The oxidative modification of low density lipoprotein (LDL) may provide a crucial link between plasma LDL and the atherosclerotic lesion. The studies presented herein define time-dependent modifications of LDL constituents caused by CuSO₄-catalyzed oxidation. Measurement of the cholesterol content of oxidized LDL by the cholesterol esterase-oxidase assay was found to be inaccurate. The enzymatic assay detected oxysterols as well as cholesterol and thus substantially overestimated the actual cholesterol content. Alteration of electrophoretic mobility and conversion of sterols into oxysterols increased in a parallel, time-dependent manner. Lipid peroxidation, judged by the thiobarbituric acid–reacting substances assay, increased early to maximal values but was not linearly related to either electrophoretic mobility or to oxysterol formation. Neither electrophoretic mobility nor oxysterol formation varied much between repeated oxidative modifications of any given LDL preparation but varied markedly among LDLs from different normolipidemic individuals, suggesting that LDL particles contain some factor conferring susceptibility or resistance to oxidation. Indeed, LDL preparations from different individuals have varying susceptibilities to oxidative modification as evidenced by the three indexes used. The major oxysterol generated was 7-ketocholesterol. Macrophage modification of LDL also resulted in the generation of oxysterols. Thus, measurement of oxysterols may afford an additional index of the oxidative modification of LDL. Since incubation of macrophages with oxidized LDL but not native LDL resulted in the accumulation of oxysterols, this could account for some of the toxic and metabolic effects of oxidized LDL on cells. (Arteriosclerosis and Thrombosis 1991;11:482–488)

Increased plasma low density lipoprotein (LDL) cholesterol concentrations contribute to the development of coronary atherosclerosis (reviewed in Reference 1). Early atherosclerotic lesions contain lipid-laden foam cells derived mainly from macrophages. Such cells apparently cannot accumulate appreciable amounts of cholesterol through the LDL receptor because synthesis of this receptor is cholesterol regulated. Also present on macrophages is another receptor, the scavenger receptor, which recognizes LDL with certain chemical modifications, does not appear to be regulated by the cellular cholesterol content, and thus might be a potential source of excess cholesterol esters within foam cells. Acetyl LDL and malondialdehyde (MDA)-modified LDL are readily taken up by this scavenger receptor, but their presence in the arterial wall remains to be determined. Recently, it has been demonstrated that oxidatively modified (Ox-) LDL also is taken up by the scavenger receptor mechanism. Oxidative modification of LDL can be produced by cells typical of the atherosclerosis lesion, that is, smooth muscle cells, endothelial cells, and macrophages and also by incubation with transition metals. Thus, Ox-LDL may be one form of modified LDL that is recognized by the scavenger receptor.

Most studies of Ox-LDL have employed LDL modified by exposure to transition metals such as iron or copper. LDL treated in this manner becomes a ligand for the scavenger receptor and is different from native LDL in several respects. Ox-LDL has increased electrophoretic mobility and fragmented apoprotein B, hydrolyzed phospholipids, increased lipid peroxide content, increased density, and lowered cholesteryl ester content. Although certain properties and biologic functions of Ox-LDL are reasonably well documented, there is a relative paucity of data on certain aspects such as the susceptibility of different LDLs to oxidative modification and the oxysterol composition of Ox-LDL.

Accordingly, the present study was undertaken to determine the time-dependent changes in LDL com-
ponents after oxidative modification of LDL obtained from 10 normolipidemic subjects. We measured lipid peroxide content, increased negative charge as evidenced by electrophoretic mobility, and lipoprotein sterol oxidation. In these studies, we compared the variation between multiple oxidations of a single LDL preparation with the variation between oxidation of different LDL preparations. We also generalized these findings to LDL modified by macrophages and found that macrophages incubated with Ox-LDL took up oxysterols contained in the lipoprotein.

Methods

Materials

2-Thiobarbituric acid, bovine serum albumin, 1-cysteine, and butylated hydroxytoluene were from Sigma Chemical Co., St. Louis, Mo.; CuSO4 was from Mallinkrodt, Inc., Paris, Ky.; cholesterol, 7-ketocholesterol, 22-hydroxycholesterol, 7-cholestanol, and 7α- and 7β-hydroxycholesterol were from Steroloids, Walton, Mass.; and silica thin-layer chromatography sheets were from Eastman Kodak Inc., Rochester, N.Y. All other chemicals were reagent grade.

