Effect of phospholipid fatty acid composition of endothelial cells on cholesterol efflux rates

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Abstract Human endothelial cells (EA.hy 926 line) were loaded with cationized low density lipoprotein (LDL) and subsequently incubated with fatty acid/bovine serum albumin complexes. The fatty acids were palmitic, oleic, linoleic, arachidonic, and eicosapentaenoic acids. The preincubations resulted in extensively modified fatty acid profiles in cell membrane phospholipids and in cellular cholesteryl esters. The cholesterol efflux from these fatty acid-modified cells was measured using 0.2 mg high density lipoprotein$_3$ (HDL_3)/ml medium. The efflux was significantly higher for the palmitic acid-treated cells, compared to all other fatty acid treatments. These differences in efflux rates were not caused by changes in the binding of HDL_3 to high affinity receptors on the EA.hy 926 cells. Efflux mediated by dimethyl suberimidate-treated HDL_3, which does not interact with high affinity HDL receptors, was similar to efflux induced by native HDL_3 after all fatty acid treatments. Our results indicate that high affinity HDL receptors are not important for HDL-mediated efflux of cell cholesterol. The fatty acid composition of the cell membrane phospholipids may be an important determinant.—**Kilsdonk, E. P. C., A. N. R. D. Dorsman, T. van Gent, and A. van Tol.** Effect of phospholipid fatty acid composition of endothelial cells on cholesterol efflux rates. *J. Lipid Res.* 1992. **33:** 1373–1382.

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High density lipoproteins (HDL) are believed to play a role in reverse cholesterol transport, i.e., the transfer of peripheral cell cholesterol back to the liver. HDL-binding membrane proteins have been visualized in different cell types (1–5), but the physiological role of these HDL-binding proteins is still unclear. Oram, Brinton, and Bierman (6) showed up-regulation of HDL-binding activity in fibroblasts and smooth muscle cells after loading the cells with cholesterol, suggesting a functional role for HDL-binding proteins in the removal of excess cell cholesterol. When HDL_3 was unable to bind to the high affinity receptors, the high affinity component of the efflux of cholesterol was also abolished (7). However, other investigators showed that there was no relation between binding of HDL and HDL-mediated efflux of cell cholesterol (8–10). The desorption of cholesterol out of the cell membrane is considered the rate limiting factor for efflux (11). In the present study we determined the effect of membrane composition on cholesterol efflux. Fatty acids added to the media of cells in culture, even at confluency, are readily incorporated in the cell membranes (12), resulting in extensively modified fatty acid profiles of the membrane phospholipids. The physical properties of the membrane, e.g., the fluidity, may change by modification of this fatty acid profile, leading to modifications of membrane-dependent cellular functions (for reviews see refs. 13 and 14). When the desorption of cholesterol out of the cell membrane is an important factor, these modifications in membrane composition will affect the cholesterol efflux. The importance of the high affinity binding of HDL for cholesterol efflux was examined in two ways. First, by determining possible differences in binding after fatty acid modification of the membrane phospholipids, and second, by measuring cholesterol efflux using modified HDL particles, which do not bind to the high affinity HDL binding sites. The conclusions of this study are based on net mass efflux of cholesterol, measured by increases of cholesterol in the medium. No isotopically labeled cholesterol was used. This was done in order to avoid the complications of isotope effects caused by exchange of labeled cell cholesterol for unlabeled lipoprotein cholesterol.

Abbreviations: apoE, apolipoprotein E; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DMS, dimethyl suberimidate; FCS, fetal calf serum; GLC, gas-liquid chromatography; HDL, high density lipoprotein; LDL, low density lipoprotein; SDS, sodium dodecyl sulfate; TC, total cholesterol; UC, unesterified cholesterol; LCAT, lecithin:cholesterol acyltransferase; ACAT, acyl-CoA:cholesterol acyltransferase.

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MATERIALS AND METHODS

Cell culture

The endothelial cell line EA.hy 926 was generously provided by Dr. C-J. S. Edgell (15). The EA.hy 926 cells were cultured in DMEM (Flow Laboratories), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U penicillin/ml, 100 μg streptomycin/ml, and HAT (100 μM hypoxanthin, Merck No. 4517; 0.4 μM aminopterin, Serva No. 13170; 16 μM thymidin, Merck No. 8206) at 37°C in 95% air and 5% CO_2. Cells were plated in multiwell dishes (35 mm: $1 \cdot 10^5$ cells/well; 22 mm: $5 \cdot 10^4$ cells/well) 4 to 5 days prior to each experiment. Medium was changed every 2 or 3 days.

The confluent monolayers were loaded with cholesterol during a 24-h incubation of the cells with cationized LDL in growth medium (50 μg LDL-cholesterol/ml medium). After enrichment with cholesterol, the cells were incubated with fatty acid/BSA complexes for 48 h. The medium was changed once during this incubation. The fatty acids used were palmitic acid (Merck No. 509), oleic acid (sodium salt, Sigma No. O-7501), linoleic acid (sodium salt, Sigma No. L-8134), arachidonic acid (Sigma No. A-9798), and eicosapentaenoic acid (Sigma No. E-2011). Stock solutions (50 mM) were prepared by dissolving the fatty acids in methanol with butylated hydroxytoluene (12.5 μg/ml). The media used for fatty acid modification contained 0.1 mM fatty acid, 2 mg BSA/ml (Sigma No. A-4378) and 0.005% n-octyl β-D-glucopyranoside (Sigma No. O-8001). An equimolar amount of NaOH was added when the acid form was used instead of the sodium salt.

