Apolipoprotein-mediated cellular cholesterol and phospholipid efflux depend on a functional Golgi apparatus

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Abstract Several studies have demonstrated that lipid-free apolipoproteins can promote cholesterol and phospholipid efflux from cells; however, the mechanisms and the role of cell-mediated pathways involved remain incompletely elucidated. We have recently demonstrated that brefeldin A or monensin, agents that disrupt Golgi apparatus structure and function, inhibit intracellular cholesterol efflux from cells to high density lipoproteins. In the present study we examined the effects of those agents on cell cholesterol and phospholipid efflux to purified apolipoprotein A-I (apoA-I) and apolipoprotein-depleted acceptors from cholesterol-loaded fibroblasts. Brefeldin A or monensin treatment of cells during incubation with apoA-I inhibited efflux of cellular cholesterol by greater than 80% compared with control cells, measured by changes in cellular cholesterol radioactivity, mass, and the substrate pool of cholesterol available for esterification by acyl coenzyme A:cholesterol acyltransferase. Inhibition of cholesterol efflux by these agents could not be overcome by increasing the apoA-I concentration and persisted during incubations up to 24 h. Similarly, brefeldin A and monensin inhibited up to 80% of apoA-I-mediated efflux of labeled phospholipids from cholesterol-loaded cells relative to controls. In contrast, lipid efflux mediated by apolipoprotein-depleted acceptors (trypsin-modified HDL and sonicated phospholipid vesicles) was not sensitive to these drugs. On the basis the known effects of brefeldin A and monensin on Golgi apparatus structure and function, these results are consistent with the notion that efflux of cell lipids by apolipoprotein-dependent mechanisms, but not by apolipoprotein-independent mechanisms, require active cellular processes involving an intact and functional Golgi apparatus.—**Mendez, A.J., and L. Uint.** Apolipoprotein-mediated cellular cholesterol and phospholipid efflux depend on a functional Golgi apparatus. *J. Lipid Res.* 1996. **37:** 2510–2524.

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The role of apolipoproteins in promoting cellular cholesterol efflux has gained increasing interest beginning with the observations of Hara and Yokoyama (1) that lipid-free apolipoproteins could deplete excess cholesterol from cultured macrophages. Other studies have confirmed and extended those observations (2–5) and the results can be briefly summarized as follows. Efflux of cell cholesterol to lipid-free apolipoproteins was observed in cells enriched with exogenous cholesterol (1, 2, 4, 5). In cells not enriched with cholesterol, free apolipoproteins had almost no capacity to stimulate efflux of cell cholesterol to the medium (4). In addition to cholesterol efflux, lipid-free apolipoproteins also promote efflux of cell phospholipids, most notably of phosphatidylcholine and sphingomyelin (1, 4, 5). Similar to efflux of cellular cholesterol, phospholipid efflux mediated by apolipoproteins depended on enriching cells with cholesterol (4). Lipid efflux resulted in (or was the result of) the association of cellular lipids with apoA-I or A-II forming particles with properties similar to pre-β HDL, assessed by the estimated composition, electrophoretic mobility, and density distribution of particles isolated from conditioned medium (1, 2, 4). Efflux of cholesterol by purified apolipoproteins was saturable with half-maximal effects occurring within the concentration range of 50–200 nM for apoA-I, A-II, and E (1, 4, 5), values within the estimated concentration range of "free" apolipoproteins present in biological fluids (6–8). These observations have been used to suggest that cholesterol efflux promoted by lipid-deficient apolipoproteins may have physiological significance (1, 9, 10).

The above studies have established that purified apolipoproteins promote cholesterol and phospholipid efflux from cholesterol-enriched cells; however, the mechanisms and the participation of cellular pathways

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; PBS, phoaphate-buffered saline; BSA, bovine serum albumin; FBS, fetal bovine serum; HDL, high density lipoprotein; LDL, low density lipoprotein.

