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Impaired Binding Affinity of Electronegative Low-Density Lipoprotein (LDL) to the LDL Receptor Is Related to Nonesterified Fatty Acids and Lysophosphatidylcholine Content[†]

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ABSTRACT

The binding characteristics of electropositive [LDL(+)] and electronegative LDL [LDL(-)] subfractions to the LDL receptor (LDLr) were studied. Saturation kinetic studies in cultured human fibroblasts demonstrated that LDL(-) from normolipemic (NL) and familial hypercholesterolemic (FH) subjects had lower binding affinity than their respective LDL(+) fractions ($P < 0.05$), as indicated by higher dissociation constant (K_D) values. FH-LDL(+) also showed lower binding affinity ($P < 0.05$) than NL-LDL(+) (K_D, sorted from lower to higher affinity: NL-LDL(-), 33.0 ± 24.4 nM; FH-LDL(-), 24.4 ± 7.1 nM; FH-LDL(+), 16.6 ± 7.0 nM; NL-LDL(+), 10.9 ± 5.7 nM). These results were confirmed by binding displacement studies. The impaired affinity binding of LDL(-) could be attributed to altered secondary and tertiary structure of apolipoprotein B, but circular dichroism (CD) and tryptophan fluorescence (TrpF) studies revealed no structural differences between LDL(+) and LDL(-). To ascertainthe role of increased nonesterified fatty acids (NEFA) and lysophosphatidylcholine (LPC) content in LDL-(-), LDL(+) was enriched in NEFA or hydrolyzed

with secretory phospholipase A_2. Modification ofLDL gradually decreased the affinity to LDLr in parallel to the increasing content of NEFA and/or LPC. Modified LDLs with a NEFA content similar to that of LDL(-) displayed similar affinity. ApoB structurestudies of modified LDLs by CD and TrpF showed no difference compared to LDL(+) or LDL(-). Our results indicate that NEFA loading or phospholipase A_2 lipolysis of LDL leads to changes that affect the affinity of LDL to LDLr with no major effect on apoB structure. Impaired affinity to the LDLr shown by LDL(-) is related to NEFA and/or LPC content rather than to structural differences in apolipoprotein B.

[1] **Abbreviations:** LDL, low-density lipoprotein; LDL(-), electro- negative LDL; LDL(+), electropositive LDL; LDLr, LDL receptor; oxLDL, oxidized LDL; acLDL, acetylated LD.; IL-8, interleukin 8; MCP-1, monocyte chemotactic protein 1; VCAM-1, vascular cell adhesion molecule 1; PAF-AH, platelet activating factor acetylhydrolase; LPC, lysophosphatidylcholine; NEFA, nonesterified fatty acids; NEFA-LDL, NEFA-modified LDL; FCS, fetal calf serum; LPDS, lipoprotein-deficient serum; apo, apoprotein; TNBS, trinitrobenzenesulfonic acid; $sPLA_2$, secretory phospholipase A_2; $sPLA_2$-LDL, $sPLA_2$- modified LDL; Lp(a), lipoprotein (a); MDA, malondialdehyde; DGPE, dipalmitoylglycerophosphodimethylethanolamine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; DiI, 1,1'- dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; K_D, dissociation constant; K_i, inhibition constant; IC_{50}, inhibitory concentration 50%; PMA phorbol myristate acetate; SRA, scavenger receptor A; TLC, thin-layer chromatography; CD, circular dichroism; TrpF, tryptophan fluorescence.

INTRODUCTION

Qualitative modification of low-density lipoproteins (LDL)[1] is considered to play a key role in the initiation and development of atherosclerosis (*1*). Although most studies focused on the modification of LDL in the arterial wall, minor subfractions of modified LDL have been detected in plasma, and its concentration has been related to cardiovas-cular risk (*2*, *3*). The presence in human plasma of a modifiedelectronegative LDL subfraction has been reported by severalgroups (*4-6*). Electronegative LDL [LDL(-)] has been suggested to be involved in atherogenesis since in cultured endothelial cells these particles induce cytotoxicity (*7*, *8*), apoptosis (*9*), and production of leukocyte recruitment mediators, such as interleukin 8 (IL-8), monocyte chemotactic protein 1 (MCP-1) (*10*, *11*), and vascular cell adhesionmolecule 1 (VCAM-1) (*12*). It has been suggested that the preferential association of platelet-activating factor acetyl- hydrolase (PAF-AH) to LDL(-) (*13*) plays a role in the atherogenicity of LDL(-) by the production of inflammationmediators such as lysophosphatidylcholine (LPC) and non-esterified fatty acids (NEFA). Further evidence of the relationship between LDL(-) and atherogenesis is that the LDL(-) proportion is increased in the plasma of subjects athigh cardiovascular risk, i.e., hypercholesterolemic, hyper- triglyceridemic,

diabetic, and hemodialysis patients (*14-18*). Several mechanisms, such as oxidation (*4*, *19-22*), nonenzymatic glycosylation (*16*, *17*), cross-linking of apoprotein B (apoB) and hemoglobin (*18*), postprandial lipemia (*23*), or increased nonesterified fatty acid (NEFA) content (*24*), have been considered to be involved in LDL(-) production. Whatever the mechanism, an increased residence time in plasma owing to a lower clearance rate could be related to the production of LDL(-). The residence time of LDL in plasma is mainly determined by its binding to the LDL receptor (LDLr). However, studies evaluating LDL(-) affinity to LDLr are scarce and controversial since higher (*25*), similar (*4*), and lower (*5*) binding affinities to those of native LDL have been reported. Our group previously reported that a higher expression of LDLr after simvastatin treatment decreased the proportion of LDL(-) in familial hypercholesterolemic (FH) patients (*14*), thereby suggesting a link between the residence time of LDL and generation of LDL(-). This link was recently strengthened by the observation that simvastatin treatment increased the affinity of LDL-(-) isolated from FH patients (*26*) measured by binding displacement experiments. The aim of the current work was to confirm by kinetic saturation experiments the affinity of LDL(-) from normolipemic (NL) and FH subjects to the LDLr and to obtain further knowledge of the molecular determinants of this binding. For this purpose, the secondary and tertiary structures of apoB were studied by circular dichroism and tryptophan fluorescence. In addition, the role of increased NEFA and lysophosphatidylcholine (LPC) content in LDL, characteristic features of LDL(-) (*10*, *11*, *13*, *20*), was also studied.

EXPERIMENTAL PROCEDURES

Subjects. FH and NL subjects were selected as described (*11*). Total cholesterol in FH patients was >8.5 mM, LDL cholesterol >5.5 mM, and triglyceride <2 mM. NL had total cholesterol <5.0 mM and triglyceride <1 mM. Hyperglycemic subjects and smokers were not included in the study. Subjects were from 32 to 52 years of age. Plasma samples were obtained in EDTA-containing Vacutainer tubes and stored at -80 ºC for binding studies since it was not possible to obtain the necessary amount of FH-LDL from fresh plasma. Structural studies were performed with fresh plasma.

Reagents. All reagents were of analytical grade and were provided by Sigma unless otherwise stated. Culture medium, fetal calf serum (FCS), and additives were obtained from BioWhittaker. Human fibroblasts and $P388_{D1}$ macrophages were obtained from the American Type Culture Collection (ATCC).