Methods

After obtaining informed consent, blood was drawn from 10 normolipidemic, apparently healthy volunteers (seven men and three women between the ages of 24 and 65 years). None of the subjects were taking any dietary antioxidants or were on any drug therapy. Low density lipoprotein (LDL) was isolated by preparative ultracentrifugation from the plasma collected in EDTA (1 mg/ml) from normolipidemic individuals. Isolated LDL was dialyzed against 0.85% NaCl, 0.01% EDTA, pH 7.4, filtered, and stored at 4°C under N2 in the dark and used within 2 weeks. Protein was measured by the method of Lowry et al using bovine serum albumin as the standard. Human monocytes were isolated by density-gradient centrifugation from blood samples derived from fasting normolipidemic subjects. Twenty milliliters of blood (anticoagulated with 10 units/ml heparin) was layered over 15 ml Ficoll-Paque (Sigma Diagnostics, St. Louis, Mo.) and centrifuged at 500g for 30 minutes at 23°C. The mixed mononuclear cell band was removed by aspiration, and the cells were washed three times with a hypotonic buffer containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. The cells were plated at 5 x 10⁶ cells/35-mm dish (Primaria brand, Falcon Labware, Becton Dickinson & Co., Oxnard, Calif.) in the same medium. After 2 hours of incubation at 37°C in 5% CO2/95% air, nonadherent cells were removed by three washes with serum-free medium. The cells were then placed in fresh medium containing 20% autologous serum and were fed twice weekly with the same medium. Monocyte-derived macrophages were used within 10–14 days of plating. The purity of the cells was confirmed by the characteristic morphology of these monocyte-derived macrophages and the positive nonspecific esterase staining of more than 95% of cells using naphthyl acetate as the substrate. The 35-mm dishes were used for the cellular oxidative modification of LDL and also for studies in which LDL and Ox-LDL were incubated with the cells to determine if the oxysterols entered the macrophages.

LDL (200 μg protein/ml) was oxidatively modified with 2.5 μM CuSO4 in phosphate-buffered saline at 37°C for the times indicated as previously described. In the cellular system, LDL (100 μg/ml) was incubated with human monocyte-derived macrophages in Hams F-10 medium (GIBCO) for 24 hours at 37°C. In both systems, oxidation was arrested by refrigeration and addition of 200 μM EDTA and 40 μM butylated hydroxytoluene.

Lipid Methods

Oxidative modification of LDL was determined by three methods. The lipid peroxide content of Ox-LDL was measured using a modification of the thiobarbituric acid–reacting substances (TBARS) assay of Buege and Aust. TBARS are expressed MDA equivalents using freshly diluted 1,1,3,3-tetramethoxypropane as the standard. LDL electrophoresis was performed at pH 8.6 in 0.05 M barbital buffer on 0.05% agarose gels as described. The gels were stained with Sudan Black B. The increased electrophoretic mobility of Ox-LDL was expressed relative to the mobility of native LDL. Total tocopherol content of four samples was measured fluorometrically.

For determination of LDL sterol content and composition, the LDL was extracted with chloroform/methanol (2:1 vol/vol). One portion of the organic phase was dried and hydrolyzed for 30 minutes at 70°C in alcoholic KOH, a second portion was analyzed directly, and a third portion was saved for enzymatic cholesterol assay. Hydrolysis was quantitative. Gas-liquid chromatography used a Shimadzu gas–liquid chromatograph (GC model mini 2, Shimadzu, Kyoto, Japan) and a packed column of OV-17. The injection port temperature was 285°C, and the column temperature was 290°C. Peak areas were measured with a Shimadzu Model GR3A chromatopac. The masses of cholesterol and other sterols were calculated from an internal recovery standard of 5α-cholestanol. Chromatography of LDL extracts also was performed on silica thin-layer chromatography plates developed in heptane/ethyl ether/acetic acid (85:15:2, vol/vol/vol). Enzymatic cholesterol assays were performed according to the Lipid Research Clinics protocol. For identification of unknown oxidative products of cholesterol, several sterols were employed as reference standards. These included 7-ketocholesterol, 22-hydroxycholesterol, 25-hydroxycholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, coprostanol, and cholestenone.

