Effect of Membrane Fatty Acyl Composition on LDL Metabolism in Hep G2 Hepatocytes†

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ABSTRACT: The mechanism by which dietary cis-unsaturated fatty acids lower plasma levels of low-density lipoprotein (LDL) cholesterol is unknown. Since plasma membrane incorporation of dietary cis-unsaturated fatty acids is known to alter the function of plasma membrane associated proteins, perhaps by increasing membrane fluidity, we examined LDL receptor function in Hep G2 hepatocytes that were unmodified, enriched with the cis-unsaturated fatty acids oleate or linoleate, or enriched with the saturated fatty acids stearate or palmitate. Hepatocytes enriched in cis-unsaturated fatty acids exhibited augmented LDL binding, uptake, and degradation in comparison to unmodified cells. In contrast, Hep G2 hepatocytes enriched in saturated fatty acids had decreased LDL binding, uptake, and degradation. Enrichment with oleate or linoleate resulted in a decrease in the calculated fatty acyl mole-weighted melting point of the plasma membrane and an increase in plasma membrane fluidity, as measured by the steady-state fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene incorporated into the plasma membrane. Conversely, stearate or palmitate enrichment resulted in an increased plasma membrane fatty acyl mole-weighted melting point and decreased plasma membrane fluidity. LDL binding, uptake, and degradation varied with plasma membrane fluidity in a highly correlated manner. Thus, one mechanism by which dietary cis-unsaturated fatty acids lower LDL cholesterol may possibly involve an alteration in membrane lipid composition or membrane fluidity that promotes enhanced LDL receptor function, thereby leading to increased hepatic clearance of LDL.

Although considerable progress in the field of low-density lipoprotein (LDL)[1] metabolism has occurred in the past 20 years, the precise molecular mechanism by which dietary cis-unsaturated fatty acids decrease serum LDL cholesterol remains unknown. Epidemiologic studies have linked diets rich in polyunsaturated fatty acids with a reduction in serum LDL cholesterol (Ahrens et al., 1957; Hegsted et al., 1965). A number of mechanisms have been postulated, including alterations in (1) fecal sterol excretion, (2) cholesterol absorption from the small intestine, (3) rates of de novo cholesterol synthesis, (4) distribution of cholesterol among various extrahepatic pools, and (5) rates of synthesis or catabolism of plasma lipoproteins (Vega et al., 1982; Spady et al., 1988). Recent studies support increased cellular LDL catabolism as the most probable mechanism by which dietary cis-unsaturated fatty acids reduce serum cholesterol.

An integral part of cellular cholesterol homeostasis, the LDL receptor, is a freely mobile transmembrane glycoprotein that could potentially be influenced by alterations in the physicochemical properties of the surrounding plasma membrane. Many plasma membrane associated proteins function only when associated with a suitable membrane lipid environment. Studies with the insulin receptor in Friend erythroleukemia and Ehrlich ascites cells, the prostaglandin E_2 receptors in rat peritoneal macrophages, the acetylcholine receptor in reconstituted membranes, and the adenylate cyclase enzyme in rat hepatocytes have demonstrated dramatic changes in membrane protein function as a result of alterations in membrane lipid composition and fluidity (Ginsberg et al., 1981; Neelands et al., 1983). As a major determinant of membrane fluidity, membrane phospholipid fatty acyl composition can be readily altered by dietary fatty acid content.

We hypothesized that the dietary cis-unsaturated fatty acid induced reduction in serum LDL cholesterol is a result of a membrane-mediated enhancement of LDL receptor function. Previous work by this laboratory has demonstrated that cis-unsaturated fatty acyl enrichment of human peripheral mononuclear cells (Loscalzo et al., 1987) and U-937 monocytes (Kuo et al., 1990) enhances cellular LDL clearance by increasing LDL uptake and degradation. Because the liver is the primary site for LDL clearance, we have altered the phospholipid fatty acyl composition of Hep G2 hepatocytes with the cis-unsaturated fatty acids oleate and linoleate and the saturated fatty acids stearate and palmitate and examined the effects of these lipid modifications on LDL metabolism.

Materials and Methods

Materials

$Na^{125}I$ was purchased from Amersham, Inc., Arlington Heights, IL. Stearate, palmitate, linoleate, oleate, heptadecanoate, and fatty acid methyl ester gas chromatography standards were obtained from NuChek Prep., Elysian, MN. Cholesterol, phosphatidylcholine, sphingomyelin, and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Sigma Chemical Co., St. Louis, MO. Human transferrin was pur-

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[1] Abbreviations: LDL, low-density lipoprotein; DPH, 1,6-diphenyl-1,3,5-hexatriene; EMEM, Earle's minimum essential medium; FAFBSA, fatty acid free bovine serum albumin; K_D, dissociation constant; B_{max}, maximum binding; PL, phospholipid; FFA, free fatty acid; NL, neutral lipid; NaPyr, sodium pyruvate; AA, nonessential amino acid solution.

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chased from Calbiochem, San Diego, CA. Earle's minimum essential medium (EMEM), fetal calf serum (FCS), sodium pyruvate (NaPyr), and nonessential amino acid solution (AA) were obtained from Gibco, Lawrence, MA. Culture flasks were obtained from Corning Glass Works, Corning, NY. Dialysis tubing was purchased from Spectrum Medical Industries, Los Angeles, CA. Iodo-Beads were obtained from Pierce Chemical Co., Rockford, IL. High-performance liquid chromatography grade hexane, 2-propanol, chloroform, methanol, petroleum ether, and diethyl ether were purchased from Aldrich Chemical Co., Milwaukee, WI. All other chemicals were reagent grade or better.