Lipoproteins

LDL (density range 1.006–1.063 g/ml) and HDL_3 (density range 1.125–1.21 g/ml) were isolated by sequential ultracentrifugation of human plasma according to Havel, Eder, and Bragdon (16). All lipoproteins were dialyzed against 0.9% NaCl with 10 mM sodium phosphate (pH 7.4) and 1 mM EDTA. Before the cell experiments the lipoproteins were dialyzed for a second time against DMEM and filter-sterilized (0.45 μm).

LDL was cationized according to Basu et al. (17). A volume of LDL (20 to 30 mg of protein) was added to an equal volume of 2 M solution of 3-dimethyl-aminopropylamine (Aldrich No. 24.005-2) in H_2O at room temperature. The pH was adjusted to 6.5 with HCl. After stirring this mixture, 100 mg of 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide (Sigma No. E-7750) was added. The pH was maintained at 6.5 with 0.1 M HCl during the reaction. After 3 h, the pH of the solution did not change anymore; the reaction mixture was left at 4°C overnight. Cationized LDL was dialyzed against 0.9% NaCl with 10 mM sodium phosphate (pH 7.4) and 1 mM EDTA, filter-sterilized (0.8 μm), and stored. Storage at 4 or −20°C did not influence the loading of the cells.

ApoE-free HDL_3 was isolated by chromatography of HDL_3 on a heparin-Sepharose column (18). Modification of HDL_3 with DMS was performed as described previously (19). The apoE-free HDL_3 (5 mg protein/ml) was incubated with 5 mg DMS/ml in a 0.09 M triethanolamine-HCl buffer (pH 9.5) for 2 h at room temperature. After the reaction, DMS-HDL_3 was reisolated on a Sephadex column (G25 Fine, 1 × 30 cm, Pharmacia). The column was eluted with 0.9% NaCl with 2 mM EDTA (pH 7.4). The cross-linking of the DMS-HDL_3 apolipoproteins was checked by electrophoresis on 12% polyacrylamide gels containing 0.1% SDS. No apoA-I monomers were visible on the cross-linked HDL_3 particles. The sizes of native HDL_3 and DMS-HDL_3 particles were measured by gel filtration as described (20), using a Superose 12 column (Prep grade, 1.0 × 30 cm, Pharmacia). The size of both HDL_3 preparations was exactly the same, indicating that cross-linking occurred only between apolipoproteins on the same HDL_3 particle. HDL_3 was iodinated using the iodine monochloride method (using $Na^{125}I$ obtained from Amersham International) as described earlier (21). The molar iodine to protein ratio of the ^{125}I-labeled HDL_3 preparations ranged from 0.22 to 0.49; 95.4 ± 0.8% of the label was protein-bound, 1.7 ± 0.6% was in the HDL lipids, and 2.9 ± 0.5% was free.

Efflux experiments

Efflux was virtually negligible in the absence of HDL. Both BSA alone and BSA plus variable amounts of LDL did not result in significant cholesterol efflux from loaded cells (not shown). After cholesterol loading and fatty acid modification, cells were rinsed twice with 2 mg BSA/ml in DMEM with penicillin/streptomycin (1 ml/well) and twice with DMEM only, plus penicillin/streptomycin, at room temperature. The assay of cholesterol efflux was started by addition of efflux medium and incubation at 37°C in 5% CO_2 and 95% air. The efflux medium consisted of DMEM, supplemented with L-glutamine, penicillin, streptomycin, 2 mg BSA/ml (Sigma No. A-4378), and lipoproteins as indicated. The efflux was ended after 24 h by cooling the culture dishes on ice. The culture media were collected and dislodged cells were spun down (500 g_{max}, 4°C). The cell media were stored at −20°C until cholesterol determinations. The wells were rinsed three times with 2 mg BSA/ml in 0.9% NaCl/50 mM Tris-HCl (pH 7.4), followed by three rinses with 0.9% NaCl/50 mM Tris-HCl (pH 7.4). The cells were scraped into 1 ml 0.9% NaCl/50 mM Tris-HCl (pH 7.4) with a rubber policeman and lipids were extracted using the method of Bligh and Dyer (22). The resulting protein pellet was dissolved in 500 μl 0.1 M

NaOH with 10% SDS (w/v). Cell protein was measured according to Lowry et al. (23). Cell lipids were dried down under nitrogen and dissolved in 300 μl 2-propanol. The lipid extracts were stored up to 1 day at −20°C and unesterified and total cholesterol were determined separately using a slight modification of the method described by Heider and Boyett (24). For determination of unesterified cholesterol the reaction mixture consisted of 0.15 mg *p*-OH-phenylacetic acid/ml (Sigma No. H-4377), 5 U peroxidase/ml (Boehringer Mannheim No. 108081), and 3.2 μg cholesterol oxidase/ml (Boehringer Mannheim No. 396818) in sodium phosphate buffer (50 mM, pH 7.4). The reaction mixture of the total cholesterol determination was as for unesterified cholesterol, plus 6.4 μg/ml cholesteryl esterase (Boehringer Mannheim No. 161772), 5 mM taurocholate (Calbiochem No. 580217) and 0.17 mM PEG-6000 (Serva No. 33137). Reaction mixture (0.75 ml) was added to 40 μl cell extract in 2-propanol. A 100 μg/ml solution of cholesterol (Sigma No. C-8253) in 2-propanol was used as a standard. After the incubation (37°C, 30 min for unesterified cholesterol and 120 min for total cholesterol assays) the reaction was ended by addition of 1.5 ml 0.5 M NaOH and fluorescence was measured with a Perkin-Elmer LS-3B fluorimeter (excitation 325 nm, emission 415 nm).

Total and unesterified cholesterol in the media were measured enzymatically using a commercially available kit (cholesterol kit No. 310328, cholesterol esterase No. 161772, Boehringer Mannheim). Cholesteryl esters were calculated by difference between total and unesterified cholesterol.

