Cholesterol and oxysterol metabolism and subcellular distribution in macrophage foam cells: accumulation of oxidized esters in lysosomes

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Abstract **Cholesterol- and cholesteryl ester-rich macrophage foam cells, characteristic of atherosclerotic lesions, are often generated in vitro using oxidized low density lipoprotein (OxLDL). However, relatively little is known of the nature and extent of sterol deposition in these cells or of its relationship to the foam cells formed in atherosclerotic lesions. The purpose of this study was to examine the content and cellular processing of sterols in OxLDL-loaded macrophages, and to compare this with macrophages loaded with acetylated LDL (AcLDL; cholesteryl ester-loaded cells containing no oxidized lipids) or 7-ketocholesterol-enriched acetylated LDL (7KCAcLDL; cholesteryl ester-loaded cells selectively supplemented with 7-ketocholesterol (7KC), the major oxysterol present in OxLDL). Both cholesterol and 7KC and their esters were measured in macrophages after uptake of these modified lipoproteins. Oxysterols comprised up to 50% of total sterol content of OxLDL-loaded cells. Unesterified 7KC and cholesterol partitioned into cell membranes, with no evidence of retention of either free sterol within lysosomes. The cells also contained cytosolic, ACAT-derived, cholesteryl and 7-ketocholesteryl esters. The proportion of free cholesterol and 7KC esterified by ACAT was 10-fold less in OxLDL-loaded cells than in AcLDL or 7KCAcLDL-loaded cells. This poor esterification rate in OxLDL-loaded cells was partly caused by fatty acid limitation. OxLDL-loaded macrophages also contained large (~40–50% total cell sterol content) pools of oxidized esters, containing cholesterol or 7KC esterified to oxidized fatty acids. These were insensitive to ACAT inhibition, very stable and located in lysosomes, indicating resistance to lysosomal esterases. Macrophages loaded with OxLDL do not accumulate free sterols in their lysosomal compartment, but do accumulate lysosomal deposits of OxLDL-derived cholesterol and 7-ketocholesterol esterified to oxidized fatty acids. The presence of similar deposits in lesion foam cells would represent a pool of sterols that is particularly resistant to removal.**—Brown, A. J., E. L. Mander, I. C. Gelissen, L. Kritharides, R. T. Dean, and W. Jessup. **Cholesterol and oxysterol metabolism and subcellular distribution in macrophage foam cells: accumulation of oxidized esters in lysosomes.** ***J. Lipid Res.*** **2000.** 41: **226–236.**

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Cholesterol- and cholesteryl ester-enriched (1), macrophage-derived 'foam' cells are an early and characteristic feature of atherosclerotic lesions. Lipid loading of macrophages in vitro can be achieved by incubation with modified forms of LDL, which bypass the tightly regulated LDL-receptor-mediated uptake route. Amongst the alternative routes, the 'scavenger' receptors that mediate uptake of AcLDL are the best characterized (2). The rapid uptake of OxLDL by cultured macrophages utilizing (at least partly) these same scavenger receptors, together with evidence for the presence of OxLDL in vivo and its cell-mediated generation in vitro, were the initial basis of the 'oxidation hypothesis' of atherogenesis (3). As a result of these observations, many studies have been made of the influence of OxLDL uptake on macrophage physiology. Yet, while it is quite clear that OxLDL is readily endocytosed by macrophages, relatively little attention has been given to the nature and extent of lipid deposition that such uptake produces. This information is necessary to understand how sterols in oxidized lipoproteins are metabolized and to generate model foam cells with a cellular sterol composition appropriate to their in vivo counterparts.

Several studies have demonstrated that the protein component (apolipoprotein B; apoB) of OxLDL is unusually resistant to lysosomal proteolysis (4–8), although hydrolysis of unmodified proteins in the same cells was (with one exception (8)) found to be normal (5–7). This is

Abbreviations: AcLDL, acetylated low density lipoprotein; ACAT, acyl-CoA:cholesterol acyltransferase; BSA, bovine serum albumin; FC, free cholesterol; CE, cholesteryl ester; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks balanced salt solution; HINBCS, heat-inactivated newborn calf serum; 7KC, 7-ketocholesterol; 7KCE, 7-ketocholesteryl ester; LDL, low density lipoprotein; LPDS, lipoprotein-deficient human serum; MPM, mouse peritoneal macrophages; OxLDL, oxidized LDL; BHT, butylated hydroxytoluene; GC, gas chromatography; HEPES, N-(2-hydroxyethly)piperazine-N′-(2-ethanesulphonic acid); PMSF, phenylmethylsulphonyl fluoride; 7KC-LDL, LDL supplemented with 7KC; 7KCAcLDL, acetylated LDL supplemented with 7KC.

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probably due to the resistance of proteins containing oxidized amino acids to lysosomal proteases, as other oxidized and lipid-free proteins, in which oxidized amino acids have been directly detected (9–11), are also resistant to lysosomal degradation in macrophages (12, 13). Detection of lysosomal accumulation of apoB in macrophages after uptake of OxLDL (14) is consistent with this hypothesis and with apoB accumulation in atherosclerotic lesions (15).

It might be predicted that lysosomal accumulation of apoB from OxLDL could be associated with accumulation of other components of OxLDL, such as some sterols and their esters. A number of studies that have examined the free and esterified cholesterol content of cells after uptake of OxLDL have observed that the relative proportion of total cholesterol that is esterified is lower than in cells comparably loaded with cholesterol derived from AcLDL (4, 5, 16–18). As ACAT activity appears to be normal in OxLDL-loaded cells (5, 16), their relatively high proportion of unesterified cholesterol might reflect inaccessibility of OxLDL-derived cholesterol to ACAT, as a result of lysosomal storage of undegraded OxLDL lipids. One group has suggested that lysosomal hydrolysis of steryl esters of OxLDL is normal, but that OxLDL-derived free sterols are trapped within the lysosomal compartment, possibly in association with undegraded sphingomyelin (17, 19). On the other hand, OxLDL enriched with liposomal unesterified cholesterol enhanced cholesterol esterification in macrophages (20), consistent with export of lysosomal free cholesterol. However, uptake of mildly oxidized LDL caused lysosomal lipid accumulation in pigeon and THP-1 macrophages (21).

It is also known that oxidation of LDL leads to a net loss in the content of unoxidized cholesterol and its esters, with matching generation of free and esterified oxysterols (16, 22). However, few studies measure cellular accumulation of oxysterols, their lysosomal processing, subcellular distribution and availability for ACAT-mediated re-esterification, after uptake of OxLDL by cells. The objective of the present study was to make a detailed examination of the content and cellular processing of sterols in OxLDL-loaded macrophages. Both cholesterol and 7-ketocholesterol (7KC) the predominant oxysterol in OxLDL (16, 22) and their esters were measured in primary mouse peritoneal macrophages and the J774A.1 cell line after uptake of OxLDL and compared with cells comparably loaded with AcLDL or AcLDL selectively enriched with 7KC (7KCAcLDL (23)). This latter system allows study of the metabolism and effects of 7KC independently of other components of OxLDL. Using subcellular fractionation, we directly demonstrate that free cholesterol and 7KC are not selectively retained within the lysosomes of OxLDL-loaded cells. We provide evidence that lysosomal hydrolysis of normal cholesteryl esters is not inhibited in OxLDL-loaded macrophages but that their cytoplasmic re-esterification may be limited by the availability of fatty acyl co-substrates for ACAT. We have previously shown that significant proportions of the esterified sterols in OxLDL-loaded cells differ chemically from those in normal LDL. In the present study we have investigated this further, defining a large intracellular pool of oxidized sterol esters which accumulate in the lysosomes of OxLDL-loaded macrophages and which are resistant to hydrolysis.

