Effects of Linoleate-Enriched and Oleate-Enriched Diets in Combination With \( \alpha \)-Tocopherol on the Susceptibility of LDL and LDL Subfractions to Oxidative Modification in Humans

Peter D. Reaven, Barbara J. Grasse, Diane L. Tribble

Abstract This report describes the effects of feeding linoleate- or oleate-enriched diets to subjects who were concurrently taking 1200 mg/d of \( \alpha \)-tocopherol on the susceptibility of low-density lipoprotein (LDL) and buoyant and dense LDL subfractions to oxidation. LDL isolated from subjects who consumed linoleate-enriched diets was more susceptible to copper-mediated oxidation, as measured by formation of conjugated dienes and lipid peroxides and loss of unsaturated fatty acids, compared with LDL isolated from subjects who consumed their usual or oleate-enriched diets. In all subjects, buoyant LDL had a higher content of \( \alpha \)-tocopherol per particle and a lower 18:2 to 18:1 ratio and was considerably more resistant to oxidation than dense LDL. Although dense LDL from all groups had comparable \( \alpha \)-tocopherol levels, dense LDL from the linoleate group was most susceptible to oxidation, followed by that from the standard diet, whereas dense LDL isolated from the oleate diet group was most resistant. In summary, high dosages of \( \alpha \)-tocopherol did not prevent enhanced susceptibility to oxidation of LDL isolated from subjects fed linoleate-enriched diets. Furthermore, dense LDL was more susceptible to oxidation than was buoyant LDL, and this effect was greatly exaggerated in the dense LDL isolated from subjects fed linoleate-enriched diets. Conversely, dense LDL isolated from subjects fed oleate-enriched diets was the most protected. If oxidation of LDL is important in the pathogenesis of atherosclerosis, then these data suggest that in people with increased amounts of small, dense LDL, dietary enrichment in oleic acid may decrease the susceptibility of their LDL to oxidation. (Arterioscler Thromb. 1994;14:557-566.)

Key Words • atherosclerosis • conjugated dienes • lipid oxidation • dense LDL • LDL subclasses • vitamin E • fatty acids

There is evidence that oxidative modification of low-density lipoproteins (LDLs) occurs in vivo\(^1\)\(^-\)\(^4\) and may increase LDL atherogenicity, as recently reviewed.\(^5\)\(^-\)\(^6\) During the generation of oxidatively modified LDL, polyunsaturated fatty acids undergo peroxidative decomposition, yielding reactive aldehydes, some of which form covalent bonds with LDL apoprotein B.\(^7\)\(^-\)\(^8\) In addition, direct adduct formation between LDL lipids and protein may occur.\(^9\) These modifications generate a particle that is recognized by the scavenger receptor(s) of the macrophage\(^10\)\(^-\)\(^11\) and lead to enhanced uptake and foam cell formation. Oxidatively modified LDL or soluble LDL oxidation products have other effects that promote atherogenesis, including monocyte chemotaxis,\(^12\) cytotoxicity,\(^13\) alteration of gene expression in arterial cells,\(^14\)\(^-\)\(^15\) and alteration of vascular tone and responsiveness (reviewed in Reference 6).

A number of factors can influence the susceptibility of LDL to oxidation. An early step in the oxidation of LDL is peroxidation of polyunsaturated fatty acids. Since linoleic acid (18:2) constitutes nearly 90% of the polyunsaturated fatty acids in LDL, it is the major substrate of LDL oxidation. Diets enriched in linoleate increase the content of linoleic fatty acid in plasma LDL and increase its susceptibility to oxidation.\(^16\)\(^-\)\(^18\) In contrast, diets enriched in oleate (18:1) and depleted of linoleate generate LDL particles that are more resistant to oxidation. A second important determinant of LDL susceptibility to oxidation is the content of endogenous antioxidants. Esterbauer et al\(^20\) have suggested that after the consumption of antioxidants in LDL, the rapid propagation phase of oxidation commences.\(^20\) Vitamin E, a potent free-radical scavenger and the most abundant antioxidant in LDL, may therefore provide important antioxidant protection to LDL. In support of this concept are studies showing that enrichment with vitamin E, both in vitro and in vivo, significantly reduces LDL susceptibility to oxidation.\(^21\)\(^-\)\(^25\) In addition to compositional features, particle size and density have been shown to influence the susceptibility of LDL to oxidation.

Several investigators have demonstrated that smaller, more dense particles are more susceptible to oxidation than are larger, more buoyant LDL.\(^26\)\(^-\)\(^29\) In studies that directly compared LDL subfractions, the susceptibility to oxidation progressively increased as LDL particle density increased.\(^26\)\(^-\)\(^28\) The goal of the current study was...
to determine the effect of diet-induced changes in LDL fatty acid composition, in combination with α-tocopherol supplementation, on the susceptibility of LDL to oxidation. Of particular interest was whether the effects of these interventions were different between buoyant and dense LDL subfractions.

