

Isolation of Low Density Lipoprotein from Atherosclerotic Vascular Tissue of Watanabe Heritable Hyperlipidemic Rabbits

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Atherogenic properties of low density lipoproteins (LDL) in vivo may reflect modification of lipoproteins associated with endothelial translocation and exposure to extracellular matrix and interstitial fluid. To examine whether modifications of LDL occur in vivo, lipoproteins were isolated from plasma and vascular tissue of Watanabe heritable hyperlipidemic (WHHL) rabbits. LDL was extracted from vascular tissue (LDL-V) by homogenization in the presence of a sodium carbonate buffer. Control experiments demonstrated that modifications did not occur under the preparative conditions used to release LDL from tissue. LDL-V contained less esterified cholesterol, but more cholesterol esters, than did LDL from plasma (LDL-P). The diameters of both LDL-V and LDL-P followed gaussian distributions, but LDL-V particles were smaller (20.3 ± 0.1 and 26.3 ± 0.1 nm). Mild lipid peroxidation was evident in LDL-V. The sphingomyelin content was increased in LDL-V, with less phosphatidylcholine than in LDL-P. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) indicated that apolipoprotein B was depleted in LDL-V, but Western blot analyses identified lower molecular weight proteins antigenically related to apolipoprotein B. LDL-V markedly stimulated cholesterol esterification in mouse peritoneal macrophages and also in rabbit alveolar macrophages, a cell type that did not respond to acetylated LDL. LDL-V was not recognized by cultured rabbit skin fibroblasts. Thus, LDL isolated from atherosclerotic vascular tissue in vivo was modified in a fashion that could confer atherogenic properties reflected by augmentation of cholesterol esterification in macrophages in vitro. (Arteriosclerosis 8:768-777, November/December 1988)

Endocytosis of low density lipoproteins (LDL) into cells via apolipoprotein B/E receptors results in activation of acyl CoA:cholesterol acyltransferase (ACAT) and increased intracellular storage of cholesterol esters. Increased cellular content of cholesterol esters reduces the endogenous production of cholesterol by inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase and prevents delivery of further exogenous cholesterol by down-regulating apolipoprotein B/E receptors.^{1,2} This mechanism precludes cholesterol overloading of cells. Despite the ability of most cell types to maintain cholesterol homeostasis, plasma concentrations of LDL are positively correlated with atherosclerosis, a disease which

is characterized by lipid-laden cells.³ To resolve this paradox, it has been hypothesized that native LDL is damaged so that particles are no longer recognized by the apolipoprotein B/E receptor, but are avidly catabolized by macrophages,⁴ the presumed precursors of foam cells in atherosclerotic lesions.^{5,6}

The first identified modification of LDL that inhibited catabolism by fibroblasts, but induced recognition by macrophages, was acetylation of apolipoprotein B, a change thought not to occur physiologically.⁴ Subsequently, numerous other modifications of apolipoprotein B that influence the metabolic behavior of LDL, primarily through alteration of lysine residues have been described.^{7,8} Recently, a sequence of events that could occur under physiological conditions has been described and modification of LDL by oxidative processes has been postulated. Based on the results of experiments performed with cells in culture, it has been suggested that translocation of LDL across the endothelium is associated with lipid peroxidation of the particles, catabolism via phospholipase A₂ of phosphatidylcholine to lysophosphatidylcholine, degradation of apolipoprotein B, and oxidative modification of lysine residues.⁹⁻¹⁶ Although many observations in vitro have supported the initial findings and suggestions by Henrikson et al.,⁹ it is unclear whether such modifications occur in vivo.

LDL-like particles have been isolated from human arteries obtained at autopsy.^{17,18,19} These particles exhibit

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increased electrophoretic mobility on agarose gels^{17,18} and stimulate cholesterol esterification in macrophages.^{20,21,22} However, artifacts or autolytic changes that could give rise to modification of LDL may occur in autopsy material.

The present study characterized LDL-like particles isolated from the vasculature of Watanabe heritable hyperlipidemic (WHHL) rabbits, an animal model of familial hypercholesterolemia.²³ This fraction was designated as LDL-V. Unlike rabbit models with diet-induced hypercholesterolemia, atherosclerosis in WHHL rabbits correlates with increased plasma concentrations of LDL cholesterol.²⁴ With the use of vascular tissue freshly excised from WHHL rabbits, LDL isolated from tissue could be compared with that isolated from plasma of the same animal under controlled conditions and with prior exclusion of intrapreparative artifacts. The results of the present study demonstrate that LDL isolated from vascular tissue is modified in a fashion that confers atherogenic properties, as manifested by the stimulation of cholesterol esterification in macrophages.

Methods

Maintenance of WHHL Rabbits

The colony of WHHL rabbits at Washington University School of Medicine was initiated from animals kindly provided by Joseph L. Goldstein (University of Texas). The animals were maintained in isolation from other colonies under aseptic conditions and were given water and standard laboratory rabbit diets ad libitum. At the time of study, the animals were between 9 and 16 months old. A total of 15 rabbits was used for this study. All procedures performed on the animals were approved by the Washington University Committee for the Humane Care of Laboratory Animals.

Extraction of Vascular Tissue

The rabbits were fasted overnight before study and were anesthetized with sodium pentobarbital (30 mg/kg). Blood was obtained from the dorsal aorta via a cannula into tubes containing EDTA (final concentration of 1.5 mg/ml). After exsanguination, aortic segments from the ascending arch to the ileal bifurcation were excised and placed in chilled saline; they were trimmed, blotted lightly, weighed, placed in Petri dishes containing sodium carbonate (100 mM)/EDTA (1 mM; pH 11.5),²⁵ minced with a scalpel, and homogenized for 45 seconds with an Ultra-Turrax (Tekmar Company, Cincinnati, OH). The homogenates were adjusted to 40 ml with sodium carbonate buffer in Teflon-capped screw-top tubes and were placed on a rotator for 30 minutes at 4°C. The tubes were centrifuged at 2500 rpm for 30 minutes at 4°C in a Beckman J6 (Beckman Instruments, Palo Alto, CA). The supernatant fraction was removed and subjected to ultracentrifugation for separation of lipoproteins.

