Atherosclerosis involves the arterial accumulation of lipid-laden “foam cells” containing oxidized and unoxidized sterols and their esters (Mattsson-Hulten, L., Lindmark, H., Diczfalussy, U., Bjorkhem, I., Ottosson, M., Liu, Y., Bondjers, G., and Wiklund, O. (1996) J. Clin. Invest. 97, 461–8). Oxidized sterols are probably critical to atherogenesis because they inhibit cholesterol removal from cells and are cytotoxic. We recently reported that there is deficient induction of cellular cholesterol efflux by apolipoprotein A-I, the main initial acceptor of cellular cholesterol from macrophages loaded in vitro with oxidized low density lipoprotein (Kritharides, L., Jessup, W., Mander, E., and Dean, R. T. (1995) Arterioscler. Thromb. 15, 276–289). There was an even more marked impairment of the release of 7-ketocholesterol which is a major oxysterol in these cells and in human atherosclerotic lesions. Here we show that hydroxypropyl-β-cyclodextrin can induce selective efflux of 7-ketocholesterol. Efflux of 7-ketocholesterol was time and concentration dependent, and the rate of its removal was 50-fold greater for hydroxypropyl-β-cyclodextrin than for apolipoprotein A-I. Over a defined range of concentrations (0–5 mg/ml), efflux of 7-ketocholesterol was preferred over that of cholesterol and occurred without cell toxicity. Efflux of free 7-ketocholesterol was associated with decreased intracellular free and esterified 7-ketocholesterol. Hydroxypropyl-β-cyclodextrin also enhanced efflux of other oxysterols. The physical solubilization of 7-ketocholesterol by the cyclodextrin was much greater than that of cholesterol, in accordance with its differential effects on efflux. These data highlight the importance of extracellular sterol solubilization in the efflux of cellular oxysterols and the mobilization of intracellular free and esterified oxysterol pools in macrophages loaded with oxidized low density lipoprotein. Synthetic sterol-solubilizing agents such as hydroxypropyl-β-cyclodextrin are thus potential prototypes for the further development of oxysterol-removing agents.

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Hydroxypropyl-β-cyclodextrin-mediated Efflux of 7-Ketocholesterol from Macrophage Foam Cells*

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Oxidation modifies low density lipoprotein (LDL)¹ such that it undergoes accelerated and unregulated uptake by macrophages, converting the latter into lipid-rich foam cells resembling those of arterial atherosclerotic lesions (1, 2). The major product of sterol oxidation found in oxidized LDL (OxLDL) is 7-ketocholesterol (7KC) (3–5). This oxysterol has also been found in whole plaque (6) and is the principal oxysterol in macrophages loaded with OxLDL in vitro (3–5). Oxysterols, including 7KC, are enriched in plaque compared with circulating lipoproteins, and there is a further relative concentration of oxysterols in plaque macrophage foam cells than in total human plaque sterols (7).

Lipid-poor apo A-I is likely to be the main initial acceptor of cellular cholesterol (8). We recently reported that there is deficient induction by apo A-I of cellular cholesterol efflux from macrophages loaded in vitro with OxLDL (9). There was an even more marked impairment of the release of 7KC, the major oxysterol in these cells (9). Intraacellular accumulation of oxysterols (including 7KC) in endothelial cells (10) and macrophages (11) can impair cholesterol efflux. Additionally, 7KC is a major contributor to OxLDL-mediated cytotoxicity (12), and oxysterols may contribute to the generation of the highly thrombogenic necrotic core of advanced atherosclerotic plaque (13). Thus, removal of oxysterols such as 7KC in vitro might facilitate physiological cholesterol efflux, prevent cell death, and hence reverse or stabilize atherosclerosis.

Cyclodextrins are cyclical sugar polymers known to solubilize a range of hydrophobic compounds within their hydrophobic core (14). They can interact with and precipitate lipoproteins, solubilize pharmacological agents, and form inclusion complexes with lipids (15, 16). β-Cyclodextrins (containing 7 α-glucopyranose units) solubilize cholesterol from erythrocyte membranes (17). Several β-cyclodextrins have been recently found to promote cellular cholesterol efflux (18), and we have independently investigated a means for facilitating removal of 7KC from OxLDL-loaded macrophages in vitro, using these agents (Part of this work has already been published in preliminary form (19)).

