LDL Isolated From Greek Subjects on a Typical Diet or From American Subjects on an Oleate-Supplemented Diet Induces Less Monocyte Chemotaxis and Adhesion When Exposed to Oxidative Stress

Sotirios Tsimikas, Athena Philis-Tsimikas, Sophoclis Alexopoulos, Farhad Sigari, Christian Lee, Peter D. Reaven

Abstract—The mechanisms underlying the cardiovascular benefits of Mediterranean-style diets are not fully understood. The high content of monounsaturated fatty acids in Mediterranean-style diets derived from oleate-rich olive oil may be beneficial in reducing low density lipoprotein (LDL) oxidation and its subsequent development of atherogenic properties. This study sought to assess the proinflammatory potential of LDL isolated from subjects consuming a diet naturally rich in olive oil. LDL was isolated from 18 Greek, 18 American, and 11 Greek-Americans subjects, all of whom were living in the United States. Fatty acid composition and vitamin E levels of LDL were determined, as was the extent of copper-mediated LDL oxidation. LDL was also mildly oxidized by exposure to fibroblasts overexpressing 15-lipoxygenase and tested in vitro for bioactivity by determining its ability to stimulate monocyte chemotaxis and adhesion to endothelial cells. To confirm that dietary fatty acids influence the proinflammatory properties of mildly oxidized LDL, LDL was also isolated from 13 healthy American subjects after consumption of an 8-week liquid diet supplemented with either oleic (n=6) or linoleic (n=7) acid and tested for bioactivity in a similar fashion. There were no differences in the baseline lipid profiles among the Greeks, Americans, or Greek-Americans. Oleic acid content in LDL was 20% higher in the Greek compared with the American or Greek-American subjects (P<0.001). The extent of in vitro LDL oxidation, measured by conjugated diene formation, was lower in the Greek subjects (P=0.02), but there was no difference in the lag time. Induction of monocyte chemotaxis and adhesion by mildly oxidized LDL was decreased by 42% in the Greek group compared with the American subjects (P<0.001). There was an inverse correlation between the oleic acid content of LDL and stimulation of monocyte chemotaxis (r=-0.64, P<0.001) and a positive correlation between the polyunsaturated fatty acid content of LDL (total linoleate and arachidonic acids levels in LDL) and stimulation of monocyte chemotaxis (r=0.51, P<0.01) in the entire cohort. There were no differences in LDL vitamin E content between the groups. In the liquid-diet groups, the oleic acid–supplemented group had a 113% higher oleic acid content in LDL and a 46% lower linoleic acid content in LDL than the linoleate-supplemented group (P<0.001), whereas the vitamin E content in LDL was equal in both groups. When exposed to oxidative stress, the LDL enriched in oleic acid promoted less monocyte chemotaxis (52% lower) and reduced monocyte adhesion by 77% in comparison with linoleate-enriched LDL (P<0.001). There was a strong, negative correlation between oleic acid content of LDL and monocyte adhesion (r=-0.73, P<0.001) and a strong, positive correlation between polyunsaturated fatty acid content of LDL and monocyte adhesion (r=0.87, P<0.001). This study demonstrates that dietary enrichment of LDL with oleic acid is realistic and readily achieved by using diets currently in use in Mediterranean countries. In addition, these data suggest that LDL enriched with oleic acid and reduced in polyunsaturated fatty acids may be less easily converted to a proinflammatory, minimally modified LDL. (Arterioscler Thromb Vasc Biol. 1999;19:122-130.)

Key Words: diet ■ lipoproteins ■ arteriosclerosis ■ lipids ■ LDL oxidation

Individuals living in countries with a high consumption of cholesterol and saturated fats have higher levels of plasma LDL cholesterol and a higher incidence of coronary heart disease.1-3 This relationship between dietary intake of cholesterol and saturated fatty acids and the development of atherosclerosis has been well demonstrated in animal and human studies.1-6 Individuals living in Mediterranean countries frequently consume diets enriched in monounsaturated fats, which is the main feature of the Mediterranean diet.7,8 In addition, Mediterranean populations consuming the traditional Mediterranean-style diet have lower levels of plasma LDL cholesterol compared with populations living in high-risk areas.3,9-12 This observation may reflect the different content of cholesterol and fatty acids in Mediterranean and high-risk areas diets.13-15

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fatty acids (MUFAs) derived mainly from olive oil. The incidence of coronary heart disease in these countries is lower than in countries with reduced levels of dietary MUFAs. It has been shown that the replacement of dietary saturated fatty acids with MUFAs decreases plasma LDL levels, which presumably contribute to the lower incidence of coronary artery disease.