Isolation of Lipoproteins

LDL fractions were isolated from three samples from each animal comprising: 1) supernatant fractions of vascular tissue homogenate (LDL-V), 2) plasma (LDL-P), and 3) plasma that had been exposed to sodium carbonate buffer (LDL-PNC). Separation of lipoprotein fractions was performed by sequential ultracentrifugation with gradients

of sodium bromide²⁶ and a Beckman L8-55 instrument equipped with a 50.3 Ti rotor. LDL was isolated at a density cut of 1.019 to 1.063 g/ml with the use of Beckman Quick-seal tubes. Isolated lipoprotein fractions were dialyzed against 100 volumes of EDTA (1 mM)/sodium chloride (0.15 M)/benzamidine (1 mM), with three changes of dialysis fluid over 24 hours. Volumes of the fractions were reduced with RCF Coniflits hollow fiber centrifugal concentrators (Molecular Dynamics, Beaverton, OR).

Cholesterol ester-rich very low density lipoprotein was harvested from a cholesterol-fed New Zealand rabbit by ultracentrifugation as described previously.²⁷

Characterization of Lipoproteins

Concentrations of triglycerides, cholesterol esters, unesterified cholesterol, and phospholipids in plasma and in isolated lipoprotein fractions were determined with commercially available enzyme kits (Wako Chemical Company, Dallas, TX). The concentrations of phospholipids and triglycerides were calculated based on mean molecular weights of 722 and 866, respectively. The concentrations of cholesterol esters were calculated from a knowledge of the concentration of unesterified cholesterol and an assumed mean molecular weight of fatty acyl residues of 1.67 times the molecular weight of cholesterol. The mass of protein in the lipoprotein fractions was determined by the method of Lowry et al.²⁸ with bovine serum albumin as a standard.

The electrophoretic mobility of the lipoproteins was assessed with agarose gels (0.5% wt/vol) stained with fat red 0. The apolipoprotein composition of the delipidated lipoprotein fractions was determined electrophoretically with sodium dodecyl sulphate gradient polyacrylamide gels (SDS-PAG) (3% to 27% wt/vol; Integrated Separation Systems, Newton, MA). The apolipoproteins were visualized after staining with Coomassie blue, and their migrations were compared with those of molecular weight standards (albumin, ovalbumin, chymotrypsin, and cytochrome c). The relative content of the apolipoproteins in lipoprotein fractions was determined after laser densitometry of stained gels (LKB model 2202 laser densitometer; LKB, Turku, Finland) and the integration of areas was measured with a Planix Tamaya 7 digital planimeter (Tamaya and Company Limited, Tokyo, Japan).

Fatty Acyl Content of Cholesterol Esters

Aliquots of LDL were extracted by the Bligh and Dyer procedure.²⁹ The extracted lipid was dried by mild warming under a stream of nitrogen before resuspension in chloroform/methanol (2:1). Resuspended lipids were spotted on silica gel thin-layer chromatography plates and were developed in petroleum ether/ethyl ether/glacial acetic acid (168:30:2). The migration of cholesterol esters was identified by spraying iodide vapor on standards that were placed at the edge of each plate. The areas of interest were scraped into Pasteur pipettes containing glass wool. The lipid was eluted by serial washing of the silica scrapings with chloroform/methanol (2:1). Samples were dried by mild warming under a stream of nitrogen.

Methylation of resuspended samples was performed by transesterification in 2 ml of boron trifluoride/methanol/

benzene (35:30:35; 2 ml) with incubation at 65°C for 45 minutes. The reaction was quenched by the addition of distilled water (1 ml), and the mixture was cooled to 0°C. Fatty acids were extracted three times with petroleum ether (2 ml for each extraction), and sodium sulphate was added. The lipid extracts were filtered through glass wool and then were dried.

The methylated lipid extracts were resuspended in chloroform/methanol (2:1) and were injected with a Hewlett Packard 7673A auto-injector (Hewlett Packard, Palo Alto, CA) onto a Hewlett Packard 5890 gas chromatograph equipped with a 3 m column containing SP2330 (Supelco, Bellefonte, PA). Fatty acids were identified with a flame ionization detector and were quantified with a Hewlett Packard integrator 3392A. Fatty acids were identified by comparing the retention time of unknowns to commercially available standards (Sigma Chemical Company, St. Louis, MO) containing fatty acids ranging from 8:0 to 22:6.

Electron Microscopy of Negatively Stained Lipoproteins

Lipoproteins were negatively stained and analyzed by electron microscopy as described by Forte and Nordhausen.³⁰ The fractions were first dialyzed against a volatile buffer containing ammonium acetate (0.125 M), ammonium carbonate (2.6 M), and EDTA (0.26 mM) for approximately 24 hours with three changes of dialyzing fluid. Dilutions of lipoproteins that contained approximately 100 µg/ml protein were mixed with equal proportions of phosphotungstate acid (2% wt/vol, pH 7.5). Aliquots of this mixture were placed on Formvar-coated, carbon-stabilized grids (Ted Pella Incorporated, Tustin, CA). The excess fluid was removed with blotting paper, and the grids were allowed to air-dry for 5 minutes. Grids were placed in a Jeol 100C electron microscope calibrated before use. The particle diameters were analyzed with a Hewlett Packard 9874A digitizer coupled to a Hewlett Packard 9121 computer. All samples were analyzed by electron microscopy within 2 days of isolation of the lipoprotein fraction.

Determination of Lipid Peroxidation

Lipid peroxidation was quantified as thiobarbituric acid-reacting substances as described by Satoh.³¹ Standards were prepared by dissolution of malondialdehyde-bis-dimethyl-acetate (Aldrich Chemical Company, Milwaukee, WI) in distilled water immediately before the commencement of the assay. Samples (100 µg protein in 0.5 ml EDTA/saline) were incubated with sulphuric acid (0.05 M) and thiobarbituric acid (0.2% wt/vol, dissolved in sodium sulphate, 2 M) for 30 minutes at 100°C. After cooling, *n*-butyl alcohol (4 ml) was added and mixtures were vigorously vortexed. The two phases were separated by centrifugation at 500 *g* for 5 minutes. The absorbance of the chromogen was determined at 530 nm with a Beckman DU-30 spectrophotometer. The results were expressed as the nanomole equivalents of malondialdehyde; the assay was linear between 0.5 and 20 nmol.

