

Oxidized type IV hypertriglyceridemic VLDL-remnants cause greater macrophage cholesteryl ester accumulation than oxidized LDL

Stewart C. Whitman, Cynthia G. Sawyez, David B. Miller, Bernard M. Wolfe, and Murray W. Huff¹

Departments of Medicine and Biochemistry, and the Robarts Research Institute at The University of Western Ontario, 100 Perth Drive, London, Ontario, Canada N6A 5K8.

Abstract We have previously shown that very low density lipoproteins (VLDL, S_f 60–400) from subjects with type IV hyperlipoproteinemia (HTG-VLDL) will induce appreciable cholesteryl ester accumulation in cultured macrophages (J774A.1). The present study examined whether copper-mediated oxidative modification of HTG-VLDL and their remnants would further enhance cholesteryl ester accumulation in J774A.1 cells. Incubation with oxidized VLDL-remnants caused the greatest increase in cellular cholesteryl ester concentrations (54-fold) relative to control cells ($P = 0.001$). HTG-VLDL and VLDL-remnants each induced similar increases in cholesteryl ester levels (32.3- and 35.8-fold, respectively; both $P = 0.001$), whereas incubation with oxidized HTG-VLDL brought about only a 20.6-fold increase in cholesteryl ester concentrations ($P = 0.014$). The increase in cellular cholesteryl ester concentrations induced by oxidized VLDL-remnants was significantly higher ($P \leq 0.04$) than that induced by all other lipoproteins tested including low density lipoprotein (LDL) and oxidized LDL which caused a 6.7- and a 35.1-fold increase ($P \leq 0.0002$ for both), respectively. Unlike HTG-VLDL and to a lesser extent VLDL-remnants, uptake of oxidized VLDL and oxidized VLDL-remnants did not require catalytically active, cell secreted lipoprotein lipase. Co-incubation with polyinosine, which blocks binding to the type I scavenger receptor, completely inhibited the cholesteryl ester accumulation induced by oxidized HTG-VLDL, oxidized VLDL-remnants and oxidized LDL ($P \leq 0.02$). We conclude that oxidation of VLDL-remnants significantly enhances macrophage cholesteryl ester accumulation compared to either HTG-VLDL, VLDL-remnants, or oxidized LDL. Uptake of oxidized VLDL and oxidized VLDL-remnants does not require catalytically active lipoprotein lipase, and involves a receptor that can be competed for by polyinosine.—Whitman, S. C., C. G. Sawyez, D. B. Miller, B. M. Wolfe, and M. W. Huff. **Oxidized Type IV Hypertriglyceridemic VLDL-remnants cause greater macrophage cholesteryl ester accumulation than oxidized LDL.** *J. Lipid Res.* 1998. 39: 1008–1020.

Supplementary key words type IV hyperlipoproteinemia • VLDL-remnants • oxidation • foam cells • scavenger receptor • lipoprotein lipase • macrophages

Cholesterol-loaded macrophages, morphologically recognized as foam cells, are characteristic of developing atherosclerotic plaques (1, 2). It has been well established that incubation of cultured macrophages with native low density lipoproteins (LDL), does not result in either significant cholesteryl ester (CE) deposition or foam cell formation (3). However, oxidation (Ox) of LDL generates a modified form of these lipoproteins whose uptake by macrophages is markedly enhanced, leading to unregulated cholesteryl ester accumulation by the macrophage and foam cell formation (4–6). Unlike native LDL, hypertriglyceridemic very low density lipoproteins (HTG-VLDL), isolated from subjects with type IV hyperlipoproteinemia (HLP) and possessing receptor competent apolipoprotein E (apoE), are capable of inducing foam cell formation without the requirement for oxidation (7–14). However, the effect of oxidation of HTG-VLDL on macrophage uptake has not been examined.

We have previously shown that the CE and triglyceride (TG) components of an HTG-VLDL particle are taken up by J774A.1 macrophages in a two-step process (8). The first step requires the interaction between VLDL and cell-secreted lipoprotein lipase (LPL). During this interaction, the TG core of HTG-VLDL is hydrolyzed extracellularly by LPL, with the resulting free fatty acids being subsequently taken up by the macrophage and re-esterified into TG within the cell. As lipolysis proceeds, the receptor binding epitopes of apoE on VLDL become exposed, allowing the CE-rich VLDL-remnant (VLDL-REM) to be taken up by the macrophage via a receptor mediated process (8).

Subjects with type IV HLP have elevated plasma levels of

Abbreviations: VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HTG, hypertriglyceridemia; HLP, hyperlipoproteinemia; LPL, lipoprotein lipase; REM, remnant; Ox, oxidation; apo, apolipoprotein; THL, tetrahydrolipstatin; CE, cholesteryl ester; FC, free cholesterol; TG, triglyceride; LPDS, lipoprotein-deficient serum; DMEM, Dulbecco's modified Eagle's medium; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

¹To whom correspondence should be addressed.

CE- and TG-enriched VLDL due to either an overproduction of this lipoprotein subfraction by the liver and/or the inability to efficiently clear these lipoproteins from the blood circulation (15, 16). Although subjects with type IV HLP have elevated levels of VLDL and VLDL-REM particles, they often have normal or reduced levels of LDL (15, 16), a class of lipoprotein whose elevation is normally associated with atherogenesis (17). While individuals with type IV HLP are at an increased risk of developing atherosclerosis (18–20), the basis for this increased risk is not fully understood. It is known that a proportion of S_f 60–400 VLDL in type IV subjects is directly removed from the circulation. In addition, after remnant formation via endothelial cell-bound LPL, the majority of HTG-VLDL remnants (S_f 12–60) are cleared, and not converted into LDL (21–24). Although the liver is the primary tissue-specific site responsible for the removal of HTG-VLDL and VLDL-REM from the blood circulation (25), macrophages, residing within the vascular intima may also facilitate removal of HTG-VLDL and VLDL-REM once these lipoproteins pass into the arterial wall. Indeed, lipoproteins in the VLDL and intermediate density lipoproteins (IDL) density range have been isolated from human aortic intima (26) and from aortic atherosclerotic plaque (27).

In the present study, we tested the hypothesis that oxidative modification of HTG-VLDL (S_f 60–400) and their remnants, isolated from subjects with type IV HLP leads to foam cell formation, *in vitro*, by a mechanism analogous to that which occurs with OxLDL. To test this hypothesis, we examined whether HTG-VLDL and VLDL-REM could be oxidized *in vitro* and whether these modified lipoproteins could induce CE-loading of cultured macrophages.

EXPERIMENTAL PROCEDURES

Subjects

Subjects were recruited from the Outpatient Endocrinology Lipid Clinic at the London Health Sciences Centre, London, Ontario. Lipoprotein phenotypes of the type IV HLP subjects were classified according to the criterion of Schaefer and Levy (28). None of the type IV HLP subjects carried the apoE2 isoform as determined by isoelectric focussing gel electrophoresis (29, 30). All type IV HLP subjects had elevated plasma cholesterol (6.68 ± 0.45 mm, mean \pm SEM) and TG (9.70 ± 0.98 mm) levels due to elevated VLDL cholesterol (4.38 ± 0.35 mm) and TG (9.12 ± 0.83 mm) concentrations. LDL preparations used in these experiments were derived from four subjects: one normolipidemic subject; one type IV HLP subject; and two type IIa HLP subjects. These studies were approved by the University of Western Ontario Health Sciences Standing Committee on Human Research and all subjects gave informed consent prior to blood sampling.

Lipoprotein isolation

Sixty to 180 ml of blood was collected from each fasted (12–14 h) subject and placed in tubes containing EDTA at a final concentration of 0.15% (w/v). Plasma was isolated by centrifugation (IEC Centra-8R centrifuge) at 2500 rpm (1000 *g*) for 25 min at 4°C. The different classes of lipoproteins were collected and washed, as described previously (31), by ultracentrifugation in a Beckman L8 ultracentrifuge. The large VLDL (S_f 60–400) sub-

class was first isolated using a Beckman 55.2 Ti rotor (1.75 h, 40,000 rpm, 12°C), and subsequently washed using a Beckman 70.1 Ti rotor (16 h, 40,000 rpm, 12°C). After isolation of the VLDL and IDL fractions ($d < 1.019$ g/ml), the LDL ($d = 1.019$ – 1.063 g/ml) fraction was isolated from the infranatant by ultracentrifugation (16 h, 50,000 rpm, 12°C) in a 55.2 Ti rotor and washed using a 70.1 Ti rotor (16 h, 50,000 rpm, 12°C). Both the VLDL (S_f 60–400) and LDL preparations were extensively dialyzed, in the dark and at 4°C, against a 200-fold excess volume of phosphate-buffered saline (PBS; 154 mm NaCl, 8 mm $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.5 mm KH_2PO_4 , 2.7 mm KCl, pH 7.4) containing 10 μM EDTA. After dialysis, the lipoprotein samples were sterilized using 0.45- μm filters and stored at 4°C.

All lipoprotein samples were analyzed for protein content by a modification of the Lowry method (32), for free fatty acids using enzymatic reagents (#990-75401) from Wako (Neuss, Germany; distributed by Immunocorp, Montreal, Quebec) and for TG, free cholesterol (FC), total cholesterol and phospholipid using enzymatic reagents from Boehringer Mannheim GmbH Diagnostica, Montreal, Quebec (TG: #450032 without free glycerol, FC: #310328, total cholesterol: #1442350 and phospholipid: #691844). Some lipoprotein preparations were analyzed for antioxidant activity, measured as α -tocopherol equivalents (Trolox, an α -tocopherol analogue), according to the method of Miller et al (33).