Methods

Participants

Eighteen healthy volunteers (9 women and 9 men) aged 22 to 61 years were recruited from the local community. Each subject was given ΔΔ-α-tocopherol (Hoffmann-La Roche, Inc) supplementation at 1200 mg/d (approximately 100 times the recommended daily allowance) for at least 3 months before randomization to diet groups. All subjects had been consuming average American diets, and none were taking lipid-lowering medications or other antioxidants. The study was approved by the Human Studies Committee of the University of California, San Diego, and was conducted in the outpatient facilities of the University of California, San Diego, General Clinical Research Center (GCRC).

Study Design

Subjects were randomized either to receive linoleate-(n=6) or oleate-(n=6) enriched diets or their usual (standard) diets for 6 weeks (n=6). Throughout the study, subjects and investigators remained blinded to the predominant type of fatty acid in the diets. Participants picked up their fully prepared diets on Monday, Wednesday, and Friday of each week. Unused portions of the diet were returned at each visit. Participants were allowed to ingest their daily diet according to their own schedule, although the entire day's allocation was to be ingested by bedtime. Subjects were instructed to refrigerate the perishable foods and the liquid portions of the diet at all times when not in use. Daily dietary records were reviewed weekly by a registered dietician to ensure adherence and to monitor acceptability.

Diet Preparation

An oleate-enriched variant of sunflower oil (Trisun 80), provided by SVO Enterprises, and conventional sunflower oil (Wesson Sunlite) were incorporated into various foods and liquid formulas. Oleic acid accounts for more than 80% of the total fatty acids in Trisun 80, whereas in Wesson Sunlite, approximately 60% of the total fatty acids are linoleic acid. Total α-tocopherol levels in the Trisun 80, whereas in Wesson Sunlite, approximately 60% of the total fatty acids are linoleic acid. Total α-tocopherol levels in the Trisun 80, whereas in Wesson Sunlite, approximately 60% of the total fatty acids are linoleic acid. Total α-tocopherol levels in the Trisun 80, whereas in Wesson Sunlite, approximately 60% of the total fatty acids are linoleic acid. Total α-tocopherol levels in the Trisun 80, whereas in Wesson Sunlite, approximately 60% of the total fatty acids are linoleic acid.

Preparation of LDL

Fasting blood samples were obtained from each subject at baseline and after 4 and 6 weeks of the diet period. Plasma was separated from blood that had been collected in EDTA (4.0 mmol/L) containing tubes and placed immediately on ice. A final concentration of 0.22 mmol/L gentamicin, 0.15 mmol/L chloramphenicol, 1 μmol/L D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone, and 2 mmol/L benzamidine was added to all plasma samples. LDL (d = 1.019 to 1.063 g/mL) was isolated by sequential ultracentrifugation as previously described and dialyzed extensively against phosphate-buffered saline containing 0.27 mmol/L EDTA (PBS-EDTA). Plasma was also divided into two equal aliquots and separated into buoyant (d = 1.026 to 1.032 g/mL) and dense (d = 1.040 to 1.054 g/mL) fractions by performing two sequential ultracentrifugation spins with each portion as follows. Aliquots were brought to the appropriate density by addition of NaBr and were centrifuged in 6-mL polycarbonate tubes in a Beckman 40.3 fixed-angle rotor at 40 000 rpm for 20 hours at 10°C. Buoyant and dense LDL isolated by this methodology corresponded to LDL subclasses LDL-I and LDL-III, respectively, as previously characterized by Musliner and Krauss. Selection of these density intervals avoids particle overlap, the absence of which was confirmed by measurement of lipoprotein particle diameters by nondenaturing 2% to 16% gradient gel electrophoresis, as previously published. Briefly, electrophoresis of samples was carried out using a Tris (0.90 mol/L)/boric acid (0.08 mol/L) buffer, pH 8.3, containing EDTA (3 mmol/L) at 10°C. Samples were adjusted to 20% sucrose, and 3 to 10 μL was applied to the gel and run at 20 V for 15 minutes, followed by 70 V for 15 minutes, and finally 125 V for 24 hours. Gels were stained with Coomassie Brilliant Blue and scanned at 555 nm with a Transdyme RFT densitometer. Molecular diameters were determined on the basis of migration distance by comparison with standards of known diameter.

Measurement of LDL-conjugated diene and lipid peroxide formation during copper sulfate (CuSO₄)–mediated oxidation was carried out immediately after LDL isolation. All other studies were completed within 2 weeks of LDL isolation. Samples were stored at 4°C in the dark until analysis. For all oxidation assays, LDL samples were dialyzed against PBS to remove EDTA.

Lipoprotein Oxidation

The formation of conjugated dienes was measured at room temperature in incubations containing 100 μg of LDL protein with 5 μmol/L CuSO₄ in 1 mL PBS. The absorbance at 234 nm (A₂₃₄) was measured at 10-minute intervals in a Uvikon 810 spectrophotometer as described. Results are expressed as the absolute increase in absorbance or as the time required for initiation of rapid oxidation (lag time). The intra-assay coefficients of variation for these measurements are less than 1%. The formation of lipid peroxides was measured by the iodometric method in separate incubations. For these experiments, LDL was incubated at 100 μg LDL protein per milliliter in PBS at 37°C, and aliquots were removed for lipid-peroxide measurement at 0, 2, 3, and 4 hours after addition of 5 μmol/L CuSO₄.