It was hypothesized that solubility may be limiting for efflux of 7KC from OxLDL-loaded macrophages and that certain cyclodextrins might efficiently solubilize 7KC and thus induce its efflux from cells. We have specifically investigated hydroxypropyl-β-cyclodextrin (hp-β-CD) in facilitating efflux of 7KC for three reasons: (a) chemically hydroxyalkylated cyclodextrins such as hp-β-CD are >50 times more water soluble than native β-cyclodextrins and do not precipitate lipoproteins; (b) hydroxyalkylated cyclodextrins are virtually nontoxic to animals, whereas native β-CD can be quite toxic; and (c) hp-β-CD.

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Atherosclerosis involves the arterial accumulation of lipid-laden “foam cells” containing oxidized and unoxidized sterols and their esters (Mattsson-Hulten, L., Lindmark, H., Diczfalussy, U., Bjorkhem, I., Ottosson, M., Liu, Y., Bondjers, G., and Wiklund, O. (1996) J. Clin. Invest. 97, 461–8). Oxidized sterols are probably critical to atherogenesis because they inhibit cholesterol removal from cells and are cytotoxic. We recently reported that there is deficient induction of cellular cholesterol efflux by apolipoprotein A-I, the main initial acceptor of cellular cholesterol from macrophages loaded in vitro with oxidized low density lipoprotein (Kritharides, L., Jessup, W., Mander, E., and Dean, R. T. (1995) Arterioscler. Thromb. 15, 276–289). There was an even more marked impairment of the release of 7-ketocholesterol which is a major oxysterol in these cells and in human atherosclerotic lesions. Here we show that hydroxypropyl-β-cyclodextrin can induce selective efflux of 7-ketocholesterol. Efflux of 7-ketocholesterol was time and concentration dependent, and the rate of its removal was 50-fold greater for hydroxypropyl-β-cyclodextrin than for apolipoprotein A-I. Over a defined range of concentrations (0–5 mg/ml), efflux of 7-ketocholesterol was preferred over that of cholesterol and occurred without cell toxicity. Efflux of free 7-ketocholesterol was associated with decreased intracellular free and esterified 7-ketocholesterol. Hydroxypropyl-β-cyclodextrin also enhanced efflux of other oxysterols. The physical solubilization of 7-ketocholesterol by the cyclodextrin was much greater than that of cholesterol, in accordance with its differential effects on efflux. These data highlight the importance of extracellular sterol solubilization in the efflux of cellular oxysterols and the mobilization of intracellular free and esterified oxysterol pools in macrophages loaded with oxidized low density lipoprotein. Synthetic sterol-solubilizing agents such as hydroxypropyl-β-cyclodextrin are thus potential prototypes for the further development of oxysterol-removing agents.

The abbreviations used are: LDL, low density lipoprotein; apo A-I, apolipoprotein A-I; 7KC, 7-ketocholesterol; hp-β-CD, hydroxypropyl-β-cyclodextrin; OxLDL, oxidized low density lipoprotein; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; 7β-OH, 7β-hydroxycholesterol; 7α-OH, 7α-hydroxysterol; LPDS, lipoprotein-deficient serum.
has been shown to solubilize both cholesterol and, importantly for our studies with 7KC, oxysterols such as 7α-hydroxycholesterol (20, 21). We here report the results of studies showing that hp-β-CD removes 7KC from macrophages loaded with OxLDL more effectively than apo A-1 and solubilizes and removes 7KC preferentially compared with cholesterol.

MATERIALS AND METHODS

Isolation of Lipoproteins—LDL (1.02 < d < 1.05 g/ml), and human lipoprotein-deficient serum (LPDS; d > 1.25, final protein concentration of 10% LPDS in Dulbecco’s modified Eagle’s medium (DMEM) of 1.5 mg/ml), were isolated from the plasma of normal subjects, healthy subjects using a discontinuous KBr gradient at 10 °C by modification of the method of Flock et al. (9, 22). LDL was dialyzed against 4 × 200 vol deoxyxygenated phosphate-buffered saline (PBS, calcium and magnesium free; Flow Laboratories) containing 0.1 mg/ml chlamphenicol and 1.0 mg/ml EDTA at 4 °C. LDL was prepared by this two-step procedure was essentially albumin free as confirmed by SDS-polyacrylamide gel electrophoresis.