Phospholipid Classes

Aliquots of LDL were extracted by the method of Bligh and Dyer,²⁹ and phospholipid classes were separated by

two-dimensional thin-layer chromatography. After the first dimension was developed in chloroform/methanol/ammonium hydroxide (103:50:10), plates were removed from the chamber and were air-dried for 15 minutes, then warmed at 80°C for 5 minutes, then cooled for 10 minutes. They were rotated 90° and were developed in the second dimension with chloroform/acetone/methanol/acetic acid/water (60:80:20:20:10). Phospholipids were visualized by exposure to iodide vapor. Phospholipids were eluted from the silica scrapings of identified spots into chloroform/methanol (2:1) and were mass-determined from a measurement of inorganic phosphate by the method of Fiske and Subbarow.³²

Radioiodination of Low Density Lipoprotein and Antibodies

LDL and affinity-purified guinea pig antirabbit IgG (Accurate Chemical and Scientific Corporation, Westbury, NY) were radioiodinated with Iodo-Beads (Pierce Chemical Company, Rockford, IL). Radiolabeled fractions were separated from unbound ¹²⁵I by passing the reaction mixture through a column (10×100 mm) containing Superose 12 prep grade (Pharmacia, Piscataway, NJ) followed by dialysis overnight. Precipitation of ¹²⁵I in the presence of trichloroacetic acid (10% wt/vol) was greater than 91%.

Western Blot Analysis

Polyclonal antibodies to apolipoprotein B were generated by immunizing Hartley guinea pigs with purified rabbit LDL. Antibodies were purified initially by precipitation with ammonium sulphate and passage through immuno-affinity columns of LDL linked to cyanogen bromide-activated Sepharose CL-4B.

For Western blot analyses,³³ electrophoresis on a gradient (3% to 25% wt/vol) SDS-PAG was performed followed by transfer of proteins to nitrocellulose paper with a Trans-Blot electrophoresis transfer cell (Bio-Rad, Richmond, VA). Nitrocellulose paper was first blocked by incubation with phosphate-buffered saline containing bovine serum albumin (3% wt/vol) for 1 hour. The blocking solution was removed and replaced with phosphate-buffered saline with albumin (3% wt/vol) and nonimmune serum (3% wt/vol) containing the affinity-purified antibody, B-4. The nitrocellulose was incubated overnight at room temperature. After the incubation, it was washed before incubation with ¹²⁵I-labeled goat antiguinea pig IgG for 2 hours; it was dried thoroughly and placed in a cassette with X-ray film for visualization of reactivity zones.

Maintenance of Fibroblasts

Primary cultures were obtained from explants of skin obtained from New Zealand rabbits. Cells were passaged each week and were maintained in MEM containing newborn calf serum (NBCS; 15% wt/vol). Cells were used between the 4th and the 15th passage. For use in metabolic assays, cells were plated at a density of 10×10⁵ cells per 35 mm well and were maintained in MEM containing NBCS for 5 days. The medium was then changed to one containing lipoprotein-deficient serum for a further 2 days, and the assays were performed on day 7.

Table 1. Properties of LDL Isolated Directly from Plasma Compared to LDL Subjected to Homogenization

	LDL isolated directly from plasma	LDL subjected to homogenization
Chemical composition (%)		
Protein	19.2±0.25	19.0±0.01
Cholesterol	23.3±0.26	22.7±0.25
Cholesterol esters	28.7±0.24	29.5±0.29
Triglycerides	10.0±0.02	10.0±0.02
Phospholipids	18.8±0.25	18.8±0.27
Phospholipid classes (%)		
Phosphatidylcholine	76.9±2.5	72.5±4.5
Sphingomyelin	18.9±3.0	22.6±3.9
Lysophosphatidylcholine	4.2±1.5	4.9±1.4
Lipid peroxidation (nmol MDA/100 µg protein)	0.57±0.12	0.43±0.24
Cholesterol esterification in macrophages at 50 µg/ml (nmol/mg)	0.61±0.13	0.78±0.08

Values are means±SE for four observations in each case. MDA=malondialdehyde.

Harvesting of Macrophages

For harvesting rabbit alveolar macrophages, pentobarbital-anesthetized male and female New Zealand rabbits (Boswells Rabbit Farm, St. Louis, MO) were exsanguinated via the abdominal aorta. Saline containing 5 U/ml heparin was introduced into the alveoli through the tracheal cannula. The lungs were lavaged five times with 50 ml fluid washes. The cells were plated in Dulbecco's minimum essential medium (DMEM) containing NBCS (20% vol/vol) at a density of 3 to 5×10^6 cells per 35 mm well. The metabolism of the cells was characterized the day after harvest.

For harvesting mouse peritoneal macrophages, Swiss-Webster mice (Charles River Laboratories Incorporated, Wilmington, MA) were peritoneally lavaged with saline.³⁴ Cells from individual mice were pooled and centrifuged (500 g for 10 minutes), were washed once with DMEM, were plated into 35 mm wells in DMEM containing NBCS (20% wt/vol) at a density of 3 to 4×10^6 cells per well in 1.5 ml of medium, and were placed in an incubator (37°C with 5% vol/vol CO₂) for 2 hours. Nonadherent cells were removed after the initial incubation, and fresh DMEM was placed in the wells. The cells were incubated overnight in DMEM containing NBCS (20% vol/vol).

Cholesterol Ester Deposition in Cultured Cells

Deposition of cholesteryl-³H-oleate was determined by the method of Brown et al.³⁴ Lipoproteins were incubated for 5 hours at 37°C with cells in medium containing ³H-oleate (specific radioactivity 3.5 dpm/pmol) complexed to fatty acid-free bovine serum albumin (Miles Scientific, Naperville, IL). For experiments with fibroblasts and macrophages, cells were incubated with MEM and DMEM, respectively, without serum. The cells were washed with Tris (10 mM)-buffered saline (0.15 M, pH 7.4) and were incubated with hexane/isopropanol (3:2; containing carrier lipid of cholesterol palmitate and triolein, both 1.5 mg/100 ml) at room temperature for 30 minutes for the extraction of neutral lipids in situ. The organic phase was removed and the extraction was repeated. Organic phases were spotted on thin-layer chromatography plates that were developed in petroleum ether/ethyl ether/glacial acetic acid (168:30:2). Cholesterol esters were visualized after exposure to iodide vapor. Areas containing cholesterol esters were scraped into vials containing Opti-fluor scintillation fluid (Packard Instruments, Downers Grove,

IL). The radioactivity was quantified with a Beckman LS 2800 scintillation counter, and the H-number technique was used to calculate quench corrections.

