

Cyclodextrins differentially mobilize free and esterified cholesterol from primary human foam cell macrophages

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Abstract Human monocyte-derived foam cell macrophages (HMFCs) are resistant to cholesterol efflux mediated by physiological acceptors. The role of the plasma membrane in regulating depletion of free cholesterol (FC) and of cholesteryl ester (CE) was investigated using cyclodextrins (CDs). HMFCs were incubated in media containing CDs (1.0 mg/ml, ~0.7 mM) with low [hydroxypropyl- β -CD (HP-CD)] or high [trimethyl- β -CD (TM-CD)] affinity for cholesterol in the presence or absence of phospholipid vesicles (PLVs). Low-affinity HP-CD caused minimal cholesterol efflux on its own, but HP-CD+PLV depleted cell FC and CE to $54.5 \pm 6.7\%$ of control by 24 h. TM-CD depleted FC at least as well as HP-CD+PLV but without depleting CE, even when combined with PLV. This was not explained by acceptor saturation, instability of PLV vesicles, de novo cholesterol synthesis, kinetically distinct cholesterol pools, or inhibition of CE hydrolysis. TM-CD did, however, deplete CE when lower concentrations of TM-CD were combined with PLV and when acetyl-CoA cholesteryl acyltransferase was inhibited. TM-CD caused much greater depletion of plasma membrane cholesterol than HP-CD without depleting plasma membrane sphingomyelin. It is concluded that differential depletion of plasma membrane cholesterol pools regulates cholesterol efflux and CE clearance in human macrophages.—Liu, S. M., A. Cogy, M. Kockx, R. T. Dean, K. Gaus, W. Jessup, and L. Kritharides. Cyclodextrins differentially mobilize free and esterified cholesterol from primary human foam cell macrophages. *J. Lipid Res.* 2003. 44: 1156–1166.

Supplementary key words cholesterol efflux • high density lipoprotein • atherosclerosis • plasma membrane

Human monocyte-derived foam cell macrophages (HMFCs) containing lipoprotein-derived free cholesterol (FC) and cholesteryl ester (CE) are a hallmark of atherosclerosis. In peripheral cells such as macrophages, excess accumulated cholesterol can be released into aqueous medium after conversion to more polar metabolites (for example, to 27OH-cholesterol) or released to a cholesterol acceptor

such as HDL or apolipoprotein A-I (apoA-I) (1–3). Data from in vitro cell culture systems suggest that sterol release after metabolism is small relative to that released to cholesterol acceptors (4). The eventual delivery of cholesterol from the peripheral tissues to the liver for clearance in the bile is referred to as the reverse cholesterol transport pathway. This is recognized as one likely route by which HDL protects against the development of atherosclerosis.

The removal of cholesterol from cells has been broadly categorized as involving several processes: aqueous diffusion, lipid-free apolipoprotein membrane microsolvubilization (5), and interaction with proteins (“receptors”) located on the cell plasma membrane. As examples of the last of these, clearance of cholesterol from foam cells to HDL and its main protein constituent, apoA-I, have been identified as requiring interactions with the scavenger receptor type I (SR-BI) and the ATP-binding cassette transporter ABCA-1 protein, respectively (6–10). However, aqueous diffusion and acceptor-cell protein interactions are not mutually exclusive. For example, efflux to phospholipid vesicles (PLVs), which can accept cholesterol after its desorption into the aqueous layer, is enhanced from cells expressing SR-BI (11).

Data from several laboratories indicate that human macrophages are resistant to cholesterol efflux, at least in part due to slow mobilization of stored CEs by hydrolysis (12–14). In addition, as the flux of cholesterol through all cellular compartments is slow in human THP-1 macrophages (14), factors other than CE hydrolysis may also be limiting. Important earlier studies indicated that variations in rates of cholesterol efflux between different cell types and or different species were largely explained by

Abbreviations: CD, cyclodextrin; CE, cholesteryl ester; FC, free cholesterol; HMFC, human monocyte-derived foam cell macrophage; HP-CD, hydroxypropyl- β -CD; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PLV, phospholipid vesicles; SPM, sphingomyelin; TM-CD, trimethyl- β -CD.

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differences in rates of efflux from the plasma membrane (15, 16). The processes that link the concentration of cholesterol in the plasma membrane with that of intracellular CE are complex (17–20), but studies generally indicate a close relationship between the cholesterol concentration of the plasma membrane and the mobilization of intracellular cholesterol.

Cyclodextrins (CDs) are cyclical oligomers of six, seven, or eight glucose molecules that can solubilize hydrophobic molecules such as cholesterol by virtue of their hydrophobic interior. Because CDs interact with the cell plasma membrane without internalization and do not disrupt membranes at low concentrations (and are thus unlike detergents) (21), they are useful for elucidating the role of plasma membrane pools of cholesterol in determining net cholesterol efflux and intracellular cholesterol metabolism (22, 23). In β -CDs, which contain seven glucose molecules and most avidly bind cholesterol, the modification of CD side chains regulates their affinity for cholesterol. This determines the concentration at which CDs can cause effective cholesterol depletion from cells. CDs such as hydroxypropyl- β -CD (HP-CD) and trimethyl- β -CD (TM-CD) used at low concentrations mediate the removal of cholesterol and oxysterols from foam cells and from isolated membranes without toxicity and apparently without significant removal of cellular phospholipids or membrane proteins (21, 22, 24, 25).

CDs can cause the net removal of cholesterol from cells in one of two ways (26). First, they can accept cholesterol from the plasma membrane and retain it in the extracellular medium, acting as depots or “sinks.” Second, at lower concentrations insufficient to alter net equilibrium between cells and medium, they can remove cholesterol from the plasma membrane and deliver it to a second acceptor of larger capacity but slower cholesterol efflux efficiency, such as PLV. In this instance, CDs act as vehicles or “shuttles” between cells and the terminal cholesterol sink (PLV). The latter process has also been identified in the removal of cholesterol by relatively small HDLs to large PLVs (27) and may apply to many other lipoproteins (28). Removal of cellular cholesterol by CDs by either mechanism permits investigation of the role of plasma membrane in net cholesterol efflux.

We have investigated cholesterol efflux from HMFC using high-affinity TM-CD (“sink”) and low-affinity HP-CD in concert with PLV (HP-CD+PLV “shuttle”) and identify the plasma membrane as a major rate-limiting site for cholesterol efflux and net CE clearance from HMFC. Significantly, we identify that depletion of cellular FC and CE are not necessarily linked. High-affinity TM-CD dissociates the clearance of FC and CE, and only HP-CD+PLV consistently depletes intracellular CE.

MATERIALS AND METHODS

Materials

Egg yolk phosphatidylcholine (PC), penicillin-streptomycin, essentially fatty-acid free BSA, and silica gel on polyester thin-

layer chromatography (TLC) plates were from Sigma Aldrich. β -CDs were from Cyclolabs (Hungary) and Sigma Aldrich. RPMI-1640 medium and glutamine were obtained from Gibco, and tissue culture plates and consumables were purchased from Falcon. [3 H]cholesterol (1 mCi/ml, specific activity 44 mCi/ μ mol) and 1-3-phosphatidyl [N-methyl- 3 H]choline,1,2-dipalmitoyl (1 mCi/ml, specific activity 83.0 mCi/ μ mol) were purchased from Amersham. Solvents were purchased from EM Science. Sephadex G50 beads were from Amersham. Buffy coats and whole human sera from normal donors were supplied by the Red Cross blood bank of NSW. Acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor Sandoz 58035 (S-58035) was a gift from Drs. Nordmann and Nadelson at Sandoz Pharmaceuticals. Ficoll was supplied by Pharmacia and Nycodenz by Nycomed.