MATERIALS AND METHODS

LDL preparation

Human LDL was isolated from healthy fasting volunteers in the presence of ethylenediaminetetraacetic acid (EDTA, 3 mm), aprotinin (90 kallikrein inhibitory units/ml; Sigma) and soybean trypsin inhibitor (20 μg/ml: Sigma) by discontinuous density gradient ultracentrifugation in the density range $\rho = 1.02–1.05$ g/ml, as previously described (24). The isolated LDL was sterilized by membrane filtration (0.45 μm), and stored in the dark in phosphate-buffered saline (PBS) containing 3.0 mm EDTA and 0.1 mg/ml chloramphenicol at 4°C under N_2. LDL preparations were used usually within 1 week of isolation. All dialyses were performed using deoxygenated buffers in filled stoppered bottles, in the dark, at 4°C, to prevent oxidation. Lipoprotein-deficient serum ($\rho > 1.25$ g/ml) was prepared as described (25).

LDL modifications

Enrichment with 7-ketocholesterol (7KC). 7KC-enriched LDL was prepared as previously described (23). Briefly, plasma from fasting normolipidemic donors containing EDTA (2 mm) was incubated for 6 h at 37°C with 7KC (final concentration 2.4 mm) added as a concentrated stock solution in ethanol (1.6%; v/v). The 7KC-enriched LDL (7KC-LDL) was isolated by density gradient ultracentrifugation as described above. The 7KC content of the LDL was routinely 25–30% of the total cholesterol content of the particle (equivalent to the 7KC content of OxLDL (22)), and this remained constant during subsequent dialyses and acetylation. 7KC-enrichment had no effect on the electrophoretic mobilities of either unmodified or acetylated LDL (23).

Oxidation. LDL was dialyzed over 24 h against 3 × 1 L PBS containing chloramphenicol (0.1 g/L) and Chelex-100 (1 g/L), then 1 × 1 litre PBS containing chloramphenicol only for the last 1 h prior to oxidation. Chelex was omitted from the final dialysis as small amounts of chelator can leach from the resin and interfere with metal ion-mediated oxidation (26). Copper oxidation was achieved by incubating LDL (400 μg protein/ml) in PBS with a sterile solution of $CuCl_2$ (final concentration 10 μm) at 37°C for 24 h (27). Some experiments were performed with 1 mg/ml LDL and 20 μm $CuCl_2$ (22) with results consistent with those presented.

Acetylation. LDL and 7KC-LDL (3–4 mg protein/ml) were acetylated as described (18). Excess reagents were removed by dialysis against 4 × 1 litre changes of PBS containing EDTA (1.0 mg/ml) and chloramphenicol (0.1 mg/ml), as described above.

The degrees of acetylation and oxidation were assessed using non-denaturing agarose gel electrophoresis on 1% Universal Agarose gels (Ciba-Corning) in Tris-barbitone buffer (pH 8.6) at 90 V for 45 min. The LDL band was visualized with Fat Red 7B stain. A relative electrophoretic mobility of >3, using native LDL as a reference, was routinely obtained for both modifications.

Isolation and culture of macrophages

Resident mouse peritoneal macrophages (MPM). Cells were isolated by lavage from QS mice (28) and plated in either 35-mm diameter tissue culture wells (Costar) at 5–6 × 10^6 cells per well or in 150-cm^2 tissue culture flasks (Falcon) at 2 × 10^8 per flask, incubated at 37°C for 1–2 h, then washed three times with prewarmed PBS before incubation with Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) LPDS, penicillin G (50

units/ml), and streptomycin (50 μg/ml) plus modified lipoproteins, as indicated.

J774A.1 macrophages. J774A.1 mouse monocyte-macrophage cells (ATCC:67-TIB, batch #F-10089), were routinely grown as adherent cultures in DMEM medium (Flow Laboratories) containing 10% (v/v) heat-inactivated newborn calf serum (HINBCS), penicillin G (50 units/ml) and streptomycin (50 μg/ml) in 150-cm^2 tissue culture flasks. Cells were scraped into suspension and re-plated at 1:3 dilution every 4/5 days. For experiments, J774A.1 cells were used at near-confluency ($2.5–3.0 \times 10^7$ per 150-cm^2 flask, or 2×10^6/35-mm well), and incubated with DMEM containing 10% (v/v) LPDS with modified lipoproteins as indicated.

Incubation of LDL with cultured macrophages

For measurements of cellular lipid accumulation and subcellular distribution, AcLDL was supplied at 50 μg protein/ml and OxLDL at 10, 25, or 50 μg/ml (2 ml medium/well or 35.0 ml/150 cm^2 flask) as indicated. In some cases non-loaded cells were prepared by incubation for 24 h in the above medium without added lipoproteins. The media were warmed to 37°C before addition to cells.

Inhibition of acyl-CoA:cholesterol acyltransferase (ACAT) was achieved in some experiments using Sandoz 58-035 (generously provided by Sandoz Pharmaceuticals, East Hanover, NJ). This was prepared as a 5.0 mg/ml stock in ethanol and used at a final concentration of 5.0 μg/ml (18).

Fatty acid supplementation of macrophages

A stock oleyl-albumin solution (12.7 mm sodium oleate in complex with 12% (w/v) bovine serum albumin; molar ratio of 7.12:1) was prepared as previously described (29) and stored at −20°C until use. Macrophages or J774A.1 cells were incubated with oleyl albumin either simultaneously with OxLDL and/or immediately after OxLDL loading. In all cases, the final concentration of BSA in the medium was 1.5 mg/ml; where necessary, the oleyl-albumin stock was diluted with an appropriate amount of fatty acid-free BSA to adjust the concentration of oleate supplied.

Lipid extraction

Cells were gently washed three times with PBS at room temperature and then cells were dissolved by addition of 0.2 m NaOH to the MPM (0.6 ml) and J774A.1 cells (1.0 ml) for 15 min at 4°C. Preliminary studies (30) confirmed that no detectable saponification of steryl esters occurs during this procedure. A sample (0.1 ml) was removed for protein assay by the bichinchoninic (BCA) acid method using BSA as standard (7). Then 0.4 ml of the MPM or 0.8 ml of the J774 cell lysate was made up to 1.0 ml with ice-cold PBS containing BHT (Sigma; final concentration 20 μm) and EDTA (2 mm final concentration), and the total was extracted into methanol (2.5 ml) then hexane (10 ml) as previously described (27). A sample of the hexane layer was evaporated to dryness, redissolved in the appropriate mobile phase (see below), and analyzed by HPLC (27) as described below. Lipoproteins (20 μg) were diluted to 1 ml with ice-cold PBS (containing 20 μm BHT and 2 mm EDTA) before extraction into methanol/hexane. Cholesteryl heptadecanoate or cholesteryl benzoate was added to the sample before extraction as internal standards. In some cases samples were saponified before extraction using methanolic KOH, as previously described (22), using cholesteryl propyl ether as internal standard.

Sterol analysis

Cholesterol and cholesteryl esters (docosahexaenoate, arachidonate, linoleate, oleate, palmitate, and stearate) were separated using reverse phase high performance liquid chromatography (HPLC) at room temperature on a C-18 column (Supelco), using an eluent of acetonitrile–isopropanol 30:70 (v/v) and 210 nm detection (27). 7KC and 7KC esters (7KCE; as listed above for cholesterol) were analyzed with a solvent system of acetonitrile–isopropanol–water 44:54:2 (v/v/v) and detection at 234 nm (22). In cells containing significant amounts of 7KCE (some of which co-elute with cholesterol in acetonitrile/isopropanol), cholesterol was measured using acetonitrile–isopropanol–water 44:54:2 (v/v/v) and detection at 210 nm (23).