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involved in these events remain unknown. Diffusion and desorption of cell cholesterol for uptake by lipid-free apolipoproteins seems an unlikely mechanism, as this model depends on the absorption of cholesterol into the lipid surface of extracellular lipoproteins and the net flux dictated by the chemical gradient of cholesterol between the cells and the extracellular acceptor (11). Other possibilities have been suggested. Hara and Yokoyama (1) suggested that efflux of cellular lipids depends on the interaction of free apolipoproteins with cell surface phospholipids. The resulting lipid:protein complexes are released to the extracellular space to form nascent HDL particles, which presumably would then act as efficient acceptors of cell cholesterol by diffusional pathways. Bielicki et al. (4) predicted a similar model; however, they found their data were better described if lipid-free apolipoproteins interact with cell membrane lipids causing the simultaneous release of cholesterol and phospholipids, with the caveat that very early efflux of phospholipid (less than 3 h) could precede cholesterol efflux to form nascent pre-β-like HDL that act as acceptors of cellular cholesterol by mechanisms predicted by the aqueous diffusion model. Studies by Forte et al. (10) also indicated that cholesterol and phospholipid efflux by apoA-I were closely linked and these authors suggested that phospholipid efflux may precede cholesterol efflux. These putative pathways predict that efflux of cell lipids by apolipoproteins depends on an interaction between the apolipoproteins and cell surface to form lipid:protein complexes and thereby deplete cellular lipids. Such models imply that cells play mostly a passive role in these events and do not actively contribute to the process of ridding excess cell cholesterol.

In several studies, efflux of cholesterol by free apolipoproteins was mostly accounted for by decreased intracellular cholesterol esters (1, 2, 5). This suggests that either lipid-free apolipoproteins deplete cell free cholesterol pools, most likely from the plasma membrane, that is then replenished by the net hydrolysis and transport of intracellular cholesterol esters or that free apolipoproteins directly stimulate the net hydrolysis of intracellular cholesteryl esters and promote depletion of cholesterol from this pool directly. Therefore, some pathway must exist to transport cholesterol from intracellular sites of storage and over-accumulation to plasma membrane sites available for efflux. We have recently demonstrated that disruption of Golgi apparatus structure and function, by treating cells with brefeldin A or monensin, prevents intracellular cholesterol efflux from cholesterol-loaded cells to HDL (12). Those data suggest an active role for the Golgi apparatus in cholesterol transport from intracellular sites of accumulation or storage to plasma membrane sites available for efflux to HDL. In the present study, we examined the effects of these agents on cellular cholesterol and phospholipid efflux to purified apoA-I to determine whether HDL and lipid-free apolipoproteins share common mechanisms in depleting excess cell cholesterol. We also examined the effects of Golgi apparatus disrupting drugs on lipid efflux to apolipoprotein-deficient acceptors. Our results showed that brefeldin A and monensin prevented both cholesterol and phospholipid efflux by greater than 80% to apoA-I but, in contrast, these agents had little effect on lipid efflux to apolipoprotein-deficient acceptors. These data are consistent with the notion that cellular lipid efflux by lipid-free apoA-I, similar to intracellular cholesterol efflux by HDL (12), involves Golgi complex-dependent events, while efflux of lipids mediated by apolipoprotein-depleted acceptors do not and the latter pathway is less effective at depleting intracellular cholesterol from cholesterol-enriched cells.

MATERIALS AND METHODS

Materials

[^{3}H]choline chloride (81–87 Ci/mmol) and carrier-free Na^{125}I (17 Ci/mg) were from DuPont NEN (Boston, MA). Lipid standards were from Sigma. Other materials used have been described in detail elsewhere (12).

Cells

Conditions for culturing human skin fibroblasts and sources of tissue culture reagents have been described (12). Fibroblasts were used between passages 5 and 12. Cellular cholesterol was labeled as described (12). Briefly, subconfluent cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and 0.2 to 0.4 μCi/ml [^{3}H]cholesterol until confluent (usually 3 days). Labeled cells were rinsed with phosphate-buffered saline (PBS) and subsequently loaded with non-lipoprotein cholesterol by incubation with DMEM containing 2 mg/ml fatty acid-free bovine serum albumin (BSA) and the indicated concentrations of cholesterol for 24 h. Cultures were incubated for an additional 40–48 h in DMEM containing 1 mg/ml BSA to allow equilibration of cholesterol pools. Cells were also enriched with cholesterol by incubation with low density lipoprotein (LDL). Subconfluent cultures were incubated with DMEM containing 2% FBS and 100 μg/ml LDL protein. After 48 h, by which time cells reached confluence, cells were rinsed with PBS containing 1 mg/ml BSA and incubated for an additional 24 h in

DMEM containing 1 mg/ml BSA to allow equilibration of cholesterol pools. Choline-containing phospholipids were labeled by incubating cells with DMEM containing 1 mg/ml BSA and 1 μCi/ml [^{3}H]choline during the final 24 h of the equilibration incubation after loading cells with cholesterol. During incubation of cells with test compounds, vehicle alone (ethanol) was added to control dishes at equal concentrations (never exceeding 0.25%).