Preparation and modification of lipoproteins

Human LDL (1.019 < d < 1.063 g/ml) and lipoprotein-deficient serum (LPDS) were isolated by sequential ultracentrifugation from normolipidemic blood donors as described (29, 30). LDL was dialyzed against phosphate-buffered saline (PBS) containing EDTA (1.0 mg/ml) and chloramphenicol (0.1 mg/ml), and sterilized by 0.45 μ m filtration. Acetylation of LDL (AcLDL) was performed as described (31).

Culture and loading of cells

Human monocytes (>95% pure by nonspecific esterase staining) were isolated by centrifugal elutriation from fresh white blood cell concentrates and plated at $1.5\text{--}2.0 \times 10^6$ cells per 22 mm-diameter culture dish in RPMI-1640 media containing penicillin and streptomycin (50 U/ml and 50 μ g/ml, respectively) at 37°C as described (14, 32, 33). Monocytes were incubated for an initial 1.5 h to establish adherence, and then the medium was exchanged with fresh RPMI-1640-containing 10% heat-inactivated whole human serum. On Day 9, the resultant human monocyte-derived macrophages were incubated with RPMI-1640 containing 10% (v/v) LPDS and 50 μ g/ml AcLDL for 48–96 h to achieve enrichment with FC and CEs typical of foam cell macrophages.

Metabolic labeling

In studies using [3 H]cholesterol-labeled HMFC, [3 H]cholesterol (1 mCi/ml, specific activity 44 mCi/ μ mol) was incorporated into AcLDL as described (14) before incubation in RPMI-1640 at a final concentration of 50 μ g/ml AcLDL and 2 μ Ci/ml [3 H]cholesterol. After 48 h loading, the cells were washed and incubated overnight in RPMI-1640 containing 0.5% (w/v) BSA to allow equilibration of intracellular [3 H]cholesterol prior to efflux incubations described below.

In order to analyze [3 H]choline-labeled phospholipids [lysophosphatidylcholine (LPC), PC, and sphingomyelin (SPM)], cholesterol-enriched HMFCs were washed with PBS and incubated in RPMI-1640 containing [3 H]choline chloride in ethanol (3 μ Ci/ml) and 0.1% BSA for 20 h. After incorporation, cells were washed in PBS and equilibrated for 1 h in RPMI containing 0.1% BSA, then washed before undertaking efflux incubations (34).

Cholesterol efflux

PLVs were prepared from egg PC by repeated sonication and extrusion as described (35, 36), and incubated with HMFC in RPMI-1640 at concentrations described (typically between 100–200 μ g phospholipid/ml). CDs were dissolved in RPMI-1640 and generally used at 1.0 mg/ml of CD. Where PLVs containing [3 H]PC were used, 40 mg of PC (400 μ l) was combined with 50 μ l of phosphatidyl [N-methyl- 3 H]choline (1 mCi/ml, 83.0 mCi/ μ mol) prior to preparation of PLV.

After loading with AcLDL, HMFCs were washed then incubated in 1 ml of efflux medium at 37°C. Efflux media contained

either RPMI-1640 alone (± 1.0 mg/ml BSA essentially fatty acid free, Sigma) or RPMI-1640 and PLV, RPMI-1640 and HP-CD \pm PLV, or RPMI-1640 and TM-CD \pm PLV. These media were incubated with HMFC for up to 24 h, at which time cells and media were extracted. Identical efflux and viability data were obtained with and without BSA in efflux media.

To determine mass efflux of cholesterol, both cells and media were extracted at specified times (up to 24 h). Aliquots of media were collected and centrifuged at 16,000 *g* for 15 min to pellet any cell debris prior to extraction of lipids. Six hundred microliters of the supernatant was extracted with 2.5 ml methanol and 5.0 ml hexane, and the hexane layer analyzed by HPLC as described below (31). HMFC monolayers were washed with PBS and lysed with 600 μ l of ice-cold 0.2 M NaOH, and lipids were extracted from 400 μ l aliquots of this lysate into methanol and hexane.

Mass cholesterol efflux was expressed as nmol cholesterol/culture, nmol/mg cell protein, or percent efflux (medium cholesterol divided by the sum of medium cholesterol and cellular cholesterol and CE in each culture). To quantify progressive efflux of [3 H]cholesterol in kinetic studies, aliquots (100 μ l) of efflux medium were removed at specified times, centrifuged to remove nonadherent cells and debris, 80 μ l aliquots analyzed by scintillation counting, and cumulative efflux expressed as a percentage of total radioactivity present in cells extracted at t_0 (14).

Lipid analysis

Four milliliters of the upper hexane phase was collected, evaporated, and redissolved in HPLC mobile phase comprising acetonitrile-isopropanol-water (44:54:2, v/v/v) or acetonitrile-isopropanol (30:70, v/v) for quantification of FC and CE, respectively (25, 30). Extraction of efflux media in all experiments established that CEs were not released into the media, confirming that cellular CE decline was not attributable to cell lysis.

For HMFC radiolabeled with [3 H]cholesterol, evaporated hexane extracts were redissolved with acetonitrile-isopropanol-water (44:54:2, v/v/v), and aliquots taken for HPLC analysis, scintillation counting, and TLC separation of [3 H]FC and [3 H]CE. Total [3 H]cholesterol in the cells was determined by scintillation counting of 10 μ l of NaOH lysate. Samples and standards were dried and redissolved in chloroform and spotted onto TLC plates, and FC and CE were separated with hexane-ether-glacial acetic acid (46:7:20:1, v/v/v). The proportion of label in [3 H]FC and [3 H]CE fractions was determined after separation by TLC using standards of FC and CE identified with 10% CuSO₄ in H₃PO₄ at 100°C.

Phospholipids were analyzed after Bligh and Dyer extraction (37) of PLV, media, cells, and cell plasma membrane fractions (see below). Phospholipid mass was determined as total phosphorous (38). Total [3 H]phospholipids in each sample were determined by scintillation counting and the percentage distribution of phospholipid classes in media or cell plasma membrane fractions determined by TLC using silica-coated glass plates (5, 34). Phospholipid classes (PC, SPM, and LPC) were separated using chloroform-methanol-ammonium hydroxide (75:25:4, v/v/v) using authentic standards of PC, SPM, and LPC. Phospholipid bands were visualized by iodine vapor, scraped, extracted in methanol, and counted by scintillation. The ratio of [3 H]PC-[3 H]SPM-[3 H]LPC on TLC was related to total [3 H]phospholipid counts to derive total counts of each phospholipid per milligram of total cell protein or per milligram of plasma membrane protein as indicated.