Cholesterol, 7KC, and individual cholesteryl esters were determined after calibration using authentic standards (27). Cholesteryl stearate was not routinely measured as it was a very minor component. 7KCE were determined using retention times and calibration data obtained from standards synthesized according to the method of Deykin and Goodman (31) as previously described (22). Results are expressed as nmol lipid per mg of cell protein or LDL protein.

Oxidized esters (i.e., sterols esterified to oxidized fatty acyl groups) in OxLDL-loaded cells were estimated as the difference between total cell sterol (i.e., cholesterol or 7KC measured after saponification) less the HPLC-detectable free sterol plus normal esters (measured in unsaponified samples, as described above) (22).

Cell viability

After incubation with modified LDLs for up to 48 h, viability was assessed in triplicate samples using Trypan blue exclusion or by lactate dehydrogenase release (32).

Subcellular fractionation

Homogenization. Macrophages (2×10^8 per 150-cm^2 flask) or J774A.1 cells ($2–3 \times 10^7$ per 150-cm^2 flask) were preloaded with lipoproteins for 24 h by incubation in RPMI containing 10% (v/v) LPDS with AcLDL or OxLDL at the concentrations indicated. Non-loaded cells were incubated for 24 h in medium without LDL. The cells were then washed twice with ice-cold Hanks buffered salt solution (HBSS), gently scraped into fresh HBSS (10 ml) and centrifuged at 145 *g* for 5 min. The pellet was washed in homogenization buffer, pH 7.0 (4°C, 0.25 m sucrose; 20 mm HEPES; 0.5 mm EDTA), re-centrifuged as above and finally resuspended in fresh homogenization buffer (1.5 ml). Whole cells were ruptured using shear force as previously described (33). J774A.1 macrophages were ruptured at a concentration of $1.6–2 \times 10^7$/ml and MPM at 1.3×10^8/ml. Quantitative breakage of the cells required 50 passes to give maximum cell disruption (80–97%) as assessed using an ethidium bromide assay (14, 33) with minimum organelle damage. The ruptured cells were centrifuged at 800 *g* for 10 min at 4°C to remove unbroken cells and nuclei. The post-nuclear supernatant was stored on ice for not more than 15 min.

Preparation of density gradients and centrifugation. Ficoll (Pharmacia) and Nycodenz (Nycomed) solutions were made up in buffer (pH 7.4) containing 0.25 m sucrose, 10 mm HEPES, 1.0 mm EDTA, 1.0 mm PMSF, 5.0 μg/ml aprotinin (Sigma), and 1.0 mm benzamidine (Sigma). A 1–22% Ficoll gradient (3.4 ml) was poured into a 5.4 ml heat-seal centrifuge tube (Beckman), and underlayered with a 45% Nycodenz (1.0 ml) cushion. The cell supernatant was gently layered on the top of the gradient and the sealed tube was centrifuged in a Beckman VTi 65.2 rotor at 240,000 *g* and 4°C for 90 min, using slow acceleration and deceleration programs.

Collection and analysis of density gradient fractions. Fractions (26 × 200 μl) were collected from the bottom of the centrifuge tube and stored on ice. Protein and aryl sulfatase assays were performed immediately on all fractions, and on aliquots of post nuclear supernatant within 2 h, as previously described (14). The refractive indices of selected samples were measured at constant temperature (23°C) using a refractometer calibrated with appropriate standard solutions.

The 26 fractions were either extracted individually into methanol/hexane as described above for whole cells, or in some cases were grouped into 5 major pools to increase sensitivity of lipid analyses. The samples were pooled as shown in Fig. 1 to separate regions of the gradient containing; free lipids, plasma membrane, endosomes, lysosomes, and the Nycodenz cushion (densities <1.02, 1.02–1.035, 1.035–1.05, 1.05–1.095, 1.095–1.25 g/ml, respectively (14)).

RESULTS

Sterol content of foam cells and of macrophages loaded with modified LDLs

We wished to determine the cellular deposition and distribution of (oxy)sterols and their esters in macrophages after uptake of three different modified lipoproteins. OxLDL contains cholesterol, oxysterols (predominantly 7KC), and their esters, including a large pool of oxidized steryl esters (22). AcLDL contains no detectable oxidized lipids, while 7KC-enriched acetylated LDL (7KCAcLDL) differs from AcLDL only in the addition of unesterified 7KC (23). The latter lipoprotein can be used to generate cholesteryl ester-loaded macrophages selectively enriched with 7KC in the absence of other oxidized lipids, thus permitting direct study of its effects on foam cell biology (23). **Table 1** presents results for both primary macrophages (MPM) and the J774A.1 macrophage cell line, each loaded for 24 h with one of the modified LDLs.

In non-loaded cells, both MPM and J774A.1 cells contained essentially only unesterified cholesterol (Table 1). With AcLDL loading, cholesteryl esters were formed, notably linoleate, with lesser amounts of oleate and palmitate, as previously shown (18, 23). In 7KCAcLDL-loaded cells, total cholesterol loading was higher than for AcLDL-loaded cells. Free and esterified 7KC also accumulated, but the proportion esterified was greater for 7KC (1:3–1:5) than for cholesterol (1:1). Steryl linoleates again contributed the majority of esters in 7KCAcLDL-loaded cells, though steryl oleates and palmitates were also substantial, both for cholesterol and 7KC.

MPM or J774A.1 cells loaded with OxLDL accumulated less than half the amount of total detectable cholesterol compared with AcLDL loading, and the distribution of cellular cholesterol between free and the specified ester pools was very different. For example, cholesteryl linoleate represented 15–25% of the total cholesterol content after AcLDL loading, but less than 1% after OxLDL loading. The relative contributions of oleate and palmitate were also lowered, though less extremely. Overall, while measured cholesteryl esters in AcLDL- and 7KCAcLDL-loaded macrophages comprised 40–60% of total cholesterol, in OxLDL-loaded cells they represented only 5–10%. OxLDL-loaded cells also contained free and esterified 7KC. As for

TABLE 1. Free and unoxidized steryl ester content of MPM and J774A.1 macrophage foam cells