Methods

Hep G2 Hepatocyte Preparation. Hep G2 hepatocytes were kindly donated by Dr. Richard Pratt of Brigham and Women's Hospital, Boston, MA. The cells were grown as a monolayer in culture with EMEM, 10% FCS, 1% AA, and 10 μM NaPyr in 95% O_2 and 5% CO_2 at 37 °C. Cells were grown to confluence and routinely split 1:5 on a weekly basis.

Low-Density Lipoprotein Preparation. Human LDL (density 1.006–1.063 g/mL) was isolated from the plasma of fasting normolipidemic volunteers by sequential ultracentrifugation in 50 mM Tris/5 mM EDTA, pH 7.4. Potassium bromide was used to adjust the solvent densities. The sample was then sequentially dialyzed with three changes of 50 mM Tris, pH 7.4 (Havel et al., 1955). Total protein content was determined by the method of Lowry and colleagues (Lowry et al., 1951). LDL cholesterol was measured by a colorimetric method using cholesterol oxidase, peroxidase, 4-hydroxybenzoate, and 4-aminophenazone (Meiattini et al., 1978). Polyacrylamide gel electrophoresis (4.3%) was performed to ensure purity of LDL. LDL prepared in this manner typically had a cholesterol-to-total protein mass ratio of 1.3 to 1.7:1.

Diferric Transferrin Preparation. Diferric transferrin was prepared by the method of Klausner and colleagues (Klausner et al., 1983). Six milligrams of transferrin was dissolved in 1 mL of 0.25 M Tris-HCl, pH 8.0, and 10 μM $NaHCO_3$, to which was added to 20 μL of 100 μM disodium nitrilotriacetate and 12.5 mM $FeCl_3$. The sample was incubated at 37 °C for 30 min and then passed over a Sephadex G-25 column, previously equilibrated with 0.15 M NaCl/0.02 M Tris-HCl, pH 7.4. The amount of iron bound by transferrin was estimated from the A_{465}/A_{280} ratio, which was routinely found to be 0.44–0.46, consistent with full saturation.

LDL and Transferrin Radioiodination. LDL was radioiodinated by the iodine monochloride method of McFarlane (McFarlane et al., 1958), as modified by Langer and coworkers (Langer et al., 1972) and Sheperd and colleagues (Sheperd et al., 1976). Iodinated in this fashion, LDL had a specific activity of 300–800 cpm/ng of protein.

Transferrin was radioiodinated by adding an Iodobead to 100 μL of 0.15 M NaCl/0.02 M Tris-HCl, pH 7.4. After incubation for 10 min at room temperature, 1 mg of iron-saturated transferrin was added to the mixture and incubated for an additional 15 min. The solution was passed over a Sephadex G-25 column, and fractions were collected. The specific activity of the diferric ^{125}I-transferrin preparations was 300–600 cpm/ng of protein (Klausner et al., 1983).

Preparation of Oleate-, Linoleate-, Stearate-, and Palmitate-Loaded Albumin. Fatty acid loaded albumin (oleate, linoleate, stearate, or palmitate) was prepared by first preparing a 250 mM fatty acid solution in absolute ethanol. An 8-mL aliquot of the fatty acid solution was adjusted to pH 7.4 using 1 N NaOH and phenolphthalein in methanol as the indicator. The ethanol was then evaporated under nitrogen on a heated stirrer (48 °C), and the resulting solid was dissolved in 20 mL of 0.9% NaCl. A 530-μL aliquot of the resulting solution of the fatty acid sodium salt (once cooled to 37 °C) was dissolved in 4 mL of ice-cold 20% fatty acid free bovine serum albumin (FAFBSA) in 0.9% NaCl, pH 7.4, while vortexing. The resulting solution was then adjusted to a final volume of 10 mL using warm (37 °C) 0.9% NaCl.

Oleate-, linoleate-, stearate-, and palmitate-loaded albumins were stored at 4 °C under nitrogen and protected from light. Purity was checked periodically by gas chromatography using methods described below.

Hep G2 Hepatocyte Up-Regulation and Fatty Acyl Modification. Hep G2 hepatocytes were grown to confluence on 24 × 16 mm wells. The medium was aspirated from freshly confluent cells and replaced with an equal volume of EMEM, 10 μM NaPyr, 1% AA, and (1) 0.2% FAFBSA, (2) 0.2% FAFBSA/0.002% oleate-loaded albumin (0.92 mol of oleate/mol of FAFBSA), (3) 0.2% FAFBSA/0.002% linoleate-loaded albumin (0.94 mol of linoleate/mol of FAFBSA), (4) 0.2% FAFBSA/0.002% stearate-loaded albumin (0.90 mol of stearate/mol of FAFBSA), or (5) 0.2% FAFBSA/0.002% palmitate-loaded albumin (0.95 mol of palimate/mol of FAFBSA). The Hep G2 hepatocytes were incubated for 24 h at 37 °C in 95% O_2/5% CO_2, after which the cell monolayers were washed twice with EMEM, 1% AA, 10 μM NaPyr, and 0.2% FAFBSA. Cell viability was determined by trypan blue exclusion. Routinely, viability was greater than 95%. Control, oleate-enriched, linoleate-enriched, stearate-enriched, and palmitate-enriched cells were always assayed in parallel in the experiments described below.