After extraction, cells were solubilized in sodium hydroxide (1 M, 1 ml). Aliquots (200 µl) were removed for assessment of cellular protein by the method of Lowry et al.²⁸ with bovine serum albumin as the standard. The results for cholesteryl-³H-oleate deposition were expressed as nanomoles of cholesteryl oleate/milligrams of cellular protein/5 hours.

Statistical Analyses

Comparisons were made with Student's *t* test (two-tailed) performed with Stats-2 (Statsoft, OK). A *p* value of greater than 0.05 was considered significant. Values are presented as means with standard error of means where applicable.

Results

Validation of Procedure for Extraction of Lipoproteins from Vascular Tissue

To ensure that exposure of aortic tissue to homogenization and sodium carbonate buffer did not produce artifactual modifications of vascular lipoproteins, exogenous plasma (5 ml) from WHHL rabbits was added to minced aortic tissue from normolipidemic New Zealand rabbits in sodium carbonate buffer. Homogenization was performed immediately. LDL was subsequently isolated by sequential ultracentrifugation. A comparison was made between LDL that was subjected to homogenization in the presence of vascular tissue in sodium carbonate buffer and LDL that was isolated directly from plasma. No demonstrable effects of the isolation procedure on the electrophoretic mobility LDL on agarose gels, on its relative chemical composition, on extent of lipid peroxidation, on relative composition of phospholipid types, or on ability to stimulate cholesterol ester deposition in cultured macrophages were detected (Table 1). Recovery of LDL cholesterol from aortic homogenates, compared with that for LDL isolated from plasma, was 92%.

A further experiment was performed: 1) to determine whether the lack of modification of LDL in the experiments described above was due to the relatively large mass of lipoprotein added to the aortic tissue, and 2) to

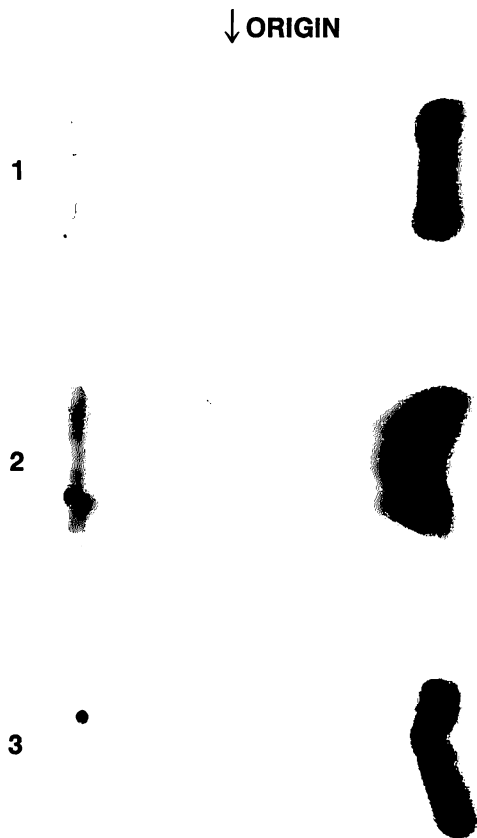


Figure 1. Electrophoretic mobility of radioiodinated low density lipoprotein (LDL) on agarose gels. ^{125}I -labeled LDL was subjected to different conditions for assessment of the effects of homogenization and tissue from two strains of rabbits on electrophoretic mobility. ^{125}I -labeled LDL (200 μg protein) was placed in sodium carbonate buffer containing either minced aortic tissue from a New Zealand rabbit, or minced tissue from a Watanabe heritable hyperlipidemic (WHHL) rabbit. The tissue was homogenized, and LDL was re-isolated as described in the Methods section. The samples analyzed by agarose gel electrophoresis were: 1) the starting material, which was not incubated with tissue, 2) ^{125}I -LDL incubated with aortic tissue from a normolipidemic New Zealand rabbit, and 3) ^{125}I -LDL incubated with aortic tissue from a WHHL rabbit.

ascertain whether homogenization in the presence of atherosclerotic tissue from a WHHL rabbit produced modifications not demonstrable with tissue from normolipidemic rabbits. Radioiodinated LDL, at a concentration of 100 μg of lipoprotein-protein per g wet weight of tissue, was added to sodium carbonate buffer containing minced aortic tissue from either normolipidemic New Zealand rabbits or WHHL rabbits. Aortic tissue was homogenized, and radioiodinated LDL was consequently re-isolated by ultracentrifugation. ^{125}I -labeled LDL re-isolated from the homogenate of New Zealand and WHHL rabbits was electrophoresed on agarose gels and compared to the starting material. The electrophoretic mobility of the LDL incubated with aortic tissue was the same as the starting material, indicating a lack of modification (Figure 1).

To further exclude preparative artifacts, all subsequent experiments examined: 1) lipoproteins isolated from vascular tissue (LDL-V), 2) lipoproteins isolated from plasma

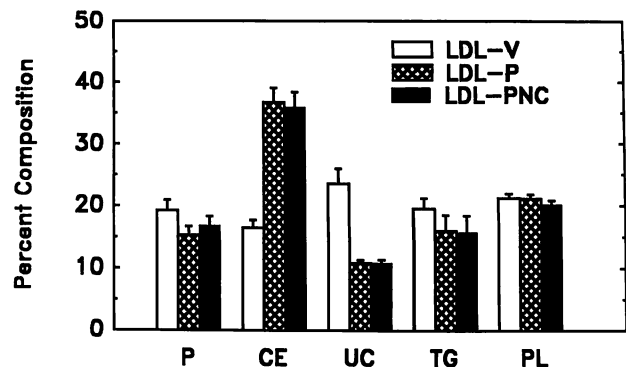


Figure 2. Chemical composition of low density lipoprotein (LDL) fractions isolated from Watanabe heritable hyperlipidemic (WHHL) rabbits. The relative chemical composition of LDL fractions isolated by ultracentrifugation at $d=1.019$ to 1.063 g/ml from aortic tissue (LDL-V), plasma (LDL-P), and plasma treated with sodium carbonate (LDL-PNC). Chemical compositions are expressed as relative percentages of protein (P), cholesterol esters (CE), unesterified cholesterol (UC), triglycerides (TG), and phospholipids (PL). Bars represent means \pm SE for eight observations.

