Oxysterol efflux from macrophage foam cells: the essential role of acceptor phospholipid

Ingrid C. Gelissen,* Kerry-Ann Rye,† Andrew J. Brown,* Roger T. Dean,* and Wendy Jessup[1,*]

Cell Biology Group,* Heart Research Institute, 145 Missenden Road, Camperdown, N.S.W. 2050, Australia, and Hanson Lipid Laboratory,† Adelaide, Australia

Abstract **Oxidized forms of cholesterol (oxysterols) are present in atherosclerotic lesions and may play an active role in lesion development. For example, 7-ketocholesterol (7KC) inhibits cholesterol efflux from macrophage foam cells induced by apolipoprotein A-I (apoA-I). Such oxysterols may promote foam cell formation in atherosclerotic lesions by preventing effective clearance of excess cholesterol. ApoA-I also induces phospholipid (PL) export from foam cells and it has been suggested that cholesterol efflux is dependent upon PL association with the apolipoprotein. In the current study, the effect of oxysterol enrichment of foam cells on phospholipid efflux was measured. Export of cellular PL to apoA-I from 7KC-enriched foam cells was inhibited to the same extent as cholesterol, indicating that the reduced cholesterol export may be a consequence of a decline in the capacity of the foam cells to generate PL/apoA-I particles capable of accepting cellular cholesterol. Incubation of foam cells with pre-formed PL/apoA-I discs increased cholesterol export from 7KC-enriched cells to levels seen in 7KC-free cells. Foam cells produced by uptake of oxidized LDL, which contain similar amounts of 7KC plus other oxidation products, expressed a more profound inhibition of PL export to apoA-I. Cholesterol efflux from these cells improved only partially by provision of PL-containing acceptors. Efflux of 7KC from both foam cell types occurred to PL/apoA-I discs but was only minimal to lipid-free apoA-I, indicating that export of this oxysterol is more dependent than cholesterol upon the presence of extracellular phospholipid.**—Gelissen, I. C., K-A. Rye, A. J. Brown, R. T. Dean, and W. Jessup. **Oxysterol efflux from macrophage foam cells: the essential role of acceptor phospholipid.** ***J. Lipid Res.*** **1999.** 40: **1636–1646.**

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Oxidized forms of cholesterol (oxysterols) have diverse and potent biological activities which may be relevant in the etiology of atherosclerosis. Analyses of human atherosclerotic plaque have indicated the presence of a number of oxysterols, of which 7-ketocholesterol (7KC) is the most abundant oxysterol of non-enzymic origin (1, 2). Interestingly, the 7KC:cholesterol ratio within plaque macrophages is substantially higher than in whole plaque homogenates or in circulating monocytes (3, 4). The contributions of such oxysterols to foam cell development and cholesterol metabolism are not fully understood. One interesting and potentially relevant property of 7KC is its capacity to inhibit reverse cholesterol transport, the process whereby peripheral cells export excess cholesterol to extracellular acceptor particles for removal and ultimate excretion via the liver. Macrophage foam cells loaded with copper-oxidized LDL (OxLDL), in which oxysterols are a significant proportion of total sterol content (5), export cholesterol less readily to lipid-free apolipoproteinA-I (apoA-I) than cells comparably loaded with acetylated LDL (AcLDL), in which cholesterol is the only sterol accumulated (6). Specific enrichment of macrophage foam cells with 7KC, by incubation with 7KC-enriched acetylated LDL (7kAcLDL), led to a similar inhibition of cholesterol efflux (7). Thus oxysterols which are present in foam cells may contribute to generation and maintenance of the foam cell phenotype by interfering with their capacity to dispose of excess cholesterol. In these studies, the export of 7KC from both OxLDL and 7kAcLDL-loaded cells to extracellular apoA-I was extremely low compared with cholesterol, both in relative and absolute terms (6, 7). The reason for this selective retention is not known, but may explain the relative enrichment of such oxysterols in foam cells in vivo.

When cells are incubated with lipid-free apolipoproteins, desorption of cellular phospholipids (PL) accompanies cholesterol export (8, 9). It has been suggested that this is a sequential process, in which the apolipoprotein first associates with PL derived from the plasma membrane to form a discoidal 'precursor' particle, which sub-

Abbreviations: 7β-OH, 7β-hydroxy cholesterol; 7kAcLDL, 7KC-enriched AcLDL; 7KC, 7-ketocholesterol; AcLDL, acetylated LDL; apoA-I, apolipoprotein A-I; BSA, bovine serum albumin; C:PL ratio, cholesterol to phospholipid ratio; DMEM, Dulbecco's modified Eagle's medium; HDL, high density lipoprotein; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; OxLDL, copper oxidized LDL; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PC/A-I discs, phosphatidylcholine/apoA-I discs; PL, phospholipid; SUV, small unilamellar vesicles.

[1] To whom correspondence should be addressed.

sequently serves as an acceptor for cell-derived cholesterol (9, 10). An alternative mechanism proposes that PL and cholesterol are simultaneously exported from cells to apoA-I by apolipoprotein-mediated solubilization of microdomains of the plasma membrane (11, 12) Whether either or both of these mechanisms apply in apoA-I-mediated export from macrophage foam cells, the capacity of 7KC to inhibit cholesterol export might be due to oxysterol-mediated inhibition of PL export from these cells. This was investigated in the present study.

PL efflux to lipid-free apoA-I was directly measured from macrophage foam cells loaded with AcLDL, 7kAcLDL, or OxLDL. Export of cellular PL from 7kAcLDL-loaded cells was inhibited to the same extent as cholesterol, relative to oxysterol-free (AcLDL-loaded) foam cells. Thus the extracellular cholesterol:PL ratio generated in the presence of apoA-I from these oxysterol-containing cells was unaltered, while the total mass of both lipids exported was reduced. The inhibition of cholesterol export from OxLDL-loaded cells was more complex. The mass of PL exported from these cells was even less than from cells containing comparable amounts of 7KC. In addition, the ratio of cholesterol:PL exported to apoA-I was lower, and cholesterol efflux was still less than the levels seen from AcLDL-loaded cells when PL-containing acceptor particles were substituted for lipid-free apoA-I.

We have previously shown that 7KC is extremely resistant to being exported to lipid-free apoA-I, when present in both OxLDL- and 7kAcLDL-loaded foam cells (6, 7). In the present study, the provision of PL-containing acceptors very significantly stimulated 7KC export from both foam cell models. These data indicate the importance of PL in stimulation of sterol export from foam cells and highlight the need to understand the types of acceptors that are available to foam cells in atherosclerotic lesions. The very existence of the foam cell phenotype indicates that sterol efflux from these cells is sub-optimal, but it is not yet known whether this is a reflection of limitations in the amount or quality of local acceptors, or a consequence of the composition of the foam cells resulting in reduced efflux of both PL and sterols.

EXPERIMENTAL PROCEDURES

Reagents

All reagents used were of analytical grade. Bovine serum albumin (BSA; essentially fatty acid-free, fraction V) and phosphatidylcholine (PC; type III-E, purified from egg yolk, approx. 99% pure) were purchased from Sigma-Aldrich (Castle Hill, N.S.W., Australia). [^{14}C]cholesterol was purchased from Amersham Life Sciences (Castle Hill, Australia). All solvents used were HPLC grade (Mallinckrodt, Biolab Scientific, Clayton, South Australia).