Isolation of Mouse Peritoneal Macrophages—Resident macrophages were isolated from C57BL/6J mice (bred at the Heart Research Institute), after asphyxiation by carbon dioxide gas, by peritoneal lavage with ice-cold DMEM (Life Technologies, Inc., catalog no. 320-1885 AD) containing 0.1 mg/ml chloramphenicol (0.1 mg/ml) and EDTA at 4°C. LDL prepared by this two-step procedure was filtered sterilized (0.2 μm) and subjected to electrophoresis in Tris-barbitone buffer (pH 8.6, 0.38% (w/v) sodium citrate, glutamine (2 mM, Life Technologies, Inc.), penicillin G (50 units/ml), and streptomycin (50 μg/ml; penicillin/streptomycin supplied by Sigma) (9). Cells were plated in 24-mm diameter culture dishes (Nunc) at 5–6 × 106 cells/well at 37 °C for 2 h to achieve adherence and then washed four times with prewarmed PBS to remove nonadherent cells before final medium containing DMEM, 10% (v/v) LPDS, and oxidized LDL was added (loading conditions are described further below).

LDL Oxidation—Before oxidation, LDL was dialyzed against 3 × 1 liter of PBS (LDL/PBS, 1/200, v/v) containing chlamphenicol (0.1 g/liter) and Chelex 100 (1 g/liter), then 1 × 1 liter PBS containing chlamphenicol only, during a total of 16 h to remove EDTA. LDL was oxidized aseptically at 400 μg of LDL protein/ml of PBS containing 10 μM cupric chloride (BDH) for 24 h at 37 °C. Oxidation was confirmed by agarose gel electrophoresis and by high performance liquid chromatography (HPLC) analysis of lipid extracts of LDL (23). After oxidation, LDL was filter sterilized (0.22 μm) and added to culture medium containing DMEM, glutamine, and antibiotics, as indicated above, and 10% (v/v) LPDS, and oxidized LDL was added (loading conditions are described further below).

Nondenaturing Agarose Gel Electrophoresis—Samples (2–4 μl) of LDL were loaded directly onto 1% Universal Agarose gels (Ciba-Corning) and subjected to electrophoresis in Tris-barbitone buffer (pH 8.6, 0.1% sodium barbitone (BDH) at 90 V for 45 min). LDL was visualized by staining with Fat Red 7B in methanol. LDL of the same preparation from the same donor that had not undergone oxidation or acetylation as used as a reference. The relative electrophoretic mobility of OxLDL was calculated using the distance traveled by the reference LDL. Satisfactory oxidation was confirmed if the relative electrophoretic mobility was >3.

Lipid-loading and Sterol Efflux from Macrophages—Adherent peritoneal macrophages were incubated in DMEM containing 10% (v/v) LPDS, 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, and OxoLDL (25 μg protein/ml) at 37 °C for 24 h. Macrophages loaded with OxoLDL were then washed in ice-cold PBS, lysed and extracted, or washed with warm PBS and incubated for a further 24 h in 2 ml of DMEM (with penicillin, streptomycin, and glutamine as specified above) containing 1.0 mg/ml bovine serum albumin (BSA; essentially fatty acid free; Sigma) with or without 25 μg/ml apo A-I or 1.0 mg/ml hp-β-CD (Aldrich) (efflux medium). Confirmatory experiments were also performed using several preparations of hp-β-CD supplied by Cyclobase (Budapest, Hungary). Efflux media were sterilized through 0.2-μm pore size filters before adding to cells. Cells and media were then separately extracted and analyzed for oxidized and unoxidized cholesterol and cholesteryl esters by HPLC.