Lipoprotein Vitamin E Content

α-Tocopherol was measured by high-performance liquid chromatography according to the method of Arnaud et al. α-Tocopherol acetate was used as an extraction internal standard and for standard-curve preparation. Actual concentrations of α-tocopherol standards were determined by measuring the absorbance of prepared solutions and calculating the concentrations on the basis of known spectral data. Sample values were determined from a standard curve of peak area ratios of sample to internal standard.

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The calculated average energy intake (mean±SD) from the total diet was 2685±801 kcal/d for the linoleate group and 2513±597 kcal/d for the oleate group, with 56% and 58%, respectively, of energy from solid food sources. As a percent of total energy intake in the linoleate diet group, 9% was monounsaturated fatty acids, 26% was polyunsaturated, and 5% was saturated.

In contrast, in the oleate diet group, 29% of total energy intake was monounsaturated fatty acids, 5% was polyunsaturated, and 6% was saturated. The standard-diet group, which contained several lower-weight subjects, had an average total energy intake of 1804±503 kcal/d with 10% as monounsaturated fatty acids, 5% as polyunsaturated, and 12% as saturated.

Mean values for body weight, plasma lipids, and lipoproteins at baseline and at 6 weeks are shown in Table 1. There were no significant changes in weight or lipid values in the standard-diet group. In the linoleate and oleate diet groups, weight decreased by approximately 3%. Total cholesterol decreased significantly in both study groups; LDL cholesterol decreased by 16.1% in the linoleate group and 13.4% in the oleate group, although only the latter reduction was significant. HDL cholesterol decreased by 7.8% and 4.0% in the linoleate and oleate groups, respectively.

### Lipoprotein and Diet Fatty Acid Compositions

Lipids from LDL and liquid-formula aliquots were extracted by a modification of the method of Folch et al. The fatty acids were transmethylated and analyzed in a Varian model 3700 gas chromatograph equipped with a column of 10% Silar SCP on a Gas Chrom Q10, 100/120 mesh. A 15:0 internal standard (pentadecanoic acid) was added to each sample before extraction, and the content of fatty acid was determined from the peak area ratios of sample to internal standard.

### Lipoprotein Lipid and Protein Compositions

Phospholipid phosphorus was analyzed according to the method of Bartlett and expressed as phosphatidylcholine equivalents. Total cholesterol and triglycerides were measured by standard enzymatic methods on a system 3500 Gilford Computer Directed Analyzer. Unesterified and esterified cholesterol contents were determined by gas-liquid chromatography on a Hewlett-Packard 5830A gas chromatograph. Protein concentrations were determined by the Lowry method that was modified to include sodium dodecyl sulfate.

### Statistical Analyses

Comparisons between diet group means were performed by ANOVA; changes within diet groups were compared by Student’s paired t tests. Repeated-measures ANOVA was used to compare differences in the formation of lipid peroxides between diet groups. Pearson’s correlation coefficients were used to evaluate the associations between measures of oxidation and composition of LDL and LDL subfractions.

### Results

Seventeen of 18 participants completed the 6-week diet study without significant problems. One subject from the oleate diet group removed herself from the study for personal reasons. Weekly food records and measurement of unused portions of the diets indicated that compliance was excellent, with greater than 90% of the provided diets being ingested by all participants. The calculated average energy intake (mean±SD) from the total diet was 2685±801 kcal/d for the linoleate group and 2513±597 kcal/d for the oleate group, with 56% and 58%, respectively, of energy from solid food sources. As a percent of total energy intake in the linoleate diet group, 9% was monounsaturated fatty acids, 26% was polyunsaturated, and 5% was saturated.

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Mean values for body weight, plasma lipids, and lipoproteins at baseline and at 6 weeks are shown in Table 1. There were no significant changes in weight or lipid values in the standard-diet group. In the linoleate and oleate diet groups, weight decreased by approximately 3%. Total cholesterol decreased significantly in both study groups; LDL cholesterol decreased by 16.1% in the linoleate group and 13.4% in the oleate group, although only the latter reduction was significant. HDL cholesterol decreased by 7.8% and 4.0% in the linoleate and oleate groups, respectively.

### α-Tocopherol and Fatty Acid Composition of LDL

LDL α-tocopherol levels (per milligram of LDL protein) were 2 to 2.5 times higher than those reported for LDL from subjects who did not receive such supplements. However, the α-tocopherol content in LDL was similar in the three groups throughout the study (Fig 1). Before the study subjects began the special diets, there were no significant group differences in the percent distribution of any measured long-chain fatty acids in LDL (Table 2). In the standard-diet group, LDL fatty acid content was similar to previously reported values and did not change during the 6-week period, reflecting the consistency of their diet. In contrast, after 6 weeks of linoleate (18:2) or oleate (18:1) diet enrichment, mean levels of 18:2 and 18:1 increased significantly (P<.001) in the linoleate and oleate diet groups, respectively. Within the oleate diet group, subjects had increases in oleic acid that ranged from 22% to 73% and small reductions in linoleic acid. In the linoleate diet group, all participants had marked reductions in 18:1 and increases in 18:2 that ranged from 14% to 57%. Thus, all 11 subjects who consumed the prepared diets had substantial changes in their LDL fatty acid content that reflected the changes in the composition of their

