Cholesterol efflux mediated by apolipoproteins is an active cellular process distinct from efflux mediated by passive diffusion

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Abstract It is becoming increasingly accepted that removal of cellular cholesterol occurs by at least two pathways, one involving the well-described aqueous diffusion mechanism and another promoted by lipid-free apolipoproteins. We compared the contribution of apolipoprotein-dependent and -independent pathways, taking into consideration the influence of cellular metabolism, on cholesterol efflux promoted by different extracellular acceptor types. The acceptors used were assumed to participate in only passive efflux by lipid-dependent mechanisms (phospholipid vesicles and trypsin-modified high density lipoproteins) or to stimulate efflux by apolipoprotein-dependent pathways (purified apolipoprotein A-I and high density lipoproteins). Apolipoprotein-mediated cholesterol efflux was only apparent in growth-arrested or cholesterol-enriched cells and required metabolic energy. In contrast, cholesterol efflux by apolipoprotein-depleted acceptors did not depend on cell growth state, cholesterol enrichment, or metabolic energy. Apolipoprotein-mediated efflux was not observed at temperatures below 22°C, while apolipoprotein-independent efflux was only reduced by 50% at 4°C compared with incubations at 37°C. Additionally, apolipoproteins promoted a more rapid and larger decrease in intracellular cholesteryl esters when measured by changes in cholesteryl ester radioactivity, mass, or the pool of cholesterol available for esterification by acyl coenzyme A:cholesterol acyltransferase. Efflux of excess cellular cholesterol by an apolipoprotein-dependent pathway appears to involve specific cellular events consistent with the properties of an active transport pathway and distinguishable from cholesterol efflux by apolipoprotein-depleted acceptors through passive mechanisms.—**Mendez, A. J.** Cholesterol efflux mediated by apolipoproteins is an active cellular process distinct from efflux mediated by passive diffusion. *J. Lipid Res.* 1997. **38:** 1807–1821.

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The ability of high density lipoproteins (HDL) to remove excess cholesterol from cultured cells is well established (reviewed in ref. 1). Studies supporting this function of HDL in vivo include demonstration that exogenously administered HDL can prevent (2) or regress (3) experimentally induced atherosclerotic plaques in rabbits and that overexpression of apolipoprotein (apo) A-I in transgenic mice prevents diet-induced atherosclerosis (4) and can reduce the extent of atherosclerosis in apoE knockout mice (5). Two main hypotheses have been proposed to explain this function of HDL. The aqueous diffusion model described by Rothblat and colleagues (1, 6, 7) predicts a passive role for HDL, solely by acting as an acceptor of desorbed cholesterol from cell membranes and the extent of efflux dictated by the chemical gradient for cholesterol between cell and lipoprotein membranes. Many studies can support such a role for HDL (1, 6, 7 and references therein). This hypothesis makes no prediction of mechanisms by which excess cholesterol from intracellular sites of accumulation is transported to the plasma membrane. If extracellular cholesterol acceptors do not stimulate the transport of intracellular cholesterol stores for subsequent removal, then a role for cellular mechanisms in directing cholesterol transport would be predicted. Thus, depletion of intracellular cholesterol would involve the removal of plasma membrane cholesterol followed by the hydrolysis of cholesteryl ester stores and transport of the liberated free cholesterol to the plasma membrane by yet unknown mechanisms. The net flux of cholesterol would therefore be dictated by the presence of extracellular acceptors and their capacity to absorb plasma membrane cholesterol.

Many studies have demonstrated that removal of cholesterol from cultured cells depends on desorption

Abbreviations: HDL, high density lipoprotein; apo, apolipoprotein; ACAT, acyl coenzyme A:cholesterol acyltransferase; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; LDL, low density lipoprotein; TLC, thin-layer chromatography.

from the membrane and is not influenced by HDL apolipoproteins (7–9). Removal of free cholesterol from cells was effected by phospholipid vesicles in the absence of apolipoproteins (e.g., 10) and by phospholipid:albumin complexes (11), suggesting an apolipoprotein-independent process. Additionally, emulsions of phospholipids and triglycerides have been shown to deplete intracellular cholesterol (as esters) from macrophages during long incubations (12). In contrast, Ho, Brown, and Goldstein (13) showed that phospholipid vesicles were unable to deplete cholesteryl ester mass from mouse peritoneal macrophages under conditions where HDL depleted 75% of the cholesteryl esters. We have shown that phospholipid vesicles can remove free cholesterol from cholesterol over-loaded cells but these vesicles had limited capacity to deplete the intracellular pool of cholesterol available for esterification by acyl coenzyme A:cholesterol acyltransferase (ACAT) unless apolipoproteins were also present (14). The reasons for these apparently contradictory results remain unknown, making the role of apolipoproteins in these events difficult to establish.

The second hypothesis involves the interaction of HDL (through its apolipoprotein moieties) with cell surface binding sites that induce an intracellular signal(s) promoting translocation of cholesterol from intracellular sites to the plasma membrane (15–17). Evidence supporting this hypothesis include demonstrating that preventing the binding of HDL to cells by chemical or protease modification of HDL apos (17–20) or by reacting HDL with antibodies against apoA-I (21–23) inhibited the ability of HDL to promote cholesterol efflux from cells. Additional studies have shown that HDL can stimulate signaling pathways and that pharmacologically induced signals may enhance cellular cholesterol efflux (24–27). Such data suggest a role for HDL-associated apolipoproteins in mediating efflux by mechanisms distinct from those predicted by aqueous diffusion. Although several HDL binding proteins have been identified (28–30) their function as cellular HDL binding sites and role in modulating cholesterol efflux remain to be established.

A direct role for apolipoproteins in promoting cholesterol efflux is supported by reports demonstrating that purified apolipoproteins can promote the net removal of cholesterol from cells that are over-loaded with cholesterol (12, 14, 31–38). Synthetic peptides mimicking the alpha-helical structure of the exchangeable apolipoproteins can also stimulate cholesterol efflux from cells, suggesting that this activity lies in a structural motif shared by the various apolipoproteins and may not depend on a specific amino acid sequence (14, 38). Cholesterol efflux from cells by free apolipoproteins has been suggested to have physiologic relevance (39, 40) as lipid-depleted apolipoproteins exist in lymph, interstitial fluids, and plasma (41–43). Incubation of cholesterol-enriched cells with apolipoproteins results in the formation of pre-beta HDL-like particles composed of apolipoproteins and cellular lipids including cholesterol, sphingomyelin, and phosphatidylcholine (31, 33, 39, 40). Thus, apolipoprotein-mediated cholesterol efflux may require the added step of phospholipid association with the protein, but events leading to particle assembly and the relationship to cholesterol removal have not been fully elucidated. Whether efflux of cellular cholesterol by apolipoproteins present on HDL particles involves similar events is unknown; however, it has been suggested that a portion of apolipoproteins dissociate from HDL and this fraction of apolipoproteins may act in promoting removal of cell lipids (20, 39, 40, 44).

It is becoming increasingly accepted that cholesterol efflux from cells occurs by more than a single mechanism (e.g., 44, 45). Recent studies from our laboratory have shown that pharmacological disruption of the Golgi apparatus prevents apolipoprotein-dependent cellular cholesterol efflux without affecting lipid-dependent efflux (36, 46). Based on these data, we suggested the involvement of a Golgi apparatus-dependent pathway for mediating lipid transport and efflux by apolipoproteins. In fibroblasts obtained from patients with Tangier disease, apoA-I failed to promote lipid efflux and cholesterol efflux by HDL was impaired, compared with normal cells (26, 37, 47), while efflux by apolipoprotein-depleted acceptors appeared unaffected (37, 47). These results are consistent with the notion that apolipoprotein and diffusional efflux are distinct mechanisms and provide evidence that a specific gene product is necessary for efficient efflux of excess cellular cholesterol by apolipoproteins.