LDL Binding Assay. Hep G2 hepatocytes were incubated with ^{125}I-LDL at 4 °C for 4 h to determine specific binding (Semenkovich et al., 1987). A range of LDL concentrations from 20 to 120 μg/mL was used in each assay to generate binding isotherms. Each assay was performed in EMEM with 1% AA, 10 μM NaPyr, and 10 mM $CaCl_2$. All reagents were chilled at 4 °C prior to use. Just prior to incubation, an aliquot of medium was removed from each well to determine total counts. Following incubation, the Hep G2 hepatocyte monolayers were washed twice with EMEM, 1% AA, 10 μM NaPyr, and 0.2% FAFBSA. Heparin-dissociable LDL bound to the cell surface was determined by incubating the cell monolayer in EMEM, 1% AA, 10 μM NaPyr, and 1500 units/mL porcine mucosal heparin at 4 °C for 1 h. After incubation, an aliquot of the medium was removed to determine heparin-dissociable receptor-specific LDL binding. The medium was then decanted, and the cells were washed twice with 0.9% NaCl; 1 N NaOH was then added to dissociate the cells. The protein content of each well was measured by using the method of Lowry (Lowry et al., 1951). Specifically bound counts were expressed as micrograms of LDL protein bound per milligram of cell protein.

LDL Uptake and Degradation Assay. The amount of ^{125}I-LDL internalized and degraded by Hep G2 hepatocytes was determined by established methods (Semenkovich et al., 1987). Hep G2 hepatocytes were incubated with ^{125}I-LDL in the presence or absence of a 20-fold excess of unlabeled LDL at 37 °C for 4 h. An ^{125}I-LDL concentration of 120 μg/mL was used. Each assay was carried out in EMEM, 1% AA, 10 μM NaPyr, and 10 MM $CaCl_2$. Just prior to incubation, an aliquot of the medium was removed from each well to determine total counts. After incubation, the medium from each well was removed and treated with 20% trichloroacetic acid to precipitate undegraded protein-bound iodotyrosine. The precipitate was removed by centrifugation at 14000*g* for

1 min. The supernatant was counted to determine the amount of acid-soluble, ether-unextractable, radioiodinated material generated by the cells and released into the aqueous medium.

The Hep G2 hepatocyte monolayers were incubated in EMEM, 1% AA, 10 μM NaPyr, and 2 mg/mL BSA at 4 °C for 30 min. The cells were washed twice with cold (4 °C) EMEM, 1% AA, and 10 μM NaPyr and then incubated at 4 °C for 1 h in EMEM, 1% AA, 10 μM NaPyr, and 1500 units/mL porcine mucosal heparin. After incubation, the cells were again washed twice with cold (4 °C) EMEM, 1% AA, and 10 μM NaPyr; 1 N NaOH was added to dissociate the cells, and the entire volume of cell suspension was counted to determine the receptor-specific uptake of ^{125}I-LDL. The protein content of each well was determined by using the method of Lowry and co-workers (Lowry et al., 1951).

LDL degradation was expressed as micrograms of LDL protein degraded per milligram of cell protein per 4-h incubation period. LDL uptake was expressed as micrograms of LDL internalized per milligram of cell protein per 4-h incubation period.

Transferrin Binding and Uptake Assay. Transferrin binding by Hep G2 hepatocytes was determined by established methods (Ciechanover et al., 1983). Hep G2 hepatocytes were grown to confluence and were then washed twice with EMEM, 1% AA, and 10 μM NaPyr. The cells were incubated in EMEM, 1% AA, 10 μM NaPyr, and 100 μM ^{125}I diferric transferrin in the presence or absence of a 20-fold excess of unlabeled diferric transferrin at 4 °C for 2 h. An aliquot of the medium was removed to determine total counts, and the monolayers were washed twice with EMEM, 1% AA, and 10 μM NaPyr; 1 N NaOH was added to dissociate the cells, and the entire volume of cell suspension from each well was counted to determine bound counts. Protein content was measured with the method of Lowry (Lowry et al., 1951). Receptor-specific transferrin binding was expressed as picomoles of transferrin bound per milligram cell protein.

Transferrin uptake was measured by growing Hep G2 hepatocytes to confluence in duplicate wells. The cells were washed twice with EMEM, 1% AA, and 10 μM NaPyr. The cells were then incubated with EMEM, 1% AA, 10 μM NaPyr, and 100 nM ^{125}I diferric transferrin at 4 °C for 2 h. The unbound ligand was removed by washing the cell monolayers twice with EMEM, 1% AA, and 10 μM NaPyr. The cells were incubated with EMEM, 1% AA, 10 μM NaPyr, and 100 μM diferric transferrin. One set of wells was incubated at 37 °C for 15 min to determine total transferrin uptake, while the other set of wells was incubated at 4 °C for 15 min to determine nonspecific transferrin uptake. Both sets of cells were then washed with EMEM, 1% AA, and 10 μM NaPyr. After being washed, 0.25% Pronase in EMEM at 4 °C was added to each well, and cells were detached by repetitive pipetting. The entire volume of cell suspension from each well was collected and centrifuged at 14000*g* for 1 min. The supernatant was decanted, and the pellet was counted. Protein content was determined by using the method of Lowry (Lowry et al., 1951). Receptor-specific transferrin uptake was expressed as picomoles of transferrin internalized per milligram of cell protein.

