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Free Fatty Acids Activate a High-Affinity Saturable Pathway for Degradation of Low-Density Lipoproteins in Fibroblasts from a Subject Homozygous for Familial Hypercholesterolemia†

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Received January 10, 1992

ABSTRACT: This paper describes a mechanism for degradation of low-density lipoprotein (LDL) in fibroblasts unable to synthesize the LDL receptor. In this cell line, long-chain free fatty acids (FFA) activated ^{125}I-LDL uptake; unsaturated FFA were the most efficient. The first step of this pathway was the binding of LDL apoB to a single class of sites on the plasma membrane and was reversible in the presence of ≥10 mM suramin. Binding equilibrium was achieved after a 60-90-min incubation at 37 °C with 1 mM oleate; under these conditions, the apparent K_d for ^{125}I-LDL binding was 12.3 μg/mL. Both cholesterol-rich (LDL and β-VLDL) and triglyceride-rich (VLDL) lipoproteins, but not apoE-free HDL, efficiently competed with ^{125}I-LDL for this FFA-induced binding site. After LDL bound to the cell surface, they were internalized and delivered to lysosomes; chloroquine inhibited subsequent proteolysis of LDL and thereby increased the cellular content of the particles. A physiological oleate to albumin molar ratio, i.e., 1:1 (25 μM oleate and 2 mg/mL albumin), was sufficient to significantly ($p < 0.01$) activate all three steps of this alternate pathway: for example, 644 ± 217 (25 μM oleate) versus 33 ± 57 (no oleate) ng of LDL/mg of cell protein was degraded after incubation (2 h, 37 °C) with 50 μg/mL ^{125}I-LDL. We speculate that this pathway could contribute to the clearance of both chylomicron remnants and LDL.

Low-density lipoproteins (LDL)[1] and chylomicron remnants bind with high affinity to the LDL receptor, which mediates endocytosis of both particles (Brown & Goldstein, 1986; Nagata et al., 1988). Besides this well-characterized receptor, less clearly delineated pathways significantly contribute to the removal of these lipoproteins (Shepherd et al., 1979; Goldstein & Brown, 1989). The issue of the mechanisms underlying these pathways has been addressed from two distinct perspectives: (1) a putative receptor for chylomicron remnants (Brown et al., 1991) and (2) a pathway for LDL receptor independent catabolism of LDL (Myant, 1990).

The normal rate of removal of chylomicron remnants in subjects homozygous for familial hypercholesterolemia (FH) (Rubinsztein et al., 1990) and in Watanabe heritable hyperlipidemic (WHHL) rabbits (Kita et al., 1982) led to the hypothesis that a specific receptor mediates the clearance of these particles. Recent reports have introduced (Herz et al., 1988) and support (Kowal et al., 1989, 1990; Beisiegel et al., 1989) the notion that the LDL receptor related protein (LRP) might be a chylomicron remnant receptor (Brown et al., 1991). This

† This work was supported by NIH Program Project HL25596, by Institutional Biomedical Support Grant BRSG SORR-5376, with funds provided by the Division of Research Resources, NIH, and by George S. Bel research grant-in-aid and fellowship awards to B.E.B. and F.T.Y., respectively.

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[1] Abbreviations: apo, apoprotein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FFA, free fatty acid(s); FH, familial hypercholesterolemia; HDL, high-density lipoprotein(s); LDL, low-density lipoprotein(s); LPDS, lipoprotein-deficient serum; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; VLDL, very low density lipoprotein(s); WHHL, Watanabe heritable hyperlipidemic.

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very large protein (600 kDa) structurally homologous to the LDL receptor was subsequently found to be identical in sequence to the α_2-macroglobulin receptor, which raised the possibility that LRP is a bifunctional receptor (Strickland et al., 1990). Despite these recent advances, the extent to which LRP, the LDL receptor, and possibly other plasma membrane receptors, e.g., the asialoglycoprotein receptor (Windler et al., 1991), contribute to the removal of chylomicron remnants remains to be defined. Furthermore, processes that could modulate LRP activity and thus determine its physiological role remain to be characterized (Brown et al., 1991).

Even less clearly defined are the mechanisms responsible for LDL receptor independent catabolism of LDL. In subjects homozygous for FH, the daily clearance of LDL is about 2-fold greater than in normal subjects (Simon et al., 1975), and occurs almost exclusively through LDL receptor independent mechanisms (Myant, 1990). In subjects with normal LDL receptor activity, these alternate mechanisms account for 20–50% of LDL catabolism (Shepherd et al., 1979; Myant, 1990), and their relative importance increases linearly with plasma LDL concentrations (Spady et al., 1986). The latter observation suggests that LDL receptor independent clearance of LDL is not limited by the adsorption of the particle to a finite number of sites on the cell surface, and could therefore result from pinocytosis (Lewis, 1931), a mechanism through which cells interiorize solutes without significant adsorption to the plasma membrane. Such a nonspecific mechanism cannot, however, explain several characteristics of LDL clearance in subjects with severe LDL receptor deficiency. In these individuals, the LDL fractional catabolic rate is faster than that of other plasma proteins, e.g., albumin (Shepherd et al., 1979; Hoeg et al., 1984), and the liver accounts for most of the LDL catabolism as it does in normal subjects (Hoeg et al., 1984). Furthermore, in WHHL rabbits, liver parenchymal cells and not phagocytic cells are responsible for LDL catabolism (Pittman et al., 1982). These observations are more readily explained by the model of adsorptive endocytosis which implies the interaction of LDL with a structure on the cell surface prior to internalization (Myant, 1990; Steinman et al., 1983).

Subsequently, lipoprotein binding sites, distinct from the LDL receptor, have been identified in liver plasma membranes and in purified endocytic vesicles (Nagata et al., 1988; Brissette & Noel, 1986; Jaeckle et al., 1989). The binding site characterized in liver plasma membranes recognizes both chylomicron remnants and LDL (Nagata et al., 1988), which led to the hypothesis that a common mechanism was responsible for the clearance of both particles in homozygous FH subjects (Nagata et al., 1988). Progress in this area of investigation has been hampered by the absence of an experimental model in which cultured cells, unable to synthesize the LDL receptor, degrade large amounts of lipoproteins (Nagata et al., 1988).

The present studies were undertaken to establish such a model. We utilized cultured fibroblasts with a large deletion in both LDL receptor alleles: these cells are unable to synthesize the LDL receptor protein (Goldstein & Brown, 1989; Hobbs et al., 1987). A fortuitous observation led us to suspect that free fatty acids (FFA) provide a signal activating the uptake of LDL in these cells. Such a finding was in keeping with previous observations that FFA regulate multiple steps of lipoprotein metabolism (Sammett & Tall, 1985; Saxena et al., 1989; Peterson et al., 1990) and control the activity of various membrane proteins (Corr et al., 1984; Pande & Mead, 1968) including the LDL receptor (Bihain et al., 1989). Here, we show that in FH fibroblasts, oleate activates a high-affinity saturable process that binds, internalizes, and degrades large amounts of LDL.

