Inhibition of cellular cholesterol efflux by 25-hydroxycholesterol

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Abstract The effect of oxysterols on efflux of cholesterol from mouse L-cell fibroblasts, rat Fu5AH hepatoma cells, J774 macrophages, and human EA.hy 926 endothelial cells was studied. Cells were preincubated with 25-hydroxycholesterol (25-OHC) either during labeling of the cells with [^{3}H]cholesterol or during equilibration after labeling. Subsequently, the release of [^{3}H]cholesterol into medium containing 0.2 mg HDL_3/ml was measured and the fractional release of cellular [^{3}H]cholesterol was calculated. Pretreatment with 25-OHC (1 μg/ml) caused a 30% reduction in [^{3}H]cholesterol efflux from L-cells during 8 h of incubation with HDL_3. 25-OHC also inhibited cholesterol efflux from Fu5AH and J774 cells, but the effect was less marked. There was only a small, nonsignificant reduction of efflux from EA.hy 926 cells. The mechanism of 25-OHC-induced inhibition of cellular cholesterol efflux was further studied in L-cells, because of their sensitivity to 25-OHC treatment. The effect of 25-OHC on cholesterol efflux was dose-dependent, with significant effects seen at 25-OHC concentrations as low as 50 ng/ml. The half-time for cholesterol efflux from 25-OHC-treated cells (5 μg/ml) was 13.0 ± 3.3 h compared to 5.7 ± 1.0 in control cells, corresponding to a 55% reduction in the rate of cholesterol release. Other oxysterols, including 7-ketocholesterol, 7α- and 7β-hydroxycholesterol, and 22(S)-hydroxycholesterol also inhibited [^{3}H]cholesterol efflux from L-cells significantly, but to a lesser degree. 25-Hydroxycholesterol (5 μg/ml) reduced efflux from both normal and cholesterol-enriched cells by 31 and 14%, respectively. Inhibition of efflux was similar when reconstituted HDL_3-apolipoprotein/phosphatidylcholine particles or small unilamellar phosphatidylcholine vesicles were used as cholesterol acceptors instead of HDL_3. The content of phospholipids, cholesterol and the FC/PL ratio of intact cells and from isolated plasma membrane vesicles were the same for control and 25-OHC-treated cells. Efflux of [^{3}H]cholesterol from plasma membranes isolated from 25-OHC-treated cells was 20% less than efflux from membranes from control cells. The difference in efflux observed in intact cells is partially explained by the reduction in efflux from the plasma membrane. In conclusion, our studies suggest that oxysterols, especially 25-hydroxycholesterol, can reduce cellular cholesterol efflux in vitro. Therefore oxysterols, either endogenous or derived from the diet, may influence cellular cholesterol efflux in vivo, the first step in reverse cholesterol transport.—**Kilsdonk, E. P. C., D. W. Morel, W. J. Johnson, and G. H. Rothblat.** Inhibition of cellular cholesterol efflux by 25-hydroxycholesterol. *J. Lipid Res.* 1995. **36:** 505–516.

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Oxysterols, oxygenated derivatives of cholesterol, have been detected in human blood (1–3) and various tissues (4) and, when given orally, enhance atherogenesis in various types of animals (5). They can by synthesized enzymatically in vivo (6, 7), or formed spontaneously upon exposure of foods to air during processing or storage (for a review see 8). In vitro, various oxysterols have been detected after lipoprotein oxidation (9) and in cultured cells (10–12). Oxysterols are known to influence a variety of biological functions in vitro. For instance, they are very potent regulators of cell sterol levels, down-regulating both the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (13, 14) and the number of low density lipoprotein receptors on cells (15) and enhancing cellular cholesterol esterification by activation of acyl-CoA:cholesterol acyltransferase (ACAT) (9, 16, 17). In addition to effects on cholesterol metabolism, antiproliferative and cytotoxic effects of oxysterols have been described (18, 19). Oxysterols also affect membrane structure and function. For example, oxysterols can alter membrane fluidity (20, 21), membrane permeability for cations (22), glucose (23), and albumin (24), and membrane-bound protein kinase C activity (25). From the examples listed above it becomes evident that oxysterols can modulate cholesterol homeostasis at the level of cholesterol uptake and intracellular metabolism. Another mechanism by which cell cholesterol levels are maintained is by the process of cholesterol efflux, the first step in reverse cholesterol transport. The rate of desorption of cholesterol from cell membranes is a function of the composition and structure of the plasma membrane (26), and therefore oxysterols could change cellular cholesterol efflux. Thus we studied the effect of oxysterols on cholesterol efflux from various types of cells.

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; HDL, high density lipoprotein; MEM, minimum essential medium; 25-OHC, 25-hydroxycholesterol; OVA, ovalbumin; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PL, phospholipid; SUV, small unilamellar vesicle.

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An effect of oxysterols on cellular cholesterol efflux could provide an additional way for the regulation of cholesterol homeostasis by oxysterols.

MATERIALS AND METHODS

Cell culture

Minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM), calf serum, L-glutamine and hypoxanthine/aminopterin/thymidine (HAT) supplement were obtained from Gibco (Grand Island, NY). RPMI-1640, heat-inactivated fetal bovine serum, and gentamicin were purchased from Sigma (St. Louis, MO). Media were buffered with sodium bicarbonate (2 g/l) and cells were cultured in a humidified incubator at 37°C with 5% CO_2 and 95% air. All media were supplemented with 2 mM of L-glutamine and 50 μg gentamicin/ml.

Mouse fibroblasts and Fu5AH rat hepatoma cells were cultured in MEM with 10% heat-inactivated fetal bovine serum and 5% fetal calf serum, respectively. Mouse J774 macrophages were cultured in RPMI-1640 with 10% heat-inactivated fetal bovine serum. EA.hy 926 human endothelial cells were kindly provided by Dr. C-J. S. Edgell (27) and cultured in DMEM, supplemented with 10% fetal bovine serum, 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine. The EA.hy 926 cell line is a permanent cell line established by hybridization of human umbilical vein endothelial cells with the permanent human lung carcinoma line A459 (27) and shows characteristics of differentiated human endothelial cells, e.g., expression of von Willebrand factor (27), tissue plasminogen activator (28), plasminogen activator inhibitor-type 1 (28), and production of prostacyclin (29). EA.hy 926 cells still expressed these typical differentiated characteristics after more than 100 cumulative population doublings (27, 28).

Efflux experiments

For efflux experiments cells were plated in 35-mm wells and grown to near confluence. Two different methods of preincubation with 25-OHC were used: method A, in which cells were labeled with [^{3}H]cholesterol and subsequently incubated with medium containing oxysterols and ovalbumin; method B, in which cells were preincubated with both [^{3}H]cholesterol label and 25-OHC at the same time. For preincubations and efflux the following media were used for the different cell types: MEM for L-cells and Fu5AH cells, RPMI for J774 macrophages and DMEM for EA.hy 926 cells.

