Sterol Efflux Is Impaired from Macrophage Foam Cells Selectively Enriched with 7-Ketocholesterol*

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The aim of the present study was to investigate whether impairment of cholesterol efflux previously found from mouse peritoneal macrophages loaded with oxidized low density lipoprotein (OxLDL) could be ascribed to the presence of oxysterols in these cells. 7-Ketocholesterol (7KC), the major oxysterol present in Ox-LDL-loaded cells, was selectively incorporated into unoxidized LDL, which was subsequently acetylated to produce a high uptake form. Mouse macrophages incubated with 7KC-enriched acetylated LDL (7kAcLDL) did not reveal cytotoxicity judged by cell protein and trypan blue exclusion. A large proportion of cellular 7KC was esterified, indicating that it is a substrate for acyl-CoA:cholesterol acyltransferase. Cholesterol efflux from mouse macrophages loaded with 7kAcLDL, using apoA-I as a sterol acceptor, was impaired in cells containing >50 nmol of 7KC/mg of cell protein compared with cells loaded with oxysterol-free acetylated LDL. Thus impairment of cholesterol efflux could be reproduced in cells loaded with 7kAcLDL containing similar proportions of 7KC as OxLDL. 7KC itself was exported very poorly, even when the levels of 7KC in the cells were low. These results suggest that oxysterols present in foam cells in vitro can affect reverse sterol transport and may be potentially important in foam cell formation in vivo.

Oxidized forms of cholesterol, oxysterols, have been studied for many years for their relevance in the etiology of atherosclerosis. Several have been found to be present in human atherosclerotic plaque (1, 2) and can have potent effects on cellular sterol metabolism (3). For example, a number of oxysterols can affect the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase and acyl-CoA:cholesterol acyltransferase (ACAT),† which are critical in maintaining cholesterol homeostasis in cells (3, 4).

Recent in vivo data demonstrate that oxysterols, including 7-ketocholesterol (7KC, cholest-5-en-3β-ol-7-one), accumulate in human foam cell macrophages from atherosclerotic plaque (5). Work from our laboratory (6) has implied that oxysterols have the potential to affect reverse cholesterol transport, a mechanism by which peripheral cells export excess cholesterol to an extracellular acceptor. Mouse peritoneal macrophages loaded with copper-oxidized low density lipoprotein (OxLDL) were less able to export intracellular cholesterol to apolipoprotein A-I (apoA-I) compared with cells loaded with acetylated LDL (AcLDL). Even more strikingly, much less 7KC was exported from these cells, both in proportional and absolute terms. 7KC is the major oxysterol present in OxLDL-loaded cells, comprising approximately 30% of the total sterol content and approximately 60% of all oxysterols present (7). These studies raised the possibility that oxysterols such as 7KC may promote foam cell formation in vivo through inhibition of reverse sterol transport.

The objective of the present study was to test the hypothesis that the oxysterol present in OxLDL-loaded cells directly inhibits sterol efflux from these cells. Since OxLDL contains a complex mixture of oxidation products of both lipid and protein (7, 8), the approach used here was to generate foam cells selectively enriched with a single oxysterol. 7KC was selected as it is the major oxysterol in both OxLDL (7, 9, 10) and OxLDL-loaded cells (7, 10). Cellular oxysterol uptake was achieved by incorporation of 7KC into LDL, which was subsequently acetylated (7kAcLDL) to promote its uptake via the scavenger receptor. Macrophage foam cells produced by this method contained both cholesterol and cholesteryl esters, as well as defined quantities of free and esterified 7KC. There was no evidence of cytotoxicity associated with oxysterol uptake achieved via this route, in contrast to previous studies where direct addition of oxysterols in solution to cell cultures led to substantial cell death (3, 11).

Cholesterol efflux to apoA-I was impaired in macrophages loaded with 7kAcLDL containing >50 nmol of 7KC/mg of cell protein when compared with cells loaded with oxysterol-free AcLDL. As measured previously in OxLDL-loaded cells, only a very small amount of 7KC was exported from 7KC-enriched cells compared with cholesterol at all 7KC loadings. These results support the hypothesis that the oxysterol content of foam cells generated in vitro directly inhibits reverse sterol transport. The presence of oxysterol in vivo may therefore be important in the formation of foam cells by affecting the cells’ ability to export sterols to extracellular acceptors.