	MPM				J774A.1			
Sterol	None (n = 3)	Ac-LDL (n = 5)	7KAc-LDL (n = 3)	OxLDL (n = 8)	None (n = 4)	Ac-LDL (n = 4)	7KAc-LDL (n = 3)	OxLDL (n = 6)
	% total				*% total*			
FC	100	41.7 ± 13.3	44.5 ± 8.6	89.6 ± 2.0	100	55.1 ± 4.9	59.4 ± 4.3	95.5 ± 0.9
CE		58.3 ± 13.2	55.5 ± 8.6	10.5 ± 2.0		44.8 ± 4.7	40.1 ± 3.7	4.50 ± 0.9
CD		1.0 ± 0.5	1.0 ± 0.2	0.2 ± 0.6		1.1 ± 0.4	0.6 ± 0.0	nd
CA		5.0 ± 1.4	4.2 ± 0.9	0.1 ± 0.1		3.8 ± 1.8	1.8 ± 0.7	0.23 ± 0.3
CL		25.1 ± 5.3	24.6 ± 1.8	0.8 ± 1.2		19.0 ± 5.4	14.8 ± 0.7	0.9 ± 0.96
CO		20.0 ± 4.3	15.8 ± 2.8	4.7 ± 1.0		13.7 ± 2.9	11.2 ± 1.9	1.5 ± 0.3
CP		5.6 ± 2.2	8.3 ± 1.6	4.8 ± 0.7		7.3 ± 1.6	12.2 ± 1.7	1.9 ± 0.4
CS		1.6 ± 1.1	1.6 ± 1.6	0.1 ± 0.2		nd	nd	nd
F7KC			16.2 ± 9.8	68.9 ± 8.0			21.4 ± 2.0	74.8 ± 1.9
7KE			83.8 ± 9.7	31.1 ± 8.0			78.6 ± 2.0	25.1 ± 2.1
7KD			1.4 ± 1.3	nd			nd	0.4 ± 0.6
7KA			3.9 ± 3.6	0.3 ± 0.8			3.1 ± 1.0	2.7 ± 3.5
7KL			29.7 ± 4.7	0.3 ± 0.7			27.2 ± 7.4	4.7 ± 4.1
7KO			23.4 ± 0.9	11.1 ± 3.1			17.8 ± 4.5	6.7 ± 1.2
7KP			19.7 ± 4.8	16.6 ± 3.5			28.7 ± 3.5	10.2 ± 9.7
7KS			5.2 ± 2.5	2.8 ± 2.1			1.9 ± 0.4	0.3 ± 0.6
	nmol/mg cell protein				*nmol/mg cell protein*			
FC + CE	45.5 ± 14.0	309 ± 52	340 ± 72	84.3 ± 29.9	31.3 ± 14.0	101 ± 22.7	188 ± 10.3	69.8 ± 31.3
F7KC + 7KE	nd	nd	101 ± 28	33.5 ± 15.5	nd	nd	111 ± 27.3	12.6 ± 3.7

Mouse peritoneal macrophages (MPM) or J774A.1 cells were incubated for 24 h in medium containing 10% (v/v) LPDS and the indicated modified LDL (25 μg protein/ml). The cells were then washed and extracted for protein and lipid analyses as described in Methods. Data provided are of individual sterols and unoxidized steryl esters, expressed as a percentage of the total measured pool (free sterol plus all detected unoxidized steryl esters, as shown above) in the cell. The absolute masses of total detected sterol (sum of free sterol plus unoxidized esters) in the various foam cells are shown at the base of the table and are expressed as nmol/mg cell protein. All data are means ± SD of the indicated number (n) of separate experiments, each of which was performed with 3–6 replicates. FC, free cholesterol; CD, cholesteryl docosahexaenoate; CA, cholesteryl arachidonate; CL, cholesteryl linoleate; CO, cholesteryl oleate; CP, cholesteryl palmitate; CS, cholesteryl stearate; CE, unoxidized cholesteryl esters (= sum of CD + CA + CL + CO + CP + CS); 7KC, free 7-ketocholesterol; 7KD, 7-ketocholesteryl docosahexaenoate; 7KA, 7-ketocholesteryl arachidonate; 7KL, 7-ketocholesteryl linoleate; 7K-O, 7-ketocholesteryl oleate; 7K-P, 7-ketocholesteryl palmitate; 7KS, 7-ketocholesteryl stearate; 7-KE, unoxidized 7-ketocholesteryl esters (= sum of 7KD + 7KA + 7KL + 7KO + 7KP + 7KS); nd, not detected.

7KCAcLDL-loaded cells, a greater proportion of 7KC than cholesterol was present as the esters listed in Table 1, suggesting that it may be a better or more accessible substrate for ACAT.

Cell viabilities were routinely >85% after 24 h uptake of all modified lipoproteins (data not shown). Thus OxLDL is not toxic to macrophages under the conditions used here.

Subcellular distribution of sterols in macrophages

There is a similarity between the steryl ester profiles of OxLDL and of cells loaded with OxLDL (22) which could reflect accumulation of undegraded lipoprotein in the cells. Previously we have shown that OxLDL reaches macrophage lysosomes (14), but that degradation of apoB is inefficient (7, 14). It is possible that lipids of OxLDL, including cholesteryl esters, are also retained lysosomally. Therefore subcellular fractionation combined with assay of sterol mass in individual organelle populations was used to directly determine the cellular location of the free and esterified sterols in lipid-loaded macrophages. Cells were ruptured by shear force and a post-nuclear supernatant was obtained by density gradient ultracentrifugation. The location of plasma membrane (ρ = 1.035 g/ml) and lysosomes (ρ = 1.095 g/ml) in this gradient were established previously (14). Endosomes (early and late), endoplasmic reticulum and Golgi (hereafter abbreviated as 'endosomes') partition at densities intermediate between plasma membrane and lysosomes (34, 35). **Figure 1** shows the distribution of protein and a lysosomal marker enzyme (aryl sulfatase) under these conditions. In some cases the fractions were combined into five major pools (as indicated in Fig. 1).

The majority of lysosomal enzyme activity was at a density of 1.095 g/ml, as previously described (14). A minor proportion routinely partitioned in the lighter region of the gradient (ρ = 1.035 g/ml) which also contains plasma membrane (14). This could represent a sub-population of light lysosomes, such as previously reported in foam cells (36) and recently in OxLDL-loaded MPM (37). However, the majority of activity at ρ = 1.035 g/ml was found to be free (non-latent) enzyme, while >80% of the more dense major peak was latent (i.e., intact lysosomes; data not shown). Free aryl sulfatase also partitions at ρ = 1.035 g/ml indicating that the minor, less dense activity is lysosomal enzyme released by rupture during cell lysis and/or plasma membrane-associated enzyme. A small proportion of this activity could be a lysosomal sub-population, but its magnitude was unaffected by loading with either AcLDL, 7KCAcLDL, or OxLDL (data not shown). Also, the buoyant density of the major lysosomal population (ρ = 1.095 g/ml) was not significantly altered by loading J774A.1 cells with increasing concentrations of OxLDL (data not shown). This is in agreement with a recent study in which OxLDL-loading of J774A.1 cells did not affect lysosomal density (37). In the same study, uptake of OxLDL by MPM was found to induce a shift in lysosomal markers to more buoyant densities, unlike the present study where MPM and J774A.1 cells behaved similarly. As the methodologies used in both studies are similar, it is presumed that differences in the degree of OxLDL accumulation between the two studies explain this discrepancy.