Lipoprotein binding experiments

After the preincubations with cationized LDL and fatty acids, the cells (in 22-mm wells) were rinsed three times with 2 mg BSA/ml and penicillin/streptomycin in DMEM at room temperature, followed by three rinses at 0°C with DMEM and penicillin/streptomycin to cool the cells. Subsequently, media (DMEM with 2 mg BSA/ml, L-glutamine, penicillin/streptomycin and lipoproteins as indicated) were added (0.5 ml/well) and the cells were incubated 4 h at 4°C in an atmosphere with 5% CO_2. After the incubations the cells were rinsed three times with 0.9% NaCl containing 2 mg BSA/ml and 50 mM Tris-HCL (pH 7.4) and three times with 0.9% NaCl containing only 50 mM Tris-HCL (pH 7.4). The cells were dissolved in 300 μl 1 M NaOH, the radioactivity was counted using a Packard Minaxi 5000 gamma counter, and protein was measured according to Lowry et al. (23). The incubation media contained 15 or 100 μg apoE-free ^{125}I-labeled HDL_3/ml (sp act 200 cpm/ng). Nonspecific binding was measured in the presence of 1 mg unlabeled HDL_3/ml. Specific binding was calculated by difference between total and nonspecific binding. Binding was expressed per mg cell protein. In competition experiments 5 μg apoE-free ^{125}I-labeled HDL_3/ml was used with 0–200 μg/ml unlabeled apoE-free HDL_3 or DMS-HDL_3.

Determination of fatty acid composition of cell phospholipids and cholesteryl esters

Cells from a 75-cm² flask (Costar No. 3375) were preincubated with 7.5 ml of cholesterol-enriched medium (growth medium plus cationized LDL) followed by fatty acid/BSA containing medium (15 ml). After the preincubation period the cells were trypsinized and resuspended in 0.9% NaCl with 50 mM Tris-HCl (pH 7.4) and 1 mM EDTA. EDTA was added to inhibit phospholipase C activity. Fatty acid profiles were determined both in phospholipids and cholesteryl esters according to Lamers et al. (25). In short, the lipids were extracted by the method of Bligh and Dyer (22) and dissolved in a small amount of chloroform–methanol 1:2. The lipid classes were separated by thin-layer chromatography on activated silica gel 60 plates (Merck No. 5721) in hexane–diethylether–acetic acid 60:40:1 with 0.02% butylated hydroxytoluene. Phospholipid and cholesteryl ester spots were scraped off and the lipids were eluted from the silica with methanol (phospholipids) or chloroform–methanol 2:1 (cholesteryl esters). The solvent was evaporated and boron trifluoride-methanol (Sigma No. B-1127) was added for transmethylation (14% BF_3 for phospholipids and 5% for cholesteryl esters). After methylation at 100°C under N_2, the methyl esters were extracted with pentane–H_2O 2:1, dried over sodium sulfate, dissolved in iso-octane, and the methyl esters were separated by GLC (split injector, 270°C; N_2 carrier gas, inlet pressure 95 kPa; CP9000 capillary column (Chrompack) equipped with a coated, fused silica capillary CP-Sil 88 column; column temperature 190°C during first 15 min, increasing to 220°C (15°C/min), total run time 31 min; flame ionization detection, 300°C).

Total phospholipid-derived phosphorus was determined using the method of Bartlett (26) in the membrane pellets of 5% trichloroacetic acid-treated cell suspensions. Inorganic potassium phosphate was used as a standard.

Statistical analysis

Analysis of variance was used for comparison of the different fatty acid treatments when possible ($n > 9$, variances identical in all treatment groups); Bonferroni *t*-tests were used for comparison among treatment groups when results of the analysis of variance were significant. The nonparametric Mann-Whitney test was used to compare two treatment groups of $n < 9$. The differences were considered statistically significant when $P < 0.05$.

TABLE 1. Cholesterol levels in EA.hy 926 cells after cholesterol enrichment and fatty acid treatment

Treatment	Total Cholesterol[a]	Unesterified Cholesterol	Unesterified Cholesterol
	μg/mg protein	*μg/mg protein*	%
Control cells	45.3 ± 4.0	39.8 ± 3.6	87.9 ± 4.6
Cholesterol-rich cells[b]			
Palmitic acid	169.8 ± 7.5[c,d]	99.0 ± 4.7[d]	57.4 ± 2.6[d,e]
Oleic acid	173.8 ± 10.7[c]	96.8 ± 5.5	55.8 ± 2.7[c,d]
Linoleic acid	166.4 ± 15.6[d]	91.5 ± 10.3	54.9 ± 2.5[c,f]
Arachidonic acid	157.0 ± 10.0[d,f,g]	96.7 ± 6.7	61.7 ± 3.0[d,e,g]
Eicosapentaenoic acid	178.0 ± 3.0[c,e,f]	90.7 ± 4.4[f]	50.8 ± 2.5[c,e,f,g]

[a]Cholesterol mass (mean ± SD).
[b]Confluent EA.hy 926 cells were perincubated for 24 h with 50 μg cationized LDL-TC/ml, followed by 48 h incubation with different free fatty acid/BSA complexes (0.1 mM fatty acid). The cells were then rinsed, scraped, and cell cholesterol was determined as described in Materials and Methods. Data are from one typical experiment (six wells per treatment).
[c]Significantly different from arachidonic acid treatment (Mann-Whitney, $P < 0.05$).
[d]Significantly different from eicosapentaenoic acid treatment (idem).
[e]Significantly different from linoleic acid treatment (idem).
[f]Significantly different from palmitic acid treatment (idem).
[g]Significantly different from oleic acid treatment (idem).