Subcellular fractionation and isolation of plasma membranes

HMFCs were washed and scraped from culture dishes, then homogenized by shear force as previously described (39, 40). Cell

lysates were layered on a 1–22% Ficoll gradient over a 45% Nycodenz cushion and centrifuged in a VTi 65.2 rotor (Beckman) at 50,000 rpm at 10°C for 90 min. Twenty-six 100 μ l fractions were collected from the bottom of each tube, fractions 15–20 corresponding to plasma membranes (40), and were confirmed by plasma membrane marker enzymes alkaline phosphatase and (Na⁺, K⁺)-ATP-ase. Using this fractionation contamination with other organelles is minimal [$<6.5\%$ endoplasmic reticulum (ER) contamination assessed by glucose 6 phosphatase, and $<10\%$ Golgi contamination assessed by UDP galactose transferase]. After isolation, aliquots of plasma membrane fractions were removed for determination of protein before the remainder was subjected to Bligh and Dyer extraction and analyzed for cholesterol mass or phospholipids (see above).

Protein estimation and cell viability

The protein content of cell lysates and of LDL samples was determined in triplicate for each cell culture or sample using the bicinchoninic acid method (Sigma) with BSA as standard (29, 32). Cell viability was assessed by light microscopy, preservation of cell protein, measurement of lactate dehydrogenase release (41), and by cellular exclusion of trypan blue (32).

Data analysis

A minimum of three separate incubations were performed for each condition in each experiment, the results of which are expressed as mean \pm SD of triplicate cultures, and all experiments described are representative of several, unless otherwise indicated. Where data from multiple experiments are pooled, results are expressed as mean \pm SEM of “n” experiments. Simple comparisons of two independent samples used unpaired Student’s *t*-test, and for multiple comparisons, one-way ANOVA with Bonferroni correction for multiple comparisons was used. In both instances, $P < 0.05$ was considered significant.

RESULTS

Side-chain substitutions modulate CD-mediated cholesterol efflux from HMFC

TM-CD and HP-CD substitutions of β -CD were directly compared for ability to induce cholesterol efflux from HMFC at 1.0 mg/ml (i.e., 0.70 mM TM-CD and 0.65 mM HP-CD) and compared with PLV (Fig. 1). Whereas HP-CD caused only minor cholesterol efflux, both TM-CD and PLV stimulated cholesterol efflux 2- to 3-fold. This differential efflux of cholesterol from HMFC to HP-CD and TM-CD is consistent with their known respective affinities for cholesterol (22), and indicates that at these concentrations TM-CD is superior to HP-CD as a cholesterol sink.

TM-CD achieves less cholesterol efflux than HP-CD when acting as a cholesterol shuttle

TM-CD and HP-CD were directly compared for their ability to induce cholesterol efflux in the presence of PLV (Fig. 1). Efflux to HP-CD+PLV was clearly greater than the sum of efflux to HP-CD and PLV added separately.

Whereas TM-CD induced more cholesterol efflux than HP-CD in the absence of PLV, TM-CD+PLV induced less efflux than HP-CD+PLV ($38.1 \pm 4.7\%$ vs. $52.9 \pm 5.3\%$, $P < 0.001$). TM-CD+PLV induced more cholesterol efflux than either TM-CD or PLV, but the effect was only approximately additive for efflux to TM-CD and PLV, not syner-

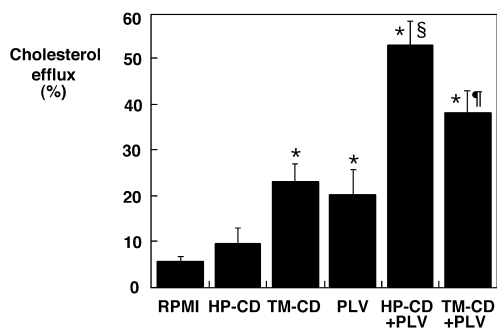


Fig. 1. Efflux to trimethyl- β -CD (TM-CD) and hydroxypropyl- β -CD (HP-CD) in the presence and absence of phospholipid vesicles (PLVs). Human monocyte-derived macrophages (HMDMs) were loaded with 50 μ g/ml acetylation of LDL (AcLDL) for 96 h [human monocyte-derived foam cell macrophages (HMFC)] and then incubated with fresh efflux media for 24 h. Efflux media contained RPMI-1640 alone (RPMI), 0.65 mM HP-CD in RPMI-1640 (HP-CD), 0.70 mM TM-CD in RPMI-1640 (TM-CD), 200 μ g/ml PLV, 0.65 mM HP-CD + 200 μ g/ml PLV (HP-CD+PLV), or 0.70 mM TM-CD + 200 μ g/ml PLV (TM-CD+PLV) in RPMI-1640. After 24 h, media were collected and lipids analyzed by HPLC, and cholesterol efflux was calculated relative to total cell cholesterol at time 0 as described in Materials and Methods. Data points represent mean \pm SEM of at least four independent experiments for each condition. Conditions marked * significantly differ from RPMI ($P < 0.001$) and HP-CD ($P < 0.01$); † and § significantly differ from PLV ($P < 0.001$); and § significantly differs from TM-CD+PLV ($P < 0.001$).

gistic. This suggests differences in the mechanisms by which TM-CD and HP-CD interact with macrophages, and indicates that at these concentrations TM-CD is superior as a cholesterol sink, but HP-CD is superior as a cholesterol shuttle.

Efflux of cholesterol is dependent on the concentrations of both HP-CD and PLV

To investigate conditions optimal for cholesterol efflux to HP-CD+PLV, the concentrations of PLV and HP-CD were systematically varied. In isolation, HP-CD at concentrations between (0.0065–0.65 mM) did not achieve significant efflux, but each of these concentrations facilitated increased cholesterol efflux when PLVs (200 μ g/ml) were present. At each concentration of HP-CD tested, efflux in the presence of PLV was greater than the sum of efflux to HP-CD and PLV added separately. PLV alone at all concentrations achieved modest cholesterol efflux (typically 15–20% over 24 h), but this was substantially increased in the presence of HP-CD to >50% at 24 h (unpublished observations). Increasing the concentration of PLV beyond 0.2 mg/ml did not improve cholesterol efflux to HP-CD+PLV. HP-CD in the amount of 0.65 mM and 0.2 mg/ml of PLV were used as standard combinations in subsequent experiments.

Differential depletion of FC and CE by TM-CD

To investigate why TM-CD \pm PLV failed to achieve as much cholesterol efflux as HP-CD+PLV, cellular lipids were analyzed in more detail. This demonstrated that TM-CD and TM-CD+PLV both depleted FC at least to the

same extent as HP-CD+PLV, but depleted CE much less efficiently (**Fig. 2**). TM-CD (0.7 mM) did not deplete cell CE in the absence of PLV in any experiments. The addition of PLV to TM-CD slightly increased FC depletion (range 0–24.1% greater depletion than TM-CD alone, $n = 5$ experiments), and in some experiments promoted mild CE depletion (range 0–18.5% depletion after 24 h, $n = 5$ experiments). This compares with the effect of HP-CD+PLV, which depleted cell CE by $54.5 \pm 6.7\%$ (mean \pm SEM, $n = 7$ experiments). Increasing PLV to 0.4 mg/ml to exclude possible saturation of PLV did not improve TM-CD-mediated clearance of CE (unpublished observations).