In non-loaded J774A.1 cells, unesterified cholesterol partitioned almost exclusively into the three major membrane-containing regions (plasma membrane, endo-

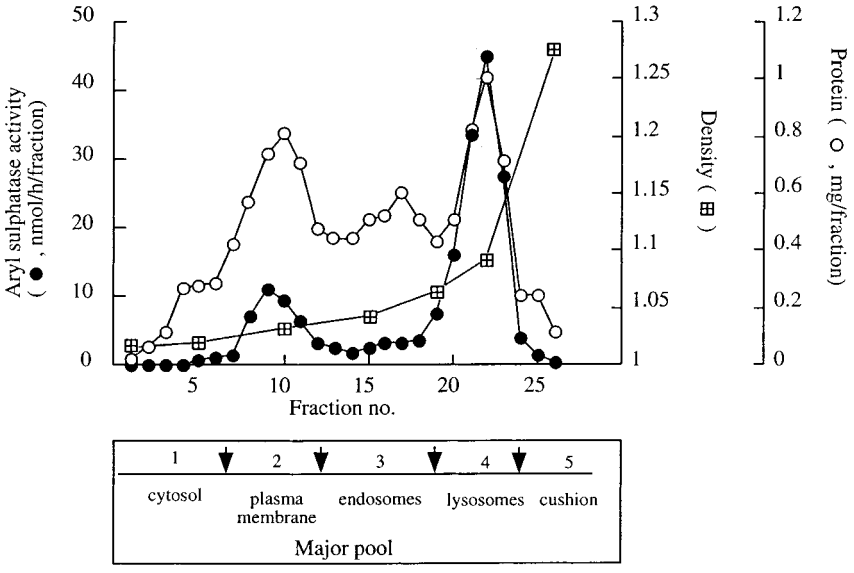

Fig. 1. Subcellular fractionation of macrophages by density gradient centrifugation. J774 A.1 cells were incubated for 24 h in RPMI containing 10% (v/v) LPDS. The cells were then washed, lysed, and fractionated on a 1% to 22% Ficoll/45% Nycodenz gradient as described in Methods. All 26 fractions were then assayed for protein (○) and aryl sulfatase activity (●), and the refractive index was used to calculate density (⊞). The arrowheads indicate the ranges of fractions which were subsequently combined in some experiments to contain the following major pools: 1, cytosolic lipids; 2, plasma membrane; 3, endosomes; 4, lysosomes; 5, Nycodenz cushion.

TABLE 2. Subcellular sterol distribution in macrophage foam cells

Loading Condition	Sterol	Major Fraction 1 Cytosolic Lipids	2 Plasma Membrane	3 Endosomes	4 Lysosomes	5 Nycodenz Cushion
	Unesterified			*% of total sterol*		
Control (3)	FC	8.2 ± 4.1	43.3 ± 5.4	23.5 ± 2.2	23.8 ± 1.1	1.2 ± 1.2
AcLDL (7)	FC	7.3 ± 5.3	43.6 ± 9.5	23.5 ± 3.3	23.8 ± 8.2	3.2 ± 3.5
7KCAcLDL (3)	FC	12.8 ± 4.8	44.7 ± 8.4	25.6 ± 1.8	13.9 ± 2.2	1.2 ± 1.1
	7KC	11.9 ± 1.6	43.8 ± 7.2	27.1 ± 0.6	16.8 ± 5.7	0.4 ± 0.4
OxLDL (4)	FC	2.7 ± 1.3	38.7 ± 3.6	25.0 ± 6.4	30.3 ± 5.6	1.9 ± 2.0
	7KC	3.2 ± 1.3	40.3 ± 5.4	24.2 ± 3.6	29.8 ± 6.3	2.2 ± 2.3
	Unoxidized esters					
AcLDL (5)	CE	82 ± 15	9 ± 10	2 ± 3	5 ± 5	0.8 ± 1.9
7KCAcLDL (3)	CE	71 ± 8	24 ± 4	5 ± 6	0.2 ± 0.4	nd
	7KCE	66 ± 6	29 ± 4	1.8 ± 1.2	2 .7 ± 2.4	nd
OxLDL (4)	CE	71 ± 24	25 ± 20	0.5 ± 1.0	4.3 ± 7.4	0.3 ± 0.6
	7KCE	57 ± 15	34 ± 13	3.6 ± 0.6	4.6 ± 1.5	1.5 ± 1.9

J774A.1 macrophages were incubated for 24 h in RPMI 1640 medium containing 10% (v/v) LPDS with either no additions (control) or with the indicated lipoproteins (25 μg protein/ml). The cells were then washed, ruptured, and subjected to density gradient centrifugation as described in Methods. The 26 fractions were assayed for density and aryl sulfatase activity and then combined into the five major pools indicated in Fig. 1. The protein and sterol contents of these fractions were determined. Data are expressed as % of total sterol (free or unoxidized esters, measured in the whole homogenate applied to the gradient) present in each fraction and are the means ± SD of several separate experiments (number indicated in parentheses). Total cellular contents of free (FC, 7KC) and esterified (CE, 7KCE) sterols are shown in Table 1. The (unoxidized) esters of cholesterol and 7KC measured are those listed in Table 1.

somal, and lysosomal fractions; major fractions 2, 3, and 4, respectively; **Table 2**). The unesterified cholesterol content of the plasma membrane fraction was a greater proportion (40–45%) of total cellular cholesterol than other individual regions of the cells, consistent with previous studies (38, 39).

After uptake of AcLDL, the subcellular distribution of free cholesterol was similar to that of non-loaded cells (Table 2). Cholesteryl esters were predominantly in the cytosolic fraction, with only a small proportion (<5% total) found in the lysosomal fraction (Table 2; **Fig. 2**). The relative proportions of individual esters in the cytosolic fraction (both MPM or J774A.1 cells) were similar to those of the whole cells (data not shown). The cytosolic location of the majority of the cellular esters in AcLDL-loaded cells is consistent with ACAT-mediated re-esterification of sterol released from the lysosome after hydrolysis of the particle.

Uptake of OxLDL by J774A.1 cells increased the cellular content of both cholesterol and 7KC (Table 1). The subcellular distribution of free cholesterol was similar to that of AcLDL-loaded cells (Table 2). The proportion of cholesterol in the lysosomal fraction of OxLDL-loaded cells was slightly greater than that of AcLDL-loaded cells (Table 2), but was identical when expressed as per mg protein (data not shown). Cholesterol distribution was closely matched by that of free 7KC (Table 2). In 7KCAcLDL-loaded cells, the distributions of free 7KC and free cholesterol were also similar (Table 2). There was no evidence to suggest that either of these free sterols was trapped in lysosomes in significant quantity in any of the foam cell models.

The majority of normal (unoxidized) cholesteryl and 7-ketocholesteryl esters measured were present in the cytosolic lipid fraction. Although the distribution of steryl esters from OxLDL- and 7KCAcLDL-loaded cells was spread over a rather broader density range than in AcLDL-loaded cells, these were always well separated from the region containing lysosomes (Table 2). Only very minor proportions of cholesteryl and 7-ketocholesteryl esters were present in the lysosomal fraction in OxLDL- or 7KCAcLDL-

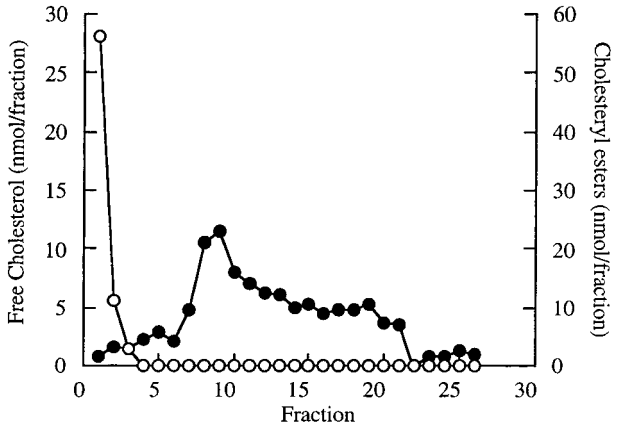

Fig. 2. Subcellular distribution of sterols in AcLDL-loaded J774A.1 macrophages. J774A.1 cells were incubated for 24 h with 50 μg/ml AcLDL in RPMI containing LPDS (10% v/v). The cells were then washed, lysed, and fractionated on a 1% to 22% Ficoll/45% Nycodenz gradient. All 26 fractions were assayed for protein and aryl sulfatase activity (not shown), and samples were extracted into hexane and assayed for cholesterol (●) and cholesteryl esters (○) by HPLC, as described in Methods. Data for each lipid are expressed as the percentage per fraction of the total applied to the gradient. Cholesteryl esters comprise the sum of the individual esters listed in Table 1.

loaded cells. As lysosomal latency in cell homogenates was routinely >80%, the presence of cellular esters at the top of the gradient cannot be accounted for by release of material from ruptured lysosomes. It can be calculated that not more than 4% of the total ester content at the top of the gradient could be accounted for by lysosomal rupture, whereas over 90% are present in this location. This indicates that the majority of the normal (unoxidized) esters of cholesterol and 7KC that are present in OxLDL-loaded cells are non-lysosomal and synthesized de novo by ACAT (see also below).