Taken together, several lines of evidence lead to the hypothesis that HDL apolipoproteins play a functional role in ridding cells of excess intracellular cholesterol and influence a process distinct from efflux mediated by diffusional processes. In the present study we compared apolipoprotein-independent and -dependent pathways in promoting cellular cholesterol efflux by using different types of acceptors, those assumed to participate in only passive efflux or that stimulate active efflux by apolipoprotein-dependent pathways. We examined the rates and extent of cholesterol transport and efflux from cultured cells and compared the influence of cell metabolism on efflux to the various acceptor types. The results demonstrate that apolipoprotein-containing acceptors are more efficient than apolipoprotein-depleted acceptors in promoting efflux of excess intra-

cellular cholesterol through mechanisms that depend on active cellular processes.

MATERIALS AND METHODS

Materials

Materials used have been described in detail elsewhere (36, 46).

Cell culture

Human skin fibroblasts were grown and maintained as previously described (46). Fibroblasts from a patient with Tangier disease were generously provided by Dr. John F. Oram, University of Washington. The inability of apolipoproteins to promote cholesterol efflux from these cells has been described (cells TG2 in reference 37). All cell incubations were done at 37°C unless otherwise noted. Cellular cholesterol was labeled by incubating pre-confluent cells in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 0.5 μCi/ml [1,2-^{3}H]cholesterol (50–60 mCi/mmol, NEN, Boston) for approximately 3 days until cells became confluent. Labeled cells were rinsed three times with phosphate-buffered saline (PBS) and cholesterol-enriched by incubation in DMEM containing 1 mg/ml fatty acid-free albumin (BSA) and 100 μg/ml low density lipoproteins (LDL) protein for 24 h, followed by a 48-h incubation in serum-free DMEM containing 1 mg/ml to allow equilibration of cholesterol pools. These conditions resulted in nearly equal specific activity of cellular free and esterified cholesterol prior to the addition of experimental medium (see appropriate figures and tables).

Lipoproteins, apolipoproteins, and phospholipid vesicles

LDL and HDL_3 (hereafter referred to as HDL) were prepared by sequential ultracentrifugation techniques in the density intervals 1.019–1.063 g/ml and 1.12–1.21 g/ml, respectively, and HDL depleted of apoE and B-containing particles as previously described (36).

ApoA-I was purified from isolated HDL as described (14).

Phospholipid vesicles were prepared from egg yolk phosphatidylcholine (Sigma Chemicals) by sonication as previously described (48).

Protein content of lipoproteins was measured by the method of Lowry et al. (49) using bovine serum albumin as the standard. Free and esterified cholesterol were measured by enzymatic assay as described by Artiss and Zak (50). Phospholipids were assayed using a commercially available kit (Biochemical Diagnostics).

HDL apolipoproteins were modified with trypsin as previously described (18, 20) except that incubations were terminated after 20 min. Previous results have shown that under these conditions there was no apparent change in the size of HDL after trypsin treatment by nondenaturing gradient gel electrophoresis (20). Additionally, the trypsin-modified HDL had similar lipid composition compared to the parent HDL. For a typical preparation, the free cholesterol to phospholipid weight ratios were 0.068 and 0.071 and the total cholesterol to phospholipid weight ratios were 0.72 and 0.71 for control and trypsin-modified HDL, respectively. In contrast, the protein to phospholipid weight ratios were 1.6 and 1.2 for control and trypsin-treated HDL, respectively, suggesting that about 25% of the HDL-associated protein was lost after protease treatment. Comparable results were obtained for at least six different trypsin-modified HDL particles prepared for the present studies that, compared with the parent HDL, showed between a 16 and 25% loss of protein relative to phospholipid.

[^{3}H]cholesterol efflux

Efflux of labeled cholesterol from cells was measured by appearance of [^{3}H]cholesterol in experimental medium after incubation as described (36, 46). Briefly, cell medium was recovered and radioactivity in aliquots of the culture medium was measured. Cell layers were washed twice with PBS containing 1 mg/ml BSA, twice with PBS, then cell lipids were extracted with hexane–isopropanol 3:2 (v:v). Cell protein was dissolved in 0.1 M NaOH and quantitated by the method of Lowry et al. (49). Lipids were separated by thin-layer chromatography (TLC) developed with heptane–diethyl ether–methanol–acetic acid 80:30:3:1.5 (v:v:v:v) to quantitate free and esterified cholesterol radioactivity by scintillation counting.

Cellular cholesterol mass

Cell free and esterified cholesterol mass were measured after TLC separation of free and ester cholesterol, as above, as described in detail (36). Appropriate spots were scraped from the TLC plates and saponified by heating at 80°C for 1 h in 1 ml 1 M KOH in 80% ethanol. Non-saponified lipids were extracted with 1.5 ml H_2O and 5 ml hexane and phases were separated by centrifugation at 1000 *g* for 10 min. Aliquots of the hexane phase were evaporated under a stream of N_2 gas in a 40°C water bath, reconstituted in isopropanol, and cholesterol mass was assayed enzymatically (51).

Cellular cholesterol esterification

Esterification of cellular cholesterol by ACAT was measured after incubation with experimental medium by the incorporation of [^{14}C]oleate into cholesteryl esters during an additional 1-h incubation at 37°C in DMEM containing 9 μM [^{14}C]oleate and 3 μM BSA (36, 52). Cell lipids and proteins were extracted as above. Lipids were separated by TLC developed with hexane–diethyl ether–acetic acid 65:20:1 (v:v:v) and appropriate spots were taken for scintillation counting. Cholesterol esterification was expressed as nanomoles of [^{14}C]oleate incorporated into [^{14}C]cholesterol esters per mg of cell protein.

Other methods

In some experiments cellular cholesterol mass, radioactivity, and cholesterol esterification were measured simultaneously in the same culture dish as described (36).

Cellular ATP was measured by a commercially available luciferase-based assay according to manufacturer's directions (Sigma Chemicals).

Statistical differences between groups were determined by Student's *t*-test and, if not, indicated significance was assumed for *P* values less than 0.05.

RESULTS

Comparison of cholesterol efflux by apolipoprotein-containing or -depleted cholesterol acceptors

The ability of different acceptors to promote [^{3}H]cholesterol efflux and deplete the substrate pool of cholesterol available for esterification by ACAT from LDL cholesterol-enriched fibroblasts was examined over time. HDL was used as an acceptor able to promote both apolipoprotein- and lipid-dependent efflux pathways. ApoA-I can only promote apolipoprotein-dependent efflux. Trypsin-modified HDL, depleted of apolipoproteins, and phospholipid vesicles, devoid of apolipoproteins, are assumed to only participate in lipid-dependent efflux pathways. HDL, trypsin-modified HDL, and phospholipid vesicles were incubated with cells at equal phospholipid concentration (25 μM) assuming that this parameter would normalize for total particle surface available to accommodate cholesterol. In the case of the vesicles, approximately half of the phospholipid molecules are found on the inner leaflet of the vesicle bilayer. However, given the rapid rate at which cholesterol can traverse a phospholipid bilayer (53), the inner leaflet surface should still be available to accommodate cholesterol. ApoA-I was incubated with cells based on protein content (5 μg/ml). All acceptors tested were able to promote efflux of [^{3}H]cholesterol from cells into the culture medium over time (**Fig. 1**). Of the acceptors tested, HDL was significantly ($P < 0.005$) more effective at promoting cholesterol efflux at all times. Trypsin-modified HDL, phospholipid vesicles, and apoA-I had apparently similar ability to promote cholesterol efflux, although differences in efflux were consistently detected when earlier times were studied (Fig. 1, inset). HDL and phospholipid vesicles promoted efflux of [^{3}H]cholesterol to a similar extent during the first 2.5 h of incubation, while apoA-I and trypsin-modified HDL were less effective. Upon further incubation, cholesterol efflux to HDL continued in a nearly linear fashion, whereas the observed rate of efflux to phospholipid vesicles began to decrease relative to HDL.