Kinetic studies of cholesterol efflux to TM-CD and HP-CD+PLV

The kinetics of differential depletion of FC and CE by TM-CD were further investigated. The rate of efflux to both TM-CD and HP-CD+PLV was nonlinear, but the rate

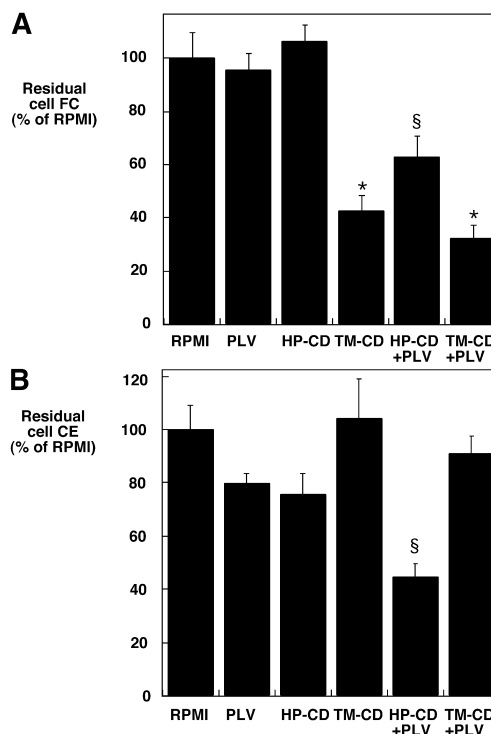


Fig. 2. Synergistic efflux to HP-CD+PLV stimulates depletion of HMFC cholesteryl ester (CE). HMDMs were loaded with 50 μ g/ml AcLDL for 96 h (HMFC), and then incubated with fresh efflux media for 24 h. Efflux media contained RPMI-1640 alone (RPMI), 0.65 mM HP-CD in RPMI-1640 (HP-CD), 0.70 mM TM-CD in RPMI-1640 (TM-CD), 200 μ g/ml PLV (PLV), 0.65 mM HP-CD + 200 μ g/ml PLV (HP-CD+PLV), or 0.70 mM TM-CD + 200 μ g/ml PLV (TM-CD+PLV) in RPMI-1640. After 24 h, cells were collected and lipids analyzed by HPLC as described in Materials and Methods. Residual free cholesterol (FC) and CE data are expressed relative to RPMI value for each experiment, and data points represent mean \pm SEM of at least three independent experiments for each condition. A: Conditions marked * significantly differ from RPMI, PLV, and HP-CD ($P < 0.001$); and condition § significantly differs from RPMI, PLV, and HP-CD ($P < 0.01$). B: Condition § significantly differs from RPMI, TM-CD, TM-CD+PLV ($P < 0.001$), PLV ($P < 0.01$), and HP-CD ($P < 0.05$).

of efflux declined more gradually with HP-CD+PLV, and much greater overall efflux was achieved by 24 h (Fig. 3). Some spontaneous loss of cell FC was observed during incubation with RPMI-1640 alone, as previously reported (42); however, TM-CD and HP-CD+PLV induced much greater depletion of FC than did RPMI-1640. The decline in cell FC induced by CDs was rapid (most occurring within 2–4 h of incubation). Depletion of CE was only detectable between 8 h and 24 h and was only seen with HP-CD+PLV. The time to 50% depletion of CE in the presence of HP-CD+PLV was 26.1 h, which is consistent with that previously described in human macrophages (13, 14). Over a series of experiments, the difference between overall cholesterol efflux to HP-CD+PLV and TM-CD was most marked when HMFC contained relatively large quantities of CE. For example, where the CE pool was large (215.0 ± 35.2 nmol CE/mg cell protein), HP-CD+PLV achieved efflux 3-fold that of TM-CD, and 2-fold that of TM-CD+PLV (24 h efflux of $60.1 \pm 1.4\%$, $16.9 \pm 1.2\%$, and $28.8 \pm 2.3\%$ respectively).

Acceptor saturation does not explain restricted efflux to TM-CD

The large number of TM-CD molecules required per molecule of cholesterol released [$\sim 1,000:1$ (22)] and the inability of PLV to substantially enhance clearance of CE by TM-CD suggested that saturation of TM-CD was not limiting efflux. To further investigate this possibility, we

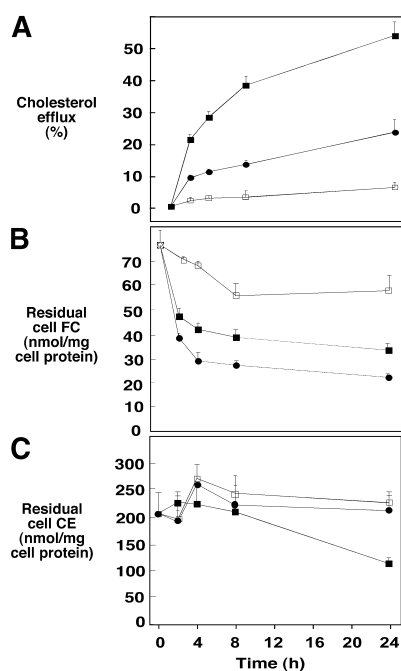


Fig. 3. Time course of cholesterol efflux to HP-CD+PLV and TM-CD. AcLDL-loaded HMFCs were incubated with efflux medium containing RPMI-1640 only (open squares), or with added HP-CD (0.65 mM) + PLV (200 μ g/ml) (closed squares) or TM-CD (0.70 mM, closed circles), for up to 24 h. Media and cells were analyzed for lipids by HPLC, and cholesterol efflux (%) was determined at each time point. A: Percent efflux. B: Residual FC in cell cultures after efflux. C: Residual CE in cell cultures after efflux.

compared the ability of fresh TM-CD \pm PLV with “cell-conditioned” TM-CD \pm PLV (previously incubated for 24 h with HMFC) to stimulate efflux from HMFC (Table 1). There was identical depletion of cell lipids by cell-conditioned and fresh media, indicating that acceptor saturation does not limit cholesterol efflux to TM-CD \pm PLV.

Kinetics of cholesterol efflux to HP-CD+PLV and TM-CD

Efflux of plasma membrane cholesterol is kinetically heterogeneous with fast and slow pools, which we hypothesized could be differentially affected by TM-CD and HP-CD. Initial kinetics of cholesterol efflux were therefore determined in cells prelabeled with [3 H]FC-enriched AcLDL to investigate if TM-CD more rapidly removed plasma membrane cholesterol than did HP-CD. Under these conditions, all cell [3 H]CEs are derived from [3 H]FC and are therefore ACAT generated. Thus, these experiments also allowed us to investigate whether TM-CD and HP-CD+PLV differed in their clearance of ACAT-derived CE.

There was rapid initial efflux of [3 H]cholesterol to both HP-CD+PLV and TM-CD, and at the earliest time point (10 min), HP-CD+PLV removed at least as much cholesterol as did TM-CD (Fig. 4). Efflux to TM-CD was nonlinear, with a declining rate between 10 min and 2 h, whereas efflux to HP-CD+PLV was almost linear over this period, indicating more limited access of cellular cholesterol to TM-CD even at early time points. At 24 h, HP-CD+PLV and TM-CD had depleted cell [3 H]FC to a similar degree, but only HP-CD+PLV depleted cell [3 H]CE, indicating that TM-CD failed to clear ACAT-generated CE. The specific activities (dpm/nmol) of FC and CE were unaffected by efflux incubations, which excludes substantial TM-CD-specific de novo synthesis of unlabeled FC or CE. While it remains possible that small amounts of cholesterol synthesis may occur under these conditions, substantial synthesis is unlikely to be a major contributor to preserved CE in HMFC exposed to TM-CD.