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1 The abbreviations used are: ACAT, acyl-CoA:cholesterol acyltransferase; 7KC, 7-ketocholesterol (cholest-5-en-3β-ol-7-one); LDL, low density lipoprotein; OxLDL, oxidized low density lipoprotein; apoA-I, apolipoprotein A-I; AcLDL, acetylated LDL; 7kAcLDL, 7-ketocholesterol-enriched LDL; 7KC, 7-ketocholesterol; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagles medium; 7kLDL, LDL enriched with 7KC; VLDL, very low density lipoprotein; HDL, high density lipoprotein; LPD/LPDS, lipoprotein-deficient plasma/lipoprotein-deficient serum; PBS, phosphate-buffered saline; REM, relative electrophoretic mobility; LCAT, lecithin:cholesterol acyltransferase; HPLC, high performance liquid chromatography.
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MATERIALS AND METHODS

Reagents

All reagents used were of analytical grade. Bovine serum albumin (BSA, essentially fatty acid-free, fraction V) was purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Dulbecco's modified Eagles medium (DMEM), RPMI 1640 medium, and L-glutamine were purchased from Life Technologies (Castle Hill, New South Wales, Australia). Neutrophils were purchased from Leucotronics (Castle Hill, New South Wales, Australia). Cholesterol was purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Prussian blue stain was purchased from Merck (Darmstadt, Germany). Heat-inactivated newborn calf serum was purchased from Biofluids (Rockville, MD). All solvents were HPLC grade (Mallinckrodt, Bio-Rad Laboratories, Purchase, NY). All reagents used were of analytical grade. Bovine serum albumin (BSA, essentially fatty acid-free, fraction V) was purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Heat-inactivated newborn calf serum was purchased from Biofluids (Rockville, MD). All solvents were HPLC grade (Mallinckrodt, Bio-Rad Laboratories, Purchase, NY).

Incorporation of 7kC into LDL

To incorporate 7kC into LDL, the method of Esterbauer et al. (13) for enriching LDL with α-tocopherol was adapted. Plasma, anti-coagulated with 2 mM EDTA, was freshly isolated from fasting healthy normolipidemic donors and mixed with a small volume of an ethanolic solution of 7kC (0.2–0.4 mg/ml) of a final 7C:cholesterol (molar ratio) equal to 1:100, and the mixture was incubated for 16 h at 37°C. Afterwards, the LDL was isolated by density gradient ultracentrifugation (see below) (14).

Lipoprotein Isolation

7kLDL and native LDL were isolated by density gradient ultracentrifugation of 7kC-enriched and normal plasma, respectively (14). Where indicated, very low density lipoprotein (VLDL, density: 1.021-1.063) and high density lipoprotein (HDL, density: 1.063-1.21) were isolated by density gradient ultracentrifugation of 7kC-enriched and normal plasma, respectively (14). Where indicated, very low density lipoprotein (VLDL, density: 1.021-1.063), high density lipoprotein (HDL, density: 1.063-1.21), and the lipoprotein-deficient plasma fraction (LPDP, density: >1.25) were also isolated (14). All salt solutions for gradient ultracentrifugation were deoxygenated and contained EDTA (1 g/l). LDL and 7kLDL were filtered sterilized (0.45 μm, Gelman Sciences), stored in the dark at 4°C under N2, and used within 7 days.

In experiments where unmodified 7kLDL was incubated with cells, it was first dialyzed to remove EDTA as described below.

LDL Modifications

Oxidation—Native LDL was dialyzed at 4°C in the dark against four changes of deoxygenated phosphate-buffered saline (PBS), containing chloramphenicol (0.1 gliters), and Cholesterol 100 resin (1 glitier) to remove EDTA. The LDL (1 mg of protein/ml of PBS) was oxidized using 20 μM cupric chloride at 37°C for 24 h. The oxidation was stopped by addition of EDTA (final concentration: 0.5 mM) and EDTA and copper removed by passing the OxLDL immediately through two Sephadex G-25 columns (PD-10; Pharmacia Biotech Inc., Uppsala, Sweden), pre-equilibrated with PBS.

Acetylation—LDL and 7kLDL were acetylated as described previously (6), dialized as described above for native LDL, and filter-sterilized (0.45 μm) before use. The 7C content of 7kLDL did not alter during the acetylation procedure (data not shown).

Agarose Gel Electrophoresis

Modification (acytlation or oxidation) of the LDL was confirmed by nondenaturing gel electrophoresis using a 1% Universal agarose gel (Ciba-Corning, Palo Alto, CA) in Tris-barbitone buffer (pH 8.6) at 90 V for 45 min. Bands were visualized using Fast Red 7B (Sigma-Aldrich).