Isolation of Total LDL, LDL(+), and LDL(-). Plasma aliquots were pooled (30-50 mL of plasma obtained from 6 to 10 subjects were used in each experiment) to obtain an amount of LDL sufficient for binding experiments. Total LDL (1.020-1.050 g/mL) was isolated by sequential flotation ultracentrifugation at 4 ºC in the presence of 1 mM

EDTA as described (*10*). Lipoprotein-deficient serum (LPDS) for cell cultures was obtained by sequential ultracentrifuga-tion (density >1.210 g/mL). Two fractions of LDL differingin their electric charge were separated by preparative anion-exchange chromatography (HiLoad 26/10 Q-Sepharose highperformance column; Amersham) using a multistep NaCl gradient in an AKTA-FPLC system (Amersham), as described (*11*). LDL(+) eluted at 0.24 M NaCl and LDL(-)at 0.60 M NaCl. Both fractions were concentrated by ultracentrifugation, and lipid and apoprotein composition wasdetermined by commercially available methods. Total cholesterol, free cholesterol, triglyceride, apoB (Roche Diagnostics), phospholipid, nonesterified fatty acids (NEFA), apoE, and apoC-III (Wako Chemicals) were determined ina Hitachi 911 autoanalyzer. The possibility of lipoprotein(a) [Lp(a)] contamination was determined by the immuno- turbidimetric method (Roche); amounts of Lp(a) lower than1% and 3% in LDL(+) and LDL(-), respectively, were obtained. Antioxidants and malondialdehyde (MDA) in LDLwere quantified by reverse-phase HPLC as described (*28*, *29*). Results were expressed as percent of LDL mass or as moles per mole of apoB. Trinitrobenzenesulfonic acid (TNBS) reactivity of free amino groups in LDL was analyzedas described (*30*). Agarose electrophoresis (Midigel; Bio- Midi) was routinely performed in all preparations to confirm increased electronegativity of LDL(-).

Preparation of Modified LDLs. Oxidized LDL (oxLDL) was obtained after incubation of PBS-dialyzed total LDL (0.2 g of apoB/L) with 10 μM $CuSO_4$ for 24 h at 37 ºC (*10*). Acetylated LDL (acLDL) was prepared by sequential additions of acetic anhydride, as described (*31*). NEFA- loaded LDL (NEFA-LDL) was obtained by incubation of total LDL (0.5 g/L of apoB) with a mixture of increasing concentrations of NEFA (0, 0.25, 0.5, and 2 mM) for 4 h at 37 ºC in the presence of albumin, as described (*24*). $sPLA_2$-treated LDL ($sPLA_2$-LDL) was obtained by incubation of total LDL (1 g/L of apoB) with increasing concentrationsof purified $sPLA_2$ (secretory PLA_2 type IIA from bee venom;Sigma reference no. P9279) (0, 1, 5, and 10 ng/mL) for 2 h at 37 ºC in the presence of 45 g/L albumin in a buffer of 5 mM HEPES, 5 mM $CaCl_2$, 2 mM $MgCl_2$, and 140 mM NaCl,as described (*32*). Modified LDLs were filtered through 0.22μm, concentrated by ultracentrifugation, and dialyzed againstbuffer A (10 mM Tris, 1 mM EDTA, pH 7.4). Nondenaturing polyacrylamide gradient gel electrophoresis (GGE) was performed to evaluate differences in electric charge (gel scanned at 1 h of electrophoresis) and differences in size (the same gel scanned at 14 h of electrophoresis). These gelswere also used to observe the presence of aggregates after LDL modification. LDLs were prestained with Sudan Black, and GGE was performed as described (*15*). ApoB integrity was assessed by SDS-PAGE in 4-15% polyacrylamide gels(Bio-Rad).

Phospholipid Measurement. Phosphatidylcholine degradation and LPC increase mediated by $sPLA_2$ were monitored by normal-phase high-performance liquid chromatography as described (*33*), with some modifications.

Briefly, 50 μL of the internal standard dipalmitoylglycerophosphodimetylethanolamine (DGPE; Sigma reference no. P0399) at 20 g/L in ethanol was added to 450 μL of LDL (0.5 g of apoB/L), and lipids were extracted according to Bligh and Dyer. The pellet was resuspended with 200 μL of hexane/2- propanol/water (6/8/1 v/v/v), and 20 μL of this solution wasinjected into a normal phase column (Hibor Lichrosorb Si60,5 μm; Merck) at a flow rate of 0.6 mL/min. The mobile phase was acetonitrile/methanol/5 mM ammonium sulfate (56/23/6 v/v/v). Peaks were detected at 206 nm using a photodiode array detector (Model 168; Beckman). Phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (SM), and lysophosphatidylcholine (LPC) peaks were resolved by this method.

Fluorescent Labeling of LDL. Total native LDL, LDL- (+), or LDL(-) fractions isolated from NL or FH were labeled with the fluorescent probe 1,1'-dioctadecyl-3,3,3',3'- tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes), as described (*34*). A stock solution of DiI was prepared by dissolving 30 mg of DiI in 1 mL of dimethyl sulfoxide (DMSO). This solution was mixed with LDL to yield a final ratio of 300 mg of DiI to 1 g of apoB. This mixture was incubated for 18 h at 37 ºC with gentle shaking. After incubation, LDL was concentrated by ultracentrifugation, filtered through 0.22 μm, and dialyzed against buffer A. Specific activity was calculated from standard solutions of DiI and DiI-LDL prepared in 2-propanol. Specific DiI-LDL activity was expressed as milligrams of DiI per gram of apoB.

Fibroblast Culture. Human fibroblasts were obtained fromskin explants. Cells were grown in a humidified incubator (5% CO_2) at 37 ºC with Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 0.1 IU/L penicillin, 100 mg/L streptomycin, and 10% fetal calf serum (FCS) (complete DMEM). Cells were grown to confluence in 75 cm^2 culture flasks. For binding experiments,confluent cells were separated with a trypsin/EDTA solution (0.05%/0.02%) and 5 x 10^4 fibroblasts/well were seeded in 12-well plates in complete medium (day 1). DMEM was replaced on day 3 and on day 5. When the cells reached confluence (day 5 or 6), complete medium was again removed and replaced by DMEM containing 10% human lipoprotein-deficient serum (LPDS) (deficient medium). Cells were incubated with deficient medium for 48 h to stimulate LDLr expression, and binding experiments were then performed.

Studies of Binding Affinity to LDLr. Binding experimentswere performed essentially as described by Innerarity et al. (*35*). Prior to the experiment, cells were placed for 30 min at 4 ºC. Cells were then switched to cold DMEM containing10% LPDS and 10 mM HEPES. All LDLs used in cell experiments were dialyzed against DMEM by gel filtration in Sephadex G-25 PD-10 columns (Amersham Biosciencies)immediately prior to assay.

