Unsaturated Fatty Acids Enhance Low Density Lipoprotein Uptake and Degradation by Peripheral Blood Mononuclear Cells

Joseph Loscalzo, Jane Freedman, M. Audrey Rudd, Irina Barsky-Vasserman, and Douglas E. Vaughan

The precise mechanism by which unsaturated fatty acids lower low density lipoprotein cholesterol is not known. Because cis-un saturated fatty acids incorporated in cell membranes increase membrane fluidity and can thereby dramatically alter membrane-dependent cellular functions, we examined the effect of linoleate and oleate incorporation in peripheral blood mononuclear cell membranes on the physical properties of the membrane and concomitantly on low density lipoprotein uptake and degradation. We found that membrane enrichment with linoleate increased the rate of low density lipoprotein degradation in both freshly isolated and derepressed mononuclear cells. Enrichment with oleate led to similar increases in degradation. "Specific" low density lipoprotein uptake by derepressed cells was also enhanced by linoleate and oleate incorporation. Enrichment with both of these fatty acids produced an increase in membrane fluidity, as indicated by a reduction in the steady-state fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene incorporated in the membrane. In contrast, stearate enrichment had little effect on uptake or degradation of low density lipoprotein, nor did it affect membrane fluidity. These data point to a novel mechanism for the reduction in low density lipoprotein produced by unsaturated fatty acids that involves their physical effects on cell membranes as it relates to metabolism of the lipoprotein. (Arteriosclerosis 7:450–455, September/October 1987)

While the cholesterol-lowering effect of unsaturated fatty acids has been appreciated for over two decades, the molecular mechanism by which unsaturated fatty acids reduce low density lipoprotein (LDL) cholesterol has eluded definition. The prevailing hypotheses argue either for a physicochemical alteration in LDL induced by the fatty acids or for an increase in the metabolism of LDL. Recent data strongly support the latter hypothesis but fail to demonstrate the actual molecular mechanism by which LDL clearance is enhanced.

Dietary unsaturated fatty acids are readily incorporated in the phospholipid fraction of cell membranes. The physical properties of the membrane, in turn, can be dramatically affected by its fatty acyl composition: increasing the cis-un saturated fatty acyl composition increases membrane fluidity (or decreases microviscosity); increasing the saturated or trans-un saturated fatty acyl composition decreases membrane fluidity (or increases microviscosity). These changes in the bulk physical properties of the membrane can then produce marked changes in membrane-dependent cellular functions. For example, enriching the membrane fatty acyl composition with linoleate inhibits the capping of surface immunoglobulin on lymphocytes, inhibits platelet aggregability, and inhibits erythrophagocytosis by macrophages.

Because of these effects on membrane function, we hypothesized that the reduction in LDL cholesterol produced by cis-un saturated fatty acids is the result of their direct effect on the membrane-mediated clearance of LDL. In order to test this hypothesis, we examined the effect on the uptake and degradation of LDL of modifying mononuclear cell membranes with cis-un saturated and saturated fatty acids. Our results indicate that cis-un saturated fatty acids do, indeed, enhance the cellular clearance of LDL and do so by increasing the rate of LDL uptake and degradation.

Methods

Materials

Na$_2$CO$_3$ was purchased from Amersham, Incorporated, Arlington Heights, Illinois. 1-$^14$C-linoleate, 1-$^14$C-stearate, and 1-$^14$C-oleate were obtained from New England Nuclear Corporation, Boston, Massachusetts. Lymphocyte separation medium (LSM) was purchased from Litton Bionetics, Incorporated, Charleston, South Carolina, and Sepacell-MN was purchased from Seprotech Corporation, Oklahoma City, Oklahoma. Thin-layer chromatography plates were obtained from Whatman Chemical
Mononuclear Cell Preparation

Whole blood was obtained from volunteer donors and was anticoagulated with 120 U/ml heparin. Mononuclear cells were prepared by mixing 2 ml whole blood with 2 ml 0.85% NaCl, after which the cell suspension was layered over 3 ml LSM.12 The suspension was then centrifuged at 400 g for 30 minutes at 20° C, and the mononuclear cells were removed from the interface of the plasma and lymphocyte separation medium. The cells were washed twice with Hanks'-balanced salt solution containing 15 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4 (Hanks'-HEPES). Cell viability was assessed by trypan blue exclusion: 90 µl of cell suspension were mixed with 10 µl of a 0.4% trypan blue solution in 0.85% NaCl, and the percentage of cells excluding the dye was determined. Routinely, 96% of the cells were viable throughout the experiments described below.