The differential effects of TM-CD and HP-CD+PLV on cholesterol clearance were confirmed by comparing the ratios of intracellular [3 H]FC and [3 H]CE after efflux. The ratio of intracellular [3 H]FC-/[3 H]CE after control medium RPMI-1640 was 0.86 ± 0.12 , after TM-CD was 0.31 ± 0.02 , and after HP-CD+PLV was 0.98 ± 0.02 , indicating preferential clearance of [3 H]FC by TM-CD.

TABLE 1. Saturation does not limit cholesterol efflux to TM-CD or TM-CD+PLV

	Residual Total Cell Cholesterol	
	Fresh Media	Cell-Conditioned Media
	nmol/mg cell protein	
RPMI	103.2 ± 8.9	
TM-CD	84.83 ± 22.8	86.1 ± 14.8
TM-CD+PLV	65.4 ± 13.0	65.1 ± 7.5

PLV, phospholipid vesicle; TM-CD, trimethyl- β -CD. Human monocyte-derived foam cell macrophages (HMFCs) were incubated with TM-CD and TM-CD+PLV for 24 h, generating “cell-conditioned” media containing 2.40 ± 0.04 nmol (TM-CD) and 3.36 ± 0.20 nmol (TM-CD+PLV) cholesterol per milliliter of medium. Cell-conditioned and fresh media containing TM-CD or TM-CD+PLV were then incubated with fresh HMFC for 24 h, and residual cell cholesterol analyzed by HPLC.

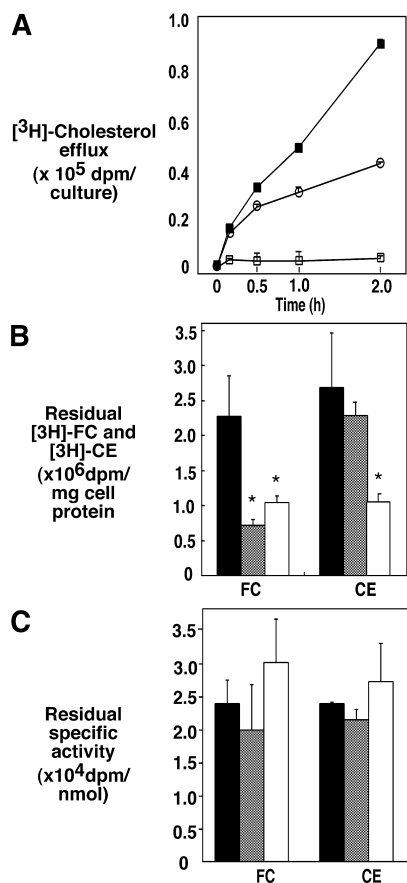


Fig. 4. Effects of HP-CD+PLV and TM-CD on initial efflux of [³H]cholesterol and specific activity of cell [³H]FC and [³H]CE. HMDMs were labeled with [³H]FC-AcLDL (final concentration 50 μg LDL protein/ml containing 2 μCi [³H]FC/ml) for 48 h, equilibrated overnight in BSA-containing RPMI-1640, and then subjected to efflux with RPMI-1640 (open square, A; black bars, B, C), HP-CD (0.65 mM) + PLV (200 μg/ml) (closed square, A; white bars, B, C), or TM-CD (0.70 mM; open circles, A; gray bars, B, C). A: Efflux of [³H]cholesterol between 0–2 h. B: Residual cell [³H]FC and [³H]CE after 24 h efflux (dpm/mg cell protein). C: Specific activity of FC and CE in cells after efflux (dpm FC/nmol FC and dpm CE/nmol CE, respectively). At the end of equilibration, before efflux (at t₀), specific activities of intracellular lipids were 2.26 ± 0.66 × 10⁴ dpm FC/nmol FC and 2.64 ± 0.38 × 10⁴ dpm CE/nmol CE. At 24 h, the percentage of efflux to each condition was RPMI 4.2 ± 0.9%, TM-CD 21.8 ± 2.65%, and HP-CD+PLV 40.9 ± 1.3%. B: Conditions marked * significantly differ from RPMI (*P* < 0.01).

The concentration of TM-CD can affect apparent synergistic cholesterol efflux and depletion of cell CE

TM-CD had achieved synergistic cholesterol efflux in previous studies using low concentrations in short-term incubations of several hours duration (43). We hypothesized that if the differential CE clearance achieved by TM-CD and HP-CD related to the amount of FC removed by TM-CD, lowering the concentration of TM-CD might reduce cholesterol efflux to that achieved by HP-CD and stimulate CE depletion to TM-CD+PLV.

Without PLV, cholesterol efflux to TM-CD was diminished by reducing its concentration (0.007–0.7 mM), but TM-CD alone did not decrease CE (unpublished observa-

tions). In the presence of PLV, decreasing the concentration of TM-CD promoted depletion of CE but diminished depletion of FC (Fig. 5). Consequently, overall cholesterol efflux to TM-CD+PLV was similar at low and high concentrations of TM-CD because of greater FC depletion at higher concentrations. These data confirmed that the depletion of FC and CE can be dissociated, and establish that depletion of CE is inhibited by higher concentrations of TM-CD.

Differential CE depletion to TM-CD and HP-CD+PLV is not explained by TM-CD-mediated cytotoxicity

A number of experiments demonstrated depletion of over 50% of cellular FC during efflux to TM-CD or HP-CD+PLV from cholesterol-enriched HMFC. Consequently, a number of measures of cell viability were performed. Trypan blue staining, cell morphology, and cell protein all suggested con-

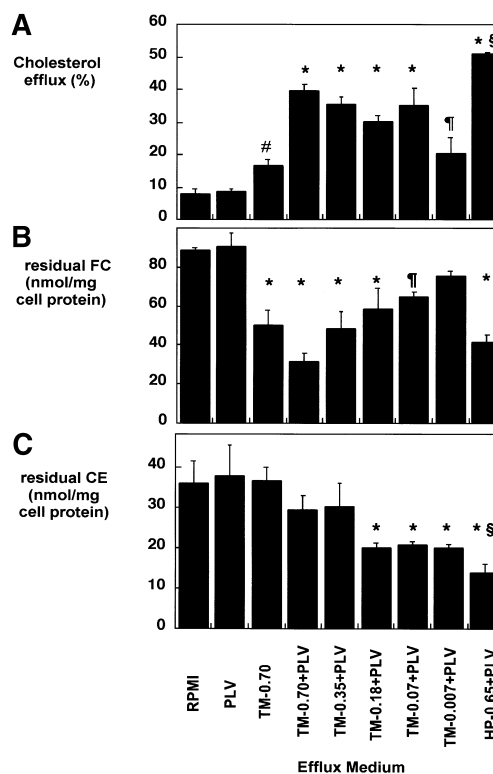


Fig. 5. CE depletion by TM-CD+PLV is stimulated by low concentrations of TM-CD. HMFCs were incubated with TM-CD (0–0.70 mM) + PLV (200 μg/ml) for 24 h. As a positive control, HMFCs were also incubated with HP-CD (0.65 mM) + PLV (200 μg/ml) (HP-CD+PLV). Cell FC and CE and medium cholesterol were determined for each culture by HPLC, and percentage cholesterol efflux calculated for each culture as described in Materials and Methods. Where error bar is not visible, it is obscured by the symbol. A: Percentage cholesterol efflux at 24 h. B: Residual cell FC at 24 h. C: Residual cell CE at 24 h. A: Conditions * significantly differ from RPMI (*P* < 0.05), condition # significantly differs from RPMI (*P* < 0.001), condition § significantly differs from all other conditions (*P* < 0.001). B: Conditions * differ from RPMI and PLV (*P* < 0.001), and condition † differs from RPMI (*P* < 0.01). C: Conditions * differ from RPMI, PLV, TM-0.70 (*P* < 0.001), and condition § differs from TM-0.70+PLV (*P* < 0.01).

sistent and preserved viability under all conditions (unpublished observations). LDH release (41) indicated a modest time-dependent decline in cell viability indistinguishable among all efflux conditions (viabilities were $75.5 \pm 3.2\%$ for RPMI-1640, $76.9 \pm 3.1\%$ for TM-CD, $76.2 \pm 3.5\%$ for TM-CD+PLV, and $76.4 \pm 0.4\%$ for HP-CD+PLV after 24 h). Thus, the poor ability of TM-CD (\pm PLV) to deplete cell CE was not attributable to cytotoxicity.