RESULTS

Cell cholesterol and phospholipid composition after preincubation with cationized LDL and free fatty acids

Table 1 shows the cholesterol levels after preincubation of the cells. Incubation with cationized LDL increased cell cholesterol levels 3-to 4-fold. In control EA.hy 926 cells almost 90% of this cholesterol was unesterified. The esterification level was much higher in the loaded cells: 40–50% was esterified. Total cell cholesterol, expressed per mg cell protein, was lowest for arachidonic acid-treated cells (significantly lower than after 16:0, 18:1, and 20:5 treatment). Preincubation with eicosapentaenoic acid resulted in the highest level of total cell cholesterol, which was significantly higher than after treatment with 16:0, 18:2, and 20:4. The percentage of cell cholesterol that was unesterified was highest for arachidonic acid-treated cells (62 ± 3%, which was significantly higher than for 18:1-, 18:2-, and 20:5-treated cells). The lowest percentage unesterified cholesterol was found after preincubation with eicosapentaenoic acid (51 ± 3%). All other fatty acid treatments gave significantly higher percentages unesterified cholesterol than eicosapentaenoic acid treatment. The absolute level of unesterified cholesterol per mg cell protein was highest in palmitic acid-treated cells (significantly different only from 20:5-treated cells).

Palmitic acid treatment also tended to increase the cell phospholipid level (13% increase, **Table 2**). Treatments with all the other fatty acids did not alter cellular phospholipid levels compared to normal confluent EA.hy 926 cells. Table 2 shows an almost 3-fold increase in the molar ratio of FC:PL in cells after enrichment with cholesterol. The FC:PL ratio ranged between 0.268 and 0.305 for the different fatty acid treatments.

Table 3 shows that the fatty acid profiles of the cell phospholipids differed greatly after the various fatty acid treatments. The control incubation, in which the cholesterol-loaded cells were incubated without fatty acids (but with the solvents), differed only slightly when compared with palmitic acid treatment: the total of saturated fatty acids in the phospholipid fraction increased from 43 to 48%. Preincubation with oleic acid resulted in 48% oleic acid in the cell phospholipids, and the total of monounsaturated fatty acids in phospholipids increased to 52%. Linoleic acid treatment gave the highest percentage of linoleic acid in cell phospholipids (compared to the other fatty acid treatments) although the increase was

TABLE 2. Phospholipid level and molar unesterified cholesterol:phospholipid ratio after cholesterol enrichment and fatty acid treatment of the EA.hy 926 cells

Treatment	Phospholipid	FC:PL (mol:mol)
	μg/mg protein	
Control cells	612 ± 18	0.098 ± 0.007
Cholesterol-rich cells		
Palmitic acid	693 ± 17	0.282 ± 0.008
Oleic acid	616 ± 11	0.269 ± 0.011
Linoleic acid	594 ± 52	0.285 ± 0.032
Arachidonic acid	590 ± 13	0.305 ± 0.011
Eicosapentaenoic acid	616 ± 23	0.268 ± 0.006

Values are means ± SD, n = 2. For preincubation conditions, see Table 1. After the preincubation, the cells were trypsinized and sonicated. The resulting cell suspensions were used for the total phosphorus, cholesterol, and protein determinations.

TABLE 3. Fatty acid composition of EA.hy 926 cell phospholipids

	Treatment[a]					
	Control	Palmitic Acid	Oleic Acid	Linoleic Acid	Arachidonic Acid	Eicosapentaenoic Acid
16:0	26.5[b]	27.0	11.1	18.7	17.7	17.3
18:0	14.2	14.8	9.1	13.3	11.9	10.7
18.0dma[c]	nd	4.0	1.2	2.3	2.6	2.1
22.0	0.9	1.1	2.5	1.2	0.9	2.9
24.0	1.0	1.2	0.8	1.0	0.9	0.5
16:1 n-7	2.9	3.3	1.7	3.2	1.8	2.1
18:1 n-9/n-7	24.6	21.4	48.2	29.0	18.3	18.2
20:1 n-9	0.4	0.5	1.3	0.6	0.3	0.2
18:2 n-6	12.1	11.2	6.7	14.2	7.7	6.9
18:3 n-3	0.1	0.1	2.0	0.5	0.3	2.8
20:3 n-6	2.1	2.1	1.4	2.2	1.2	1.1
20:4 n-6	5.6	6.4	8.1	6.7	24.3	5.2
20:5 n-3	0.8	0.9	0.6	1.0	0.3	16.1
22:4 n-6	0.6	0.5	0.4	0.5	9.1	1.0
22:5 n-3	1.9	1.9	1.6	1.8	1.0	11.1
22:6 n-3	3.0	2.8	1.8	2.4	0.8	0.8
Others	3.4	1.1	1.5	1.4	0.9	1.0
% Saturated	42.9	48.2	24.9	36.7	34.1	33.8
% Monounsaturated	30.4	25.8	52.4	33.7	21.0	21.2
% Polyunsaturated	26.7	26.1	22.7	29.6	44.8	45.1
Unsaturation index[d]	119	114	132	128	186	212

[a]Data are from one typical experiment, one flask (75 cm²) of cells was used for each fatty acid treatment (preincubations as in Table 1). At the end of the preincubation the cells were trypsinized, lipids were extracted, phospholipids were separated and methylated for GLC analysis. The variation between replicate GLC injections was < 2%. Even the relatively small differences between 16:0 and 18:2 treatments were reproduced in two separate experiments.

[b]Values are % of total fatty acids in the phospholipid fraction; fatty acids were listed when in one of the treatments the percentage was >1%; nd, not detectable.

[c]18:0 Dimethyl acetal.