Experimental Procedures

Materials

$Na^{125}I$ was purchased from Amersham (Arlington Heights, IL). Free fatty acids, bovine serum albumin (A2153), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), chloroquine, and 1,2-cyclohexanedione were obtained from Sigma Chemicals (St. Louis, MO). Fatty acyl derivatives (oleyl alcohol and oleyl acetate) were purchased from NuChek Prep Inc. (Elysian, MN). Sodium suramin was obtained from FBA Pharmaceuticals (West Haven, CT). Pronase was purchased from CalBiochem (San Diego, CA). Dulbecco's modified Eagle's medium (DMEM), trypsin, penicillin–streptomycin, glutamine, fetal bovine serum (FBS), and trypan blue were obtained from Gibco (Grand Island, NY). Enzymatic colorimetric kits for determination of FFA and phospholipids were purchased from Biochemical Diagnostics, Inc. (Edgewood, NY); those for triglycerides and cholesterol were obtained from Sigma Chemicals and Boehringer Mannheim Diagnostics (Indianapolis, IN), respectively.

Cells. Homozygous FH (GM 00486A) fibroblasts were obtained from NIGMS human genetic mutant cell repository (Camden, NJ). This cell line presents a 10-kb deletion involving the first exon and promoter region of the LDL receptor gene (Hobbs et al., 1987).[2] Observations presented here were all obtained with this FH cell line. However, the FFA-activated pathway characterized in this paper was also evidenced in other FH cell lines (i.e., GM2000E, GM00701B, and GM00488C). The cells were plated in 36-mm dishes (Costar, Cambridge, MA) at a density of 150×10^3 cells per dish, and grown in a 5% CO_2, humidified incubator. FH fibroblasts achieved confluency after 4–5 days of culture in DMEM supplemented with 2 mM glutamine, 100 units/mL penicillin, 100 units/mL streptomycin, and 20% (v/v) FBS.

Lipoproteins. Very low density lipoproteins (VLDL) were isolated by ultracentrifugation (20 h, 4 °C, Beckman 50.2 Ti rotor, 245000*g*) from plasma collected at 4 °C from overnight-fasted normolipidemic subjects. The top fraction [density (d) < 1.006 g/mL] was separated by density gradient ultracentrifugation (Beckman SW41 rotor) into $VLDL_1$ (S_f 100–400), $VLDL_2$ (S_f 60–100), and $VLDL_3$ (S_f 20–60) as described by Gianturco and Bradley (1986).

β-VLDL were purified from plasma of rabbits fed normal rabbit chow supplemented with 0.5% (w/w) cholesterol as described by Kowal et al. (1989).

LDL were isolated from fresh plasma obtained from the local blood bank by sequential ultracentrifugation ($1.025 < d < 1.055$ g/mL) and iodinated to a specific activity ranging between 115 and 200 cpm/ng as previously described (Bihain et al., 1989; Goldstein et al., 1983). ^{125}I-LDL were used within a week of their isolation and filtered through 0.2-μm filters (Gelman, Ann Arbor, MI) immediately prior to use.

Selected LDL preparations were derivatized on apoB arginine residues by incubation with 0.15 M 1,2-cyclohexanedione in 0.2 M borate buffer (pH 8.1) as described by Innerarity et al. (1986). Derivatized LDL were separated from unreacted cyclohexanedione by gel filtration on Sephadex G-25 (PD-10, 1 × 10 cm) (Pharmacia, Piscataway, NJ), filtered (0.2 μm), and used within 1 h of their preparation. Proteolysis of LDL apoB was obtained by incubation (18 h, 37 °C, in the dark, under N_2) of 2.5 mg of LDL with 750 μg of pronase in

[2] J. Davignon and S. Lussier-Cacan, personal communication.

1 mL of phosphate-buffered saline: 5.4 mM KCl, 11.8 mM KH_2PO_4, 8 mM Na_2HPO_4, and 140 mM NaCl, pH 7.4 (PBS). Pronase-treated LDL were reisolated by ultracentrifugation at $d < 1.063$ g/mL (40 000 rpm, 20 h, 4 °C, Beckman 40.3 Ti rotor, 143000*g*), desalted by passing through PD10 columns, filtered (0.2 μm), and used within 1 h.

High-density lipoproteins (HDL) were prepared from plasma of overnight-fasted normolipidemic subjects by sequential ultracentrifugation ($1.085 < d < 1.21$ g/mL). HDL were dialyzed against 50 mM NaCl and 0.24 mM EDTA, pH 7.4, applied to a heparin–agarose column (1 cm × 60 cm) equilibrated in the same buffer, and eluted by gravity. The unbound fraction was collected and found by gel electrophoresis (Phastsystem, Pharmacia) to contain no apoE.

Lipoprotein-deficient serum (LPDS) ($d > 1.21$ g/mL) was prepared by ultracentrifugation (48 h, 4 °C, Beckman 50.2 Ti rotor, 245000*g*) and dialyzed against 0.15 M NaCl, pH 7.4, before use.

Lipoprotein triglyceride, cholesterol, and phospholipid concentrations were determined enzymatically following manufacturers' instructions. The protein content of lipoproteins was measured using Markwell's modification of Lowry's procedure (Markwell et al., 1981).

Methods

Incubation with FFA. FFA from a stock solution (100 mM in 2-propanol) were added to FH cells incubated with 1 mL of DMEM containing 2 mg of albumin and 10 mM HEPES (final pH 7.4). Care was taken to avoid direct contact between 2-propanol and the cell monolayer, and to prevent FFA from forming a film at the surface of the culture media. This was achieved by tilting the culture plate and introducing the pipet tip below the air–water interface. Final FFA concentrations, determined enzymatically in selected experiments, were found to be within 10% (mean variation 6.9%) of the predicted concentrations. Control studies showed that the small volume of 2-propanol added (up to 10 μL/mL) had no effect on lipoprotein uptake.

FFA in excess of albumin have been previously reported to exert cytotoxic effects which were attributed to detergent properties (Spector, 1986; Pande & Mead, 1968). To test whether FFA added to FH cells solubilized cellular protein, we determined the cell protein content after incubations with increasing oleate concentrations. Oleate (up to 2 mM) did not alter the protein content of cells incubated in DMEM containing 2 mg/mL albumin and supplemented (Figure 1, open circles) or not supplemented (closed triangles) with 50 μg/mL LDL. However, in dishes incubated with PBS containing 2 mg/mL albumin (closed circles), oleate (0.5 mM) induced a dramatic loss of cellular protein. All ^{125}I-LDL binding and uptake assays reported in this paper were conducted in DMEM containing 2 mg/mL albumin. Under these conditions, incubations with FFA did not detectably alter the cellular protein content (see figure legends). In addition, cell viability assessed by staining with 0.1% trypan blue was greater than 93% (2 mM oleate) and 95% (no oleate) after 2-h incubations at 37 °C in DMEM containing 2 mg/mL albumin.