ApoA-I, lipoproteins, and phospholipid vesicles

ApoA-I was purified from isolated high density lipoprotein (HDL) as previously described (13) and quantified by absorbance at 280 nm (14).

LDL and HDL were isolated by standard sequential ultracentrifugation methods in the density intervals 1.02–1.063 and 1.12–1.21 g/ml, respectively. Isolated lipoproteins were centrifuged a second time at the higher density to wash and concentrate the fractions, then dialyzed against PBS containing 1 mM EDTA. HDL was subjected to heparin-agarose chromatography to remove apoE and B-containing particles as described (15). HDL was depleted of intact apolipoproteins by trypsin treatment (trypsin-modified HDL) as described (16). Proteins were quantitated by the method of Lowry et al. (13). ApoA-I and HDL were iodinated by the ICl method of MacFarlane as modified by Bilheimer, Eisenberg, and Levy (17). Specific activities ranged from 350 to 600 cpm/ng protein and 250 to 500 cpm/ng protein for apoA-I and HDL, respectively.

Small unilamellar phospholipid vesicles were prepared by sonication of egg yolk phosphatidylcholine in PBS as previously described (18).

Cholesterol efflux

Efflux of labeled cholesterol from cells was measured by appearance of [^{3}H]cholesterol into experimental medium after incubation as described (12). Briefly, cell medium was centrifuged for 10 min at 1500 *g* and radioactivity in aliquots of the culture medium was measured and assumed to represent [^{3}H]cholesterol (12). 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. (13). 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.

Cell free and esterified cholesterol masses were measured after TLC separation of free and esterified cholesterol, as above. 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. Nonsaponified 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 (19).

Cholesterol esterification

Esterification of cellular cholesterol by acyl coenzyme A:cholesterol acyltransferase (ACAT) was measured by the incorporation of [^{14}C]oleate into cholesteryl esters (20). After incubation with experimental medium, cells were rinsed once with PBS then incubated for 1 h in DMEM containing 9 μM [^{14}C]oleate and 3 μM BSA for 1 h at 37°C. 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]cholesteryl esters per mg of cell protein.

Simultaneous assay of cellular cholesterol mass, radioactivity and esterification

In some experiments cellular cholesterol mass, radioactivity, and cholesterol esterification were measured simultaneously in the same culture dish. For these studies, cells were plated in 35-mm dishes and labeled with [^{3}H]cholesterol and enriched with cholesterol (described in the appropriate figure legends) as above. After incubation with experimental medium for the indicated times, medium was removed and [^{3}H]cholesterol efflux was determined as above. Cell layers were rinsed once and incubated with [^{14}C]oleate medium to measure ACAT activity as above. Cell layers were extracted, lipids were separated by TLC, and free and esterified cholesterol spots were collected, then saponified and extracted with hexane as described for determination of cholesterol mass. This approach allowed for quantitation of labeled cholesterol recovery at each step of the sample workup and to directly correlate changes among the measured variables. Cellular free and esterified cholesterol mass and radioactivity were measured in aliquots of the hexane phase. Recovery of [^{3}H]cholesterol in the hexane extract was 96 ± 4 and 94 ± 5 % (mean ± SD of 160 dishes from 5 different experiments) for free and esterified cholesterol, respectively. Cholesterol mass measurements were corrected for recovery of label. Incorporation of [^{14}C]oleate was determined by counting aliquots of the lower aqueous phase after saponification and extraction. Based on the partition of [^{14}C]oleate radioactivity between the hex-

ane and aqueous phase, saponification efficiency exceed 98%.