Prior exposure to TM-CD does not inhibit efflux to HP-CD+PLV

To determine if 0.7 mM TM-CD caused irreversible changes to the composition or structure of HMFC plasma membrane, cells were preincubated with either RPMI-1640 or TM-CD for 1 h before measuring efflux to HP-CD+PLV for 24 h (Fig. 6). TM-CD did not significantly inhibit subsequent cholesterol efflux (unpublished observations) or depletion of FC or CE by HP-CD+PLV.

Liposome stability is not affected by TM-CD

The integrity of PLV in the presence of TM-CD under usual and extreme conditions was tested to exclude the possibility that TM-CD destabilized PLV structure and that this limited cholesterol efflux. PLVs prepared using egg PC and [3 H]PC were incubated with RPMI, 0.7 mM TM-CD, 70 mM TM-CD, or 1% Triton-X100 (as positive control for PLV destabilization) for 24 h, then subjected to gel filtration (44). Whereas treatment with Triton did cause substantial redi-

tribution of PLV lipids to smaller particles, 0.70 mM TM-CD only slightly ($<10\%$) increased the amount of phospholipid in smaller size particles. PLVs are added at 200 $\mu\text{g}/\text{ml}$, and 100 $\mu\text{g}/\text{ml}$ is saturating for cholesterol efflux (unpublished observations); thus loss of 10% of PLV phospholipid is unlikely to inhibit cholesterol efflux by TM-CD+PLV.

Differential CE depletion to TM-CD and HP-CD+PLV requires ongoing cholesterol esterification in the presence of TM-CD

CEs synthesized in cells are subject to a continuous cycle of hydrolysis via neutral CE hydrolase and reesterification via ACAT (45). Consequently, the inability of TM-CD to mediate CE clearance, despite FC clearance, could be due to inhibition of CE hydrolysis or require ongoing cholesterol esterification via ACAT, and this was investigated by incubating cells with the ACAT inhibitor S-58035 during efflux. Under these conditions, RPMI, TM-CD \pm PLV, and HP-CD+PLV all caused equivalent depletion of cell CE (Fig. 7). This suggests that TM-CD does not inhibit hydrolysis of CE, and that cell CEs remaining after TM-CD exposure require synthesis by ACAT. They also indicate that HP-

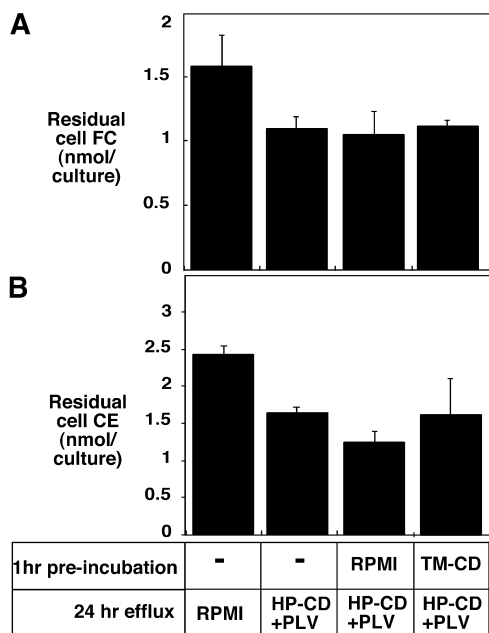


Fig. 6. Preincubation with TM-CD does not inhibit cholesterol efflux to HP-CD+PLV. HMFCs were incubated with RPMI-1640 (RPMI), or HP-CD (0.65 mM) + PLV (200 $\mu\text{g}/\text{ml}$) (HP-CD+PLV) for 24 h. Parallel cultures of HMFC were also preincubated with either RPMI-1640 alone or with TM-CD (0.70 mM) for 1 h before washing and incubating with HP-CD+PLV for 24 h. Cell FC and CE and medium cholesterol were determined for each culture after 24 h by HPLC. A: Residual cell FC at 24 h. B: Residual cell CE at 24 h. All three HP-CD+PLV-incubations generated identical efflux at 24 h (unpublished observations).

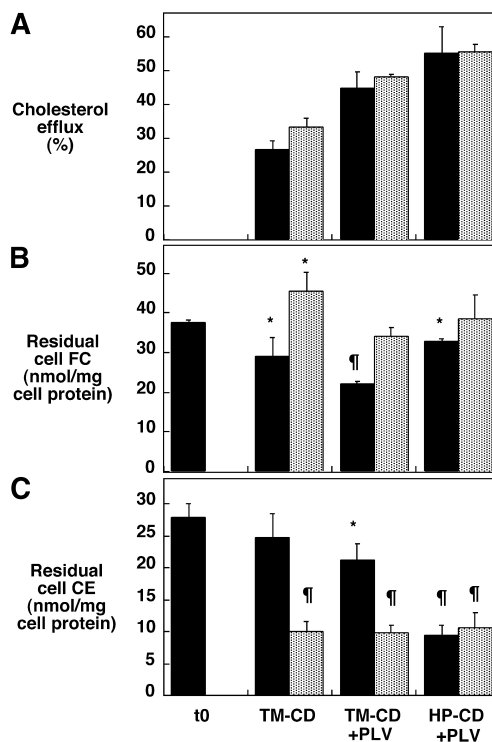


Fig. 7. Acyl-CoA:cholesterol acyltransferase (ACAT) inhibition during efflux permits CE depletion by TM-CD. HMFCs were generated by incubating HMDM with AcLDL (T_0), then incubated for 24 h with TM-CD (0.70 mM), TM-CD (0.70 mM) + PLV (200 $\mu\text{g}/\text{ml}$) (TM-CD+PLV), and HP-CD (0.65 mM) + PLV (200 $\mu\text{g}/\text{ml}$) (HP-CD+PLV) in the presence (gray bars) or absence (solid bars) of 5.0 $\mu\text{g}/\text{ml}$ ACAT inhibitor S-58035. Cell FC and CE were determined for each culture by HPLC as described in Materials and Methods. After 24 h in RPMI with ACAT inhibitor, cell CE content was 12.7 ± 0.9 nmol/mg cell protein, which was not significantly different from that of TM-CD, TM-CD+PLV, or HP-CD+PLV, as shown in B. Conditions marked * and ‡ are significantly different from T_0 (respectively $P < 0.05$ and $P < 0.001$).