### Table 1. Comparison of Weight, Lipid, and Lipoprotein Levels at Baseline and 6 Weeks

<table>
<thead>
<tr>
<th>Diet</th>
<th>Body Weight, kg</th>
<th>Total Chol, mg/dL</th>
<th>LDL Chol, mg/dL</th>
<th>HDL Chol, mg/dL</th>
<th>Triglycerides, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline Final</td>
<td>Baseline Final</td>
<td>Baseline Final</td>
<td>Baseline Final</td>
<td>Baseline Final</td>
</tr>
<tr>
<td>Standard (n=6)</td>
<td>70.1 69.4</td>
<td>210.6 211.2</td>
<td>133.4 132.2</td>
<td>55.8 53.5</td>
<td>106.8 127.7</td>
</tr>
<tr>
<td>Mean SD</td>
<td>70.1 9.5</td>
<td>14.8 17.4</td>
<td>16.2 22.2</td>
<td>15.2 13.0</td>
<td>26.7 57.1</td>
</tr>
<tr>
<td>Oleate (n=5)</td>
<td>76.6 74.5</td>
<td>168.5 147.6*</td>
<td>103.5 89.6*</td>
<td>46.0 44.2</td>
<td>87.2 69.0</td>
</tr>
<tr>
<td>Mean SD</td>
<td>76.6 3.8</td>
<td>15.8 15.2</td>
<td>11.6 9.5</td>
<td>7.7 4.9</td>
<td>29.8 22.6</td>
</tr>
<tr>
<td>Linoleate (n=6)</td>
<td>71.7 70.1</td>
<td>203.0 172.8*</td>
<td>124.6 104.5</td>
<td>59.7 55.0</td>
<td>93.2 67.3*</td>
</tr>
<tr>
<td>Mean SD</td>
<td>71.7 13.9</td>
<td>11.8 17.3</td>
<td>16.3 12.0</td>
<td>14.2 13.4</td>
<td>37.2 29.5</td>
</tr>
</tbody>
</table>

Chol indicates cholesterol; LDL, low-density lipoprotein; and HDL, high-density lipoprotein.

*Significant difference compared with the baseline value at P<.05.
Baseline 4wk 6wk

**FIG 1.** Bar graph showing low-density lipoprotein (LDL) α-tocopherol levels during the study period. Six hundred milligrams of α-tocopherol was given twice a day for 3 months before and for the duration of the study to subjects in all three diet groups (standard (○), oleate (●), and linoleate (□)). α-Tocopherol levels in LDL (mean±SD) are shown at baseline, 4 weeks, and 6 weeks (wk). No significant differences were present between groups.

Effects of Diet on Parameters of LDL Susceptibility to Oxidation

The rate and extent of conjugated-diene formation was measured for each individual LDL sample (d=1.019 to 1.063 g/mL) at room temperature in the presence of 5 μmol/L Cu²⁺ (Fig 2). At baseline, there were no differences in lag times of LDL oxidation among the three study groups. However, after 4 and 6 weeks, lag time values in the linoleate diet group were less than those in the other two groups and by 6 weeks had decreased significantly compared with baseline.

On completion of the study, there was a significant correlation between lag time and preoxidation levels of LDL α-tocopherol (r=−.59, P<.05) and the LDL 18:2 to 18:1 ratio (r=−.58, P=.05) for all subjects.

The time course of LDL lipid-peroxide formation as measured by the iodometric method during Cu²⁺-mediated oxidation is shown in Fig 3. The rate and extent of lipid-peroxide formation varied substantially between individuals and from assay to assay (note the large standard deviations and the differences in peak lipid-peroxide values). However, despite this variation, mean levels of lipid peroxides after 4 and 6 weeks of dietary supplementation were consistently higher in the linoleate diet group at each time point, with differences achieving statistical significance at the 4-week interval (panels B and C).

Effects of Oxidation on LDL Fatty Acid Composition

At the end of the study, determinations of fatty acids were performed on all LDL samples from each dietary group before and after 16 hours of Cu²⁺-mediated oxidation (Fig 4). Values are presented as a percentage of the preoxidation amounts rather than absolute units because of differences in the initial amounts of fatty acids within LDL from the three groups. In response to oxidation, marked decreases in the LDL content of 18:2 and 20:4 occurred in all diet groups, but a significantly greater percent loss of unsaturated fatty acids occurred in the LDL from the linoleate diet group (8% decrease in 18:1, P=.07; 75% decrease in 18:2, P=.06; and a 95% decrease in 20:4, P=.05) compared with the other diet groups. The absolute fatty acid loss due to oxidation was also significantly greater in the linoleate diet group.