However, with a greater understanding of the mechanisms contributing to the development of atherosclerosis, it has become clear that dietary fat composition may also influence the development of atherosclerosis by mechanisms other than lowering plasma cholesterol levels. Several lines of evidence suggest that the oxidation of lipoproteins plays an important role in the early events leading to atherosclerotic lesion formation. When LDL becomes oxidized it takes on a variety of atherogenic properties. For example, when LDL is minimally oxidized (mm-LDL), it is capable of generating foam cells. The susceptibility of LDL to oxidation is influenced by a variety of factors, including its antioxidant and fatty acid content. Polyunsaturated fatty acids (PUFAs) in LDL are readily oxidized, and their replacement by MUFAs appears to reduce LDL oxidation.

Previous studies assessing dietary fat composition and LDL oxidation have been carried out in artificial settings, such as metabolic wards or communal living conditions, and have instituted highly restrictive dietary guidelines. In general, these studies have utilized carefully prepared, liquid-formula or solid diets highly enriched in MUFAs, have usually provided the subjects with all the required foods, and have closely monitored subjects to ensure compliance. These investigations have demonstrated that in comparison with PUFA-enriched diets, feeding MUFA-enriched diets to both normcholesterolemic and hypercholesterolemic subjects reduces LDL susceptibility to oxidation. Similar results were found when MUFA diets were compared with carbohydrate-enriched diets. It is unknown whether a more realistic level of MUFA enrichment will lead to similar results. In this study, we evaluated whether LDL isolated from Greek subjects who were consuming a “typical” unsupplemented Mediterranean-style diet rich in olive oil was more resistant to oxidation and less readily converted to a bioactive mm-LDL.

Methods

Study Subjects

There are 2 studies described in this article. The first study includes 3 groups of subjects. Eighteen Greek subjects were compared with 18 age- and sex-matched American controls and 11 Greek-Americans. The Greek subjects were recruited from 3 towns in northwestern Greece on the Ionian coast (Igoumenitsa, Plataria, and Faskomilia). Approximately half of the subjects have olive groves on their property and produce their own olive oil seasonally; the others consumed olive oil that was produced locally. Although strict dietary records were not obtained, all of the Greek subjects indicated that they used olive oil exclusively for all of their cooking and dietary needs that required oil. The parents of all Greek-American subjects were of Greek origin. Three subjects were born in Greece and had lived in the United States for >15 years. The others were either first- (n=5) or second- (n=3) generation Greek-Americans. They were recruited into the study to assess for possible ethnic differences in LDL susceptibility to oxidation. The American subjects had lived in southern California for most of their lives and were consuming a typical American diet.

The second study included 13 subjects from the United States who had participated in a previous trial testing the effects of dietary modifications on the susceptibility of LDL to oxidation. These healthy, mildly hypercholesterolemic subjects consumed a liquid diet enriched in either oleate (n=6) or linoleate (n=7) as their main source of nutrition for 8 weeks. The liquid diets were prepared according to the method of Mattson and Grundy by using oleate-enriched sunflower oil (Trisun 80) and conventional sunflower oil. The details of this study protocol have been described previously.

Plasma samples prepared from these subjects at the end of the 8-week study had been frozen at −70°C for 5 years before being thawed and used in the current study. LDL was isolated from frozen samples, fatty acid contents of LDL and LDL vitamin E levels were measured, and the LDL was subsequently used in the monocyte chemotaxis and adhesion assays as described below. None of the data presented in this article have been published previously.

Subjects from both studies were without acute or chronic medical conditions, and none were taking lipid-lowering medications or antioxidant supplements. These studies were approved by the Human Studies Committee of the University of California, San Diego, and were conducted in part at the outpatient facilities of the University of California, San Diego, General Clinical Research Center.

Preparation of LDL

Fasting blood samples were obtained from each subject into tubes containing EDTA (4.0 mmol/L) and placed immediately on ice, and the plasma was then separated. A final concentration of 0.22 mmol/L gentamicin, 0.15 mmol/L chloramphenicol, and 1 μmol/L 3-phenylalanyl-L-propyl-L-arginine chloromethyl ketone was added to all plasma samples and all solutions used subsequently during LDL isolation. The Greek plasma samples were sealed in an airtight container and shipped on ice to the United States within 24 hours. All plasma samples were frozen at −70°C in sucrose (17.5 μmol/L final concentration) to cryopreserve the LDL as described previously.