Phosphatidylcholine efflux

After labeling with [^{3}H]choline, cells were washed 4 times with PBS containing 1 mg/ml BSA, then once in DMEM prior to addition of experimental medium. After incubation, efflux medium was collected, centrifuged to remove cell debris as above, and aliquots were taken for extraction by the method of Folch, Lees, and Sloane Stanley (21). Cell lipids were extracted with 2 ml isopropanol for 18 h (4), then dishes were rinsed twice with 1 ml isopropanol and extracts were pooled. Extracts were evaporated to dryness under a stream of N_2 gas in a 50°C water bath. The residue was dissolved with $CHCl_3$–CH_3OH 2:1 (v/v) and phospholipid classes were spotted on Whatman (Clinton, NJ) silica gel linear K TLC plates with a pre-absorbent zone and development in $CHCl_3$–CH_3OH–H_2O 65:35:4 (v/v/v). Phospholipids were identified by iodine staining and co-migration with authentic standards. After allowing the iodine stain to evaporate, appropriate spots were scraped from the plates and radioactivity was measured by scintillation counting.

Cell surface binding of apoA-I and HDL

Cell binding of iodinated apoA-I and HDL was measured essentially as previously described (20). Cholesterol-loaded fibroblasts were washed twice with PBS containing 1 mg/ml BSA and chilled on ice during a third wash. Cells were then incubated at 0°C (on ice) with HEPES-buffered DMEM containing 1 mg/ml BSA and either 1 μg/ml ^{125}I labeled apoA-I or 5 μg/ml ^{125}I labeled HDL protein for 3 h. Cells were washed 5 times with ice-cold PBS containing 1 mg/ml BSA, then twice with PBS. Cell layers were dissolved in 0.1 M NaOH and aliquots were taken to quantitate radioactivity and protein.

Statistics

Comparisons between groups were analyzed by paired Student's *t*-test. When not indicated, a statistically significant difference was assumed for P values less than 0.05.

RESULTS

ApoA-I-mediated cholesterol efflux from cells

The ability of apoA-I to promote cholesterol efflux from cells labeled with [^{3}H]cholesterol and incubated with increasing concentrations of cholesterol (to enrich cholesterol pools) was determined by simultaneously measuring changes in cholesterol radioactivity, mass, and ACAT-mediated cholesterol esterification (**Fig. 1**). Incubation with exogenous cholesterol increased the proportion of radioactivity in cellular cholesteryl esters (concomitant with a decrease in free [^{3}H]cholesterol), and increased free and esterified cholesterol mass and ACAT activity. Efflux of cellular [^{3}H]cholesterol to medium containing 1 mg/ml BSA was less than 2% of total [^{3}H]cholesterol and decreased slightly after enriching cells with cholesterol. Addition of apoA-I to the medium stimulated efflux of cholesterol from cells based on appearance of [^{3}H]cholesterol in the medium, a decrease in cellular esterified cholesterol radioactivity and mass, and a decrease in the substrate pool of cholesterol available for esterification by ACAT relative to cells incubated without apoA-I. Labeled cholesterol efflux by apoA-I was mostly accounted for by a decrease in cellular [^{3}H]cholesteryl esters and only small changes in free [^{3}H]cholesterol were observed. ApoA-I reduced cellular cholesteryl ester mass while free cholesterol mass was slightly (but consistently) decreased, similar to the changes observed for [^{3}H]cholesterol. Finally, apoA-I decreased cholesterol esterification by ACAT at all levels of cellular cholesterol enrichment. The magnitude of apoA-I-mediated efflux by each measure was increased with cellular cholesterol enrichment.

Further analysis of these results demonstrated a strong correlation between several of the measured parameters. For all conditions studied a positive correlation was found between cellular esterified cholesterol mass and radioactivity ($r = 0.951$, $P < 0.001$), esterified cholesterol mass and ACAT activity ($r = 0.830$, $P < 0.001$), and [^{3}H]cholesteryl esters and ACAT activity ($r = 0.943$, $P < 0.001$). A significant correlation was also observed between free cholesterol mass and ACAT activity and between free cholesterol mass and esterified cholesterol mass. Efflux of [^{3}H]cholesterol did not show any correlation between other results as efflux to DMEM containing 1 mg/ml BSA was low and unchanged for all conditions studies. However, apoA-I-mediated [^{3}H]cholesterol efflux was significantly correlated with apoA-I-mediated changes (relative to medium containing 1 mg/ml BSA) in cellular cholesteryl ester mass ($r = 0.955$, $P < 0.05$), cholesteryl ester radioactivity ($r = 0.988$, $P < 0.02$) and ACAT activity ($r = 0.996$, $P < 0.01$).