Bovine milk lipoprotein lipase isolation

Bovine skim milk lipoprotein lipase (LPL) was partially purified by a modification of the method of Socorro and Jackson (34) as described previously (35). The LPL activity in each eluted fraction was determined by measuring the amount of free fatty acids released from a predetermined amount of a commercially obtained TG emulsion (Intralipid, Pharmacia Inc., Mississauga, Ontario). The LPL activity assay was conducted in 16×100 mm borosilicate glass tubes (Fisher Scientific, Pittsburgh, PA) by adding, in order, the following: 300 μl LPL buffer (0.15 m NaCl, 0.2 m Tris, pH 8.2), 5% (w/v) fatty acid-free bovine serum albumin (FAF-BSA; fraction V, Sigma, St. Louis, MO), 12% (v/v) normolipidemic human plasma (as a source of apoC-II), 10 μl deionized H_2O , 10 μl lipase solution and 100 μl Intralipid containing 1.82 μmol of TG. After incubation for 30 min at 37°C in a shaking water bath, the reaction was stopped by adding 2 ml isopropyl alcohol–3 N H_2SO_4 40:1 (v/v), 1 ml H_2O and 2.5 ml hexane, with vigorous vortexing for 1 min. The phases were allowed to separate by standing for 20 min at room temperature. The hexane layer was removed into new 16×100 mm glass test tubes, and the hexane extraction step was then repeated once more. The pooled hexane fractions were evaporated under N_2 . One ml of chloroform was added to each tube, and a 100 μl aliquot was taken for subsequent fatty acid determination. To this aliquot, 750 μl of a 1% (v/v) Triton X-100 in chloroform solution was added; the solution was mixed, dried under N_2 , and the amount of free fatty acids released was determined using a spectrophotometric based free-fatty acid assay kit (see above). One unit of LPL activity is defined as 1 μmol of free fatty acid released per ml of enzyme solution per h. To examine whether oxidation *a*) inhibited the ability of HTG-VLDL to serve as a substrate for LPL, and/or *b*) inhibited apoC-II from acting as a cofactor for LPL, we performed two different *in vitro* LPL activity assays using a slight modification of the LPL activity assay described above. In the first assay we tested the ability of bovine milk LPL to release free fatty acids from either native HTG-VLDL or OxHTG-VLDL. In this LPL activity assay, HTG-VLDL and OxHTG-VLDL at concentrations of 500 and 1000 μg TG/reaction were added in place of the intralipid. In the second *in vitro* LPL activity assay, HTG-VLDL and OxHTG-VLDL at concentrations of 18 and 36 μg total protein/reaction were used in place of human plasma as the source

of apoC-II. In this second assay, intralipid (1.82 μmol of TG) was used as the substrate for LPL.

VLDL-remnant preparation

Remnant-like particles of HTG-VLDL (S_f 60–400) were formed in vitro under sterile conditions by incubating the HTG-VLDL with LPL (0.2 units per 50 μg total lipoprotein cholesterol) in the presence of a 5% (w/v; final concentration) solution of FAF-BSA in PBS. TG-hydrolysis was allowed to proceed at 37°C for 8 h. The VLDL-REM were reisolated by adjusting the reaction buffer to a final density of 1.019 g/ml followed by ultracentrifugation in a Beckman 70.1 Ti rotor (16 h, 40,000 rpm, 12°C). The VLDL-REM preparations were dialyzed and sterilized by filtration as stated above. Percent TG-hydrolysis was calculated using the following formula:

$$\% \text{ hydrolysis} = 100 - \left[\frac{(\text{TG}/\text{CE of VLDL-REM} \times 100)}{(\text{TG}/\text{CE of native-VLDL})} \right]$$

In all remnant forming assays, the percentage of TG hydrolyzed ranged from 30% to 40%.

Oxidation of lipoproteins

The dialyzed and sterile HTG-VLDL, VLDL-REM, and LDL preparations were oxidized in vitro following a modification of the protocol described by Steinbrecher, et al. (36). Briefly, reactions were performed under sterile conditions by incubating the lipoprotein preparation (200 μg protein/ml) with CuSO_4 (5.0 μM) in EDTA-free PBS for 48 h at 37°C and in the absence of light. The reactions were stopped by immediately placing the samples on ice followed by the addition of EDTA (200 μM) and butylated hydroxytoluene (40 μM). The oxidized lipoprotein preparations were then reisolated by adjusting the reaction buffer to a final density of either 1.063 g/ml (OxHTG-VLDL and OxVLDL-REM) or 1.10 g/ml (OxLDL), followed by ultracentrifugation using a Beckman 70.1 Ti rotor spun for 16 h at 50,000 rpm and 12°C. The OxHTG-VLDL, OxVLDL-REM, and OxLDL preparations were dialyzed and sterilized by filtration as stated above. Filtration of the oxidized preparations also served to remove any aggregated lipoproteins.

The lipoprotein-oxidation reactions were monitored by assaying for conjugated diene formation. The conjugated diene assays were conducted in parallel with the oxidation reactions by following the protocol of Kleinvelde et al. (37). The conjugated diene curves were produced by incubating (37°C) the lipoprotein preparations (50 μg protein/ml) with CuSO_4 (5.0 μM) in EDTA-free PBS and continuously monitoring the changes in absorbance at 234 nm for 20 h.

Some of the native and oxidized HTG-VLDL, VLDL-REM, and LDL preparations were dialyzed against ammonium bicarbonate, lyophilized and delipidated as described previously (38). Apolipoproteins were resolubilized and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 3%-15% acrylamide gels containing β -mercaptoethanol (39). Gels were stained with Coomassie blue R-250.

Cell culture

J774A.1 cells, a murine macrophage-like cell line that secretes LPL but not apoE (40, 41), were used in this study. The J774A.1 cells were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in culture as outlined previously (8). For all experiments in the present report, J774A.1 cells were plated in six-well (35 mm) culture plates (Falcon, VWR, Mississauga, Ontario.) in 2.0 ml of Dulbecco's modified Eagle's medium (DMEM) (low glucose) (Gibco, Burlington, Ontario) containing 10% fetal bovine serum (Sigma) and grown for 2–3 days. When the monolayers had become 70–80% confluent,

the media were replaced with DMEM containing 5% lipoprotein-deficient serum (LPDS, prepared as outlined previously (8)); the LPDS was assayed for apoC-II and apoE (21) and found to be free of these apolipoproteins. The final albumin concentration in the media was 0.13%. For each lipoprotein preparation, between 50 and 300 μg of total lipoprotein cholesterol per ml media was added to duplicate wells of cells which were then incubated for 16 h at 37°C. The LPL inhibitor tetrahydrolipstatin (THL) (brand name: orlistatTM; provided by Hoffmann-LaRoche Pharmaceuticals Ltd, Montreal, Canada) was used at a concentration of 1.0 μM . At this concentration, THL inhibited the activity of 0.25 units of bovine milk LPL by greater than 95% (data not shown). Previous studies demonstrated that J774A.1 macrophages secrete 0.25 units of LPL/24 h (8). The THL stock solution was made up in dimethyl sulfoxide (DMSO) and then diluted with DMEM media plus LPDS prior to being added to the cells. Control dishes received an equal volume (not exceeding 10 μl /well) of DMSO alone. In experiments in which the polynucleotides polyinosine and polycytosine (Pharmacia Biotech, Mississauga, Canada) were used, various concentrations (5 μg to 100 μg /ml media) of the polynucleotides in deionized H_2O were added in volumes no greater than 40 μl . Control dishes received polynucleotide (100 μg /ml media) in the absence of any lipoprotein.

Quantitative analysis of cellular lipids

The cell-lipoprotein incubations were terminated by two washes of Tris buffer (0.15 M NaCl, 50 mM Tris, 0.2% (w/v) FAF-BSA, pH 7.4) and two additional washes with Tris buffer without FAF-BSA. The lipids were extracted in situ with two 30-min incubations using 1.0 ml of hexane-isopropanol 3:2 (v/v). The solvents from each extraction were pooled for analysis. To each dish, 1.0 ml of 0.1 N NaOH was added and incubated overnight at room temperature to digest the cells. Cell protein was determined by a modification of the Lowry method (32). Cellular total cholesterol, FC, and TG mass were determined spectrophotometrically by a modification of a method described previously (42), using enzymatic reagents from Boehringer Mannheim (see above), and a V_{max} Kinetic 96 multi-well microplate reader (Molecular Devices, Mississauga, Ontario). Briefly, each hexane-isopropanol sample was evaporated to dryness under N_2 , and resuspended in 1.2 ml of a chloroform-Triton mixture (0.5% Triton v/v). The solvent was evaporated again under N_2 , and finally resolubilized in 300 μl of deionized H_2O (final sample concentration, 2% triton). Two 50- μl aliquots of each sample were then pipetted into individual wells of a 96 multi-well, flat-bottom, microtiter plate (Nunc, Gibco) and assayed for total cholesterol mass at 490 nm. Two more 50- μl aliquots of each sample were also pipetted into individual wells of a second 96 multi-well microtiter plate and assayed for FC mass at 490 nm. CE mass was calculated by taking the difference between the total and FC mass values. To determine the TG-mass of each sample, 75 μl of each sample was first mixed with 50 μl of a 2% triton (in deionized H_2O) (v/v) solution and then two 50- μl aliquots were pipetted into individual wells of a third 96 multi-well microtiter plate and assayed for TG-mass at 490 nm. Cholesterol and triglyceride standards (range between 1 and 20 μg /well) used to generate standard curves were processed in a fashion identical to that of the experimental samples. Cellular lipid results are reported as μg of cellular lipid (CE, TG, or FC) per μg of cell protein.

Statistical analysis

In each experiment, duplicate cell culture wells were used for each specific lipoprotein preparation, with the resulting values combined to give a mean value for that particular experiment. Mean values from separate experiments were then used to calculate a group mean \pm SEM for each condition. The "n" referred

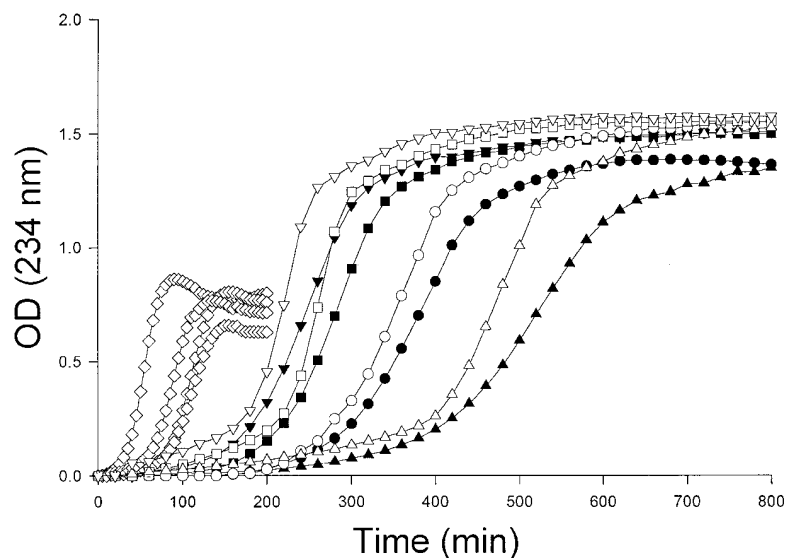


Fig. 1. Conjugated diene curves generated from the oxidation of HTG-VLDL, VLDL-REM, and LDL. Line graph shows conjugated diene formation curves generated from the oxidation of HTG-VLDL (solid symbols) and corresponding VLDL-REM (open symbols), with each symbol (∇ , \blacksquare , \square , \bullet , \circ , \blacktriangle , \triangle) representing a lipoprotein preparation from one subject (HTG-VLDL and the corresponding VLDL-REM; $n = 4$ for each). The four curves generated from the oxidation of LDL (open diamond symbols, \diamond) were derived from plasma obtained from four subjects, which were distinct from the subjects used to obtain HTG-VLDL. The conjugated diene curves were produced by incubating (37°C) the lipoprotein preparations ($50 \mu\text{g protein/ml}$) with CuSO_4 ($5.0 \mu\text{M}$) in EDTA-free PBS and continuously monitoring the changes in absorbance at 234 nm for 20 h.

to for each experiment indicates the number of different patients' samples used to determine each experimental parameter. Statistical significance between control and experimental group mean values was assessed using a Student's t test. A two-tailed $P \leq 0.05$ was considered statistically significant.