Plasma samples from each group were then thawed simultaneously, and LDL was isolated by sequential ultracentrifugation as previously described and dialyzed extensively against PBS containing 0.27 mmol/L EDTA (PBS-EDTA). Plasma total cholesterol, LDL cholesterol, triglycerides, and HDL were measured by enzymatic assays on an automated Abbott VP Super System bichromatic analyzer. Measurement of LDL conjugated-diene formation during copper-mediated oxidation was carried out immediately after isolation, as described below. LDL samples were subsequently stored at 4°C in the dark, and all other studies were completed within 1 week of isolation.

Fatty Acid Composition

Lipids from LDL were extracted by a modification of the method of Folch et al. The fatty acids were trans-methylated and analyzed in a Varian gas chromatograph model 3700 equipped with a column of 10% Silab 5CP on a Gas Chrom QII, 100/120 mesh. A 15:0 internal standard (pentadecanoic acid) was added to each sample before extraction, and calculations of fatty acid amounts were determined from peak area ratios of sample to internal standard.

Vitamin E Content

α-Tocopherol was measured by high-performance liquid chromatography as described previously. α-Tocopherol acetate was prepared in 100% ethanol and used as an extraction internal standard and for standard-curve preparation. Actual concentrations of α-tocopherol...
were determined by measuring the absorbance of prepared solutions and calculating the concentrations based on known spectral data. Calculations were determined from a standard curve of peak area ratios of sample to internal standard.

**Oxidation of LDL**

LDL oxidation was assessed as both the lag time before initiation of rapid oxidation of LDL particles and as the extent of conjugated-diene formation. Seventy-five micrograms of LDL protein was incubated with 2.5 μmol/L CuSO₄ in 1 mL of PBS at 30°C, and the absorbance at 234 nm was measured continuously in a Uvikon 810 spectrophotometer as described.⁴² For data presentation, the first derivative of the rapid phase of oxidation was calculated (slope), and its intercept with the x-axis (lag time) determined; OD at 234 nm was then determined for assessment of maximal conjugated-diene formation. In some experiments, lipid peroxidation was also assessed by determining the formation of thiobarbituric acid–reactive substances (TBARS) in the samples, as described by Yagi.²⁹

**Cell Culture Procedures**

Murine fibroblasts expressing high levels of intracellular 15-lipoxygenase (15-LO) were established by infection with a retroviral vector as previously described.³⁰ Cells were grown in Dulbecco’s modified essential medium with high glucose, containing 10% FCS and G418 sulfoxide, 30% serum, and 60% RPMI-1640 medium. The monocytes were then frozen at −80°C, nonadherent cells were washed off, leaving a highly enriched monocyte preparation. The remaining adherent cells were then thawed free of serum, and LDL (250 μg/mL) was then incubated with the fibroblasts at 37°C for 20 hours in Hams F-10 medium to induce mild oxidation and generate mm-LDL,³¹ which was subsequently used in the monocyte chemotaxis and adhesion assays described below.

**Monocyte Chemotaxis Assay**

Human monocytes were isolated by a modification of the method of Boyum.³² In brief, mononuclear cells were recovered from the interface formed during ultracentrifugation of the white blood cell pellet on a Histopaque gradient. Cells were then washed in PBS containing 0.02% EDTA-PBS, resuspended in autologous serum (10%) and RPMI-1640 medium, and plated. After a 2-hour incubation at 37°C, nonadherent cells were washed off, leaving a highly enriched monocyte preparation. The remaining adherent cells were then gently released by using 0.2% EDTA-PBS in 10% dimethyl sulfoxide, 30% serum, and 60% RPMI-1640 medium. The monocytes were then frozen at −70°C and stored in LN₂ until use for subsequent monocyte chemotaxis assays. Chemotaxis assays were performed in Neuroprobe chambers with a polycarbonate filter (Poretics) of 5-μm-pore size separating the upper and lower chambers. Approximately 100,000 monocytes were added to each upper well, the lower wells were filled with 28 μL of supernatant (1:20, in 0.1% BSA–Tyrode’s salt solution) from the fibroblast incubation experiments, and the chambers were treated as previously described by Berliner et al.³³ The number of monocytes per high-power field that had migrated to the lower surface of the filter was then counted by using a light microscope.