Determination of Cellular Lipid Composition. After being washed twice in 0.9% NaCl, confluent Hep G2 hepatocytes were dissociated from the wells by adding 1 N NaOH. The resulting cell suspension was extracted with hexane/2-propanol (3:2 v/v). A heptadecanoate internal standard was added to the lipid extract. The fatty acyl groups were transesterified with methanolic HCl and extracted into petroleum ether. The petroleum ether was then evaporated by using N_2 gas, and the lipids were resuspended in chloroform. Separation and identification were performed by gas–liquid chromatography using a Chrompack CP Sil 88 capillary column in a Hewlett Packard 5890A gas chromatograph equipped with an automated injector, flame ionization detector, and Hewlett Packard 3393 integrator. Separation was performed in the isothermal mode at 210 °C with an injection volume of 5 μL, helium as the carrier gas, and a split ratio of 60:1. Fatty acid methyl ester GC standards were used to determine retention times.

In order to determine incorporation into particular lipid classes, Hep G2 hepatocytes were dissociated from the wells and extracted in hexane/2-propanol (3:2 v/v) to which a C17:0 standard was added. The solvent was evaporated under N_2, and the remaining solute was resuspended in 100 μL of hexane. The samples were applied to 20 × 20 Whatman LK6DF silica gel thin-layer chromatography plates. The plates were developed with hexane/diethyl ether/acetic acid (60:39:1 v/v) and lipids visualized. With this system, neutral lipids run near the solvent front, free fatty acids run in the middle, and phospholipids remain near the origin. The spots were scraped from the plates, collected, and reextracted in hexane/2-propanol (3:2 v/v). The samples were then transesterified, extracted, and identified by using the system described above.

Cellular cholesterol and cholesteryl ester content were determined by the *p*-hydroxyphenylacetic acid method of Gamble and colleagues (Gamble et al., 1978). Phosphatidylcholine and sphingomyelin contents were analyzed with the thin-layer chromatography technique of Rouser and co-workers (Rouser et al., 1966).

Determination of Plasma Membrane Fatty Acyl Content. Hep G2 hepatocyte plasma membranes were prepared at 4 °C by established methods (Scully et al., 1982; Segel et al., 1979). Hep G2 hepatocytes were dissociated from culture flasks by using trypsin–EDTA. The resulting cell suspensions were sedimented at 200*g* for 15 min and washed twice with 0.9% NaCl. The cells were resuspended in 15 mL of lysis medium (1 mM $NaHCO_3$/0.5 mM $CaCl_2$, pH 7.4) and disrupted in a homogenizer (25 strokes). The cell lysate was diluted to 50 mL and centrifuged at 500*g* for 20 min. The supernatant was removed and saved. The pellet was resuspended in 25 mL of lysis medium and rehomogenized. This suspension was then centrifuged at 500*g* for 20 min, and the supernatant was removed. The two supernatants were combined and sedimented at 12500*g* for 20 min. The pellet was resuspended in 10 mL of lysis medium and mixed with 30 mL of 53% sucrose prepared in lysis medium to yield a final concentration of 40% sucrose. Twenty milliliters of the 40% sucrose was layered under 15 mL of a 30% sucrose solution. This sucrose step gradient was centrifuged at 55000*g* for 4 h using a Ti70 rotor in a Beckman L8-70 ultracentrifuge. Following centrifugation, the 10-mL fraction just above the interface was removed from the 30% sucrose layer and diluted with 25 mL of lysis medium. This solution was then sedimented at 45000*g* for 1 h. The pellet was extracted with hexane/2-propanol (3:2 v/v), and determination of fatty acyl content was performed as described above. This method results in less than 5% contamination with endoplasmic reticular and mitochondrial membranes (Scully et al., 1982; Segel et al., 1979).

Plasma Membrane Fluidity Measurements. Hep G2 hepatocyte plasma membrane vesicles were incubated with DPH (Shinitzky et al., 1978) at a concentration of 1 μM for 1 h at 25 °C, after which the suspension was washed twice with Hanks'–HEPES buffer, pH 7.4. DPH at a concentration of

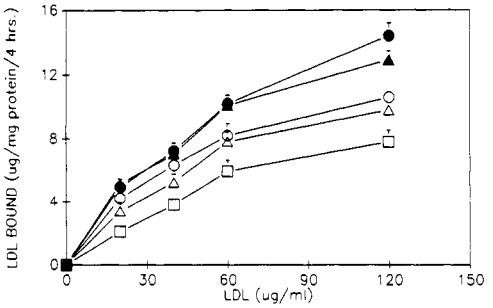

FIGURE 1: Specific binding of ^{125}I LDL by Hep G2 hepatocytes modified with specific fatty acids. Binding assays were performed as described under Methods. Data represent the average ± SEM of five experiments with control (open circles), oleate-enriched (closed circles), linoleate-enriched (closed triangles), stearate-enriched (open triangles), and palmitate-enriched (open squares) cells performed in parallel and in duplicate.

1 μM results in polarization values that are independent of the DPH concentration while the fluorescence intensity is directly proportional to the DPH-to-membrane protein ratio. Fluorescence measurements were performed with a Spex Flourolog-2 spectrofluorometer (Spex Industries, Inc., Edison, NJ) equipped with a thermostat-controlled cell holder and a polarization accessory. The steady-state fluorescence polarization was measured at 7.5, 15, 25, and 37 °C by exciting the membrane vesicle suspension at 360 nm and recording the emission at 430 nm. The polarization of fluorescence emission was calculated from the equation:

$$P = (I_{vv} - GI_{vh})/(I_{vv} + GI_{vh})$$

where P = 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 $G = I_{hv}/I_{hh}$. Values were corrected for turbidity by using the method of Lentz (Lentz et al., 1979).