Washing Procedure. Cell monolayers were washed at 4 °C, 3 times with 2 mL/dish of PBS containing 2 mg/mL albumin and twice with 2 mL/dish of PBS, over a 20–30-min period. This procedure minimized (1) the amount of ^{125}I-LDL spontaneously released into the media during subsequent washes and (2) nonspecific ^{125}I-LDL binding.

Binding, Uptake, and Degradation Studies. The cell monolayers were rinsed twice at room temperature with 2 mL/dish of PBS and then incubated with ^{125}I-LDL and FFA in 1 mL/dish of DMEM containing 2 mg of albumin. The amount of ^{125}I-LDL bound to the cell surface after incubation at 4 °C was determined by radioisotopic counting (LKB gamma counter) of the cells solubilized in 0.1 N NaOH. After incubations at 37 °C, the amounts of ^{125}I-LDL bound to the cell surface were determined by incubation (60 min, 4 °C) with ≥10 mM suramin in PBS, pH 7.4, and subsequent measurement of the radioactivity in the media. In experiments at 37 °C, the amount of radioactivity remaining with the cells after displacement with suramin provided an estimate of internalized ^{125}I-LDL. Degraded ^{125}I-LDL was measured by precipitation of the incubation media with 20% (w/v) trichloroacetic acid (TCA) at 4 °C followed by extraction with chloroform of the supernatant supplemented with KI and H_2O_2, as previously described (Bihain et al., 1989; Goldstein et al., 1986). The background value for LDL degradation was determined by precipitation of samples incubated under identical conditions in dishes containing no cells. The cell protein content was measured by Markwell modification of Lowry's procedure (Markwell et al., 1981).

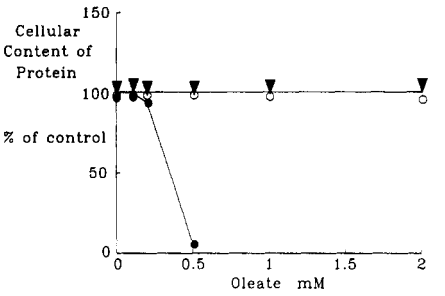

FIGURE 1: Effect of FH fibroblast incubations in DMEM or PBS with increasing oleate concentration on the cellular protein content. The cell monolayers were rinsed twice with 2 mL/dish of PBS and then incubated for 2 h at 37 °C with the indicated concentrations of oleate in 1 mL/dish of (1) DMEM containing 2 mg of albumin (▼), (2) the same media supplemented with 50 μg of LDL (○), or (3) PBS containing 2 mg of albumin (●). The cell monolayers were washed at 4 °C and solubilized in 0.1 N NaOH, and the protein content was determined. Results are the mean of duplicate determinations. The protein content measured in the absence of oleate was 148 and 135 μg/dish incubated with DMEM and PBS, respectively.

RESULTS

FFA Increased LDL Uptake in FH Fibroblasts. Investigations reported here were prompted by the observation that oleate increased ^{125}I-LDL uptake when added to the media of FH fibroblasts. This oleate-induced uptake of ^{125}I-LDL increased as a function of time and oleate concentrations (Figure 2A), as well as LDL concentrations up to 50 μg/mL (Figure 2B). With LDL concentrations ≥50 μg/mL, this process reached saturation. Comparison of the stimulatory effect of different FFA indicated that those with chain lengths ≥12 carbons increased ^{125}I-LDL uptake in FH fibroblasts (Figure 3). Unsaturated FFA were consistently more efficient than their saturated analogues (circles). FFA analogues substituted on the carboxylic group with uncharged residues [oleyl acetate or oleyl alcohol (open symbols)] also demonstrated the stimulatory effect. Since oleate is both an efficient stimulator of this phenomenon and one of the most abundant FFA in human plasma (Spector, 1986), this compound was chosen to characterize the mechanism responsible.

Oleate-Induced Uptake of LDL Led to Their Delivery to Lysosomes. To determine whether FFA-induced uptake of LDL bears potential as a final step for lipoprotein catabolism, we measured TCA-soluble non-iodide ^{125}I in the incubation media of FH fibroblasts and observed the release of LDL

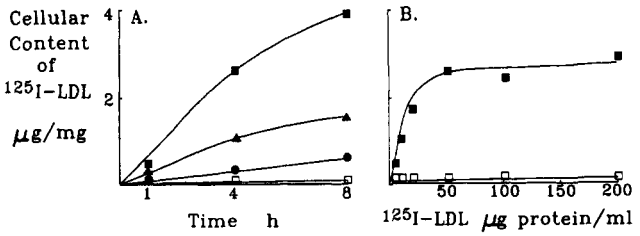

FIGURE 2: Effect of time and LDL concentration on oleate-induced uptake of ^{125}I-LDL in FH fibroblasts. FH fibroblasts were incubated at 37 °C for (A) the indicated times with 20 μg/mL ^{125}I-LDL (specific activity, 140 cpm/ng) and increasing concentrations of oleate [0 (□), 0.6 (●), 0.8 (▲), or 1 (■) mM] or (B) 4 h with (■) or without (□) 1 mM oleate and increasing concentrations of ^{125}I-LDL. The cell monolayers were washed and solubilized in NaOH, and their radioactive content was determined. Each point represents the average of duplicate samples. In experiment A, the average protein contents after 8-h incubation without and with oleate were 182 and 179 μg/dish, respectively. In experiment B, the average protein contents ± standard deviations (n = 12) were 167 ± 39 (no oleate) and 155 ± 25 (1 mM oleate) μg/dish.

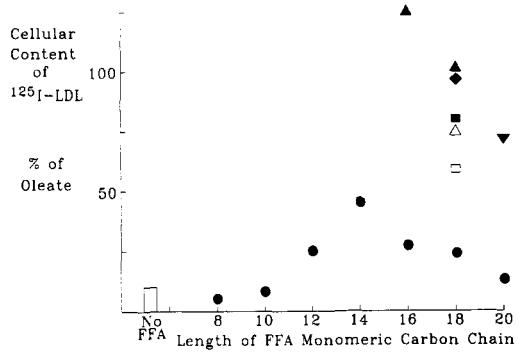

FIGURE 3: Comparison of the effect of different FFA and FFA analogues on ^{125}I-LDL uptake in FH fibroblasts. FH fibroblasts were incubated at 37 °C for 2 h with 20 μg/mL ^{125}I-LDL (specific activity, 170 cpm/ng) in the absence (open bar) or presence of FFA (1 mM) with increasing chain length and degree of unsaturation [saturated FFA (●); unsaturated FFA: one double bond (▲), two double bonds (■), three double bonds (♦), four double bonds (▼)] or uncharged FFA analogues [acetyl-18:1 (△), OH-18:1 (□)]. After this, the cell monolayers were washed and solubilized in NaOH, and their radioactive content was determined. The data, pooled from three different experiments, are expressed relative to the effect of oleate set at 100%.

