in a Uvikon 810 spectrophotometer (25). Results are expressed as the increase in absorbance above the initial value.

LDL Degradation and Uptake. Resident mouse macrophages were harvested from the peritoneal cavity by lavage. Cells were plated on 24-well cluster dishes at a density of 1.4 × 10^6 per well with RPMI medium containing 10% (vol/vol) fetal calf serum. Cells were incubated overnight prior to use in a medium containing 5 mg of lipoprotein-deficient serum (LPDS) per ml. Degradation, cell association, and total uptake were determined as described by Goldstein *et al.* (26) after incubation of macrophages with 10 μg of ^{125}I-LDL protein per ml of medium for 5 hr at 37°C. Human skin fibroblasts were cultured on 12-well cluster dishes and incubated 24 hr in Dulbecco's modified Eagle's medium containing 5 mg of LPDS per ml and 10–25 μg of ^{125}I-LDL protein per ml of medium.

RESULTS

Effects of Diets on Fatty Acid Composition of LDL. The oleate-rich diet contained >83% oleate (18:1) and only 8% linoleate (18:2); the conventional sunflower oil diet contained <20% oleate and >67% linoleate (Table 1). Fatty acid compositions of LDL from rabbits fed these diets for 10 weeks are shown in Table 1. The values from rabbits fed these diets for 6 weeks were similar. The LDL from rabbits fed conventional sunflower oil contained 24% oleate; this was more than doubled on the oleate-rich diet (50% of total fatty acids). The LDL linoleate in the sunflower oil-fed rabbits was 39% of total fatty acids and fell to 16% on the oleate-rich diet. The fat in the standard chow was similar in composition to conventional sunflower oil, and the LDL composition on the chow diet reflects that. Despite the major substitution of oleate for linoleate in LDL on switching to the oleate-rich diet, there were only minor differences in the concentrations of cholesterol and triglycerides in the plasma on the sunflower oil and oleate-rich diets, respectively (triglycerides: 38 ± 10 and 39 ± 10 mg/dl, respectively; cholesterol: 87 ± 30 and 96 ± 32 mg/dl, respectively).

Effects on Susceptibility of LDL to Lipid Peroxidation. When these LDL samples were subjected to oxidative conditions *in vitro* (5 μM Cu^{2+} in Ham's F-10 medium at room temperature), a dramatic difference was seen in the rate of lipid peroxidation as indicated by diene conjugation (*A* at 234 nm) (Fig. 1). The LDL from rabbits fed conventional sunflower oil was readily oxidized, as seen by the steep increase in the rate of formation of conjugated dienes. In contrast, the oleate-enriched LDL showed a much slower rate of oxidation. At 14 hr, the increment in conjugated dienes in the oleate-rich LDL was only one-third to one-fourth that in the linoleate-rich LDL. This large difference in the formation of conjugated dienes is concordant with the differences in fatty acid composition measured before and after Cu^{2+} oxidation (Table 1). Oxidation of the LDL from sunflower oil-fed rabbits was associated with a decrease of linoleate from 39% to 11%; in the oleate-rich LDL, which contained only 17% linoleate at the start, there was also a decrease (to 6.7%), but the percentage of oleate remained essentially unchanged (50% versus 52%).

Oxidation of LDL is accompanied by the generation of TBARS that roughly reflects oxidation of polyunsaturated fatty acids but can be derived in part from other materials. LDL from the animals fed sunflower oil and standard chow for 6 weeks showed a large increment in TBARS, whereas the LDL from animals fed the oleate-rich diet showed only a very modest increase in TBARS (Fig. 2*A*), a result consonant with the lower rate of formation of conjugated dienes (Fig. 1).

Effects on Susceptibility of LDL to "Biological Modification." The increased atherogenicity of oxidized LDL is believed to rest in large part on the generation of a new configuration such that the modified LDL acquires a high affinity for the acetyl LDL or scavenger receptor(s) on macrophages. This is what we refer to as "biological modification," and it is assessed by