Method A. Cells were labeled for 24 h with medium containing 0.5 μCi [^{3}H]cholesterol/ml and 2.5% FBS (2 ml of medium/well). [1,2-^{3}H]cholesterol was obtained from NEN (NET-139, Boston, MA) and checked for purity by thin-layer chromatography prior to use (30). This labeling medium also contained cholesterol/phosphatidylcholine dispersions (at 200 μg cholesterol/ml, mol/mol ratio > 2) for experiments using cholesterol-enriched cells (31). Subsequently, pre-labeled cells were incubated with 25-OHC (5 ng to 5 μg/ml, as indicated for each experiment, Sigma) and 0.2% ovalbumin (OVA, w/v) for another 24 h. This period allows for equilibration of radioactive cholesterol in cellular cholesterol pools. Ovalbumin was used instead of the more commonly used albumin, because preliminary experiments showed that albumin promoted efflux of oxysterols to the medium, whereas ovalbumin did not. Other sterols, which were added instead of 25-OHC in some experiments, were: 22(S)-hydroxycholesterol, cholesterol 5α,6α-epoxide, cholestane-3β,5α,6β-triol (obtained from Sigma) and 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol (Steraloids).

Method B. Cells were preincubated for 24 h in medium containing 0.5 μCi [^{3}H]cholesterol/ml, 2.5% FBS, and the indicated concentration of 25-OHC. Subsequently, monolayers were incubated with medium plus 0.2% OVA for 2–24 h to allow for equilibration of the radioactive isotope in the various cellular cholesterol pools. An ACAT-inhibitor, Sandoz 58-035, was added during both labeling and equilibration for both preincubation methods A and B (1 μg/ml, 0.1% DMSO final concentrations; Sandoz compound 58-035 was a gift from Dr. John Heider). Radiolabeled cholesterol and oxysterols were added to the medium in ethanol (final concentration 0.1%).

After labeling and oxysterol preincubations, cells were rinsed three times with medium supplemented only with gentamicin and were then switched to medium with HDL_3 (0.2 mg of phospholipid/ml) and 1 μg Sandoz 58-035/ml (2 ml/well). This efflux medium was buffered with both sodium bicarbonate (2 g/l) and HEPES (50 mM). Aliquots of medium were removed at various time points during the 8-h incubation. These aliquots were filtered to remove any floating cells in 96-well, 0.45 μm filtration plates (Millipore, Bedford, MA) using a Millipore multiscreen vacuum manifold and subsequently 50 μl was sampled for liquid scintillation counting. Monolayers were rinsed three times with PBS after incubation and cellular lipids were extracted with isopropanol overnight (32). An internal standard (cholesteryl methyl ether, Sigma) was added to the wells prior to extraction and cholesterol was measured by gas–liquid chromatography using a 50% phenylmethyl polysiloxane column (33). An extra set of cells was harvested at the beginning of the efflux period to determine initial cell [^{3}H]cholesterol and cholesterol mass. Cell protein was determined on the remaining monolayer using a modification of the method of Markwell et al. (34). Cell protein was dissolved in a solution of 0.1 N NaOH and 1% SDS, and duplicate aliquots were taken out for protein determinations.

Efflux is expressed as the fraction of initial [^{3}H]cholesterol remaining in the cells at each time point. Every time point is the mean of triplicate wells. These data were fitted to a single exponential model using a nonlinear regression model as previously described (35). The equation used was: $y = A \cdot e^{-B.t} + C$, in which y is the fraction of initial cell [^{3}H]cholesterol remaining in the cells and t is the incubation time in h. Rate constants for efflux k_e and half-times for efflux could be calculated using the following formulas: $k_e = A \times B$ and $t_{1/2} = \ln 2/k_e$.

The nonparametric Mann-Whitney test was used to compare treatments. Differences were considered statistically significant when $P < 0.05$.

Isolation of L-cell plasma membranes and efflux of membrane [^{3}H]cholesterol

L-cells were grown in 100-mm dishes to near confluence and preincubated 24 h with MEM containing 1.0 μCi [^{3}H]cholesterol/ml, 2.5% FBS and 1 μg Sandoz 58-035/ml and subsequently switched to medium containing 0.2% OVA and Sandoz 58-035 plus or minus 5 μg 25-OHC/ml for 24 h (5 ml/dish). Cells were rinsed three times with PBS after the preincubations and incubated with PBS containing 1.0 mM $CaCl_2$, 0.5 mM $MgSO_4$, 50 mM formaldehyde (Fischer Scientific) and 2 mM D,L-dithiothreitol (Sigma) using a modification of the method of Scott et al. (36). This treatment results in the formation of plasma membrane vesicles, which are shed from the cells. Vesicles were collected after 2 h of incubation. Cells were rinsed twice with alkaline, hypertonic PBS (PBS with 0.3 M NaCl, pH 8.6) to detach loosely bound vesicles. This rinse with hypertonic phosphate-buffered saline results in a large increase in the yield of plasma membrane vesicles (37). Plasma membrane vesicles were pooled and centrifuged at 500 rpm for 10 min to remove any whole cells (Beckman GPR tabletop centrifuge). The supernatant from the low-speed spin was centrifuged for 1 h at 40,000 g in a SW-27 rotor at 4°C and the plasma membranes in the pellet were resuspended in either medium (for efflux experiments) or ice-cold saline (for compositional analyses). To obtain homogenates from intact L-cells for compositional analyses, duplicate 100-mm dishes with L-cells were rinsed and scraped into saline and homogenized (10 strokes in a Potter homogenizer). Protein, alkaline phosphodiesterase I and N-acetyl-β-glucosaminidase activity of cell homogenates and plasma membrane fractions were measured as described by Markwell et al. (34) and Beaufay et al. (38), respectively. Marker enzyme activities were expressed as the change in absorbance units (absorbance at 400 nm, corrected for absorbance of reagent blank) per h per mg of protein. Lipids were extracted from both whole cell and membrane preparations (39); cholesterol and phosphorous were determined by the methods of Johnson et al. (33) and Sokoloff and Rothblat (40), respectively.

Efflux of [^{3}H]cholesterol from plasma membrane vesicles from two to three 100-mm dishes (approximately 1 mg of protein) was measured in 2 ml of medium containing HDL_3 at a concentration of 0.2 mg of phospholipid/ml, incubated at 37°C on a rotary shaker (20–30 rpm) for up to 8 h. Aliquots were removed at various time points. Release of membrane [^{3}H]cholesterol was measured after 0.22 μm filtration of the aliquots (Millipore filtration plates MAGV N22 50). Data are expressed as described above for efflux of cellular cholesterol.