degradation products (Figure 4, closed triangles) 10–20 min after the oleate-induced increased in ^{125}I-LDL uptake (closed circles). This delay suggested that a precursor–product relationship existed between these two parameters. To examine this possibility, the medium was removed after 30-min incubation with ^{125}I-LDL and oleate, and the cells were further incubated for 60 min at 37 °C in DMEM containing 5% LPDS. This chase period resulted in an 80% reduction in cell-associated ^{125}I-LDL (closed circle), and in the release of an equivalent amount of ^{125}I-LDL degradation products into the media (closed triangle). Addition of 100 μM chloroquine (open symbols, dashed lines) at the beginning of the chase period prevented both the reduction in cell-associated ^{125}I-LDL (open circle) and the further release of TCA-soluble ^{125}I into the incubation media (open triangle). Furthermore, chloroquine added concomitantly with oleate and ^{125}I-LDL inhibited in a dose-dependent manner oleate-induced degradation of ^{125}I-LDL (Figure 5, closed triangles),[3] and thereby increased

[3] A reduction of protein content was observed with cells incubated with both >5 μM chloroquine and >0.5 mM oleate. To prevent this loss, oleate concentration was reduced to 0.2 mM when FH fibroblasts were incubated with both chloroquine and oleate.

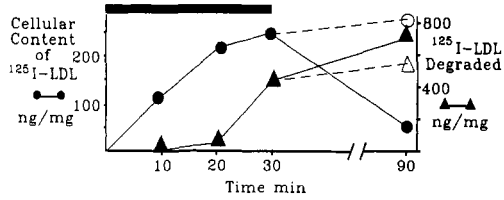

FIGURE 4: Time course of oleate stimulatory effect on ^{125}I-LDL uptake and degradation in FH fibroblasts. FH fibroblasts (181 μg of protein/dish) were incubated at 37 °C with 50 μg/mL ^{125}I-LDL (specific activity, 151 cpm/ng) and 1 mM oleate for up to 30 min (solid bar). After this, the medium was then replaced with DMEM containing 5% (v/v) LPDS (●, ▲) or with DMEM containing 5% LPDS and 100 μM chloroquine (○, △, dashed lines). ^{125}I-LDL degradation products (▲, △) present in the incubation media were determined as described under Experimental Procedures. The cell monolayers were washed and solubilized in NaOH, and their radioactive content was measured (●, ○). Each point is the mean of duplicate determinations. The amounts of ^{125}I-LDL degradation products shown at the 90-min time point represent the amounts measured after 30-min incubation plus those released into the media after 60-min chase.

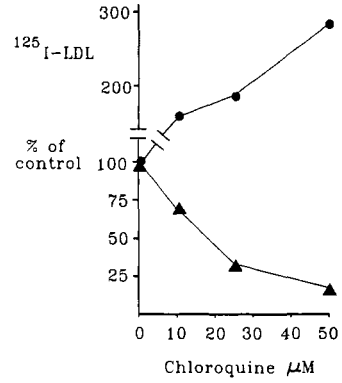

FIGURE 5: Effect of increasing chloroquine concentration on oleate-induced uptake and degradation of ^{125}I-LDL in FH fibroblasts. FH fibroblasts were incubated with 20 μg/mL ^{125}I-LDL (specific activity, 176 cpm/ng) for 90 min at 37 °C in the presence or absence of 0.2 mM oleate and the indicated concentrations of chloroquine. After this, ^{125}I-LDL degradation products (▲) in the incubation media were determined as described under Experimental Procedures; the cell monolayers were washed and solubilized in NaOH, and their radioactive content was measured (●). The data represent the differences between dishes incubated with and without oleate and are the means of duplicate determinations. The average protein content ± standard deviation (n = 8) of dishes incubated without and with oleate was 136 ± 5 and 137 ± 4 μg/dish, respectively.

the amount of ^{125}I-LDL associated with the cells (closed circles). These data indicated that oleate-induced uptake of LDL leads to their degradation in the lysosomal compartment.

Oleate Increased ^{125}I-Specific Binding at 4 °C to FH Fibroblasts. We questioned whether the first step of this pathway involved LDL binding to a limited number of sites on the plasma membrane. To characterize such binding, experiments were conducted at 4 °C, a temperature at which internalization of the plasma membrane is very slow (Steinman et al., 1983). As previously reported, oleate added to FH fibroblasts incubated at 4 °C was unable to consistently increase ^{125}I-LDL binding (Bihain et al., 1989). In contrast, FH fibroblasts that were preincubated with oleate at 37 °C and then incubated at 4 °C with both oleate and ^{125}I-LDL displayed a 7-fold increase in ^{125}I-LDL binding capacity (Figure 6, closed circles), as compared to cells treated identically except that oleate was omitted (open circles). An excess of unlabeled LDL (1 mg/mL) competed with ^{125}I-LDL for this oleate-induced binding (closed triangles). In cells incubated with oleate, the difference between total ^{125}I-LDL binding and nonspecific binding (measured in the presence of unlabeled LDL) represented ^{125}I-LDL specific binding (dashed

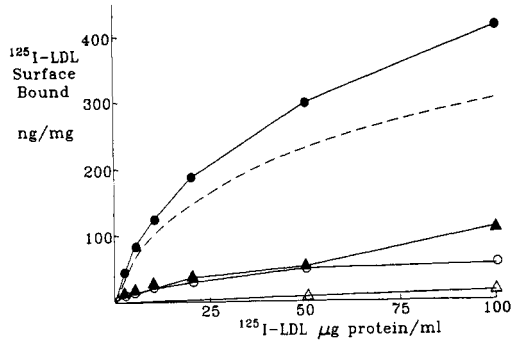

FIGURE 6: ^{125}I-LDL binding at 4 °C to FH fibroblasts preincubated at 37 °C with and without oleate. FH fibroblasts were incubated for 1 h at 37 °C without (○, △) or with 1 mM oleate (●, ▲). The cells were placed for 30 min in a cold room and then incubated for 60 min at 4 °C with oleate and ^{125}I-LDL (specific activity, 146 cpm/ng) in the presence (△, ▲) or absence (○, ●) of 1 mg/mL unlabeled LDL. The cell monolayers were washed and recovered, and their radioactive content was determined. Each point is the average of duplicate determinations. After incubation with oleate, the difference between the amounts of ^{125}I-LDL bound to cells incubated without and with unlabeled LDL represents ^{125}I-LDL-specific binding (dashed line). The average protein content ± standard deviation of dishes incubated with and without oleate was 119 ± 5 and 122 ± 8 μg/dish, respectively.