Lipoprotein isolation and modification

Low density lipoprotein (LDL; d 1.02–1.055 g/ml), high density lipoprotein (HDL; d 1.063–1.21 g/ml) and lipoprotein-deficient serum (LPDS; d > 1.25 g/ml) were prepared from fasting plasma by density gradient ultracentrifugation as previously described (13, 14). All salt solutions for gradient ultracentrifugation were deoxygenated and contained EDTA–Na_2 (1 g/l). LDL and HDL were filter sterilized (0.45 μm), stored at 4°C, and used within 7 days. LPDS was stored at −20°C until required. Before addition to cells, HDL was dialyzed at 4°C against phosphate-buffered saline (PBS) containing chloramphenicol (0.1 g/l) and pre-washed Chelex (1.0 g/l) to prevent artifactual oxidation (15) to remove EDTA–Na_2.

Acetylated LDL (AcLDL) was prepared as described (6). Oxidized LDL (OxLDL) was prepared by incubating LDL (1 mg protein/ml) with cupric chloride (20 μm) in PBS for 24 h at 37°C (7). Before addition of the cupric chloride, the LDL had been dialyzed against PBS containing chloramphenicol and pre-washed Chelex resin in order to remove EDTA–Na_2. AcLDL and OxLDL were dialyzed extensively at 4°C against PBS containing chloramphenicol and pre-washed Chelex resin, and filter-sterilized (0.45 μm) before addition to cells. Acetylation and oxidation of the LDL was confirmed by non-denaturing agarose gel electrophoresis (6).

7KC-enriched acetylated LDL (7kAcLDL) was prepared as described previously (7). Briefly, fasting plasma was incubated for 6 h at 37°C with an ethanolic solution of 7KC (2.4 mm final plasma 7KC concentration). After incubation, the LDL was isolated by ultracentrifugation and acetylated as described above. The 7KC concentration used was adjusted to enrich LDL with a 7KC content comparable to that of OxLDL (i.e., approx. 30% of total LDL sterol) (5). The average 7KC content achieved in the LDLs used for the current studies was 27.8 ± 0.5%.

ApoA-I isolation

ApoA-I was purified from human HDL by fast protein liquid chromatography using a Q Sepharose Fast Flow column (Pharmacia Biotechnology, Uppsala, Sweden) and lyophilized, as described in (16). The purity of the apoA-I was confirmed on a 20% SDS-PAGE gel by detection of a single band of molecular mass 28 kDa. The lyophilized stock was stored at −20°C until reconstitution.

ApoA-I was reconstituted at approximately 8–10 mg/ml using 3 m guanidine-HCl/10 mm Tris/0.01% (w/v) EDTA–Na_2, pH 8.2, and dialyzed over 5 days against 5 × 1 L exchanges of 0.15 m NaCl/10 mm Tris-HCl/0.005% (w/v) EDTA–Na_2/0.006% (w/v) NaN_3 (pH 7.4). The protein was filter sterilized (0.45 μm) and stored in the dark at 4°C. ApoA-I was used within 3 months of reconstitution.

Preparation of PC/A-I discs and small unilamellar vesicles (SUV)

Phosphatidylcholine/apoA-I (PC/A-I) discs were prepared according to the cholate dialysis method (17). The molar ratio of PC to apoA-I in the discs ranged from 62:1 to 95:1. Small unilamellar vesicles (SUV) which contained only PC were prepared as described by Jonas (18). Briefly, PC in hexane (100 mg/ml) was dried under a stream of N_2 and suspended in 0.15 m NaCl/10 mm Tris-HCl/0.006% (w/v) NaN_3 (pH 7.4) by vortexing. The solution was sonicated using a titanium probe (Soniprep 150, MSE) for 3-min periods followed by 2-min intervals, for a total of 1–1.5 h until the solution was optically clear. The PC solution was kept on ice during the sonication procedure. After sonication, the SUV were centrifuged in an Eppendorf centrifuge for 1 h (16,000 g; 8°C) to remove any particulate material (18) and used within 24 h. The PC concentration of the SUV was determined after filter sterilization (0.45 μm) as described below.

Sterol and phospholipid analysis

Samples were extracted and prepared for HPLC analysis as previously described (5, 6). Briefly, lipids were extracted from LDLs, cell lysates, or efflux media using methanol/hexane. The sterols, which partitioned into the hexane phase, were dried by evaporation and redissolved in HPLC mobile phase. OxLDL-loaded cell lysates were analyzed before and after saponification

as described (5). Cholesterol, cholesteryl esters, 7KC, and 7KC-esters were analyzed by HPLC as previously described (5, 19) using a Supelco reverse-phase C18 column (5 μm particle size). Cholesterol and cholesteryl esters were detected at 210 nm after elution with acetonitrile–isopropanol 30:70 (v/v). 7KC and 7KC-esters were detected at 234 nm after elution with acetonitrile–isopropanol–water 44:54:2 (v/v). Oxidized esters (i.e., sterols esterified to oxidized fatty acyl groups) in OxLDL-loaded cells were estimated as the total sterol (cholesterol or 7KC measured after saponification) less the HPLC-detectable free and esterified sterol (measured in unsaponified samples).

7β-Hydroxy cholesterol and 7KC in cells and media of OxLDL-loaded cells were also measured by normal-phase HPLC as described in detail in Brown et al. (1). Lipids from cells and media were extracted using ether–hexane, dried, and redissolved in heptane–isopropanol 95:5 (v/v). 7β-Hydroxy cholesterol and 7KC were separated on two silica columns in series using a mobile phase of hexane–isopropanol–acetonitrile 95.8:3.9:0.3 (v/v/v). Free and total sterols were measured before and after saponification and the total esterified sterol content was measured as the difference between these two assays.

PL in cellular efflux media was estimated using the method of Bartlett (modified in ref. 20) measuring total phosphorus, after lipid extraction of the media according to Bligh and Dyer (21). Briefly, total lipids were extracted using chloroform–methanol 1:1. The chloroform layer was washed with fresh phosphorus-free upper phase to remove any residual inorganic phosphorus or precipitated proteins that could cause background absorbance in the phosphorus assay. The chloroform layer was dried and total phosphorus was measured according to Sokoloff and Rothblat (20). Samples of DMEM containing BSA (1 mg/ml) were taken through the lipid extraction and phosphorus procedure as blank controls. [^{14}C]cholesterol was added prior to the lipid extraction to correct for losses during extraction.

PL in HDL, PC/A-I discs, and SUV were measured using a commercially available kit (Boehringer-Mannheim).

Mouse macrophage isolation, cholesterol loading, and efflux

Mouse peritoneal macrophages from 6-week-old Quackenbush-Swiss mice were isolated and loaded with cholesterol and cholesteryl esters as previously described in detail (6, 7). Resident macrophages were collected by peritoneal lavage and plated at 5–6 × 10^6 cells per 35-mm diameter culture dish. After adhering and washing, the cells were incubated for 24 h in Dulbecco's modified Eagle's medium (DMEM) containing LPDS (1.5 mg protein/ml), and 25 μg/ml AcLDL, OxLDL, or 7kAcLDL. After lipid loading, the cells were washed and incubated overnight (16–18 h) in DMEM containing BSA (1 mg/ml) to allow sufficient time for normal lysosomal degradation of the LDL (6). None of these loading conditions adversely affected cell viability, measured by lactate dehydrogenase release and by trypan blue exclusion (data not shown, 7).

The macrophage foam cells were further incubated in efflux media containing either BSA only (1 mg/ml) or BSA plus apoA-I, PC/A-I discs, SUV, or HDL at the indicated concentrations. After the efflux period, media and cells were collected separately and analyzed for sterols and cell protein (7). In experiments where whole HDL was used as acceptor, the free cholesterol content of the HDL was analyzed separately and subtracted from the total cholesterol recovered in the media. All cell loading and efflux experiments were carried out in triplicate cultures.