(LDL-P), and 3) lipoproteins isolated from plasma that had been incubated with sodium carbonate (LDL-PNC).

Characteristics of Low Density Lipoprotein Isolated from Vascular Tissue

All WHHL rabbits used in this study had readily discernable atherosclerotic lesions on the intimal surface of their aortas. The mean weight of the aortic segments studied was 1581 ± 226 mg. The mass of LDL-V protein isolated from the tissue was 328 ± 29 $\mu\text{g/g}$ wet weight ($n=15$). Thus, a mean of 518 μg LDL-protein per animal was obtained from vascular tissue.

The chemical compositions of LDL-V, LDL-P, and LDL-PNC are shown in Figure 2. LDL-V had a decrease in cholesterol esters compared with LDL isolated from plasma, with an increase in the content of unesterified cholesterol. There were no significant changes in the relative content of protein, triglyceride, or phospholipid.

LDL-V and LDL-P were visualized by negative staining and electron microscopy (Figure 3). The particle sizes for both lipoprotein fractions fitted gaussian distributions, but LDL-P was significantly larger than LDL-V (26.3 ± 0.1 and 20.3 ± 0.1 nm, $n=200$, $p<0.01$).

The fatty acyl residues of cholesterol esters of LDL from plasma were relatively enriched in cholesteryl linoleate, as has been described by others.³⁵ LDL-V also contained a high percentage of cholesteryl linoleate, although oleate and myristate residues were more prominent compared to LDL-P (Figure 4).

LDL-P demonstrated typical β -mobility when subjected to electrophoresis on agarose and was not modified by exposure to sodium carbonate. LDL-V manifested two electrophoretic mobilities on agarose, with a major component migrating faster than the β -position and a minor component that stayed at the origin (Figure 5).

Lipid peroxidation of LDL-P and LDL-PNC were at the lowest limit of the thiobarbituric assay procedure used. However, as Figure 6 shows, lipid peroxidation was

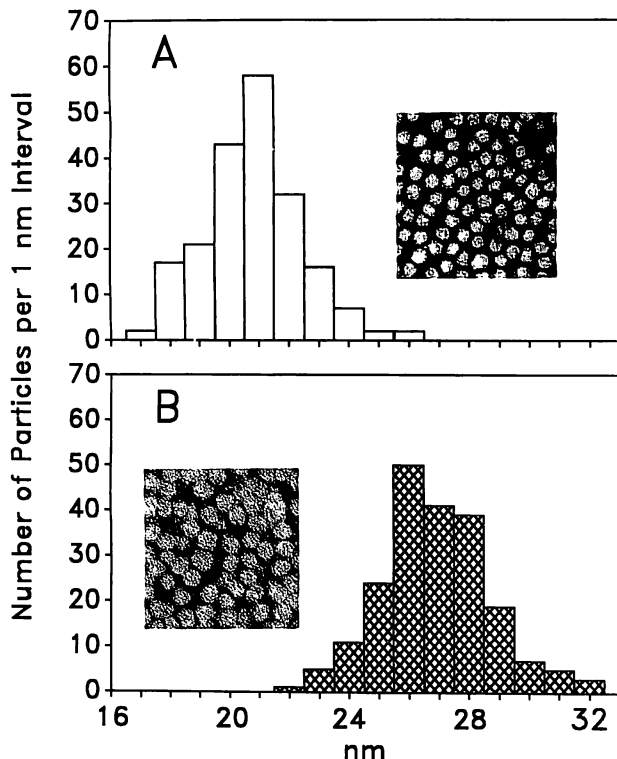


Figure 3. Low density lipoprotein (LDL) visualized by electron microscopy with negative staining. Electron micrographs of LDL isolated from vasculature (A) and LDL isolated from plasma (B) at the same magnification, 72 475 \times . Histograms represent the distribution of sizes for 200 randomly selected particles.

readily demonstrable for LDL-V (1.53 ± 0.17 nmol malonaldehyde equivalents/100 μ g protein; $n=4$).

Phospholipid compositions of LDL-P and LDL-PNC were similar, with the predominant type being phosphatidylcholine (77.8 ± 3.7 and 71.3 ± 3.1 , respectively, $n=7$). Other demonstrable phospholipids were sphingomyelin (16.9 ± 3.7 and 20.7 ± 3.3) and lysophospholipid (5.2 ± 1.4 and 7.89 ± 1.7). LDL-V was significantly depleted of phosphatidylcholine (40.6 ± 6.4 , $n=7$, $p < 0.01$) and enriched with sphingomyelin (48.9 ± 6.6 , $p < 0.01$) without any significant change in relative lysophosphatidylcholine content (Figure 7).

The results of SDS-PAGE of LDL-P and LDL-PNC (Figure 7) demonstrated that, as expected, apolipoprotein B was the major constituent of this lipoprotein class ($88\% \pm 2\%$, $n=3$). In addition, apolipoprotein E ($5\% \pm 1\%$ of total protein) was present in this fraction. In contrast, LDL-V apparently was depleted of apolipoprotein B. LDL-V exhibited other bands of M_r 73, 53, 49, and 28 kDa. The protein of M_r 28 kDa was assumed to be apolipoprotein A-I due to the similarity of its molecular weight and that of the authentic protein.

Western blot analyses were performed to determine whether the lower molecular weight proteins of LDL-V were immunologically related to apolipoprotein B. With antibody B-4, the major band recognized in LDL-P and LDL-PNC corresponded to apolipoprotein B-100, with some reactivity of the lower molecular weight bands that were assumed to be cleavage products. In LDL-V, reac-

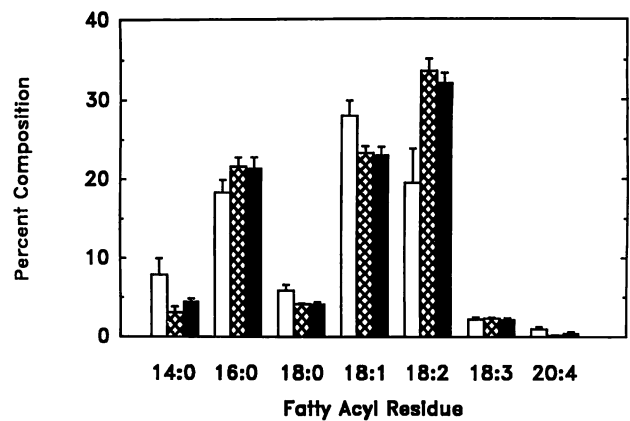


Figure 4. Fatty acyl content of cholesterol esters of low density lipoprotein (LDL). The fatty acyl residues of cholesterol esters are shown for LDL from vasculature (open bars), LDL from plasma (cross-hatched bars), and LDL from plasma treated with sodium carbonate (filled bars). Bars represent the means \pm SE for five observations.