[d]Unsaturation index = Σ (% of each fatty acid · amount of double bonds per FA).

small. Arachidonic and eicosapentaenoic acid treatments both lead to very high amounts of the respective polyunsaturated fatty acids in the cell phospholipid fraction (24 and 16%, respectively). Not only the percentages of these added fatty acids increased, but there was also a marked increase of the elongation products of arachidonic and eicosapentaenoic acids, resulting in 9% 22:4 n-6 and 11% 22:6 n-3, after the respective treatments. Palmitic acid treatment resulted in a 4% increase in dimethylacetal esters from stearic acid, which is also an elongation product. For the other fatty acid treatments there was no clear increase of elongation products. In summary, the preincubations of the EA.hy 926 cells with the various fatty acids resulted in five types of endothelial cells with clearly different fatty acid profiles of the cell phospholipid fraction.

There was also an effect on fatty acid profiles of the cell cholesteryl esters (**Table 4**). Independent of the fatty acid type used for modification, the cell cholesteryl esters contained 27–38% linoleic acid, which was mostly derived from the cationized LDL used for cholesterol enrichment. Although the differences caused by the fatty acid treatments were less than in the phospholipid fraction, the effect of the added fatty acids was visible in the cholesteryl ester moieties. Palmitic acid treatment gave 23% 16:0 in the cell cholesteryl esters. The added oleic acid was incorporated very well in the cholesteryl esters: 41% cholesteryl oleate after oleic acid addition. Linoleic acid resulted in 38% cholesteryl linoleate in the cell cholesteryl ester fraction. Preincubation with arachidonic acid gave 17% 20:4 n-6 in the cholesteryl esters and 7% of its elongation product, 22:4 n-6. Eicosapentaenoic acid treatment resulted in a cholesteryl ester fraction with 11% 20:5 n-3 and 5% 22:5 n-3.

HDL_3-mediated efflux and HDL_3 binding of fatty acid-modified cholesterol-enriched EA.hy 926 cells

Efflux of cell cholesterol to HDL_3 was measured after 12, 24, and 36 h of incubation (**Fig. 1**). The increase of unesterified and total cholesterol in the incubation media was identical (not shown). The amount of cholesterol leaving the cells increased in time for all the treatment

TABLE 4. Fatty acid composition of EA.hy 926 cell cholesteryl esters

	Treatment[a]					
	Control	Palmitic Acid	Oleic Acid	Linoleic Acid	Arachidonic Acid	Eicosapentaenoic Acid
16:0	17.5[b]	23.1	15.2	19.4	14.2	15.7
18:0	2.6	2.7	2.5	2.8	1.8	2.5
22:0	2.5	0.4	0.4	0.4	0.4	1.0
16:1 n-7	4.0	3.6	3.3	4.3	3.8	3.6
18:1 n-9/n-7	23.3	24.5	40.8	27.9	20.1	19.9
20:1 n-9	0.1	3.6	1.8	0.4	nd	nd
22:1 n-9	1.0	3.4	0.9	0.4	0.4	0.5
24:1 n-9	nd	3.7	1.2	0.4	nd	0.2
18:2 n-6	40.0	29.6	26.7	37.9	33.0	34.4
18:3 n-3	4.0	0.6	0.7	0.7	0.6	1.0
20:4 n-6	2.3	1.3	1.4	1.9	17.1	2.5
20:5 n-3	0.3	0.3	0.2	0.2	nd	11.0
22:4 n-6	0.5	nd	0.5	0.4	6.5	nd
22:5 n-3	nd	0.2	0.5	nd	nd	5.2
Others	3.1	3.0	3.9	2.9	2.1	2.5
% Saturated	22.6	30.2	22.5	26.2	19.6	23.1
% Monounsaturated	28.4	37.6	46.5	32.5	23.7	23.5
% Polyunsaturated	49.0	32.2	31.0	41.3	56.8	53.4
Unsaturation index[c]	141	108	119	124	185	185

[a]The same cells were used for fatty acid analysis of cholesteryl esters and phospholipids (see Table 3).
[b]Values are % of total fatty acids in the cholesteryl ester fraction, fatty acids were listed when in one of the treatments the percentage was >1%; nd, not detectable.
[c]Unsaturation index = Σ (% of each fatty acid · amount of double bonds per FA).

groups. The efflux of cholesterol had different rates for the various fatty acid treatments. After 12 and 24 h of incubation, the efflux was highest for palmitic acid-treated cells; respectively, 10.3 ± 0.5% and 16.7 ± 0.3% of initial cell cholesterol was released from the cells (means ± SEM, Mann-Whitney, $P < 0.01$). After 36 h of incubation with HDL_3, 21.2 ± 1.5% of the cell cholesterol was transferred to the medium. The differences in efflux between the palmitic acid-treated cells and the other fatty acid-treated cells decreased at this time point, but efflux after palmitic acid treatment remained significantly higher than after treatment with 18:1, 20:4, and 20:5 (Mann-Whitney, $P < 0.05$). No significant correlations were observed between the efflux and initial cell cholesterol levels or grade of esterification.