Other analytical methods

The protein contents of LDLs, cell lysates, apoA-I, and PC/A-I discs were measured using the bicinchoninic acid method (Sigma-Aldrich) with BSA as standard. Standards were prepared in PBS for LDLs, apoA-I, and PC/A-I discs or 0.2 m sodium hydroxide for cell lysates. Absorbance at 562 nm was measured after incubation at 60°C for 1 h.

Statistics and presentation of data

Data were compared using unpaired *t*-tests (25). Sterol efflux in most cases is presented as proportional (%) efflux for each respective sterol, calculated as: 100 × (free sterol in medium)/(free sterol in medium + free sterol in cells + sterol esters in cells). We have previously shown that proportional efflux is very reproducible between experiments and independent of total sterol loading over a wide range of loadings characteristic for each type of model foam cell (6, 7). Where appropriate, mass efflux data are also presented.

RESULTS

PL efflux from foam cells after incubation with lipid-free apoA-I

Table 1 shows the lipid content of the three types of macrophage foam cells used in this study. Loading with AcLDL produced a 4- to 5-fold increase in cellular cholesterol over non-loaded cells, of which approximately half was esterified. 7kAcLDL loading produced free and esterified cholesterol levels similar to AcLDL but also to free and esterified 7KC. In the present study the total 7KC content was ~25% of the total sterol and 85% was esterified. OxLDL-loaded cells contained free and esterified cholesterol plus 7KC (Table 1) and other oxysterols (not shown); 30% and 20% of total cell sterols, respectively (5). About 70% of the 7KC in OxLDL-loaded cells was esterified. The majority of esterified sterols in OxLDL-loaded cells contained oxidized fatty acyl groups (oxidized esters, Table 1).

We have previously shown that mouse peritoneal macrophages loaded with OxLDL are less able to export intracellular cholesterol to lipid-free apoA-I compared to cells

TABLE 1. Lipid composition of model macrophage foam cells

Loading Condition	Non-loaded	AcLDL	OxLDL	7kAcLDL
Cholesterol				
Total (nmol/mg)	57.0 ± 3.9	309 ± 52	220 ± 45	344 ± 61
% Esterified				
Normal	2.5 ± 3.4	42 ± 13	5.0 ± 1.9	53 ± 6
Oxidized	nd	nd	50 ± 15	nd
7-Ketocholesterol				
Total (nmol/mg)	nd	nd	107 ± 46	111 ± 16
% Esterified				
Normal	nd	nd	15 ± 1	85 ± 8
Oxidized	nd	nd	56 ± 15	nd
Phospholipids				
Total (nmol/mg)	91 ± 25	164 ± 26	149 ± 15	170 ± 28

Mouse macrophages were loaded for 24 h with 25 μg/ml of either AcLDL, OxLDL, 7kAcLDL, or no added lipoprotein (non-loaded). After loading they were incubated overnight in medium containing BSA (1mg/ml), then extracted and analyzed for cellular sterols and phospholipid. Total phospholipid, cholesterol and 7-ketocholesterol are expressed as nmol/mg cell protein; esterified sterols are expressed a percentage of the total cellular pool of the respective sterol. Data are means ± SD of four independent experiments for each condition except for non-loaded phospholipids, which are the mean and range of two experiments. nd, not detected.

loaded with AcLDL. Even more strikingly, only a very small proportion of 7KC, the major oxysterol in OxLDL-loaded cells, was exported to apoA-I (6). Similarly, selective enrichment of cholesteryl ester-loaded foam cells with 7KC (achieved by loading with 7kAcLDL) produced a similar inhibition of cholesterol export and low accompanying oxysterol release (7). When cells are incubated with lipid-free apolipoproteins, PL as well as cholesterol are exported to the media (8, 9). To determine whether impaired sterol efflux is associated with a comparable reduction in PL efflux, media from cells loaded with AcLDL, OxLDL, and 7kAcLDL, after exposure to lipid-free apoA-I, were analyzed for total PL content (**Table 2**).

While there was some variation in the total mass of cholesterol and PL exported between individual experiments from the three foam cell types (data not shown), the data standardized for cell protein were very reproducible. Standardization was necessary because the yields of primary cells varied between experiments. Also, we and others have found differences in cell survival during loading and subsequent culture between cells loaded with different modified forms of LDL. Interestingly, OxLDL-loaded cells survive better than AcLDL-loaded cells (23–25). However, we have previously shown that selective enrichment of mouse peritoneal macrophages with 7KC does not affect cell viability (7).

The mass of PL exported from OxLDL- and 7kAcLDL-loaded cells was substantially less than from AcLDL-loaded cells (Table 2). Thus the presence of oxidized lipids in macrophage foam cells is associated with reduced PL export to lipid-free A-I compared to cells containing similar amounts of cholesterol but no oxysterols. This was partially reproduced by the selective introduction of 7KC as the only oxidized lipid (Table 2).

To assess whether the impaired PL efflux from oxysterol-enriched cells (Table 2) was reflected by a lower cellular PL pool, total PLs were measured in AcLDL-, Ox-LDL-, and 7kAcLDL-loaded cells (Table 1). All of the loading conditions produced an increased cellular phospholipid content relative to non-loaded cells, consistent with previous studies (10, 26) but the total cellular PL pool was similar among the three foam cell models. Therefore, the lower PL efflux from oxysterol-enriched cells after incubation with apoA-I (Table 2) cannot be explained simply by a corresponding reduction in the cellular PL pool after loading.

The cholesterol:PL molar ratio (C:PL) of the exported lipids to lipid-free apoA-I (Table 2) from AcLDL-loaded cells (C:PL = 2.94 ± 0.38) is consistent with previous studies using similar efflux conditions, including AcLDL-loaded mouse peritoneal macrophages (C:PL = 2.2) (8) and non-loaded CHO cells (C:PL = 2.9) (9). The C:PL ratio for 7kAcLDL-loaded cells was not significantly different from that of AcLDL-loaded cells (3.03 ± 0.89 vs. 2.94 ± 0.38, respectively; Table 2). Thus 7KC enrichment of macrophage foam cells reduced the mass of cholesterol and PL exported, but not the relative proportions of each. This is consistent with the possibility that the reduced cholesterol export may be a consequence of a loss in the capacity of the foam cells to generate PL-apoA-I particles capable of accepting cellular cholesterol either simultaneously or subsequently. In contrast, OxLDL-loading reduced both PL mass and the C:PL ratio exported to apoA-I (1.10 ± 0.84 vs. 2.94 ± 0.38 for AcLDL-loaded; Table 2). Thus OxLDL-loading may not only impair PL desorption but, additionally and possibly independently, inhibit export of cholesterol into these particles. Alternatively, OxLDL-loading may alter the membrane lipid composition of cells and hence the functional activity of the membrane domains suggested to be involved in efflux (12).

Comparison of sterol efflux to lipid-free A-I vs. PC/A-I discs

The previous experiments indicated that impaired efflux of cholesterol from both 7kAcLDL-loaded and OxLDL-loaded cells was associated with reduced PL export from these cells. To determine whether impairment of cholesterol efflux was still seen when PL was supplied exogenously with the apoA-I, efflux was also measured in the presence of preformed PC/A-I discs. We have previously established that 20 μg/ml of lipid-free apoA-I is sufficient to induce maximum cholesterol and 7KC efflux from each mouse peritoneal macrophage foam cell type (6, 7). **Figures 1A** and **1B** show the dose-dependent efflux of cholesterol and 7KC to PC/A-I particles. The concentration required to induce maximum efflux was higher, particularly for 7KC efflux (Fig. 1B; 75–100 μg apoA-I protein/ml). Therefore PC/A-I discs were used at 100 μg apoA-I/ml in subsequent experiments.