Cells were lysed by incubation in ice-cold 0.2 M NaOH for 15 min at 4 °C; then 0.4 ml of lysate was mixed with 0.6 ml of PBS, butylated hydroxytoluene (final concentration 2 μM, and EDTA (final concentration, 200 μM) and extracted into methanol (3 ml) and then by 100 ml as described previously (9). Cell extracts were stored at −80 °C until analysis which was usually performed within 7 days. 1.5 ml of efflux medium were removed and spun in an Eppendorf centrifuge at 4 °C for 2 min at 14,000 rpm (16,000 × g) to remove any cellular debris. One ml of the supernatant was mixed with 20 μl butylated hydroxytoluene, 2 mM EDTA, and 10 μl of trifluoroacetic acid and was extracted into methanol/hexane. Preliminary experiments had shown that the cholesterol standards were poorly resolved from DMEM containing BSA, but that this could be overcome (with 100% extraction efficiency) by the addition of trifluoroacetic acid. Both internal and external cholesterol benzoate and standards of analyzed compounds were confirmed to be extracted with 100% efficiency using this system, and values of cell and medium extracts were corrected for extraction of internal standard. Samples were stored at −80 °C until analysis by HPLC.

Lipid Analysis by Reversed Phase HPLC—Eight ml of each hexane extract were evaporated to dryness under vacuum, and the sample was redissolved in appropriate mobile phase for each reversed phase HPLC separation. Two analyses were performed for each hexane extract, one at 210 nm (unoxidized lipids) and one at 234 nm (oxidized lipids). Samples were injected using a Shimadzu SIL-10A autoinjector. Unoxidized cholesterol and cholesteryl esters were separated at room temperature on a C18 column (Supelco; 25-cm length, 0.46-cm diameter, 5-mm particle size and 0.5-cm guard column), using an eluent of acetoni-trile/isopropl alcohol (water (44/56, 2/v/v) as solvent and detected at 234 nm (0.2%)). All solvents used were of HPLC grade (Mallinkrodt). Quantities of sterols and sterol esters were expressed as nmol per mg of cell protein, and were calculated using standard curves established for individual lipids.

Protein Estimation—The protein content of LDL samples and cell lysates was measured using the bichoninic acid method (Sigma) using BSA as standard. Samples were incubated for 60 min at 60 °C, and the absorbance at 562 nm was determined. All samples were assayed in duplicate or triplicate for each culture dish.

Preparation of Human Apolipoprotein A-I— Purified, LCAT-free, human apolipoprotein A-I (apo A-I) was isolated, delipidded and lyophilized as described previously (24). Lyophilized apo A-I was stored at −20 °C until reconstitution in 5 mM guanidine hydrochloride (Sigma) and dialysis against 5 × 1 L 10 mM Tris Buffer (containing 150 mM NaCl, 0.05 g/liter EDTA, and 0.06 g/liter NaNO3) over 5 days at 4 °C. The purity of the reconstituted apo A-I was confirmed by SDS-polyacrylamide gel electrophoresis by detection of a single band with a molecular weight of 28,000. Reconstituted apo A-I was sterilized through a 0.2 μm pore size filter and stored at 4 °C until use.

Cell Viability—Cell viability was routinely assessed by light microscopic morphology, by estimation of cell protein, and by absence of cell staining with 0.5% trypan blue (Life Technologies, Inc.) after 2 min of incubation. Counting was performed from at least two high power fields in representative dishes.

Physical Solubilization of Sterols by hp-β-CD—Stock solutions of cholesterol and 7KC in ethanol were added to PBS (final ethanol concentration 1% v/v) to give a final sterol concentration of 0.05 μM. After 15 min at room temperature, hp-β-CD was added to the turbid sterol suspension from 10 μM hp-β-CD stocks in PBS, at concentrations of 0–5 mM (final volume, 3 ml). In selected experiments, the concentration of hp-β-CD was increased to 10 mM. After a further 5 min incubation, absorbance at 300 nm was determined as a marker of turbidity. PBS containing matched volumes of ethanol as the analytic solution was used as a blank. The absorbance due to ethanol or hp-β-CD alone was negligible. The above times were chosen on the basis of preliminary kinetic studies of the generation of turbidity and the sterol solubilization by hp-β-CD. Turbidity was linearly related to the concentration of sterol at the concentrations of sterols studied. From graphs of turbidity with respect to hp-β-CD concentration, the concentration of hp-β-CD required for 50% solubilization in turbidity was interpolated.

Presentation of Data—A minimum of three separate incubations were performed for each experiment. Individual calculations were made for each cell culture dish from which cells or efflux media were removed, and results for each experiment are expressed as the mean ± S.D. of triplicate incubations. All experiments described are representative of several.