The effects of each acceptor on changes in cellular free and esterified [^{3}H]cholesterol and the substrate pool of cholesterol available for esterification were measured in the same experiment (**Fig. 2**). All of the acceptors decreased cellular [^{3}H]cholesterol over time, relative to incubations with DMEM containing only BSA, and this decrease accounted for the majority of cholesterol appearing in the medium when HDL, trypsin-modified HDL, and phospholipid vesicles were present. In contrast, most of the cholesterol appearing in the medium after incubation with apoA-I was accounted for by a decrease in cellular [^{3}H]cholesteryl esters compared to incubations with DMEM alone. Although HDL promoted significantly greater cholesterol efflux than apoA-I, these acceptors were equally effective at depleting cellular [^{3}H]cholesteryl esters at the concentrations tested. Trypsin-modified HDL had a limited capacity to deplete cellular [^{3}H]cholesteryl esters compared to DMEM containing only albumin. Phospholipid vesicles decreased cholesteryl esters over the course of the study, however, at an apparently slower rate and to a lesser extent than the apolipoprotein-containing acceptors. Similar to changes in cellular [^{3}H]cholesteryl esters, HDL and apoA-I had nearly identical effects on decreasing the substrate pool of cholesterol available for esterification by ACAT. Trypsin-modified HDL and phospholipid vesicles also decreased cholesterol esterification, but again, at slower rates and to a lesser extent than HDL or apoA-I. For example, semi-log plots of the fractional decrease (relative to incubations with DMEM) in cellular [^{3}H]cholesteryl esters or of the fractional decrease in cholesterol esterification over time demonstrated a linear relationship for all acceptors, however, the relative rates were 3- to 4-fold faster for HDL and apoA-I than for trypsin-modified HDL or phospholipid vesicles (not shown).

In these experiments cellular free and esterified cho-

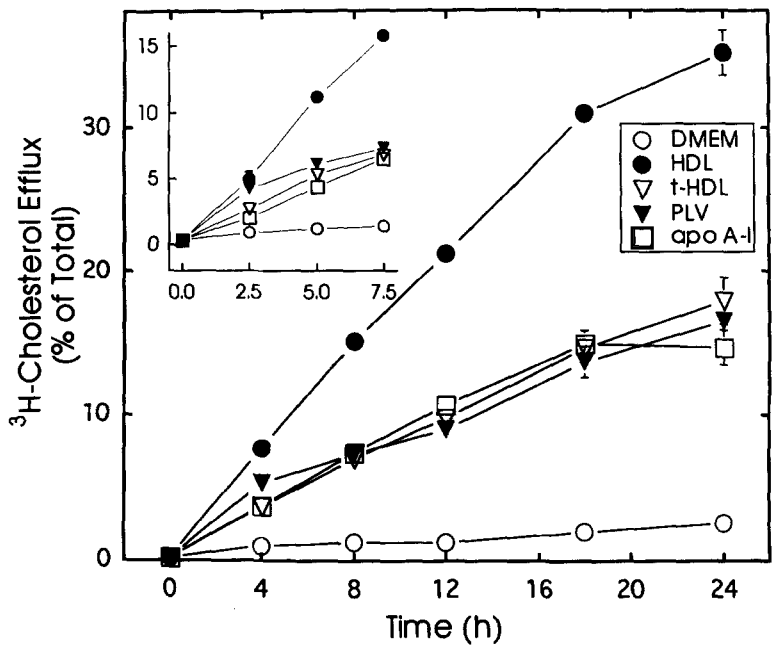

Fig. 1. Time course of cellular cholesterol efflux from LDL cholesterol-enriched fibroblasts by different acceptor types. Cells labeled with [^{3}H]cholesterol and enriched with LDL cholesterol as described under Methods were incubated with DMEM containing 1 mg/ml BSA (DMEM, ○) alone or with the following additions: 25 μM phospholipid of HDL (HDL, ●), trypsin-modified HDL (t-HDL, ▽) or phospholipid vesicles (PLV, ▼) or with 5 μg/ml of apoA-I (□) for the indicated times at 37°C. After incubation, efflux medium was removed and cells incubated with DMEM containing 9 μM [^{14}C]oleate and 3 μM BSA for 1 h at 37°C. Cell lipids and proteins were extracted as described under Methods. Cellular lipids were separated by TLC to quantitate [^{3}H]cholesterol and ^{3}H/^{14}C-cholesteryl esters. Cholesterol efflux is expressed as the percent of total (cell + medium) [^{3}H]cholesterol appearing in the medium. The inset shows results from a separate experiment conducted under identical conditions but at shorter times. Results are the mean ± SD of three dishes; missing error bars are contained within the symbols. For all dishes, recovery of cell protein and [^{3}H]cholesterol averaged (n = 126) 26.4 ± 2.6 μg/dish and 24632 ± 2647 cpm/dish, respectively, and was similar for all conditions. Specific activity of free and esterified cholesterol was 20721 ± 413 and 19117 ± 870 cpm/μg cholesterol determined in a parallel set of dishes (n = 6) incubated under identical conditions prior to the addition of experimental medium.

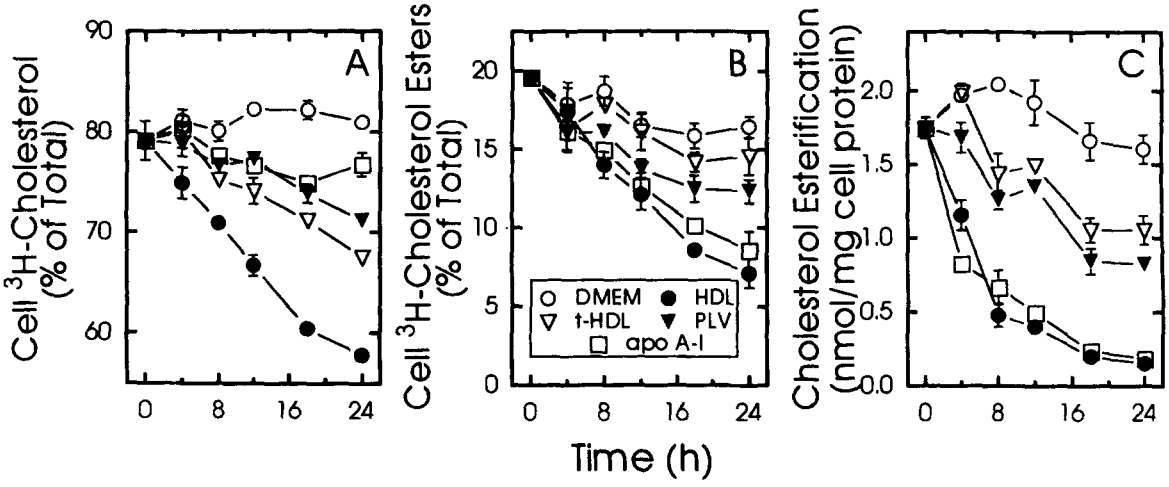

Fig. 2. Changes in cellular free and esterified cholesterol in LDL cholesterol-enriched fibroblasts by different acceptor types. Cells and conditions used were described in the legend to Fig. 1. Panels A and B: Cell [^{3}H]cholesterol and [^{3}H]cholesteryl ester, respectively, are expressed as the percent of total (cell + medium) [^{3}H]cholesterol. Panel C: Cholesterol esterification is expressed as nmoles of [^{14}C]oleate incorporated into cholesteryl esters per mg of cell protein. All results are the mean ± SD of three dishes.