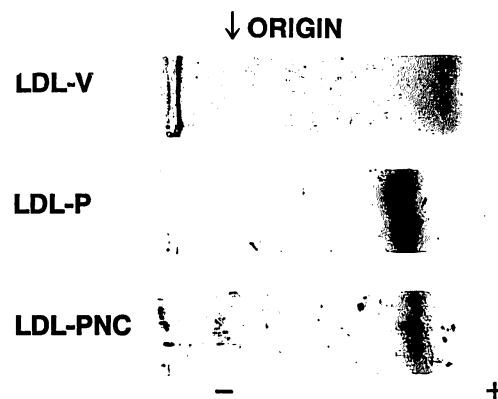


Figure 5. Electrophoretic mobility of low density lipoprotein (LDL) of Watanabe heritable hyperlipidemic (WHHL) rabbits. Mobility for LDL from vasculature (LDL-V), LDL from plasma (LDL-P), and LDL from plasma treated with sodium carbonate (LDL-PNC). Electrophoresis was performed on agarose gels (0.5% wt/vol), and fractions were visualized after staining with fat red O.

tivity was noted with proteins of M_r 59 and 73 kDa, with a mild reactivity against apolipoprotein B-100 (Figure 8).

Cellular Metabolism of Low Density Lipoprotein

LDL-P stimulated esterification of ^3H -oleate into cholesteryl- ^3H -oleate in rabbit skin fibroblasts, although incubation with LDL-V did not produce any such stimulation (Figure 9). In contrast, incubation of mouse peritoneal macrophages with LDL-V produced a dramatic stimulation of cholesteryl- ^3H -oleate deposition from a baseline value of 0.9 ± 0.1 to 38.4 ± 2.4 nmol/mg cellular protein ($n=3$), while LDL-P and LDL-PNC produced only a small augmentation of ACAT activity (Figure 10A).

Incubation of LDL-V with rabbit alveolar macrophages stimulated deposition of cholesteryl- ^3H -oleate from a basal level of 0.9 ± 0.5 to 3.2 ± 0.7 nmol/mg cellular protein when incubated with lipoproteins at 50 μ g protein/ml. Neither LDL-P or LDL-PNC produced augmented ACAT activity. In fact, both these fractions appeared to decrease the cellular deposition of cholesteryl- ^3H -oleate (Figure 10B).

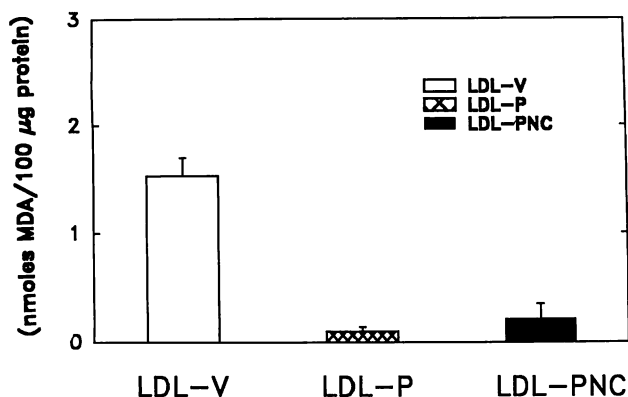


Figure 6. Lipid peroxidation of low density lipoprotein (LDL). Lipid peroxidation (nmol malondialdehyde equiv/100 µg protein) was determined on LDL from vasculature (LDL-V), LDL from plasma (LDL-P), and LDL from plasma treated with sodium carbonate (LDL-PNC). Bars represent means±SE for five observations.

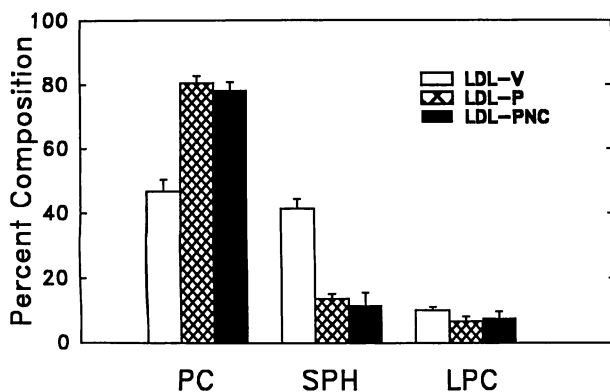


Figure 7. Phospholipid composition of low density lipoprotein (LDL). Phospholipid composition was determined by two-dimensional thin-layer chromatography for LDL from vasculature (LDL-V), LDL from plasma (LDL-P), and LDL from plasma treated with sodium carbonate (LDL-PNC). Bars represent means±SE for seven observations. PC=phosphatidylcholine, SPH=sphingomyelin, LPC=lysophosphatidylcholine.

Incubation of rabbit alveolar macrophages with LDL-P or acetylated LDL (with concentrations up to 50 µg protein/ml) did not augment deposition of cholesteryl-³H-oleate (Figure 11). Despite the lack of effect of acetylated LDL on ACAT activity in these cells, specific lipoprotein fractions such as cholesterol ester-rich VLDL did produce a pronounced, and concentration-related, increase in cholesteryl-³H-oleate deposition.

Discussion

The present study examined the composition and functional properties of LDL-like particles in atherosclerotic vascular tissue in vivo of WHHL rabbits compared with LDL, which is present in plasma from the same animals. LDL-V was modified with respect to chemical composition, particle size, phospholipid composition, lipid peroxidation, and degradation of apolipoprotein B. LDL-V also augmented cholesterol esterification in cultured macrophages, but not with cultured fibroblasts.