Table 1. Fatty acid composition of rabbit diets and of rabbit LDL samples

Diet	Rabbit LDL	Distribution of fatty acids (%)						
		16:0	16:1	18:0	18:1	18:2	18:3	20:4
		Composition of diets fed to rabbits						
Oleate-rich diet	—	4.1	0.2	3.2	83.1	8.2	0.2	0.9
Sunflower oil diet	—	7.1	0.1	4.5	19.8	67.1	0.6	0.7
Standard chow diet	—	20.3	0.9	3.0	20.4	45.5	8.8	1.1
		Composition of rabbit LDL						
Oleate-rich diet	Native	16.7	4.2	9.8	49.5	16.5	0.5	2.8
	Oxidized	21.3	5.7	11.6	51.5	6.7	—	3.3
Sunflower oil diet	Native	18.8	4.0	11.3	24.0	39.0	0.3	2.6
	Oxidized	33.8	4.5	16.2	33.2	11.1	—	1.2
Standard chow diet	Native	24.9	3.1	13.3	22.5	30.2	2.6	3.4
	Oxidized	43.7	3.7	17.7	28.4	4.9	—	1.6

Lipids from 0.5 g of each diet or 200 μg of LDL protein [before (native) and after (oxidized) Cu^{2+}-induced oxidation] were extracted in chloroform and transmethylated, and the fatty acid methyl esters were analyzed by gas chromatography as described. 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; 20:4, arachidonic acid.

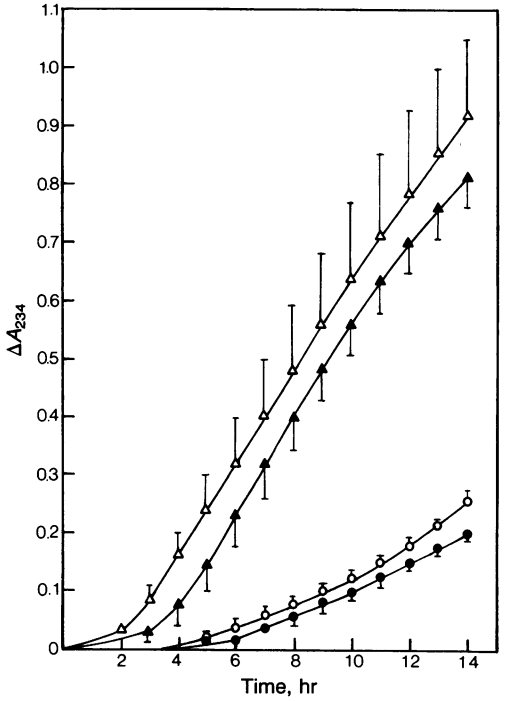

FIG. 1. Rate of formation of conjugated dienes. LDL (100 μg/ml) was incubated in Ham's F-10 medium containing 5 μM Cu^{2+} at room temperature. Absorbance was measured continuously in a Uvikon 810 spectrophotometer. Each point represents the mean ± SD of triplicate measurements. LDL from rabbits fed a diet of 10% (wt/wt) conventional sunflower oil for 6 weeks (▲) or 10 weeks (△) was compared with LDL from rabbits fed a diet of 10% (wt/wt) oleate-rich oil for 6 weeks (●) or 10 weeks (○).

measuring the amount of uptake and degradation of modified LDL by mouse peritoneal macrophages over a period of 5 hr. After being subjected to 16 hr of Cu^{2+}-induced oxidation, the LDL from animals fed either standard chow or conventional sunflower oil for 6 weeks showed more than a 10-fold increase in the amount of degradation products generated (Fig. 2*B*). Remarkably, oleate-rich LDL showed virtually no increased degradation by macrophages.

We have previously reported that oxidized LDL, unlike native LDL or acetyl LDL, tends to accumulate intracellularly in macrophages—i.e., degradation does not keep pace with uptake (12). For this reason, both degradation and cell association were measured when evaluating LDL from the animals fed the diets for 10 weeks. Both the total uptake and the degradation (trichloroacetic acid-soluble radioactivity) were much greater in the case of LDL isolated from rabbits fed either conventional sunflower oil or standard chow compared with LDL from animals on the oleate-rich diet (Fig. 3). The total uptake (sum of that degraded and that associated with the cells) was about 7-fold greater. To be certain that this enhanced uptake was attributable to the acetyl LDL receptor (or other receptors specific for oxidatively modified LDL), we tested for competition between oxidized LDL (from sunflower oil-fed animals) and other ligands for that receptor (27). All of these ligands reduced macrophage degradation by 70% or more (Table 2). Native LDL failed to compete, as expected.