Results

Time-Dependent Modification of Low Density Lipoprotein by Copper Sulfate Oxidation

To study time-dependent changes in LDL exposed to CuSO4, LDL was isolated from 10 different donors.
FIGURE 1. Line plots showing effects of CuSO₄ treatment on electrophoretic mobility of low density lipoprotein (LDL). LDL preparations were isolated from 10 individuals and subjected to the oxidation protocol described in “Methods” (panels A and B). Aliquots of single LDL preparations, 3a-c and 5a-c, were subjected to three separate oxidation protocols (panels C and D). At the times indicated (hr.), oxidation was arrested by refrigeration and addition of 200 μM EDTA and 40 μM butylated hydroxytoluene, and lipoproteins were subjected to 1% agarose gel electrophoresis. Data are expressed as mobility relative to native LDL, so that a lipoprotein with a relative electrophoretic mobility of two migrated twice as far as native LDL.

and subjected to the oxidation protocol described in “Methods.” Two LDL preparations, labeled “3” and “5” in all the figures, were treated by three separate oxidation protocols over a 10-day period, after isolation of the LDL. In this way, variation caused by the properties of different LDLs could be separated from the assay variation. Electrophoretic mobility of LDL, sterol oxidation, and production of TBARS were measured as a function of time. The electrophoretic mobility of LDL increases when the particle is subjected to CuSO₄ treatment due to the increased negative charge. The results in Figure 1 show that the electrophoretic mobility of all 10 preparations of LDL increased as a function of time. The variation in electrophoretic mobility was greatest at early time points but still persisted even after 24 hours. The change in electrophoretic mobility of single preparations subjected to triplicate oxidations (3a-c, 5a-c) were minimal, indicating that the variation resided in some property of the LDL particle and not in the electrophoretic technique. The coefficient of variation of this technique in repeated oxidations of a single patient’s LDL averaged 3.5% for all time points.

The data of Figure 2 show the time course for production of TBARS. These data show that there is no gradual time-dependent increase in MDA formed. The TBARS assay indicated that the increase in lipid peroxide content of the different LDLs generally peaked at 8 hours. Analysis of repeated oxidations of a single LDL sample (3a-c, 5a-c) showed that the technique has a coefficient of variation averaging 10%.

**Oxysterol Products of Low Density Lipoprotein Oxidation**

Oxidation of LDL produced a series of oxidative products of cholesterol as revealed by gas-liquid chromatography. The major oxidative product was 7-ketocholesterol. The presence of 7-ketocholesterol was detected as a 4.37-minute retention time peak, which was identical to that of authentic 7-ketocholesterol. Further, thin-layer chromatography revealed the major oxidative product to have a mobility identical to that of 7-ketocholesterol. Another large oxidative product had a retention time of 1.98 minutes and was not identified, but it was not 22-hydroxycholesterol, 25-hydroxycholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, coprostanol, or cholestenone. In some but not all preparations, a small peak with a retention time of 6.7 minutes was noted and tentatively identified as 22-hydroxycholesterol. No peaks had retention times identical to those of 25-hydroxycholesterol, 7α- or 7β-hydroxycholesterol, coprostanol, or cholestenone. A typical gas chromatogram of LDL before and after oxidation is depicted in Figure 3. The oxysterols were contained in the lipoprotein since 92% as much...
oxysterol as cholesterol could be reisolated at d=1.090 g/ml by ultracentrifugation of Ox-LDL.

The results of Figure 4 show the time course for conversion of lipoprotein sterols into oxysterols for five of the LDL preparations analyzed above. 7-Ketocholesterol made up from one half to two thirds of total oxysterols and increased in parallel with total oxysterols. The data show that oxysterol content increased in a time-dependent manner, with the greatest variability being noted at early time points but with still considerable variability at 24 hours. Free sterol was only slightly more susceptible to oxidation than esterified cholesterol. Again, the triplicate oxidations of a single LDL preparation (3 a–c, 5 a–c) showed much less variability, indicating that the analysis was consistent but that individual LDL preparations varied in their response to oxidative modification. The coefficient of variation in oxysterol formation in repeated oxidations of a single patient’s LDL averaged 8.5%.