(A) Saturation Kinetic Studies. DiI-LDL was filtered through 0.22 μm to prevent the presence of large aggregates. Increasing concentrations (2.5-100 mg of apoB/L) of each type of DiI-LDL, in triplicate, were then incubated with fibroblasts for 3 h at 4 ºC under gentle shaking. Nonspecificbinding was determined by adding a 10-fold excess of unlabeled LDL, in duplicate. Nonspecific binding was subtracted from total binding to calculate specific binding. At the end of the incubation, cells were washed twice with PBS containing 2 g/L bovine serum albumin (BSA) and twice with PBS alone. Then, 1 mL of 2-propanol was addedto each well, and plates were shaken for 15 min. The extractwas centrifuged at 3000 rpm for 15 min, and 200 μL was transferred to a 96-well plate. Fluorescence was measuredin a Perkin-Elmer LS-50B spectrofluorometer with excitationand emission wavelengths at 520 and 578 nm, respectively,as described (*34*). Cells on the plates were dissolved in 1 N NaOH for protein determination by the method of Bradford (*36*). Scatchard plots deduced from the saturation kineticswere used to determine the dissociation constant (K_D) (*37*).

(B) Binding Displacement Studies. A similar procedureas in saturation kinetics described above was used. In thiscase, a fixed concentration of total DiI-LDL from NL or FHsubjects (25 mg of apoB/L) was added to the cells, andincreasing concentrations of unlabeled LDL (0-200 mg of apoB/L) were used to displace the binding of DiI-LDL. Theamount of unlabeled LDL necessary to displace 50% of thebinding (IC_{50}) was determined and then used to calculatethe inhibition constant (K_i) according to Cheng-Prusoff (*38*).

Macrophage Culture. Murine macrophages of the cell line $P388D_1$ were grown in 75 cm^2 culture flasks in RPMI medium containing 10% FCS supplemented with glutamine (2 mM), 0.1 unit/L penicillin, and 100 mg/L streptomycin (complete medium). Confluent cells were scraped, and 5 $x10^4$ cells/well were seeded in six-well plates containing 1mL of complete medium (day 1). On days 3 and 5, completemedium was replaced by fresh medium. Phorbol myristateacetate (PMA) (50 μg/L) was added on day 5, and the cellswere incubated for 48 h to induce scavenger receptor A (SRA) expression (*39*). On day 7, medium was replaced bydeficient medium (RPMI containing 10% LPDS, without PMA), and LDLs were added to cells to measure the intracellular accumulation of esterified cholesterol.

Intracellular Accumulation of Esterified Cholesterol. LDLswere dialyzed against buffer A by gel filtration and filtered through 0.22 μm. Fifty milligrams of apoB/L of each LDL was added to cells for 72 h. On day 10, cells were washed twice with PBS containing 2 g/L BSA and twice with PBS alone and detached with a cell scraper. An aliquot of the cellular solution was used to determine the protein concentration by the method of Bradford. A second aliquot of cellswas centrifuged in a glass tube at 2000 rpm for 10 min, andthe supernatant was discarded. Lipids were extracted with hexane/2-propanol (3/2) by shaking for 30 min, and the mixture was centrifuged at 2000 rpm for

10 min. The supernatant was transferred to a conic-bottom glass tube and evaporated with N_2. Esterified cholesterol, triglyceride, freecholesterol, and phospholipids were separated by thin-layerchromatography (TLC) in silica gel plates (LK5DF 5 20;Whatman) as follows. The pellet was resuspended with 40 μL of chloroform, and 5 μL was spotted 1 cm from the edgeof the plate. Heptane/diethyl ether/acetic acid (74:24:4) wasused as the first solvent to 10 cm of the plate and heptaneas the second solvent to 14 cm. The plate was dried with a hot air stream and dipped in the staining solution (10 g of phosphomolybdate, 10 mL of sulfuric acid, 200 mL ofethanol) for 1 min. Plates were then incubated in an oven at 100 ºC for 7 min. A standard curve using cholesteryl linoleate, triolein, and free cholesterol (at 1, 2.5, 5, and 12.5g/L) was performed in each experiment to measure theamount of intracellular esterified cholesterol accumulated inmacrophages. The optical density of the bands was quantifiedin a Gel Doc densitometer (Bio-Rad).

Structural Studies.

(A) Circular Dichroism. Circular dichroism (CD) spectra of native and modified LDLs (50 mg of protein/L in PBS) were determined in a JASCO J-715 spectropolarimeter in the far-UV region (190-250 nm) as described (*40*). The study was also performed in buffer A. An 0.2 cm quartz cuvette was used, and the temperature wasmaintained at 25 ºC. Twenty spectra were averaged for each measurement, and the buffer blank was subtracted. Theresulting spectra were noise-reduced by the J-715 Standard Analysis program (Jasco) prior to their deconvolution. CD spectra were deconvoluted using the DICHROWEB facility(Birkbeck University, London) to calculate the relative contribution of α-helix, β -sheet, turns, and random coilsecondary structures (*41*, *42*). The CDSSTR analysis program with reference set number 4 and a mean residue weight of 113 were used.

(B) Tryptophan Fluorescence. Tryptophan fluorescence emission spectra (310-370 nm) of native and modified LDLs (50 mg of protein/L in PBS) were determined in a Perkin-Elmer LS-50B spectrofluorometer using 295 nm excitation, as described (*40*). Ten spectra were averaged for each measurement.

Statistical Analysis. Results are expressed as the mean ± SD. A Sigma Stat 2.0 statistical package was used for statistical analysis. Differences between groups were testedwith a Wilcoxon T test for paired data [LDL(+) vs LDL(-)] and a Mann-Whitney U test for unpaired data. A *P* value <0.05 was considered significant.

RESULTS

Characterization of LDL(+) and LDL(-) Binding to LDLr. Apolipoprotein and lipid composition of LDL sub- fractions concurs with previously published results (*10*, *11*, *26*). LDL(-) presented a higher content of triglyceride, free cholesterol, NEFA, apoE, and apoC-III compared with LDL-(+) in both NL and FH samples (Table 1). No evidence of

oxidative modification in LDL(-) was observed, since TNBS reactivity, MDA, and antioxidant content were similar between LDL(+) and LDL(-) (Table 1). Representativesaturation kinetics of LDL(+) and LDL(-) isolated from both groups of subjects are depicted in Figure 1. The insert shows the Scatchard plot from which K_D was calculated. These experiments demonstrated that LDL(-) from bothgroups had lower binding affinity to LDLr than LDL(+), as higher K_D values indicate (Table 2). Interestingly, LDL(+) from FH also had lower affinity than LDL(+) from NL. Plasma freezing did not modify the affinity of LDL sub- fractions since K_D values obtained with fresh samples in preliminary experiments with LDL subfractions from NL subjects (n=2); LDL(+), 12.3 ± 2.3 nM; LDL(-), 31.9 ± 3.3 nM) were similar to that observed in frozen samples. The impaired affinity of LDL(-) was confirmed by binding displacement studies (Figure 2). In these experiments, acLDL and oxLDL were poor displacers of DiI-LDL whereas LDL(-) displaced the binding of DiI-LDL with lower efficiency than LDL(+). The calculation of IC_{50} and K_i according to Cheng-Prussoff confirmed results obtained by saturation kinetics (Table 2); i.e., LDL(-) from both groups presented lower affinity to the LDL receptor than LDL(+). In addition,LDL(+) from FH also displayed lower affinity than LDL- (+) from NL subjects. In addition to the recognition of LDLby LDLr, several cellular receptors for modified lipoproteinshave been described. Since increased electronegativity is a requisite for LDL to scavenger receptor (SR) recognition andfoam cell formation (*43*), the ability of LDL(-) to inducethe accumulation of cholesteryl esters in the macrophage cellline P388D_1, known to express the SR type A (SRA), was examined. Acetylated LDL induced the intracellular ac-cumulation of cholesteryl esters in macrophages 6-fold compared to LDL(+) from both groups, but LDL(-) failed to increase the accumulation of cholesteryl esters (Figure3), indicating that the increase in negative charge of LDL(-) is not sufficient to be recognized by SRA.