Cholesterol acceptors

Various types of acceptor particles were used to induce efflux of cellular cholesterol. HDL_3 (d 1.125–1.21 g/ml) was isolated by sequential ultracentrifugation of human plasma according to Hatch and Lees (41). ApoE-free HDL_3 was isolated by chromatography of HDL_3 on a heparin-Sepharose column (42). Small unilamellar phosphatidylcholine vesicles and apoHDL/PC complexes were prepared from 1-palmitoyl-2-oleoyl-*sn*-glycerophosphocholine (Avanti Polar Lipids Inc.) and lipid-free HDL apolipoproteins as described by McLean and Phillips (43) and Jonas (44), respectively. ApoE-free HDL_3, SUVs, and apoHDL/PC particles were dialyzed against medium; subsequently lipids were extracted (39) and phosphorus was determined according to Sokoloff and Rothblat (40).

RESULTS

Exposure of cells to 25-hydroxycholesterol reduced cholesterol efflux from mouse L-cell fibroblasts, Fu5AH rat hepatoma cells, and murine J774 macrophages (**Table 1**). The magnitude of inhibition of efflux varied among the different cell lines, with the most pronounced effect observed in L-cells. The two preincubation methods used for exposing cells to oxysterol (see Methods) produced similar results: the 8-h fractional release of L-cell [^{3}H]cholesterol was reduced 33 and 28%, when preincubated with methods A and B, respectively. Whereas in L-cells inhibition of efflux by 25-OHC was relatively stable over an 8-h incubation period after removal of 25-OHC, the inhibitory effect of 25-OHC rapidly decreased in Fu5AH cells upon removal of the oxysterol. For both Fu5AH and J774 cells 25-OHC reduced efflux about 8% after 8 h of incubation. In the case of J774 cells, only preincubation method B could be used (i.e., 25-OHC exposure in the presence of FBS), as exposure to 25-OHC using preincubation method A led to pronounced cell toxicity, as reflected by a 15% decrease of both cell protein and [^{3}H]cholesterol for cells exposed to oxysterol. Macrophages appeared to be more sensitive to 25-OHC-mediated cell toxicity than the other cells, as no indication of toxicity was observed in the other cell types exposed to 25-OHC. In contrast to the other cell types, EA.hy 926

TABLE 1. Effect of 25-hydroxycholesterol on [^{3}H]cholesterol efflux from various cell types

Cell Type	Preincubation Method 25-OHC conc.	Efflux Time	Percentage [^{3}H]FC Released from Cells		% Inhibition of Efflux by 25-OHC
			Control	25-OHC	
		h			
L-cell mouse fibroblasts	A. 1 μg/ml	1.5	13.3 ± 0.3	8.2 ± 0.5[a]	38
		4.5	35.3 ± 0.6	21.7 ± 1.4[a]	38
		8	49.1 ± 1.2	33.0 ± 2.1[a]	33
	B. 1 μg/ml	2	20.8 ± 0.9	11.9 ± 0.8[a]	43
		3.8	32.8 ± 1.2	20.8 ± 0.8[a]	37
		8	50.2 ± 1.2	36.1 ± 0.9[a]	28
Fu5AH hepatoma cells	A. 1 μg/ml	1	12.7 ± 0.3	12.1 ± 0.4	4.7
		1.6	18.1 ± 0.3	17.7 ± 0.1	2.2
		8	46.1 ± 0.4	45.9 ± 0.5	0.4
	A. 5 μg/ml	8	42.4 ± 0.8	39.1 ± 0.5[a]	7.8
	B. 5 μg/ml	1.1	21.2 ± 0.3	15.8 ± 0.5[a]	26
		2.2	36.0 ± 1.0	28.5 ± 0.4[a]	21
		4.2	52.0 ± 0.3	44.4 ± 0.8[a]	15
		8	63.4 ± 0.9	57.9 ± 1.2[a]	8.7
EA.hy 926 cells	A. 5 μg/ml	8.1	13.2 ± 0.5	12.8 ± 0.7	3.0
J774 macrophages	B. 5 μg/ml	4	20.6 ± 0.1	18.7 ± 0.9[a]	9.2
		8	34.4 ± 0.3	31.7 ± 1.1[a]	7.8

Cells were pretreated with [^{3}H]cholesterol and oxysterols by either method A or B (see Materials and Methods) and subsequently incubated with medium containing HDL_3 (0.2 mg phospholipid/ml) and 1 μg Sandoz 58-035/ml. Efflux is expressed as the percentage release of initial cell [^{3}H]cholesterol to the medium (means ± SD, incubations in triplicate for every treatment).

[a]Significantly different fractional [^{3}H]cholesterol release from 25-OHC-treated cells, when compared with control cells (non-parametric Mann-Whitney test, $P < 0.05$).

cells showed only a small nonsignificant reduction in efflux.

Effect of oxysterols on L-cell cholesterol efflux

As L-cells exhibited the greatest sensitivity to 25-OHC, inhibition of cholesterol efflux was further studied in L-cells. The time-course of [^{3}H]cholesterol efflux from control and 25-OHC-treated L-cell fibroblasts is shown in **Fig. 1.** Half-times for efflux of cellular [^{3}H]cholesterol in this typical experiment were 6.8 and 13.2 h for control and treated cells, respectively. The dose-response curves for the effect of 25-OHC on [^{3}H]cholesterol efflux from

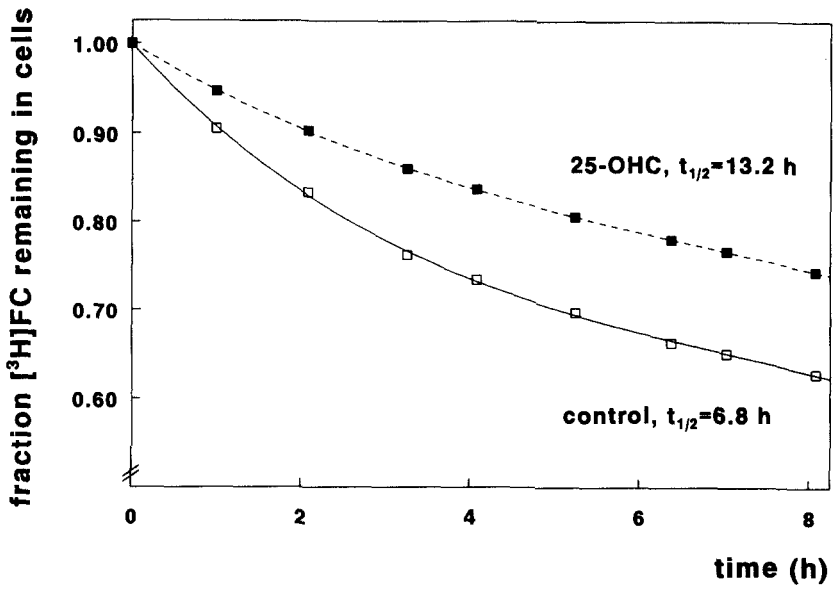

Fig. 1. Time-course of L-cell [^{3}H]cholesterol efflux to HDL_3. L-cells were labeled with [^{3}H]cholesterol and subsequently preincubated with 0.2% OVA and either 0 (–□–) or 5 (–■–) μg 25-OHC/ml. Efflux was in the presence of HDL_3 (0.2 mg phospholipid/ml) for 8 h. ACAT inhibitor, Sandoz 58035, was added during the entire experiment. Values are means ± SD for triplicate wells; error bars are within the markers if not apparent.