RESULTS

HTG-VLDL, like LDL, was susceptible to oxidative modification after exposure to CuSO_4 as assessed by the formation of conjugated dienes (**Fig. 1**). The conjugated diene curves for HTG-VLDL ($n = 7$), VLDL-REM ($n = 7$), and LDL ($n = 4$) were analyzed for lag time, diene formation rate, maximal diene formation, and the time between the initiation of oxidation and maximal diene formation. HTG-VLDL (compared to LDL) was found to have a 4-fold longer lag time (262 ± 49 vs 64 ± 12 min, $P = 0.004$), took 6-fold longer to reach maximal diene production

(740 ± 26 vs 134 ± 15 min, $P = 0.0001$), and produced a 2-fold greater amount of dienes (974 ± 27 vs 524 ± 29 nmol/g of lipoprotein protein, $P = 0.0001$) but at half the rate (5.40 ± 0.65 vs 9.33 ± 1.39 nmol/g/min, $P = 0.02$). Oxidation of VLDL-REM produced a conjugated diene curve that had a very similar lag time (261 ± 46 vs 262 ± 49 min, $P = 0.5$) and took the same time to reach maximal diene production (745 ± 38 vs 740 ± 26 min, $P = 0.5$) as that of HTG-VLDL. However, compared to HTG-VLDL, oxidation of VLDL-REM produced a similar amount of conjugated dienes (1053 ± 6 vs 974 ± 27 nmol/g) but at 1.5-fold the rate (8.36 ± 1.39 vs 5.40 ± 0.65 nmol/g/min, $P = 0.02$).

HTG-VLDL, VLDL-REM, and LDL were prepared from plasma obtained from three different type IV hypertriglyceridemic patients and analyzed for total antioxidant activity (33). The mean antioxidant activities (nmol/mg of lipoprotein protein) were: HTG-VLDL, 213 ± 86 ; VLDL-REM,

TABLE 1. Characteristics of lipoproteins used in the cell studies

Lipoprotein Class	C/TG	TG/CE	C/Protein	TG/Protein	PL/Protein	FFA/Protein
<i>ratio</i>						
Type IV HTG-VLDL						
Native ($n = 7$)	0.16 ± 0.04	13.39 ± 2.06	2.10 ± 0.20	14.06 ± 1.82	2.85 ± 0.48	0.82 ± 0.33
Oxidized ($n = 7$)	0.16 ± 0.02	23.42 ± 6.85	2.38 ± 0.14	15.48 ± 1.96	3.38 ± 0.34	0.75 ± 0.29
Type IV VLDL REM						
Native ($n = 7$)	0.18 ± 0.02	8.44 ± 1.33^a	1.95 ± 0.31	11.34 ± 2.47^a	2.09 ± 0.53	2.98 ± 1.74
Oxidized ($n = 7$)	0.19 ± 0.04	11.66 ± 1.07^b	3.18 ± 0.34^b	18.30 ± 1.87^b	4.10 ± 0.47^b	4.81 ± 2.48
LDL ^c						
Native ($n = 4$)	5.84 ± 1.21		1.87 ± 0.12	0.32 ± 0.05	0.97 ± 0.06	
Oxidized ($n = 4$)	1.24 ± 1.33		0.97 ± 0.09^d	0.78 ± 0.05	0.98 ± 0.12	

Values are expressed as means \pm SEM; C, cholesterol; TG, triglyceride; PL, phospholipid; FFA, free fatty acids; CE, cholesteryl ester; VLDL, very low density lipoprotein (S_f 60–400); VLDL-REM, 30–40% TG-hydrolysis; LDL, low density lipoprotein. The value for n refers to the number of different patients' lipoprotein samples used in the determination of each parameter.

^aSignificantly different from HTG-VLDL $P \leq 0.03$.

^bSignificantly different from VLDL-REM $P \leq 0.003$.

^cLDL was isolated from one normolipidemic, one Type IV, and two Type IIA subjects.

^dSignificantly different from LDL $P \leq 0.015$.

202 ± 85, and LDL, 54 ± 13. The corresponding lag times (min), obtained from the conjugated diene curves, for these lipoprotein preparations were: HTG-VLDL, 493 ± 157; VLDL-REM, 444 ± 95, and LDL, 84 ± 5. The lag times were correlated with the total antioxidant activity ($r = 0.98$, $P = 0.0001$).

The compositions of the lipoproteins used in the present study are summarized in **Table 1**. The process of *in vitro* remnant formation resulted in a 38.4% reduction ($P = 0.003$) in the TG to CE ratio and a 19.4% reduction ($P = 0.03$) in the TG to protein ratio (Table 1). Oxidation of HTG-VLDL caused no significant change in any of the compositional characteristics measured (Table 1). Oxidation of VLDL-REM caused a 38% increase ($P = 0.0007$) in the TG to CE ratio, a 63% increase ($P = 0.001$) in the total cholesterol to protein ratio, a 61.4% increase ($P = 0.002$) in the TG to protein ratio, and a 96.4% increase ($P = 0.003$) in the phospholipid to protein ratio (Table 1). Oxidation of LDL caused a significant decrease in the total cholesterol to protein ratio ($p = 0.015$; Table 1). The latter observation has been reported previously by other laboratories (36, 43).

Incubation of J774A.1 cells with HTG-VLDL resulted in up to a 32.3-fold increase in cellular CE concentrations relative to control cells incubated in lipoprotein-deficient media ($P = 0.001$; **Fig. 2A**). In contrast, oxidation of HTG-VLDL induced a smaller increase in cellular CE concentrations when compared to its native counterpart (20.6-fold compared to control cells, $P = 0.014$; **Figure 2A**). The difference in cellular CE accumulation between HTG-VLDL and OxHTG-VLDL was significant at each of the concentrations tested ($P \leq 0.044$; **Figure 2A**).

Incubation of cells with HTG-VLDL resulted in as high as an 8.5-fold increase in cellular TG concentrations when compared to control cells (**Fig. 2B**, $P = 0.0002$). This finding is consistent with observations reported previously by this laboratory (8). Incubation of cells with OxHTG-VLDL also resulted in up to a 6.9-fold increase in cellular TG concentrations ($P \leq 0.0007$; **Fig. 2B**). The difference between TG levels induced by HTG-VLDL and OxHTG-VLDL was statistically significant at the lowest and two highest incubation concentrations examined ($P \leq 0.05$; **Fig. 2B**) and could not be explained by any differences in TG content of the lipoprotein preparation (Table 1).

Incubation of J774A.1 cells with VLDL-REM induced up to a 35.8-fold increase in cellular CE concentrations when compared to control cells ($P = 0.001$; **Fig. 2A**). This level of cellular CE accumulation was found to be similar to that induced by HTG-VLDL ($P = 0.10$; **Fig. 2A**). Oxidative modification significantly enhanced (up to a 54-fold increase) the cellular CE loading by VLDL-REM ($P = 0.0002$; **Fig. 2A**). This enhanced uptake was statistically significant ($P \leq 0.03$) at the higher concentrations (200 and 300 μg total lipoprotein cholesterol/ml media) examined (**Fig. 2A**).

VLDL-REM were found to induce up to an 8.7-fold increase in cellular TG concentrations when compared to control cells ($P = 0.0005$; **Fig. 2B**). OxVLDL-REM caused up to a 6.9-fold increase in cellular TG concentrations ($P = 0.0007$; **Fig. 2B**), however, this increase was significantly

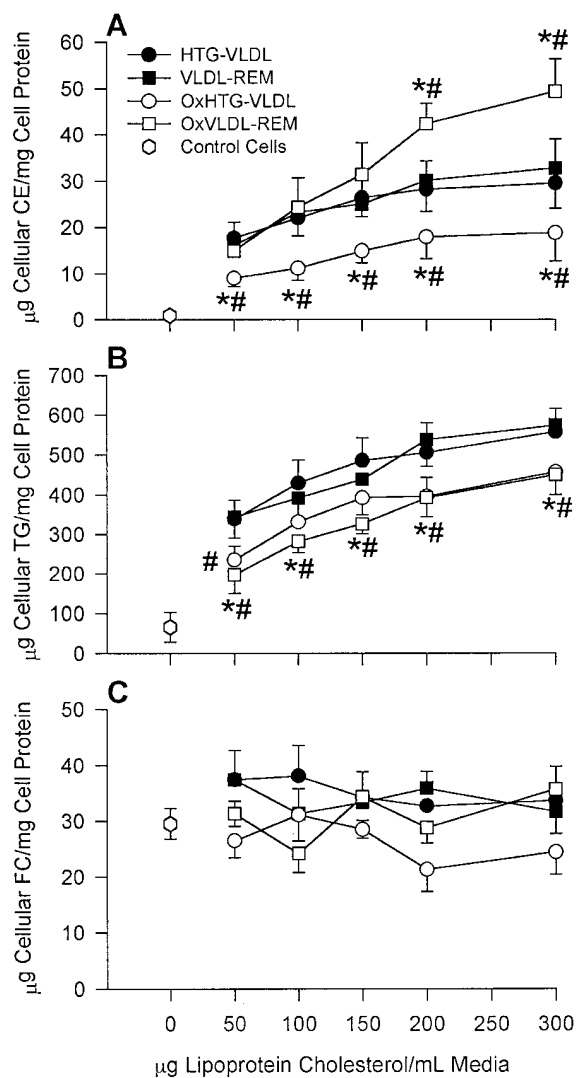


Fig. 2. Esterified cholesterol, triglyceride and free cholesterol content of J774A.1 cells incubated with HTG-VLDL, VLDL-REM, OxHTG-VLDL, or OxVLDL-REM. Esterified cholesterol (A), triglyceride (B), and free cholesterol (C) content of J774A.1 macrophages exposed for 16 h to HTG-VLDL (\bullet), VLDL-REM (\blacksquare), OxHTG-VLDL (\circ), and OxVLDL-REM (\square) (50–300 μg lipoprotein cholesterol/ml media; $n = 4$ for each preparation). The value of n refers to the number of different patients' lipoprotein samples analyzed. Each lipoprotein preparation from each patient was analyzed in duplicate. The values represented by each symbol are the mean \pm SEM, for all patients' lipoprotein preparations. *, $P \leq 0.05$ relative to HTG-VLDL; #, $P \leq 0.05$ relative to VLDL-REM. CuSO_4 -mediated oxidation of the lipoproteins was performed as described under Methods. Cellular CE, FC, and TG levels were determined as described under Methods.