Fatty acid supplementation enhances sterol esterification in OxLDL-loaded cells

Because free sterols are not restricted to the lysosomes and ACAT is active in OxLDL-loaded cells, an alternative explanation for the relatively low ester content of OxLDL-loaded cells could be a limited supply of fatty acyl co-substrates for ACAT. The very striking similarity of the cholesteryl ester profile of macrophages loaded with either AcLDL or OxLDL (Table 1) and the ester profile of the lipoproteins themselves (22) initially suggested that the major fatty acyl substrates for sterol re-esterification after uptake of modified LDLs are also lipoprotein-derived. As OxLDL contains relatively few normal esters (either of cholesterol or oxysterols) it is possible that insufficient substrate for ACAT was supplied with the lipoprotein. To investigate this, additional oleic acid was supplied to cells, either together with OxLDL or in a chase period immediately after loading (**Table 3**). This produced an increase in the content of cholesteryl oleate and 7-ketocholesteryl oleate. At the concentration tested (180 μm), 4-fold and 2.3-fold increments in cholesteryl oleate were produced in J774A.1 and MPM, respectively, plus 8.4- and 1.2-fold increments in 7-ketocholesteryl oleate, respectively. Thus fatty acyl substrate limitation may normally contribute to the low ester content of these cells. However, other factors may also influence their low ester content. This is suggested by the observations that: *a*) supplementation was even more efficient when the fatty acid was added in a 24-h chase period immediately after loading (Table 3); *b*) even though supplementation was substantial, it did not generate the proportions of steryl esters formed in AcLDL-loaded cells (cf. Table 1); and *c*) supplementation of oleyl esters in these cells was to some extent counterbalanced by a decrease in the palmitoyl ester content of the cells. Similar results were obtained after supplementation with linoleic, palmitic, or arachidonic acids (L. Kritharides, unpublished results).

TABLE 3. Sterol esterification during OxLDL uptake in the presence of added free oleate

	Peritoneal Macrophages[a] Oleate Concentration			J774.A1 Cells[b] Oleate Concentration	
Sterol	0 μm	180 μm	0/180 μm[c]	0 μm	180 μm
	nmol/mg cell protein			*nmol/2 × 10⁶ cells*	
FC	126 ± 4	112 ± 29	100 ± 13	42.6 ± 6.8	34.3 ± 1.5
CO	4.7 ± 1.0	10.5 ± 1.4	16.5 ± 1.0	1.1 ± 0.3	5.3 ± 2.1
CP	5.4 ± 0.8	2.4 ± 0.4	1.95 ± 0.2	0.7 ± 0.1	0.8 ± 0.2
7KC	52.5 ± 0.7	33.2 ± 6.6	27.7 ± 3.7	7.5 ± 1.6	5.6 ± 0.4
7KO	5.2 ± 0.2	8.9 ± 0.6	17.6 ± 0.8	0.4 ± 0.0	3.3 ± 2.1
7KP	8.2 ± 0.2	2.3 ± 0.4	3.4 ± 0.3	0.9 ± 0.3	0.6 ± 0.1

Peritoneal macrophages (MPM, 5 × 10^6) or J774.A1 cells (2 × 10^6) were incubated with OxLDL (25 μg/ml) in DMEM containing sodium oleate (at the indicated final concentration) complexed to albumin (1.5 mg/ml) for 24 h. The cells were then either washed, extracted, and their lipid content analysed by HPLC as described in Methods, or incubated for a further 24 h in fresh medium before extraction and analysis. FC, free cholesterol; CO, cholesteryl oleate; CP, cholesteryl palmitate; 7KC, 7-ketocholesterol; 7KO, 7-ketocholesteryl oleate; 7KP, 7-ketocholesteryl palmitate.

[a] Data are expressed as nmol/mg cell protein and are means ± SD of triplicate observations.

[b] Data are expressed as nmol/2 × 10^6 cells and are means ± SD of triplicate observations.

[c] Cells loaded for 24 h with OxLDL in the absence of sodium oleate, followed by a 24 h chase in medium containing 180 μm oleate.

Oxidized steryl esters in OxLDL-loaded macrophages

An additional explanation for the apparently limited esterification of sterols in OxLDL-loaded cells might be that novel esters are formed that are not identified by our HPLC methods. For example, oxidized fatty acids are known to be generated during LDL oxidation and to be present in atherosclerotic tissue (27, 40). Therefore total cholesterol and 7KC were measured in cell extracts after gentle saponification (see Methods; (22)) and compared with the amount of sterol (free plus 'normal' (unoxidized) esters) measured by HPLC (see Methods). This revealed the presence of a substantial additional pool of esterified sterol in both OxLDL and in macrophages loaded with OxLDL (22). As shown in **Table 4**, 40–50% of both cholesterol and 7KC were present in the form of such esters, which we assume contain oxidized fatty acyl elements inasmuch as *a*) they are not resolved by our current HPLC analyses which detect all major cholesteryl and 7-ketocholesteryl esters of unoxidized fatty acids; and *b*) they are present only in OxLDL and in OxLDL-loaded cells and not in AcLDL or 7KCAcLDL or in macrophages loaded with these lipoproteins (data not shown). We have not yet characterized the components of this pool but it is likely that they are multiple and diverse, in view of the wide range of products generated during fatty acid oxidation (41). Cholesteryl linoleate peroxides and hydroxides, which our HPLC analyses can detect (27), are present at trace or undetectable levels in OxLDL-loaded macrophages (data not shown), and therefore are not significant components of this pool. When the magnitude of the oxidized ester pool in OxLDL-loaded cells is considered, then their total esterified cholesterol content becomes comparable to that in AcLDL-loaded cells (i.e., 50–60% total cholesterol; Tables 1 and 4). Table 4 shows that the size of the cellular oxidized ester pool is stable for at least 48 h after OxLDL uptake.