Cholesterol and 7KC efflux from AcLDL-, 7kAcLDL-,

TABLE 2. Sterol and phospholipid analyses of efflux media

Foam Cell	Acceptor	n	Medium Lipid			C:PL Ratio
			CHOL	7KC	PL	
			nmol/mg cell protein			
AcLDL	BSA	2	21.3 ± 2.9		22.3 ± 2.1	0.95 ± 0.05
OxLDL	BSA	2	2.5 ± 1.0	0.4 ± 0.1	1.7 ± 0.9	1.58 ± 0.27
AcLDL	apoA-I	4	230 ± 32		78.3 ± 6.6	2.94 ± 0.38
OxLDL	apoA-I	2	18.0 ± 5.7	2.1 ± 1.0	20.5 ± 10.6	1.10 ± 0.84
7kAcLDL	apoA-I	3	126 ± 34	7.6 ± 3.4	43.0 ± 11.9	3.03 ± 0.89

Mouse macrophages were loaded for 24 h with 25 μg/ml of either AcLDL, OxLDL, or 7kAcLDL, equilibrated overnight in medium containing BSA (1 mg/ml), then incubated for 24 h in efflux medium containing BSA (1 mg/ml) ± lipid-free apoA-I (25 μg/ml). The efflux media were analyzed for cholesterol and total phospholipid. Values are means of the indicated number of separate experiments ± SD or 1/2 range (where n = 2).

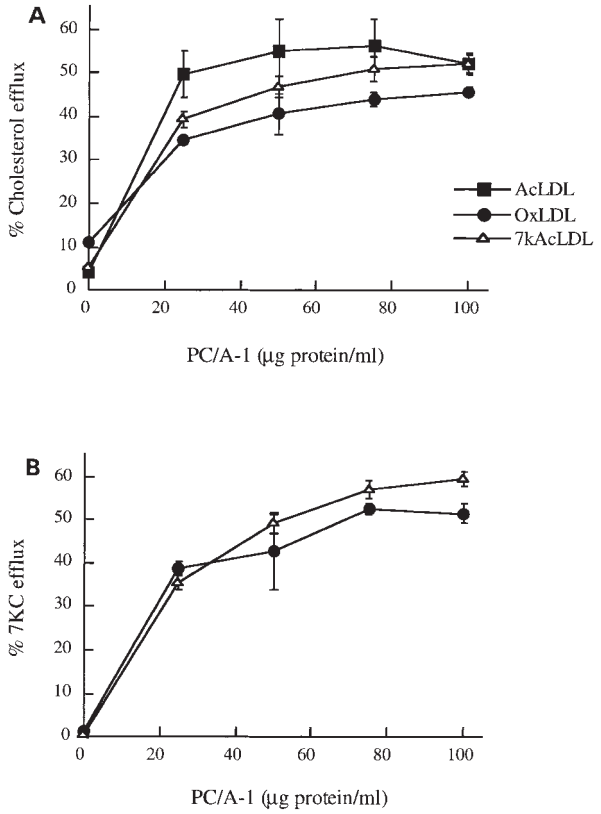

Fig. 1. Efflux of cholesterol (A) and 7KC (B) from cells loaded with modified LDLs in the presence of varying concentrations of PC/A-I discs. Mouse macrophages were loaded for 24 h with 25 μg/ml of either AcLDL, OxLDL, or 7kAcLDL. After loading they were equilibrated overnight in medium containing BSA (1 mg/ml), and then incubated for 24 h in efflux medium containing the indicated concentrations of PC/A-I discs. Cholesterol (A) and 7KC (B) efflux are expressed as the amount of cholesterol or 7KC in the medium relative to the total cholesterol or 7KC pool (including cholesteryl or 7KC esters) in cells and media. Results are mean ± standard deviation of triplicate cultures. Total sterol masses (nmol/mg cell protein, sum of free sterol and sterol esters; mean ± SD; n = 3) in the cultures were: AcLDL-loading, cholesterol 179 ± 32; OxLDL-loading: cholesterol 77 ± 16; 7KC 35 ± 3 (oxidized esters not measured); 7kAcLDL-loading: cholesterol 153 ± 29; 7KC 57 ± 10.

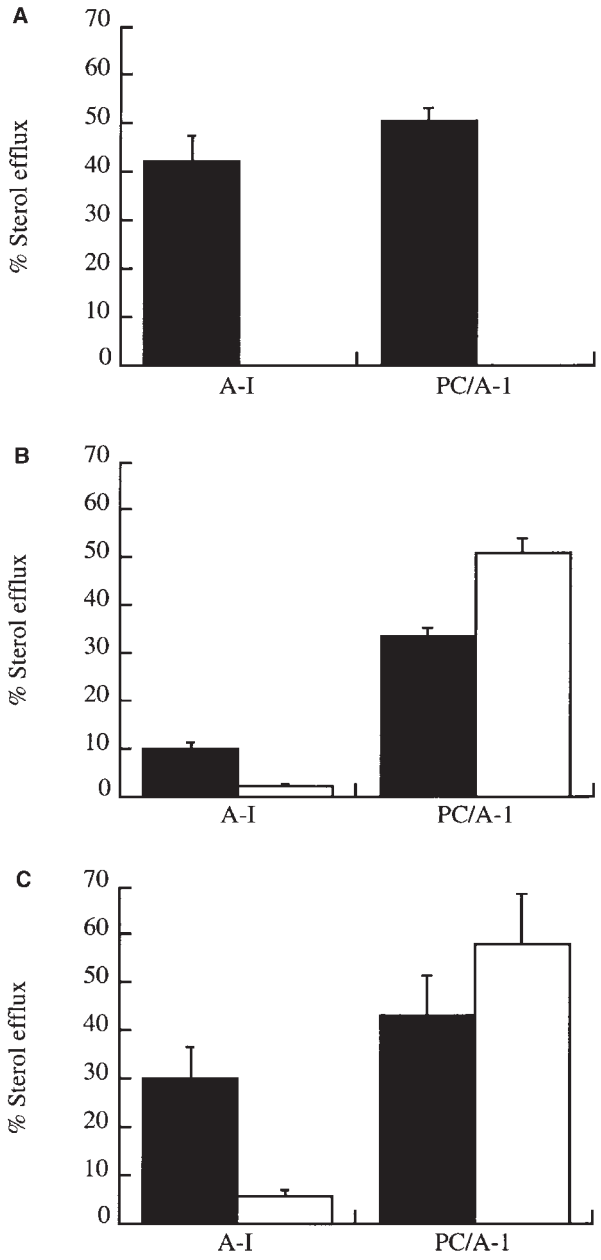

Fig. 2. Efflux of cholesterol (■) and 7KC (□) from cells loaded with modified LDLs in the presence of apoA-I or PC/A-I discs. Mouse macrophages were loaded for 24 h with 25 μg/ml of AcLDL (A), OxLDL (B) or 7kAcLDL (C), equilibrated overnight in medium containing BSA (1 mg/ml), followed by 24 h in efflux medium containing apoA-I (25 μg/ml) or PC/A-I discs (100 μg protein/ml). Cholesterol and 7KC efflux is expressed as the amount of cholesterol or 7KC in the medium relative to the total cholesterol or 7KC pool (including cholesteryl or 7KC esters) in cells and media. Results are mean ± standard deviation of four independent experiments, each performed with triplicate cultures. To standardize for differences in experimental conditions such as different batches of cells, basal efflux to acceptor-free medium was subtracted for each individual experiment before the data were combined.

and OxLDL-loaded cells to lipid-free apoA-I and PC/A-I discs were compared. In **Fig. 2**, data from four independent experiments are presented and in each case, 'basal' efflux to BSA alone has been subtracted to facilitate comparison between experiments. The proportional cholesterol efflux from AcLDL-loaded cells to apoA-I was 42 ± 5%, consistent with previous studies (6, 7). PC/A-I discs mediated cholesterol efflux slightly but significantly greater (50 ± 3% for PC/A-I discs, n = 4; $P < 0.05$; Fig. 2A).