TABLE 1. Effect of different acceptors on efflux of cellular cholesterol mass and radiolabel

Efflux Medium	[^{3}H]Cholesterol			Cholesterol Mass		Cholesterol Specific Activity	
	Medium	Cell Free	Cell Ester	Free	Ester	Free	Ester
	% of total			*μg/mg cell protein*		*cpm/μg*	
DMEM	1.9 ± 0.1	83.8 ± 0.4	14.3 ± 0.4	47.3 ± 0.7	8.9 ± 0.7	16449 ± 554	14860 ± 797
HDL	24.4 ± 1.1	69.5 ± 1.2	6.1 ± 0.3	36.8 ± 1.3[a]	4.0 ± 0.3[b]	15777 ± 473	13105 ± 809
t-HDL	11.5 ± 0.6	76.0 ± 1.5	12.5 ± 0.9	46.3 ± 2.4	8.8 ± 0.9	15954 ± 351	13815 ± 1209
PLV	10.7 ± 0.7	79.4 ± 2.1	9.9 ± 1.5	40.5 ± 1.5[a]	6.6 ± 0.7[c]	17469 ± 329	14339 ± 780
ApoA-I	10.8 ± 0.3	82.3 ± 0.4	6.9 ± 0.3	43.2 ± 1.2	4.5 ± 0.3[b]	16561 ± 871	13417 ± 1050

Fibroblasts were labeled with [^{3}H]cholesterol and enriched with LDL cholesterol as described in the legend to Fig. 1. Cultures were incubated with DMEM containing 1 mg/ml BSA alone (DMEM) or containing 25 μM phospholipid of HDL, trypsin-modified HDL (t-HDL), or phospholipid vesicles (PLV), or 5 μg/ml of apoA-I protein and incubated for 18 h at 37°C. After incubation, medium was collected to measure radioactivity and cells were analyzed for free and esterified (Ester) cholesterol mass and radioactivity as described under Methods. Results are the means ± SD of four culture dishes for each condition.

[a] $P < 0.02$, [b] $P < 0.001$, compared to DMEM.

[c] $P < 0.025$ compared to DMEM and $P < 0.01$ compared to HDL.

lesterol were labeled to nearly equal specific activity (see legends) and we assumed that changes in radioactivity reflected changes in cholesterol mass. This assumption was largely confirmed in a parallel study in which cholesterol mass and radioactivity changes were compared for each of the acceptors after a 24-h incubation (**Table 1**). [^{3}H]cholesterol efflux and changes in cellular cholesterol radioactivity paralleled those of the previous experiment and accurately reflected changes in cellular cholesterol mass. The specific activity of free and esterified [^{3}H]cholesterol approached equality, but was slightly lower for esterified than for free cholesterol, a consistent observation in several experiments. Nevertheless, these data show that mass and radiolabeled cholesterol efflux were highly correlated and that radiolabeled cholesterol accurately reflected changes in cholesterol mass under the conditions used. Also worth noting is that in the LDL cholesterol-enriched cells there were no measurable changes in the specific activity of free or esterified cholesterol after incubation with any of the extracellular cholesterol acceptors compared to incubations with DMEM alone.

In the above studies, the apolipoprotein-depleted acceptors were incubated at equal concentration of particle phospholipid, assuming that this measure normalizes for particle surface available to accept cholesterol. However, given the structural differences between HDL and sonicated phospholipid vesicles, this assumption may be incorrect. Therefore, the concentration of vesicles, relative to HDL, may have been limiting and accounted for the observed differences in the above experiments. In addition, the lower extent of cholesterol efflux promoted by phospholipid vesicles and trypsin-modified HDL, compared with HDL, could account for the reduced ability to decrease intracellular cholesteryl esters and the substrate pool of cholesterol available for esterification by ACAT. To address these issues we compared the dose response of each acceptor on the ability to promote [^{3}H]cholesterol efflux and decrease cholesterol esterification in LDL cholesterol-loaded fibroblasts (**Fig. 3**). All of the acceptors promoted [^{3}H]cholesterol efflux from cells in an apparently dose-dependent fashion. Saturation was clearly attained when apoA-I was used as the cholesterol acceptor, and was approached when HDL or trypsin-modified HDL were used. Phospholipid vesicles continually increased [^{3}H]cholesterol efflux over the range of concentrations studied. For HDL, trypsin-modified HDL and the phospholipid vesicles, the majority of efflux was accounted for by a decrease in cellular [^{3}H]cholesterol. HDL was more effective at reducing [^{3}H]cholesteryl esters at each dose compared with the apolipoprotein-depleted acceptors, and the latter were unable to reduce cellular [^{3}H]cholesteryl esters to the level achieved with HDL. The bulk of efflux mediated by apoA-I was due to depletion of cellular [^{3}H]cholesteryl esters, similar to the decrease produced by HDL. HDL and apoA-I also had the greatest effect on reducing the pool of cellular cholesterol available for esterification by ACAT. Trypsin-modified HDL and phospholipid vesicles did reduce cholesterol esterification in a dose-dependent fashion, although the extent of this effect was significantly less than observed for apoA-I or HDL. Most notably, in spite of increasing [^{3}H]cholesterol efflux at the higher doses of phospholipid vesicles, there was not a concomitant decrease in either cellular [^{3}H]cholesteryl esters or cholesterol esterification. When HDL and phospholipid vesicles were compared at concentrations having a similar extent of cholesterol efflux (e.g., 25 and 100 μM, respectively, both promoting the efflux of 29% of the labeled cholesterol), HDL was still significantly more effective at decreasing cellular cholesterol esters and ACAT activity. These data suggest a dissociation between the pools of cholesterol available for efflux to the