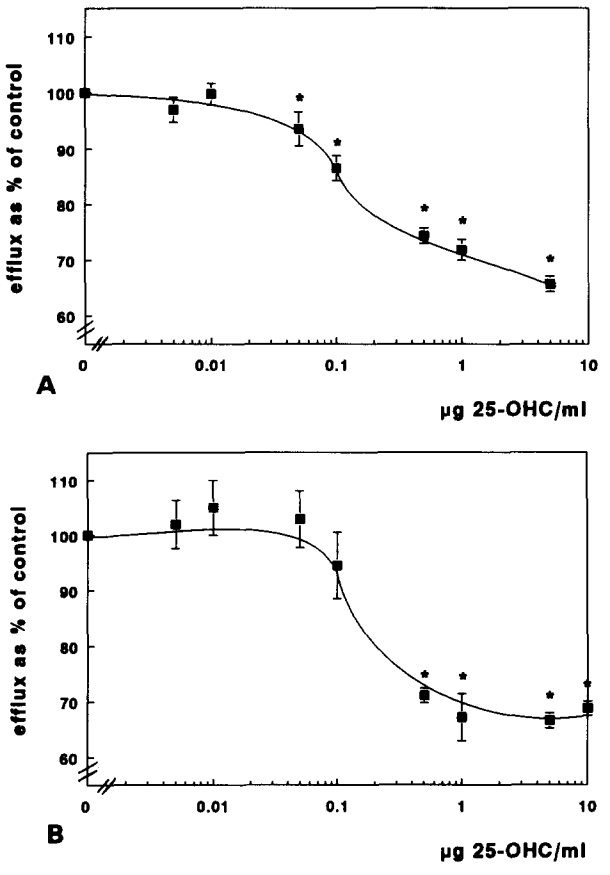

Fig. 2. Effect of 25-OHC dose on L-cell [^{3}H]cholesterol efflux using either preincubation method A or B. Cells were incubated with efflux medium containing HDL_3 (0.2 mg phospholipid/ml) and 1 μg Sandoz 58-035/ml for 8 h. Data are expressed as percentage of the fractional release of initial cellular [^{3}H]cholesterol of control cells (means ± SD, n = 3). Fractional release of initial cellular [^{3}H]cholesterol for control cells was 50.2 ± 1.2 and 49.1 ± 1.2% after 8 h of incubation for methods A and B, respectively. *Significantly different from incubation with HDL_3 alone (non-parametric Mann-Whitney test, $P < 0.05$).

L-cells for preincubation methods A and B are given in **Fig. 2.** The inhibition of efflux by 25-OHC is similar for both preincubation methods, with significantly decreased efflux observed at concentrations of 25-OHC as low as 0.05 μg/ml for method A and 0.5 μg/ml for method B, respectively. As simultaneous exposure of cells to 25-OHC and FBS during the preincubation period (method B) resulted in differences between control and 25-OHC-treated cells in both [^{3}H]cholesterol uptake and cell cholesterol mass, complicating the interpretation of efflux data, the protocol in which oxysterols were added after labeling of the cells (method A) was selected for the following experiments.

The effect of 25-OHC on the rate constant and half-time for L-cell [^{3}H]cholesterol efflux in a number of different experiments is given in **Table 2.** To obtain rate constants, the fraction of [^{3}H]cholesterol remaining in the cells was fitted to a single exponential decay model ($r >$ 0.99). The half-time for efflux from 25-OHC-treated cells increased 1.7-fold (1 μg/ml) and 2.3-fold (5 μg/ml). These effects correspond to reductions in the rate of cholesterol release of 41% and 55%, respectively.

TABLE 2. Effect of 25-hydroxycholesterol on rate constant and half-time for efflux of L-cell cholesterol

Preincubation	n[a]	k_e	$t_{½}$
		h^{-1}	h
Control	6	0.124 ± 0.022	5.7 ± 1.0
1 μg 25-OHC/ml	3	0.073 ± 0.010	9.7 ± 1.2
5 μg 25-OHC/ml	4	0.056 ± 0.013	13.0 ± 3.3

Cells were labeled with [^{3}H]cholesterol for 24 h and subsequently pretreated with 25-OHC for another 24 h. Then cells were incubated with HDL_3 (0.2 mg phospholipid/ml) for 8 h. ACAT inhibitor, Sandoz 58-035, was added during both preincubations and efflux period. Medium aliquots were taken out at various time points during the time course and efflux was expressed as the fraction of [^{3}H]cholesterol remaining in the cells. Values for k_e and $t_{½}$ (means ± SD) were obtained as described in Materials and Methods.

[a]The number of experiments; each experiment was done in triplicate.

Table 3 shows the effect of various oxysterols on L-cell [^{3}H]cholesterol efflux. 7α-Hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, and 22(S)-hydroxycholesterol reduced efflux significantly at 5 μg/ml. However, 25-OHC was more effective than all other oxysterols tested.

In all of the efflux experiments described above the extracellular acceptor for cholesterol was HDL_3. To determine whether the oxysterol-mediated reduction in cholesterol efflux was influenced by the nature of the acceptor, a series of incubations was conducted using different exogenous acceptors. As expected from previous studies (45), the acceptors differed in their efficiencies for removing cellular cholesterol from control L-cells (HDL_3 and

TABLE 3. Effects of various oxysterols on L-cell [^{3}H]cholesterol efflux

Oxysterol	Efflux of Cell [^{3}H]Cholesterol as % of Control
Control (0.1% EtOH only)	100.0 ± 3.5
7α-OH cholesterol	90.5 ± 1.0[a]
7β-OH cholesterol	92.2 ± 1.3[a]
7-Keto cholesterol	85.8 ± 0.6[a]
22(S)-OH cholesterol	91.4 ± 1.7[a]
25-OH cholesterol	72.5 ± 0.5[a]
Cholestantriol	102.9 ± 2.8
Cholesterol 5α,6α-epoxide	98.2 ± 0.7

L-cells were labeled with [^{3}H]cholesterol and 2.5% FBS for 24 h, and subsequently incubated with 5 μg oxysterol/ml in 0.2% ovalbumin for another 24 h. Efflux was started by adding 2 ml of medium with HDL_3 (0.2 mg phospholipid/ml). Sandoz 58-035 was added throughout the entire experiment. The amount of cell [^{3}H]cholesterol released to the medium after 8 h of incubation was measured and expressed as a % of the efflux from control cells. Data are the means ± SD of 3 wells for every treatment.