less than that induced by VLDL-REM. The difference between VLDL-REM and OxVLDL-REM was statistically significant at all of the incubation concentrations examined ($P \leq 0.02$). Again, this difference could not be explained by differences in the incubation conditions based on TG concentrations between VLDL-REM and OxVLDL-REM (Table 1). Incubation of macrophages with increasing concentrations of HTG-VLDL, VLDL-REM, OxHTG-VLDL,

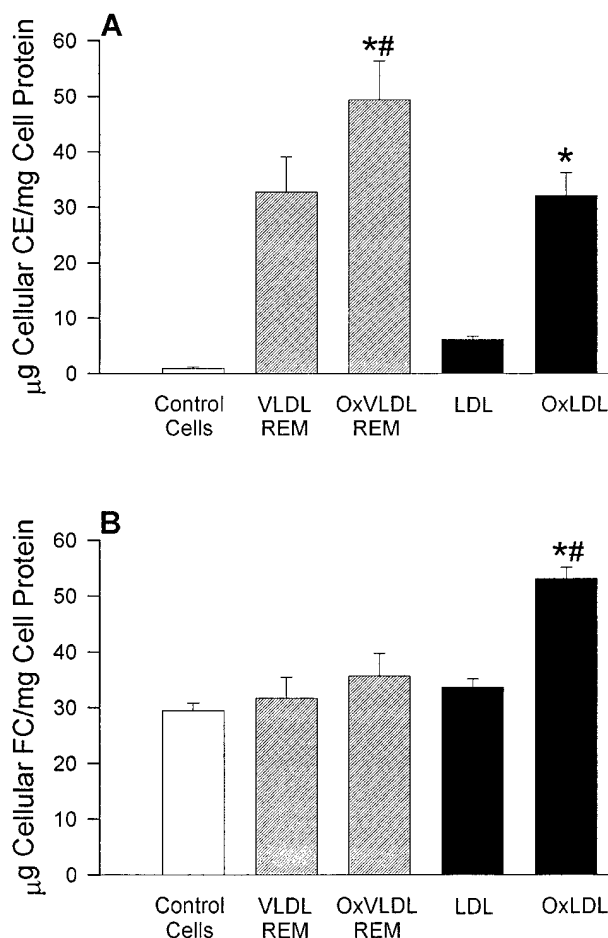


Fig. 3. Esterified cholesterol and free cholesterol content of J774A.1 cells incubated with VLDL-REM, OxVLDL-REM, LDL, or OxLDL. Esterified cholesterol (A) and free cholesterol (B) content of J774A.1 macrophages exposed for 16 h to VLDL-REM, OxVLDL-REM, LDL, and OxLDL (300 µg lipoprotein cholesterol/ml media; $n = 4$). The value of n refers to the number of different patients' lipoprotein samples analyzed. Each lipoprotein preparation from each patient was analyzed in duplicate. The values represented by each bar are the mean \pm SEM, for all patients' lipoprotein preparations. (A) *, $P \leq 0.018$ relative to its native counterpart; #, $P = 0.039$ relative to OxLDL. (B) *, $P \leq 0.0003$ relative to LDL, VLDL-REM, and OxVLDL-REM. CuSO_4 mediated oxidation of the lipoproteins was performed as described under Methods. Cellular CE and FC levels were determined as described under Methods.

and OxVLDL-REM did not change the cellular FC concentrations ($P \geq 0.07$; Fig. 2C).

Native LDL (300 µg total cholesterol/ml media) caused only a 6.7-fold increase in cellular CE concentrations relative to control cells ($P = 0.0002$; Fig. 3A). Although the LDL preparations used were derived from four subjects with different lipid phenotypes, no significant difference was found in cellular CE uptake between preparations. In contrast to native LDL, OxLDL (300 µg total cholesterol/ml media) caused a 35.1-fold increase in cellular CE concentrations ($P = 0.0001$; Fig. 3A). Although the increase in cellular CE levels induced by OxLDL was very large, it was significantly lower than that induced by OxVLDL-REM ($P = 0.039$; Fig. 3A). In addition, no significant dif-

ference was seen between the amount of cellular CE accumulation induced by OxLDL versus VLDL-REM ($P = 0.47$, Fig. 3A) and HTG-VLDL ($P = 0.36$, Figs. 2A and 3A). Incubation with OxLDL was found to cause a 1.8-fold increase in cellular FC concentrations compared to control cells ($P = 0.0001$; Fig. 3B). There was no change in cellular FC with any of the other lipoproteins examined.

The polynucleotide polyinosine, but not polycytosine, has been shown previously to inhibit type I scavenger receptor-mediated binding, uptake, and degradation of chemically modified LDL by both cultured mouse peritoneal macrophages (44) and cultured CHO cells (45) and of copper OxLDL and β -VLDL by liver Kupffer cells in vivo (46, 47). A dose-response relationship for the effect of polyinosine (5–100 µg/ml of media) on lipid accumulation in J774A.1 cells by OxHTG-VLDL, OxVLDL-REM, and OxLDL was examined. A significant reduction in both cellular CE and TG concentrations was observed at all concentrations and maximal inhibition was achieved at 100 µg/ml of media (data not shown). Co-incubation of polyinosine (100 µg/ml of media) with OxHTG-VLDL, OxVLDL-REM, and OxLDL inhibited (72%–95%) the ability of these oxidized lipoprotein preparations to induce cellular CE accumulation in cultured J774A.1 macrophages ($P \leq 0.02$; Fig. 4A). In addition, polyinosine also inhibited (66%–81%) the ability of OxHTG-VLDL and OxVLDL-REM to induce cellular TG accumulation ($P \leq 0.03$; Fig. 4B). In contrast to the oxidized lipoproteins, co-incubation of polyinosine with HTG-VLDL, VLDL-REM, and LDL had no effect on the ability of these native lipoprotein preparations to induce either cellular CE or TG accumulation ($P \geq 0.3$; Figs. 4A and 4B).

Co-incubation of various concentrations of polycytosine with OxHTG-VLDL, OxVLDL-REM, OxLDL, or their native counterparts had no significant effect on the ability of these lipoprotein preparations to induce cellular CE and TG accumulation ($P \geq 0.14$; Figs. 4C and 4D, respectively).

As described previously, LPL-mediated TG-hydrolysis is an important initial step in the uptake of both the TG and CE components of HTG-VLDL (8). We therefore examined whether a similar two-step mechanism was required for the uptake of OxHTG-VLDL and OxVLDL-REM. In these experiments THL, a potent competitive inhibitor of LPL, was used to inhibit the catalytic activity of LPL secreted by the J774A.1 macrophages. Co-incubation of HTG-VLDL with THL inhibited the accumulation of HTG-VLDL-CE (by 91%) and HTG-VLDL-TG (by 100%) by the cultured macrophages ($P = 0.0001$ for both; Figs. 5A and 5B). Co-incubation of partially hydrolyzed (30–40% TG-hydrolysis) VLDL-REM with THL caused a smaller but significant reduction in cellular CE (by 49%) and TG levels (by 75%, $P \leq 0.003$ for both; Figs. 5A and 5B). In contrast, co-incubation of OxHTG-VLDL and OxVLDL-REM with THL did not affect the ability of these modified lipoproteins to induce increases in both cellular CE and TG concentrations ($P = 0.6$; Figs. 5A and 5B).

To examine whether oxidation *a*) inhibited the ability of HTG-VLDL to serve as a substrate for LPL, and/or *b*) inhibited apoC-II from acting as a cofactor for LPL, we performed two different in vitro LPL activity assays. In the