**Monocyte Adhesion Assay**

The assay used was a modification of the method of Navab et al.³⁴ Native LDL or LDL conditioned by incubation with fibroblasts in F-10 medium for 20 hours was transferred to confluent, porcine aortic endothelial monolayers in 96-well tissue culture plates. After a 4-hour incubation at 37°C, the supernatant was removed and the endothelial monolayers washed twice with RPMI 1640. THP-1 cells (a monocyte-like cell line) were placed on the endothelial cells at 40,000 cells per well, and the plates were incubated for 20 minutes at 37°C. The suspension was removed, and the cells were vigorously washed 3 times to remove all but the firmly adherent THP-1 cells. The number of adherent THP-1 cells was determined in 4 high-power fields per well, and the results of 6 to 8 separate wells were averaged for each experiment.

To determine whether LDL isolated from frozen plasma yielded results similar to those of LDL isolated from fresh plasma in our monocyte chemotaxis and THP-1 cell adhesion assays, we performed the following experiments. Plasma aliquots from 2 individuals were either kept in the dark at 4°C or frozen at −70°C for 3 days. LDL was then isolated from each sample and conditioned on the 15-LO fibroblasts as described above. The level of chemotactic activity and THP-1 cell adhesion stimulated by the cell-conditioned LDL was nearly identical to that for LDL prepared from fresh or thawed plasma.

**Statistical Analysis**

Initial analyses confirmed that the data were normally distributed. One-way ANOVA was used to compare values between multiple groups, and post hoc comparisons between specific groups were performed by Student’s unpaired t test. Data are presented as mean±SE.

**Fatty Acid Composition of Olive Oil**

Greek olive oil that is typical of that produced along the northwestern Ionian coast region was tested for its fatty acid content. It contained 73% MUFAs, essentially all in the form of oleic acid, 13% PUFAs, and 14% saturated fatty acids. The fatty acid composition is comparable to other commercially available olive oils.³⁵

**Fatty Acid Content of LDL**

The fatty acid composition of LDL was determined for all patients and is shown in Table 2. The mean oleic acid content in LDL was 20% higher in the Greek subjects than in the Americans or Greek-Americans. (Oleic acid content represented 24.2% of the total fatty acid content of LDL in the Greek subjects compared with 19.6% in the Americans and 19.5% in the Greek-Americans, P<0.001.) Correspondingly, total measured PUFAs in LDL were lower in the Greek subjects. There was also a small but significant increase in palmitic acid content in the Greek subjects compared with that in the Greek-Americans only, but there was no significant difference in palmitoleate or stearic acid content. These

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**TABLE 1. Age, Sex, Lipid, and Lipoprotein Levels**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Greeks</th>
<th>Americans</th>
<th>Greek-Americans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>18</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>Male/female</td>
<td>6/12</td>
<td>6/12</td>
<td>4/7</td>
</tr>
<tr>
<td>Age, y</td>
<td>59±9</td>
<td>57±9</td>
<td>46±18</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.6±1.3</td>
<td>5.7±1.3</td>
<td>5.2±1.6</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.5±0.8</td>
<td>3.6±0.9</td>
<td>3.3±1.0</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.5±0.3</td>
<td>1.5±0.4</td>
<td>1.4±0.4</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.3±0.3</td>
<td>1.2±0.3</td>
<td>1.1±0.3</td>
</tr>
</tbody>
</table>

Baseline characteristics of the subjects participating in the natural-diet study are shown. The Greek-Americans were slightly younger as a group but did not differ from the Greeks or Americans in their measured lipid parameters. Data presented are mean±SE. There were no significant differences between the groups.
differences in the LDL fatty acid content reflected the higher content of oleic acid and the lower content of PUFAs in Greek olive oil. The ratio of 18:1 to 18:2 in LDL was higher in the Greek subjects, at 0.71, versus 0.52 in the Americans and 0.51 in the Greek-Americans (P<0.001 by ANOVA), confirming an overall proportional rise in MUFA content. When linoleic and arachidonic acid contents in LDL were combined as a measure of the total PUFA content, there was still a higher ratio of MUFAs to PUFAs in the Greek subjects (0.58 in the Greeks) versus 0.41 in the Americans and 0.40 in the Greek-Americans (P<0.001 by ANOVA).