RESULTS

Comparative Efflux of 7-Ketocholesterol and Cholesterol— Medium containing only BSA did not cause significant efflux of either cholesterol or 7KC from OxoLDL-loaded macrophages (Fig. 2 and Table I). The inclusion of 5–10% hp-β-CD led more efflux of 7KC and cholesterol than BSA, but the percentage of 7KC effluxed to apo A-I was much lower than that of cholesterol (see Table I). In contrast, hp-β-CD (1.0 mg/ml) much
more effectively induced the release of 7KC as compared with cholesterol, even though the available intracellular pool of 7KC was much smaller than that of cholesterol (as is evident in the molar ratios of cholesterol to 7KC in cells). Efflux to apo A-I of both cholesterol and 7KC from mouse peritoneal macrophages is maximal at apo A-I concentrations of &lt;25 μg/ml (9, 19, 25). As indicated below, efflux to hp-β-CD increases with concentrations &gt;1.0 mg/ml.

Preliminary studies using gas chromatography, as described previously (5), with mass spectrometric detection confirmed efflux of 7KC and cholesterol as described above and also detected enhanced efflux of 7β-hydroxycholesterol (7β-OH) and 7a-hydroxycholesterol (7a-OH) from OxLDL-loaded macrophages in the presence of hp-β-CD (7β-OH and 7a-OH are not detected by the HPLC procedures used in the present study). For each nanomol of 7a-OH exported to BSA, 1.0 nmol was exported to BSA-hp-β-CD; while for 7β-OH, for each nanomol exported to BSA, 2.0 nmol were exported to apo A-I and 26 nmol were exported to BSA-hp-β-CD. In the presence of BSA-hp-β-CD, 2.3 nmol of 7β-OH and 0.11 nmol of 7a-OH were exported per nmol of cholesterol exported. These data demonstrate preferential efflux of both 7β-OH and 7a-OH compared with cholesterol in the presence of hp-β-CD (allowing for basal cholesterol efflux in the presence of control medium BSA) and, together with the above data regarding efflux of 7KC, suggest generally superior efflux of oxysterols to hp-β-CD than to BSA or apo A-I.

Kinetic Studies of 7-Ketocholesterol Release—Kinetic studies showed that the release of 7KC from OxLDL-loaded cells to hp-β-CD was essentially complete in 4 h whereas release to apo A-I was linear over 24 h (Fig. 2). The maximal rates of removal of 7KC by 25 μg/ml apo A-I and 1 mg/ml hp-β-CD were 8.4 &times; 10⁻² and 4.1 nmol/mg/h, respectively, emphasizing the efficiency of action of the cyclodextrin. The data of Table I indicate that the molar ratio of 7KC to hp-β-CD in efflux medium of cells exposed to 1 mg/ml hp-β-CD reaches ~1:1000 (730 μM hp-β-CD and 0.6 μM 7KC at 24 h) and are similar to those seen by others for efflux of cholesterol at higher concentrations (2–10 mM) of cyclodextrin (18).

To establish whether the release of 7KC was principally limited by the available hp-β-CD concentration or by cellular factors, macrophages were incubated with control medium or 1 mg/ml hp-β-CD and then exposed to fresh control medium or hp-β-CD at 4 and 8 h for a total exposure of 12 h (Fig. 3). There was substantial efflux of 7KC to hp-β-CD in each successive period. When hp-β-CD was withdrawn at 4 or 8 h, no additional efflux occurred, precluding nonspecific leakage of 7KC to control medium following initial exposure of cells to cyclodextrin.
Cyclodextrin-mediated Efflux of 7-Ketocholesterol

**Effect of Cyclodextrin Concentration on Sterol Efflux and Cell Viability**—In Table I, efflux of cholesterol to control medium containing only BSA is hardly exceeded by that achieved with 1 mg/ml hp-β-CD and BSA (2.30 ± 0.80 versus 2.93 ± 1.03 nmol/mg cell protein, respectively). The comparative effects of increasing hp-β-CD concentration on cholesterol and 7KC efflux were determined (Fig. 4). Efflux of both cholesterol and 7KC increased as concentrations increased up to 10 mg/ml, but this highest concentration was toxic to cells after 24 h. At concentrations up to 5 mg/ml, viability was always >90% (co-