Although LDL isolated from plasma has been characterized extensively,³⁶ this material may not directly bathe

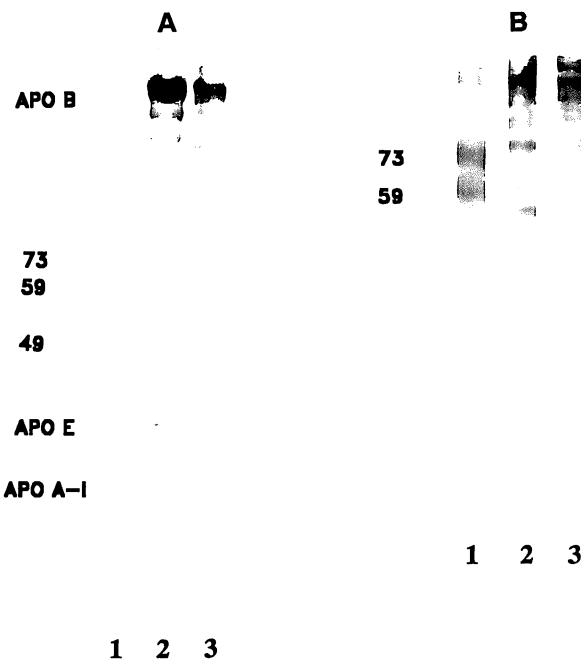


Figure 8. A. Sodium dodecyl sulphate polyacrylamide gradient gel electrophoresis of low density lipoprotein (LDL). All lanes were loaded with 20 µg protein. B. Western blot analysis of proteins of LDL. Blots were incubated with antibody B-4 to detect proteins immunologically related to apolipoprotein B. Lanes 1, LDL from vasculature; Lanes 2, LDL from plasma; and Lanes 3, LDL from plasma treated with sodium carbonate.

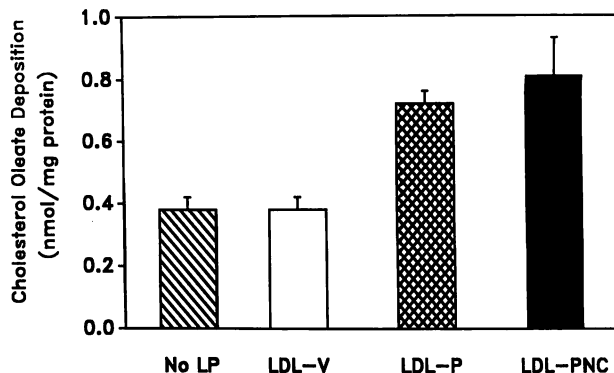


Figure 9. Effect of low density lipoprotein (LDL) fractions on acyl CoA cholesterol acyl transferase (ACAT) activity in cultured rabbit skin fibroblasts. Extent of cholesterol ester deposition is shown for no lipoprotein (No LP), LDL from vasculature (LDL-V), LDL from plasma (LDL-P), and LDL from plasma treated with sodium carbonate (LDL-PNC). All were present at 10 µg protein/ml. Histograms represent means±SE for three observations at each concentration.

cellular elements of vascular tissue involved in atherogenesis. Recent morphologic findings demonstrate that endothelialization of vasculature occurs only in the late phases of the atherosclerotic process.^{37,38,39} Therefore, early in atherosclerotic lesion formation, lipoproteins must traverse the endothelial barrier and interact with cells in the subendothelial space.⁴⁰ The atherogenic potentials of lipoproteins may be enhanced by chemical modification of apoproteins either during translocation or after translocation to the subendothelial space. Consequently, studies of the characteristics of lipoproteins in interstitial fluid that

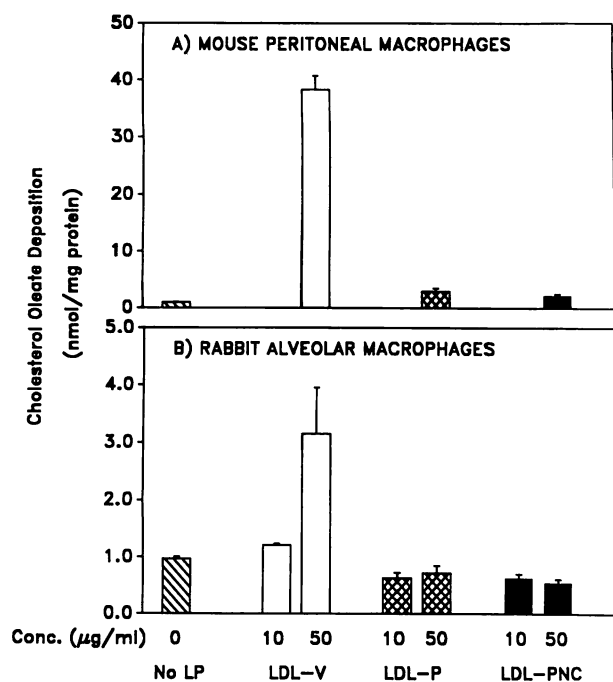


Figure 10. Effect of low density lipoprotein (LDL) fractions on acyl CoA cholesterol acyl transferase (ACAT) activity in cultured macrophages. Extent of cholesterol ester deposition in mouse peritoneal macrophages (A) and rabbit alveolar macrophages (B) is shown for no lipoprotein (No LP), LDL from vasculature (LDL-V), LDL from plasma (LDL-P), and LDL from plasma treated with sodium carbonate (LDL-PNC) at the indicated concentrations. Histograms represent means \pm SE for three observations at each concentration.

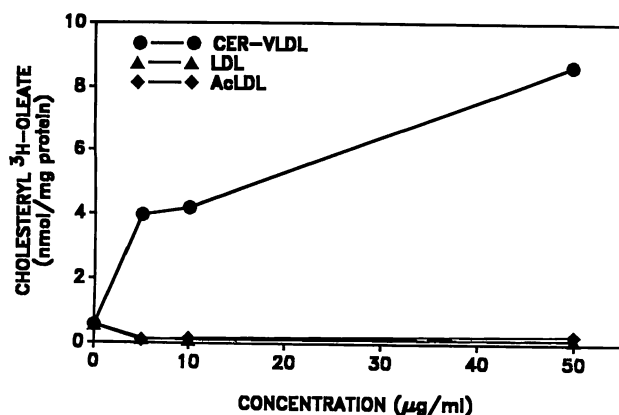


Figure 11. Effect of low density lipoprotein (LDL), acetylated LDL, and cholesterol ester-rich very low density lipoprotein (CER-VLDL) on acyl CoA cholesterol acyl transferase (ACAT) activity in cultured rabbit alveolar macrophages. Concentration-response curves were constructed for each lipoprotein in the presence of alveolar macrophages harvested from New Zealand rabbits. Cells were incubated with lipoproteins for 5 hours before extraction and quantitation of cholesterol 3 H-oleate. Points represent the means of three observations.