To test whether the oleate-rich LDL also resisted cell-induced oxidation, it was incubated with monolayers of rabbit aortic EC, and aliquots of the medium were transferred at 8, 16, and 24 hr to plates of mouse peritoneal macrophages for measurement of the rate of uptake. The oleate-rich LDL was just as resistant to biological modification induced by cells as it was to Cu^{2+}-induced oxidation and biological modification (Fig. 4).

We considered the possibility that the resistance of oleate-rich LDL to biological modification might reflect a difference in the configuration of apolipoprotein B (apoB) in such LDL. However, measurements of degradation by cultured human skin fibroblasts of the linoleate-rich and the oleate-rich LDL failed to show any difference (14.9 and 13.4 μg degraded per mg of cell protein per 24 hr, respectively). We also considered the possibility that the differences in fatty acid composition might in some way hinder the presumptive change in configuration of apoB that results in high affinity binding to the acetyl LDL receptor (11). However, acetylation of linoleate-rich or oleate-rich LDL samples resulted in comparable enhancement of their degradation by macrophages (35.8 and 34.3 μg/mg of protein per 5 hr, respectively).

DISCUSSION

The conditions used for oxidation of LDL in these experiments are extreme (16 hr of incubation in the presence of 5 μM Cu^{2+}), and yet the LDL from rabbits fed the oleate-rich diet showed only very modest degrees of lipid peroxidation and almost no biological modification (i.e., very little enhancement of degradation in a subsequent incubation with macrophages). In contrast, LDL from rabbits fed either standard chow or conventional sunflower oil (both rich in linoleate) showed marked increases in lipid peroxidation and in biological modification (7-fold to 10-fold increases in rates of degradation by mouse peritoneal macrophages after oxi-

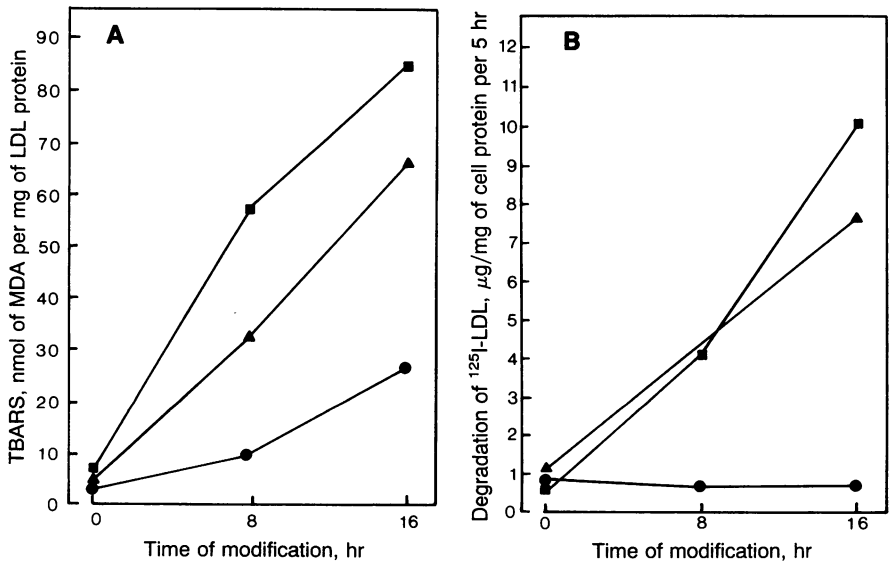

FIG. 2. Production of TBARS during oxidative modification (*A*) and the subsequent degradation of the modified LDL by macrophages (*B*). ^{125}I-LDL was prepared from rabbits fed standard chow (■), conventional sunflower oil diet (▲), or oleate-rich diet (●) for 6 weeks. ^{125}I-LDL was incubated with 5 μM Cu^{2+} in Ham's F-10 medium at 37°C at a concentration of 100 μg of LDL protein per ml. At the indicated times, aliquots were removed for the measurement of TBARS or assayed for macrophage degradation at 10 μg of LDL protein per ml of Dulbecco's modified Eagle's medium containing 5 mg of lipoprotein-deficient serum per ml for 5 hr. Each point represents the mean of duplicate assays. Values are from one experiment but are representative of five separate experiments yielding similar results. MDA, malondialdehyde.