The relative electrophoretic mobility (REM) was calculated as the distance travelled by the oxidized or acetylated LDL relative to reference native LDL. An REM of >3 was used as an indicator of satisfactory modification. 7kLDL had the same REM as native LDL.

Apolipoprotein A-I Isolation

The apoA-I was purified from human HDL by fast protein liquid chromatography using a Q-Sepharose Fast Flow column (Pharmacia Biotech Inc.) and lyophilized, as described in detail in Rye (15). The purity was confirmed by detection of a single band of molecular mass 28 kDa. The purified lyophilized apoA-I was stored at −20°C until use.

ApoA-I was reconstituted at approximately 10 mg/ml in 3 × guanidine hydrochloric acid solution and dialyzed overnight. The reconstituted apoA-I solution was filter-sterilized (0.45 μm), stored in the dark at 4°C, and used within 3 months.

Mouse Macrophage Isolation and Stimulation

Six-week-old Quackenbush-Swiss mice were asphyxiated by carbon dioxide inhalation. Resident macrophages were collected by peritoneal lavage using ice-cold DMEM containing 0.38% (w/v) sodium citrate, 10 units/ml penicillin G, 50 μg/ml streptomycin (50 μg/ml). The cells were then washed three times with prewarmed PBS to remove nonadherent cells. The cells were then incubated for 1 h with DMEM containing penicillin G and streptomycin, human lipoprotein-deficient serum (1.5 mg of protein/ml), pre-stimulated with ultracentrifuged (14), and 25 μg/ml AcLDL, OXLDL, or 7kAcLDL. The cells were then washed three times with prewarmed PBS and incubated overnight (14–16 h) in DMEM containing BSA (1 mg/ml), L-glutamine (2 mM), penicillin G (50 units/ml), and streptomycin (50 μg/ml).

Sterol Efflux

Cells loaded as described above were incubated for 24 h in efflux medium containing DMEM supplemented with penicillin G (50 units/ml), streptomycin (50 μg/ml), L-glutamine (2 mM), and either BSA only (1 mg/ml) or BSA plus apo-A-I (25 μg/ml). After the efflux period, medium samples were collected and centrifuged (16,000 g, 4°C, 10 min) to remove any detached cells. The adherent cells were lysed at 4°C for 15 min using 0.6 ml of ice-cold 0.2% sodium hydroxide before lipid and protein measurements as described below. All cell loading and efflux experiments were carried out in triplicate cultures.

Loading and Subcellular Fractionation of J774A.1 Macrophages

J774A.1 mouse monocyte-macrophage cells (ATCC; 67-TIB, batch F-10089) were routinely grown as adherent cultures in DMEM containing 10% (v/v) heat-inactivated newborn calf serum, penicillin G (50 units/ml), streptomycin (50 μg/ml) in 150-mm2 tissue culture flasks (17). Cells were used at near-confluence (2.5–3.0 × 10^5/150-mm2 flask) and preloaded with lipoproteins for 24 h by incubation in RPMI 1640 medium containing 10% (v/v) LPDS with 25 μg/ml AcLDL or 7kAcLDL. Nonloaded cells were incubated for 24 h in medium without LDL. The cells were washed, resuspended, ruptured by shear force, and fractionated on a 1–22% sucrose gradient with 45% Nycodenz cushion as described previously (17). 26 fractions were collected and samples extracted individually into methanol/hexane and analyzed by HPLC as described below. Samples were also assayed for protein and arylsulfatase B, a lysosomal marker enzyme. Identification of this and other organelles is described elsewhere (17). Lysosomal enzyme latency was assessed by separate assays of cell homogenates in the presence of 0.25 μM sucrose versus 0.01% (v/v) Triton X-100 and was >75% under all loading conditions.

Sterol Analysis

Lipids were extracted and prepared for HPLC analysis as described previously (18). Briefly, lipids were extracted from lipoproteins, efflux medium, cell lysates, or subcellular fractions in a total aqueous volume of 1 ml, containing 2% EDTA and 20 μM butylated hydroxytoluene, and using 2.5 ml of methanol followed by 10 ml of hexane. The hexane extracts were evaporated and redissolved in the appropriate mobile phase (described below). In experiments where all major lipoprotein classes isolated from plasma were assayed, a rapid lipid extraction procedure was employed as described in Brown et al. (7).