directly bathes cells may be more informative than studies of plasma particles.⁴¹

To ensure that the characteristics of LDL-V were not artifacts induced by the procedures used to isolate lipoprotein from vascular tissue, plasma lipoproteins were exposed to the highly alkaline sodium carbonate buffer and to vigorous aeration during the homogenization pro-

cedure that released lipoproteins from vascular tissue. To simulate the conditions of the isolation procedure, this procedure was performed in the presence of aortas from normolipidemic rabbits, a tissue from which a significant mass of LDL-V could not be isolated. No comparative artifacts were encountered for any of the parameters measured in this study. In addition to the inability of the sodium carbonate buffer to modify LDL, it also had the advantage of releasing lipoprotein rapidly from vascular tissue while preventing proteolysis.²⁵

Lipid peroxidation is usually detected as thiobarbituric acid-reacting substances. In the present study, it was quantified by measurement of thiobarbituric acid-reacting substances as described by Satoh,³¹ and mild peroxidation of lipid in LDL-V was evident. Although this method is not as sensitive as some others,⁴² it has the advantage of yielding results relating to lipid peroxidation only, while other assays will also react with sialic acid residues that may confound results for material isolated from tissue.

Lipid peroxidation of LDL has been correlated with the ability of these particles to interact with macrophages in culture.^{11,12} It has also been demonstrated that the oxidative processes that lead to modification of LDL in vitro may be inhibited by probucol, leading to a reduction of cholesterol ester deposition.^{43,44} Recently, Montgomery et al.⁴⁵ have dissociated the extent of lipid peroxidation of LDL detected as thiobarbituric acid-reacting substances from induction of cholesterol esterification in cultured macrophages. The extent of oxidation of LDL in their study was similar to that in our study, but it was somewhat lower than those reported by others.^{10,11} However, the mild lipid peroxidation may indicate an important initiator of a cascade of events that lead to the LDL detected in the present in vivo study. Lipid initially subjected to lipid peroxidation may be removed from the particle before modification. In addition, lipid peroxidation products may have been lost during ultracentrifugation and the concentrating steps required to isolate LDL-V.¹⁴ This may explain our observation of modest lipid peroxidation.

Haberland et al.,⁴⁶ using immunocytochemical methods, reported recently that a monoclonal antibody directed against maleylated proteins detected peroxidized products that co-localize with apolipoprotein B in atherosclerotic tissue from WHHL rabbits. These data corroborate the findings of the present study.

The proportions of phosphatidylcholine and sphingomyelin in LDL-V differed significantly from those of LDL-P. A small, but not statistically significant, increase of lysophospholipids was also present. Lysophospholipids may have been generated from the original particles, but physiological concentrations of albumin in interstitial fluid would have promoted transfer or efflux of lysophospholipids in extracellular fluid. Generation of lysophospholipid during modification of LDL is indicated by the reduced content of its precursor, phosphatidylcholine. The relative increase in sphingomyelin implies an augmented phospholipase A₂ activity because the enzyme does not cleave the sn-2 carbon of sphingomyelin.

On SDS-PAGE, LDL-V was greatly depleted of intact apolipoprotein B, although the relative content of total protein was not significantly changed. A caveat to this

apparent depletion is that the content of apolipoprotein B was based on staining with Coomassie blue, which may not accurately assess material that has undergone oxidation.⁴⁷ Since new proteins were not present in LDL-V in large amounts, most of the products of apolipoprotein B fragmentation must have originated from LDL particles. Indeed, Western blots distinguished two bands of proteins of apparent lower molecular weight that were antigenically related to apolipoprotein B. The protein with M_r 49 kDa has not been characterized.

LDL-V augmented cholesterol ester deposition in cultured mouse peritoneal macrophages, a cell type known to possess scavenger receptors.⁴ LDL-V also produced cholesterol ester accumulation in cultured rabbit alveolar macrophages, in contrast to the effects of acetylated LDL. The processing of LDL-V by macrophages could have differed from that for acetylated LDL, either as a consequence of interaction at different receptors or of differences in intracellular processing. In addition, some of the stimulation of cholesterol esterification in macrophages may have occurred because of enhancement of the unesterified cholesterol/phospholipid ratio that would favor the flux of cholesterol into cells by physicochemical mechanisms.^{48,49} Cholesterol transport of this type has been shown to stimulate ACAT activity in cultured cells.⁵⁰ In agreement with Parthasarathy et al.¹² data from the present study do not implicate lysophospholipid as the determinant in LDL responsible for the cellular interactions, because this phospholipid type is not significantly elevated in LDL-V. Fragmentation of apolipoprotein B alone does not lead to recognition of LDL by cultured macrophages, although fragmentation in the presence of oxidative conditions produces stimulation of cholesterol esterification in this cell type.¹⁵

An important question arising from the detection of these particles is their location within the tissue. During the preliminary experiments performed for this study, no material was detected when homogenization was performed in the presence of a Tris buffer.^{18,21} Perhaps, due to their avid catabolism by macrophages, LDL-V may not accumulate in significant quantities in the interstitium, or they may be too highly bound to extracellular matrix elements to be released by aqueous buffers alone. The results from the present study were acquired in the presence of sodium carbonate buffer, which is known to release bound proteins from membrane surfaces.²⁵ Although these studies could mean that the characterized fraction was bound, further study is required to define the location of these particles.

In summary, the present study described LDL-like particles from in vivo vascular tissue that are modified in a manner similar to those described when LDL is subjected to potential modifiers in vitro. The modified particles may play a role in initiating or potentiating atherosclerosis. Indeed, probucol may prevent the oxidation of LDL, and this drug has recently been demonstrated to exert anti-atherosclerotic effects that are independent of a hypolipidemic action.^{51,52} Accordingly, pharmacological inhibition of specific modifications of LDL in vivo offers promise for prevention and toward a

theory of atherosclerosis that defines the role of lipoprotein perturbations in atherogenesis.

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Index Terms: atherosclerosis • Watanabe heritable hyperlipidemic rabbits • low density lipoproteins • lipoprotein modification • phospholipids • lipid peroxidation • apolipoprotein B • cholesterol esterification • macrophages