In OxLDL-loaded cells, cholesterol efflux to apoA-I (10 ± 1%) was significantly lower than from AcLDL-loaded cells (6) and increased substantially in the presence of PC/A-I discs (34 ± 2%; Fig. 2B; $P < 0.001$) although this did not increase efflux to the level of AcLDL-loaded cells. Cholesterol efflux from 7kAcLDL-loaded foam cells also improved significantly after incubation with PC/A-I discs compared to lipid-free apoA-I (Fig. 2C; 30 ± 7% and 43 ± 8% for apoA-I and PC/A-I discs, respectively; $P < 0.05$). Cholesterol efflux induced by PC/A-I from 7kAcLDL-loaded cells was similar to that induced from AcLDL-loaded cells (43 ± 8% vs. 50 ± 3%, respectively; difference not significant), indicating that

the suppression of cholesterol efflux induced by 7KC can be overcome by providing an exogenous supply of PL.

Consistent with earlier studies (6, 7), efflux of 7KC from both OxLDL- and 7kAcLDL-loaded cells to lipid-free A-I was very low (2 ± 0.5% and 5 ± 2%, respectively). Incubation with PC/A-I discs induced a massive increase in 7KC efflux from both OxLDL- and 7kAcLDL-loaded cells to 51 ± 3 and 58 ± 10%, respectively (Fig. 2B and 2C). Thus the provision of exogenous PL stimulated 7KC proportional efflux to approximately the same levels as cholesterol efflux.

Kinetics of efflux of cholesterol and 7KC to PC/A-I discs

To study the kinetics of cholesterol and 7KC release in the presence of exogenous PL, macrophages loaded with either AcLDL, OxLDL, or 7kAcLDL were incubated with PC/A-I discs (100 μg/ml) for the indicated times (**Fig. 3**). Cholesterol efflux from AcLDL- and 7kAcLDL-loaded cells (Fig. 3A), expressed as a proportion of the total cholesterol pool, was linear as found previously for efflux induced by lipid-free apoA-I (27). Cholesterol efflux from OxLDL-loaded cells was slower and less linear. The rate of proportional 7KC export from 7kAcLDL-loaded macrophages was similar to that of cholesterol (Fig. 3B). However, 7KC efflux from OxLDL-loaded cells displayed different kinetics compared to 7kAcLDL (Fig. 3B), showing rapid efflux in the first 6 h followed by a decline in the rate over the later timepoints. Interestingly, in OxLDL-loaded cells, the absolute mass of 7KC exported from the cells exceeded that of cholesterol over the first 12 h (data not shown), although the size of the 7KC pool is much smaller (less than 30% of total sterol) than that of cholesterol in these cells. In 7kAcLDL-loaded cells the mass release of cholesterol and 7KC was proportional to the cellular content of the two sterols (data not shown).

Figure 4 indicates changes in several individual cellular pools of sterol during exposure to PC/A-I discs. In AcLDL-loaded cells, 60% of intracellular cholesterol was initially esterified (Fig. 4A). Export of cholesterol was accompanied by a large decline in intracellular cholesteryl esters and a lesser decline in intracellular free cholesterol (Fig. 4A). A similar initial cholesterol distribution and pattern of redistribution during efflux was found in 7kAcLDL-loaded cells (Fig. 4B). In OxLDL-loaded cells, the cellular cholesterol content was initially quite different. Thus a larger proportion (55%) of the cell cholesterol was unesterified and only a very minor component (5%) appeared as cholesteryl esters. The residual 40% of the cellular cholesterol was esterified to oxidized cholesteryl esters (cholesterol esterified to oxidized fatty acyl group(s)). During efflux to PC/A-I discs (Fig. 4C), this oxidized cholesteryl ester pool initially declined slightly (~25% during 0–6 h) and then remained unaffected for the remainder of the incubation. Between 6–24 h the majority of cholesterol loss was from unesterified cellular cholesterol although the small unoxidized cholesteryl ester pool was also significantly depleted.

In 7kAcLDL-loaded cells, like cholesterol, the majority of cellular 7KC was initially esterified and this pool contributed most of the 7KC exported to PC/A-I discs (Fig. 4D). The distribution of 7KC in OxLDL-loaded cells was also quite similar to cholesterol; unesterified and oxidized esters of 7KC were the major cellular pools of the oxysterol (Fig. 4E). 7KC efflux from OxLDL-loaded cells was mostly derived from the unesterified 7KC pool which was rapidly depleted in the first 6 h.

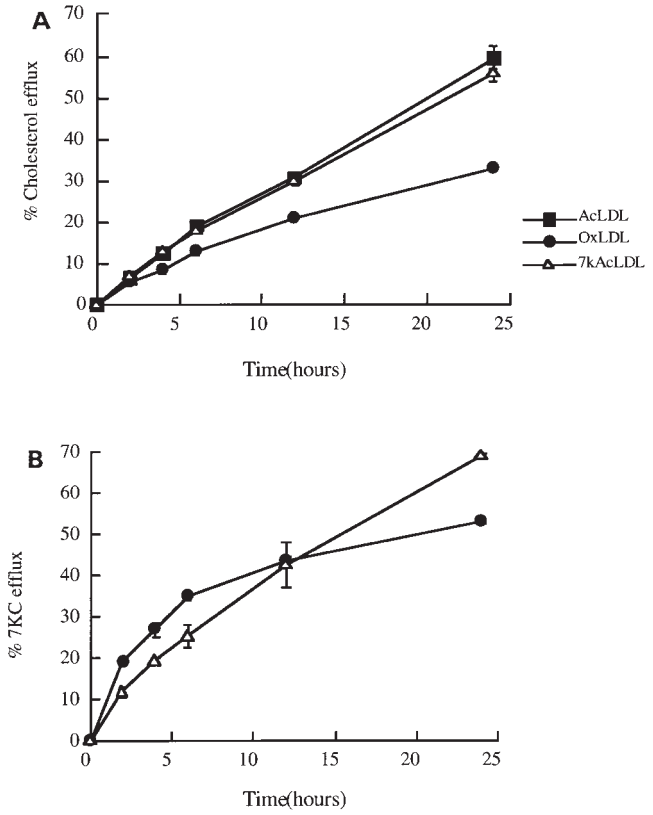

Fig. 3. Rates of efflux of cholesterol (A) and 7KC (B) from cells loaded with modified LDLs in the presence of PC/A-I discs. Mouse macrophages were loaded for 24 h with 25 μg/ml of either AcLDL (■), OxLDL (●), or 7kAcLDL (△). After loading they were equilibrated overnight in medium containing BSA (1 mg/ml), and then incubated for 24 h in efflux medium containing PC/A-I discs (100 μg protein/ml). Cholesterol (A) and 7KC (B) efflux is expressed as the amount of cholesterol or 7KC in the medium relative to the total cholesterol or 7KC pool (including cholesteryl or 7KC esters) in cells and media. Results are mean ± standard deviation of triplicate cultures. Total sterol mass (nmol/mg cell protein, sum of free sterol and sterol ester; mean ± SD; n = 3) present in cultures at T = 0: AcLDL-loading, cholesterol 367 ± 62; OxLDL-loading, cholesterol 279 ± 55, 7KC 156 ± 32; 7kAcLDL-loading, cholesterol 385 ± 115, 7KC 158 ± 41.