Cholesterol, cholesteryl esters, 7kC, and 7kE esters were analyzed by HPLC as described previously (7, 18) using a Supelco reverse-phase C18 column (0.46 × 25 cm, 2-cm Pellicular column, 5 μm particle size, Sigma-Aldrich) run at room temperature with a flow rate of 1 ml/min. Cholesterol and cholesteryl esters were determined at 210 nm after elution with acetonitrile/isopropanol (30/70, v/v), while 234 nm detection and a mobile phase of acetonitrile/isopropanol/water (44/54/2, v/v) were used for 7kC and 7kE esters (7, 18). The latter HPLC conditions can also be used to detect cholesterol linoleate hydroperoxide, a product of early lipid oxidation (18).

In cells containing significant quantities of 7kC, 7kE esters were generated by the cells (see results) that eluted close to cholesterol in acetonitrile/isopropanol/water (44/54/2, v/v). Under these conditions, the retention time of cholesterol was approximately 8 min, while the 7kC esters eluted between 10 and 17 min.
In one experiment where the incorporation of 7KC into the major lipoprotein fractions was measured, plasma total cholesterol, triglycerides, and HDL cholesterol (after dextran sulfate-MgCl₂ precipitation (19)) were also measured enzymatically using commercial kits (CHOD-PAP for cholesterol, GPO-PAP for triglycerides, Boehringer Mannheim, Castle Hill, New South Wales, Australia). The analyses were carried out by a lipid reference laboratory (Department of Biochemistry, Royal Prince Alfred Hospital, Camperdown, New South Wales, Australia). The cumulative cholesterol content of the individual lipoprotein subfractions measured by HPLC was 90 ± 1% (mean ± standard deviation, n = 4) of the total plasma cholesterol value measured by the reference laboratory.

In the results, the 7KC content of 7KC-enriched LDL (before and after acetylation) is expressed as a proportion of the total LDL sterol, which includes cholesterol, cholesteryl esters, and 7KC.

Cell Viability

Cell viability was measured by trypan blue exclusion staining. Counting was performed in triplicate cultures by two independent observers, whose results were averaged.

Protein Determination

The protein contents of apoA-I, cell lysates, subcellular fractions, and LDL samples were measured using the bicinchoninic acid method (Sigma-Aldrich) with BSA as standard. Standards were prepared in PBS for apoA-I and LDL, 0.2 M sodium hydroxide for whole cell lysates, and nanopure water for subcellular fractions. Absorbance at 562 nm was measured after incubation at 60 °C for 1 h.

Statistics

Where indicated, data were compared statistically using a two-sample unpaired t test (20). A p value of <0.05 (two-tailed) was considered significant. Data are presented as mean ± standard deviation unless stated otherwise.

RESULTS

Selective Incorporation of 7KC into Plasma Lipoproteins—Direct incubation of isolated LDL with 7KC as described by Addis et al. (21) resulted in incorporation of only a small proportion of the supplied 7KC (~3.5%, e.g. approximately 40 nmol of 7KC/mg of LDL after incubation with 1200 nmol/mg of LDL and subsequent re-isolation by ultracentrifugation). As an alternative approach, whole plasma samples obtained from two female and two male donors after an overnight fast were incubated with 7KC (400 nmol/ml of plasma) and VLDL, LDL, HDL and the LPDP fractions isolated. The cholesterol and 7KC contents of these fractions were measured by HPLC (Fig. 1). The relative distribution of 7KC between the major lipoproteins was closely related to the distribution of total cholesterol (as a proportion of total plasma cholesterol) in these fractions. Thus, for LDL, the proportion of added 7KC that was incorporated into this fraction was dependent upon the percentage of the donor’s total plasma cholesterol present as LDL, rather than the absolute LDL cholesterol concentration per se. To minimize donor to donor variation in 7KC incorporation into LDL, plasma from a single donor (M1) was used for all subsequent experiments.

In one experiment where plasma was incubated with 400 nmol of 7KC/ml, the 7KC ester content of the VLDL, LDL, HDL, and LPDP fraction were analyzed (Fig. 2). The presence of 7KC esters suggests that free 7KC is a substrate for plasma lecithin:cholesterol acyltransferase (LCAT). Only a small proportion of the 7KC (~5%) was esterified, the largest proportion being present in HDL. In LDL, only a very small proportion of the 7KC was esterified (less than 2% of total 7KC in this fraction).