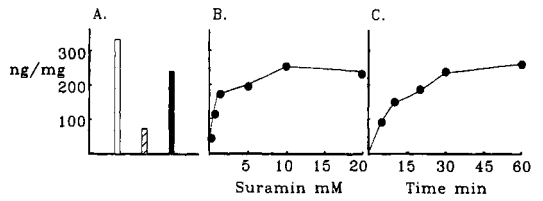

FIGURE 7: Releasing effect of suramin on ^{125}I-LDL bound at 4 °C to FH fibroblasts preincubated at 37 °C with oleate. FH fibroblasts were incubated for 60 min at 37 °C with 1 mM oleate; the cells were cooled to 4 °C and further incubated for 60 min at this temperature with both 1 mM oleate and 20 μg/mL ^{125}I-LDL (specific activity, 134 cpm/ng). After this, the cell monolayers were washed and then divided in three sets represented as sets A–C. The cells of set A were directly solubilized in NaOH, and their radioactive content was determined (open bar); the amount of ^{125}I-LDL nonspecifically bound was determined in dishes incubated with 1 mg/mL unlabeled LDL (hatched bar); ^{125}I-LDL specific binding represents the difference between total and nonspecific binding (solid bar). Cells from set B were incubated at 4 °C for 60 min with the indicated concentrations of suramin in PBS; the media were then recovered, and their radioactive content was determined. Cells from set C were incubated at 4 °C for the indicated times with 20 mM suramin in PBS, and the media radioactive content was determined. Each point is the mean of duplicate determinations. Average cellular protein content ± standard deviation was 178 ± 6, 184 ± 8, and 184 ± 10 μg/dish for the first, second, and third set of cells, respectively.

line). The latter parameter tended to saturate with increasing LDL concentrations, which was consistent with oleate revealing a limited number of LDL binding sites.

Suramin Released Specifically Bound ^{125}I-LDL from Oleate-Induced Binding Sites. Sulfated glycosaminoglycans and other polyanionic compounds have been shown to release LDL bound to the LDL receptor and to provide an estimate, after incubations at 37 °C, of the amount of LDL specifically bound to the surface of normal fibroblasts (Goldstein et al., 1976; Schneider et al., 1985). We thus searched for conditions that completely reversed oleate-induced binding of ^{125}I-LDL to the surface of FH fibroblasts. Screening experiments established that suramin, a polysulfated aromatic sodium salt, released LDL from oleate-induced binding sites. To characterize this effect of suramin, FH fibroblasts were preincubated at 37 °C with 1 mM oleate and then incubated at 4 °C with both oleate and ^{125}I-LDL. The amount of ^{125}I-LDL bound to the cell surface was determined by solubilization of one set of cells in NaOH (Figure 7A, bar graph). A second and third set of cells, which contained the same amount of ^{125}I-LDL bound to the cell surface, were used to characterize the releasing effect of suramin (Figure 7B,C). The data indicated that suramin released, in a time- and dose-dependent manner, up to 85% of surface-bound ^{125}I-LDL. The maximum amount of ^{125}I-LDL released by suramin corresponded to that estimated as specifically bound by competition with unlabeled LDL (Figure 7A, solid bar). Incubations with suramin did not reduce the cellular protein content (Figure 7, legend). Therefore, the releasing effect of suramin most likely resulted from disruption of ^{125}I-LDL binding to oleate-induced binding sites.

We then tested whether suramin inhibition of ^{125}I-LDL binding could prevent uptake and proteolytic degradation of the particle. Suramin (10 mM), added simultaneously with ^{125}I-LDL (20 μg/mL) and oleate (1 mM) to FH fibroblasts incubated for 90 min at 37 °C, inhibited oleate-induced uptake and oleate-induced degradation of ^{125}I-LDL by 87% and 90%, respectively. These data were consistent with ^{125}I-LDL binding to the cell surface preceding uptake and degradation of the particle. An estimate of the amount of ^{125}I-LDL specifically bound to the cell surface after incubation with oleate at 37 °C could therefore be obtained by subsequent displacement with suramin.

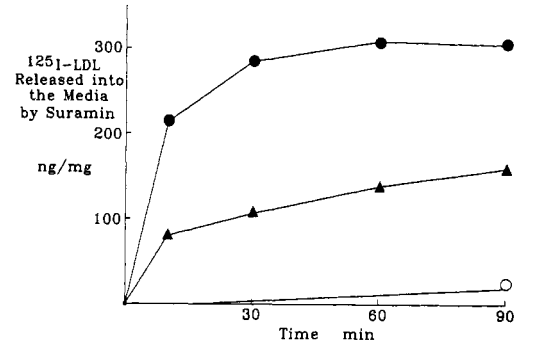

FIGURE 8: Effect of time and oleate concentration on the amount of ^{125}I-LDL specifically bound to FH fibroblasts. FH fibroblasts were incubated at 37 °C for the indicated times with 20 μg/mL ^{125}I-LDL (specific activity, 134 cpm/ng) and no oleate (○), 0.5 mM oleate (▲), or 1 mM oleate (●). After this, the cell monolayers were washed and incubated for 60 min at 4 °C with 10 mM suramin in PBS. The media were removed, and their radioactive content was determined. Each point represents the mean of duplicate determinations. The average protein content ± standard deviation was 128 ± 3, 114 ± 16, and 125 ± 12 μg/dish for dishes incubated without, with 0.5, and with 1 mM oleate, respectively.

Kinetic Characteristics of Oleate-Induced ^{125}I-LDL Binding to FH Fibroblasts. This method was used next to establish conditions in which oleate-induced binding of ^{125}I-LDL reached equilibrium. Figure 8 shows that in FH fibroblasts incubated at 37 °C with 0.5 mM oleate (closed triangles), the amount of ^{125}I-LDL that could be released by subsequent incubation with suramin, i.e., ^{125}I-LDL specifically bound to the cell surface, continued to increase throughout the duration of the experiment. In cells incubated with 1 mM oleate (closed circles), however, the amounts of specifically bound ^{125}I-LDL reached a maximum at 60 min and remained stable thereafter.

The kinetic characteristics of ^{125}I-LDL binding were then defined under these conditions of binding equilibrium. Figure 9 shows that the amount of specifically bound ^{125}I-LDL increased with LDL concentrations up to 20 μg/mL, and then saturated (Figure 9A, closed circles). An excess of unlabeled LDL reduced this binding (closed triangles) to values similar

Table I: Composition of Lipoprotein Subfractions Used in the Competition Experiment Shown in Figure 11[a]

lipoprotein subfraction	protein		cholesterol		triglycerides		phospholipid	
	mg/mL	%	mg/mL	%	mg/mL	%	mg/mL	%
VLDL (S_f 100–400)	1.3	7.8	1.0	5.8	12.1	72.2	2.4	14.2
VLDL (S_f 60–100)	0.7	9.9	0.5	7.8	4.6	68.5	0.9	13.9
VLDL (S_f 20–60)	1.2	10.7	1.0	9.0	6.9	64.2	1.7	16.1
β-VLDL	2.0	6.2	19.3	60.7	5.7	18.0	4.8	15.2
LDL	39.1	25.2	59.4	38.2	16.6	10.7	40.3	25.9
HDL	6.9	50.7	2.0	14.3	0.8	5.6	4.0	29.5

[a] Lipoproteins were isolated and characterized as described under Experimental Procedures. Each value represents the average of duplicate determinations; the percent value was calculated on a weight basis.