Structural Studies. Apoprotein B-100 (apoB) is the major natural ligand for the LDLr. Specific epitopes of apoB enriched in Lys are determinants in the binding to its receptor, and the maintenance of apoB structure is necessary for an optimal interaction (*44*). It was previously reported that apoB in LDL(-) presents a loss of secondary structure and conformation (*40*). For this reason we explored the possibility that our LDL(-) samples also presented structuralmodifications in the secondary and tertiary structures of apoB determined by CD and TrpF, respectively. A representative experiment of seven independent determinations of CD and TrpF spectra including LDL(+), LDL(-), and oxLDL from normolipemic subjects is shown in Figure 4A. CD spectra of LDL(+) and LDL(-) were almost identical in all the experiments. The deconvolution of CD spectra indicates a similar relative proportion of α-helix, β-sheet, β-turns, and random coil secondary structures between LDL(+), LDL(-), and oxLDL (Table 3). These dataroughly agree with those of most authors although a great dispersion of the relative proportion of each secondary structure is reported in the literature (*45*). The high dependence of the CD spectrum on environmental conditions, such as ionic strength, pH, or temperature (*46*), could contribute to explaining these discrepancies. Thus, the same LDL samples dialyzed in buffer A (low ionic strength) showed different proportions of each

structure [LDL(+) α-helix, 50.3 ± 1.6; β-sheet, 18.0 ± 1.2; β-turn, 15.5 ± 1.4; random coil, 16.3 ± 1.8; LDL(-) α-helix, 52.5 ± 1.4; β-sheet, 19.1 ± 1.3; β-turn, 13.4 ± 1.9; random coil, 15.5 ± 0.8; $n = 2$] compared with samples in PBS (Table 3), although without differences between LDL(+) and LDL(-). The increased β-conformation in PBS could be due to stabilization of the packing of this amphipathic structure on the surface of the particle at high ionic strength.

Concerning TrpF, similar spectra were obtained with LDL(-) and LDL(+) (Figure 4B), and no buffer effect was observed (data not shown). The emission maximum wavelength of LDL(-) was not shifted with respect to LDL(+) (Table 3) as would occur if the Trp environment hydrophobicity had changed due to tertiary structural rearrangementsof apoB. The oxLDL spectrum presented a 10-fold lower emission fluorescence (Figure 4B) and was red shifted (Table4). The decrease in intensity in oxLDL was due to oxidationof Trp residues, whereas the red shift indicated changes in the tertiary packing of apoB in oxidized LDL.

Effect of NEFA Loading and sPLA$_2$ Treatment on the Binding Affinity of LDL to LDLr. Increased NEFA associated with LDL is a hallmark of LDL(-) (*10*, *11*, *13*, *26*). The NEFA content of LDL modifies several characteristics of LDL surface, including electric charge and fluidity (*24*, *27*),with these properties being known to affect binding to LDLr. LPC is also increased in LDL(-) compared to LDL(+) (*13*, *20*). With the aim of studying a putative role of increased content of these polar lipids in the decreased binding affinityof LDL(-), the NEFA content in LDL(+) was increased by two methods: incubation with a mixture of NEFA andlipolysis mediated by sPLA$_2$. sPLA$_2$ treatment also increased the content of LPC in LDL(+). Approximately 20-30% of LPC and NEFA generated by hydrolysis of PC remained bound to LDL particles in our assay conditions. Both methods rendered a progressive increase in NEFA associated with LDL (Table 4) and electrophoretic mobility due to electric charge (Figure 5A). However, whereas NEFA loading did not modify the remaining lipid components of LDL(+) (data not shown), sPLA$_2$ treatment modified the relative content of phospholipids in LDL(+) (Table 5). LDL size progressively decreased after sPLA$_2$-mediated lipolysisand NEFA loading (Figure 5B). The possibility that oxidative modification could affect LDL after incubation with NEFA or sPLA$_2$ was ruled out by measuring TNBS reactivity, MDA, and antioxidant content (Table 4). Modification of LDL(+) by both methods promoted a progressive loss of affinity to the LDLr, demonstrated by binding displacement experiments, which was more evident in sPLA$_2$-treated LDL(Figure 6). At a NEFA content similar to that observed in LDL(-) (NEFA-LDL at 0.25 mM and PLA$_2$-LDL at 1 ng/mL), the loss of affinity was similar to that displayed by LDL(-). CD and TrpF (Table 3) studies revealed that no change in secondary or tertiary structure, measurable by these methods, occurred after LDL modification, suggesting that major conformational changes in apoB are not required to explain the decreased affinity of LDL(-) to the LDLr.

DISCUSSION

Reports studying the affinity of LDL(-) to the LDLr are scarce, contradictory, and not conclusive (*4*, *5*, *7*, *25*). These studies evaluated the affinity of LDL(-) from normolipemic (NL) subjects and were based on binding displacement experiments. Our results confirm the impaired affinity of LDL(-) to the LDLr by saturation kinetic and binding displacement experiments in both NL and FH subjects, in accordance with previous reports by our laboratory (*26*) andothers (*4*). LDL(-) was poorly recognized by LDLr, and in addition, macrophage SRA receptors did not bind this modified lipoprotein (*4*), suggesting that its residence in plasma would increase and, as a consequence, the inflammatory action of LDL(-) particles would persist for a longertime. Furthermore, this increased presence in circulation is likely involved in further modification of LDL(-), since all of the mechanisms suggested to promote LDL(-) formation,including oxidation (*4*, *19-22*), glycosylation (*16*, *17*), phospholipase modification (*13*), cross-linking with hemoglobin (*18*), or NEFA enrichment (*24*), would be exacerbated with increasing residence time. This possibility is supportedby the observation that simvastatin therapy in FH subjects, known to increase plasma clearance of LDL, decreases the proportion of LDL(-) (*14*, *26*); interestingly, simvastatin also improved affinity of LDL(-) to the LDLr, suggesting that a decrease in the time in blood of LDL reduces its modification(*26*).

Determinants of impaired binding of LDL(-) are currently unclear. The binding of LDL to its receptor is mediated by positively charged Lys residues in apoB that show affinity to negatively charged Cys residues in the binding domainof LDLr. The abolition of Lys in apoB causes the loss of affinity of modified LDLs such as oxidized or acetylated LDL (*44*). However, TNBS reactivity of LDL(-) and LDL-(+) was similar, indicating that Lys residues are intact in LDL(-). This was supported by the lack of evidence of oxidative modification in LDL(-), in accordance with previous reports by our group (*10*, *11*, *26*) and others (*5-7*, *25*).