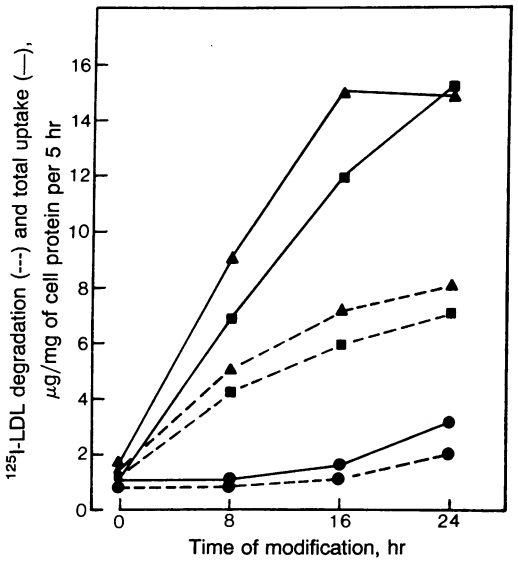

FIG. 3. Degradation and total uptake of Cu^{2+}-oxidized ^{125}I-LDL. ^{125}I-LDL was prepared from rabbits fed for 10 weeks with oleate-rich oil (●), conventional sunflower oil (▲), or standard chow (■) and then oxidized for the indicated times as noted in the legend to Fig. 2. An aliquot was then further incubated at 37°C with macrophages (10 μg of LDL protein per ml of Dulbecco's modified Eagle's medium). After 5 hr, the amount of ^{125}I-labeled degradation products in the medium (---) and the total uptake by the cells (—) were determined (sum of degradation and the amounts of ^{125}I-LDL associated with cells). Each point represents the mean of duplicate assays.

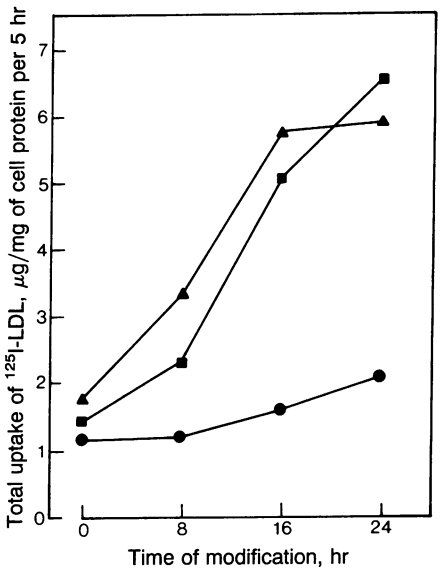

FIG. 4. Modification of LDL by incubation with cultured EC. ^{125}I-LDL was prepared from rabbits fed oleate-rich oil (●), conventional sunflower oil (▲), or standard chow (■) for 10 weeks. Each LDL was incubated at 37°C with a monolayer of rabbit aortic EC in Ham's F-10 medium for the indicated times. Aliquots of the medium containing EC-modified ^{125}I-LDL were then incubated with macrophages for 5 hr (10 μg of LDL protein per ml of Dulbecco's modified Eagle's medium). Total uptake of ^{125}I-LDL was determined (cell-associated ^{125}I-LDL plus ^{125}I-labeled degradation products in the medium). Each point represents the mean of duplicate assays. At least three separate trials were performed with similar results.

dation) (Figs. 2*B* and 3). Similar results were obtained when the oxidation was cell-induced (Fig. 4). This degree of resistance to lipid peroxidation and biological modification has previously been seen only in the case of LDL isolated from animals or patients treated with probucol, a potent lipophilic antioxidant (28).

It is important to ask whether the resistance to oxidative modification of oleate-rich LDL in the present studies might have been due to something other than, or in addition to, the fact that it is rich in oleate and relatively poor in linoleate. We considered the possibility that oleate-rich sunflower oil might contain more antioxidant materials than conventional sunflower oil and that therefore LDL from the rabbits fed the former might be better protected. To test this, we mixed LDL from animals fed the oleate-rich diet with LDL from animals fed conventional sunflower oil and then subjected the mixture to oxidative conditions. The presence of the oleate-rich LDL did not protect the linoleate-rich LDL from oxidation (data not shown).

We also considered the possibility that the configuration of the oleate-rich LDL might be radically different and that this might somehow inhibit biological modification. However, studies with fibroblasts showed that the LDL receptor did not distinguish oleate-rich LDL from ordinary LDL. Finally, after acetylation, both the oleate-rich and the linoleate-rich LDL were recognized comparably by the acetyl LDL receptor. In short, these data suggest that introducing monounsaturated fatty acids in place of polyunsaturated fatty acids protects LDL against oxidative modification by simply reducing the number of polyunsaturated fatty acids available as targets for peroxidation.