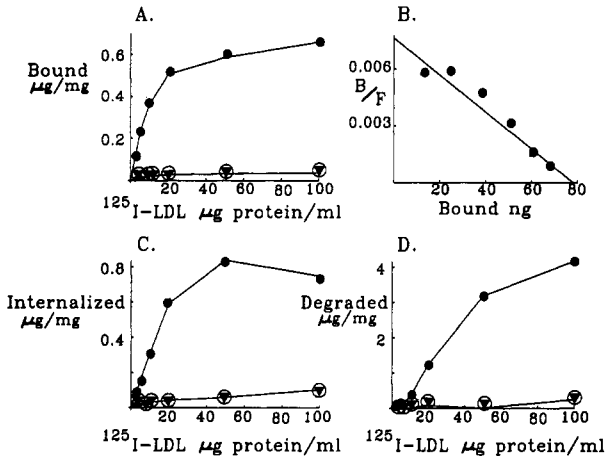

FIGURE 9: Binding, Scatchard transformation, uptake, and degradation of LDL in FH fibroblasts incubated with oleate as a function of ^{125}I-LDL concentration. FH fibroblasts were incubated for 90 min at 37 °C with (●) or without (○) 1 mM oleate and the indicated concentrations of ^{125}I-LDL (specific activity, 151 cpm/ng). An excess of unlabeled LDL (1 mg/mL) was added to one set of cells incubated with oleate (▼). After this, the cell monolayers were washed, and ^{125}I-LDL binding, uptake, and degradation were determined as detailed under Experimental Procedures. Each point is the average of duplicate samples. The dotted line represents specific binding, uptake, and degradation estimated by competition with unlabeled LDL. Scatchard transformation derived from the amount of ^{125}I-LDL released into the media by suramin (A, ●) revealed a single class of binding sites (K_d = 12.3 μg/mL, B_{max} = 78.4 ng, r = 0.97). Average cellular protein content ± standard deviation was 104 ± 4, 117 ● 2, and 96 ± 3 μg/dish for dishes incubated with oleate, with oleate and excess unlabeled LDL, and without oleate, respectively. ^{125}I-LDL concentrations used in this representative experiment were selected to provide equal number of points (n = 3) above and below the estimated K_d.

to those measured in the absence of oleate (open circles). Scatchard transformation of suramin-released ^{125}I-LDL is shown in Figure 9B. Both this analysis and curve-fitting of the data using the computer program LIGAND (Munson & Rodbard, 1980) showed that a single class of sites was responsible for oleate-induced binding of LDL. Consistent with the notion that membrane proteins account for this binding, treatment of FH fibroblasts at 4 °C with 600 μg/mL trypsin prior to incubation at 37 °C with ^{125}I-LDL and oleate inhibited oleate-induced uptake of LDL by more than 60% (data not shown).

After incubation at 37 °C and subsequent displacement with suramin, the amounts of LDL that remained associated with FH fibroblasts (Figure 9C) exceeded those specifically bound to the cell surface (Figure 9A). This contrasted with the results observed after incubation with ^{125}I-LDL at 4 °C. Suramin released into the media up to 85% of surface-bound ^{125}I-LDL (Figure 7). The simplest explanation for this difference was that during incubations at 37 °C (Figure 9), LDL were internalized and thus resisted subsequent displacement by suramin. This interpretation was supported by the observation of ^{125}I-LDL degradation products in the media of

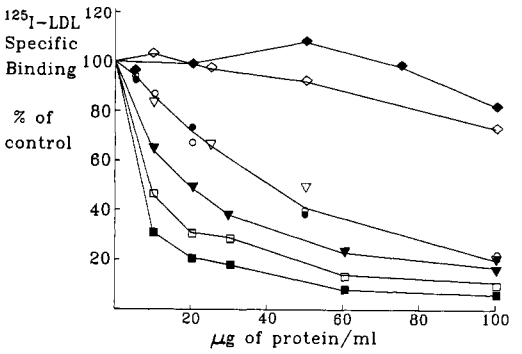

FIGURE 10: Competition of unlabeled lipoproteins with ^{125}I-LDL for binding to the oleate-induced binding site. FH fibroblasts were incubated for 2 h at 37 °C with 1 mM oleate, 20 μg/mL ^{125}I-LDL, and the indicated concentrations of unlabeled lipoproteins [$VLDL_1$ (■), $VLDL_2$ (□), $VLDL_3$ (▼), rabbit β-VLDL (○), LDL (●), Pronase-treated LDL (◊), cyclohexanedione-modified LDL (▽), HDL (♦)]. After this, the cell monolayers were washed and incubated for 60 min at 4 °C with 20 mM suramin in PBS. The media were removed, and their radioactive contents were determined. Each point is the average of duplicate determinations. Pronase-treated LDL were added on the basis of their protein content determined before proteolysis. β-VLDL, LDL, and cyclohexanedione LDL are represented by a single line because the duplicate for each point overlapped.

FH fibroblasts incubated with oleate at 37 °C (Figure 9D, closed circles). The amounts of LDL degradation products released into the media (1) tended to saturate with high ^{125}I-LDL concentrations, (2) were markedly reduced by an excess of unlabeled LDL (closed triangles), and (3) were barely detectable after FH fibroblast incubation in the absence of oleate (open circles).

Various Classes of Lipoproteins Containing ApoB Competed with ^{125}I-LDL for Binding to the Oleate-Induced Binding Site. Competition experiments were conducted to delineate the potential contribution of this pathway to the clearance of different classes of lipoproteins. These studies showed (Figure 10) that, unlike apoE-free HDL (closed diamonds) which did not efficiently compete with ^{125}I-LDL, both cholesterol-rich [rabbit β-VLDL (open circles) and LDL (closed circles)] and triglyceride-rich [$VLDL_1$ (closed squares), $VLDL_2$ (open squares), and $VLDL_3$ (closed triangles)] lipoproteins bind with high affinity to the oleate-activated binding site. The data suggested that the affinity of triglyceride-rich lipoproteins was slightly higher than that of those rich in cholesterol and that VLDL affinity for the oleate-induced binding site decreased with its progressive loss of triglycerides (Table I and Figure 10).

LDL treatment with pronase reduced their affinity for the oleate-induced binding site (Figure 10, open diamonds), consistent with LDL's sole apoprotein, i.e., apoB, contributing to the binding of this particle. Derivatization of LDL with cyclohexanedione did not detectably affect their affinity for the oleate-induced binding site (open triangles); this contrasted with an 8-fold reduced affinity of the same cyclohexanedione LDL preparation for the LDL receptor (data not shown).