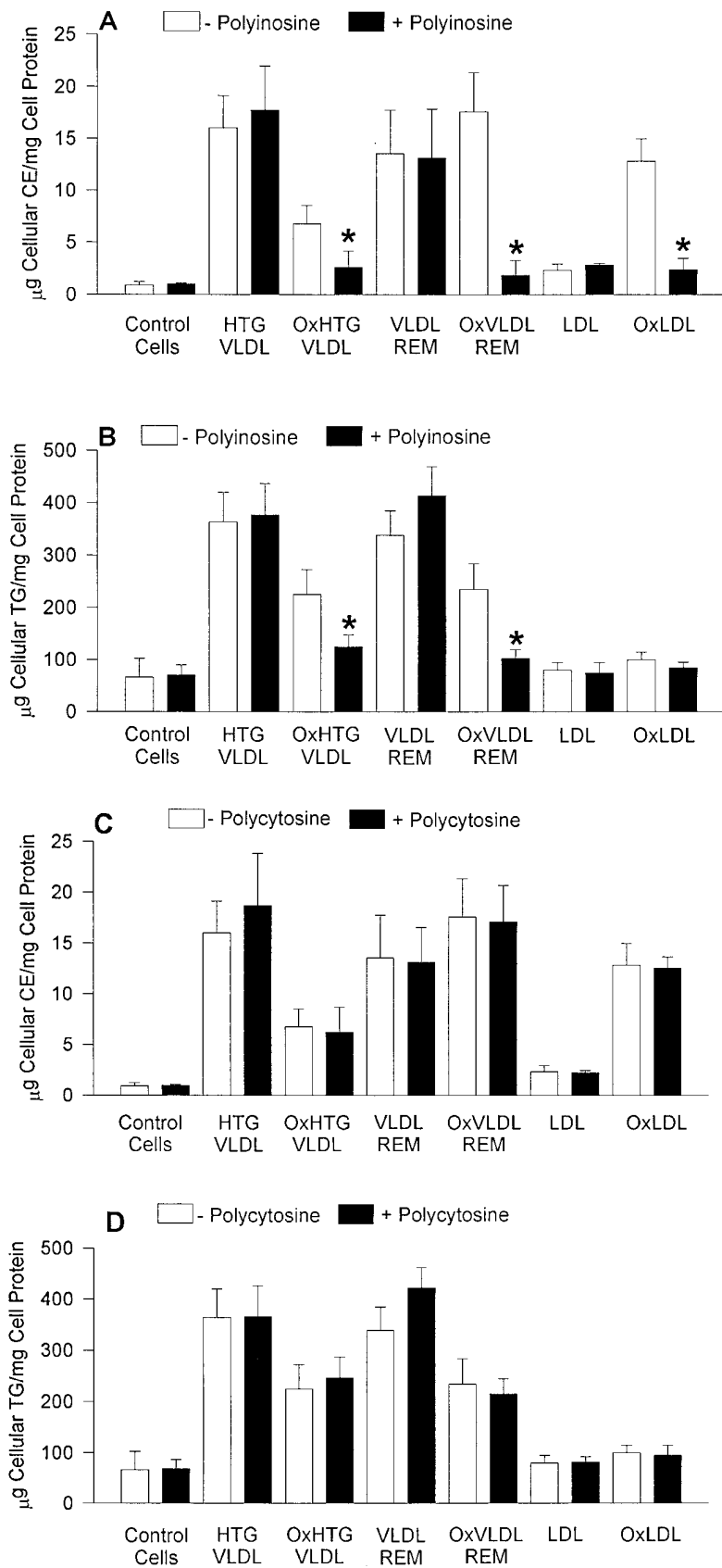


Fig. 4. Esterified cholesterol and triglyceride content of J774A.1 cells incubated with HTG-VLDL, VLDL-REM, LDL, OxHTG-VLDL, OxVLDL-REM, or OxLDL in the absence and presence of polyinosine or polycytosine. Esterified cholesterol (A, C) and triglyceride (B, D) content of J774A.1 macrophages exposed for 16 h to OxHTG-VLDL, OxVLDL-REM, OxLDL, or their native counterparts (50 μg lipoprotein cholesterol/ml media; $n = 4$) in the absence (\square) or presence (\blacksquare) of either polyinosine (A, B) or polycytosine (C, D) (100 μg of polynucleotide/ml of media). The value of n refers to the number of different patients' lipoprotein samples analyzed. Each lipoprotein preparation from each patient was analyzed in duplicate. The values represented by each bar are the mean \pm SEM for all patients' lipoprotein preparations. In (A) and (B) polyinosine significantly (*) inhibited the increases in cellular CE ($P \leq 0.02$) and TG ($P \leq 0.03$) concentrations induced by OxHTG-VLDL, OxVLDL-REM, and OxLDL. In (C) and (D) polycytosine had no effect on cellular CE and TG concentrations induced by any of the lipoprotein preparations ($P \geq 0.14$). CuSO_4 -mediated oxidation of the lipoproteins was performed as described under Methods. Cellular CE and TG levels were determined as described under Methods.

first assay we tested the ability of bovine milk LPL to release free fatty acids from either native HTG-VLDL or OxHTG-VLDL. An exogenous source of apoC-II (normal

plasma) was added to fully activate LPL. Compared to HTG-VLDL, OxHTG-VLDL at concentrations of 500 and 1000 μg TG/reaction reduced the rate of LPL-mediated

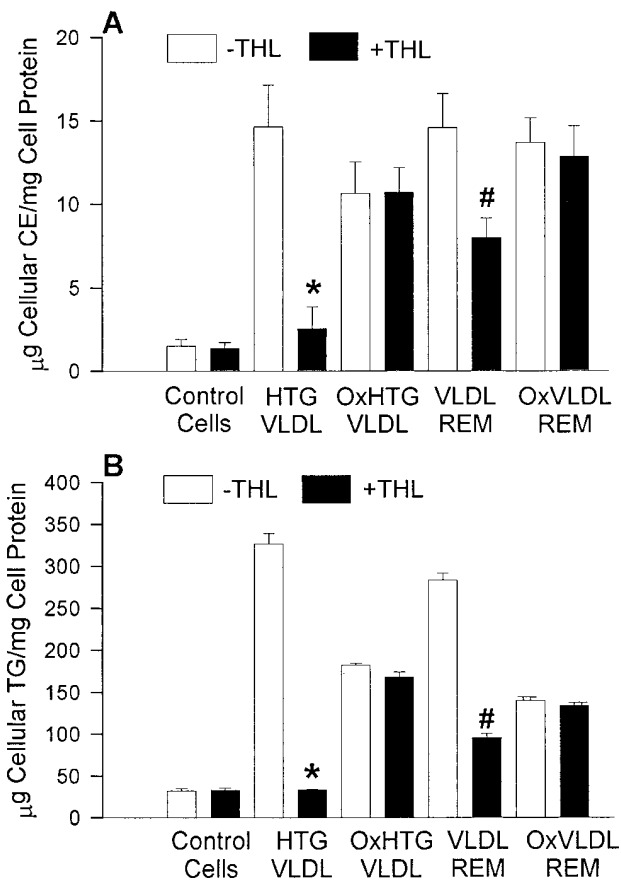


Fig. 5. Esterified cholesterol and triglyceride content of J774A.1 cells incubated with HTG-VLDL, VLDL-REM, OxHTG-VLDL, or OxVLDL-REM in the absence and presence of the lipoprotein lipase inhibitor tetrahydrolipstatin. Esterified cholesterol (A) and triglyceride (B) content of J774A.1 macrophages exposed for 16 h to HTG-VLDL, VLDL-REM, OxHTG-VLDL, and OxVLDL-REM (50 µg lipoprotein cholesterol/ml media; $n = 3$) in the absence (□) or presence (■) of tetrahydrolipstatin (THL; 1 µM), a competitive inhibitor of lipoprotein lipase. The value of n refers to the number of different patients' lipoprotein samples analyzed. Each lipoprotein preparation from each patient was analyzed in duplicate. The values represented by each bar are the mean \pm SEM for all patients' lipoprotein preparations. (A) *, $P = 0.0001$ relative to HTG-VLDL in the absence of THL; #, $P = 0.003$ relative to VLDL-REM in the absence of THL. (B) *, $P = 0.0001$ relative to HTG-VLDL in the absence of THL. #, $P = 0.001$ relative to VLDL-REM in the absence of THL. CuSO_4 mediated oxidation of the lipoproteins was performed as described under Methods. Cellular CE and TG levels were determined as described under Methods.

free fatty acid release by 53% and 47%, respectively (both $P = 0.001$, Fig. 6A). In the second in vitro assay, HTG-VLDL and OxHTG-VLDL were used as the source of apoC-II for the LPL-mediated release of free fatty acids from a TG emulsion. As shown in Fig. 6B, using OxHTG-VLDL as the source of apoC-II (16 and 36 µg protein/reaction) resulted in a 67% and a 60% reduction, respectively, in the rate of LPL-mediated free fatty acid release ($P = 0.001$) compared to HTG-VLDL. Native and oxidized HTG-VLDL, VLDL-REM, and LDL preparations from three type IV hypertriglyceridemic patients were delipidated and the apolipoproteins were separated by SDS-

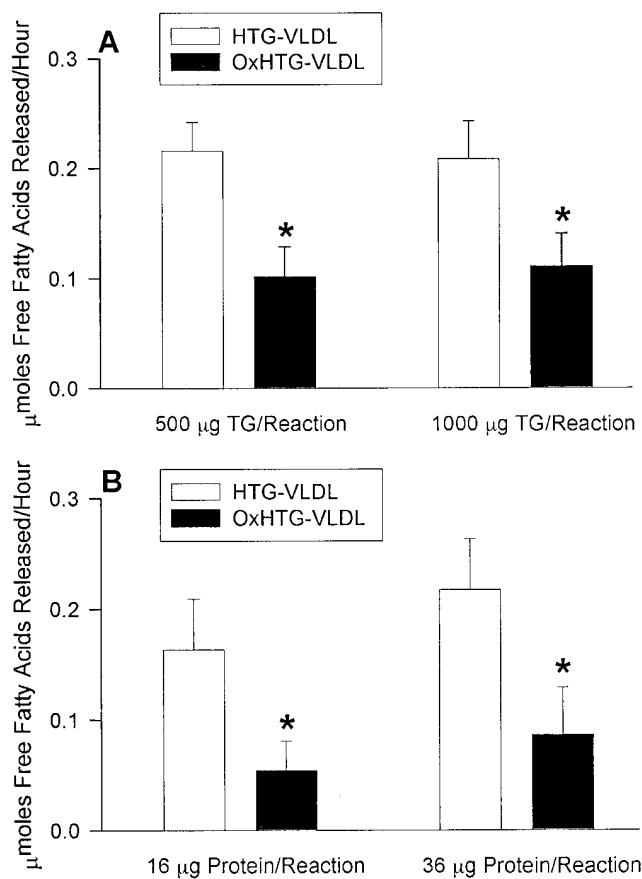


Fig. 6. In vitro activity assay used to examine whether OxHTG-VLDL can act as a substrate for bovine milk lipoprotein lipase. (A) The release of free fatty acids from either native HTG-VLDL or OxHTG-VLDL ($n = 4$ for each) after incubation with bovine milk LPL using an exogenous source of apoCII (normal plasma) to fully activate LPL. Compared to HTG-VLDL, OxHTG-VLDL at concentrations of 500 and 1000 µg TG/reaction reduced the rate of LPL-mediated free fatty acid release by 53% and 47%, respectively (* $P = 0.001$). (B) The release of free fatty acids from 1.82 µmol of a TG emulsion by bovine milk LPL using HTG-VLDL and OxHTG-VLDL ($n = 4$ for each) as the source of apoC-II. Using OxHTG-VLDL as the source of apoC-II (16 and 36 µg protein/reaction) resulted in a 67% and a 60% reduction, respectively, in the rate of LPL-mediated free fatty acid release (* $P = 0.001$) compared to HTG-VLDL. The value of n refers to the number of different patients' lipoprotein samples analyzed. Each lipoprotein preparation from each patient was analyzed in duplicate. The values represented by each bar are the mean \pm SEM for all patients' lipoprotein preparations.

PAGE. As shown in Fig. 7, compared to their native counterparts, oxidized HTG-VLDL and VLDL-REM showed a marked depletion in apoC as well as apoE. ApoB-100 for OxHTG-VLDL, OxVLDL-REM, and OxLDL was depleted in each sample and fragmentation of apoB was observed as described previously for rabbit β -VLDL (46) and normal human VLDL (48).

DISCUSSION

In this present study, we tested the hypothesis that oxidative modification of HTG-VLDL (S_f 60–400) and their

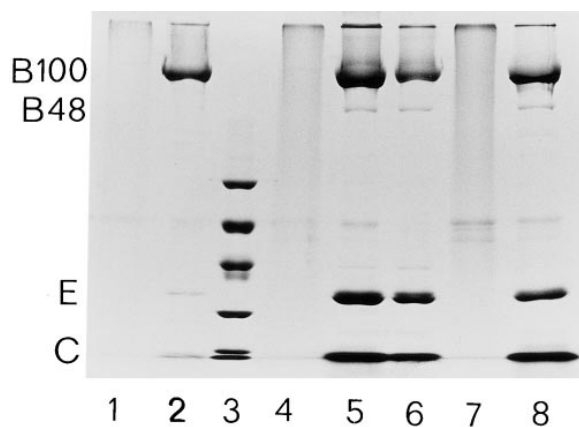


Fig. 7. SDS-PAGE analysis of native and oxidatively modified HTG-VLDL, VLDL-REM, and LDL. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of HTG-VLDL, OxHTG-VLDL, VLDL-REM, OxVLDL-REM, LDL, and OxLDL apolipoproteins ($n = 4$ patients' samples) as described under Methods. Gels were stained with Coomassie blue. A representative gel containing samples from one patient is shown. Various bands were identified according to molecular weight markers. Lane 1, oxidized (Ox) LDL; lane 2, native LDL; lane 3, molecular weight markers; lane 4, OxHTG-VLDL, lanes 5 and 6, native HTG-VLDL; lane 7, OxVLDL-REM, and lane 8, VLDL-REM. B-100, B-48, E, and C refer to apolipoproteins B-100, B-48, E, and C. Molecular weight markers, phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,000), and α -lactalbumin (14,400).

remnants, isolated from subjects with type IV HLP, would lead to foam cell formation, *in vitro*, by a mechanism analogous to that which occurs with OxLDL. Our findings demonstrate for the first time that OxVLDL-REM are more effective than OxLDL at inducing CE accumulation in cultured macrophages. In addition, we also found that native HTG-VLDL and their remnants are equally as effective as OxLDL at inducing macrophage CE accumulation. This study is also the first to directly compare the susceptibility of human HTG-VLDL, VLDL-REM, and LDL to oxidation *in vitro*. Our findings clearly show that oxidative modification of TG-hydrolyzed HTG-VLDL resulted in significantly greater macrophage CE accumulation than any other human (native or oxidized) lipoproteins examined.