RESULTS

Effect of Fatty Acyl Enrichment on LDL and Transferrin Binding. The effect of incorporating oleate, linoleate, stearate, or palmitate on specific LDL binding by upregulated Hep G2 hepatocytes is shown in Figure 1. Cis-unsaturated fatty acyl enrichment was associated with increased specific LDL binding at maximal total LDL concentrations. Oleate and linoleate enrichment resulted in a significant increase in LDL binding, 14.4 ± 0.8 μg of LDL/mg of cell protein and 12.9 ± 0.6 μg of LDL/mg of cell protein, respectively, compared with 10.6 ± 0.5 μg of LDL/mg of cell protein in control cells, 9.8 ± 0.3 μg of LDL/mg of cell protein in stearate-enriched cells, and 7.8 ± 0.8 μg of LDL/mg of cell protein in palmitate-enriched cells. Oleate-enriched cells bound 126% ($p < 0.005$) and linoleate-enriched cells bound 122% ($p < 0.02$) as much LDL as control cells. In contrast, stearate-enriched cells bound only 92% (p = NS) and palmitate-enriched cells bound only 74% ($p < 0.02$) as much LDL as control cells (Table I). The difference in binding between oleate-enriched cells and linoleate-enriched cells was not statistically significant. Palmitate-enriched cells bound only 80% as much LDL as stearate-enriched cells ($p < 0.05$). Scatchard analysis of the binding data is shown in Table II. Specific LDL binding represented 70% of total binding at maximal total LDL concentrations used.

Transferrin binding was not altered by fatty acyl modification of Hep G2 hepatocytes. At maximal total concentrations, oleate- and linoleate-enriched cells bound 2.04 pmol of transferrin/mg of cell protein and 1.87 pmol/mg of cell protein, respectively. Similarly, control cells bound 1.96 pmol/mg of cell protein while stearate-enriched cells bound 1.91 pmol/mg of cell protein, and palmitate-enriched cells bound 1.95 pmol/mg of cell protein of diferric transferrin.

Table I: Binding, Uptake, and Degradation of LDL by Hep G2 Hepatocytes[a]

	binding	uptake	degradation
control	10.6 ± 0.5	4.4 ± 0.2	2.0 ± 0.3
oleate	14.4 ± 0.8[c]	5.9 ± 0.3[c]	3.2 ± 0.3[b]
linoleate	12.9 ± 0.6	9.7 ± 0.5[d]	7.1 ± 0.3[d]
stearate	9.8 ± 0.3[b]	2.7 ± 0.2[d]	0.7 ± 0.1[d]
plamitate	7.8 ± 0.8[b]	3.2 ± 0.1[d]	0.8 ± 0.3[d]

[a] Binding values are expressed as micrograms of LDL bound per milligram of protein. Uptake and degradation values are expressed as micrograms of LDL per milligram of protein per 4 h. All assays were performed with 120 μg/mL LDL. Each value represents the mean ± SEM of five experiments performed in parallel and in duplicate. [b] $p <$ 0.02 compared with control. [c] $p < 0.005$ compared with control. [d] $p <$ 0.001 compared with control.

Table II: Scatchard Analysis of Binding Data[a]

	K_D (μg/mL)	B_{max} (μg/mg of protein)	r	p
control	84	16.4	0.736	0.005
oleate	74[c]	24.2[d]	0.5363	0.046
linoleate	60[b]	21.0[b]	0.6018	0.034
stearate	81	16.8	0.5529	0.066
palmitate	143[b]	12.0[b]	0.6529	0.038

[a] Values are derived from Scatchard plots of data from five binding experiments (Figure 1). Each experiment was performed in duplicate. K_D = dissociation constant; B_{max} = maximum binding; r = correlation coefficient. [b] $p < 0.02$ compared with control. [c] $p < 0.05$ compared with control. [d] $p < 0.005$ compared with control.

Effect of Fatty Acid Incorporation on LDL and Transferrin Uptake. The effect of incorporating oleate, linoleate, stearate, or palmitate on LDL uptake by Hep G2 hepatocytes is shown in Table I. After a 4-h incubation, oleate enrichment resulted in the uptake of 5.9 ± 0.3 μg of LDL/mg of cell protein, and linoleate enrichment resulted in uptake of 9.7 ± 0.5 μg/mg of cell protein compared to 4.4 ± 0.2 μg/mg of cell protein in control cells, 2.7 ± 0.2 μg/mg of cell protein in stearate-enriched cells, and 3.2 ± 0.1 μg/mg of cell protein in palmitate-enriched cells. Oleate-enriched cells internalized 133% ($p < 0.005$) and linoleate-enriched internalized 218% ($p <$ 0.001) as much LDL as control cells. In contrast, stearate-enriched cells internalized 61% ($p < 0.001$) and palmitate-enriched cells internalized 73% ($p < 0.001$) as much LDL as control cells. Within the cis-unsaturated fatty acyl enrichment group, oleate-enriched cells internalized 61% as much LDL as linoleate-enriched cells ($p < 0.001$). Within the saturated fatty acyl enrichment group, stearate-enriched cells internalized 84% as much LDL as palmitate-enriched cells ($p <$ 0.05).

Fatty acyl modification of Hep G2 hepatocytes did not alter receptor-specific uptake of diferric transferrin. Control cells internalized 0.083 pmol of transferrin/mg of cell protein, while oleate-enriched cells internalized 0.071 pmol/mg of cell protein, and linoleate-enriched cells internalized 0.088 pmol/mg of cell protein. Similarly, stearate-enriched cells, internalized 0.101 pmol of transferrin/mg of cell protein, and palmitate-enriched cells internalized 0.072 pmol/mg of cell protein.