Sterol efflux to other PL-containing acceptors

The complete or partial normalization of cholesterol export from 7kAcLDL- and OxLDL-loaded cells, respectively, to PC/apoA-I discs suggests that the compromise in cholesterol export to lipid-free apoA-I may be a consequence of insufficient capacity of the cells to provide extracellular PL. This was further investigated by extending the range of acceptors used to include several PL-containing particles to stimulate cholesterol export. **Figure 5** compares lipid-free apoA-I, PC/A-I discs, PC-only containing small unilamellar vesicles (SUV) and whole HDL. The apolipoprotein and PL concentrations of these various acceptors are compared in **Table 3**. The SUV concentration was matched to the PC

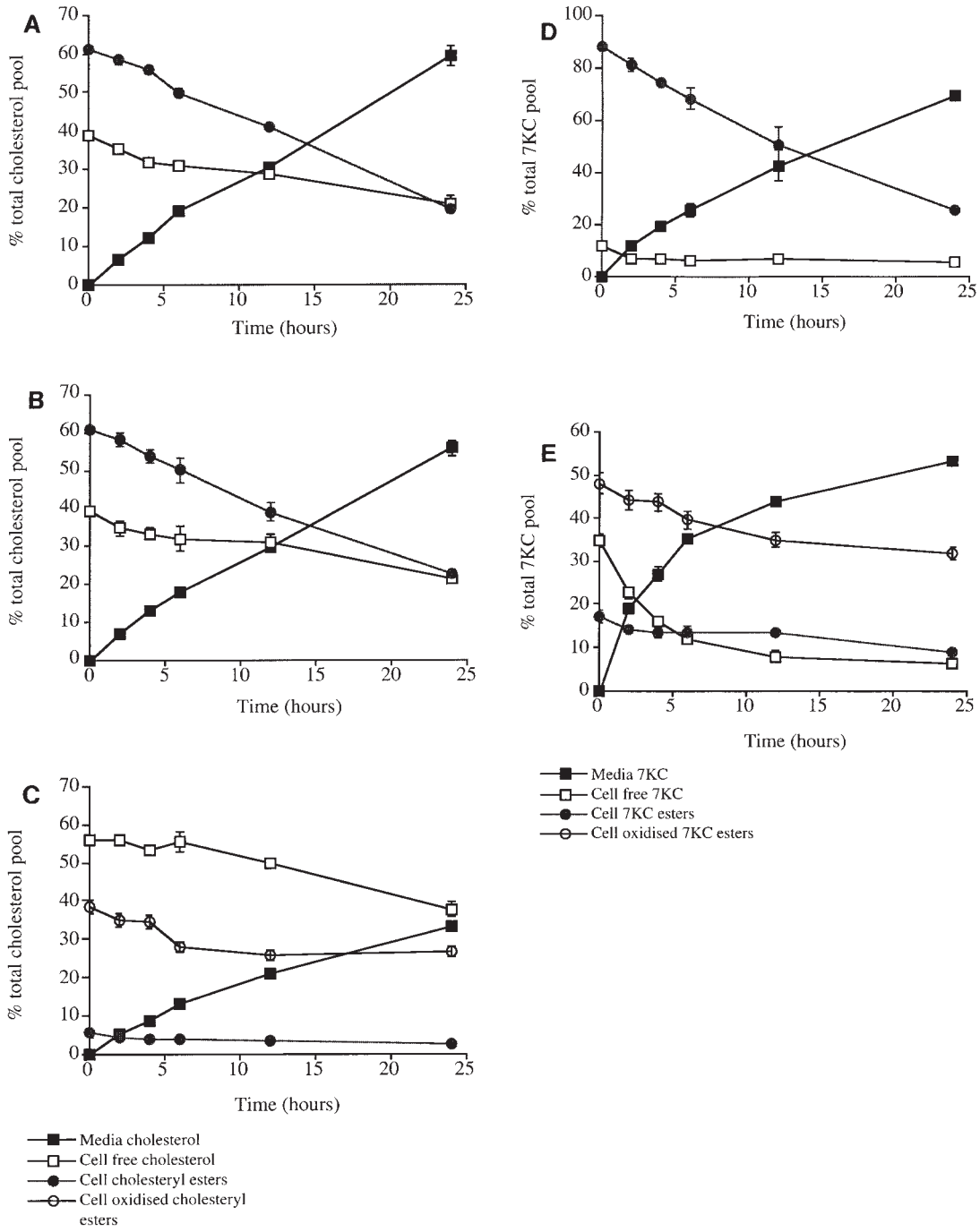

Fig. 4. Intracellular distribution of free and esterified sterol during efflux in cells loaded with modified LDLs in the presence of PC/A-I discs. Mouse macrophages were loaded for 24 h with 25 μg/ml of either AcLDL, OxLDL, or 7kAcLDL. After loading they were equilibrated overnight in medium containing BSA (1 mg/ml), and then incubated for 24 h in efflux medium containing PC/A-I discs (100 μg protein/ml). The individual sterols are expressed as a proportion of their respective total sterol pools. (A) Cholesterol pool in AcLDL-loaded cells, (B) Cholesterol pool in 7kAcLDL-loaded cells, (C) Cholesterol pool in OxLDL-loaded cells, (D) 7KC pool in 7kAcLDL-loaded cells, (E) 7KC pool in OxLDL-loaded cells. Total sterol mass (nmol/mg cell protein, sum of free sterol and sterol ester; mean ± SD for triplicate cultures) present in cultures at T = 0: AcLDL-loading, cholesterol 367 ± 62; OxLDL-loading, cholesterol 279 ± 55, 7KC 156 ± 32; 7kAcLDL-loading, cholesterol 385 ± 115, 7KC 158 ± 41.

concentration of media containing PC/A-I discs (265 μg PC/ml). HDL was added at the same apolipoprotein concentration as PC/A-I discs (100 μg protein/ml). OxLDL-loaded cells and efflux media were also analyzed for 7β-hydroxy cholesterol (7β-OH), present in OxLDL and OxLDL-loaded cells (approx. 7–8% of total OxLDL sterol) (5) and after 7KC, the next major non-enzymic oxysterol present in atherosclerotic plaque (1, 2, 28).