Copper-induced oxidation of LDL results in the modification of apoB-100 (3, 36, 49), which reduces its affinity for the LDL receptor and increases its affinity for the scavenger receptor. Unlike the LDL receptor, the scavenger receptor is not down-regulated by increases in cellular CE concentrations (49, 50), and therefore the uptake of OxLDL by this pathway results in unregulated accumulation of cellular CE and subsequent foam cell formation. The early stages of the oxidative process can be monitored by assaying for the formation of conjugated dienes, which are transiently formed during the conversion of polyunsaturated fatty acids to reactive aldehydes (37). In this study we showed that both HTG-VLDL and their remnants, like LDL, can undergo oxidation. However, the kinetics of oxidation are different for the various lipoprotein preparations examined. LDL underwent oxidation much sooner

than both HTG-VLDL and VLDL-REM, although, once oxidation was initiated, the rate of conjugated diene formation in VLDL-REM proceeded at the same rate as the oxidation of LDL, which was twice as fast as for HTG-VLDL. During oxidation of both HTG-VLDL and VLDL-REM a greater number of conjugated dienes (and hence reactive aldehydes) were generated than during the oxidation of LDL, with the mean number of conjugated dienes generated being greater for the VLDL-REM than for HTG-VLDL and LDL.

The difference between VLDL and LDL lag times in the conjugated diene kinetic curves was most likely due to the fact that the larger VLDL particles contained more antioxidant molecules, such as α -tocopherol, per lipoprotein particle than did LDL (51, 52). The magnitude of the lag phase of a typical conjugated diene curve for LDL has been shown to be directly proportional to the amount of antioxidants present within the target lipoprotein (52–54). Of the various antioxidants in a given lipoprotein subclass, α -tocopherol has been shown to be among the most potent (52–54). In the present study we found that the lag times for HTG-VLDL and HTG-VLDL remnants were 4- to 5-fold longer than for LDL and that the lag times were directly correlated with the lipoprotein antioxidant content ($r = 0.98$). These results indicate that the greater number of antioxidant molecules per particle would protect both HTG-VLDL and VLDL-REM to a greater extent than LDL against the initiation of oxidation.

More conjugated dienes were produced during the oxidation of HTG-VLDL and VLDL-REM than during oxidation of LDL. VLDL and their remnants are physically larger than LDL, and therefore contain more polyunsaturated fatty acids, the substrate for oxidation, than LDL. One explanation to account for the observation that conjugated dienes were formed at twice the rate in LDL compared to HTG-VLDL is that the surface phospholipid monolayer of the smaller, denser, LDL particles had an enhanced vulnerability to oxidation by the external agent CuSO_4 . This idea of an enhanced susceptibility to oxidation based on size and density has been shown to exist between different LDL subfractions (55) and is consistent with the observation that the rate of conjugated diene formation of VLDL-REM was greater than that observed for HTG-VLDL and found not to be significantly different from that of LDL.

OxLDL has received considerable attention with respect to its ability to induce foam cell formation (56). However, in the last decade several groups have examined the atherogenic potential of OxVLDL. These studies have used either human VLDL isolated from normolipidemic (48, 51, 57–59) and hypercholesterolemic (Type IIa) subjects (58), or β -VLDL isolated from hypercholesterolemic rabbits (46, 60, 61). Human VLDL is capable of being oxidized *in vitro* as assessed by increases in VLDL electrophoretic mobility (57), the formation of thiobarbituric acid-reactive substances (TBARS) (57–59), and/or the formation of hydroperoxides (51, 57). Jurgens, Ashy, and Esterbauer (57) were the first to examine both VLDL and LDL, isolated from normolipidemic subjects, under simi-

lar oxidative conditions (exposure to CuCl_2). In their study, OxVLDL and OxLDL were found to have very similar electrophoretic mobilities and levels of TBARS. To date, only one human study has directly examined whether OxVLDL could be taken up more avidly by cultured macrophages than native VLDL (48). In that study CuSO_4 mediated oxidation of VLDL, isolated from normolipidemic subjects, resulted in a 4-fold increase in degradation of the radioiodinated lipoprotein by mouse peritoneal macrophages when compared to native VLDL (48).

β -VLDL from cholesterol-fed rabbits has also been shown to undergo both cell-mediated and copper-mediated oxidative modification (46, 60–62). Endothelial cell-mediated oxidation of β -VLDL significantly enhanced its degradation by both macrophages (60) and smooth muscle cells (SMC) (61). Copper-mediated oxidation of β -VLDL was also shown to enhance its degradation by both macrophages (60) and liver Kupffer cells (46). In two separate studies, the ability of oxidized β -VLDL (60) and oxidized IDL (62) to be degraded by cultured macrophages was compared to that of OxLDL. In the first study Parthasarathy et al. (60) showed that even though copper-mediated oxidation of β -VLDL ($d < 1.006$ g/ml) enhanced its rate of degradation by mouse peritoneal macrophages 2.5-fold compared to native β -VLDL ($d < 1.006$ g/ml), its rate of degradation was still less than that of OxLDL (reported previously by this group to be up to 10-fold greater than native LDL (4, 55, 63)). In the second study, Haratz et al. (62) showed that SMC-induced oxidation of hypercholesterolemic rabbit IDL ($d 1.006$ – 1.019 g/ml) enhanced its degradation by J774A.1 macrophages up to 2.4-fold greater than SMC-modified LDL. Although the findings of Haratz et al. (62) contrast with those of Parthasarathy et al. (60), we feel that both of these previous studies are consistent with the findings of the present study. Like Parthasarathy et al. (60), we found that OxHTG-VLDL caused significantly less cellular CE accumulation than OxLDL, and as implied by Haratz et al. (62) we found that if HTG-VLDL was allowed to undergo remnant formation prior to oxidation, the resulting OxVLDL-REM actually caused greater macrophage CE accumulation than OxLDL. The difference between OxVLDL, OxVLDL-REM, and OxLDL in their ability to induce macrophage CE accumulation, as shown in the present study for human lipoproteins and collectively by others for rabbit β -VLDL (62), demonstrates that the lipoprotein with the most atherogenic potential, *in vitro*, is OxVLDL-REM.

Although it is generally accepted that LDL have access to the artery wall and directly contribute to atherogenesis, there is increasing evidence that VLDL and in particular VLDL remnants are also atherogenic. The relative atherogenic potential, *in vivo*, is most likely dependent on their plasma concentrations, plasma transport rates, and rates of infiltration into the arterial intima. Subjects with type IV HLP have elevated plasma concentrations of HTG-VLDL and VLDL-REM; however, they often have normal or reduced plasma levels of LDL (15, 16). If HTG-VLDL and especially VLDL remnants infiltrate the arterial intima, these lipoproteins may contribute to the athero-

genic process. Infiltration of lipoproteins into the arterial wall appears to be proportional to their plasma concentrations (64, 65). It is known that lipoproteins in the VLDL + IDL density range can be isolated from normal aortic intima (26) or from aortic atherosclerotic plaques (27). In the latter study, Rapp et al (27) found that greater than one-third of the cholesterol content of plaque lipoproteins was associated with particles in the VLDL + IDL range. In those patients with hypertriglyceridemia, the plaque particles in the VLDL + IDL range had a triglyceride content similar to their plasma counterparts. HTG-VLDL and their remnants have atherogenic potential without a requirement for oxidation and *in vitro*, they can be readily taken up by macrophages to produce foam cells (7–14). VLDL + IDL, extracted from normal aortic intima were found to stimulate cholesterol esterification in mouse peritoneal macrophages 3- to 6-fold greater than aortic LDL and 10- to 20-fold greater than plasma LDL (26). The results of the present study extend these findings and suggest that oxidation of VLDL-REM further enhances its ability to cause macrophage cholesteryl ester accumulation.

OxLDL was the only lipoprotein preparation examined that increased cellular FC levels. At the highest concentration tested, OxLDL caused approximately a 30% increase in cellular FC levels when compared to the other lipoprotein preparations. Maor and Aviram (66) have shown that incubation of J774A.1 macrophages with OxLDL led to macrophage accumulation of FC as a result of lysosomal trapping of the lipoprotein hydrolyzed CE. These investigators (66) concluded that this lysosomal trapping may have been mediated in some way by oxysterols generated by the oxidative process and brought into the cell as a component of the OxLDL particle. Our results with OxLDL were consistent with this idea; however, neither OxHTG-VLDL nor OxVLDL-REM caused a similar increase in cellular FC levels. Reasons for this difference are unknown.

Incubating cultured macrophages with OxHTG-VLDL or OxVLDL-REM did not result in the same increase in cellular TG concentrations when compared to their non-oxidized counterparts. We have previously shown that cellular uptake of the TG and CE components of HTG-VLDL occurs by two different mechanisms (8). The first step is initiated extracellularly by cell-secreted LPL, which mediates hydrolysis of the VLDL-TG to free fatty acids which are then taken up by the cell and re-esterified into TG. The second step involves receptor-mediated uptake of the resulting CE-rich VLDL-REM, a process dependent on receptor-competent apoE (8). A possible explanation for the reduction in the ability of OxHTG-VLDL to induce cellular TG accumulation could be due to a decrease in extracellular OxVLDL-TG hydrolysis. Oxidative modification of HTG-VLDL may in some way inhibit the ability of LPL to associate with OxHTG-VLDL by either inactivating lipoprotein-associated apoC-II or by inhibiting the binding of LPL to the lipoprotein altogether. The idea that OxHTG-VLDL can no longer serve as a substrate for LPL was supported by the *in vitro* LPL activity assay data which indicated that the oxidation process was not only inhibit-

ing the ability of apoC-II associated with OxHTG-VLDL to function as a cofactor for LPL, but that OxHTG-VLDL was itself unable to act as a substrate for catalytically active LPL. SDS-PAGE analysis of OxHTG-VLDL and OxVLDL-REM confirmed that these particles were markedly depleted in apoC as well as apoE. Fragmentation of apoB was observed. Other studies have shown that oxidation of VLDL from normolipidemic subjects (48) and β -VLDL from cholesterol-fed rabbits (46) resulted in the fragmentation of the apoC-II as well as apoB-100 and apoE. We observed a greater uptake of OxVLDL-REM, compared to OxHTG-VLDL, even though the loss of apoC, E, and fragmentation of apoB appeared similar. These findings are consistent with the concept that epitopes of modified apolipoproteins on OxVLDL-REM were more accessible for recognition at the cell surface and that enhanced exposure of these epitopes on OxHTG-VLDL could not be increased by hydrolysis via macrophage LPL.