Table 5 shows the average efflux (mean nmol cholesterol ± SEM/mg cell protein) after fatty acid treatment of the cholesterol-enriched cells after four separate experiments. Initial cell cholesterol was measured in only two of the four experiments (not shown) and was comparable with the data shown in Table 1. Efflux was 90.6 ± 1.9 for palmitic acid-treated cells, 66.3 ± 1.8 for oleic acid-treated cells, 63.9 ± 3.9 for linoleic acid-treated cells, 60.7 ± 2.5, for arachidonic acid-treated cells, and 52.8 ± 2.6 after eicosapentaenoic acid treatment. Palmitic acid treatment resulted in significantly more

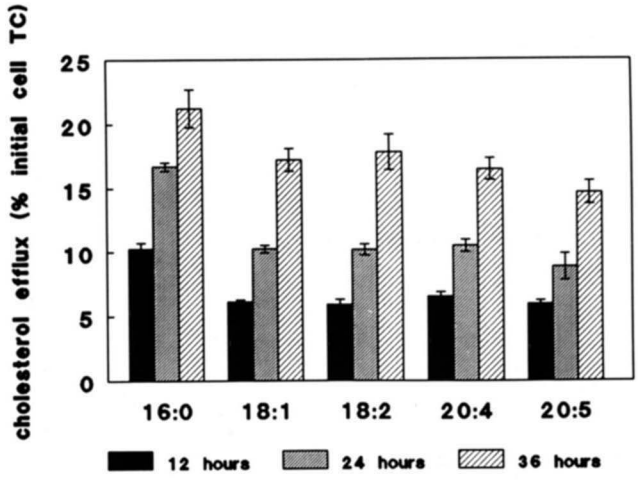

Fig. 1. Efflux of cell cholesterol after 12, 24, and 36 h of incubation with 0.2 mg HDL_3/ml, expressed as the percentage of the initial cell cholesterol per well appearing in the medium (data are from one typical experiment, means ± SEM, n = six wells). The cholesterol-enriched cells were preincubated with palmitic acid, oleic acid, linoleic acid, arachidonic acid, or eicosapentaenoic acid. For each fatty acid treatment efflux was significantly different between the three time points (Mann-Whitney, $P < 0.02$). For each fatty acid treatment, cell protein (μg per well) after 0, 12, 24, and 36 h of incubation was, respectively: palmitic acid 257 ± 11, 250 ± 4, 251 ± 6, and 258 ± 5; oleic acid 226 ± 12, 234 ± 11, 219 ± 7, and 226 ± 8; linoleic acid 219 ± 21, 229 ± 12, 239 ± 7, and 236 ± 6; arachidonic acid 225 ± 20, 211 ± 5, 216 ± 10, and 233 ± 9; eicosapentaenoic acid 217 ± 6, 234 ± 6, 263 ± 13, and 248 ± 8 (means ± SD). For initial cell cholesterol levels, see Table 1.

TABLE 5. HDL_3-mediated efflux of cholesterol from cholesterol-enriched fatty acid-modified EA.hy 926 cells

Treatment (n)	Cholesterol Increase in Medium[a]
	nmol/mg cell protein/24 h
Palmitic acid (30)	90.6 ± 1.9[b]
Oleic acid (11)	66.3 ± 1.8
Linoleic acid (18)	63.9 ± 3.9
Arachidonic acid (14)	60.7 ± 2.5
Eicosapentaenoic acid (10)	52.8 ± 2.6

[a]Values are means ± SEM from 4 separate experiments (n = total number of wells).
[b]Significantly different from other fatty acid treatments (Bonferroni *t*-test, $P < 0.001$).

efflux than the other four fatty acid treatments (Bonferroni *t*-test, $P < 0.001$).

The binding of apoE-free HDL_3 to the fatty acid-treated cells was measured at 15 and 100 μg HDL_3 protein/ml (**Fig. 2**). Both total binding and nonspecific binding were determined (nonspecific binding was measured with an excess of 1 mg unlabeled HDL_3/ml). Specific binding is defined as the difference between total binding and nonspecific binding. At 15 μg/ml no differences between the fatty acid treatments were found for total HDL_3 binding or specific HDL_3 binding. At higher HDL_3 concentrations (i.e., 100 μg/ml, above the K_D, where most of the high affinity HDL_3 sites are occupied) the palmitic acid-treated cells showed a significantly higher total and "specific" binding than all the other treatments (Bonferroni *t*-test, $P < 0.05$).

To test whether binding to the high affinity HDL receptor was important for cholesterol efflux, we used DMS-HDL_3. This modified HDL does not compete with binding of native HDL_3 to high affinity binding sites, as is shown in **Table 6** (see also ref. 19). **Fig. 3** shows the HDL_3- or DMS-HDL_3-mediated cholesterol efflux for three different fatty acid modifications. The efflux was similar for both HDL_3 preparations after all treatments. The efflux was significantly higher for palmitic acid-modified cells, if compared to oleic or eicosapentaenoic acid treatment using DMS-HDL_3 as well as native HDL_3 (Mann-Whitney, $P < 0.025$).

DISCUSSION

These results demonstrate that HDL-mediated efflux of cell cholesterol is dependent on the type of fatty acid used to modify the fatty acid profiles of cell phospholipids and cholesteryl esters. Binding of apoE-free HDL_3 to high affinity receptors on the cell membranes does not seem to be involved in the efflux process. This conclusion is based on two observations: *1)* the specific binding of HDL_3 at 15 μg/ml was not changed after fatty acid treatment and did not correlate with cholesterol efflux; and *2)* DMS-HDL_3, which does not bind to high affinity HDL receptors on cell membranes, gave efflux similar to that of native HDL_3 after each fatty acid treatment. The lack of relation between HDL binding and HDL-mediated cholesterol efflux was also reported by Karlin et al. (8), Johnson et al. (9), and Mendel and Kunitake (10), using a variety of cell types. These conclusions were based on studies involving modified HDL (8, 9) and comparison of HDL-concentration dependencies of HDL binding and HDL-mediated efflux (8, 10). However, Brinton et al. (7) concluded from their experiments with fibroblasts, in which tetranitromethane-modified HDL particles were used, that HDL binding to high affinity receptors is necessary to induce efflux from cholesterol-loaded cells.