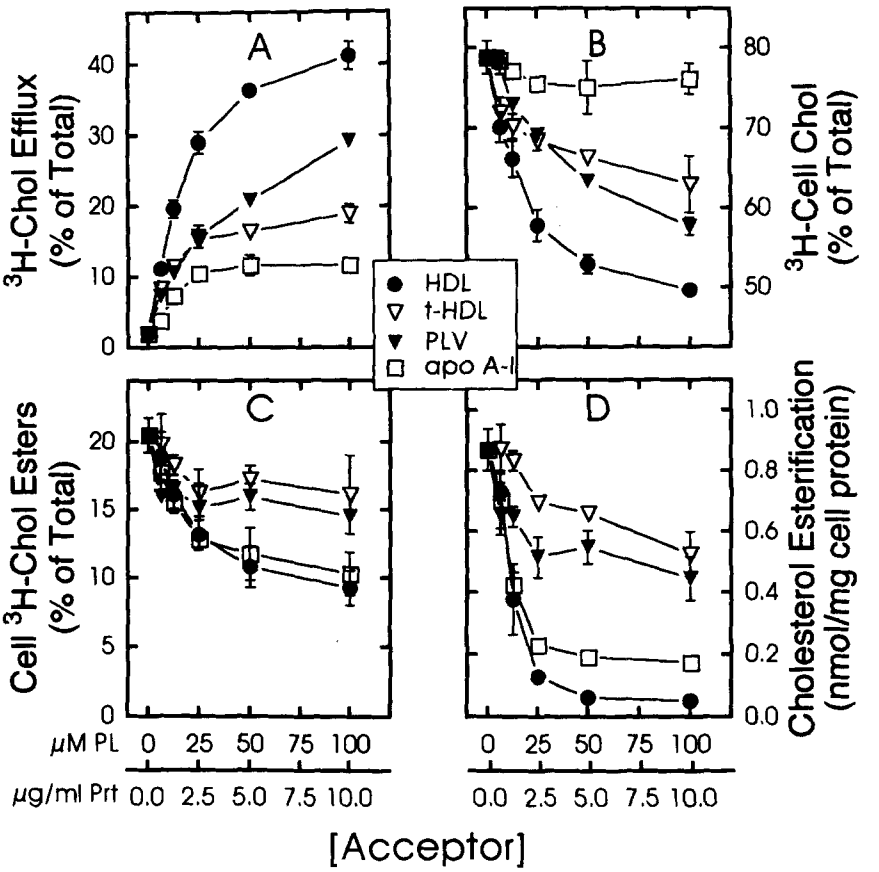

Fig. 3. Effect of acceptor dose on cellular cholesterol and cholesterol esterification in LDL cholesterol-enriched fibroblasts. Cultures were grown, labeled with [^{3}H]cholesterol, and enriched with LDL as described in the legend to Fig. 1. After the incubation to arrest cell growth and equilibrate cellular cholesterol pools, cells were incubated with the indicated concentrations of HDL (●), trypsin-modified HDL (t-HDL, ▽) or phospholipid vesicles (PLV, ▼) based on phospholipid content (μM PL) or apoA-I (□) based on protein content (μg/ml Prt) in DMEM containing 1 mg/ml BSA for 16 h at 37°C. Medium and cells were analyzed as described in the legend to Fig. 1 and under Methods [^{3}H]cholesterol results are expressed as the percent of total ^{3}H radioactivity. Panel A: [^{3}H]cholesterol (Chol) efflux. Panel B: Cellular free [^{3}H]cholesterol. Panel C: Cellular [^{3}H]cholesteryl esters. Panel D: Cholesterol esterification expressed as nmoles of [^{14}C]oleate incorporated into cellular cholesterol esters per mg of cell protein. All results are the mean ± SD of three dishes. For all dishes, recovery of cell protein and [^{3}H]cholesterol averaged (n = 96) 17.6 ± 1.2 μg/dish and 16289 ± 1768 cpm/dish, respectively, and was similar for all conditions.

lipid-containing acceptors and the ability to reduce intracellular cholesterol pools. Additionally, these results are consistent with the notion that apolipoproteins are necessary to selectivity reduce a pool of intracellularly derived cholesterol.

Comparison of cholesterol efflux by different acceptor types from growing cells

Previous studies have demonstrated that apolipoprotein-mediated efflux of cellular cholesterol is reduced or absent in cells not enriched with cholesterol (33, 36), consonant with the idea that apolipoproteins mediate efflux of excess cellular cholesterol and not directly of plasma membrane-associated cholesterol. Cholesterol efflux by the various cholesterol acceptors used above was compared from growing, pre-confluent, [^{3}H]cholesterol-labeled fibroblasts. Under these culture conditions, up to 90% of cellular cholesterol is reported to be present in the plasma membrane of fibroblasts (54). In cells not enriched with cholesterol, [^{3}H]cholesterol efflux was largely similar for HDL, trypsin-modified HDL, and phospholipid vesicles, with the vesicles exhibiting a greater capacity to promote efflux at the highest concentration tested (**Fig. 4**). In contrast, apoA-I showed little cholesterol efflux activity, stimulating efflux less than 2-fold compared with efflux to DMEM containing 1 mg/ml of BSA, whereas in cholesterol-enriched cells a greater than 10-fold increase was consistently observed (e.g., Fig. 1). Parallel cultures were incubated with 50 μM HDL, trypsin-modified HDL, or

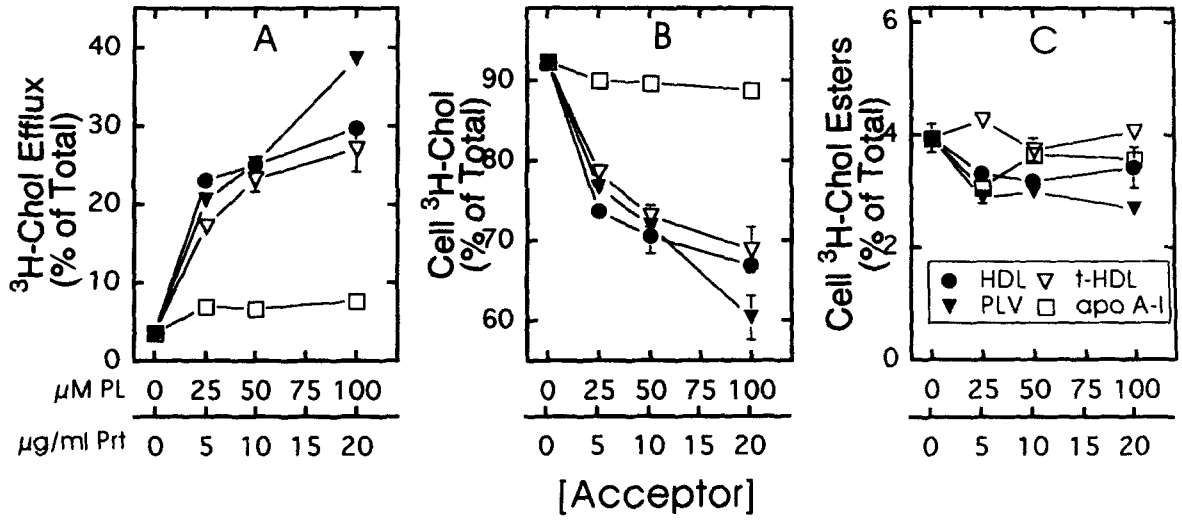

Fig. 4. Cellular cholesterol efflux from non-confluent, growing fibroblasts by different acceptor types. Fibroblasts were plated and maintained in DMEM containing 10% fetal bovine serum and 0.5 μCi/ml [^{3}H]cholesterol. When cells reached approximately 50% confluence, they were rinsed 5 times with PBS, followed by a 30-min wash incubation with DMEM (no additions) at 37°C. Cells were then incubated with the indicated concentrations of HDL (●), trypsin-modified HDL (t-HDL, ▽) or phospholipid vesicles (PLV, ▼) based on phospholipid content (μM PL) or apoA-I (□) based on protein content (μg/ml Prt) in DMEM containing 1 mg/ml BSA for 16 h at 37°C. Medium and cells were analyzed for [^{3}H]cholesterol as described under Methods. Results are expressed as the percent of total ^{3}H radioactivity. Panel A: [^{3}H]cholesterol (Chol) efflux. Panel B: Cellular free [^{3}H]cholesterol. Panel C: Cellular [^{3}H]cholesteryl esters. Results are the mean ± SD of three dishes. For all dishes, recovery of cell protein and [^{3}H]cholesterol averaged (n = 48) 9.4 ± 1.3 μg/dish and 13660 ± 1492 cpm/dish, respectively.

phospholipid vesicles based on phospholipid content or with 10 μg/ml of apoA-I and cholesterol mass measured after an 18-h incubation (**Table 2**). The low levels of cholesteryl esters present in cells under these conditions were below the sensitivity of the assay but could account for less than 1.2 μg cholesterol/mg cell protein, equivalent to less than 5% of total cholesterol mass, in agreement with the radiolabel data. None of the acceptors had a significant effect on cellular free cholesterol mass compared with cells incubated in DMEM alone. However, under these conditions, HDL, trypsin-modified HDL, and phospholipid vesicles reduced the specific activity of cellular [^{3}H]cholesterol. For HDL and trypsin-modified HDL this may have resulted from the exchange of cholesterol between the cells and the lipoproteins (containing 2.3 μg cholesterol/dish free cholesterol) without promoting a net flux of cholesterol out of the cells. For phospholipid vesicles, devoid of cholesterol, efflux of radiolabeled cholesterol into the medium must represent a net flux of cholesterol from the cells. Thus, lower cholesterol specific activity in cells incubated with phospholipid vesicles could arise if sterol synthesis maintained cell cholesterol levels within the range of control cells. Indeed, when medium was lipid extracted and cholesterol mass was measured, medium from cells treated with phospholipid vesicles contained 0.8 μg cholesterol/dish compared to a cell cholesterol content of 4.3 μg/dish and sufficient to account for a 20% decrease in specific activity. In contrast, medium from dishes incubated with HDL or trypsin-modified HDL showed no measurable change in free or esterified cholesterol mass after incubation with cells.