As shown by Esterbauer and coworkers (25), the rate of generation of diene conjugates in LDL is initially slow but after a variable induction time (lag period) reaches a maximum, which continues then for some time. The addition of antioxidants lengthens the induction time but does not affect the maximum slope of the curve. In the present instance, the oleate-rich LDL showed a somewhat longer induction time than the linoleate-rich LDL but the slope of the steep part of the curve was greatly reduced (Fig. 1). We believe the latter effect is attributable not to antioxidants but to the decreased content of polyunsaturated fatty acids. Whether or not the longer induction time is due to differences in antioxidant content or due also to the low content of polyunsaturated fatty acids is not certain. The vitamin E content of Trisun 80 is not different from that of conventional sunflower oil (R. Yodice, personal communication).

Previous studies have shown that treatment with probucol is antiatherogenic in the LDL receptor-deficient rabbit independently of its cholesterol-lowering effects (6, 7). This antiatherogenic effect was presumed to result largely from the ability of probucol to protect LDL against oxidative modification, although additional metabolic effects of probucol cannot be ruled out. If indeed protection of the LDL

Table 2. Competition for macrophage degradation of Cu^{2+}-oxidized ^{125}I-LDL by ligands for the scavenger receptor(s)

Additions	Degradation, μg/mg of cell protein per 5 hr
Native ^{125}I-LDL alone	1.02 ± 0.1
Cu^{2+}-oxidized ^{125}I-LDL alone	9.82 ± 0.05
+ unlabeled native LDL	9.80 ± 0.12
+ fucoidin	1.94 ± 0.08
+ poly(inosinic acid)	0.67 ± 0.07
+ unlabeled EC-modified LDL	0.56 ± 0.05
+ unlabeled Cu^{2+}-oxidized LDL	1.70 ± 0.15

^{125}I-LDL (100 μg/ml) from NZW rabbits fed conventional sunflower oil was incubated with 5 μM Cu^{2+} in Ham's F-10 medium at 37°C for 20 hr. The oxidized ^{125}I-LDL was then incubated with macrophages at 37°C (5 μg of LDL protein per ml) for 5 hr. Unlabeled LDL, fucoidin, poly(inosinic acid), or EC-modified LDL was added at 10-fold excess, and Cu^{2+}-oxidized LDL was added at 6-fold excess. The results represent the mean ± SD of three determinations.

against oxidation were the major factor, other interventions that similarly protect LDL against oxidative modification would be similarly antiatherogenic. Specifically, substitution of oleic acid for polyunsaturated fatty acids in the diet could reduce the atherogenicity of LDL. Thus, if the oxidative modification hypothesis is valid (1), the present findings could have exciting implications. The use of diets rich in monounsaturated fats has been widely discussed, and there is an emerging consensus that such diets may be preferable for purposes of lowering plasma cholesterol levels since they do not lower high density lipoprotein levels whereas polyunsaturated fat-rich diets do (16, 17). Moreover, there is no evidence that monounsaturated fat-rich diets are anything but safe; indeed, longevity tends to be higher and coronary heart disease rates tend to be lower in countries with high dietary intakes of oleate (29). Consequently, there would be no bar to implementation of a public health program to encourage the use of fats rich in monounsaturated fats. Of course, additional research is needed before such recommendations can be made. First, it must be shown that oleate-rich diets not only protect the LDL against oxidation, as shown in these studies, but also slow the progression of atherosclerosis. In addition, it remains to be shown whether a sufficient degree of oleate enrichment in LDL can be achieved in humans. If it can, then clinical studies will ultimately be needed to test the oxidative modification hypothesis in man. One strategy would be to use known antioxidants (e.g., probucol, vitamin E, or β-carotene). The present studies suggest an alternative, and possibly preferable, strategy to test the hypothesis in man—namely, to utilize oleate-rich diets rather than an antioxidant. Conceivably *both* could be utilized simultaneously. In fact, reducing the polyunsaturated fatty acid content of LDL should reduce the "consumption" of antioxidants and make them more effective at any given level of intake. The possibility of carrying out a critical test of the oxidative hypothesis in man using naturally occurring foods, rather than drugs, is an attractive one.

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