**TABLE 2.** LDL (d=1.019 to 1.063 g/mL) Fatty Acid Content at Baseline and After 6 Weeks of Diet Modification

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Standard Diet (n=6)</th>
<th>Oleate Diet (n=5)</th>
<th>Linoleate Diet (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Final</td>
<td>Baseline</td>
</tr>
<tr>
<td>16:0</td>
<td>21.0±1.4</td>
<td>21.0±0.8</td>
<td>21.7±1.9</td>
</tr>
<tr>
<td>16:1</td>
<td>2.2±1.1</td>
<td>2.6±1.0</td>
<td>2.6±1.0</td>
</tr>
<tr>
<td>18:0</td>
<td>6.8±5</td>
<td>7.2±0.7</td>
<td>6.9±0.9</td>
</tr>
<tr>
<td>18:1</td>
<td>19.8±1.0</td>
<td>19.8±1.1</td>
<td>20.4±2.3</td>
</tr>
<tr>
<td>18:2</td>
<td>43.2±2.0</td>
<td>42.5±2.1</td>
<td>41.3±4.8</td>
</tr>
<tr>
<td>20:4</td>
<td>7.0±1.9</td>
<td>6.9±1.4</td>
<td>6.9±1.9</td>
</tr>
</tbody>
</table>

LDL indicates low-density lipoprotein. Comparison between diet groups in their content of fatty acids is presented as percent of total fatty acids in LDL. Values are mean±SD. No significant differences were present between groups at baseline.

*Significant differences compared with the standard-group final value at P<.05.
†Significant differences compared with the standard-group final value at P<.001.
Fe Ê Une plots showing lipid-peroxide formation in copper-oxidized low-density lipoprotein (LDL). LDL (100 µg/mL) samples from subjects who ate either a standard diet (a) or an oleate- (b) or linoleate- (c) supplemented diet were incubated in phosphate-buffered saline with 5 µmol/L Cu²⁺, and aliquots were removed for measurement of lipoperoxides at 0, 2, 3, and 4 hours (H). Values are mean±SD for the baseline (a), 4-week (b), and 6-week (c) visit.*Linoleate diet group time course significantly different compared with the other diet groups. (P<.05), whereas there was no difference in fatty acid loss between the oleate and standard-diet groups.

Composition of LDL Subfractions

To allow an overall comparison between buoyant and dense subfractions, compositional data from all diet groups were combined (Table 3). There were significantly greater amounts of dense LDL in plasma compared with buoyant LDL. Triglyceride, free cholesterol, phospholipid, and total lipid contents (as a percent of total LDL mass) were significantly less in the dense fraction, whereas cholesteryl ester and protein were higher. These values for the buoyant and dense LDL fractions are consistent with those previously reported. α-Tocopherol content per milligram of LDL protein (or per LDL particle) was nearly twofold higher in buoyant LDL compared with dense LDL. α-Tocopherol levels per milligram of total lipid also were higher in buoyant LDL (data not shown), although the α-tocopherol to polyunsaturated fatty acid content was similar in buoyant and dense LDL. The composition of dense LDL subfractions from each diet group are shown in Table 4. There were no notable differences in lipid or vitamin E content in the three diet groups. However, the total polyunsaturated fatty acid content per LDL particle was higher and the α-tocopherol to polyunsaturated fatty acid ratio significantly lower in the dense LDL of the linoleate group. A similar pattern was seen in the buoyant LDL subfractions from each diet group (Table 5).

Effect of Diets on LDL Subfraction Fatty Acid Composition

The fatty acid composition of buoyant and dense LDL is shown in Fig 5. As in unfractionated LDL, 18:2 is significantly higher and 18:1 significantly lower in both subfractions from the linoleate group and 18:1 is increased in both subfractions from the oleate diet.

Table 3. Characteristics and Composition of Combined Buoyant and Combined Dense Fractions

<table>
<thead>
<tr>
<th></th>
<th>Buoyant LDL</th>
<th>Dense LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total LDL mass, mg/mL plasma</td>
<td>0.92±0.47</td>
<td>1.32±0.48†</td>
</tr>
<tr>
<td>TG content*</td>
<td>11.2±2.4</td>
<td>6.9±2.1†</td>
</tr>
<tr>
<td>Free cholesterol*</td>
<td>8.3±0.5</td>
<td>6.8±0.7†</td>
</tr>
<tr>
<td>Cholesteryl ester*</td>
<td>37.5±2.6</td>
<td>41.3±2.3†</td>
</tr>
<tr>
<td>Phospholipid*</td>
<td>21.3±1.0</td>
<td>19.9±1.4†</td>
</tr>
<tr>
<td>Protein*</td>
<td>22.1±1.65</td>
<td>25.0±0.8†</td>
</tr>
<tr>
<td>α-Tocopherol/LDL protein, µg/mg</td>
<td>13.4±4.4</td>
<td>5.6±1.2†</td>
</tr>
<tr>
<td>α-Tocopherol/PUFAs, µg/mg</td>
<td>22±7</td>
<td>22±6</td>
</tr>
<tr>
<td>18:2/18:1</td>
<td>2.42±2.0</td>
<td>2.95±1.5†</td>
</tr>
</tbody>
</table>

LDL indicates low-density lipoprotein; TG, triglycerides; and PUFAs, polyunsaturated fatty acids (18:2+20:4). Values from buoyant LDL fractions from all three diet groups (N=15) were combined (buoyant LDL) as were values for dense LDL fractions (dense LDL). *Percent of total LDL mass. †Significant compared with the buoyant-group value at P<.05.
TABLE 4. Comparison of Composition of Dense LDL Fractions (d=1.040 to 1.054 g/mL) After 6 Weeks of Dietary Modification