In contrast to HTG-VLDL and to a lesser extent VLDL-REM, OxHTG-VLDL, and OxVLDL-REM did not require lipolysis by macrophage-secreted LPL to induce particle uptake and CE accumulation. The addition of the LPL inhibitor THL had no effect on the increase in either cellular TG or CE concentrations induced by OxHTG-VLDL or OxVLDL-REM. This is consistent with the observation that both of these lipoproteins were poor substrates for LPL. Thus the increased cellular TG induced by both OxHTG-VLDL and OxLDL-REM suggests that particles were taken up intact by macrophages without prior lipolytic modification by LPL. A non-catalytic role for LPL in enhancing cellular uptake of these oxidized lipoproteins, as demonstrated for LDL uptake by THP-1 macrophages (67), could not be excluded.

A possible candidate receptor responsible for mediating uptake of OxHTG-VLDL and OxVLDL-REM is the scavenger type I receptor. This idea is based on evidence that the scavenger receptor is directly responsible for the uptake of chemically modified LDL (44, 49, 50) and copper-oxidized β -VLDL (46). Polyinosine is known to bind competitively to the scavenger type I and II receptors and prevent binding and uptake of scavenger receptor ligands (44, 45). Our co-incubation studies with polyinosine showed that the scavenger receptor(s) may play an active role in the uptake of these oxidized lipoproteins as polyinosine significantly inhibited the cellular CE and TG accumulation induced by OxHTG-VLDL, OxVLDL-REM, and OxLDL. In support of these findings, de Rijke, Hesses, and van Berkel (46) have shown that polyinosine can inhibit both the *in vivo* association and *in vitro* association/degradation of iodinated oxidized rabbit β -VLDL by liver Kupffer cells, a macrophage-like cell known to express the scavenger type I receptor (68).

In conclusion, the present experiments clearly demonstrated that both human HTG-VLDL and their remnants could be oxidatively modified *in vitro*. However, only OxVLDL-REM were capable of enhancing macrophage cellular CE accumulation above that of VLDL-REM and HTG-VLDL. In addition, OxVLDL-REM caused significantly greater CE accumulation than equal concentra-

tions of OxLDL. The present experiments further demonstrated that HTG-VLDL and VLDL-REM could, without modification, significantly stimulate macrophage CE accumulation to the same extent as the known atherogenic particle OxLDL. The enhanced cellular CE accumulation induced by OxVLDL-REM compared to VLDL-REM, suggested that the former may be the more atherogenic, *in vivo*, and may provide an additional potential mechanism to explain atherosclerosis associated foam cell formation in patients with hypertriglyceridemia. We hypothesize that *in vivo*, in the arterial intima, HTG-VLDL and/or partially catabolized HTG-VLDL interact with macrophage-secreted LPL, thereby trapping the lipoprotein within the extracellular matrix and to the macrophage cell surface. After further TG hydrolysis by LPL, remnant uptake by macrophage may occur by several pathways. 1) Particles are taken up by receptor-mediated endocytosis, as described previously (8) and/or 2) the anchored VLDL-REM may undergo cell-induced oxidation by one or more of the possible cellular mechanisms that have been proposed for LDL modification (47, 69, 70). Oxidative modification of the VLDL-REM would allow these modified lipoproteins to be readily taken up via macrophage scavenger receptors, resulting in CE accumulation and foam cell formation. The latter pathway may be predominant when the macrophage LDL-receptor is down-regulated. ■

We are grateful to Sandra Kleinstiver for expert technical assistance. This work was supported by a Medical Research Council of Canada Grant (MT8014) to M. W. H. S. C. W. is a recipient of a Medical Research Council of Canada Studentship. D. B. M. is a recipient of a Heart and Stroke Foundation of Canada Research Fellowship. M. W. H. is a Career Investigator of the Heart and Stroke Foundation of Ontario. A preliminary report of this work was presented at the 68th Scientific Sessions of the American Heart Association, November, 1995, Anaheim, CA and printed in abstract form in *Circulation*. 1995. **92**: I-289.

Manuscript received 11 June 1997 and in revised form 29 December 1997.

REFERENCES

1. Schaffner, T., K. Taylor, E. J. Bartucci, K. Fischer-Dzoga, J. H. Beeson, S. Glagov, and R. W. Wissler. 1980. Arterial foam cells with distinctive immunomorphologic and histochemical features of macrophages. *Am. J. Pathol.* **100**: 57-80.
2. Gerrity, R. G. 1981. The role of the monocyte in atherogenesis I. Transition of blood-borne monocytes into foam cells in fatty lesions. *Am. J. Pathol.* **103**: 181-190.
3. Fogelman, A. M., I. Shechter, J. Seager, M. Hokom, J. S. Child and P. A. Edwards. 1980. Malondialdehyde alteration of low density lipoproteins leads to cholesteryl ester accumulation in human monocyte-macrophages. *Proc. Natl. Acad. Sci. USA*. **77**: 2214-2218.
4. Henriksen, T., E. M. Mahoney, and D. Steinberg. 1981. Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: recognition by receptors for acetylated low density lipoproteins. *Proc. Natl. Acad. Sci. USA*. **78**: 6499-6503.
5. Hoff, H. F., and J. A. O'Neil. 1991. Oxidation of LDL: role in atherogenesis. *Klin. Wochenschr.* **69**: 1032-1038.
6. Witztum, J. L., and D. Steinberg. 1991. Role of oxidized low density lipoprotein in atherogenesis. *J. Clin. Invest.* **88**: 1785-1792.
7. Huff, M. W., A. J. Evans, C. G. Sawyez, B. M. Wolfe, and P. J. Nestel. 1991. Cholesterol accumulation in J774 macrophages induced by