Table II: Effect of Physiological FFA:Albumin Molar Ratio on ^{125}I-LDL Binding, Uptake, and Degradation in FH Fibroblasts[a]

oleate added (μM)	binding (ng/mg)	uptake (ng/mg)	degradation (ng/mg)
0	16 ± 3	34 ± 4	33 ± 57
25	30 ± 7[a]	50 ± 5[b]	644 ± 217[b]

[a] FH fibroblasts were incubated for 2 h at 37 °C with 50 μg/mL ^{125}I-LDL (specific activity, 115 cpm/ng) in the presence or absence of 25 μM oleate. After this, the cell monolayers were washed, and ^{125}I-LDL binding, uptake, and degradation were determined as described under Experimental Procedures. The results are the means ± standard deviations of six determinations. The *t*-test determined the significance of differences between cells incubated with and without oleate: [a] $p < 0.01$. [b] $p < 0.001$.

Thus, the arginine residues of apoB, although critical in mediating binding to the LDL receptor, appeared less important for binding to the oleate-induced binding site.

An Oleate:Albumin Molar Ratio of 1:1 Increased Binding, Uptake, and Degradation of ^{125}I-LDL in FH Fibroblasts. The oleate-activated pathway achieved conditions of binding equilibrium only with oleate concentrations exceeding largely the albumin binding capacity. To test whether FFA in equilibrium with albumin could also serve as a signal activating this alternate pathway, oleate was added to DMEM containing 2 mg/mL albumin to achieve a final concentration of 25 μM. This represented a FFA to albumin molar ratio of 1:1, which is typically observed in normal human plasma (Spector, 1986). Under these conditions, ^{125}I-LDL binding, uptake, and degradation were significantly ($p < 0.01$) increased (Table II). All three steps of the process activated by 25 μM oleate were inhibited by the addition of either unlabeled LDL (Figure 11, solid bar) or suramin (hatched bar). We thus concluded that the pathway characterized using oleate concentrations exceeding albumin binding capacity can also be activated by FFA in equilibrium with albumin at the molar ratio of 1:1.

Discussion

The main conclusion to be drawn from these observations is that cells unable to synthesize the LDL receptor degrade, when incubated with oleate, large amounts of ^{125}I-LDL. Indeed, in short-term incubations reported here, FH fibroblasts degrade LDL at rates equal to or slightly faster than those of normal fibroblasts expressing maximum LDL receptor activity (Goldstein et al., 1983).

Two lines of evidence indicate that lysosomes are responsible for this oleate-induced proteolysis of LDL. First, chloroquine, a known inhibitor of lysosomal function (De Duve, 1963), prevented the appearance of TCA-soluble ^{125}I in the incubation media, thereby increasing the cellular content of ^{125}I-LDL. Second, the release of TCA-soluble ^{125}I occurred 10–20 min after the increase in cell-associated LDL; this delay is consistent with the latency for delivery of internalized macromolecules into lysosomes (Berg et al., 1985).

The following observations lead us to believe that oleate-induced degradation of LDL is the consequence of oleate-induced binding of the particle to a limited number of sites on the cell surface: (1) Both LDL degradation and uptake tended to saturate with increasing LDL concentrations. (2) An excess of unlabeled LDL suppressed both cellular uptake and degradation of ^{125}I-LDL. (3) In pulse–chase experiments, the amount of ^{125}I-LDL released into the media as TCA-soluble material was equivalent to the decrease in ^{125}I-LDL cellular content. (4) Addition of chloroquine at the beginning of the chase period prevented both the decrease in ^{125}I-LDL cellular content and the release into the media of TCA-soluble ^{125}I. (5) Suramin, a polyanionic compound found to reverse ^{125}I-LDL specific binding to the cell surface, also induced the parallel inhibition of ^{125}I-LDL uptake and degradation. This analysis leads to the conclusion that oleate-induced degradation of LDL in FH fibroblasts is mediated by a three-step pathway involving binding to the cell surface, rapid internalization, and subsequent delivery into lysosomes.

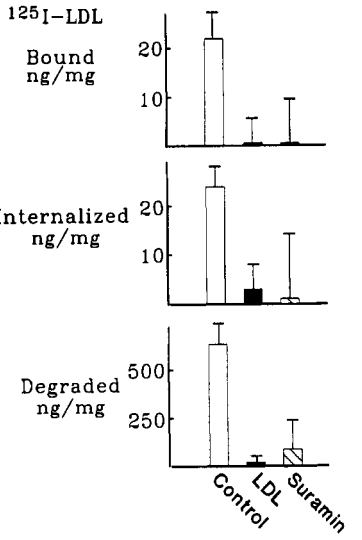

FIGURE 11: Effect of unlabeled LDL and suramin on the increase in ^{125}I-LDL binding, uptake, and degradation induced by 25 μM oleate in FH fibroblasts. FH fibroblasts were incubated for 2 h at 37 °C with 50 μg/mL ^{125}I-LDL (specific activity, 115 cpm/ng) with or without 25 μM oleate in media supplemented or not with either unlabeled LDL (1 mg/mL) or suramin (20 mM). The cell monolayers were washed, and ^{125}I-LDL binding, uptake, and degradation were determined as detailed under Experimental Procedures. The data represent the means and standard deviations ($n = 3$) of the differences between dishes incubated with and without (25 μM) oleate (open bar), or between dishes incubated with and without oleate and supplemented with unlabeled LDL (solid bar) or suramin (hatched bar). The average protein content ± standard deviation of dishes incubated without and with oleate was 101 ± 11 and 103 ± 12 μg/dish, respectively.

Scatchard transformation of ^{125}I-LDL binding to the plasma membrane of FH fibroblasts incubated with oleate is consistent with a single class of high-affinity binding sites. The inhibitory effects on this binding of cell preincubation with trypsin and of LDL pretreatment with pronase, as well as the reversibility of binding induced by suramin, are consistent with ionic interactions between membrane protein(s) and LDL apoB. Further, the oleate-induced binding site demonstrates some degree of specificity. Indeed, the oleate-induced binding of ^{125}I-LDL was (1) not inhibited by 1000-fold molar excess of albumin, (2) only marginally reduced by apoE-free HDL, and (3) more efficiently competed for by triglyceride-rich than by cholesterol-rich lipoproteins. It therefore appears that FFA reveal, in FH cells, the activity of a receptor which can mediate lipoprotein endocytosis.

LDL was chosen as primary ligand for these studies because they contain solely apoB which does not exchange among lipoproteins, and, unlike apoE, displays only few nonspecific interactions with the cell monolayer (Elovson et al., 1985). This selection, motivated by technical considerations, does not imply that apoB is the only apoprotein able to bind to the putative FFA-activated receptor. In fact, preliminary data indicate that recombinant apoE complexed to lipid emulsions is also able to compete with ^{125}I-LDL for binding to the oleate-induced binding site (Yen and Bihain, unpublished data). Investigations are currently under way to determine which apoproteins in each lipoprotein subfraction are responsible for binding and to define the binding properties of the different apoE isoforms.