The relationship between the amount of 7KC supplied to plasma and the corresponding enrichment of the LDL fraction was tested by incubating whole plasma with concentrations of 7KC up to 2 μmol/ml plasma. The 7KC content of the LDL after isolation and subsequent acetylation (7kAcLDL) is shown in Fig. 3. There was a dose-dependent relationship between the amount of 7KC added to plasma and the corresponding incorporation of 7KC into LDL, which was initially linear (up to 500 nmol/ml of plasma). Incorporation was nonlinear at higher concentrations of added 7KC, indicating that saturation of the particles was being reached. At 7KC concentrations of ≥2 μmol/ml of plasma, a faint precipitate, presumably 7KC, was
visible in the plasma after incubation with 7KC, again suggesting that the plasma solubility limit for 7KC was approached. This precipitate disappeared after filtering the LDL (0.45 μm) after ultracentrifugation. The maximum 7KC incorporation into LDL achieved under the conditions described was 30% of total LDL sterol (or 659 nmol/mg of LDL protein). There were small but noticeable concentrations of 7KC (up to 27% of total sterol or 659 nmol/mg of LDL protein) and then incubated overnight in DMEM + BSA (1 mg/ml). The cells were lysed in 0.2 M sodium hydroxide and protein measured as described under “Materials and Methods.” Results of two independent experiments (open and filled circles) are presented. Data are means ± S.D. of triplicate determinations. Total 7KC in culture refers to free + esterified 7KC content of the cells.

To study whether the incorporation of 7KC into LDL changed the composition of the particle in terms of free cholesterol content, plasma was incubated with 7KC up to 2 μmol/ml and the cholesterol to cholesteryl ester ratio of the LDL after acetylation measured. This ratio (ranges 0.29–0.30 and 0.30–0.33 for two independent experiments) was not significantly affected by the addition of 7KC, even when 7KC comprised as much as 27% of the total LDL sterol (or 659 ± 17 nmol/mg of LDL protein).

Macrophage Uptake and Metabolism of 7kAcLDL—Mouse peritoneal macrophages incubated with 7kAcLDL for 24 h were microscopically (phase contrast × 400) of similar appearance to cells incubated with AcLDL (6), containing peripherally distributed lipid inclusions. There were no visible signs of cytotoxicity. The cell protein content of macrophage cultures after incubation with 7kAcLDL (25 μg/ml) containing increasing concentrations of 7KC (up to 27% of total LDL sterol or 659 ± 17 nmol/mg of LDL protein) are presented in Fig. 4. The recovery of cell protein was unaffected by incubation with 7kAcLDL. Similarly, trypan blue exclusion staining of mouse macrophages after incubation with 7kAcLDL (7KC comprised 26% of LDL sterol or 720 ± 25 nmol of 7KC/mg of LDL protein) showed that 80–90% of the cells were viable. This is similar to the viability routinely found for AcLDL and OxLDL-loaded cells in our laboratory (6).

The sterol compositions of the macrophages loaded with AcLDL or 7kAcLDL (both prepared from the same plasma) are compared in Table I. In the 7kAcLDL substrate supplied to the cells, the majority of the 7KC was unesterified (7KC ester content 1.0 ± 0.02% of total 7KC). However after loading the macrophages with 7kAcLDL, the majority of the 7KC present in the cells was esterified (90.8 ± 0.4%), indicating that 7KC is a substrate for ACAT. The total cholesteryl content (free + esterified) was similar for both AcLDL and 7kAcLDL-loaded cells, although the proportion of free cholesterol was higher in the 7kAcLDL-loaded cells. However, the proportion of the total sterol pool (i.e. cholesterol + 7KC) that was esterified was not significantly different between the two cases (70.9 ± 4.0% and 67.1 ± 1.8% for AcLDL and 7kAcLDL, respectively). Interestingly, in the 7kAcLDL-loaded macrophages, more than 90% of the 7KC pool was esterified compared with <60% for the cholesterol pool, indicating that 7KC may be a preferred substrate for ACAT. The ester profiles of the AcLDL and 7kAcLDL-loaded cells were similar with lineolate and deplete the major fatty acyl chains esterified to both cholesterol and 7KC.

Sterol Efflux to ApoA-I—We have shown previously that sterol efflux from OxLDL-loaded macrophages to apoA-I is impaired compared with AcLDL-loaded cells. To test whether this inhibition is also seen in macrophage foam cells in which 7KC is the only oxidized lipid present, efflux was measured from cells loaded with 7kAcLDL. The use of apoA-I as a sterol acceptor was based on observations of lipid-poor apoA-I in interstitial fluid and evidence that such particles function as an initial cholesterol acceptor in reverse cholesterol transport (22, 23).