Effect of Fatty Acid Incorporation in LDL Degradation. The effect of membrane incorporation of oleate, linoleate, stearate, or palmitate on LDL degradation by Hep G2 hepatocytes is shown in Table I. During a 4-h incubation, oleate-enriched cells degraded 3.17 ± 0.29 μg of LDL/mg of cell protein, and linoleate-enriched cells degraded 7.09 ± 0.34

Table III: Cellular Fatty Acyl Composition (mol %)[a]

	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C20:4
control	0.75 ± 0.43	0.90 ± 0.32	26.4 ± 1.0	15.8 ± 0.4	42.1 ± 1.7	12.7 ± 1.2	0.63 ± 0.69
oleate	0.66 ± 0.47	1.3 ± 0.21	23.8 ± 1.8[b]	12.7 ± 1.8[c]	48.9 ± 1.8[e]	11.2 ± 1.1	1.40 ± 1.0
linoleate	0.40 ± 0.40	1.2 ± 0.65	26.8 ± 1.1	18.8 ± 1.5[c]	30.8 ± 1.1[e]	20.1 ± 1.1[e]	2.35 ± 1.35
stearate	0.83 ± 0.65	1.3 ± 0.80	22.6 ± 1.5[d]	22.7 ± 1.6[e]	39.3 ± 1.4[b]	12.3 ± 0.6	0.87 ± 0.63
palmitate	1.0 ± 0.2	1.0 ± 0.1	36.3 ± 2.6[e]	10.1 ± 1.2[e]	38.4 ± 2.0[b]	10.7 ± 1.6	2.3 ± 1.3

[a] Data represent average ± SD of four experiments. Experiments were performed as described under Methods. [b] $p < 0.05$. [c] $p < 0.002$. [d] $p < 0.001$. [e] $p < 0.001$ (all p values in comparison to control).

Table IV: Plasma Membrane Fatty Acyl Composition (mol %)[a]

	C14:0	C16:0	C18:0	C18:1	C18:2	C20:4
control	1.1	14.2	15.2	55.8	6.4	7.2
oleate	1.1	12.3	13.4	61.7	6.2	5.3
linoleate	1.0	8.4	15.6	58.5	10.1	6.3
stearate	0.9	11.6	19.3	55.4	6.4	6.3
palmitate	0.4	34.0	12.9	43.5	4.4	4.8

[a] Data represent average of two experiments. Experiments were performed as described under Methods.

μg of LDL/mg of cell protein, while control cells degraded 2.04 ± 0.26 μg of LDL/mg of cell protein. Stearate-enriched cells degraded 0.69 ± 0.11 μg of LDL/mg of cell protein, and palmitate-enriched cells degraded 0.79 ± 0.30 μg of LDL/mg of cell protein. Oleate-enriched cells degraded 155% ($p < 0.02$) and linoleate-enriched cells degraded 348% ($p < 0.001$) as much LDL as control cells. In contrast, stearate-enriched cells degraded 34% ($p < 0.001$) and palmitate-enriched cells degraded 39% ($p < 0.001$) as much LDL as control cells. Comparison of oleate- and linoleate-enriched cells shows that oleate-enriched cells degraded only 45% as much LDL as linoleate-enriched cells ($p < 0.001$). Stearate-enriched cells degraded 87% as much LDL as palmitate-enriched cells (p = NS).

Quantitation of Cellular Fatty Acyl Incorporation. The cellular fatty acid profile of Hep G2 hepatocytes is shown in Table III. Oleate-enriched cells had a 2.6 mol % decrease in cellular palmitate content ($p < 0.05$), a 3.1 mol % decrease in cellular stearate ($p < 0.02$), and a 6.8 mol % increase in cellular oleate ($p < 0.001$). Linoleate-enriched cells had a 7.4 mol % increase in cellular linoleate ($p < 0.001$), accompanied by an 11.3 mol % decrease in cellular oleate ($p < 0.001$) and a 3.0 mol % increase in cellular stearate ($p < 0.02$). Cells incubated with stearate had a 3.8 mol % decrease in cellular palmitate ($p < 0.01$), a 6.9 mol % increase in cellular stearate ($p < 0.001$), and a 2.8 mol % decrease in cellular oleate ($p < 0.05$). Palmitate-loaded cells had a 9.9 mol % increase in cellular palmitate ($p < 0.001$), a 5.7 mol % decrease in cellular stearate ($p < 0.001$), and a 3.7 mol % decrease in cellular oleate ($p < 0.05$). Thin-layer chromatographic analysis of these cells showed that oleate enrichment partitioned among the phospholipid, free fatty acid, and neutral lipid classes in a 4.2:2.1:1 ratio, respectively. Linoleate enrichment partitioned in a 4.1:1.5:1 ratio. Stearate enrichment partitioned in a 3.8:1.8:1 ratio, while palmitate enrichment partitioned in a 3.4:1.7:1 ratio.

Cellular cholesterol and cholesteryl ester contents were not altered by fatty acid enrichment. The average total cellular cholesterol content was 25.2, 22.8, 24.1, 24.0, and 23.7 μg of cholesterol/mg of cell protein in control, oleate-enriched, stearate-enriched, linoleate-enriched, and palmitate-enriched cells, respectively (n = 2). The average cholesteryl ester content was 4.69, 4.72, 4.41, 4.55, and 4.60 μg of cholesteryl ester/mg of cell protein in control, oleate-enriched, stearate-enriched, linoleate-enriched, and palmitate-enriched cells, respectively (n = 2). The cholesterol-to-phospholipid content of the Hep G2 hepatocytes was not altered by fatty acyl enrichment. The C/P ratio of control cells was 1.37 while that of oleate-, linoleate-, stearate-, and palmitate-enriched cells was 1.35, 1.35, 1.40, and 1.38, respectively. Similarly, the phosphatidylcholine-to-sphingomyelin ratio was not altered in fatty acyl modified Hep G2 cells; this ratio in control, oleate, stearate, linoleate, and palmitate cells was 0.950, 0.810, 0.861, 0.929, and 0.904, respectively.