These results show that apoA-I promotes cholesterol efflux from cells and efflux was increased when cell cholesterol levels were elevated, in agreement with previous reports (4). In addition, there was statistically significant correlation between all measures of intracellular cholesterol reduction and the ability of apoA-I to promote efflux, implying that changes in radioactive cho-

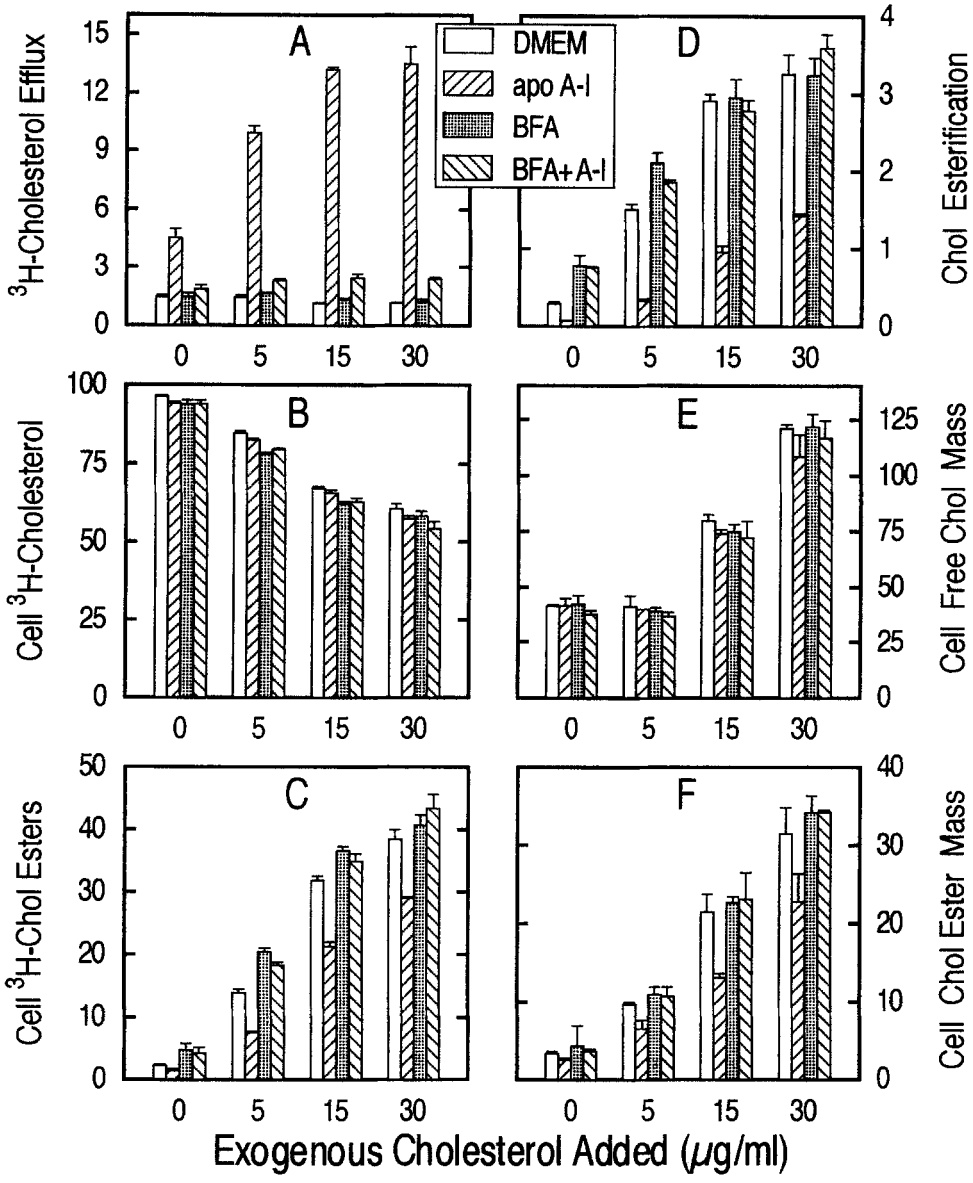

Fig. 1. Effect of enriching cells with cholesterol on apoA-I-mediated cholesterol efflux in control and brefeldin A-treated cells. Human skin fibroblasts were labeled with [^{3}H]cholesterol during growth and maintained to confluence as described under Methods. Cells were then incubated with DMEM containing 2 mg/ml BSA and the indicated concentrations of exogenous cholesterol for 24 h at 37°C, followed by incubation for 40 h with DMEM containing 1 mg/ml BSA. Cells were incubated with DMEM containing 1 mg/ml BSA alone (□, DMEM) or with 3.4 μM brefeldin A (■, BFA) or with 5 μg/ml apoA-I (▨) or with BFA and apoA-I (▦) for 20 h. [^{3}H]cholesterol efflux, cellular [^{3}H]cholesterol distribution, cellular cholesterol mass, and cholesterol esterification by ACAT were determined in the same culture dish as described under Methods. Panel A: [^{3}H]cholesterol efflux; panel B: cell unesterified [^{3}H]cholesterol; and panel C: cell [^{3}H]cholesterol esters are expressed as the percent of total (medium + cell) [^{3}H]cholesterol radioactivity. Panel D: Cellular cholesterol (Chol) esterification was determined by a 1-h incubation with [^{14}C]oleate medium after removal of experimental medium as described under Methods and results are expressed as nmoles of oleate incorporated into cholesteryl esters per mg of cell protein. Panels E and F: Cellular free and esterified cholesterol mass, respectively, expressed as μg cholesterol/mg cell protein. For all panels results are the means ± SD of three dishes. Recovery of cell protein was 97 ± 5 and 95 ± 5 μg/dish (mean ± SD, n = 24) for control and BFA treated cells, respectively and were similar (no statistical differences) for all conditions of cholesterol loading. Recovery of total [^{3}H]cholesterol was 53123 ± 5994, 43512 ± 4451, 46703 ± 3225, and 47497 ± 5763 cpm/dish (mean ± SD, n = 12 for each group) for cells incubated with 0, 5, 15, or 30 μg/ml of cholesterol, respectively, and similar for control and BFA-treated cells.