Low Density Lipoprotein Preparation

Human LDL (density 1.020 to 1.063 g/ml) was isolated from the plasma of normolipidemic, fasting volunteers by differential ultracentrifugation as described previously.13 LDL prepared in this way routinely had a total cholesterol-to-total protein mass ratio of 1.5:1.

Lipoprotein-Deficient Serum Preparation

Lipoprotein-deficient serum (LPDS) (density > 1.215 g/ml) was obtained from the plasma of healthy subjects by differential ultracentrifugation as described previously.13

Low Density Lipoprotein Radiolodination

LDL was radiiodiated by the ICI method of McFarlane14 as modified by Langer et al.15 and Shepherd et al.16 Radioiodinated in this fashion, LDL had a specific activity of 600 to 900 cpm/ng.

Fatty Acid Modification of Mononuclear Cells

Mononuclear cells were modified with fatty acids according to the method of Klausner et al.7 Cell suspensions in Hanks'-HEPES were incubated with 30 µM linoleate, stearate, or oleate (below the critical micelle concentrations) for 60 minutes at 25° C, after which the cells were washed with Hanks'-HEPES twice. Cells were then used directly in assays of LDL uptake and degradation. Control cells underwent sham modification with an equal volume of ethanol vehicle.

Low Density Lipoprotein Degradation Assay

Proteolytic degradation of LDL was assessed essentially according to published methods.17-19 An aliquot of 2 x 10^5 freshly isolated cells in Hanks'-HEPES (pH 7.4) was incubated with 32 µg/ml of radioiodinated LDL at 37° C for 12 hours. Derepressed cells were also studied after 24 hours of incubation in 10% LPDS. These cells subsequently underwent fatty-acid modification as described above, then were incubated in Hanks'-HEPES (pH 7.4) with 9.5 µg/ml LDL at 37° C for 4 hours. At the end of the incubation, an aliquot of the medium was obtained for determination of LDL degradation products that were not free iodide.17 All results are expressed as “high affinity” LDL degradation, determined by subtracting the nonspecific degradation (i.e., 125I-LDL degradation in the presence of a 50-fold excess of unlabelled LDL) from total 125I-LDL degradation. The results are expressed as nanograms of LDL protein degraded/hr/mg cell protein.

Low Density Lipoprotein Uptake Assay

Cellular accumulation of LDL was measured according to standard published methods.18-20 Derepressed cells were used in all cases because of the very low levels of uptake noted in freshly isolated mononuclear cells. Chloroquine at 20 µM was used to inhibit LDL degradation16,18 in an incubation medium containing 2 x 10^6 cells in Hanks'-HEPES (pH 7.4) with 9.5 µg/ml LDL at 37° C. At the end of the 4-hour incubation, the cells were pelleted in a microfuge at 8,700 g for 1 minute through a cushion of Hanks'-HEPES with 20% sucrose to separate the cell-associated LDL from the free LDL. The cell pellet was isolated by cutting the tip of the microfuge tube with a razor blade and counting the radioactivity in a gamma counter. All results are expressed as “high affinity” LDL uptake” determined by subtracting the nonspecific uptake (i.e., 125I-LDL uptake in the presence of a 50-fold excess of unlabelled LDL) from the total 125I-LDL uptake. The results are expressed as nanograms of cell-associated LDL protein/hr/mg cell protein.