**LDL Vitamin E Levels**

There was no significant difference in the LDL vitamin E levels among the Greek, American, and Greek-American subjects (Greeks, 5.6±0.3 μg/mg LDL protein versus American, 5.2±0.2 μg/mg LDL protein, versus Greek-Americans, 5.9±0.4 μg/mg LDL protein).

**LDL Oxidation**

There were no significant differences in lag times between the 3 groups (102±20 minutes for the Greeks, 106±22 for the Americans, and 105±22 for the Greek-Americans, P=0.6). The extent of LDL oxidation was measured by the peak OD at 234 nm of generated conjugated dienes (which primarily reflect the total substrate available for oxidation) during oxidation of LDL. There was a significant difference among the groups as measured by ANOVA (P<0.02). There was a significantly lower amount of conjugated dienes generated in LDL from Greek subjects compared with LDL from the Greek-Americans (peak OD of 1.29±0.03 versus 1.46±0.05, P<0.008), but no significant difference was present between the Greek and American subjects (peak OD of 1.29±0.03 versus 1.34±0.05, P=0.20). Because there were no significant differences in fatty acid composition, vitamin E levels, or susceptibility to oxidation between LDL samples from the Americans and Greek-Americans, these 2 American groups were combined for further analysis of copper-mediated LDL oxidation. The combined American and Greek-American group had a significantly higher peak OD value, consistent with a greater extent of oxidation, than did the Greek subjects (peak OD of 1.29±0.03 versus 1.39±0.05, P=0.017).

Figure 1A shows that there was a moderately strong correlation between the 18:2 content and the peak OD (r=0.63, P=0.005) and a trend toward a negative correlation between 18:1 and peak OD (r=−0.32, P=0.11) in the Greek subjects. Similar results were noted in the combined American and Greek-American group in Figure 1B (18:2 and peak OD, r=0.53, P=0.003; and 18:1 and peak OD r=−0.35, P=0.07). When all subjects were combined there was a strong, positive correlation between the 18:2 content and the peak OD (r=0.60, P<0.001) and a strong, negative correlation between 18:1 content and peak OD (r=−0.44, P=0.002).

**Table 2. Fatty Acid Composition of LDL**

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Natural-Diet Subjects</th>
<th>Liquid-Diet Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Greeks (n=18)</td>
<td>Americans (n=18)</td>
</tr>
<tr>
<td>Palmitic (16:0)</td>
<td>24.8±0.4*</td>
<td>23.9±0.5</td>
</tr>
<tr>
<td>Palmitylaidic (16:1)</td>
<td>2.6±0.4</td>
<td>3.2±0.2</td>
</tr>
<tr>
<td>Stearic (18:0)</td>
<td>8.8±0.4</td>
<td>8.3±0.3</td>
</tr>
<tr>
<td>Oleic (18:1)</td>
<td>24.2±0.7†</td>
<td>19.6±0.5</td>
</tr>
<tr>
<td>Linoleic (18:2)</td>
<td>34.0±1.0†</td>
<td>38.0±1.2</td>
</tr>
<tr>
<td>Arachidonic (20:4)</td>
<td>7.6±0.4†</td>
<td>9.4±0.4</td>
</tr>
<tr>
<td></td>
<td>Greeks-Americans (n=11)</td>
<td>Americans (n=6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Greek-Americans (n=7)</td>
</tr>
<tr>
<td>Palmitic (16:0)</td>
<td>22.4±0.4</td>
<td>22.0±1.3</td>
</tr>
<tr>
<td>Palmitylaidic (16:1)</td>
<td>2.6±0.1</td>
<td>...</td>
</tr>
<tr>
<td>Stearic (18:0)</td>
<td>8.2±0.2</td>
<td>7.3±0.5</td>
</tr>
<tr>
<td>Oleic (18:1)</td>
<td>19.5±0.8</td>
<td>8.3±0.3</td>
</tr>
<tr>
<td>Linoleic (18:2)</td>
<td>38.3±1.6</td>
<td>35.6±1.6†</td>
</tr>
<tr>
<td>Arachidonic (20:4)</td>
<td>10.4±0.4</td>
<td>9.4±0.4</td>
</tr>
</tbody>
</table>

Fatty acid composition of LDL from the natural diet groups (American and Greek subjects) and from the liquid diet group (oleate and linoleate groups) are shown. Data are presented as the percent of total fatty acids in LDL and represent the mean±SE.