Another mechanism that could impair the binding of LDL(-) is an abnormal conformation of apoB. This possibility was explored by CD and TrpF, since it had previously been described that LDL(-) presents loss of secondary and tertiarystructures of apoB (*40*). Our results indicate that the overallsecondary structure of apoB in LDL(-) is essentially identical to that of LDL(+). Since major differences between LDL(+) and LDL(-) are density and triglyceride content, our data agree with those of other authors indicating that differences in size, density (*47*), and triglyceride content (*48*,*49*) do not result in changes in the secondary structure of LDL measured by CD. Concerning oxLDL, it was previouslyreported that oxidative modification of LDL (*50*) did not modify the secondary structure of LDL. Our data disagree with those of Parasassi et al. (*40*), who reported loss of α-helix and increase in random coil in LDL(-) compared to LDL(+). An explanation for these differences is unclear although it could be related to differences in the density rangeused to isolate total LDL. Thus, we isolated LDL at 1.019-1.050 g/mL to avoid the presence of contaminating Lp(a) whereas Parasassi et al. isolated LDL at 1.019-1.063 g/mL.In this context, Chi Chen et al. reported that LDL fractions from hypertriglyceridemic subjects with density higher than 1.048 g/mL showed a CD spectrum slightly different than LDL from NL subjects (*51*).

Regarding TrpF, Parasassi et al. described a fluorescenceintensity 10-fold lower in LDL(-) than in LDL(+) (*40*); this strong decrease is consistent with extensively oxidized LDL, as Figure 4 shows, but not with LDL(-). Indeed, the maximum of emission fluorescence was the same in LDL(+) and LDL(-), indicating that the hydrophobic environment of Trp residues did not differ between LDL(-) and LDL(+). Nevertheless, the lack of differences by CD and TrpF does not imply that slight tertiary structural differences affecting the ligand binding domain of apoB could not existin LDL(-). Lund-Katz et al. (*47*) reported that LDL subspecies presenting identical CD spectra (i.e., the same overall secondary structure) differed in the conformation of Lys residues evaluated by nuclear magnetic resonance; thus,those authors reported that the lightest and densest LDL subspecies (which were also the most electronegative particles measured by electrophoretic mobility) presented a lower number of active Lys than those of intermediate density (which also showed lower electronegativity). In particular, these LDL subspecies with abnormal density, increased electronegativity, and low number of active Lys showed decreased affinity to the LDLr, which concurs with our data.Further studies evaluating the specific environment of Lys residues are required to obtain deeper insight into the molecular determinants of decreased binding affinity of LDL(-).

Modification of LDL(+) by NEFA loading or $sPLA_2$ lipolysis promoted a concentration-dependent loss of affinity to the LDLr, with $sPLA_2$ treatment being more effective than NEFA loading. The enhanced effect of $sPLA_2$ should be attributed to major changes in composition, electric charge,size, and aggregation level induced by phospholipolysis, withthese properties being determinant in LDL binding to the LDLr (*44*, *47*, *52*, *53*). Kleinman et al. (*54*) previously reported that $sPLA_2$-mediated lipolysis altered the interaction of LDL with cells, thereby abolishing the specific interaction with the LDLr and increasing the nonspecific binding. Our findings are somewhat similar to those described by Kleinman et al. although the loss of affinity in our study was muchmore moderate, undoubtly due to the fact that we modified LDL(+) much more slightly (approximately 20% PC was hydrolyzed at the highest concentration of 10 ng/mL $sPLA_2$) than Kleinman et al. (100% of PC in LDL was hydrolyzed) (*54*). LDL(+) modified with a concentration of 1 ng/mL $sPLA_2$ showed binding affinity similar to that displayed by LDL(-) although size, electric charge, total phospholipids, and phosphatidylcholine and LPC content were almost unchanged compared to nonmodified LDL(+). Interestingly, at this concentration of $sPLA_2$ the NEFA content in modifiedLDL(+) (22.4 ± 5.4 mol/mol of apoB) was similar to that observed in LDL(-) (24.5 ± 9.9 mol/mol of apoB), suggesting that NEFA plays a major role in the binding affinity of LDL to the LDLr. This is supported by results obtained with NEFA-modified LDL. A NEFA content of approximately 20-30 mol/mol of apoB in LDL (obtained after incubation of LDL with 0.25 mM NEFA) was enough to decrease the affinity without additional changes in composition, electric charge, size, or aggregation level. It isnoteworthy that a very high content of NEFA in LDL did not result in the total loss of affinity since LDL(+) incubatedwith 2 mM NEFA (639 mol of NEFA/mol of apoB) showeda similar displacement capacity to LDL(+) incubated with 0.5 mM NEFA (40 mol of NEFA/mol of apoB). This observation points to a threshold of

the NEFA content abovewhich the binding affinity of LDL is not modified. It is interesting to note that both an increase (NEFA loading) anda net loss of lipids ($sPLA_2$ lipolysis) in LDL result in decreased binding affinity.

The mechanism by which NEFA loading decreases bindingof LDL(-) is currently unknown. The loss of affinity was observed without modification of the secondary and tertiarystructures of apoB measured by CD and TrpF, which supports the notion that major structural changes in apoB are not necessary to decrease binding to the LDLr. Nevertheless, local structural changes in the binding domain of apoB cannot be ruled out. Electronegatively charged NEFA could form ionic bonds with the positively charged Lys residues, thereby interfering in the recognition between Lys of apoB and Cys of the LDLr. A further possibility is that negative NEFA in LDL could directly interfere by repulsion with negative Cys of the LDLr.

In summary, LDL(-) from NL and FH subjects shows impaired binding to the LDLr, a characteristic that could lead to decreased "in vivo" clearance. Lower plasma clearance results in increased residence time in blood which, in turn, could induce further modifications of LDL(-), resulting in an increase in its inflammatory and atherogenic potential. This would represent a positive feedback mechanism that could be involved in the increase in LDL(-) plasma concentration, particularly in situations in which LDL receptor impairment exists, such as in familial hypercholesterolemia. Increased NEFA content in LDL(-) appears as a major factor in its impaired affinity to the LDLr, although the exact molecular mechanism is currently unknown. Furtherstudies are required to elucidate the molecular mechanisms involved in the NEFA-mediated inhibition of LDL affinity to its receptor.