[a]Significantly different from control cells (non-parametric Mann-Whitney test, $P < 0.05$).

apoHDL/PC particles > small unilamellar vesicles > BSA). However, 25-OHC inhibited efflux by 31, 42, and 35%, for HDL_3, apoHDL/PC, and SUV particles, respectively. Thus, the inhibition of cholesterol efflux by 25-OHC was similar regardless of the type of extracellular cholesterol acceptor. **Table 4** shows the effect of 25-OHC on the reduction of sterol mass in L-cells. Initial cell cholesterol concentrations were the same for control and 25-OHC-treated cells. However, desmosterol content was significantly lower in 25-OHC-treated cells, presumably due to the inhibition of HMG-CoA reductase by 25-OHC. ApoHDL/PC particles and SUVs, both cholesterol-free acceptors, decreased cell cholesterol mass significantly after 8 h of incubation in both experiments (see Table 4). 25-OHC reduced the loss of cellular cholesterol and desmosterol compared to the sterol loss from control cells for all acceptors. When the decrease in cellular sterol mass is expressed as a percentage of initial cell sterols (cholesterol, desmosterol, and 25-hydroxycholesterol, if present), the decrease of sterol efflux is most clear in experiment 2 (see Table 4, 5 μg 25-OHC/ml pretreatment): efflux is 31 and 13% of total cell sterols for control and 25-OHC-treated cells, respectively. The 25-OHC-induced reduction in net efflux was similar in magnitude to the reduction in efflux of cellular label (shown in **Fig. 3**), demonstrating that radiolabeled cholesterol in the cells closely tracked the actual mass of cell cholesterol when a cholesterol-free acceptor was used to avoid bidirectional flux of cholesterol. As cholesterol enrichment influences cellular metabolic processes, we examined the effect of 25-OHC on efflux from L-cells enriched with cholesterol (**Fig. 4**). 25-OHC also inhibited efflux in enriched cells, although the magnitude of reduction was less than in control cells.

Effect of 25-hydroxycholesterol on L-cell plasma membrane composition and plasma membrane [^{3}H]cholesterol efflux

The inhibitory effect of oxysterols on efflux of cell cholesterol may be related to cellular cholesterol esterification, as oxysterols are known to increase cellular acyl-CoA:cholesterol acyltransferase (ACAT) activity. We routinely added the ACAT-inhibitor Sandoz compound 58-035 to all preincubation and efflux media to prevent formation of [^{3}H]cholesteryl esters. Stimulation of ACAT by an oxysterol could reduce the size of the cholesterol pool available for efflux. The presence of active ACAT enzyme might enhance the shift of membrane cholesterol to internal pools, making this cholesterol less available for efflux, which could result in an even larger inhibition of cholesterol efflux. To test this possibility, cholesterol efflux was measured in L-cells preincubated with [^{3}H]cholesterol and 25-OHC, either in presence or absence of the ACAT inhibitor Sandoz 58-035. Inhibition of efflux, however, was identical for cells treated with or without the inhibitor (data not shown).

As a large portion of cell cholesterol resides in the plasma membrane (46), and as the efflux of cholesterol occurs through the release of cholesterol molecules from the plasma membrane (26), a likely site of action of 25-OHC is the plasma membrane. To test whether exposure of cells to oxysterols changes plasma membrane composition and in that way alters efflux characteristics, a series of experiments was conducted using plasma membrane preparations from control and 25-OHC-treated L-cells. **Table 5** shows the chemical composition of L-cell plasma membranes. Two marker enzymes, alkaline phosphodiesterase I activity (APD) for plasma membranes and *N*-acetyl-β-

TABLE 4. Effect of 25-hydroxycholesterol on efflux of sterol mass from L-cells

	Control			25-OHC-Treated			
Acceptor	Cholesterol[a]	Desmosterol	Total Cell Sterol	Cholesterol	Desmosterol	25-OHC	Total Cell Sterol
Experiment 1							
t = 0 h	12.71 ± 0.50	7.37 ± 0.34	20.08 ± 0.84	12.41 ± 0.55	3.08 ± 0.30[b]	1.38 ± 0.05[b]	16.87 ± 0.90[b]
Sterol loss after 8 h:							
ApoHDL/PC discs	7.31 ± 0.68[c]	5.69 ± 0.39[c]	13.00 ± 1.07[c]	5.07 ± 0.97[b,c]	2.39 ± 0.54[b,c]	1.38 ± 0.05[b,c]	8.84 ± 1.56[b,c]
SUV	3.53 ± 0.72[c]	2.02 ± 0.46[c]	5.55 ± 1.18[c]	2.68 ± 0.77[c]	0.27 ± 0.27[b]	1.38 ± 0.05[b,c]	4.33 ± 1.09[c]
Experiment 2							
t = 0 h	12.41 ± 1.24	6.21 ± 2.66	18.62 ± 3.90	11.63 ± 0.67	1.80 ± 0.43[b]	2.07 ± 0.23[b]	15.50 ± 1.33
Sterol loss after 8 h:							
ApoHDL/PC discs	3.24 ± 1.28[c]	3.02 ± 2.93[c]	6.26 ± 4.21[c]	0.98 ± 0.85[b,c]	0.08 ± 0.57[b]	0.92 ± 0.27[b,c]	1.98 ± 1.69

[^{3}H]cholesterol-labeled L-cells were preincubated for 24 h in medium containing 0.2% OVA with or without 25-OHC (1 and 5 μg/ml were used for 25-OHC-treated cells in experiments 1 and 2, respectively). Subsequently, efflux was measured by incubation of the cells with cholesterol-free acceptors, either reconstituted particles made from HDL-apolipoproteins and phosphatidylcholine (apoHDL/PC discs, at 0.2 and 0.1 mg phospholipid/ml in exps. 1 and 2, respectively) or small unilamellar phosphatidylcholine vesicles (SUV) at 0.3 mg phospholipid/ml. Throughout the entire experiment all media contained 1 μg Sandoz 58-035/ml.

[a]Values are expressed as μg sterol/mg cell protein of triplicate wells (means ± SD). In control cells no 25-OHC could be detected.

[b]Significantly different from control cells (non-parametric Mann-Whitney test, $P < 0.05$).

[c]Significant loss of cell sterol, when compared with t = 0 h (non-parametric Mann-Whitney test, $P < 0.05$).