*P<0.01, Greeks compared with Greek-Americans.
†P<0.001, Greeks compared with Americans and Greek-Americans.
‡P<0.001, oleate- versus linoleate-enriched diets.

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**Figure 1. Scatterplot showing the correlation between the LDL fatty acid content and the extent of conjugated-diene formation in Greek subjects (A) and the combined Greek-American and American subjects (B).**
Monocyte Chemotaxis and Adhesion Assays

Monocyte chemotactic activity induced by mild oxidation of LDL by fibroblasts overexpressing 15-LO was assessed in LDL from 10 Greek subjects and 10 age- and sex-matched American LDL samples. (Greek-American LDL samples were not studied.) We have previously shown that incubation of LDL with fibroblasts overexpressing 15-LO, but not with control cells, leads to the generation of mm-LDL that stimulates monocyte chemotactic activity and monocyte adhesion to endothelial cells. This process has been shown to be dependent on lipoprotein oxidation, because the presence of antioxidants, such as vitamin E and probucol, or transition-metal chelators inhibit the generation of bioactivity. Although many lines of evidence suggest that 15-LO may be an important contributor to oxidation of lipoproteins in vivo, there is still controversy regarding this issue. In this study, the 15-LO fibroblasts were being used primarily as a reliable system for generating a mildly oxidized and bioactive lipoprotein.

After modification by 15-LO cells, LDL from Greek subjects induced 42% less monocyte chemotactic activity compared with LDL from American subjects (P < 0.001, Figure 2). Consistent with previously published studies, development of chemotactic activity required conditioning on 15-LO cells, because neither LDL conditioned in medium alone nor “native” LDL samples (tested in a subset of 5 subjects per group) demonstrated significant chemotactic activity (data not shown). When values from all 20 subjects were combined, there was a strong, negative correlation between 18:1 levels and monocyte chemotactic activity (r = −0.64, P < 0.001, Figure 3). There was also a positive correlation between the content of 20:4 (r = 0.62, P = 0.003) and 18:2 (r = 0.36, P = 0.11) and monocyte chemotactic activity, as well as between total PUFAs (18:2 and 20:4) in LDL and the increase in monocyte chemotactic activity (r = 0.51, P < 0.01, Figure 3). Lipid peroxidation as measured by the TBA assay on 15-LO–conditioned LDL samples was lower in the Greek group, but this difference was not statistically significant (10.8 ± 0.4 nmol/mg LDL protein in the Greeks versus 12.0 ± 0.8 nmol/mg LDL protein in the Americans, P = 0.25). In a subset of subjects (5 subjects per group), we also tested the ability of modified LDL to stimulate THP-1 cell adhesion to endothelial cells. LDL isolated from Greek subjects and incubated on 15-LO cells stimulated significantly less THP-1 cell adhesion to endothelial cells than did conditioned LDL from American subjects (21.7 ± 12.2 versus 45.0 ± 7.3 THP-1 cells per field, P = 0.01). LDL samples from both diet groups that were conditioned in medium alone induced only low levels of THP-1 cell adhesion that were not significantly different between groups.

To confirm that the high content of dietary oleic acid and lower content of PUFAs but not other components in the Mediterranean diet may be directly responsible for the decreased bioactivity of LDL, we studied LDL samples from 13 subjects fed oleic or linoleic acid–enriched diets that were prepared by the Clinical Research Nutrition Unit at the University of California, San Diego. The composition of these diets has been described previously. The oleic acid content of LDL from oleate-supplemented subjects was 113% higher than in LDL from the linoleate-supplemented subjects (25.6% of the total fatty acid content in LDL versus 12.0%, respectively, P < 0.001). In contrast, the linoleate content in LDL from the linoleate-supplemented subjects was 46% higher than that in LDL from the oleate-supplemented subjects (52.1% versus 35.6%, P < 0.001, Table 2). Of note, the fatty acid composition of these LDL samples was quite similar to that originally measured on LDL isolated from these same plasma samples 5 years previously. In addition, the fatty acid composition of LDL from subjects fed liquid-formula diets enriched in oleic acid was very similar to that of LDL from Greek subjects eating a diet naturally enriched in olive oil (Table 2). The vitamin E content in LDL was not significantly different between the oleate- and linoleate-supplemented groups.