CD+PLV does not stimulate CE hydrolysis, as cells exposed to ACAT inhibitor contained similar residual CEs after incubation with RPMI (12.7 ± 0.90 nmol/mg cell protein) or HP-CD+PLV (10.7 ± 2.4 nmol/mg cell protein).

ACAT inhibition did not substantially improve net clearance of total cell cholesterol to TM-CD+PLV because cell FC increased under these conditions. In three separate experiments with ACAT inhibitor, efflux to HP-CD+PLV versus TM-CD+PLV was $57.2 \pm 3.7\%$ versus $41.2 \pm 2.0\%$, $62.7 \pm 1.0\%$ versus $54.2 \pm 1.1\%$, and $55.3 \pm 2.1\%$ versus $48.0 \pm 0.6\%$ ($P < 0.01$ for comparison of HP-CD+PLV vs. TM-CD+PLV in each experiment). These results indicate that impaired clearance of CE is not the only factor limiting efflux to TM-CD (\pm PLV) relative to HP-CD.

HP-CD acting as a sink achieves depletion of cell CE

Cells were incubated with higher concentrations of HP-CD sufficient to stimulate cholesterol efflux even without PLV (Fig. 8). HP-CD differed from TM-CD as it promoted significant CE clearance at high concentrations in the absence of PLV, i.e., when acting as a sink. In the presence of PLV, CE depletion was maximal at 0.65 mM HP-CD, whereas FC depletion increased with increasing concentrations of HP-CD. Thus, removal of cholesterol by CDs does not in itself necessarily impede depletion of cellular CE, and suggests a qualitative difference in the response to TM-CD and HP-CD.

TM-CD depletes plasma membrane cholesterol in human macrophages

Qualitative differences in the effects of TM-CD and HP-CD on cells could be attributable to differential depletion

of plasma membrane cholesterol or phospholipids. Massive depletion of SPM from the plasma membrane by sphingomyelinase has been shown to inhibit cholesterol efflux and to promote cholesterol esterification by ACAT (46), and recently, methyl CDs have been shown to promote phospholipid transfer between phospholipid bilayers (47). We therefore investigated whether TM-CD selectively depleted SPM from the plasma membrane (Table 2) and whether there were differences in cholesterol depletion from the plasma membrane achieved by HP-CD and TM-CD.

Both TM-CD and HP-CD stimulated release of cell phospholipids in the presence of PLV. Efflux media did contain relatively greater proportions of lysoPC than did isolated plasma membranes ($\sim 15\%$ vs. $< 5\%$ of total phospholipids, respectively), but the proportions of phospholipid subclasses in efflux media and in plasma membranes were not altered by exposure to TM-CD or HP-CD. Importantly, the cholesterol content in isolated plasma membranes was much lower after exposure to TM-CD or TM-CD+PLV than after other conditions ($P < 0.01$ for HP-CD+PLV vs. TM-CD \pm PLV). The difference in depletion of plasma membrane cholesterol achieved by TM-CD \pm PLV relative to HP-CD+PLV was much greater than differences in whole-cell FC depletion (e.g., see Figs. 2, 5). These data indicate that TM-CD depletes pools of plasma membrane FC not depleted by HP-CD, which supports the possibility that certain pools of plasma membrane cholesterol modulate CE clearance in HMFC.

DISCUSSION

These studies demonstrate that the plasma membrane is a major rate-limiting factor for cholesterol efflux and CE depletion from HMFC, and establish differential CE depletion as an important qualitative difference in the consequences of efflux to CDs with different affinity to cholesterol. This demonstrates for the first time that the properties of an acceptor that are optimal for depletion of FC need not be optimal for depletion of CE and overall cholesterol clearance, and that FC depletion and CE depletion can be dissociated.

CDs are sterol-solubilizing agents (21). Their efficient ability to access plasma membrane cholesterol has been attributed to a combination of properties: their small size that increases the frequency of collisions with desorbed cholesterol molecules; their ability to directly access the plasma membrane, evading large surface structures that limit access to comparatively larger PLVs; and their excellent water solubility that facilitates penetration of the extracellular water layer (22, 48). Their interaction with PLVs during the process of shuttling combines efficient CD-mediated solubilization of membrane cholesterol with the greater cholesterol-binding capacity of PLV, and relies upon the relatively low affinity CDs have for cholesterol (K_d).

The determination of factors that restrict efflux from primary HMFC has hitherto been indirect. This has substantially underestimated the importance of the plasma

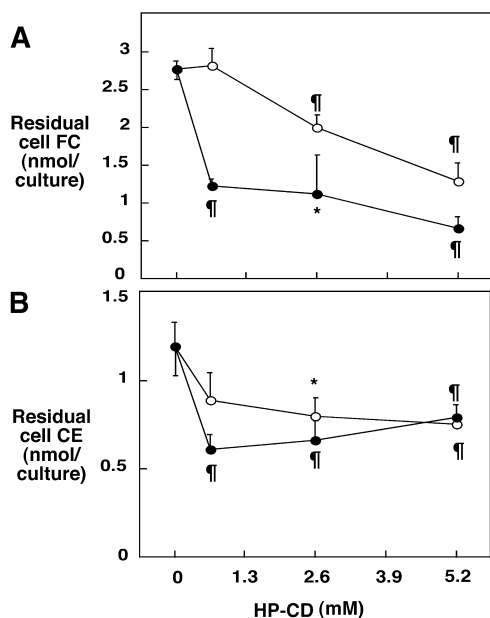


Fig. 8. HP-CD mediates CE clearance at concentrations sufficient to cause depletion of cell FC. HMFCs were incubated with RPMI-1640 control, HP-CD alone (0.65–5.2 mM) (open circles), or HP-CD (0.65–5.2 mM) + PLV (200 μ g/ml) (closed circles). A: Residual FC. B: Residual CE. Values marked † and * significantly differ from control RPMI ($P < 0.01$ and $P < 0.02$, respectively).

TABLE 2. TM-CD depletes plasma membrane cholesterol in human foam cell macrophages

	RPMI Mean (SD)	PLV	TM-CD	TM+PLV	HP-CD	HP+PLV
Medium PL						
A: % PL efflux	9.34 (0.89)	10.5 (0.51)	8.76 (0.65)	27.7 (0.85) ^a	6.62 (0.40)	22.7 (1.27) ^a
B: PL subclass (% distribution)						
LysoPC	15.9 (5.0)	13.7 (4.5)	14.1 (3.4)	9.7 (3.0)	19.1 (6.7)	9.8 (1.3)
SM	26.1 (2.7)	23.1 (2.1)	29.5 (2.6)	27.3 (1.1)	25.7 (4.4)	26.3 (2.6)
PC	58.0 (7.5)	63.2 (6.5)	56.4 (4.4)	62.9 (4.1)	55.3 (11.0)	63.9 (1.6)
Plasma membrane PL						
C: PL subclass (%)						
LysoPC	1.2 (0.6)	0.8 (0.4)	1.5 (0.9)	1.7 (1.0)	1.4 (1.1)	1.7 (1.2)
SM	21.4 (0.6)	22.5 (4.2)	21.9 (1.6)	20.4 (2.4)	24.0 (1.2)	26.0 (0.2)
PC	77.5 (0.1)	76.8 (4.6)	76.6 (2.5)	77.9 (1.4)	74.6 (0.2)	72.4 (1.3)
D: FC-PL content (molar ratio)	0.55 (0.097)	0.59 (0.034)	0.29 (0.042) ^a	0.29 (0.045) ^a	0.61 (0.10)	0.63 (0.035)