Mouse macrophages were loaded with 7kAcLDL containing varying amounts of 7KC (up to 27% of total sterol or 659 ± 17 nmol/mg of LDL protein). There were small but noticeable differences in total sterol loading among the different 7kAcLDL cultures (Fig. 5A). This is consistent with our previous findings that loading cells with AcLDL can lead to substantial variations in the mass of sterol accumulation (6). The mass of cholesterol in the medium (Fig. 5A) decreased only when large quantities of 7KC were present in the cells (10 nmol/culture). This drop in cholesterol mass in the medium corresponded with an increase in intracellular cholesterol and cholesteryl esters. 7KC enrichment of macrophages caused a concentration-dependent increase in the mass of 7KC and 7KC esters in the cells (Fig. 5B). The absolute amount of 7KC in the medium was small, i.e. <1 nmol/culture. As expected (6, 24), no esters (cho-
lesterol or 7KC) were detected in the medium under any conditions.

We have previously found that despite variations in total sterol loading, the proportional efflux from AcDL- and 7kAcDL-loaded cells to apo A-I is remarkably consistent (6). Proportional efflux is calculated as: cholesterol (or 7KC) in medium (nmol) / cells/culture) were incubated with 25 μg/ml AcDL or 7kAcDL (7KC content is 30% of total LDL sterol or 1043 ± 49 nmol/ml of protein) for 24 h. After washing and overnight incubation with medium containing BSA (1 mg/ml), media and cells were analyzed for sterol content by HPLC. Sterols are expressed as nmol/mg cell protein (bold figures) and are means ± standard deviation of triplicate cultures. The values in parentheses are the proportional cholesterol efflux (cholesterol or 7KC) as percent of the total ester (either cholesterol or 7KC) pool.

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circles
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made use of the same cell line, this was considered appropriate. The previous studies (25, 26) use of primary murine peritoneal macrophages prohibitive, the large number of cells required for this procedure made the efflux of these sterols to apoA-I seen in some conditions. Since cells loaded with 7kAcLDL and could explain the impaired sary to determine if lysosomal sterol accumulation occurred in macrophages (6) disagree with this hypothesis, it was neces-
some of 7kAcLDL-loaded macrophages (Table III). This, to-
the distribution of 7KC was identical to that of cholesterol. Table III. As expected, free cholesterol partitioned predomi-
nantly into membrane-rich regions of the cell with the greatest propor-
ion in the plasma membrane. In 7kAcLDL-loaded cells, the proportion of 7KC esterified (80% ± 3%) was greater than cholesterol. This is consistent with our findings in primary macrophages (Table I) in which the propor-
tion of cellular 7KC esterified also exceeded that of cholesterol.

The subcellular distributions of free sterols are presented in Table III. As expected, free cholesterol partitioned predomin-
antly into membrane-rich regions of the cell with the greatest propor-
ton in the plasma membrane. In 7kAcLDL-loaded cells the distribution of 7KC was identical to that of cholesterol. Both cholesteryl and 7KC esters were present only in the lowest density ("cytosol") regions of the gradient (data not shown), indicating that both AcLDL and 7kAcLDL were completely degraded lysosomally and re-esterified in the cytoplasm, presumably via ACAT. Most importantly, there was no evidence for retention of either cholesterol or 7KC in the lysosomes of 7kAcLDL-loaded macrophages (Table III). This, together with the substantial degrees of extralysosomal esterifi-
cation of both sterols, suggest that they are freely able to exit from lysosomes.

Incubation of Cells with 7kLDL—The use of 7kAcLDL was adopted as a practical method for generating cholesterol-loaded
The esterification of a large proportion of the cellular 7KC, selective transfer of 7KC from LDL to the plasma membrane, and LDL sterol as 7KC or 7KC as a percentage of the total sterol pool in cells and media are expressed as nmol/mg of cell protein and ± S.D. of triplicate cultures unless stated otherwise.

**TABLE II**

| Sterol Efflux from Macrophage Foam Cells |
|-----------------------------------------|
| DMEM + BSA                               |
| AcLDL | OxLDL | 7kAcLDL | AcLDL | OxLDL | 7kAcLDL |
| **Cells**                               |
| Cholesterol 130 ± 13                     | 120 ± 17 | 186 ± 20 | 85 ± 13 | 115 ± 15 | 155 ± 17 |
| Cholesterol esters 227 ± 35              | 9 ± 1    | 213 ± 19 | 135 ± 20 | 9 ± 3 3* | 198 ± 23 |
| 7KC 37 ± 5                               | 12 ± 1   | 106 ± 14 | 17 ± 2  | 41 ± 3  | 22 ± 3  |
| 7KC esters                               | 3 ± 1    | 7 ± 2    | 172 ± 15 | 22.7 ± 7.6 | 117 ± 25 |
| **Mean of duplicate ± range.**           |

**DISCUSSION**

Oxysterols have been tested in many in vitro studies for their effects on cellular sterol metabolism. The commonly used route of delivery has been direct addition of oxysterols to the cell culture medium (3, 11). As oxysterols are carried in lipoproteins in vivo (27), a more physiological delivery system was developed by selectively incorporating the oxysterol of interest into LDL.