The enrichment of the confluent endothelial cells with cationized LDL caused an increase in cellular unesterified cholesterol and cholesteryl esters as well as in the FC:PL ratio. It was possible to measure a net mass cholesterol efflux from cells to HDL_3 particles. This efflux was measured as the increase of cholesterol in the medium which is far more accurate than the measurement of the decrease of cellular cholesterol. Measurement of total as well as unesterified cholesterol increase gave similar results, indicating that cholesterol leaves the cells in the unesterified form and that no esterification of cholesterol in the medium took place during the incubation. The measurement of LCAT activity confirmed this conclusion: LCAT activity was absent in the HDL preparation used, which

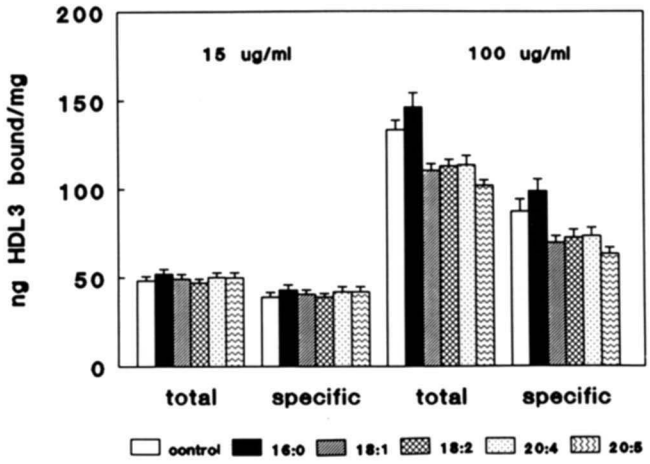

Fig. 2. Binding of apoE-free HDL_3 to EA.hy 926 cells after fatty acid treatment of cholesterol-loaded cells. The binding was measured at 15 and 100 μg ^{125}I-labeled HDL_3/ml for 4 h at 4°C. Specific binding is the difference of total and nonspecific binding (measured with an excess of 1 mg unlabeled HDL_3/ml). The data are means ± SEM from four and three separate experiments at 15 and 100 μg/ml, respectively (n = three wells for each fatty acid treatment in each experiment). Analysis of variance showed no significant differences between the fatty acid treatments for total and specific binding at 15 μg HDL_3/ml. At 100 μg/ml of HDL_3 total as well as specific binding to palmitic acid-treated cells was higher than to 18:1-, 18:2-, 20:4-, and 20:5-treated cells ($P < 0.02$). Total binding to control cells is higher in comparison with 18:1 and 20:5 treatment ($P < 0.05$). Specific binding to control cells is higher than to 20:5-treated cells ($P < 0.05$).

TABLE 6. Competition of ^{125}I-labeled HDL_3 binding by unlabeled native HDL_3 or DMS-HDL_3

Competitor Concentration	% ^{125}I-Labeled HDL_3 Bound[a]	
	Native HDL_3	DMS-HDL_3
μg protein/ml		
0	100 ± 5	100 ± 5
5	80 ± 10	118 ± 4
10	68 ± 6	109 ± 3
25	58 ± 3	102 ± 6
50	47 ± 4	109 ± 6
100	39 ± 2	114 ± 9
200	28 ± 1	77 ± 3

[a]Binding was measured with 5 μg apoE-free ^{125}I-labeled HDL_3/ml and 0–200 μg of unlabeled native HDL_3 or DMS-HDL_3/ml. The data are expressed as % of the amount of binding without competitors (means ± SEM from two separate experiments, n = three wells for each experiment).

was isolated by ultracentrifugation. The fact that no increase in cholesteryl esters in the culture medium was measured after 24 h of efflux also indicated that no extracellularly bound cationized LDL was released from the cells during the incubation (27, 28). Stein, Halperin, and Stein (28) reported that extracellular cationized LDL is very tightly bound to the plasma membrane.

The various types of fatty acids used resulted in specific changes in fatty acid profile of cell phospholipids. Addition of unsaturated fatty acids to the incubation media had more effect on fatty acid profiles than addition of saturated fatty acids (Table 3). This may have been due to endogenous phospholipase A_2 activity of the cells, resulting in a preferential incorporation of unsaturated fatty acyl chains on the *sn*2 position. It is likely that unsaturated fatty acids present at the *sn*2 position of phospholipids have a faster turnover than saturated fatty acids, mostly present at the *sn*1 position. The data presented in Fig. 1 show that the different fatty acid modifications resulted in changes in rates of cholesterol efflux. After 36 h of incubation the differences among the various treatments were smaller than after 12–24 h of incubation. This may be explained by a reversal of the cell phospholipid fatty acid profiles, resulting from substantial exchange of phospholipids between cells and HDL particles after long-term incubations (13).

Cell protein per well was virtually constant during the 36 h incubation with HDL_3 (see legend Fig. 1). These data indicate that palmitic acid had no toxic effects on the cells. Therefore, cell death cannot have caused apparent cholesterol efflux after palmitic acid treatment. The small differences in initial cholesterol levels observed after the various fatty acid treatments (Table 1) are too small to explain the differences in efflux as shown in Table 5, assuming that the efflux is linear with initial cholesterol levels. The differences in efflux rates among the fatty acid treatments may have been caused by efflux of cholesterol derived from different kinetic pools of membrane cholesterol, as proposed recently by Mahlberg and Rothblat (29): different plasma membrane microdomains (30) may represent different pools of cholesterol from which efflux occurs with different rates. Modification of membrane acyl composition may affect the packing of cholesterol in different microdomains of the plasma membrane, thereby facilitating efflux from certain microdomains with fast kinetic cholesterol pools.