SUV and HDL were able to induce substantial cholesterol efflux from AcLDL-loaded cells (Fig. 5A), as has been found previously (29, 30), to an extent comparable to PC/A-I discs or lipid-free apoA-I. With 7kAcLDL-loaded

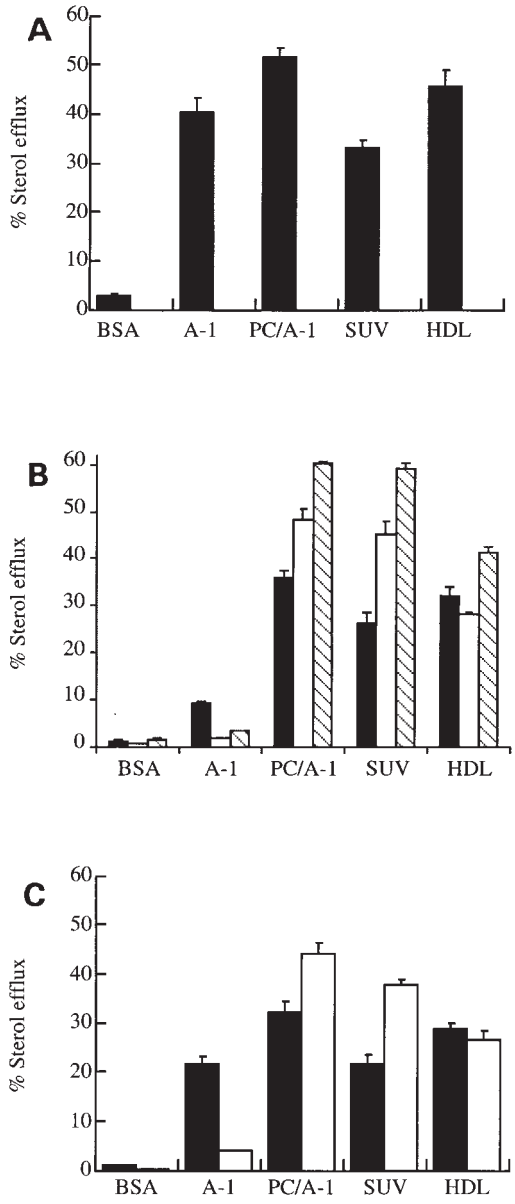

Fig. 5. Efflux of cholesterol (■), 7KC (□), and 7β-OH (▨) to various acceptors from cells loaded with modified LDLs. Mouse macrophages were loaded for 24 h with 25 μg/ml of either AcLDL (A), OxLDL (B), or 7kAcLDL (C). After loading they were equilibrated overnight in medium containing BSA (1 mg/ml), and then incubated for 24 h in efflux medium containing BSA alone or BSA and apoA-I (25 μg/ml), PC/A-I discs (apoA-I 100 μg/ml; PC 265 μg/ml), SUV (PC 265 μg/ml), or HDL (protein 100 μg/ml; PL 33 μg/ml). Sterol efflux is expressed as the amount of cholesterol, 7KC or 7β-OH in the medium relative to the total cholesterol, 7KC or 7β-OH pool (including their respective esters) in cells and media. Results are mean ± standard deviation of triplicate cultures. Total sterol mass (nmol/mg cell protein, sum of free sterol and sterol ester; mean ± SD for triplicate cultures) present in cultures after efflux to BSA: AcLDL-loading, cholesterol 538 ± 92; OxLDL-loading, cholesterol 177 ± 12, 7KC 91 ± 5; 7kAcLDL-loading, cholesterol 416 ± 6, 7KC 124 ± 12.

(Fig. 5B) and OxLDL-loaded foam cells (Fig. 5C), SUV and HDL behaved similarly to PC/A-I discs, inducing substantial cholesterol and 7KC efflux. As for 7KC, efflux of 7β-OH from OxLDL-loaded cells (Fig. 5B) to apoA-I was very low (3 ± 0.3%) and increased greatly to all PL-containing acceptors. This indicates that as for 7KC efflux, the presence of extracellular acceptor PL is also important for inducing 7β-OH efflux from OxLDL-loaded cells.

TABLE 3. ApoA-I and phospholipid composition of different acceptors

	ApoA-I	PC/A-I	PUV	HDL
ApoA-I[a]	25 (0.88)	100 (3.53)	nd	100[b]
Phospholipid[a]	nd	265 (342)	265 (342)	33 (42.6)

[a] Results are expressed as μg (or nmol) per ml of efflux media. Molecular weights: ApoA-I, 28,331; PL, 775. Each culture well contains 2 ml of media.
[b] Total HDL protein; for this donor, apoA-I comprised 50% of total HDL protein; nd, not detected.

DISCUSSION

Oxysterols such as 7KC are present in atherosclerotic plaque in low but significant amounts (1, 2, 28) and are concentrated in plaque foam cells relative to both circulating monocytes and surrounding extracellular lipid (3, 4). Oxysterols display a range of potentially proatherogenic biological activities in vitro (reviewed in ref. 3), suggesting that their presence in vivo may play an active role in lesion development. One interesting potential proatherogenic characteristic shown in vitro is their inhibitory effect on cholesterol efflux (6, 7, 31, 32).

In the current study, we investigated the mechanism of oxysterol-inhibited sterol efflux from macrophage foam cells. Incubation of cells with lipid-free apoA-I stimulates PL desorption and the formation of PL/apoA-I particles (8, 9). It has been suggested that such PL export is an essential component of cholesterol efflux, either by generation of a nascent PL/apoA-I particle into which cholesterol subsequently partitions (9, 10), or by physically chaperoning concomitant cholesterol export through desorption of PL/cholesterol-containing domains of the plasma membrane (11, 12). The present study has shown that PL efflux to lipid-free apoA-I from 7KC-containing foam cells is reduced relative to oxysterol-free foam cells. The mechanism of 7KC-mediated suppression of PL efflux to apoA-I was not investigated in the present study, but several possibilities exist. First, the presence of 7KC in the plasma membrane may directly affect its properties. For example, 7KC affects the packing of PC headgroups in bilayers which might inhibit subsequent interaction of these PL with extracellular acceptors (33). Second, oxysterols such as 7KC may affect PL biosynthesis and/or availability for export. Enrichment of cells with cholesterol increases its export to apoA-I (34), a process that may be affected by 7KC. Alterations in the types and relative quantities of various PL available in the plasma membrane may also influence sterol export. Finally, oxysterols may inhibit the expression of membrane proteins such as SR-B1 and cave-

olin, a major component of caveolae. These cholesterol and sphingomyelin-rich microdomains of the plasma membrane have been implicated as sites of cholesterol efflux (35). Treatment of fibroblasts with oxysterols including 7KC led to a reduction in cholesterol efflux to plasma (32) and reduced caveolin mRNA levels. At present the existence of caveolae and even caveolin in macrophage foam cells remains controversial but merits further investigation.

The restoration of cholesterol efflux from 7kAcLDL-loaded cells incubated with PC/A-I discs to levels similar to oxysterol-free foam cells is consistent with a limitation in availability of cell-derived PL induced by 7KC. However, it is also possible that PC/A-I discs and lipid-free apoA-I induce cholesterol efflux by different mechanisms which are differentially sensitive to 7KC. It has been suggested that the mechanisms of cholesterol efflux to apolipoprotein and non-apolipoprotein acceptors are distinct: lipid-free/poor apolipoproteins utilizing an energy-dependent, possibly receptor-mediated interaction with the plasma membrane while non-apolipoprotein acceptors such as cyclodextrins and PL liposomes as well as HDL and fully lipidated apoA-I stimulate an energy-independent, aqueous diffusion mode of efflux (30, 36–39).

In the present study, all PL-containing acceptors were able to induce substantial cholesterol efflux from 7KC-containing foam cells. The provision of PL per se, as in the case of SUV, is clearly sufficient to stimulate substantial cholesterol export from these cells. In the present study, SUV were almost as efficient as PC/A-I discs in stimulating cholesterol efflux from macrophages. This is rather different from some other cell types, where PC liposomes are relatively poor acceptors of cellular cholesterol (40), but is consistent with previous measurements of PC-liposome-stimulated cholesterol efflux from cholesterol-loaded human monocyte-derived macrophages (29) and J774 macrophages (41) where efflux rates were closer to, though lower than, PC/A-I mediated cholesterol export.