TABLE 2. Effects of different cholesterol acceptors on cholesterol mass and radioactivity in growing cells

Efflux Medium	Free Cholesterol	Specific Activity
	μg/mg protein	*cpm/μg*
DMEM	31.1 ± 3.5	43903 ± 2349
HDL	29.8 ± 1.2	32012 ± 490[a]
Trypsin-HDL	30.7 ± 0.9	36625 ± 1282[a]
PLV	28.1 ± 1.3	36145 ± 858[a]
ApoA-I	31.0 ± 0.9	41638 ± 2988

Human skin fibroblasts were treated exactly as described in the legend to Fig. 4. Cells were incubated with DMEM containing 1 mg/ml BSA alone (DMEM) or with 50 μM HDL, trypsin-modified HDL (trypsin-HDL), or egg yolk phospholipid vesicles (PLV) based on phospholipid content, or with 10 μg/ml apoA-I protein for 18 h at 37°C. After incubation, medium and cells were analyzed for cholesterol mass and radioactivity as described under Methods. Results are the means ± SD of four dishes.

[a] $P < 0.01$ compared to incubations with DMEM.

Effects of energy poisons on cholesterol efflux

Whether efflux of excess cellular cholesterol requires metabolic energy remains unknown. The effects of ATP depletion, by use of the energy poisons NaF (an inhibi-

tor of glycolysis) and KCN (uncouples oxidative phosphorylation), was examined on cholesterol efflux by HDL, trypsin-modified HDL, phospholipid vesicles and apoA-I from either growing, normal fibroblast cultures or growth-arrested and LDL cholesterol-enriched normal cells or LDL cholesterol-enriched fibroblasts derived from a patient with Tangier disease. The latter cell line has been demonstrated to lack apolipoprotein-mediated cholesterol efflux activity (37) and served as an additional control to examine the effects of energy poisons under conditions where mainly passive diffusion events are involved in cholesterol efflux. ATP depletion was accomplished by pre-incubating cells with KCN and NaF prior to addition of acceptors. Control studies showed that after pre-treatment, there was a 48–63% decrease in ATP levels compared with control cells. In these and other similar experiments, no evidence of cell toxicity was observed based on similarities in the recovery of cell protein and [^{3}H]cholesterol or by microscopic examination. Treating cells with energy poisons consistently increased the proportion of labeled cholesteryl ester compared with control cells (e.g., see legend to **Fig. 5**), the reasons for this were not further investigated. As above, HDL, trypsin-modified HDL, and the phospholipid vesicles could promote efflux of labeled cholesterol from growing cells, while apoA-I was without effect (Fig. 5). Treating cells with energy poisons had no measurable effects on cholesterol efflux to trypsin-modified HDL or phospholipid vesicles but caused a 10–15% reduction in HDL-mediated cholesterol efflux from cells under these conditions. When cells were enriched with cholesterol by incubation with LDL, efflux to apoA-I became apparent and was inhibited by up to 80% in cells treated with energy poisons. HDL-mediated cholesterol efflux from cholesterol-enriched cells was inhibited by up to 50% by treatment with energy poisons compared with control incubations. In contrast, energy poisons had little or no effect on cholesterol efflux mediated by either trypsin-modified HDL or phospholipid vesicles under the same culture conditions. It is worth noting that energy inhibitors reduced cholesterol efflux from cholesterol-enriched cells to HDL to the level of efflux obtained by trypsin-modified HDL. When efflux was measured in fibroblasts from a patient with Tangier disease, apoA-I had almost no capacity to stimulate cholesterol efflux in agreement with previous observations (37), and thus energy poisons were without effect. HDL-mediated efflux was decreased in Tangier cells compared to normal cells enriched with cholesterol, and the extent of efflux and the effects of energy poisons resembled that observed in growing, normal cells. Trypsin-modified HDL and phospholipid vesicles also promoted cholesterol efflux from the patient's cells, and treatment with energy poisons caused an approximately 10% decrease in efflux, as observed for HDL, compared with control incubations. These results suggest that apolipoprotein-mediated cholesterol efflux, observed only in normal fibroblasts upon enrichment with cholesterol, depends on metabolic energy. Efflux mediated by lipid-containing acceptors, observed in normal and Tangier disease cells regardless of cholesterol enrichment, is independent of metabolic energy.

Effects of temperature on cholesterol efflux

The ability of HDL, apoA-I and phospholipid vesicles to promote [^{3}H]cholesterol efflux from LDL cholesterol-enriched cells was measured at different temperatures between 4 and 37°C (**Fig. 6**). At 37°C, efflux as a percent of total [^{3}H]cholesterol was greatest for HDL followed by phospholipid vesicles then apoA-I, similar to previous results. Cholesterol efflux to all acceptors, including DMEM, decreased with decreasing temperature and the relative capacity of the acceptors to promote cholesterol efflux changed. At temperatures below 37°C, phospholipid vesicles were the most effective cholesterol acceptors. Decreasing the incubation temperature had a greater effect on efflux by HDL and apoA-I than by phospholipid vesicles. For example, decreasing the temperature to 30°C inhibited efflux (relative to 37°C incubations) by 57, 60, and 28% for HDL, apoA-I, and phospholipid vesicles, respectively. At temperatures below 22°C, [^{3}H]cholesterol efflux from cells to apoA-I was completely absent, HDL efflux was decreased by greater than 90%, however, the vesicles still showed appreciable cholesterol efflux activity, reduced by only 50% compared with incubations at 37°C. Interestingly, efflux from cells to medium containing 1 mg/ml BSA, although having a much lower magnitude of cholesterol efflux than the other acceptors, showed a temperature-dependent decrease in cholesterol efflux similar to that for phospholipid vesicles.

DISCUSSION

In the present study we examined the hypothesis that cholesterol efflux from cells occurs by both passive and active mechanisms. Our results suggest that two apparently distinct pathways may be involved. One pathway most likely involves the well-described aqueous diffusion mechanism (1, 6, 7). Efflux by this pathway depends on the presence of suitable extracellular cholesterol acceptors that contain a lipid surface (e.g., phospholipid vesicles or trypsin-modified HDL) or other cholesterol binding sites (e.g., cyclodextrins, ref.