After incubation of plasma with 7KC, traces of 7KC esters were found in some of the lipoprotein fractions. 7KC and other oxysterols have been shown to be available for esterification by human plasma LCAT in vitro using nonphysiologically high enzyme activities (28). This study presents further evidence that the formation of 7KC esters in plasma is possible in principle when physiological levels of the enzymes are present. However the 7KC ester concentrations are likely to be low in circulating lipoproteins considering the low levels of oxysterols present in plasma (27, 29).

A high-uptake form of the 7KC-enriched LDL could be produced by acetylation, which made it possible to load macrophages simultaneously with cholesterol and cholesteryl esters and controlled amounts of oxysterol. 7KC was supplied to the cells in the free form for practical purposes, as 7KC esters are not available commercially and in our experience are more difficult to incorporate into LDL.

An often cited effect of oxysterols such as 7KC is their apparent cytotoxicity to cells in vitro (3, 11) when administered to cells in solution. After loading mouse macrophages with 7kAcLDL, no apparent cytotoxicity was measured using several criteria, indicating that the route of oxysterol delivery may be of importance in its influence on the target cell. A study by Clare et al. (11) showed that 7KC provided to human monocyte-derived macrophages as a solution was cytotoxic, whereas co-incubation with cholesterol reduced the toxicity of 7KC. The exposure of cells to single oxysterols in the absence of cholesterol may be an unlikely scenario in vivo, considering that oxysterols are transported in cholesterol-rich lipoproteins such as LDL, VLDL, and chylomicrons (27).

7KC was esterified by intracellular ACAT, and moreover the data suggested that 7KC may be a preferred substrate over cholesterol. This is in disagreement with a study of 7KC esterification in J 774 cells (25). After loading their cells with OxLDL (25), very little 7KC esters were detected, and it was concluded that 7KC is not a good substrate for ACAT. However, in OxLDL and OxLDL-loaded mouse peritoneal and J 774 cells, there may be a limit in fatty acids available for esterification (6), as a large
Subcellular distribution of unesterified cholesterol and 7KC in J 774A.1 macrophages

J 774A.1 macrophages were incubated for 24 h in RPMI 1640 medium containing 10% (v/v) lipoprotein deficient serum and AcLDL or 7kAcLDL (25 µg of protein/ml) as described under "Materials and Methods." Data are expressed as a percentage of the total cellular sterol content in each of the major cell fractions and are means ± S.D. of five (no added lipoprotein), four (AcLDL), or three (7kAcLDL) independent experiments. FC = free (unesterified) cholesterol; 7KC = free (unesterified) 7KC.

| Sterol supplied | Cytosol | Plasma membrane | Endosomal | Lysosomal | Cushion |
|-----------------|---------|-----------------|-----------|-----------|---------|
| None            | FC      | 14.6 ± 14.8     | 33.3 ± 14.0| 24.8 ± 10.0| 18.2 ± 10.3| 8.8 ± 10.0|
| AcLDL           | FC      | 9.0 ± 6.2       | 46.2 ± 7.4| 25.1 ± 1.7 | 20.1 ± 2.4 | 1.7 ± 1.6 |
| 7kAcLDL         | FC      | 12.8 ± 4.8      | 44.7 ± 1.8| 25.6 ± 1.8 | 13.9 ± 2.2 | 1.2 ± 1.1 |
| 7KC             | FC      | 11.9 ± 1.6      | 43.8 ± 7.2| 27.1 ± 0.6 | 16.8 ± 5.7 | 0.4 ± 0.4 |

![Image](97x416 to 260x569)

**Fig. 8. Sterol content of cells incubated with 7kLDL.** Macrophages were incubated for 24 h with LDL containing 7KC (27% of total LDL sterol or 757 ± 31 nmol/mg of LDL protein). 7kLDL concentrations in the incubation medium were: solid bars = 12.5 µg/mL; hatched bars = 25 µg/mL. Values are means ± S.D. of triplicate cultures. Chol = cholesterol; CE = cholesteryl ester; 7KC = 7-ketocholesterol; 7KEs = 7KC esters.

proportion of those in OxLDL are modified during copper-catalyzed oxidation (7). It is not known if these oxidized fatty acids are recognized by ACAT. We also have evidence that an additional supply of free fatty acids such as oleate and linoleate to the culture medium of OxLDL-loaded cells increases the 7KC ester content of the cells.