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Table 1: Lipid and Apoprotein Composition and Oxidative Parameters of LDL(+) and LDL(-) from NL and FH Subjects

	NL-LDL(+)[f]	NL-LDL(-)[f]	FH-LDL(+)[f]	FH-LDL(-)[f]
total cholesterol[a]	40.1 ± 1.4	39.9 (1.5	40.7 (0.9	41.7 (0.8[d,e]
free cholesterol[a]	11.8 ± 0.3	12.7 ± 0.4[d]	11.8 ± 0.4	12.1 ± 0.4[d]
triglyceride[a]	7.0 ± 1.2	8.3 ± 1.4[d]	5.0 ± 1.0[e]	7.1 ± 1.3[d,e]
phospholipid[a]	26.1 ± 0.8	26.5 ± 1.4	26.3 ± 0.7	25.6 ± 1.0[e]
apoB[a]	26.8 ± 1.5	25.3 ± 2.0[d]	28.0 ± 1.3[e]	25.7 ± 1.1[d]
apoE[b]	0.03 ± 0.03	0.82 ± 0.79[d]	0.01 ± 0.01	0.24 ± 0.22[d,e]
apoC-III[b]	0.04 ± 0.01	0.44 ± 0.40[d]	0.03 ± 0.03	0.10 ± 0.06[d,e]
NEFA[b,g]	10.8 ± 5.2	24.5 ± 9.9[d]	12.5 ± 3.6	18.0 ± 5.8[d]
MDA[b,g]	0.46 ± 0.08	0.40 ± 0.03	0.51 ± 0.018	0.50 ± 0.13
R-tocopherol[b]	8.8 ± 1.1	9.6 ± 3.8	11.4 ± 3.9	11.6 ± 4.4
R-carotene[b]	0.05 ± 0.01	0.06 ± 0.04	0.06 ± 0.03	0.07 ± 0.04
§-carotene[b]	0.24 ± 0.11	0.26 ± 0.13	0.30 ± 0.12	0.32 ± 0.16
lycopene[b]	0.36 ± 0.11	0.49 ± 0.21	0.56 ± 0.31	0.58 ± 0.31
TNBS reactivity[c,g]	100	103.5 ± 3.3	101.5 ± 1.9	104.2 ± 3.5

[a] % of total LDL mass. [b] mol/mol of apoB. [c] %. [d] $P < 0.05$ vs LDL(+). [e] $P < 0.05$ vs NL. [f] Results are the mean ± SD of 10 independentexperiments. [g] Abbreviations: NEFA, nonesterified fatty acids; MDA, malondialdehyde; TNBS, trinitrobenzenesulfonic acid.

Table 2: Dissociation Constants (K_D), Inhibition Constants (K_i), and Inhibitory Concentration To Displace 50% of Binding (IC_{50}) of LDL(+) and LDL(-) Subfractions from NL and FH Subjects[a]

	K_D (nM)[b]	K_i (nM)[b]	IC_{50} (mg of apoB/L)[b]
NL-LDL(+)[c]	10.9 ± 5.7	5.2 ± 2.1	17.3 ± 2.1
NL-LDL(-)[c]	33.0 ± 11.4[d]	18.2 ± 6.2[d]	32.1 ± 11.0[d]
FH-LDL(+)[c]	16.7 ± 7.0[e]	7.9 ± 2.1	19.7 ± 5.4
FH-LDL(-)[c]	24.4 ± 7.1[d]	15.9 ± 6.4[d]	32.2 ± 12.9[d]

[a] Human fibroblasts were cultured, and saturation kinetics (K_D) or binding displacement (K_i and IC_{50}) experiments were performed as described in Experimental Procedures. Data are the mean SD calculated from five (K_D) or six (K_i and IC_{50}) independent experiments.

[c] Abbreviations: NL, normolipemic; FH, familial hypercholesterolemic. [d] $P < 0.05$ vs LDL(+). [e] $P < 0.05$ vs NL.

Table 3: Emission Maximum of Trp Fluorescence (TrpF) and Relative Proportion of Each Type of Secondary Structure Determined byCircular Dichroism of LDL Subfractions and Modified LDLs

	TrpF (nm)[a]	α-helix[a]	β-sheet[a]	β-turn[a]	random coil[a]
LDL(+)	338.3 ± 1.8	33.4 ± 1.8	26.3 ± 3.9	18.1 ± 1.5	22.1 ± 2.2
LDL(-)	338.9 ± 2.0	34.7 ± 2.7	28.3 ± 1.5	17.0 ± 1.1	20.0 ± 1.2
oxLDL	345.4 ± 7.9[b]	33.3 ± 2.5	28.6 ± 2.1	18.0 ± 1.3	20.1 ± 1.3
NEFA modification					
0.25 mM	338.7 ± 1.2	32.4 ± 2.3	30.8 ± 1.2	16.4 ± 2.4	20.4 ± 1.8
0.5 mM	338.6 ± 0.9	33.1 ± 0.9	30.5 ± 3.1	16.3 ± 2.5	20.1 ± 1.7
2 mM	339.6 ± 1.6	33.6 ± 1.0	30.0 ± 1.4	17.3 ± 2.0	19.2 ± 1.9
$sPLA_2$ modification					
1 ng/mL	338.5 ± 3.2	32.0 ± 0.9	26.5 ± 2.1	18.4 ± 1.7	23.1 ± 0.9
5 ng/mL	338.5 ± 3.0	33.5 ± 0.9	26.0 ± 2.3	18.1 ± 1.5	22.4 ± 2.6
10 ng/mL	338.2 ± 2.0	34.1 ± 1.5	26.9 ± 3.0	18.1 ± 1.4	20.9 ± 1.9

[a] Results are the mean ± SD of six independent experiments for LDL(+), LDL(-), and oxLDL and three independent experiments for NEFA-and sPLA2-modified LDLs. [b] $P < 0.05$ vs LDL(+).

Table 4: NEFA, Malondialdehyde, and Antioxidant Content Associated with LDL after Modification with NEFA or sPLA2

	NEFA[a,b]	MDA[a,b]	α-tocopherol[a]	lycopene[a]	α-carotene[a]	β-carotene[a]	TNBS[a,b]
[NEFA][c]							
0 mM	9.9 ± 2.1	0.46 ± 0.08	6.0 ± 1.2	0.24 ± 0.12	0.04 ± 0.02	0.20 ± 0.05	99 ± 9
0.25 mM	18.9 ± 2.8[d]	0.53 ± 0.23	6.1 ± 1.1	0.26 ± 0.07	0.04 ± 0.01	0.19 ± 0.06	110 ± 13
0.5 mM	40.3 ± 6.4[d]	0.37 ± 0.11	5.9 ± 1.1	0.22 ± 0.15	0.04 ± 0.01	0.18 ± 0.06	108 ± 5
2 mM	639.4 ±	0.41 ± 0.33	6.4 ± 1.0	0.25 ± 0.05	0.04 ± 0.01	0.19 ± 0.07	105 ± 6
[sPLA2][c]							
1 ng/mL	22.4 ± 5.2[d]	0.47 ± 0.21	6.4 ± 1.5	0.25 ± 0.03	0.03 ± 0.01	0.17 ± 0.04	109 ± 12
5 ng/mL	56.4 ± 6.6[d]	0.45 ± 0.11	6.0 ± 0.8	0.19 ± 0.12	0.04 ± 0.01	0.16 ± 0.05	104 ± 9
10 ng/mL	66.6 ± 9.6[d]	0.40 ± 0.14	5.8 ± 1.6	0.16 ± 0.09	0.04 ± 0.01	0.15 ± 0.04	95 ± 6

[a] Results are the mean ± SD of six independent experiments and are expressed as mol/mol of apoB.

[b] Abbreviations: NEFA, nonesterified fatty acids; MDA, malondialdehyde; TNBS, trinitrobenzenesulfonic acid.

[c] Concentration in the incubation mixture.

[d] $P < 0.05$ vs inferior NEFA or sPLA2 concentration.