**Mobilization of Intracellular Oxysterol Esters by Cyclodextrin-mediated Oxysterol Efflux**—We have previously found that 7-ketocholesterol oleate and palmitate are the most abundant detectable esters of 7KC in OxLDL-loaded macrophages (5, 9). The mobilization of intracellular 7KC and 7-ketocholesterol oleate and palmitate in response to efflux of 7KC was investigated. As demonstrated above, most efflux of 7KC to hp-β-CD occurred within 4 h, and cell viability was preserved at hp-β-CD concentrations ≤5.0 mg/ml. Because shorter incubations with cyclodextrins are associated with less cell toxicity (18), in the experiments described below, OxLDL-loaded macrophages were exposed to ≤5.0 mg/ml hp-β-CD for 4 h. As plasma membrane cholesterol concentrations rise or fall, there is movement and redistribution of cholesterol to other cellular compartments and activation or inhibition of cellular acyl CoA:cholesterol acyltransferase and neutral cholesteryl ester hydrolase (26–28). To permit sufficient time for equilibration of intracellular lipid pools, after exposure of cells to hp-β-CD for 4 h, cells were washed and incubated in BSA for 24 h before analysis. Although there was no apparent decline in cholesteryl esters, there was a clear concentration-dependent decline in intracellular 7-KC esters as well as free 7KC following incubation with these concentrations of hp-β-CD (Fig. 5). Both esterified and unesterified intracellular 7KC decreased, even though only unesterified 7KC was released into the medium. A lack of decline in cholesteryl esters (not shown) was associated with a much lesser decline in cell-free cholesterol than in cell-free 7KC (Fig. 5B). This implies that at least to some degree, the extent of free sterol efflux caused by hp-β-CD is related to the extent...
Cyclodextrin-mediated Efflux of 7-Ketocholesterol

Fig. 5. Decline in cellular 7-ketocholesterol and 7-ketocholesterol esters during cyclodextrin-mediated efflux. OxLDL-loaded macrophages were incubated for 4 h with DMEM containing BSA and specified concentrations of hp-β-CD; then cells were washed and incubated for 24 h in DMEM containing BSA alone to allow for equilibration of intracellular sterol pools. Cells were lysed, extracted into hexane, and analyzed by HPLC as indicated under "Materials and Methods." A, 7-ketocholesterol ester; B, 7-ketocholesterol. All results calculated as percentage of sterol mass remaining in cells after incubation with BSA alone for entire 28 h (mean ± S.D.).

DISCUSSION

OxLDL and oxysterols in particular may contribute to various component processes of atherogenesis. These include monocyte recruitment, cell toxicity, endothelial dysfunction, and inhibition of cholesterol efflux (2, 9, 10, 12, 29, 30). Facilitating the removal of oxysterols may therefore be important for reversing or limiting these consequences in vivo. These data are the first to identify that hp-β-CD removes 7KC from OxLDL-loaded macrophages, thereby overcoming the deficient efflux of 7KC mediated by apo A-I. They have also identified a relative selectivity of hp-β-CD in removing 7KC in preference to cholesterol and demonstrate that the efflux of oxysterols (such as 7KC) by cyclodextrins promotes the mobilization of intracellular oxysterol esters.

Cyclodextrins function as encapsulating and solubilizing agents for hydrophobic compounds. Cyclodextrin-mediated cellular sterol efflux would therefore be expected to be independent of binding to cellular proteins such as high density lipoprotein-binding proteins (31) but would more probably arise from the capacity of these agents to solubilize sterols derived from the plasma membrane. The diffusion of sterols from the plasma membrane across the unstirred water layer is rate limiting for efflux (28). We hypothesized that limited solubilization of 7KC may restrict its diffusion from OxLDL-loaded macrophages to apo A-I and have demonstrated that hp-β-CD enhances efflux of 7KC at least in part by enhancing its solubility.