In an effort to determine the biologic relevance of these findings, we determined the oxysterol content of LDL that had been modified in a cell-oxidation system using human monocyte-derived macrophages. In two experiments, we found that 20% and 32% of total lipoprotein sterol was converted to the oxysterol, 7-ketocholesterol, after macrophage oxidation of LDL. To ascertain whether the oxysterols entered the cells, both LDL and Ox-LDL were incubated with human macrophages. The sterols accumulated in the cells were then quantified. It is evident from Figure 5 that Ox-LDL caused a greater accumulation of cellular cholesterol, mainly esterified cholesterol. In addition, significant quantities of 7-ketocholesterol were detected in the cells incubated with Ox-LDL but not native LDL.

The variability among LDL preparations from different patients was remarkable and seemingly might be explained by endogenous LDL antioxidants. Vitamin E is a potent antioxidant that is concentrated in lipoproteins; therefore, we measured the vitamin E levels in blood samples from four patients whose LDL varied widely in susceptibility to oxida-
Figure 4. Line plots showing conversion of lipoprotein cholesterol to oxysterol by CuSO₄ treatment. Lipoproteins prepared as described in the legend to Figure 1 were extracted in chloroform/methanol (2:1, vol/vol) containing 5-cholestane as an internal recovery standard. The organic phase of the extract was dried under N₂, resolubilized in a small volume of heptane, and injected into the gas chromatograph directly (free sterol, panels A, C, and E) or after hydrolysis of cholesteryl esters with alcoholic KOH (total sterol, panels B, D, and F). Percentage of oxidized sterol was calculated by dividing the sum of all noncholesterol sterol peaks by all cholesterol plus noncholesterol sterol peaks times 100. Data shown are the average of duplicate injections that did not vary by more than 10%.

The vitamin E contents of the four patients’ LDLs were 27.5±4, 35.5±11.5, 28.0±5.0, and 36.5±5.0 μg/ml. In contrast, a cumulative oxidation susceptibility score made by adding the areas under the time-course curves for electrophoretic mobility, free and total oxysterol contents varied far more. For the same four patients, the oxidation susceptibility scores were 128.7, 160.3, 280.0, and 335.6 arbitrary units, respectively.

The data of Figure 6 show the relation among the three parameters that were measured. In this analysis, the data were plotted and fitted to an appropriate line. The results showed that the alteration in electrophoretic mobility and the oxidation of sterols were related in a nearly linear manner, whereas neither parameter could be simply related to generation of TBARS. The relation between TBARS and either electrophoretic mobility or oxysterol content was clearly nonlinear and best fit a second-degree polynomial. The relation between sterol oxidation and electrophoresis could be fit to a second-degree polynomial but fit about as well to a simple linear regression. The relation is clearly nearly linear or linear. The improvement in fit between the linear regression and the second-degree polynomial was quantified by comparing the regression coefficients, r.

The relation of TBARS to electrophoretic mobility improved such that r increased from 0.752 for a linear regression to 0.948 for a second-degree polynomial. The relation between TBARS to oxysterol formation improved such that r increased from 0.636 to 0.867.
FIGURE 6. Scatterplots showing correlation between lipid peroxidation, oxysterol formation, and electrophoretic mobility of low density lipoprotein treated by CuSO₄. Data points from experiments in Figures 1–3 are plotted. Lines drawn are those that best fit the data, and all are represented by a second-degree polynomial. Regression coefficients are 0.948 for panel A, 0.867 for panel B, and 0.948 for panel C.

The relation between oxysterol formation and electrophoretic mobility was only slightly improved; \( r \) increased only from 0.932 to 0.948.

**Enzymatic Cholesterol Assays Versus True Cholesterol Content**

During the course of these experiments, it became clear that estimates of the cholesterol content of Ox-LDL measured by enzymatic methods and by gas chromatography were different. Since this problem was not encountered when the cholesterol content of native LDL was measured, we reasoned that some characteristics of the oxidized lipoprotein interfered with the enzymatic assay. The data of Table 1 show experiments designed to explore this possibility. In these experiments, identical aliquots of the lipid extract were analyzed either by the enzymatic method or by gas chromatography. The data showed that the enzymatic assay was detecting oxysterols as well as cholesterol. The estimate of cholesterol by the enzymatic method matched the sum of all sterol peaks detected by gas chromatography; 700.0 versus 693.5, 550.0 versus 451.5, and 900.0 versus 989.5.