Fatty Acyl Incorporation and Identification

To determine the extent of incorporation of fatty acids in the mononuclear cell membranes and the particular lipid classes into which they were incorporated, 1-14C-labelled fatty acid (40 to 60 mCi/mmol) was mixed with the corresponding unlabelled fatty acid at a ratio of 1:10 (labelled/unlabelled) at 30 µM final concentration. After incubation at 25° C for 60 minutes, the cells were washed twice with Hanks'-HEPES (pH 7.4) and were resuspended in the same solution. The cellular lipids were then extracted with hexane/2-propanol (3:2, vol/vol),22 were dried down under nitrogen gas, and were separated by thin-layer chromatography by use of Whatman LKB6 silica gel plates. The lipids were separated into three classes (free fatty acid, phospholipid, and neutral lipid) by use of the solvent system, hexane/diethyl ether/acetic acid (60:39:1, vol/vol/vol). With this system, neutral lipids run near the solvent front, free fatty acids run in the middle, and phospholipids run near the origin.23 The spots were visualized with iodine vapor, were scraped, and were counted in an LKB liquid
scintillation counter model 1211 (LKB Instruments, Gaithersburg, Maryland) using Aquasol (Dupont Company, Boston, Massachusetts).

Membrane Fluidity Measurements

The cells were incubated with DPH7,24 at a final concentration of 2 μM for 20 minutes at 25° C, after which the cells were washed twice with Hanks'-HEPES (pH 7.4). Fluorescence measurements were performed with a Spex Fluorolog-2 spectrofluorimeter (Spex Industries, Incorporated, Edison, New Jersey) equipped with a thermostat-controlled cell holder and a polarization accessory. The steady-state fluorescence polarization was measured by exciting the cell suspension at 360 nm and recording the emission at 430 nm. The polarization of fluorescence emission was calculated from the equation:

$$P = \frac{I_{vh} - G_{h}}{I_{vh} + I_{h}}$$

where P is polarization, I is the fluorescence intensity, the first and second subscripts refer to the plane of polarization of the excitation and emission beams, respectively (v = vertical, h = horizontal), and G = I_{vh}/I_{h}. For each measurement of P, the emission intensities were corrected for contributions from scattering by measurement of these intensities in a blank containing all the solution constituents except fluorophore.

Analytic Methods

The protein concentration was determined by the method of Lowry et al.26 LDL cholesterol was measured by a colorimetric method that used cholesterol oxidase, peroxidase, 4-hydroxybenzoate, and 4-aminophenazone.26

Results

Effect of Fatty Acid Incorporation by Mononuclear Cells on Low Density Lipoprotein Degradation

The effect of incorporating linoleate, stearate, or oleate on "specific" LDL degradation by freshly isolated mononuclear cells is shown in Figure 1. Linoleate incorporation enhanced the rate of LDL degradation by 300%. Oleate was essentially as effective, enhancing the rate of degradation by 282%. Stearate enrichment of mononuclear cells appeared to have a minimal effect on LDL degradation, producing rates that were 98% of the control. Derepressed cells were also studied in this assay in which upregulation of LDL receptors was induced to enhance the overall rate of degradation. In another experiment (Figure 2) mononuclear cells were derepressed by preincubation in 10% LPDS for 24 hours, after which fatty acid modifications were performed as described above. In these derepressed cells, linoleate incorporation also enhanced "specific" LDL degradation by 182% compared with control cells. Oleate was, again, essentially as effective (Figure 3), enhancing degradation by 162%, while stearate enrichment failed to have any effect on degradation, producing rates that were 99% of control. Increasing the cell number twofold and fivefold (to 4 x 10^5/ml and 1 x 10^8/ml, respectively) in this assay increased the extent of degradation 1.8-fold and 5.1-fold, respectively, for control cells, 1.6-fold and 5.0-fold, respectively, for stearate-modified cells, 1.9-fold and 4.9-fold, respectively, for linoleate-modified cells, and 1.8-fold and 5.2-fold, respectively, for oleate-modified cells, compared with the standard assay performed with 2 x 10^5 cells/ml with each cell population studied. Increasing incubation time was also associated with a linear increase in activity regardless of the type of fatty acid modification.
MEMBRANE FATTY ACIDS, LDL METABOLISM, AND LYMPHOCYTES