It is well known that oxysterols, including 7KC, desorb into the aqueous phase from lipid layers such as the cell surface, more rapidly than does cholesterol. For example, this is expressed in the more rapid movement of 25-hydroxycholesterol than cholesterol from red blood cells to plasma lipoproteins (32) and by the faster transfer of oxysterols than cholesterol between phospholipid liposomes (33). Consequently, if cholesterol and 7KC were to associate with the cyclodextrin cavity with equal affinity, the potential for faster desorption of 7KC could explain the more rapid accumulation of 7KC than of cholesterol in cell culture media containing hp-β-CD. Since apo A-I induces less efficient release of 7KC than cholesterol from macrophages, it is apparent that factors other than desorption are important in regulating efflux of 7KC from OxLDL-loaded macrophages. In the case of hp-β-CD, we assessed the possibility that an additional crucial factor might be the solubilization of sterols, as must occur subsequent to desorption for net mass efflux to be observed, lest the sterol resorb to the cell membrane. We demonstrated that hp-β-CD far more efficiently solubilized 7KC from aqueous suspension in comparison with cholesterol, an observation that paralleled its more effective induction of cellular efflux of 7KC than of cholesterol. Apo A-I did not induce demonstrable solubilization of sterols in aqueous
cholesteryl esters in OxLDL-loaded and 7KC-AcLDL-loaded guest molecules, it is not surprising that even higher ratios of are substantially greater than that by cholesterol. In the complex system of J774 cells (37). However, the effect of cyclodextrin-derivatives has the potential to prevent the inhibition of sterol efflux of 7KC and other oxysterols from macrophages. This implies differential affinity for the cyclodextrin cavity for different sterols in aqueous solution, but these data also suggest that simple solubilization does not readily explain the sterol-binding properties of apo A-I.

We have observed that cells and hp-β-CD reach an equilibrium with respect to efflux of 7KC quickly but not instantaneously, consistent with the data of Kilsdonk et al. (18) for cholesterol efflux. This equilibrium did not result in nonspecific leakage of sterols from the cells following defined short incubations with hp-β-CD, but it required the ongoing supply of cyclodextrin. Physicochemical complexes between cholesterol and β-cyclodextrins vary in stoichiometry from 1:1 to 1:3 (34, 35), but near complete sequestration of a sterol or other "guest" by cyclodextrins in solution is usually achieved only at high cyclodextrin-guest concentration ratios (≥50:1), even with preferred guests such as phenolphthalein (36). Our solubilization and cell data indicate that guest occupancy of hp-β-CD by 7KC is substantially greater than that by cholesterol. In the complex system involving intact cells and many different potential guest molecules, it is not surprising that even higher ratios of hp-β-CD to sterol are observed. More selective and efficient acceptors of 7KC derived from hp-β-CD may lower this ratio.

Most important is our observation that intracellular 7-ketocholesterol esters in OxLDL-loaded and 7KC-AcLDL-loaded cells decrease in response to free 7KC efflux to hp-β-CD. This indicates that synthetic sterol-capturing agents such as cyclodextrins can cause net hydrolysis of intracellular esters simply by accepting sterols from the cell membrane. For oxysterols in particular, this fact is novel in confirming that oxysterol efflux almost certainly promotes the hydrolysis of oxysterol esters by cytosolic hydrolase, in a manner analogous to that described by apo A-I and high density lipoprotein for cholesterol esters. Thus, at least for oxysterols such as 7KC, binding of apolipoproteins to specific cell protein receptors is not essential for some mobilization of intracellular esters. In addition, the extent of decline in detectable cell unesterified 7KC and 7KC ester in response to incubation with hp-β-CD implies that much intracellular 7KC in OxLDL-loaded mouse peritoneal macrophages is not sequestered lysosomally, as had been suggested in J774 cells (37). However, the effect of cyclodextrin-mediated efflux on other unquantified pools of steryl esters (5) requires further investigation.

Presently understood hp-β-CD plasma-membrane interactions (15, 17) suggest that hp-β-CD promotes the release of 7KC without partitioning into the plasma membrane. The capacity of synthetic agents such as hp-β-CD to overcome deficient oxysterol efflux may facilitate oxysterol transfer to lipoproteins. In vivo oxysterol efflux by hp-β-CD or its derivatives has the potential to prevent the inhibition of sterol efflux and cytotoxicity caused by oxysterols and could facilitate the reversal of the foam cell phenotype by normal reverse cholesterol transport mechanisms.

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