lesterol reflect changes in cell cholesterol mass. It is worth noting that under the conditions used for labeling cell cholesterol and the subsequent loading of cells with exogenous cholesterol, free and esterified cholesterol do not attain equal specific activities, although the specific activity of each pool reaches a constant level that remains unchanged during incubation with efflux medium. This may be due to artificially high levels of free cholesterol produced by this loading protocol and may reflect cholesterol mass that may not be cell associated. For example, loading cells with non-lipoprotein cholesterol has been reported to result in the presence of micro-crystalline cholesterol deposition on the cell surface when examined by electron microscopy (20) potentially leading to apparently lower specific activity of cellular free cholesterol relative to cholesteryl esters. In contrast, cholesteryl ester levels and the substrate pool of cholesterol available for esterification by ACAT were also increased in a dose-dependent manner by this loading protocol, and reflect cholesterol that is taken up and metabolized within cellular compartments, demonstrating that intracellular levels of cholesterol were effectively increased. Also worth noting is that even in quiescent and growth-arrested cells not exposed to exogenous cholesterol, free and esterified cholesterol did not attain equal specific activities, suggesting that other factors involved in determining cellular cholesterol distribution may also be involved (e.g., ref. 22).

The effects of brefeldin A, a compound that inhibits Golgi apparatus-mediated protein transport (23), on apoA-I-mediated cholesterol efflux were examined in the same study (Fig. 1). Brefeldin A treatment increased cellular cholesteryl esters and ACAT activity compared with controls pre-incubated with 0 or 5 μg/ml cholesterol but had no observable effects on ACAT activity in cells enriched with higher concentrations of cholesterol. Brefeldin A did not affect [^{3}H]cholesterol efflux from cells to DMEM containing 1 mg/ml BSA but prevented apoA-I-mediated efflux of cholesterol from cholesterol-enriched cells by greater than 90% due to the inability of apoA-I to deplete cellular free or esterified cholesterol mass or radiolabel and prevented the apoA-I-mediated decrease in cholesterol esterification compared with controls. These results suggest that apoA-I-mediated efflux of cellular cholesterol depends on a brefeldin A-sensitive pathway.