Table 5: Effect of LDL(+) Lipolysis by Secretory Phospholipase A_2 (sPLA2) on Phospholipids and Nonesterified Fatty Acid (NEFA) Content

[sPLA2][b]	TPh[a,c]	PC[a,c]	PE[a,c]	SM[a,c]	LPC[a,c]	NEFA[a]
0 ng/mL	24.3 ± 1.5	676 ± 71	114 ± 27	286 ± 49	24 ± 10	8.3 ± 5.1
1 ng/mL	23.1 ± 1.3	618 ± 78[d]	106 ± 17	273 ± 22	29 ± 8[d]	22.4 ± 5.2[d]
5 ng/mL	21.7 ± 1.0[d]	554 ± 122[d]	81 ± 12[d]	295 ± 39	50 ± 20[d]	56.4 ± 6.6[d]
10 ng/mL	20.8 ± 0.5[d]	404 ± 67[d]	71 ± 13[d]	296 ± 49	93 ± 29[d]	66.6 ± 9.6[d]

[a] Results are the mean ± SD of nine independent experiments. NEFA and individual phospholipids are expressed as mol/mol of apoB and total phospholipids as % of total LDL mass.

[b] Concentration in the incubation mixture.

[c] Abbreviations: TPh, total phospholipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; LPC, lysophosphatidylcholine. [d] $P < 0.05$ vs inferior sPLA2 concentration.

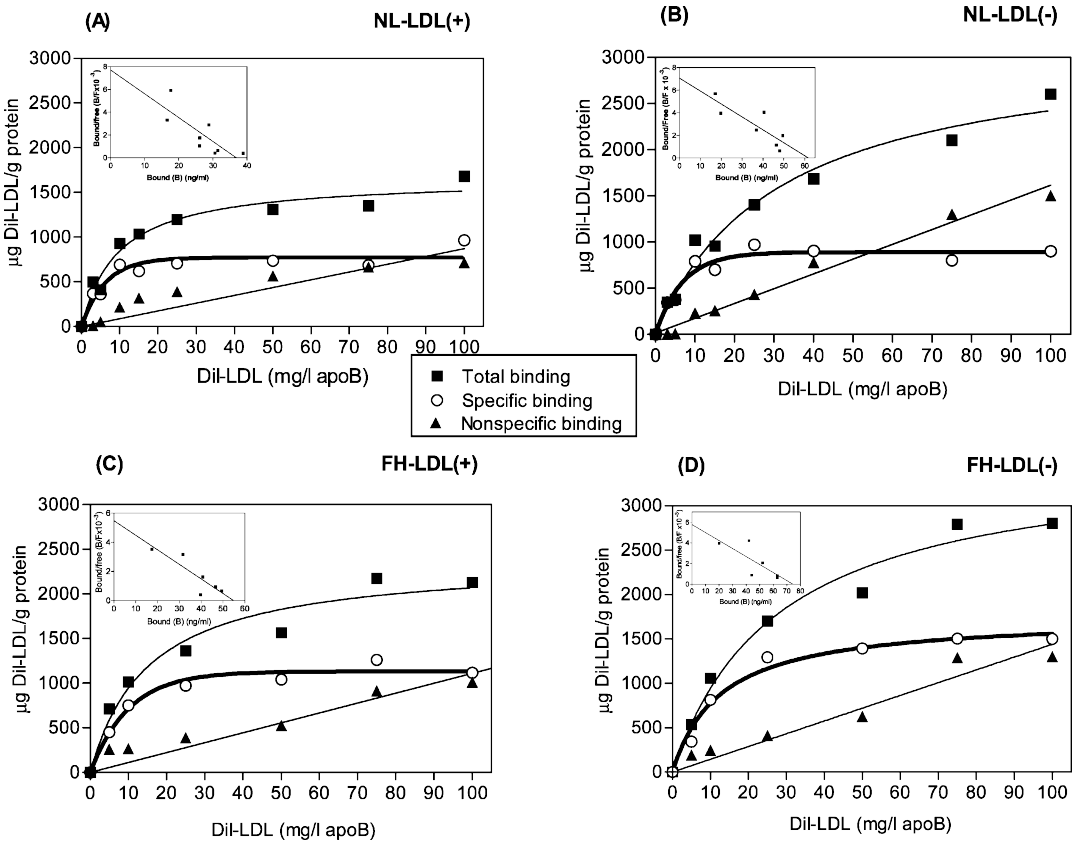

FIGURE 1: Representative saturation kinetic experiments of LDL(+) (A, C) and LDL(-) (B, D) subfractions isolated from normolipemic (NL) (A, B) and familial hypercholesterolemic (FH) (C, D) subjects. Human skin fibroblasts were cultured, and experiments were developed as described in Experimental Procedures. Increasing amounts of LDL(+) and LDL(-) labeled with DiI were incubated, in triplicate, with fibroblasts for 3 h at 4 ºC under gentle shaking. Nonspecific binding was determined by adding a 10-fold excess of unlabeled LDL, in duplicate. Nonspecific binding was subtracted from total binding to calculate specific binding. The inserts show the Scatchard plot deduced from saturation kinetics that was used to calculate K_D.

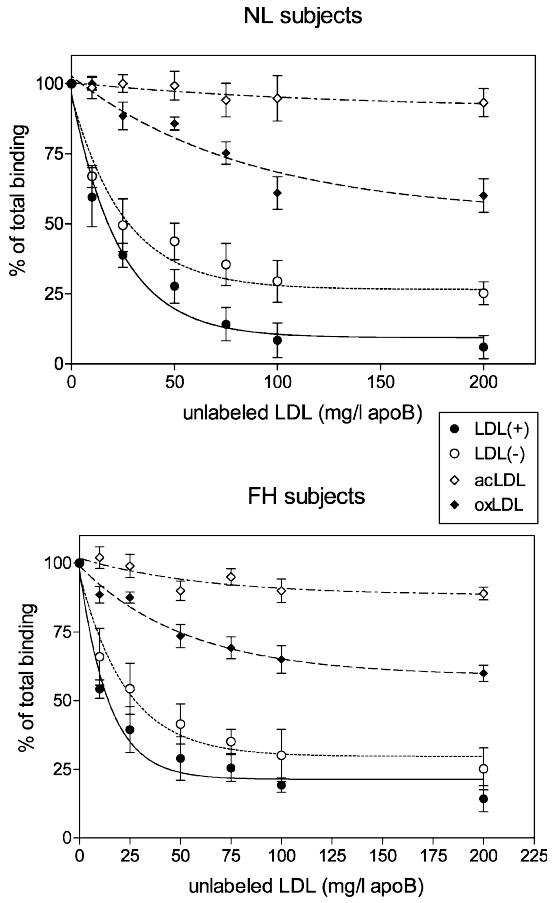

FIGURE 2: Binding displacement studies. Human skin fibroblasts were cultured, and experiments were developed as described in Experimental Procedures. Total DiI-LDL (25 mg/L) binding was displaced with increasing concentrations of unlabeled LDL(+), LDL(-), acetylated LDL (acLDL), and oxidized LDL (oxLDL). LDLs were incubated with fibroblasts for 3 h at 4 °C under gentle shaking. The upper panel corresponds to normolipemic (NL) subjects and the lower panel to familial hypercholesterolemic (FH) subjects. Results are the mean of six independent experiments.