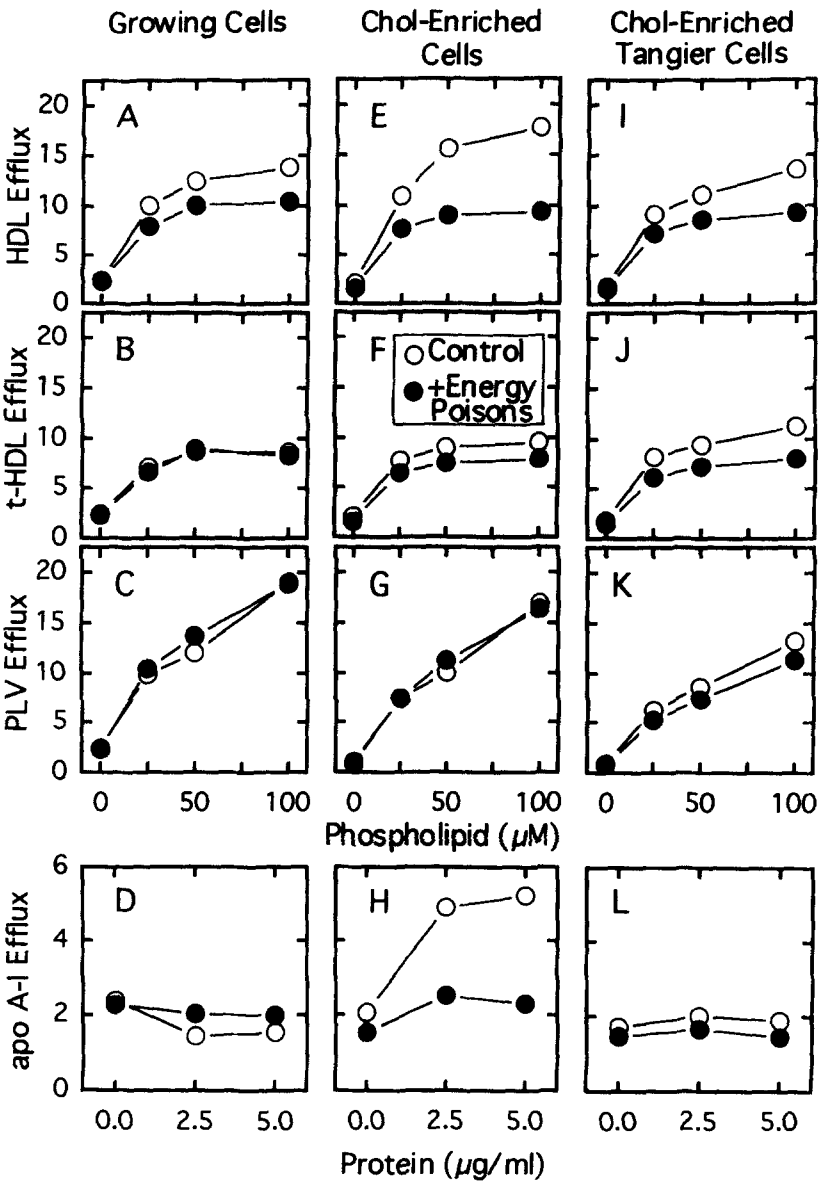

Fig. 5. Effects of energy poisons on the efflux of cellular cholesterol by different acceptor types from growing fibroblasts and cholesterol-enriched normal or Tangier disease fibroblasts. Panels A–D: Normal human skin fibroblasts were labeled as described in the legend to Fig. 4 under conditions of cell growth. Panels E–H: Normal fibroblasts. Panels I–L: Fibroblasts obtained from a patient with Tangier disease; cells were labeled and enriched with LDL-cholesterol as described in the legend to Fig. 1. Cells were pre-incubated for 30 min at 37°C with DMEM containing 1 mg/ml BSA alone (○, Control) or with 2 mM KCN and 10 mM NaF (●, +Energy Poisons). HDL, trypsin-modified HDL (t-HDL), and phospholipid vesicles (PLV) were added to medium based on phospholipid and apoA-I based on protein to obtain the indicated concentrations. Cells were incubated for 6 h at 37°C, and cholesterol efflux (for each acceptor as indicated on the ordinate) was measured as described under Methods and expressed as the percent of total [^{3}H]cholesterol. Results are the means of duplicate incubations and representative of 3 to 5 experiments for each acceptor using normal cells and of 2 experiments for the Tangier disease cells. For panels A–D, cell protein was 10.4 ± 1.0 and 9.7 ± 1.0 and [^{3}H]cholesteryl esters accounted for 3.4 and 5.2% of total counts for control and treated cells, respectively. For panels E–H, cell protein was 22.9 ± 1.1 and 26.7 ± 1.1 and [^{3}H]cholesteryl esters accounted for 9.7 and 12.1% of total counts for control and treated cells, respectively. For panels I–L, cell protein was 27.6 ± 1.2 and 27.0 ± 0.7 and [^{3}H]cholesteryl esters accounted for 6.4 and 9.6% of total counts for control and treated cells, respectively.

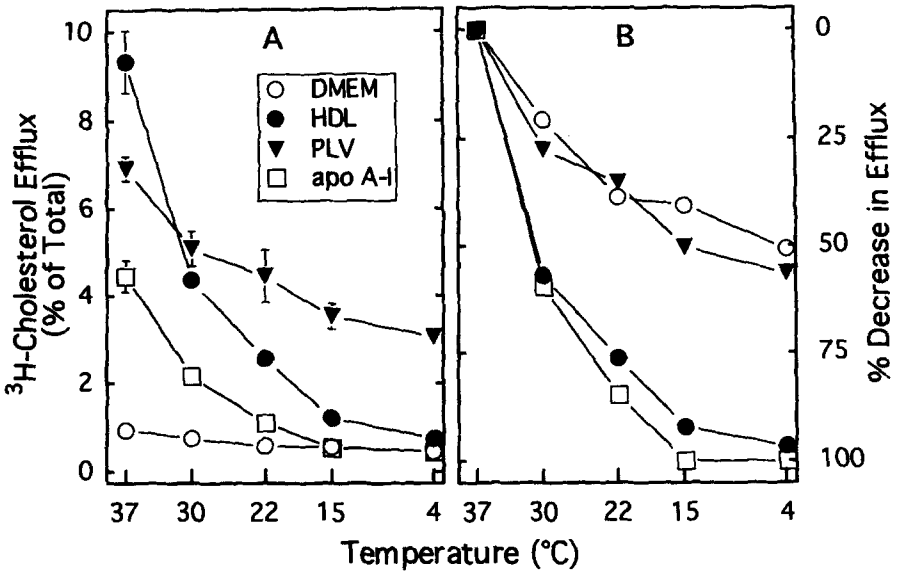

Fig. 6. Effect of incubation temperature on cellular cholesterol efflux from LDL cholesterol-enriched fibroblasts. Cultures were grown, labeled with [^{3}H]cholesterol and enriched with LDL as described in the legend to Fig. 1. After the incubation to arrest cell growth and equilibrate cellular cholesterol pools, cells were preincubated at the indicated temperatures for 15 min, then efflux medium (equilibrated to the appropriate temperature) was added to the cells: DMEM containing 1 mg/ml BSA and buffered with 50 mM HEPES (DMEM, ○); 50 μM phospholipid of HDL (●), or phospholipid vesicles (PLV, ▼) or 5 μg/ml apoA-I (□). Incubations were for 6 h. Medium and cells were analyzed as described in the legend to Fig. 1 and under Methods. Panel A: [^{3}H]cholesterol efflux results are expressed as the percent of total ^{3}H radioactivity. Recovery of cell protein, [^{3}H]cholesterol radioactivity, and the percent of label present in cholesteryl esters were 21.0 ± 1.7 μg/dish, 30026 ± 2087 cpm/dish, and 12.5 ± 1.4%, respectively, for all conditions and not affected by incubation temperature. Results from two independent experiments were combined and are the mean ± SD of six dishes. Panel B: Results expressed as the percentage decrease in efflux for the acceptors at each temperature relative to incubations at 37°C (=100%).