<table>
<thead>
<tr>
<th></th>
<th>Standard Diet (n=6)</th>
<th>Oleate Diet (n=4)</th>
<th>Linoleate Diet (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG content*</td>
<td>7.6±2.7</td>
<td>7.3±0.6</td>
<td>5.8±1.8</td>
</tr>
<tr>
<td>Free cholesterol*</td>
<td>6.7±0.8</td>
<td>7.3±0.8</td>
<td>6.7±0.7</td>
</tr>
<tr>
<td>Cholesteryl ester*</td>
<td>42.1±2.4</td>
<td>39.9±1.5</td>
<td>41.6±2.5</td>
</tr>
<tr>
<td>Phospholipid*</td>
<td>19.3±0.6</td>
<td>19.8±1.8</td>
<td>20.8±2.5</td>
</tr>
<tr>
<td>Protein*</td>
<td>24.4±0.8</td>
<td>25.8±0.2</td>
<td>25.1±0.6</td>
</tr>
<tr>
<td>α-Tocopherol/LDL protein, µg/mg</td>
<td>6.2±1.1</td>
<td>4.8±0.8</td>
<td>5.2±0.6</td>
</tr>
<tr>
<td>Total PUFAs/LDL protein, mg/mg</td>
<td>244.9±16.5</td>
<td>215.0±19.2</td>
<td>322.3±7.3†</td>
</tr>
<tr>
<td>α-Tocopherol/PUFAs, µg/mg</td>
<td>25±4</td>
<td>22±3</td>
<td>18±5†</td>
</tr>
<tr>
<td>18:2/18:1</td>
<td>2.0±0.2</td>
<td>1.2±0.1</td>
<td>5.2±1.8†</td>
</tr>
</tbody>
</table>

LDL indicates low-density lipoprotein; TG, triglycerides; and PUFAs, polyunsaturated fatty acids (18:2+20:4). Total LDL mass in milligrams per milliliter of plasma values are as follows: standard diet, 1.39±0.23; oleate diet, 1.33±0.90; and linoleate diet, 1.24±0.30.

TABLE 5. Comparison of Composition of Buoyant LDL Fractions (d=1.026 to 1.032 g/mL) After 6 Weeks of Dietary Modification

<table>
<thead>
<tr>
<th></th>
<th>Standard Diet (n=6)</th>
<th>Oleate Diet (n=4)</th>
<th>Linoleate Diet (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG content*</td>
<td>11.4±3.2</td>
<td>10.7±2.2</td>
<td>11.3±1.9</td>
</tr>
<tr>
<td>Free cholesterol*</td>
<td>8.1±0.4</td>
<td>8.4±0.5</td>
<td>8.3±0.7</td>
</tr>
<tr>
<td>Cholesteryl ester*</td>
<td>38.0±2.7</td>
<td>36.5±1.9</td>
<td>37.6±3.1</td>
</tr>
<tr>
<td>Phospholipid*</td>
<td>21.3±0.8</td>
<td>20.7±0.5</td>
<td>21.0±1.4</td>
</tr>
<tr>
<td>Protein*</td>
<td>21.2±1.0</td>
<td>23.7±1.1</td>
<td>21.8±1.9</td>
</tr>
<tr>
<td>α-Tocopherol/LDL protein, µg/mg</td>
<td>13.1±1.3</td>
<td>11.0±0.3</td>
<td>12.3±2.5</td>
</tr>
<tr>
<td>Total PUFAs/LDL protein, mg/mg</td>
<td>588±44</td>
<td>491±22</td>
<td>740±116†</td>
</tr>
<tr>
<td>α-Tocopherol/PUFAs, µg/mg</td>
<td>22±3</td>
<td>22±3</td>
<td>17±5</td>
</tr>
<tr>
<td>18:2/18:1</td>
<td>1.7±0.2</td>
<td>1.02±0.1</td>
<td>4.1±1.1</td>
</tr>
</tbody>
</table>

LDL indicates low-density lipoprotein; PUFAs, polyunsaturated fatty acids (18:2+20:4). Total LDL mass in milligrams per milliliter of plasma values are as follows: standard diet, 1.14±0.49; oleate diet, 0.73±0.43; and linoleate diet, 0.81±0.45.

Discussion

Our study was designed to answer three questions: (1) Does the enrichment of LDL in both α-tocopherol and...
oleate lead to a greater degree of protection against in vitro oxidation than does \( \alpha \)-tocopherol supplementation alone? (2) Does enrichment of LDL in linoleate increase LDL susceptibility to oxidation despite concurrent supplementation with \( \alpha \)-tocopherol? (3) Do the effects of diet and \( \alpha \)-tocopherol supplementation differ among LDL density subfractions?

Considering first the effect of these interventions on unfractionated LDL, several points are demonstrated. LDL enriched in both \( \alpha \)-tocopherol and oleic acid was not more resistant to oxidation than LDL enriched in \( \alpha \)-tocopherol alone. This suggests that for \( \alpha \)-tocopherol–enriched LDL, there is no additional benefit (as measured by our in vitro assays of oxidation) of enrichment with oleic acid. However, it is possible that in subjects who take lower doses of \( \alpha \)-tocopherol, diets enriched in oleic acids may still further reduce the susceptibility of LDL to oxidation.