Table V: DPH Fluorescence Polarization Values[a]

	temp (°C)			
	7.5	15	25	37
control	0.341	0.285	0.279	0.233 ± 0.001
oleate	0.304	0.304	0.256	0.230 ± 0.002[b]
linoleate	0.303	0.268	0.240	0.228 ± 0.001[b]
stearate	0.354	0.342	0.317	0.239 ± 0.002[c]
palmitate	0.368	0.345	0.296	0.240 ± 0.003[c]

[a] Data for 7.5, 15, and 25 °C are the average of two experiments. Data for 37 °C are the average ± SD of four experiments. Experiments were performed as described under Methods. [b] $p < 0.05$. [c] $p < 0.01$ (all p values in comparison to control).

The plasma membrane fatty acyl composition of modified Hep G2 hepatocytes is shown in Table IV. Oleate-enriched cells had a 5.9 mol % increase in membrane oleate. Linoleate-enriched cells had a 3.7 mol % increase in membrane linoleate in association with a 2.7 mol % decrease in membrane oleate and a 5.8 mol % increase in membrane palmitate. Stearate-enriched cells had a 4.1 mol % increase in membrane stearate and a 2.6 mol % decrease in membrane palmitate. Cells enriched with palmitate exhibited a 19.8 mol % decrease in membrane palmitate accompanied by a 2.3 mol % decrease in membrane stearate, a 12.3 mol % decrease in membrane oleate, and a 2.0 mol % decrease in membrane linoleate.

Effect of Fatty Acyl Incorporation on Membrane Fluidity. The steady-state fluorescence polarization of DPH incorporated into Hep G2 hepatocyte plasma membrane vesicles was measured to assess the effect of fatty acyl incorporation on bulk membrane fluidity (Table V). The mole-weighted melting point of the plasma membrane based upon fatty acyl composition was calculated as a secondary indicator of bulk membrane fluidity. The membrane melting points for control, oleate-, linoleate-, stearate-, and palmitate-modified cells were 25.6, 25.0, 23.0, 27.2, and 35.4 °C, respectively. These data indicate that cis-unsaturated fatty acyl enrichment of Hep G2 hepatocytes is associated with lower DPH fluorescence polarization values and lower mole-weighted membrane melting points, both indicative of a more fluid membrane environment. In addition, membrane enrichment with linoleate resulted in

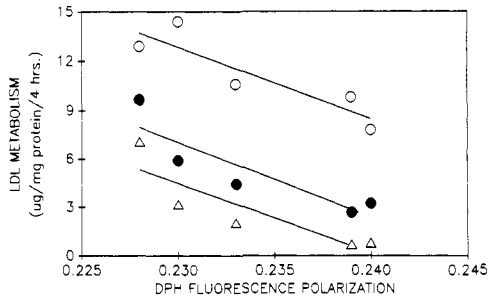

FIGURE 2: Plot of LDL binding at 4 °C (open circles), receptor-specific LDL uptake at 37 °C (closed circles), and receptor-specific LDL degradation at 37 °C (triangles) versus DPH fluorescence polarization at 37 °C ($r_{binding} = 0.898$, $p = 0.037$; $r_{uptake} = 0.893$, $p = 0.040$; $r_{deg} = 0.875$, $p = 0.050$).

increased plasma membrane fluidity compared to membrane enrichment with oleate. In contrast, saturated fatty acyl enrichment of Hep G2 hepatocytes with either stearate or palmitate results in decreased membrane fluidity as demonstrated by higher DPH polarization values and higher mole-weighted melting points.

Discussion

The mechanism by which dietary cis-unsaturated fatty acids reduce serum LDL cholesterol is unknown. Since the classical LDL receptor is the primary mediator for cellular clearance of LDL, a deficiency or dysfunction of this receptor leads to elevated circulating levels of LDL and, consequently, promotes atherogenesis. A number of steps in the processing of receptor-bound LDL can be defective. Investigators have described receptors with decreased LDL affinity, alterations in receptor clustering in coated pits, changes in endosome production, and reduced rates of LDL degradation in lysosomes (Goldstein et al., 1985; Jolleshaug et al., 1983; Lehrman et al., 1985; Zannis et al., 1985; Hobles et al., 1986). All of these processes occur within the membrane environment and, therefore, could be affected by the lipid composition of the membrane and its physicochemical properties.

Because the liver is the organ primarily responsible for clearance of LDL particles, Hep G2 hepatocytes were chosen to model closely hepatocyte function in vitro. In this study, the phospholipid fraction of Hep G2 hepatocytes has been selectively enriched with the cis-unsaturated fatty acids, oleate, or linoleate. As a result, global plasma membrane fluidity, as assessed by both DPH fluorescence polarization and calculated mole-weighted fatty acyl melting points, was increased. Other variables determining membrane fluidity, such as the cholesterol:phospholipid ratio and the phosphatidylcholine: sphingomyelin ratio, were not altered. In association with this fatty acyl enrichment, the data demonstrate a stepwise increase in LDL binding, uptake, and degradation as a function of membrane fluidity. Conversely, plasma membrane enrichment with the saturated fatty acids, stearate or palmitate, resulted in decreased membrane fluidity and decreased LDL binding, uptake, and degradation. LDL binding, uptake, and degradation in Hep G2 hepatocytes varied with DPH fluorescence polarization in a highly correlated linear manner (Figure 2).