The rapid stimulatory effect of FFA on LDL degradation in FH fibroblasts could result from their interaction with the cells or the LDL. FH fibroblasts preincubated with oleate at 37 °C and then cooled to 4 °C and washed to remove unbound FFA retained significantly increased LDL binding capacity (Yen and Bihain, unpublished data). Therefore, our current working hypothesis is that FFA alter the conformation of a putative receptor, unmasking its ability to bind and mediate lipoprotein endocytosis. The previous observation that FFA rapidly alter the conformation of the LDL receptor (Bihain et al., 1989) provides a precedent for such a mechanism. Stimulation of LDL degradation in FH fibroblasts occurred optimally with concentrations of FFA exceeding albumin binding capacity. These concentrations did not affect the viability of cells incubated in DMEM. Recent studies of the physical properties of FFA have shown that at pH 7.4, FFA in excess of albumin form oil and/or lamellar phases (Cistola et al., 1988, 1986). It is thus not surprising that FFA, which below pH 9 do not form micellar phases, cannot solubilize membrane proteins. The cytotoxic effect of FFA observed on cells incubated with PBS resulted therefore most likely not from FFA detergent properties but, as previously suggested (Cistola et al., 1986, 1988), from disruption of calcium and/or proton gradients (Cistola et al., 1986, 1988; Messineo et al., 1984; Watras et al., 1984).

Lamellar structures of FFA have been observed during hydrolysis of triglyceride-rich lipoproteins by lipoprotein lipase (Blanchette-Mackie & Scow, 1981; Wetzel & Scow, 1984). It is thus tempting to speculate that the local accumulation of FFA produced by lipolytic enzymes provides the signal to recruit a receptor, which could serve for the clearance of lipoproteins undergoing rapid lipolysis, i.e., chylomicron remnants. We therefore questioned whether the LRP/α_2-macroglobulin receptor was responsible for the oleate-induced degradation of lipoproteins (data not shown). Testing for competition between LDL and α_2-macroglobulin–methylamine (kindly provided by Dr. D. Strickland) indicated that in FH fibroblasts incubated with oleate, these two ligands do not compete with each other. Further, oleate had no effect on binding of α_2-macroglobulin–methylamine to its receptor (Yen and Bihain, unpublished data). This absence of competition does not rule out that one of the potential ligand binding domains of LRP (Myant, 1990; Kowal et al., 1989) accounts for the oleate-induced binding site. Further testing of this hypothesis requires a more definitive characterization of the putative FFA-activated receptor.

Another possible role for such a receptor which retains the ability to bind cyclohexanedione-modified LDL and to be activated by FFA circulating in equilibrium with albumin would be to contribute to LDL receptor independent clearance of LDL. This hypothesis bears the potential to reconcile the apparent discrepancies in the conclusions of the in vivo turnover studies (Myant, 1990). Indeed, it has to be explained why LDL receptor independent catabolism of LDL, which is characterized by some degree of tissue and ligand specificity, does not display saturability. If LDL binding to the putative oleate-activated receptor occurs by competition with rapidly lipolyzed lipoproteins or under conditions in which the receptor is not maximally recruited, i.e., low FFA concentrations (Williams, 1991), no saturation would be observed over a broad LDL concentration range. It is also striking that in the liver sinusoid, conditions are such that lipolytic products are delivered directly into the environment bathing parenchymal cells. Finally, hepatocytes are responsible for the removal of a large fraction of FFA, which circulate in equilibrium with albumin (Potter et al., 1989). The possibility therefore exists that the pathway characterized here provides a model to study a mechanism which in vivo contributes to both chylomicron clearance and LDL receptor independent catabolism of LDL.

Acknowledgments

We are grateful to Dr. Paul S. Roheim for his constant support and many useful suggestions. We greatly appreciate many fruitful discussions with Drs. Lex H. T. van der Ploeg and Christopher J. Mann, as well as their critical review of the manuscript. The suggestions and critical comments of Dr. Joseph L. Goldstein contributed to an important part of the progress of investigations reported here and are gratefully acknowledged.

Registry No. Oleic acid, 112-80-1; cholesterol, 57-88-5.

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Thermodynamics of Ion Binding to Phosphatidic Acid Bilayers. Titration Calorimetry of the Heat of Dissociation of DMPA†

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Received October 2, 1991; Revised Manuscript Received February 21, 1992

ABSTRACT: The heat of dissociation of the second proton of 1,2-dimyristoylphosphatidic acid (DMPA) was studied as a function of temperature using titration calorimetry. The dissociation of the second proton of DMPA was induced by addition of NaOH. From the calorimetric titration experiment, the intrinsic pK_0 for the dissociation reaction could be determined by applying the Gouy-Chapman theory. pK_0 decreases with temperature from ca. 6.2 at 11 °C to 5.4 at 54 °C. From the total heat of reaction, the dissociation enthalpy, ΔH_{diss}, was determined by subtracting the heat of neutralization of water and the heat of dilution of NaOH. In the temperature range between 2 and 23 °C, ΔH_{diss} is endothermic with an average value of ca. 2.5 kcal·mol^{-1} and shows no clear-cut temperature dependence. In the temperature range between 23 and 52 °C, ΔH_{diss} calculated after subtraction of the heat of neutralization and dilution is not the true dissociation enthalpy but includes contributions from the phase transition enthalpy, ΔH_{trans}, as the pH jump induces a transition from the gel to the liquid-crystalline phase. The ΔC_p for the reaction enthalpy observed in this temperature range is positive. Above 53 °C, the pH jump induces again only the dissociation of the second proton, and the bilayers stay in the liquid-crystalline phase. In this temperature range, ΔH_{diss} seems to decrease with temperature. The thermodynamic data from titration calorimetry and differential scanning calorimetry as a function of pH can be combined to construct a complete enthalpy-temperature diagram of DMPA in its two ionization states.

Differential scanning calorimetry (DSC)[1] has been used extensively for the determination of thermotropic properties of lipid model membranes composed of pure or mixed lipids and for the determination of the nature of lipid-protein interactions [for reviews, see Mabrey and Sturtevant (1979), McElhaney (1982, 1986), Bach (1984), Jones (1988), and Blume (1988, 1991)]. However, reaction calorimetry (RC) and differential titration calorimetry (DTC) have rarely been

† This work was supported by the Deutsche Forschungsgemeinschaft (Bl 182/7-1) and the Fonds der Chemischen Industrie.

[1] Abbreviations: DSC, differential scanning calorimetry; DTC, differential titration calorimetry; RC, reaction calorimetry; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPA, 1,2-dimyristoyl-*sn*-glycero-3-phosphoric acid; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; PA, phosphatidic acid; PG, phosphatidylglycerol; PS, phosphatidylserine.

0006-2960/92/0431-4636$03.00/0