In contrast, LDL isolated from the linoleate diet group was oxidized more rapidly and had more extensive fatty acid decomposition despite a similarly high content of \( \alpha \)-tocopherol. Thus, by providing LDL with more substrate for oxidation, a diet sufficiently enriched in linoleic fatty acids can overcome the antioxidant effects of high dosages of \( \alpha \)-tocopherol. It also follows that at lower levels of \( \alpha \)-tocopherol supplementation, a diet enriched in polyunsaturated fatty acids may increase the susceptibility of LDL to oxidation even more.

Buoyant LDLs (from all diet groups) were markedly resistant to oxidation, and there was no evidence of an accelerated oxidation phase (propagation) during the 6 hours of monitored conjugated-diene formation. This did not appear to be due to prior oxidation, because the baseline optical density values were similar to those of dense LDL. Furthermore, exposure of buoyant LDL to more harsh oxidizing conditions did lead to oxidation (data not shown). In contrast, oxidation of dense LDL occurred within the 6-hour measurement period for samples from all diet groups. Although the basis of these differences in rates of oxidation between buoyant and dense LDL is not clear, there are several striking differences in composition between the two fractions. The average content of \( \alpha \)-tocopherol per dense LDL particle was less than half that in buoyant LDL, and the 18:2 to 18:1 ratio was significantly higher. Both factors correlated well with the rate and extent of dense LDL.

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**Figures**

**Fig 5.** Bar graphs showing fatty acid content of low-density lipoprotein (LDL) subfractions. LDL lipids were extracted, and amounts of 18:1, 18:2, and 20:4 were measured as described in “Methods.” Data represent the mean±SD values from all buoyant (A) and dense (B) LDL samples as a percent of total LDL fatty acid content for the standard (•), oleate (a), and linoleate (e) diet groups. *Significant difference compared with values for the standard-diet group at \( P < .05 \).

**Fig 6.** Line plots showing measurement of conjugated-diene formation in low-density lipoprotein (LDL) subfractions during copper-mediated oxidation. LDL (100 \( \mu \)g/mL) was incubated in phosphate-buffered saline medium containing 5 \( \mu \)mol/L \( \text{Cu}^{2+} \), and absorbance at 234 nm was measured at 1-minute (min) intervals in a UVikon 810 spectrophotometer. In A, buoyant (c) and dense (e) fractions from all three diet groups are combined. In B, dense LDL fractions from the standard (•), oleate (a), and linoleate (e) diet groups are shown separately. Values are mean±SD.
conjugated-diene formation. Although differences between LDL subfractions in the content of vitamin E per particle have been noted before,26-28 the presence of similar vitamin E to lipid ratios in these LDL fractions has led some investigators to minimize the importance of the reduced absolute content of vitamin E. However, the absolute content of vitamin E per LDL particle may be relevant to the predominate LDL subclasses'29 susceptibility to oxidation, because there may be a threshold level of vitamin E particles necessary to substantially retard LDL oxidation.41 It is also possible that there were differences between LDL subfractions that we did not measure, such as the content of carbohydrate side chains or other antioxidants that may account for differences in the susceptibility of buoyant and dense LDL to oxidation. In particular, because the standard group maintained their usual diets, their LDL subfractions may have varied from those of the linoleate and oleate diet groups in constituents other than fatty acids. Variations in oxidation rates of LDL subfractions have also been previously attributed to differences in protein or free cholesterol content.16 Although this was not evident in our study, possibly as a result of our manipulations of the participants' diets and α-tocopherol intake.

Particularly striking were the differences in the rate and extent of oxidation of dense LDL among the three diet groups. Most susceptible to oxidation was linoleate-enriched dense LDL, followed by dense LDL from the standard-diet group, whereas dense LDL from the oleate diet group was least susceptible. Although dense LDL from all diet groups had comparable α-tocopherol levels, the content of 18:1 and 18:2 differed markedly, as did the α-tocopherol to 18:2 and α-tocopherol to 18:1 ratios. The α-tocopherol to 18:2 ratio was lowest in the linoleate diet group and highest in the oleate diet group, and this ratio accounted for nearly 65% of the variance (r² = .64) in maximal conjugated-diene formation that occurred in all dense LDL samples. Although other factors not measured in this study may contribute to LDL susceptibility to oxidation, these results indicate that the contents of linoleic and oleic acids in dense LDL are important determinants of its oxidation.