**Discussion**

The oxidative modification of LDL may provide a crucial link between plasma LDL and the atherosclerotic lesion. Ox-LDL could promote atherogenesis by behaving as a cytotoxin, a monocyte chemoattractant, an inhibitor of macrophage motility, or a stimulator of cholesteryl ester accumulation after uptake by the scavenger receptor mechanism. In the present experiments, we have characterized the time course of oxidation of LDL components and incidentally found a source of error in measurement of cholesterol in Ox-LDL.

The present data show that in the presence of a transition metal or after macrophage modification, the sterol component of LDL is oxidized in parallel with other lipoprotein components. In accord with the findings in the present report, Zhang et al have recently documented that after copper oxidation of a single LDL sample, 7-ketocholesterol was the predominant oxysterol generated. Since oxysterols are potent regulators of cellular processes, the presence of these sterols in Ox-LDL would be expected to contribute significantly to the actions of the lipoprotein on cells. These data seemingly have relevance to the in vivo situation since cellular modification of LDL by macrophages leads to oxysterol formation and since Ox-LDL results in an increase in the content of both cholesteryl esters and 7-ketocholesterol in macrophages. These data also indicate that the oxysterol content of Ox-LDL is a good indicator of lipoprotein modification and closely parallels changes in electrophoretic mobility. Our studies also confirm earlier reports that indicate that Ox-LDL is deficient in true cholesterol and suggest that previous studies may have overestimated the true cholesterol content of oxidized lipoproteins. Thus, Ox-LDL is

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Enzyme assay (µg/mg)</th>
<th>Total Cholesterol (µg/mg)</th>
<th>7-ketocholesterol (µg/mg)</th>
<th>Remaining sterols (µg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>700</td>
<td>693.5</td>
<td>334.5</td>
<td>242.5</td>
</tr>
<tr>
<td>2</td>
<td>550</td>
<td>451.5</td>
<td>221.5</td>
<td>131.0</td>
</tr>
<tr>
<td>3</td>
<td>900</td>
<td>989.5</td>
<td>731.0</td>
<td>119.0</td>
</tr>
</tbody>
</table>

Low density lipoprotein (LDL) was oxidized for 24 hours as described in "Methods." At this time, lipoprotein lipids were extracted in chloroform/methanol, and aliquots of the organic phase were taken for either enzymatic cholesterol assay or analysis by gas chromatography (GC). Both the enzymatic assay and GC were performed as described in "Methods." Results shown are expressed per milligram LDL protein, are the average of duplicate runs or injections, and varied by not more than 11%.
even more deficient in cholesterol than previously recognized. It is possible that such a particle could exert major biologic effects through oxysterols or lipid peroxides in addition to transporting cholesterol into cells.

Our data show that each patient's LDL has a particular "oxidation profile." Some patients, LDLs are oxidized quite rapidly and completely, while others are very resistant to oxidation. An obvious explanation for the variability in the oxidation of individual LDLs is that some preparations of LDL contain more susceptibility or resistance factors than other LDLs. Since both the electrophoretic behavior of the lipoprotein and the oxysterol content vary proportionally, such a factor must affect the lipoprotein generally and not be specific for a single component. LDL can take up and bind lipophilic drugs such as probucol, and it also contains significant amounts of α-tocopherol, β-carotene, and other lipophilic components of plasma. α-Tocopherol as well as β-carotene are known antioxidants that might act as endogenous oxidation resistance factors. However, in our studies here as well as in a recent report, it appears that the α-tocopherol content of various LDLs could not be used to predict their relative resistance to oxidation. Hence, future studies should be directed at identifying the role of other endogenous antioxidants present in LDL in its propensity to oxidative modification.

In conclusion, this study has documented two other properties of Ox-LDL that might be relevant to atherosclerosis. First, oxysterols are generated after the oxidative modification of LDL, and these could induce toxic and metabolic effects on cells. Second, we documented a wide variability in the susceptibility of different LDLs to oxidative modification. Thus, if the oxidation of LDL proves to be a key step in atherogenesis, an enhanced propensity of an individual's LDL to oxidation could predispose that person to premature atherosclerosis.

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Key Words • oxysterols • lipid peroxides • low density lipoproteins
Varying susceptibility of different low density lipoproteins to oxidative modification.
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