FC, free cholesterol; HP-CD, hydroxypropyl- β -CD; PC, phosphatidylcholine. HMFCs that were metabolically labeled with 3 μ Ci/ml [3 H]choline in RPMI and 0.1% BSA (see Materials and Methods) were incubated for 24 h in efflux medium containing RPMI-1640, TM-CD (0.70 mM), TM-CD (0.70 mM) + PLV (200 μ g/ml) (TM-CD+PLV), HP-CD (0.65 mM), or HP-CD (0.65 mM) + PLV (200 μ g/ml) (HP-CD+PLV). At 24 h, aliquots of media were removed, efflux of [3 H]labeled phospholipids (PL) was determined after Bligh and Dyer extraction, cells were washed, scraped, homogenized, and subjected to subcellular fractionation, and plasma membrane fractions analyzed for total PL mass and for percent distribution of PL subclasses (see Materials and Methods). The percent distribution of [3 H]choline-labeled phospholipids [lysophosphatidylcholine, sphingomyelin, and PC] was determined by TLC. Rows A and B are values from efflux medium at 24 h. A: Total [3 H]choline-phospholipid efflux (% of t_0). B: Percent distribution of individual [3 H]choline-phospholipids. C, D: Values from plasma membranes at 24 h. C: Percent distribution of plasma membrane [3 H]choline-phospholipids. D: Cholesterol-phospholipid ratio in plasma membrane fraction. The specific activity of cellular phospholipids was $15,502 \pm 1,127$ dpm/nmol before efflux.

^a Conditions marked are significantly different from corresponding RPMI incubation ($P < 0.01$).

membrane because of the relative inability of physiological acceptors to cause significant cholesterol efflux. In our laboratory, saturating concentrations of apoA-I and HDL cause modest depletion of CE from HMFC (<15% per 24 h), but this depletion is commensurate with the limited cholesterol efflux from primary HMFC achieved by these agents (total efflux 10–20% at 24 h). In contrast, HP-CD+PLV depleted CE by >50% over 24 h. This effect is consistent with either direct stimulation of CE hydrolysis or CE depletion secondary to efficient removal of plasma membrane cholesterol. Under conditions in which ACAT was inhibited, equal depletion of CE was achieved with HP-CD+PLV and control medium RPMI-1640. This indicates that HP-CD+PLV depletes CE by stimulating cholesterol efflux, not by stimulating CE hydrolysis.

The detection of synergy between CD and PLV predictably requires a relatively low level of efflux to individual components; however, previous studies have not described any important qualitative differences in the consequences of efflux to TM-CD acting as a sink and HP-CD+PLV where the CD acts as a shuttle (26, 43). Differences between this study and previous reports may be due to our use of primary HMFCs, which are inherently resistant to cholesterol efflux and may be more sensitive to redistributions of plasma membrane cholesterol, and our deliberate use of net CE clearance as one of the measures of efficacy. The latter is important in HMFCs, given the slow CE hydrolysis in these cells, which cannot be assessed from experiments of <8 h duration.

The differences in the effects of low- and high-affinity CDs are also important because of recent application of these agents to study diverse biological phenomena, including the actions of raft-related proteins. A number of studies have manipulated cellular and plasma membrane cholesterol by the use of high concentrations of high-affinity CDs such as methyl CD (49), and high concentrations of

HP-CD (50) (10- to 80-fold higher than the present study). Given our observations with nontoxic and low-dose CDs, we anticipate that the use of such high concentrations of high-affinity CDs acting as sinks may dramatically perturb normal cholesterol trafficking pathways and CDs, because a class cannot be assumed to be biologically equivalent.

It is most notable in the present studies that TM-CD permits ongoing cholesterol reesterification despite concurrent depletion of FC, as previous studies have shown a very close relationship between plasma membrane cholesterol content and cholesterol esterification in the ER (51). This suggests that TM-CD may remove certain plasma membrane pools of FC that do not affect ACAT activity, or may remove plasma membrane cholesterol from ACAT-inaccessible pools and redeliver it to ACAT-accessible pools while still causing net FC depletion. Previous studies have shown that solutions of CDs that are saturated with respect to cholesterol can deliver cholesterol to macrophages, and that this cholesterol can be esterified by ACAT (14, 43, 52).


Net efflux to TM-CD was modestly increased by ACAT inhibition, but greater CE clearance occurred at the expense of diminished FC clearance, suggesting that other unknown properties of TM-CD restrict cholesterol efflux. It is unlikely that TM-CD cannot transfer cholesterol to PLV, as a number of agents with high cholesterol affinity shuttle cholesterol to PLV very effectively (43), and the present study clearly shows synergy between lower concentrations of TM-CD and PLV in stimulating cholesterol efflux and CE depletion.

The apparent dissociation of FC and CE depletion with TM-CD may occur via several routes. It is known that plasma membrane pools of cholesterol are structurally and kinetically heterogeneous (53, 54). TM-CD may remove cholesterol from domains within the plasma membrane where it is more strongly bound (e.g., rafts), and subsequently allow cholesterol to reenter the plasma

membrane at other sites with greater access to ACAT (54). Our data showing much greater depletion of plasma membrane cholesterol by TM-CD (\pm PLV) than by HP-CD+PLV support the possibility that TM-CD accesses pools of plasma membrane cholesterol that are not accessed by HP-CD. The removal of selected pools of plasma membrane cholesterol, which may represent only a small proportion of the total, has been shown to affect intracellular protein translocation (55). It is therefore possible that such cholesterol depletion may directly affect the location of ACAT or its regulatory proteins inside the cell. Such depletion may also perturb other cholesterol-regulatory proteins, such as the more recently described ABCA1.

Using [3 H]cholesterol-labeled cells (Fig. 4), it appears that neither CE mass nor CE specific activity were increased by TM-CD (relative to control incubations), indirectly indicating that ACAT activity is not increased by this agent; however, we have not directly measured changes to ACAT activity induced by CDs. Interpretation of ACAT activity using conventional assays would be complicated by the solubilizing effects of CDs on ACAT substrates (cholesterol and fatty acids) when these are added to culture media, which will indirectly alter apparent ACAT activity. Direct experimental measurement of ACAT activity in whole cells, and investigating if CDs affect cellular fatty acid metabolism and directly regulate ACAT activity, are important future investigations.

Selective depletion of SPM from the plasma membrane can potentially perturb the condensing and cholesterol-binding properties of sites preferentially enriched in SPM (such as membrane rafts). By analogy with the effects of sphingomyelinase treatment of cells, this would be expected to increase cholesterol internalization, traffic to the ER, and cytoplasmic esterification (46). We did not identify significant depletion of SPM from the plasma membrane under these experimental conditions. Recent studies show that the rate of cellular SPM synthesis is markedly increased by depletion of cell cholesterol by CDs (56). Consequently, depletion of SPM in cell membranes would require substantial SPM efflux that exceeds the cellular capacity for synthesis and replenishment.

In summary, high-affinity CDs appear to dissociate the link between plasma membrane cholesterol concentration, net cholesterol efflux, and intracellular cholesterol esterification. It is likely that plasma membrane cholesterol is an important regulator of net cholesterol efflux and CE metabolism in human macrophages. 

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