Several results from this study deserve further comment. In subjects who take a high dose of α-tocopherol, diet enrichment with linoleic or oleic fatty acid only modestly influences oxidation of unfractonated LDL (d = 1.019 to 1.063 g/mL). In contrast, diet enrichment with these fatty acids reduces the susceptibility of dense LDL to oxidation. This suggests that buoyant LDL particles and intermediate-density LDL particles (d = 1.032 to 1.040 g/mL, which were not isolated in this study) in the incubation mixture may influence measurement of the rate and extent of formation of conjugated dienes in smaller, more dense LDL. Our findings may simply reflect the fact that in our subjects a large portion of the plasma LDL was relatively more buoyant, and hence the oxidation of dense LDL particles was overshadowed. This result is consistent with that of a previous study, in which we found that the rate of oxidation of unfractonated LDL was correlated with the diameter of the predominate LDL subclass.29

The results of this study have important implications. Several epidemiological studies have shown LDL of greater density to be associated with an increased risk for coronary artery disease. Austin et al23 demonstrated that for men and women there was overall a threefold higher risk for coronary artery disease in those with increased amounts of dense LDL. In a study of Framingham subjects, individuals with coronary artery disease had a predominance of dense LDL in their plasma compared with control subjects.44 Similar findings were reported in a study by Crouse et al.45 Greater intake by artery wall macrophages of dense LDL as a result of enhanced susceptibility to oxidation is one potential explanation for these findings,26-28 although both in vitro and animal studies have suggested additional mechanisms. Stender and Zilversmit46 and Nordestgaard and Zilversmit47 have demonstrated that entry of lipoproteins into the artery wall is size dependent, with smaller particles, such as dense LDL, gaining entry more readily. Additionally, the sialic acid content of lipoproteins may influence their interactions with artery wall proteoglycans,48 with reduced sialic acid content being associated with increased avidity of proteoglycan binding. Small, dense LDL has less total carbohydrate and a lower sialic acid content than larger LDL particles.48 These properties are probably important, because oxidation of dense LDL is unlikely to occur in plasma because of abundant plasma antioxidants. On the other hand, if dense LDL enters the arterial wall more readily and/or is retained preferentially within the intima,48,49 the ease of oxidation of dense LDL may be critically relevant. For these reasons, therapies directed toward reducing LDL oxidation should focus on dense LDL in particular. The current results suggest that diets enriched in monounsaturated fatty acids alone or in combination with antioxidants such as vitamin E may be a particularly safe and effective method of achieving this goal.

Previous diet studies have in general used specially prepared liquid diets to alter the LDL fatty acid composition.16-19 While these studies have helped demonstrate the feasibility of altering LDL susceptibility to oxidation through dietary manipulations, the specific diets are not practical for general use. The current study demonstrates that standard baked goods and other food items can be sufficiently enriched in oleate to create a more practical, palatable diet that will effectively increase the 18:1 content of LDL. The amount of total calories per day that must be replaced by monounsaturated fatty acids to reduce LDL susceptibility to oxidation may be less than previously thought. Abbey et al.19 have recently shown that diets with less than 20% of total daily calories enriched in monounsaturated fatty acids or polyunsaturated fatty acids may influence LDL susceptibility to oxidation. With the marked effect that linoleate- or oleate-enriched diets appear to have on the oxidation of dense LDL, it is conceivable that even more modest changes in dietary fatty acids will influence atherogenesis.

In summary, whereas diet-induced differences in susceptibility of unfractonated LDL (representing a mixture of buoyant, intermediate, and dense LDL) to oxidation were small when large supplements of vitamin E were given concurrently, the effect on the susceptibility of dense LDL to oxidation was substantial. Interventions designed to reduce lipoprotein oxidation should also assess differences in efficacy between LDL subfractions. α-Tocopherol supplementation alone, even at
high doses, did not prevent dense LDL from undergoing oxidation. In contrast, oleate-enriched diets decreased the extent of oxidation of dense LDL and therefore may be an effective method of reducing oxidation of this subtraction. This may be particularly relevant for individuals with familial combined hyperlipidemia, hypertriglyceridemia, or diabetes, who have an increased amount of dense LDL.\textsuperscript{28-30,51} Individuals with this LDL pattern, also called the type B phenotype,\textsuperscript{44} appear to be at greater risk for coronary artery disease and may benefit most from therapy that can specifically reduce the oxidation of dense LDL.

Acknowledgments

This work was supported by grant HL-14197 from the National Heart, Lung, and Blood Institute (SCOR); GCRC Program MO1 RR00827 of the National Center for Research Resources, National Institutes of Health; and the American Philosophical Society. Part of this work was conducted at the Lawrence Berkeley Laboratory through the US Department of Energy under contract No. DE-AC03-76SF00098. We thank Dr Joseph Witzum for his advice and comments regarding the manuscript; Haven Webb, Joellen Barnett, Elizabeth Miller, Pat Blanche, and Laura Holt for important assistance in the conduct of these studies; and Annie Durning-Canty, Eva Brzezinski, Carol Walsh, and Ann Lake from the GCRC Nutrition Unit for excellent assistance in diet preparation and nutrient analysis. We also thank SVO Enterprises for supplying Trisun 80 oil and for additional financial support; Protein Technologies International for providing soy protein; and Hoffmann-La Roche, Inc, for additional financial support; Protein Technologies International for providing soy protein; and Hoffmann-La Roche, Inc, for additional financial support.

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Effects of linoleate-enriched and oleate-enriched diets in combination with alpha-tocopherol on the susceptibility of LDL and LDL subfractions to oxidative modification in humans.

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Arterioscler Thromb Vasc Biol. 1994;14:557-566
doi: 10.1161/01.ATV.14.4.557

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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