- triglyceride-rich lipoproteins: a comparison of VLDL from subjects with Types III, IV and V hyperlipoproteinemia. *Arterioscler. Thromb.* **11**: 221-233.
8. Evans, A. J., C. G. Sawyez, B. M. Wolfe, P. W. Connelly, G. F. Maguire, and M. W. Huff. 1993. Evidence that cholesteryl ester and triglyceride accumulation in J774 macrophages induced by very low density subfractions occurs by different mechanisms. *J. Lipid Res.* **34**: 703-717.
 9. Bates, S. R., B. A. Coughlin, T. Mazzone, J. Borensztajn, and G. S. Getz. 1987. Apoprotein E mediates the interaction of beta-VLDL with macrophages. *J. Lipid Res.* **28**: 787-797.
 10. Ishibashi, S., N. Yamada, H. Shimano, N. Mori, H. Mokuno, T. Gotohda, M. Kawakami, T. Murase, and F. Takaku. 1990. Apolipoprotein E and lipoprotein lipase secreted from human monocyte-derived macrophages modulate very low density lipoprotein uptake. *J. Biol. Chem.* **265**: 3040-3047.
 11. Granot, E., and S. Eisenberg. 1990. ApoE-3-specific metabolism of human plasma very low density lipoproteins in cultured J-774 macrophages. *J. Lipid Res.* **31**: 2133-2139.
 12. Gianturco, S. H., W. A. Bradley, A. M. Gotto, J. D. Morrisett, and D. L. Peavy. 1982. Hypertriglyceridemic very low density lipoproteins induce triglyceride synthesis in mouse peritoneal macrophages. *J. Clin. Invest.* **70**: 168-178.
 13. Lindqvist, P., A. Ostlund-Lindqvist, J. L. Witztum, D. Steinberg, and J. A. Little. 1983. The role of lipoprotein lipase in the metabolism of triglyceride-rich lipoproteins by macrophages. *J. Biol. Chem.* **258**: 9086-9092.
 14. Sacks, F. M., and J. L. Breslow. 1987. Very low density lipoproteins stimulate cholesterol ester formation in U937 macrophages: heterogeneity and biologic variation among normal humans. *Arteriosclerosis* **7**: 35-46.
 15. Sane, T., and E. A. Nikkila. 1988. Very low density lipoprotein triglyceride metabolism in relatives of hypertriglyceridemic probands. *Arteriosclerosis* **8**: 217-226.
 16. Grundy, S. M., H. Y. I. Mok, L. Zech, D. Steinberg, and M. Berman. 1979. Transport of very low density lipoprotein triglycerides in varying degrees of obesity and hypertriglyceridemia. *J. Clin. Invest.* **63**: 1274-1283.
 17. Breslow, J. L., and M. Dammerman. 1995. Genetic basis of lipoprotein disorders. *Circulation* **91**: 505-512.
 18. Barbir, M., D. Wile, I. Trayner, V. R. Aber, and G. R. Thompson. 1988. High prevalence of hypertriglyceridemia and apolipoprotein abnormalities in coronary artery disease. *Br. Heart J.* **60**: 397-403.
 19. Carlson, L. A., and L. E. Bottiger. 1985. Risk factors for heart disease in men and women. Results of the 19-year follow-up of the Stockholm Prospective Study. *Acta Med. Scand.* **218**: 207-211.
 20. Castelli, W. P. 1986. The triglyceride issue: a view from Framingham. *Am. Heart J.* **112**: 432-437.
 21. Huff, M. W., W. C. Breckenridge, W. L. Strong, and B. M. Wolfe. 1988. Metabolism of apolipoproteins C-II, C-III and B in hypertriglyceridemic men. Changes after heparin-induced lipolysis. *Arteriosclerosis* **8**: 471-479.
 22. Shepherd, J., and C. J. Packard. 1987. Metabolic heterogeneity in very low density lipoproteins. *Am. Heart J.* **113**: 503-508.
 23. Reardon, M. F., N. H. Fidge, and P. J. Nestel. 1978. Catabolism of very low density lipoprotein apoprotein B in man. *J. Clin. Invest.* **61**: 850-860.
 24. Packard, C. J., A. Munro, R. Lorimer, A. M. Gotto, and J. Shepherd. 1984. Metabolism of apolipoprotein B in large triglyceride-rich very low density lipoproteins of normal and hypertriglyceridemic subjects. *J. Clin. Invest.* **74**: 2178-2192.
 25. Havel, R. J., and R. L. Hamilton. 1988. Hepatocytic lipoprotein receptors and intracellular lipoprotein catabolism. *Hepatology* **8**: 1689-1704.
 26. Yla-Herttuala, S., O. Jaakkola, C. Ehnholm, M. J. Tikkanen, T. Solakivi, T. Sarkioja, and T. Nikkari. 1988. Characterization of two lipoproteins containing apolipoproteins B and E from lesion-free human aortic intima. *J. Lipid Res.* **29**: 563-572.
 27. Rapp, J. H., A. Lespine, R. L. Hamilton, N. Colyvas, A. H. Chaumeton, J. Tweedie-Hardman, L. Kotite, S. T. Kunitake, R. J. Havel, and J. P. Kane. 1994. Triglyceride-rich lipoproteins isolated by selected-affinity anti-apolipoprotein B immunosorption from human atherosclerotic plaques. *Arterioscler. Thromb.* **14**: 1767-1774.
 28. Schaefer, E. J., and R. I. Levy. 1985. Pathogenesis and management of lipoprotein disorders. *N. Engl. J. Med.* **312**: 1300-1310.
 29. Bouthilleir, D. C. F., C. F. Sing, and J. Davignon. 1983. Apolipoprotein E phenotyping with a single gel method: application to the study of informative matings. *J. Lipid Res.* **24**: 1060-1069.
 30. Evans, A. J., M. W. Huff, and B. M. Wolfe. 1989. Accumulation of an apoE-poor subfraction of very low density lipoprotein in hypertriglyceridemic men. *J. Lipid Res.* **30**: 1691-1701.
 31. Evans, A. J., C. G. Sawyez, B. M. Wolfe, and M. W. Huff. 1992. Lipolysis is a prerequisite for lipid accumulation in HepG2 cells induced by large hypertriglyceridemic very low density lipoproteins. *J. Biol. Chem.* **267**: 10743-10751.
 32. Markwell, M. A. K., S. M. Haas, L. L. Bieber, and N. E. Tolbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **87**: 206-210.
 33. Miller, N. J., C. Rice-Evans, M. J. Davies, V. Gopinatham, and A. Milner. 1993. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin. Sci.* **84**: 407-412.
 34. Socorro, L., and R. L. Jackson. 1985. Monoclonal antibodies to bovine milk lipoprotein lipase. *J. Biol. Chem.* **260**: 6324-6328.
 35. Huff, M. W., A. J. Evans, B. M. Wolfe, P. W. Connelly, G. F. Maguire, and W. L. P. Strong. 1990. Identification and metabolic characteristics of an apolipoprotein C-II variant isolated from a hypertriglyceridemic subject. *J. Lipid Res.* **31**: 385-396.
 36. Steinbrecher, U. P., J. L. Witztum, S. Parthasarathy, and D. Steinberg. 1987. Decrease in reactive amino groups during oxidation of endothelial cell modification of LDL. *Arteriosclerosis* **7**: 135-143.
 37. Kleinvelde, H. A., H. L. M. Hak-Lemmers, A. F. H. Stalenhoef, and P. N. M. Demacker. 1992. Improved measurement of low density lipoprotein susceptibility to copper-induced oxidation: application of a short procedure for isolating low density lipoprotein. *Clin. Chem.* **38**: 2066-2072.
 38. Huff, M. W., and D. E. Telford. 1984. Characterization and metabolic fate of two very low density lipoprotein subfractions separated by heparin-Sepharose chromatography. *Biochim. Biophys. Acta.* **796**: 251-261.
 39. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.
 40. Koo, C., T. L. Innerarity, and R. W. Mahley. 1985. Obligatory role of cholesterol and apolipoprotein E in the formation of large cholesterol-enriched and receptor-active high density lipoproteins. *J. Biol. Chem.* **260**: 11934-11943.
 41. Werb, Z., and J. R. Chin. 1983. Onset of apoprotein E secretion during differentiation of mouse bone marrow-derived mononuclear phagocytes. *J. Biol. Chem.* **97**: 1113-1118.
 42. Carr, T. P., C. J. Andresen, and L. L. Rudel. 1993. Enzymatic determination of triglyceride, free cholesterol, and total cholesterol in tissue lipid extracts. *Clin. Biochem.* **26**: 39-42.
 43. Barenghi, L., S. Bradamante, G. A. Giudici, and C. Vergani. 1990. NMR analysis of low-density lipoprotein oxidatively-modified in vitro. *Free Radical Res. Comm.* **8**: 175-183.
 44. Brown, M. S., S. K. Basu, J. R. Falck, Y. K. Ho, and J. L. Goldstein. 1980. The scavenger cell pathway for lipoprotein degradation: specificity of the binding site that mediates the uptake of negatively charged LDL by macrophages. *J. Supramol. Struct.* **13**: 67-81.
 45. Pearson, A. M., A. Rich, and M. Krieger. 1993. Polynucleotide binding to macrophage scavenger receptor depends on the formation of base-quartet-stabilized four-stranded helices. *J. Biol. Chem.* **268**: 3546-3554.
 46. deRijke, Y. B., E. M. A. J. Hessels, and T. J. C. vanBerkel. 1992. Recognition sites on rat liver cells for oxidatively modified β -very low density lipoproteins. *Arterioscler. Thromb.* **12**: 41-49.
 47. Hazen, S. L., F. F. Hsu, D. M. Mueller, J. R. Crowley, and J. W. Heinicke. 1996. Human neutrophils employ chlorine gas as an oxidant during phagocytosis. *J. Clin. Invest.* **98**: 1283-1289.
 48. Keidar, S., M. Kaplan, M. Rosenblat, G. J. Brook, and M. Aviram. 1992. Apolipoprotein E and lipoprotein lipase reduce macrophage degradation of oxidized very-low-density lipoprotein (VLDL), but increase cellular degradation of native VLDL. *Metabolism* **41**: 1185-1192.
 49. Brown, M. S., and J. L. Goldstein. 1983. Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. *Annu. Rev. Biochem.* **52**: 223-262.
 50. Goldstein, J. L., Y. K. Ho, S. K. Basu, and M. S. Brown. 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. Natl. Acad. Sci. USA.* **76**: 333-337.

51. Mohr, D., and R. Stocker. 1994. Radical-mediated oxidation of isolated human very-low-density lipoprotein. *Arterioscler. Thromb.* **14**: 1186-1192.
52. Esterbauer, H., M. Dieber-Rotheneder, G. Waeg, G. Striegl, and G. Jurgens. 1990. Biochemical, structural, and functional properties of oxidized low-density lipoprotein. *Chem. Res. Toxicol.* **3**: 77-92.
53. Esterbauer, H., J. Gebicki, H. Puhl, and G. Jurgens. 1992. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radical Biol. Med.* **13**: 341-390.
54. Esterbauer, H., H. Puhl, M. Dieber-Rotheneder, G. Waeg, and H. Rabl. 1991. Effect of antioxidants on oxidative modification of LDL. *Ann. Med.* **23**: 573-581.
55. Steinbrecher, U. P., S. Parthasarathy, D. S. Leake, J. L. Witztum, and D. Steinberg. 1984. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc. Natl. Acad. Sci. USA.* **81**: 3883-3887.
56. Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witztum. 1989. Beyond cholesterol: modifications of low density lipoproteins that increase its atherogenicity. *N. Engl. J. Med.* **4**: 915-924.
57. Jurgens, G., A. Ashy, and H. Esterbauer. 1990. Detection of new epitopes formed upon oxidation of low density lipoprotein, lipoprotein [a] and very low density lipoprotein. *Biochem. J.* **265**: 605-608.
58. Lavy, A., G. J. Brook, G. Dankner, A. B. Amotz, and M. Aviram. 1991. Enhanced in vitro oxidation of plasma lipoproteins derived from hypercholesterolemic patients. *Metabolism.* **40**: 794-799.
59. Mohan, P. F., and D. Desai. 1994. Very low density and low density lipoproteins induce nitric oxide synthesis in macrophages. *Biochem. Biophys. Res. Commun.* **3**: 1047-1054.
60. Parthasarathy, S., M. T. Quinn, D. C. Schwenke, T. E. Carew, and D. Steinberg. 1989. Oxidative modification of beta-very low density lipoprotein: potential role in monocyte recruitment and foam cell formation. *Arteriosclerosis.* **9**: 398-404.
61. Horrigan, S., J. H. Campbell, and G. R. Campbell. 1991. Oxidation of beta-very low density lipoprotein by endothelial cells enhances its metabolism by smooth muscle cells in culture. *Arterioscler. Thromb.* **11**: 279-289.
62. Haratz, D., O. Stein, R. Shwartz, E. M. Berry, and Y. Stein. 1988. Preferential metabolism by macrophages of conditioned rabbit hypercholesterolemic remnant lipoproteins. *Biochim. Biophys. Acta.* **959**: 127-133.
63. Parthasarathy, S., U. P. Steinbrecher, J. Barnett, J. L. Witztum, and D. Steinberg. 1985. Essential role of phospholipase A₂ activity in endothelial cell-induced modification of low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **82**: 3000-3004.
64. Nicoll, A., R. Duffield, and B. Lewis. 1981. Flux of plasma lipoproteins into human arterial intima. *Atherosclerosis.* **39**: 229-242.
65. Shaikh, M., R. Wootton, B. G. Nordestgaard, P. Baskerville, J. S. Lumley, A. E. La Ville, J. Quiney, and B. Lewis. 1991. Quantitative studies of transfer in vivo of low density, S_f 12-60, and S_f 60-400 lipoproteins between plasma and arterial intima in humans. *Arterioscler. Thromb.* **11**: 569-577.
66. Maor, I., and M. Aviram. 1994. Oxidized low density lipoprotein leads to macrophage accumulation of unesterified cholesterol as a result of lysosomal trapping of the lipoprotein hydrolyzed cholesterol ester. *J. Lipid Res.* **35**: 803-819.
67. Obunike, J. C., I. J. Edwards, S. C. Rumsey, L. K. Curtiss, W. D. Wagner, R. J. Deckelbaum, and I. J. Goldberg. 1994. Cellular differences in lipoprotein lipase-mediated uptake of low density lipoproteins. *J. Biol. Chem.* **269**: 13129-13135.
68. vanBerkel, T. J. C., Y. B. deRijke, and J. K. Kruijt. 1991. Different fate in vivo of oxidatively modified-LDL and acetylated-LDL in rats. Recognition by various scavenger receptors on Kupffer and endothelial liver cells. *J. Biol. Chem.* **266**: 2282-2289.
69. Chait, A., and J. W. Heinecke. 1994. Lipoprotein modification: cellular mechanisms. *Curr. Opin. Lipidol.* **5**: 365-370.
70. Heinecke, J. W. 1997. Mechanisms of oxidative damage of low density lipoprotein in human atherosclerosis. *Curr. Opin. Lipidol.* **8**: 268-274.