Role of ACAT in esterification of cholesterol and 7KC in the OxLDL-loaded cells

Addition of the ACAT inhibitor 58-035 during OxLDL uptake and a subsequent 24 h chase inhibited the accumulation of normal cholesteryl and 7KC esters by 79 and 94%, respectively (**Table 5**). It was only slightly less effec-

TABLE 4. Normal and oxidized steryl ester pools in OxLDL-loaded macrophages

Time after OxLDL Uptake	Cholesterol			7KC		
	Unesterified	Normal Ester	Oxidized Ester	Unesterified	Normal Ester	Oxidized Ester
h		*% total*			*% total*	
0 (n = 2)	47.0 ± 22.0	6.8 ± 4.9	44.0 ± 23.7	32.0 ± 17.1	19.6 ± 5.8	48.4 ± 22.9
24 (n = 4)	48.1 ± 11.0	6.3 ± 1.9	43.3 ± 4.7	30.6 ± 11.7	21.7 ± 6.9	47.7 ± 7.5
48 (n = 4)	51.5 ± 9.5	5.8 ± 0.5	42.7 ± 9.7	34.6 ± 0.8	20.7 ± 2.0	44.1 ± 2.8
OxLDL* (n = 3)	40.1 ± 0.4	4.8 ± 0.2	55.1 ± 0.5	26.8 ± 2.8	19.4 ± 0.9	53.9 ± 3.6

Mouse peritoneal macrophages were incubated for 24 h with OxLDL (25 μg protein/ml) in DMEM containing LPDS (2.5 mg protein/ml), then washed and incubated for the times indicated in medium without lipoprotein. Cells were then lysed and the sterol contents of aliquots of cell lysates and OxLDL* determined before and after cold alkaline saponification. Measurements of free sterols and of normal and oxidized esters were as described in Methods. Data are means ± SD of the indicated numbers of separate experiments (in parentheses) each of which was performed with 3–6 replicate cultures. The intracellular contents of cholesterol and of 7KC and their esters are expressed as percentages of the total pool of each sterol within the cells or the OxLDL. These did not change significantly during the period of the experiments.

tive when presented only during uptake (52 and 79% inhibition, respectively; data not shown). This is similar to the sensitivity of cholesteryl esters to 58-035 in AcLDL-loaded macrophages, which were completely ablated after a 24 h chase (Table 5) but less completely immediately after loading (84% inhibition, data not shown and ref. 18). However, the oxidized ester pools of both cholesterol and 7KC in OxLDL-loaded cells were much less affected by ACAT inhibition (26 and 7%, respectively). This suggests that esters of sterol plus oxidized fatty acids are not generated by the activity of ACAT, but derive directly from the donor oxidized lipoprotein. The insensitivity of the oxidized esters to ACAT inhibition, their derivation from endocytosed OxLDL, and their inferred long half-lives (Tables 4 and 5) suggest that the location of these esters is likely to be lysosomal.

Subcellular location of oxidized sterol esters in OxLDL-loaded macrophages

To confirm the subcellular location of oxidized sterol esters in OxLDL-loaded cells, macrophages were loaded for 24 h with OxLDL and then subjected to subcellular fractionation. Fractions were analyzed for free sterol and for sterol esterified to normal and oxidized esters. **Figure 3** compares the subcellular distribution of these three forms for cholesterol and 7KC. As shown in Table 2, less than 5% of normal cholesteryl and 7-ketocholesteryl esters co-localized with lysosomes (Figs. 3A vs. 3B). In contrast, the majority (70–80%) of oxidized esters were lysosomal (Fig. 3C). Together with their insensitivity to ACAT inhibition and long half-lives, these data suggest that oxidized sterol esters in OxLDL-loaded cells represent an undegraded fraction of the endocytosed lipoprotein which remains sequestered in lysosomes after uptake.

DISCUSSION

The first part of this study showed that free cholesterol and 7KC are readily released from OxLDL after endocytic uptake by macrophages and hydrolysis of unoxidized sterol esters. Maor and Aviram (17) indicated that lysosomal steryl ester hydrolysis of OxLDL was normal, but concluded that the liberated free sterols remain trapped within

TABLE 5. Relative sensitivities of normal and oxidized esters in OxLDL-loaded macrophages to inhibition of ACAT

LDL Species	Treatment	Sterol	Total nmol/mg protein	Unesterified nmol/mg protein	Normal Esters		Oxidized Esters	
					nmol/mg protein	% Inh	nmol/mg protein	% Inh
OxLDL	−58-035	Chol	80.0 ± 12	37.7 ± 3.3	6.6 ± 1.7		35.7 ± 13	
	+58-035	Chol	70.0 ± 10	42.4 ± 4.6	1.4 ± 0.6	79	26.5 ± 9	26
	−58-035	7KC	54.0 ± 8.2	13.5 ± 1.2	16.3 ± 1.8		24.2 ± 8.0	
	+58-035	7KC	44.4 ± 4.6	21.1 ± 1.4	0.8 ± 0.1	94	22.4 ± 3.9	7
AcLDL	−58-035	Chol	91 ± 8.1	48 ± 0.2	44 ± 7.9		nd	
	+58-035		87 ± 8.7	89 ± 8.7	0 ± 3.8	100	nd	

Mouse peritoneal macrophages were loaded for 24 h with OxLDL or AcLDL (25 μg protein/ml) in DMEM containing 2.5 mg/ml LPDS in the presence or absence of 58-035 (5 μg/ml). The cells were then washed and incubated for a further 24 h in lipoprotein-free medium ±58-035. Normal and oxidized esters and free sterols were measured as described in Methods. Data are expressed as nmol/mg cell protein and are means ± SD (n = 3–6) from single experiments representative of three separate experiments. Chol, cholesterol; 7KC, 7-ketocholesterol. % Inh, inhibition of esterification in the presence of 58-035, expressed as a percentage of the ester content of the appropriate uninhibited cell ester pool; nd, not detected.

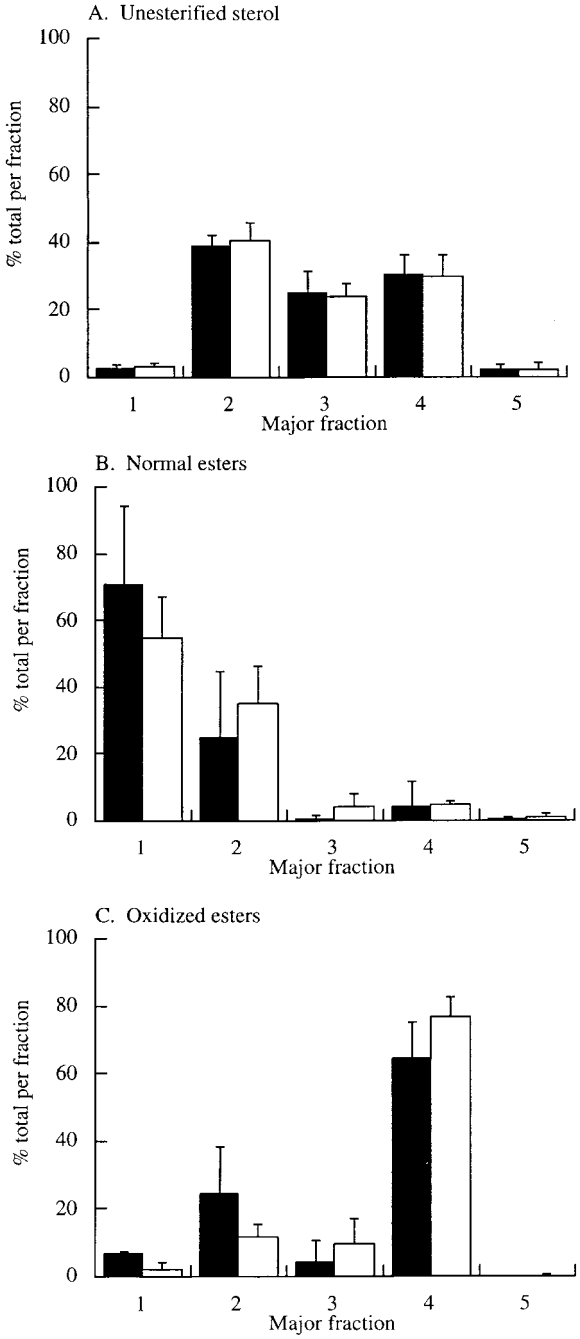

Fig. 3. Subcellular distribution of sterols in OxLDL-loaded J774A.1 macrophages. J774A.1 cells were incubated for 24 h with 25 μg/ml OxLDL in RPMI containing LPDS (10% v/v). The cells were then washed, lysed, and fractionated on a 1% to 22% Ficoll/45% Nycodenz gradient. All 26 fractions were assayed for density, protein, and aryl sulfatase activity (not shown) and then pooled into 5 major fractions as indicated in Fig. 1. Samples of each major fraction were extracted and assayed for (A) free (B) normal (unoxidized) esters, and (C) oxidized esters of cholesterol (solid bars) and 7KC (open bars) as described in Methods. Data for each category of sterol are expressed as the percentage per major fraction of the total applied to the gradient and are the means ± SD of 4 separate experiments.

this compartment. In contrast, we found no evidence for significant selective retention of either free cholesterol or 7KC in macrophage lysosomes. It was found that cholesterol enrichment of all membrane fractions of the cells occurred after uptake of OxLDL, similar to that induced by AcLDL uptake, and that the relative distribution of free 7KC was identical to that of cholesterol.