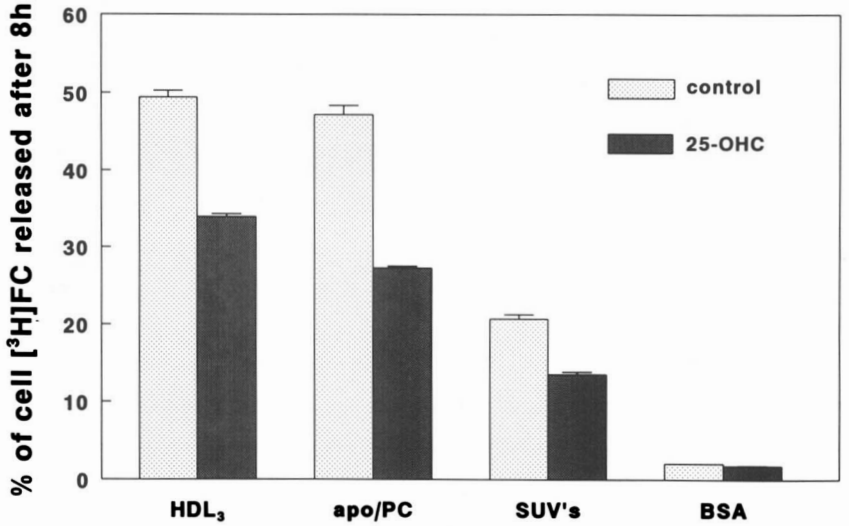

Fig. 3. Effect of 25-OHC on L-cell [^{3}H]cholesterol efflux to various cholesterol acceptors. [^{3}H]cholesterol-labeled L-cells were incubated 24 h in medium containing 0.2% OVA plus or minus 1 μg 25-OHC/ml (2 ml/well). The following acceptors were used: apoE-free HDL_3 (0.2 mg phospholipid/ml), apoHDL/PC particles (0.2 mg phospholipid/ml), small unilamellar PC vesicles (0.3 mg phospholipid/ml), or BSA (0.2% w/v). All media, during preincubation as well as the efflux period, contained 1 μg Sandoz 58-035/ml. Efflux is expressed as the percentage release of initial cellular [^{3}H]label after 8 h of incubation (means ± SD, n = 3).

glucosaminidase activity (NABGase) for lysosomes, were measured to determine the purity of the plasma membrane preparations. The ratio of APD to NABGase activity increased 13-fold for membrane vesicles isolated from control cells. Plasma membrane preparations from 25-OHC-treated cells were isolated with an even higher purity, as the ratio of APD to NABGase increased 34-fold. Cholesterol and phospholipid content increased 3.1- and 1.5-fold, respectively, for control vesicles, and 3.0- and 1.5-fold, respectively, for vesicles from 25-OHC-treated

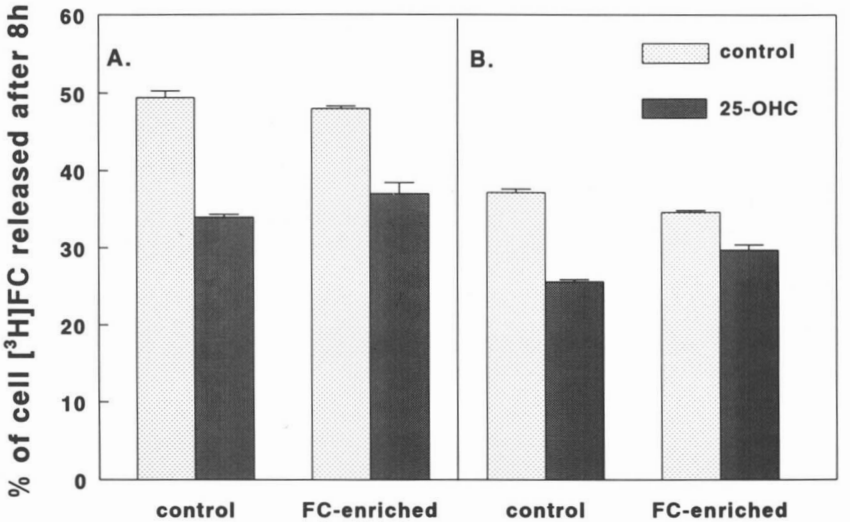

Fig. 4. Effect of 25-OHC on [^{3}H]cholesterol efflux from control and cholesterol-enriched L-cells. Cells were labeled with [^{3}H]cholesterol in medium containing either 2.5% serum (control cells) or 2.5% serum plus cholesterol/phospholipid dispersions (cholesterol-enriched cells). Subsequently, cells were incubated in medium with 0.2% OVA and 1 μg 25-OHC/ml for 24 h in experiment A, or with 5 μg 25-OHC/ml for experiment B. Efflux was induced by HDL_3 (0.2 mg phospholipid/ml) and expressed as the release of initial cell [^{3}H]cholesterol into the medium (means ± SD, n = 3). All media contained 1 μg/ml of the ACAT inhibitor Sandoz compound 58-035. Initial cell cholesterol levels were: A: non-loaded cells 12.7 ± 0.5 μg/mg for control and 12.4 ± 0.6 for 25-OHC-treated cells; loaded cells 22.2 ± 0.3 μg/mg for control and 23.9 ± 0.5 μg/mg for 25-OHC-treated cells. B: non-loaded cells 12.4 ± 1.2 μg/mg for control and 11.6 ± 0.7 μg/mg for 25-OHC-treated cells; loaded cells 20.3 ± 0.9 μg/mg for control and 19.1 ± 0.4 μg/mg for 25-OHC-treated cells.

TABLE 5. Composition of intact L-cells and L-cell plasma membrane vesicles

	Intact Cells		Plasma Membrane Vesicles	
	Control	25-OHC	Control	25-OHC
Experiment 1				
Marker enzyme activity in δA_{400}/(h × mg protein):				
APD	2.3 ± 0.5	2.4 ± 0.8	11.1 ± 0.4	11.0 ± 1.8
NABGase	9.4 ± 0.1	8.4 ± 1.7	2.9 ± 0.2	1.1 ± 0.1
Ratio APD/NABGase	0.3	0.3	3.8	10.3
Chemical composition in μg lipid/mg protein:				
Phospholipid	147 ± 13	128 ± 28	216 ± 10	191 ± 10
Cholesterol	9.2 ± 1.9	8.9 ± 2.0	28.8 ± 1.4	26.5 ± 0.4
FC/PL (mol/mol)	0.13	0.14	0.27	0.28
Desmosterol	2.2 ± 0.4	1.5 ± 0.4	6.2 ± 0.3	3.9 ± 0.5
25-Hydroxycholesterol		0.6 ± 0.2		0.9 (n = 1)
Total sterols	11.4 ± 2.3	11.0 ± 2.6	35.0 ± 1.7	31.3 ± 0.9
Experiment 2				
Marker enzyme activity in δA_{400}/(h × mg protein):				
APD			9.3 ± 1.4	7.7 ± 0.5
NABGase	N.D.	N.D.	3.6 ± 0.4	0.8 ± 0.1
Ratio APD/NABGase			2.6	9.9
Chemical composition in μg lipid/mg protein:				
Phospholipid			187 ± 22	153 ± 4
Cholesterol			33.4 ± 2.5	27.4 ± 2.6
FC/PL (mol/mol)			0.36	0.36
Desmosterol	N.D.	N.D	6.9 ± 1.4	3.2 ± 0.3
25-Hydroxycholesterol				2.8 ± 0.4
Total sterols			40.2 ± 3.9	30.6 ± 3.3

Confluent L-cells in 100-mm dishes were labeled with [^{3}H]cholesterol for 24 h and subsequently preincubated with 5 μg 25-OHC/ml for 24 h. Plasma membranes were isolated and composition of cell homogenates and plasma membrane vesicles was determined as described in Materials and Methods. Values are means ± SD (n = 3); N.D., not determined.

cells. 25-OHC pretreatment had no effect on plasma membrane FC/PL ratio (mol/mol): 0.27 versus 0.28 for control and 25-OHC treatment, respectively.