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Table 1. Extent of Incorporation of Fatty Acids In Cellular Lipids

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Total lipid</th>
<th>Phospholipid fraction</th>
<th>Free fatty acid fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linoleate</td>
<td>5.53</td>
<td>3.42</td>
<td>2.11</td>
</tr>
<tr>
<td>Oleate</td>
<td>5.40</td>
<td>3.30</td>
<td>1.90</td>
</tr>
<tr>
<td>Stearate</td>
<td>3.04</td>
<td>1.00</td>
<td>2.04</td>
</tr>
</tbody>
</table>

Values are ng/10^8 cells.

Table 2. Effect of Fatty Acid Modification on Membrane Fluidity

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Control</th>
<th>Linoleate</th>
<th>Oleate</th>
<th>Stearate</th>
</tr>
</thead>
<tbody>
<tr>
<td>5°C</td>
<td>0.310</td>
<td>0.267</td>
<td>0.270</td>
<td>0.301</td>
</tr>
<tr>
<td>10°C</td>
<td>0.261</td>
<td>0.231</td>
<td>0.238</td>
<td>0.249</td>
</tr>
<tr>
<td>25°C</td>
<td>0.248</td>
<td>0.192</td>
<td>0.200</td>
<td>0.232</td>
</tr>
<tr>
<td>35°C</td>
<td>0.220</td>
<td>0.169</td>
<td>0.174</td>
<td>0.224</td>
</tr>
</tbody>
</table>

DPH = 1,6-diphenyl-1,3,5-hexatriene.

Figure 3. Effect of membrane fatty acid enrichment on low density lipoprotein (LDL) uptake by derepressed human peripheral blood mononuclear cells. The rate of "specific" uptake of LDL (9.5 μg/ml) was measured in a 1-ml assay volume containing 2 x 10^5 control (C), stearate-enriched (S), linoleate-enriched (L), or oleate-enriched (O) cells in Hanks' HEPES (pH 7.4) as described in Methods. Incubations were performed at 37°C for 4 hours. Values represent the mean ± standard deviation of three experiments each performed in triplicate.

Effect of Fatty Acid Incorporation on Low Density Lipoprotein Uptake by Mononuclear Cells

The effect of incorporating linoleate, stearate, or oleate on "specific" LDL uptake was also studied. Mononuclear cells whose LDL receptors were derepressed by incubation for 24 hours in 10% LPDS were subsequently enriched in fatty acid, then incubated with LDL in the presence of chloroquine to inhibit degradation. "Specific" cell-associated LDL was localized to the neutral lipid fraction.

Quantitation of Incorporation of Fatty Acids in Mononuclear Cell Lipid Fraction

The extent of incorporation of each of the fatty acids in the cellular lipids is listed in Table 1. Cells incubated with 30 μM linoleate incorporated 5.53 ng/10^8 cells after 1 hour of incubation at 25°C; 3.42 ng/10^8 cells were found in the phospholipid fraction, while 2.11 ng/10^8 cells remained as free fatty acid. Incubating cells with 30 μM stearate led to incorporation of 5.04 ng/10^8 cells, of which 1.00 ng/10^8 cells were found in the phospholipid fraction and 2.04 ng/10^8 cells remained as free fatty acid. Incubation with 30 μM oleate produced incorporation of 5.40 ng/10^8 cells, of which 3.30 ng/10^8 cells were found in the phospholipid fraction and 1.90 ng/10^8 cells remained as free fatty acid. In each case, less than 2% of the total fatty acid incorporated was localized to the neutral lipid fraction.