55) to accommodate cholesterol desorbed from the cell surface. Cellular cholesterol efflux by this pathway was not influenced by the growth state of the cells or the apolipoprotein content of the acceptor. Furthermore, it did not require metabolic energy, and demonstrated a temperature dependence distinct from that of apolipoprotein-containing acceptors. In growing cells or cells not enriched with cholesterol, this pathway predominates and may be an important mechanism for maintaining cellular cholesterol homeostasis. A second putative pathway was only apparent in growth-arrested cells and became more pronounced when cells were also enriched with cholesterol. This pathway required the presence of apolipoproteins in the extracellular cholesterol acceptor, depended on metabolic energy, and showed a more complex temperature dependence compared with the apolipoprotein-depleted acceptors. Taken together these results are suggestive of an active transport pathway involved in apolipoprotein-mediated cholesterol efflux. Additionally, the present studies demonstrated that efflux mediated by apolipoproteins promoted a more rapid and larger decrease in intracellular cholesteryl esters when measured by changes in cholesteryl ester radioactivity, mass, or the pool of cholesterol available for esterification by ACAT. This pathway may be of importance in either preventing or regressing the accumulation of excess cellular cholesterol.

The results also show that purified apoA-I could only participate in efflux by the active pathway while phospholipid vesicles and trypsin-modified HDL promote efflux predominantly by the passive pathway. HDL could promote efflux by both pathways. Thus the use of a combination of acceptors can provide useful experimental tools for examining cholesterol efflux from cells by these different mechanisms.

Apolipoprotein-mediated cholesterol efflux appears to involve specific cellular events distinct from mechanisms involved in efflux of cell cholesterol by the apolipoprotein-depleted acceptors. This notion is supported by several lines of evidence. First, apolipoprotein-mediated cholesterol efflux was only apparent in growth arrested or cholesterol-enriched cells, in agreement with earlier reports (33, 36), whereas efflux to other acceptors could be observed under all conditions of cell culture. HDL and apoA-I had no effect on cellular cholesterol mass after incubation with growing

cells while significantly decreasing cholesterol mass after incubation with cholesterol-enriched cells. The near inability of apoA-I to promote cholesterol efflux from growing cells and data demonstrating that certain cell types are resistant to apolipoprotein-mediated cholesterol efflux (40, 55, 57) suggests that efflux does not result from nonspecific interaction with membrane lipids. Second, metabolic energy was required for cholesterol efflux by apolipoprotein-containing acceptors but not for efflux by lipid-containing acceptors. Third, the effects of incubation temperature on cholesterol efflux to apolipoprotein-containing and -depleted acceptors were different. The temperature dependence of cholesterol efflux to phospholipid vesicles is consistent with a previous report concluding that cholesterol efflux mediated by phospholipid vesicles occurs by an aqueous diffusion process (58). In contrast, the temperature dependence of cholesterol efflux by apoA-I and HDL showed a more complex behavior, suggesting that additional pathways or mechanisms are also involved. In this respect, the temperature dependence of cholesterol efflux by the apolipoprotein-containing acceptors is reminiscent of Golgi apparatus-mediated protein transport that was effectively blocked at temperatures below 20°C (59, 60). Fourth, our previous results have distinguished acceptor-dependent efflux of excess cellular cholesterol based on sensitivity to drugs that inhibit Golgi apparatus structure and function (36). Apolipoprotein-dependent efflux required a functional Golgi apparatus transport system while efflux by lipid-containing acceptors was independent of this cellular function. We used those data to suggest that cholesterol from sites of storage is transported to sites available for removal by apolipoproteins through Golgi apparatus-mediated pathways (36, 46). Recently, Smith and coworkers (27) confirmed the effects of Golgi apparatus disruption on apolipoprotein-mediated cholesterol efflux in a macrophage cell line, but hypothesized that the effects were due to the loss of a cellular protein that interacts with extracellular apolipoproteins. These hypotheses await further validation. The notion that apolipoprotein-mediated efflux occurs independently of diffusional efflux is also supported by the studies of Tsujita and Yokoyama (61). Treating macrophage cultures with probucol resulted in the selective inhibition of apolipoprotein-mediated efflux under conditions that did not affect cholesterol exchange between cells and lipoproteins (61). Francis et al. (62) showed that products resulting from the peroxidase-mediated tyrosylation of HDL depleted cells of cholesteryl esters and increased the rate of free cholesterol efflux. These authors suggested that a mechanism exists to deplete cell cholesteryl esters and ACAT substrate without necessitating prior free cholesterol removal (62). Finally, the absence of apolipoprotein-dependent cholesterol efflux from fibroblasts of Tangier disease patients without an apparent defect in the ability of apolipoprotein-depleted acceptors to promote efflux (26, 37, 47) are among the strongest evidence to date for the involvement of a specific genetic factor in apolipoprotein-mediated transport and efflux of cellular lipids.

As discussed above, cellular cholesterol efflux has been described as a physical–chemical process involving the desorption of cholesterol from the cell membrane followed by diffusion through the surrounding aqueous space and adsorption by an appropriate acceptor, usually a lipoprotein particle such as HDL (1). This process can be viewed as an exchange mechanism and the direction and extent of efflux is dictated by the chemical gradient of free cholesterol between cells and the acceptors. In contrast, we propose that apolipoprotein-mediated cholesterol efflux from cholesterol-enriched-cells may be viewed as an active transport process, having the characteristics of active transport as described by Lehninger (63). The first identifying characteristic of an active transport process is that it depends on a source of metabolic energy. This requirement was fulfilled by showing that apolipoprotein-dependent efflux of cholesterol was inhibited by greater than 85% when ATP production was inhibited with fluoride and azide. A second property of active transport systems of cells is that they are specific for a given solute. While this criteria referred to ion or amino acid transports, for apolipoprotein-mediated cholesterol efflux an analogy may be made if intracellular cholesterol is considered the 'solute'. ApoA-I had a greater capacity to reduce intracellular cholesterol than the apolipoprotein-depleted acceptors, and thus, specificity lies in the ability to preferentially deplete this pool of cholesterol relative to the bulk of free cholesterol present in cells. A third property of active transport is that the system can be saturated with the substance being transported. This criterion was clearly met when efflux of cholesterol by apoA-I from cholesterol-enriched cells was measured showing that the process occurs in a dose-dependent and saturable fashion. A fourth property of active transport systems is that they have directionality. Although diffusional efflux can be bi-directional, apolipoprotein-mediated efflux from growth-arrested and cholesterol-enriched cells led to a net reduction in cellular cholesterol mass. Finally, active transport systems may be selectively poisoned. As yet no true antagonists of apolipoprotein-mediated cholesterol efflux have been discovered. However, for the purpose of this discussion, we can extend the definition of selectively poisoned to include 'poisoning' of the acceptor particle. Our earlier work (18, 20) and the present studies demonstrate that the proteolysis of HDL-associated apolipoproteins pre-

vents efflux of excess cholesterol from cholesterol-enriched cells. Therefore, the apolipoprotein-mediated efflux component can be selectively 'poisoned' without apparently affecting efflux mediated by lipid-dependent mechanisms.

We interpret our data to indicate that apolipoprotein-mediated efflux of excess cellular cholesterol occurs by an active transport pathway that appears to be distinct from aqueous diffusion mechanisms. Details of the mechanisms involved require further elucidation. How this pathway becomes activated remains unknown. The possibility of a cell surface receptor has been implied by many studies, yet the unambiguous identification of a receptor protein is lacking. An additional possibility is that apolipoprotein-mediated efflux of cholesterol occurs by interaction with non-protein cell surface sites. Refined elucidation of cell surface binding sites and their unambiguous relationship to initiating the events involved in apolipoprotein-mediated cholesterol efflux need to be established. Are signaling cascades required to stimulate transport and efflux of excess cellular cholesterol? If activation by signals is not involved might cholesterol be "recycled" between storage pools and efflux-accessible sites and efflux dictated by the presence of an appropriate acceptor. Again, specific mechanisms need to be more completely elucidated. Last, the identification of specific cellular proteins involved (e.g., transfer proteins; proteins involved in vesicular transport; the Tangier disease gene product) and their regulation are needed to completely understand the processes involved in apolipoprotein-mediated efflux of excess cellular cholesterol.

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