After incubation on 15-LO cells, the conditioned LDL samples were tested for their ability to induce monocyte chemotaxis and THP-1 cell binding to endothelial cells. The monocyte chemotactic activity of oleate-enriched LDL was 52% lower (P < 0.001) than that of linoleate-enriched LDL (Figure 4A). Similarly, after conditioning on 15-LO cells, oleate-enriched LDL reduced THP-1 cell adhesion to endothelial cells by 77% (P < 0.001) compared with linoleate LDL (Figure 4B). There was a strong, positive correlation between the content of PUFAs (18:2 and 20:4) in LDL and the
increase in monocyte chemotaxis ($r=0.56$, $P<0.001$) as well as a negative correlation between 18:1 levels and monocyte chemotaxis ($r=-0.45$, $P<0.001$). A strong, negative correlation was also noted between 18:1 levels in LDL and THP-1 cell adhesion ($r=0.73$, $P<0.001$), and a positive correlation was present between PUFAs (18:2 and 20:4) and THP-1 cell adhesion ($r=0.87$, $P<0.001$, Figure 5A and 5B, respectively). Measures of lipid peroxidation (TBARS) on 15-LO–conditioned LDL samples were not significantly different between groups (12.6±0.9 nmol/mg LDL protein in the oleate-supplemented subjects versus 14.2±0.8 in the linolate-supplemented subjects, $P=NS$).

**Discussion**

The reduced risk of cardiovascular disease in Mediterranean countries has been well documented. Although lower cholesterol levels may be partially responsible for this finding, other aspects of the Mediterranean diet or lifestyle may contribute. Given the important role that lipoprotein oxidation may play in the development of atherosclerosis, it is conceivable that dietary factors that reduce this process may be important. The results of this study suggest that the Mediterranean diet is sufficiently enriched in 18:1 and reduced in 18:2 compared with the American diet to reduce LDL oxidation and its induction of monocyte chemotaxis and monocyte adhesion. It has been suggested that other components in the Mediterranean diet, eg, nonsaponifiable fats in olive oil, may contain antioxidant activity. Although we cannot rule out the possibility that compounds such as oleuropein and other antioxidants that have been detected in small amounts in olive oil are responsible for the reduced oxidation of LDL in Greeks, there are several pieces of evidence that argue against this possibility. First, these study results are quite consistent with findings from several previous dietary trials comparing MUFA-enriched diets to PUFA- or carbohydrate-rich diets in their ability to reduce LDL oxidation. In many of these studies, enrichment of LDL with oleic acid was achieved through use of fats and oils other than olive oil, yet LDL was still less extensively oxidized when exposed to copper sulfate or other oxidant stress. Of relevance, the Greek individuals in this study had levels of oleic acid enrichment in LDL that, on average, approached that of LDL isolated from individuals from these dietary trials, pointing out the relevance of results from many of the dietary feeding studies. Second, the extent of LDL oxidation in the current experiments as well as in previously published studies correlates well with the linoleic acid (18:2) fatty acid content of LDL and inversely with oleic acid (18:1) content. Finally, in the second set of studies reported in this article, olive oil was not used;
instead, a similar source of fat calories was used for both groups and essentially identical results were obtained.

Although it is conceivable that there may be other differences aside from the diets, including genetic or other cultural factors, between Greeks and Americans that might explain differences in lipoprotein oxidation, we could find no evidence for this possibility. Greek-Americans living in the United States had values of LDL oxidation similar to those of Americans, and cigarette and medication usage was minimal in all groups.