Although supplementation of foam cells with 7KC alone (7kAcLDL-loading) reduced the mass of PL and cholesterol efflux to lipid-free apoA-I, the C:PL ratio exported was similar to oxysterol-free foam cells. This suggests that the export of cholesterol and PL are related, although it does not indicate whether the formation of PL/apoA-I particles precedes sterol efflux or whether cholesterol and PL desorb simultaneously from membrane microdomains ('microsolubilization') (36, 42). The failure of 7KC to also efflux under these conditions suggests that if simultaneous efflux occurs from specific membrane domains, they would have to be relatively depleted of oxysterol, as a similar proportion of the free cholesterol and 7KC pools in macrophages is present in the plasma membrane (7).

In OxLDL-loaded cells, the phospholipid mass and the C:PL ratio of lipid exported from OxLDL-loaded cells to apoA-I were significantly lower than from AcLDL- or 7kAcLDL-loaded cells, suggesting that both PL desorption and subsequent movement of cholesterol into the extracellular acceptor complex were inhibited. Cholesterol efflux from these cells could be somewhat improved by incubation with PC/A-I, however efflux was still less than levels seen from AcLDL-loaded cells. An important characteristic of OxLDL, which may limit cholesterol availability for export in OxLDL-loaded cells, is the presence of a relatively large pool of cholesteryl esters with oxidized fatty acyl chains (5). We have evidence that these oxidized esters are predominantly lysosomal and very slowly hydrolyzed (43; A. J. Brown, E. L. Mander, I. C. Gelissen, L. Kritharides, R. T. Dean, and W. Jessup, unpublished observations), quite unlike the cytoplasmic cholesteryl esters containing normal fatty acyl chains present in AcLDL and 7kAcLDL-loaded cells. Several other studies have also reported lysosomal sterol sequestration in OxLDL-loaded macrophages (44–46). During efflux to PC/A-I discs, depletion of the oxidized cholesteryl ester pool occurred slowly over the first few hours and then not at all. This indicates that availability of oxidized esters for efflux is limited and probably reflects a lysosomal ester pool resistant to hydrolysis. However, if the oxidized ester pool is ignored in the calculation of cholesterol efflux to PC-A-I, then the rates of efflux of cholesterol from OxLDL-loaded cells improve significantly. For example, in the experiment in Fig. 4, cholesterol efflux increases from 33% to 40%. This suggests that in OxLDL-loaded cells, both impaired phospholipid desorption as well as lysosomal sequestration of cellular cholesterol contribute to the resistance observed in cholesterol export to apoA-I. It will be important to determine whether such oxidized esters are present in lesion foam cells, as their capacity to export from such a pool of cholesterol may be similarly compromised. There is some evidence that esters containing oxidized linoleate and more advanced aldehydic products of fatty acid oxidation are present in atherosclerotic plaque (1, 45, 47). Their cellular location and significance in foam cell formation remain to be determined.

The much lower rate of efflux of 7KC to lipid-free apoA-I indicates a selectivity in sterol export induced by this acceptor. We have recently observed a similar negligible 7KC efflux to lipid-free apolipoprotein J, although, like apoA-I, this apolipoprotein is an efficient stimulator of cholesterol efflux from macrophage foam cells (27). Clearly, the oxysterol is available for export, as its proportional efflux from 7kAcLDL- and OxLDL-loaded cells matches or exceeds that of cholesterol in the presence of PL-containing acceptors such as PC/A-I discs, SUV, and HDL. This implies a requirement for extracellular PL, which is not adequately served by the PL exported from these cells in the presence of apoA-I. It is possible that the types of PL desorbed or the size and quantity of the particles generated from cell-derived PL are not optimal for 7KC export. For example, the PL:A-I ratio achieved during incubation of initially lipid-free apoA-I with AcLDL-loaded foam cells was ~3, while that of preformed PC/A-I discs was >60. The different types and fatty acid content of the phospholipids may also be significant. This is under investigation.

Also of interest were the differences in kinetics of 7KC efflux from OxLDL- and 7kAcLDL-loaded cells to PC/A-I. 7KC efflux from OxLDL-loaded cells was more rapid at earlier timepoints while 7KC efflux from 7kAcLDL-loaded

cells was slower but sustained. In OxLDL-loaded cells, a large proportion of 7KC is unesterified, unlike 7kAcLDL-loaded cells where the majority is esterified. Oxysterol analyses of plaque tissue have shown that the majority of 7KC present in plaque is also esterified (>90% (3)) while preliminary analyses from our laboratory suggest that the same is true for foam cells re-isolated from plaque tissue (D. M. van Reyk and A. J. Brown, unpublished observations). Hydrolysis of esterified 7KC may be a rate-limiting step that promotes 7KC accumulation in plaque foam cells.

The quantitative importance of such pathways for sterol export from foam cells in vivo depends on the types of acceptors available locally and little detailed information is presently available. Castro and Fielding (48) identified a lipid-poor apoA-I fraction in plasma (preβ-HDL) as a likely first acceptor for cellular cholesterol efflux. These particles are believed to originate from remodelling of HDL, as very lipid-poor apoA-I can dissociate from plasma HDL in vitro (49). In plasma, about 5% of the total apoA-I pool is estimated to be lipid-free or poor (50, 51). The relative levels of lipid-poor versus lipid-replete apolipoproteins in peripheral tissues, and particularly in the intima, are less clearly understood. Small or 'lipid-free' apoA-I fractions have been found in peripheral lymph (52, 53) and apoA-I-containing lipoproteins of d > 1.21 g/ml that contain small quantities of PL (average PC/A-I molar ratio of 2.5–3.0) have been isolated from human aortic tissues (54). Smith (55) found that the majority of apoA-I in human intima is in a very high density fraction. Thus it seems likely that lipid-free or poor apoA-I exists in intimal tissues in significant amounts. An important issue is likely to be the relationship between the PL:apoA-I ratio of exogenous acceptors and their capacity to mediate sterol export from foam cells. From the present study, we would predict that both lipid-free and lipid-poor apoA-I (comparable to that generated by exposure of apoA-I to 7KC-enriched foam cells) are unable to mediate significant export of 7-ketocholesterol from foam cells. Further, the consequent accumulation of oxysterols such as 7KC in foam cells could lead to further impairment of the capacity of these cells to export cholesterol. Preliminary data (E. Oates, T. Sloane, C. Dass, R. T. Dean, W. Jessup, and L. Kritharides, unpublished results) indicate that human foam cells are more sensitive to the inhibitory action of 7KC than their murine counterparts. Apart from plasma-derived apoA-I-containing acceptors, other apolipoproteins such as apoJ and/or apoE may also be important for inducing sterol efflux from foam cells. In the case of endogenously synthesized and secreted apo E, the amounts of cell-derived phospholipids could also be limiting.

In summary, we have shown that PL as well as cholesterol efflux is impaired from oxysterol-enriched foam cells. The inhibition of cholesterol efflux could be largely overcome by supplying exogenous PL. The likelihood that oxysterols could limit cholesterol efflux in foam cells in vivo will depend on the nature of acceptors available in the environment of developing foam cells, which presently is not known. The relatively poor capacity of 7KC to efflux to lipid-free (or lipid-poor) apoA-I could explain the relative enrichment of these oxysterols in human foam cells. The likely effect of physiological levels of oxysterols (up to 5% total sterol) in human foam cells remains to be determined. If oxysterols play a significant role in foam cell development, the design of agents specifically targeting oxysterol removal becomes important.

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