The fatty acid composition of cell cholesteryl esters also changed during the preincubation with the various fatty acids. It is unlikely that this will influence the rate of cholesterol efflux in our experiments. It is controversial whether the rate of cholesteryl ester hydrolysis varies for the different types of cholesteryl esters. There are reports suggesting a substrate specificity of neutral cholesterol esterase for unsaturated cholesteryl esters (31, 32), but there are also articles denying any preference (33, 34). It was reported that the clearance of cholesteryl esters from oleic acid- or linoleic acid-treated cells was higher than the clearance from palmitic acid-modified cells (34). The authors concluded that this was due to a more isotropic condition of cytoplasmic lipid droplets, resulting from a higher triacylglycerol content and more unsaturated fatty acyl chains in the cholesteryl esters, facilitating the action of neutral cholesteryl ester hydrolases. So it is unlikely that the higher efflux rate from palmitic acid-treated cells, observed in our experiments, can be explained by increased hydrolysis of cholesteryl palmitate.

Although there was no significant correlation between the free cholesterol levels at t = 0 and cholesterol efflux during 24 h, the palmitic acid-treated EA.hy 926 cells had a relatively high free cholesterol content (Table 1). This

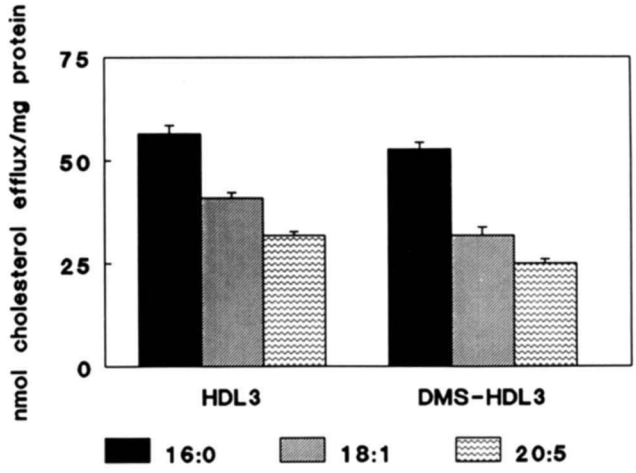

Fig. 3. Efflux of cell cholesterol after 24 h of incubation with 0.2 mg native HDL_3/ml (left) or 0.2 mg DMS-HDL_3/ml (right). Cholesterol-enriched cells were preincubated with palmitic acid, oleic acid, or eicosapentaenoic acid. The efflux was measured as the increase of cholesterol in the medium, and expressed/mg cell protein (data are from one experiment, means ± SEM, n = six wells). Efflux was significantly different between the three fatty acid treatments for both native HDL_3 and DMS-HDL_3 (Mann-Whitney, $P < 0.025$).

could be due to a lower ACAT activity after this treatment, as for palmitic acid-treated J774 cells (35). A lower ACAT activity renders more cholesterol directly available for efflux. McCloskey et al. (35) did not report whether the lower ACAT activity of palmitic acid-treated macrophages was due to a lower enzyme activity, or to changes in the availability of the cholesterol substrate in the membranes of the endoplasmatic reticulum. In contrast, Murthy et al. (36) measured a higher ACAT activity in CaCo-2 cells after palmitic acid treatment.

Other investigators, measuring exchange of cholesterol, observed a relatively low cholesterol exchange between dipalmitoylcholine vesicles and vesicles of egg phosphatidylcholine (37). This indicates that net mass efflux of cholesterol from cholesterol-enriched cells involves mechanisms different from cholesterol exchange processes. We did not measure whether the various fatty acid treatments lead to different amounts of phospholipid subclasses. It is conceivable that the amount of sphingomyelin, containing relatively high amounts of saturated fatty acids, may change. Although a high sphingomyelin content of membranes or vesicles decreases the cholesterol exchange (37, 38), the [^{3}H]cholesterol efflux from sphingomyelinase-treated fibroblasts remained unchanged (39).

The physical properties of the cell membrane can be expected to influence membrane-dependent cellular functions, like cholesterol desorption and receptor activities. Platelet aggregation (40), the phagocytotic capacities of peritoneal macrophages (41), binding properties of the insulin receptor (42), and the affinity as well as maximal binding of LDL to the LDL-receptor on peripheral blood mononuclear cells (43), U937 monocytes (44), and HepG2 cells (45) are influenced by membrane fatty acid composition. We could not detect any differences in the binding of apoE-free HDL_3 to fatty acid-modified cells at low HDL_3 concentrations (15 μg/ml). At this concentration, binding is mostly to high affinity binding sites. At higher HDL_3 concentrations (100 μg/ml), the low affinity binding becomes more important. This concentration is 3–5 times the K_D. We measured a K_D of 37 μg/ml for untreated EA.hy 926 cells (not shown) and others reported a value of about 20 μg/ml (46). We found small (statistically significant) differences at HDL_3 concentrations of 100 μg/ml. These results suggest that there may be differences in the low affinity binding sites after fatty acid treatment of cholesterol-enriched cells.

The results of the DMS-HDL_3-mediated efflux experiments also show that binding of HDL_3 to specific high affinity receptors is not important for efflux of cell cholesterol because for the three fatty acid treatments, efflux with HDL_3 and DMS-HDL_3 was similar (Fig. 3). This is in agreement with results obtained by other investigators (9).

Our experiments demonstrate that efflux of cholesterol from cholesterol-loaded EA.hy 926 cells is possible without binding of HDL_3 to specific high affinity binding sites. The composition of the cell membranes is likely to be important in determining the rate of efflux. The degree of saturation of fatty acids in membrane phospholipids could influence the distribution of cholesterol-poor and cholesterol-rich domains in cell membranes, which may play a role in cholesterol efflux (29, 30).

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