Effect of Fatty Acid Modification on Membrane Fluidity

To assess the effect of fatty acid incorporation on bulk membrane fluidity, the steady state fluorescence polarization of DPH incorporated in mononuclear cell membranes was measured. These data are shown in Table 2 and indicate that at each of the four temperatures at which the measurements were made, cells incubated with linoleate or oleate had significantly reduced DPH fluorescence polarization, which is indicative of a more fluid membrane microenvironment. At 5°C linoleate reduced the polarization by 14%, while oleate did so by 15%; at 25°C linoleate reduced polarization by 23% and oleate by 19%; and at 35°C linoleate reduced polarization by 23% and oleate by 21%. At all temperatures, stearate failed to alter polarization values to any significant degree compared with controls.

Discussion

The cellular clearance of LDL is primarily mediated by the LDL receptor that recognizes epitopes on apoprotein B-100. A deficiency or dysfunction of this receptor leads to elevated circulating concentrations of LDL and thereby promotes atherogenesis. Several steps in the pathway of processing receptor-bound LDL can be defective, thereby decreasing cellular clearance. Among these are synthesis of receptors of reduced affinity for LDL, alterations in the rate of formation of coated pits, abnormalities in endosome production, and reduced rates of LDL degradation in lysosomes. Importantly, all of these processes occur within, or are directly associated with, the membrane milieu of the cell and, in the context of well-defined precursors in other cell surface receptor systems, should be affected by the lipid composition of that environment.

In this study, we have shown that by enriching the membranes of human peripheral blood mononuclear cells with the cis-unsaturated fatty acids, linoleate and oleate, the uptake and degradation of normal human LDL is markedly enhanced. The mechanism by which fatty acid enrichment leads to enhanced LDL uptake and degradation has not yet been precisely defined by these studies. Clearly, these
data do show that the cellular clearance of LDL is specifically and dramatically enhanced when fatty acids of a class known to enhance whole body clearance of LDL are incorporated into the membrane.

-cis-Unsaturated fatty acids impart disorder to a membrane milieu primarily by intercalating among the saturated and trans-saturated fatty acyl groups forming relatively gel-like microdomains.8,9 These local changes in order and fluidity may modulate certain membrane functions, particularly those mediated by specific receptors within these domains.41-43 In other receptor systems, changes in the cells’ receptor affinity or number were produced by modifying the lipid composition and fluidity.10,38,37,36 We did not specifically address LDL’s surface binding to mononuclear cells in this study because peripheral blood mononuclear cells have only a few binding sites. We measured “specific” degradation and “specific” uptake (reflecting both surface-associated and internalized LDL) to identify the likely site of enhancement of LDL clearance afforded by fatty acid enrichment. Our results suggest that degradation is enhanced by cis-un saturated fatty acid enrichment by an increase in LDL per cell. From these data, we cannot distinguish among which mechanism (altered receptor affinity, number, or rate of internalization) causes this increased association.

Our method of modification of membrane lipid composition produced small changes in total membrane lipid composition. Interestingly, in contrast to ex vivo changes in cholesterol content (the other major determinant of membrane fluidity), the percentage change in fatty acyl composition producing the same degree of change in membrane fluidity and function was much more modest. For example, a 54% increase in platelet cholesterol/phospholipid ratio led to a 47% reduction in EC20 for thrombin-induced platelet aggregation,38 while as little as a 5% increase in platelet linoleic content led to an 60% inhibition of platelet aggregation.10 The biophysical explanation for the effect of such modest manipulation of membrane lipid composition may relate to the fact that specific annaular9 or boundary36 lipids that are relatively in exchangeably bound to membrane proteins form local microdomains9 that can significantly change membrane protein conformation or function. Jurtshuk et al.44 demonstrated this principle as early as 1963 when they reported that optimal activation of mitochondrial 7-hydroxybutyric acid dehydrogenase requires an unsaturated acyl lecithin in its microenvironment.

This report demonstrates for the first time that the membrane milieu in which protein determinants of cholesterol metabolism function can alter that metabolism. The lipid microenvironment can dramatically modulate the normal processes of LDL binding, internalization, lysosomal incorporation, and degradation. Since this microenvironment can be modified by dietary means and by intermediary metabolism, these results suggest a novel mechanism for altering plasma LDL cholesterol levels that can be regulated by exogenous lipids themselves. This is clearly worthy of further investigation.45

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