Plasma membrane vesicles from control and 25-OHC-treated L-cells, with identical FC/PL ratios, were incubated with HDL_3 (0.2 mg phospholipid/ml) to measure efflux of membrane [^{3}H]cholesterol. The half-time for efflux of membrane [^{3}H]cholesterol was calculated as described for intact cells. Membrane cholesterol efflux was inhibited by 25-OHC pretreatment of the cells by 21, 21, and 18% in three separate experiments (**Table 6**). However, average inhibition of efflux by 25-OHC was

TABLE 6. Half-times for [^{3}H]cholesterol efflux from L-cells: whole cells versus plasma membranes vesicles

	Half-time for Efflux (h)		
	Control	25-OHC	% Inhibition of Efflux
Experiment 1			
Cells	6.7	11.7	42
Membranes	9.7	12.3	21
Experiment 2			
Cells	N.D.	N.D.	
Membranes	9.6	12.3	21
Experiment 3			
Cells	6.9	17.5	60
Membranes[a]	12.5	15.1	18
AVG (± SD)			
Cells (n = 2)	6.8 ± 0.1	14.6 ± 4.1	51
Membranes (n = 3)	10.6 ± 1.6	13.2 ± 1.6	20

L-cells were labeled with [^{3}H]cholesterol and subsequently preincubated with 5 μg 25-OHC/ml for 24 h. Plasma membranes were isolated as described in Materials and Methods. In three separate experiments, cells or membranes were incubated in triplicate with HDL_3 (0.2 mg phospholipid/ml) for 8 h. Efflux was expressed as fraction of initial [^{3}H]cholesterol remaining in cells or membranes, fitted to a single exponential decay model and half-times for cholesterol efflux were calculated from these equations; N.D., not determined.

[a]In experiment 3, 12-well tissue culture dishes with membrane vesicle suspension were incubated in a humidified incubator (30 rpm), while in the first two experiments vesicles were incubated in capped glass vials (20 rpm).

51%, when measured for intact cells. Thus, although we observed a reduction in efflux of cholesterol from plasma membrane vesicles derived from oxysterol-treated cells, the magnitude of the reduction was approximately half of that observed with whole cells.

In order to examine how fast 25-OHC exerts an effect on efflux and whether 25-OHC had to be given to intact cells to affect efflux, we added 25-OHC to the efflux medium instead of pretreating cells with oxysterols. **Figure 5A** shows that efflux was reduced detectably after 2 to 3 h of incubation of intact cells with 25-OHC and HDL_3, compared to cells incubated with HDL_3 only. In Fig. 5B however, when isolated plasma membranes vesicles were used, 25-OHC did not reduce membrane [^{3}H]cholesterol efflux.

DISCUSSION

Exposure of cells to oxygenated sterols has been shown to produce a wide variety of effects, particularly as related to cellular sterol metabolism (13–17). For example, exposure of cells to oxysterols results in a down-regulation of the cell HMG-CoA reductase and LDL receptor activities and a concomitant increase in the activity of ACAT. Such changes in sterol metabolism can influence the deposition of cholesterol and cholesteryl esters in cells of the vessel wall. Our present study has demonstrated that oxysterols can modulate cell cholesterol efflux. This inhibition of efflux produced by oxysterols could also contribute to cholesterol retention in cells.

The effectiveness of 25-OHC in reducing cholesterol efflux differed among cell types. The reason for the differences in sensitivity may be related to both the efficiency of oxysterol uptake and oxysterol metabolism by the various cell types. For example, we have observed that in hepatoma cells 25-OHC can be metabolized to more polar compounds (unpublished results). In addition, cellular 25-OHC concentrations decrease upon incubation with HDL_3, because 25-OHC is also released to cholesterol acceptors in the incubation medium. The metabolism and/or efflux of oxysterols is consistent with the observation that the inhibitory effect was most pronounced early in the time-course, and decreased with prolonged incubation of the cells with HDL_3. The decline in inhibition of cholesterol efflux over the time-course could not be prevented by the addition of 25-OHC (up to 20 μg/ml) to the efflux medium (not shown). However, 25-OHC will associate with the HDL_3 particles in the efflux medium and will therefore be less available to the cells. In addition, it is possible that, because of the association of 25-OHC with the HDL_3 particles in the medium, there is a change in HDL_3 structure which might have affected cellular cholesterol efflux. However, a more likely explanation for the decline of inhibition over time is that

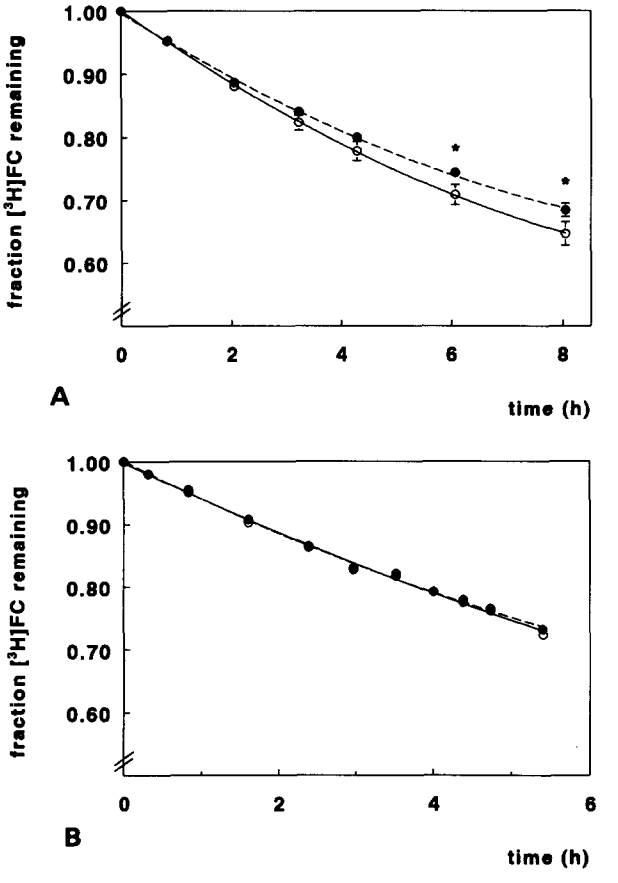

Fig. 5. Direct effect of 25-OHC on [^{3}H]cholesterol efflux from intact L-cells or L-cell membranes. [^{3}H]cholesterol labeled L-cells (panel A) or membrane vesicles (panel B) were incubated with either HDL_3 alone (—○—) or with HDL_3 plus 5 μg 25-OHC/ml (—●—) and efflux was measured as described earlier. Values are means ± SD; error bars are within the markers if not displayed (n = 3). *Significantly different from incubation with HDL_3 alone (non-parametric Mann-Whitney test, $P < 0.05$).