Scatchard analysis of the binding data demonstrates alterations in both maximum LDL binding and LDL receptor affinity with either cis-unsaturated or saturated fatty acyl enrichment of the Hep G2 hepatocyte plasma membrane. Because cellular cholesterol and cholesteryl ester contents did not change, a simple increase in LDL receptor number in response to a change in cellular cholesterol stores cannot be invoked as an explanation for our observations. In contrast to the LDL receptor, transferrin receptor function was not altered by fatty acyl enrichment of the plasma membrane and alteration in membrane fluidity. This would suggest that our observations are not simply the result of a global increase in membrane fluidity or the result of a generalized alteration in the function of the clathrin-coated pit-associated proteins. Instead, enhanced LDL receptor function may occur specifically as a consequence of changes in the LDL receptor protein membrane microdomain, such as local alterations in membrane fluidity, which occur in association with the changes in global membrane fluidity, as a result of or requirements for specific fatty acyl moieties in the lipid annulus of the LDL receptor.

In other receptor systems, such as the platelet thrombin receptor, the acetylcholine receptor, and the β-adrenergic receptor, modification of both local and global membrane lipid composition and fluidity has been demonstrated to result in changes in receptor affinity for its ligand, receptor number, and coupled receptor functions (Papot et al., 1978; Jandon et al., 1983; Fang et al., 1987; North et al., 1983). Increases in the cholesterol:phospholipid ratio in the platelet membrane, with a resultant decrease in membrane fluidity, produced a 40% decrease in the thrombin receptor high-affinity association constant and a 60% increase in the number of thrombin receptor sites (Jandon et al., 1983). Functional correlation was demonstrated with a decrease in the thrombin concentration required for half-maximal aggregation from 0.35 to 0.17 nM. The acetylcholine receptor has been studied in the setting of reconstituted membranes with altered lipid composition. Ion flux activity initiated by ligand binding to the acetylcholine receptor requires the presence of both cholesterol and negatively charged phospholipids. With the use of fatty acid spin-label probes in a variety of reconstituted membranes, a correlation between membrane fluidity and both ligand-induced transitions and ion flux responses has been demonstrated (Miller et al., 1986; Aloia et al., 1988). Studies using Fourier transform infrared spectroscopy have shown that the membrane lipids necessary for the ion flux activity of the receptor also have a role in stabilizing the α-helical secondary structure essential for the optimal conformation of the acetylcholine receptor (Miller et al., 1986; Aloia et al., 1988). In reticulocyte ghosts, increasing membrane fluidity by phospholipid methylation facilitates β-adrenergic coupling with adenylate cyclase to generate cyclic AMP (Hirata et al., 1979). Other examples of transmembrane proteins whose functions are modulated by membrane fluidity and membrane lipid composition include Ca^{2+}-ATPase, Na^+–K^+-ATPase, rhodopsin, and 5′-nucleotidase (Hesbeth et al., 1976; Kimelberg et al., 1972; O'Brien et al., 1977; Merisko et al., 1981).

A number of hypotheses have been proposed to explain these observations. It has been suggested that cryptic functional sites on the receptor or cryptic receptors exist as reservoirs for adjustment of function in response to perturbations in membrane fluidity (Shinitsky, 1984). Others have suggested that lipid-induced displacement of the receptor, either vertically or laterally as a result of changes in membrane order, could contribute to changes in receptor conformation (Borochov & Shinitsky, 1976). Mathematical analyses have implied a direct relationship between receptor turnover and membrane fluidity (Shinitsky, 1984). The optimal fluidity hypothesis proposes that the overt maximal velocity of receptor function may possess a peak value at a specific membrane fluidity (Shinitsky, 1984). The exact role of any of these proposed mechanisms is still unknown.

We have shown that increasing Hep G2 hepatocyte membrane fluidity by enrichment with cis-unsaturated fatty acids is associated with enhanced LDL binding, uptake, and deg-

radation. However, the specific role of membrane fluidity in modulation of LDL receptor function is unknown. Possibilities include a fluidity-induced change in LDL receptor conformation toward a more optimal state for LDL binding or clathrin interaction, or a functional requirement for specific fatty acyl moieties in the LDL receptor lipid annulus. Clearly, LDL receptor function in the context of a more fluid membrane requires further study. It is still unknown whether other enzymes involved in cholesterol homeostasis, such as HMG-CoA reductase or acyl-CoA:cholesterol acyltransferase, can also be affected by alterations in the microsomal membrane milieu.

This study demonstrates that modification of plasma membrane composition by fatty acyl incorporation can dramatically alter LDL receptor function. In the setting of cis-unsaturated fatty acyl incorporation into the plasma membrane of Hep G2 hepatocytes, LDL binding, uptake, and degradation were greatly enhanced. In contrast, saturated fatty acyl enrichment of the plasma membrane resulted in decreased LDL binding, uptake, and degradation. LDL receptor function varied directly with membrane fluidity, as determined by DPH fluorescence polarization. This may be one mechanism by which humans and animals fed diets rich in unsaturated fatty acids manifest enhanced hepatic LDL clearance. Our results suggest a novel molecular mechanism by which exogenous lipids can alter hepatic LDL clearance.

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Registry No. Oleate, 112-80-1; linoleate, 60-33-3; stearate, 57-11-4; palmitate, 57-10-3.

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