Uptake of isotropic cholesteryl ester droplets by macrophages leads to their deposition in a minor population of lysosomes of low density (ρ = 1.035 g/ml) and the release of cholesterol from these organelles is slow (42). However, this is quite different from the present study, in which the vast majority of the OxLDL which is taken up by macrophages initially enters lysosomes of normal density (ρ = 1.095 g/ml) (14) and from which sterol export appears normal. We found no evidence of an increase in the proportion of low density lysosomes after uptake of either AcLDL or OxLDL.

That lipolysis of a significant component of OxLDL can be measured under conditions in which proteolysis of apoB is severely impaired is further evidence that no general inactivation of lysosomal hydrolases is induced as a consequence of the uptake of oxidized lipoprotein particles, in contrast to other reports (8, 37, 43). The re-esterification of the free cholesterol and 7KC due to cytoplasmic ACAT is also effective, resulting in normal esters of the two sterols that also show a parallel distribution in the cytoplasmic fraction of the cellular homogenates. ACAT inhibition interfered with the generation of these esters, confirming that they originated in the cytoplasm (and not by retention in lysosomes of unhydrolyzed LDL components). Gross differences in the proportion of sterols esterified with, for example, linoleic acid, in AcLDL- versus OxLDL-loaded cells attested to the limitation by substrate availability in the latter cells. This could be partially overcome by the supply of large amounts of unoxidized fatty acid to the cells.

There are few reports of newly formed oxysteryl esters in OxLDL-loaded cells (see also ref. 18). The specificities of re-esterification of cholesterol and 7KC were distinct, suggestive of substrate selectivity of the enzymes involved. Interestingly, 7KC appeared to be as good, if not better, than cholesterol as a substrate for ACAT, as we have previously suggested in 7KCAcLDL-loaded macrophages (23). Thus, while approximately equal molar quantities of cholesterol and 7KC were esterified in OxLDL-loaded MPM and J774A.1 cells, the ratio of 7KCE:7KC was higher than the CE:FC ratio in both cell types. This is in contrast to another study (17) in which the converse was suggested. However, very different analytical methods were used, based on TLC separation of radiolabeled lipids derived from [^{3}H]- and [^{14}C]cholesteryl linoleate. As oleate and palmitate are by far the most abundant unoxidized esters of both cholesterol and 7KC that were measured in OxLDL-loaded cells (this study), the earlier study involves a substantial underestimate of the cellular ester content. In addition, steryl esters of lipid core aldehydes, such as cholesteryl and oxysteryl 9-oxononanoate, co-migrate with unesterified cholesterol on TLC (44). As these oxidized esters are

generated in OxLDL and are resistant to acid hydrolysis by macrophage homogenates (44) this may explain the apparent accumulation of unesterified cholesterol in lysosomes after uptake of OxLDL.

In agreement with our previous work (22), we have identified oxidized esters as a major sterol pool in OxLDL-loaded macrophages, representing up to 50% of total sterol content in these cells. These represent sterols esterified with oxidized fatty acids. This pool contributes the major fraction of esterified sterol in OxLDL cells, representing as much as 5-fold greater amounts (on a molar basis) than unoxidized esters in the same cells. When this pool is included in calculations of macrophage sterol loading by OxLDL, total esterified cholesterol is equivalent in amount to that in AcLDL-loaded cells, in contrast to previous impressions based on incomplete analyses (4, 5, 17, 45).

We have not yet attempted chemical characterization of these oxidized esters, but predict that they may be complex and numerous. A diverse range of oxidation products of linoleate and arachidonate have been found in oxidized LDL (46–48). Interestingly, products of oxidized cholesteryl linoleate are poorly degraded by macrophages and resistant to acid hydrolysis in cell lysates (44). A major proportion of these esters contained scission products of fatty acids terminating in carbonyl groups (lipid core aldehydes) such as 9-oxononanoyl cholesterol (44, 48). Oxidation products such as these may correspond to the lysosomally accumulated oxidized esters that we have identified in the present study.

The accumulation of these oxidized esters in cells was far less dependent on ACAT than was that of normal esters. After loading with OxLDL, the oxidized esters were stable for at least 48 h. The direct demonstration of location within the lysosomal fraction of OxLDL-loaded macrophages confirms that these esters represent a lysosomal pool of poorly degraded esters derived directly from the endocytosed lipoprotein, confirming our earlier prediction (14) that some of the lipid components of OxLDL, like the apolipoprotein, are poorly handled by lysosomes, in spite of normal hydrolase activities being present. Thus there is differential handling of normal and oxidized esters in lysosomes, indicative of substrate specificity in the esterases involved.

Lysosomal accumulation of cholesteryl linoleate hydroperoxides and hydroxides occurs in macrophages incubated with mildly oxidized AcLDL, associated with retarded lysosomal hydrolysis of unoxidized cholesteryl esters (49). This probably reflects general inactivation of acid esterase activity by such early oxidation products. Similarly, it was recently reported that mildly oxidized LDL (2 h copper oxidation; likely to contain large amounts of cholesteryl linoleate hydroperoxides (22)) stimulated lysosomal cholesteryl ester accumulation in macrophages (21). These esters are also likely to be normal esters and the early oxidation products reported by Kritharides et al. (49). In heavily oxidized LDL, as used in the present study, cholesteryl ester hydroperoxides have decomposed to more advanced oxidation products (22, 27), and general inhibition of normal ester hydrolysis in lysosomes did not occur. Thus oxidized LDL may promote lysosomal sequestration of esters by different mechanisms depending on its degree of oxidation; mildly oxidized LDL may cause general inhibition of ester hydrolysis, while heavily oxidized LDL contains oxidized esters that are resistant to hydrolysis.

The presence of a large and relatively inert pool of cholesterol and oxysterols in OxLDL-loaded macrophages raises several important questions of relevance to atherosclerosis. It is not known whether such oxidized esters are present in foam cells, although some products have been detected in extracts of atherosclerotic lesions (44, 48). If oxidized LDL is the major source of foam cell cholesterol, as is often suggested, then oxidized esters probably constitute a large proportion of their cellular sterol pool. Sterols sequestered in lysosomes are likely to be resistant to removal by normal acceptors, promoting the development and persistence of the foam cell phenotype. Consistent with this, we have recently shown that oxidized cholesteryl esters are resistant to efflux to a range of extracellular acceptors (50). Furthermore, lysosomal accumulation of other particulate materials has been shown to increase macrophage secretion of several degradative enzymes that are active against components of extracellular matrix (51). It is possible that accumulation of oxidized esters may elicit similar potentially proatherogenic effects. These observations open many important avenues for further study.

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