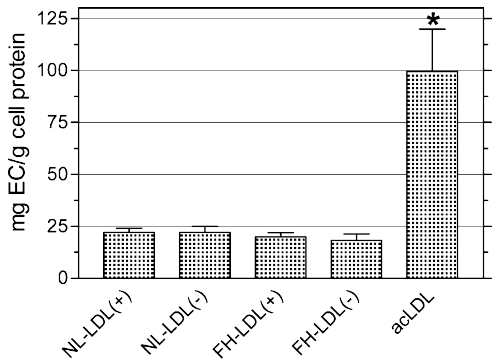

FIGURE 3: Intracellular accumulation of esterified cholesterol (EC) induced by LDL(+), LDL(-) from normolipemic (NL) and familialhypercholesterolemic (FH) subjects, and acetylated LDL (acLDL)in murine macrophages of the cell line P388D_1. Cells were cultured,and experiments were developed as described in Experimental Procedures. PMA-stimulated cells were incubated with 50 mg of apoB/L of LDLs for 72 h at 37 ºC. Lipids were extracted and separated by thin-layer chromatography, and esterified cholesterolwas quantified by densitometry. Results are the mean of six independent experiments. The asterisk indicates $P < 0.05$ vs LDL- (+) and LDL(-).

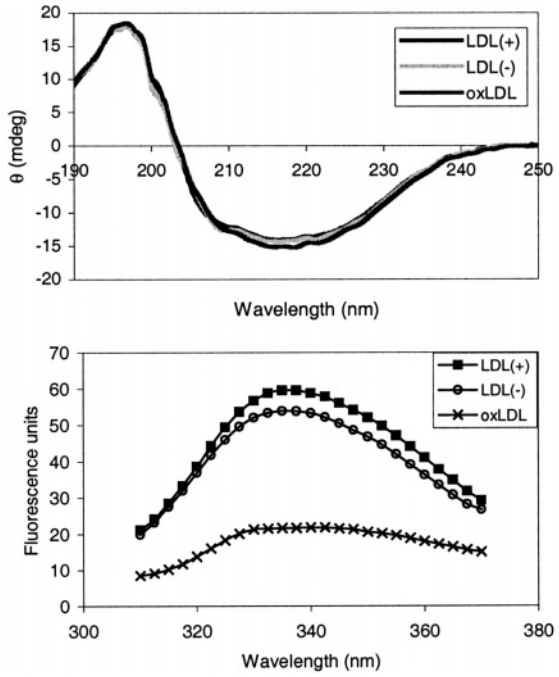

FIGURE 4: Representative circular dichroism (CD) (upper panel) and tryptophan fluorescence (TrpF) (lower panel) spectra of LDL-(+), LDL(-), and oxidized LDL (oxLDL) from normolipemic subjects. Spectra were measured as described in Experimental Procedures. PBS-dialyzed LDL was at 0.05 g of protein/L, and CD spectra were measured using a quartz cuvette of 0.2 cm.

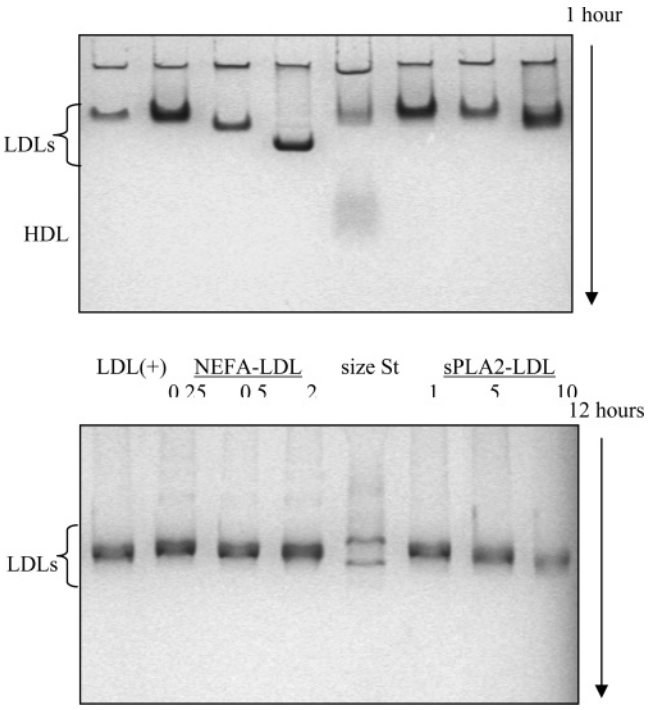

FIGURE 5: Representative nondenaturing polyacrylamide gradient (2-16%) gel electrophoresis (GGE) of LDL(+) modified by nonesterified fatty acid loading (NEFA-LDL) or secretory phospholipase A_2 lipolysis (sPLA$_2$-LDL). LDL(+) was modified as described in Experimental Procedures. Modified LDLs were prestained with Sudan Black, and 5 μL of LDL was electrophoresed at 100 V in a cold room using a Mini Protean II system (Bio-Rad). After 1 h of electrophoresis, the gel was scanned in a Gel Doc 2000 system (upper gel). At that moment LDLs ran exclusively depending on the electric charge of the particle, since the pore sizeis not restrictive. The gel was then allowed to continue electrophoresis for 12 h at 100 V (lower gel). At that moment, LDLs ran depending on size, since particles had reached the position at which the pore of the polyacrylamide net is restrictive. A mixture of plasmas with four LDL bands of known diameter was used as the size standard.

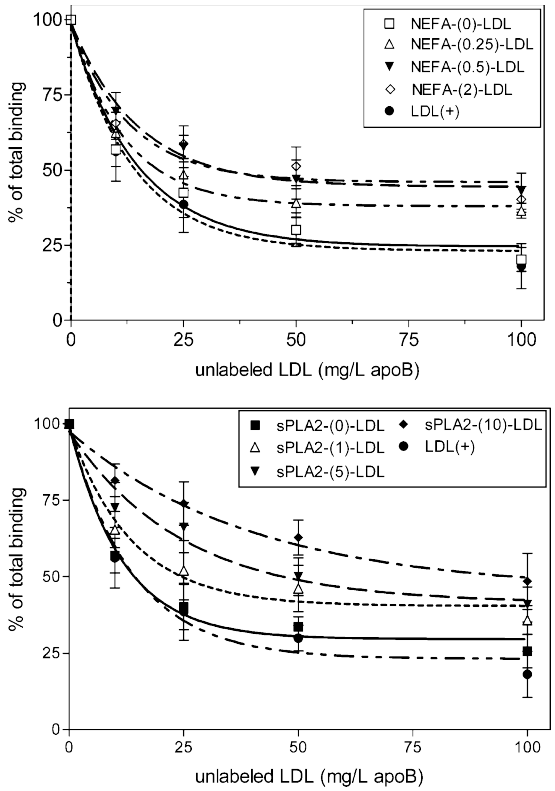

FIGURE 6: Binding displacement experiments using LDL(+) modified by nonesterified fatty acid loading (NEFA-LDL) (upper panel) or secretory phospholipase A_2 lipolysis (sPLA$_2$-LDL) (lowerpanel). Total DiI-LDL (25 mg/L) binding was displaced withincreasing concentrations of unlabeled modified LDLs. Human skinfibroblasts were cultured, and experiments were developed asdescribed in Experimental Procedures. LDLs were incubated with fibroblasts for 3 h at 4 ºC under gentle shaking. Results are the mean of six independent experiments.