25-OHC is only inhibiting initial rates, but not the final distribution of labeled cholesterol between cells and medium when equilibrium is achieved. Therefore the difference between control and treated cells will diminish as incubations continue and the distribution of labeled cholesterol in the cell and medium pools approach equilibrium. In support of this explanation is the observation that the difference between control and 25-OHC-treated cells is reduced rapidly in studies with Fu5AH cells (see Table 1); these cells have fast initial efflux and rapidly achieve equilibrium. L-cells exhibited the greatest sensitivity to 25-OHC, demonstrating approximately 30% inhibition of efflux when pretreated with 25-OHC concentrations above 1 μg/ml. Significant inhibition of [^{3}H]cholesterol efflux was achieved at 0.05 μg/ml (see Fig. 2A), a concentration which has been shown to alter HMG-CoA reductase activity (16, 47, 48), LDL receptor expression (15) and ACAT activity (16, 17) in other cell types (mainly human fibroblasts). A possible explanation

for the prolonged effectiveness and greater sensitivity of L-cells to the 25-OHC-induced inhibition of efflux might be related to the desmosterol present in these cells (49). However, in J774 macrophages, which also synthesize desmosterol instead of cholesterol (50), the inhibitory effect of 25-OHC is modest.

Although 25-OHC was the most effective of the oxysterols tested, a number of them produced significant inhibition of efflux (Table 3). No single structural feature of the tested oxysterols appears to be linked to their efficiencies as cholesterol efflux inhibitors; however, the compounds that functioned as efflux inhibitors have all been shown to reduce HMG-CoA reductase activity and/or stimulate ACAT activity (5, 9, 13, 14, 16). In cholesterol-enriched cells, in which HMG-CoA reductase and LDL receptor activity are decreased and ACAT activity is increased, oxysterol pretreatment still inhibited cellular cholesterol efflux, showing no direct link between reduction of efflux and these activities.

The concentration of oxysterols resulting in a significant inhibition of cellular [^{3}H]cholesterol efflux in our experiments is comparable with oxysterol concentrations detected in human and rabbit plasma (51). Fig. 2A showed that 25-OHC concentrations of 0.05 μg/ml and more significantly inhibited cholesterol efflux. Recently Stalenhoef et al. (51) reported that plasma of normocholesterolemic volunteers contained 1.36 μmol of total oxysterols per ml (circa 0.6 μg/ml). Hypercholesterolemic plasma of WHHL rabbits contained 3.7 μg total oxysterols/ml of which 2.0 and 0.14 μg/ml were 7α-hydroxycholesterol and 25-hydroxycholesterol, respectively.

A number of different mechanisms by which oxysterols reduce cholesterol efflux can be proposed. Possible mechanisms would be: *1)* a change in the activity of the putative HDL receptor, *2)* a shift of cholesterol out of the plasma membrane into an internal pool, and *3)* a change in the structure of the plasma membrane. An oxysterol-mediated change in HDL receptor activity seems unlikely for a number of reasons. First, efflux of membrane cholesterol has been shown to be independent of HDL binding to cells (52, 53), and the labeling protocol in the present studies results in the bulk of labeled cholesterol being present in the plasma membrane (54). Second, treatment of cells with 25-OHC has been shown to upregulate the specific high affinity binding of HDL to various cell types (55, 56), which should increase efflux rather than reduce it. Third, we observed comparable inhibition of cellular cholesterol efflux regardless of the type of extracellular cholesterol acceptor. This is particularly relevant in the case of SUVs, particles containing no apoproteins; SUVs therefore cannot specifically interact with membrane receptors and yet oxysterol treatment reduced cholesterol efflux to SUVs.

Exposure of cells to a number of oxysterols results in an increase in the activity of ACAT (16, 17), and it has been suggested that the stimulation of ACAT activity is, in part, related to a shift in the distribution of cholesterol among intracellular pools (57, 58). Thus, it is possible that 25-OHC treatment resulted in the movement of cholesterol out of the plasma membrane into an intracellular ACAT pool that was less available for efflux. This mechanism seems unlikely for two reasons: first, the reduction of efflux obtained upon exposure of L-cells to 25-OHC was observed both in the presence and absence of active ACAT; and second, an examination of the cholesterol/phospholipid ratio in plasma membrane vesicles obtained from control and 25-OHC-treated cells demonstrated a similar composition; an observation inconsistent with a shift of cholesterol out of the plasma membrane.

Even though we could not demonstrate any change in the cholesterol/phospholipid ratio in plasma membrane fractions, exposure of cells to oxysterols may produce more subtle changes in membrane structure. A number of studies have demonstrated that incorporation of oxysterols into both native and artificial membranes can result in changes in the physical properties of membranes (20–25). These changes, which are often detected by changes in membrane fluidity, can occur without modifications in chemical composition, and probably reflect a redistribution of cholesterol and phospholipid among different membrane domains (21, 59). The possible redistribution of cholesterol among different microdomains within membranes could lead to the appearance of different kinetic pools of cholesterol in efflux experiments, as proposed previously (59, 60). However, oxysterol treatment did not change the qualitative results when efflux data were fitted to curves describing the release of cell-derived cholesterol to either a one- or to a two-pool kinetic model. Data from whole cell, as well as from membrane experiments, were fitted equally well to a model describing one kinetic pool for both control and 25-OHC-treated cells. Our observation that the half-time for efflux was reduced in membrane preparations obtained from 25-OHC-treated cells is consistent with a membrane modification mechanism. The observation that the magnitude of efflux reduction was less with isolated membranes than with intact cells indicates that either the procedure used for membrane isolation reduced the oxysterol effect, or that an additional mechanism, unrelated to plasma membrane effects, also contributed to the reduction of cholesterol efflux observed in 25-OHC-treated cells. If a shift in plasma membrane structure is occurring, it happens relatively quickly, as we could already detect inhibition of efflux after 2 to 3 h of exposure of cells to oxysterol (Fig. 5). However, cellular uptake is required, because treatment of isolated membrane vesicles with 25-OHC had no effect on efflux.

The results from the present study add another component to the array of effects produced by oxysterols on cellular cholesterol metabolism. Although we have not yet

identified the specific mechanism by which oxysterols reduce cholesterol efflux, the data are most consistent with a mechanism linked to changes in membrane structure. Whether this type of change is directly associated with other metabolic events elicited by 25-OHC remains to be determined. It is clear, however, that oxidized forms of cholesterol, present in tissues, could contribute to the cellular accumulation and retention of cholesterol because of an inhibition of efflux.

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