It has been demonstrated that mm-LDL is quite bioactive and in vitro is capable of stimulating gene expression and synthesis of a variety of proinflammatory proteins in several cell types present in the artery wall. Injection of mm-LDL in animal models indicates that these bioactive effects appear to be maintained in vivo. Therefore, it has been suggested that the generation of mm-LDL may be a very important early step in atherogenesis. Recent studies by several investigators have implicated oxidative decomposition products of lipoprotein phospholipids as possible proinflammatory agents responsible for some of the bioactivity of mm-LDL. In particular, several oxidation products of 1-palmitoyl-2-arachidonoyl-sn-glyero-3-phosphocholine appear capable of stimulating monocyte chemotaxis and transmigration, as well as stimulation of monocyte adhesion to endothelial cells. We have recently shown that oxidation products of other PUFAs such as 18:2, when incorporated into phospholipids in liposomes, may also be proinflammatory. This observation may explain the strong correlation found in the current study between total LDL PUFA content and induction of monocyte chemotaxis by LDL conditioned on 15-LO cells. In this light it is not surprising that 18:1-enriched and 18:2- and 20:4-depleted LDL from Greek subjects was not only less extensively oxidized by exposure to copper but was also significantly less able to stimulate monocyte chemotaxis and adhesion compared with LDL from Americans. This latter effect appeared to be related to the LDL fatty acid composition, as further studies on samples from individuals fed specially prepared diets enriched in 18:1 and depleted in 18:2 revealed strikingly similar results; ie, both monocyte chemotaxis and stimulation of monocyte adhesion to endothelial cells were dramatically reduced. An important difference in these latter studies is that the diets differed only in the quantity of 18:1 or 18:2 in their diets. In addition, as in the first set of studies, bioactivity measurements of monocyte chemotaxis and adhesion were correlated positively with content of 18:2 and 20:4 in LDL and inversely with 18:1. The mechanism by which oleic acid–enriched diets decrease lipoprotein susceptibility to oxidation is presumably related to the resulting decrease in linoleic acid content within lipoproteins. However, recent work from our laboratory demonstrates that liposomes derived from synthetic phospholipids that are progressively enriched in oleic acid while keeping linoleic acid content steady induce less monocyte chemotaxis and adhesion when exposed to mild oxidative stress, suggesting that oleic acid may have an additional independent mechanism of action.

Interestingly, despite having significantly reduced measures of bioactivity, oleic acid–enriched samples had only modestly lower levels of TBARS than did 18:2-enriched LDL. This apparent disassociation between the extent of lipid peroxidation and bioactivity that occurs with mild cell-mediated oxidative stress has been noted previously. However, generation of bioactive LDL is dependent on LDL oxidation, as we have previously demonstrated that lipid oxidation and loss of PUFAs occur and that the addition of antioxidants or transition metal chelators inhibits generation of bioactivity. 15-LO cells may be inducing generation of unique (or greater levels of) bioactive compounds in oxidation-susceptible 18:2-enriched LDLs that are not detected by TBARS measurements. Alternatively, the TBARS assay may be insufficiently sensitive or specific to detect modest but important differences in lipid oxidation products between LDL samples.

Although it has been well demonstrated that replacement of dietary saturated fat with monounsaturated fat has beneficial effects on plasma cholesterol levels, these current studies point out several potential advantages of diets enriched in 18:1 and depleted in 18:2 on lipoprotein oxidation and its biological consequences. These findings may offer additional insight into understanding possible mechanisms underlying the reduced cardiovascular risk in Mediterranean countries. The Seven Countries and the Framingham Heart Studies were crucial in defining the epidemiological association between dietary fat intake, hypercholesterolemia, and atherosclerosis. However, there have been few controlled studies assessing the effect of consuming a Mediterranean-style diet on cardiovascular outcomes. As a whole, these studies have suggested that patients who adhere to a Mediterranean-style diet composed of a higher proportion of legumes, fruits, vegetables, and grains; less red meat; more fish; and a significant reduction in saturated fats replaced by mono-unsaturated or polyunsaturated fats have a reduced incidence of coronary heart events. In particular, a recent clinical trial of the Mediterranean diet conducted in patients with a previous myocardial infarction provides evidence of the ability of these diets to reduce subsequent cardiovascular events. Importantly, this reduction in cardiovascular events was independent of changes in plasma cholesterol levels.

Previous dietary studies have shown that in comparison with PUFA-enriched diets, MUFA-enriched diets result in enrichment of LDL with MFAs that reduce LDL susceptibility to oxidation. The current study of “natural” diets clearly demonstrates that the level of dietary enrichment with oleic acid necessary to obtain these benefits is realistic and readily achieved with diets currently in use in Mediterranean countries. In addition, this study suggests that LDL enriched with 18:1 and depleted in 18:2 and 20:4 may be less readily converted to proinflammatory mm-LDL, which has the ability to enhance early events in atherosclerosis such as monocyte chemotaxis and adhesion.

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Exposed to Oxidative Stress

Oleate-Supplemented Diet Induces Less Monocyte Chemotaxis and Adhesion When LDL Isolated From Greek Subjects on a Typical Diet or From American Subjects on an

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