Maor et al. (25, 26) further suggested that the presence of 7KC in macrophages leads to lysosomal trapping of both cholesterol and 7KC. In contrast, subcellular fractionation of J 774 cells loaded with 7kAcLDL demonstrated that the presence of 7KC did not lead to lysosomal sequestration of either free sterol, as we have also shown previously for OxLDL-loaded cells (6). The discrepancy between different results must arise from the techniques used, such as methods of oxysterol delivery, use of radioabeled tracers versus mass determination of lipids, and methods for sterol separation (TLC versus HPLC). Of these, the method for oxysterol delivery may be particularly important, as we have noted that the supply of 7KC in free solution has cytotoxic effects on J 774A.1 macrophages which were not seen using 7kAcLDL.

Cholesterol efflux from cells loaded with 7kAcLDL, containing 7KC concentrations similar to OxLDL, was inhibited, indicating that the impairment of cholesterol efflux from OxLDL-loaded cells can be partially reproduced when 7KC is the only oxysterol present. A report (30), studying the effects of oxysterols on cholesterol efflux, also found that 7KC delivered to cells in solution inhibited cholesterol efflux, although the effect was very small. However, in this case there was no measure of the amount of 7KC present in the cells, making comparison with the present study difficult.

There are currently two hypotheses proposed to explain the regulation of cholesterol efflux. One suggests that the rate-limiting step is the desorption of cholesterol from the membrane into the aqueous phase, a receptor-independent process that is influenced by the distribution of cholesterol in the membrane (31). The other suggests that the transfer of cholesterol from intracellular compartments to the plasma membrane is the rate-limiting step, a process that is regulated by the binding of HDL to an as yet unidentified receptor (32). It is possible that the effects of 7KC on cholesterol efflux are dependent on alterations in properties of the plasma membrane. In monocyte studies, 7KC was shown to display less of a condensing effect than cholesterol and also reduced glucose permeability (33). 7KC may change the plasma membrane composition in such a way as to disturb the desorption of cholesterol from the membrane and its interaction with the acceptor particle. The role of the acceptor itself could also be of importance. Free apoA-I has been shown to interact with the plasma membrane and to associate with membrane phospholipids and cholesterol to form an HDL-like particle in the medium (24). It is not known whether this process could be influenced by the presence of oxysterols in the membrane. However, we presented data to eliminate the possibility of an extracellular effect of 7KC on cholesterol efflux to apoA-I.

As found previously with OxLDL-loaded cells, efflux of 7KC was only minimal compared to cholesterol efflux, even when only low levels of 7KC were present in the cells. Several studies suggest that 7KC exchanges more rapidly than cholesterol between lipid containing vesicles or membranes and acceptor particles (33, 34). If the inhibition of 7KC efflux is due to impaired passive desorption from the membrane, this indicates differences between the behavior of cell membranes and vesicle systems. Another possibility is that apoA-I is selective for cholesterol but not for 7KC.

7KC also selectively accumulated in cells after incubation with nonacetylated LDL containing 7KC under conditions where no significant change in the cholesterol and cholesteryl ester content of the cells was observed. 7KC could have entered the cells via exchange, particularly since it is more rapidly exchanged than cholesterol in membrane systems (34) and since there is a large concentration gradient between the outside and the inside of the cell. Tabas (35) has postulated that the cell surface pathway of delivery and subsequent esterification by ACAT may be an efficient way in which foam cell formation can be induced. It may provide a pathway for oxysterols such as 7KC to enter and accumulate in cells without the need for uptake via the scavenger receptor pathway.

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2 L. Kritharides and W. J essup, unpublished observations.
3 W. J essup, unpublished observation.
In conclusion, a novel method for incorporating an oxysterol into LDL has been presented. Oxysterol-enriched LDL was used to increase the 7KC content of mouse macrophages either with or without increases in intracellular cholesterol and cholesterol ester content. Loading of cells with 7KC containing LDL that was acetylated showed that cholesterol efflux from these cells was impaired. Efflux of 7KC from these cells was minimal compared with cholesterol efflux. An impairment of these cells was impaired. Efflux of 7KC from these cells provides a possible mechanism for the accumulation of 7KC seen in human foam cells present in atherosclerotic plaque.

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