A similar experiment as described above was performed using cells enriched with cholesterol derived from LDL and the ability of apoA-I and HDL to promote efflux was examined in control or brefeldin A-treated cells (**Table 1**). In control incubations, apoA-I and HDL promoted efflux of cell cholesterol determined by the appearance of [^{3}H]cholesterol in the culture medium, decreased radioactivity and mass of cellular free and esterified cholesterol, and a decrease in the pool of cholesterol available for esterification by ACAT compared to cells incubated with medium containing 1 mg/ml BSA. In brefeldin A-treated cells [^{3}H]cholesterol efflux from cells to medium containing apoA-I or HDL was significantly ($P < 0.01$) reduced compared to control cells. Decreased efflux could mostly be accounted for by a diminished ability of these acceptors to deplete intracellular cholesteryl esters (mass or radioactivity) and the pool of cholesterol available for esterification by ACAT. Similar to results observed in Fig. 1, brefeldin A treatment of cells caused an apparent increase in ACAT activity relative to controls; however, the extracellular acceptors were unable to effect any change in cholesterol esterification. These data agree with results obtained using cells loaded with non-lipoprotein cholesterol and our previous observations regarding the effects of brefeldin A on HDL-mediated cholesterol efflux (12) indicating that the method or extent of cholesterol enrichment (while affecting absolute levels of cellular cholesterol, distribution of radioactive cholesterol, and relative ACAT activity) is not a primary determinant in brefeldin A-mediated inhibition of apolipoprotein-mediated cholesterol efflux from cells.

The time course of [^{3}H]cholesterol efflux by apoA-I from cholesterol-loaded cells was examined in control, brefeldin A or monensin-(another compound that also affects Golgi apparatus structure and function [24]) treated cells (**Fig. 2**). Under control conditions, [^{3}H]cholesterol efflux to apoA-I increased in a nearly linear fashion over time. At earlier times the majority of efflux was due to depletion of cellular free [^{3}H]cholesterol. After 12 h, there was little further decrease in cellular free cholesterol and most of the cholesterol appearing in the medium was accounted for by depletion of [^{3}H]cholesteryl esters. Brefeldin A treatment had no significant effect on cellular free or esterified [^{3}H]cholesterol levels or on the low level of cholesterol efflux to medium containing 1 mg/ml BSA in the absence of apoA-I. Addition of brefeldin A during incubation with apoA-I significantly inhibited [^{3}H]cholesterol efflux ($P < 0.02$ compared with controls at all times), by 40% after 3 h of incubation and by 85 ± 2% (mean ± SD) at all other times. Incubating cells with monensin decreased cellular esterified [^{3}H]cholesterol and increased free [^{3}H]cholesterol over time compared with controls ($P < 0.05$ at all times greater than 6 h), with an apparent new steady state attained after 18 h. In spite of increased [^{3}H]cholesterol levels, efflux of [^{3}H]cholesterol from cells to apoA-I was inhibited by 87 ± 3% at all times in monensin-treated cells relative to control cells ($P < 0.01$), comparable to effects of brefeldin A. These data show that brefeldin A and monensin have profound effects on the ability of apoA-I to pro-

TABLE 1. Effect of brefeldin A on apoA-I- and HDL-mediated cholesterol efflux from LDL-cholesterol-loaded human skin fibroblasts

	[^{3}H]Cholesterol			Cell Cholesterol Mass		
	Efflux	Cell UC	Cell CE	Cell UC	Cell CE	ACAT Activity
	% of total ^{3}H			*ug/mg cell protein*		*pmol/mg cell protein*
DMEM	2.2 ± 0.1	87.9 ± 0.7	9.9 ± 0.8	55.8 ± 3.0	8.6 ± 0.6	450 ± 55
+5 μg/ml apoA-I	11.2 ± 1.0	83.0 ± 0.7	5.9 ± 0.5	48.2 ± 1.7	4.2 ± 0.5	62 ± 9
+50 μg/ml HDL	34.4 ± 0.5	60.9 ± 0.6	4.7 ± 0.2	50.7 ± 1.8	2.7 ± 0.3	30 ± 2
2 μM Brefeldin A	3.1 ± 0.4	84.8 ± 0.5	12.7 ± 0.8	50.8 ± 0.4	10.3 ± 0.7	638 ± 45
+5 μg/ml apoA-I	3.6 ± 0.1	85.0 ± 0.3	11.4 ± 0.4	49.4 ± 1.8	7.8 ± 0.5	663 ± 49
+50 μg/ml HDL	20.3 ± 0.3	71.3 ± 0.4	8.5 ± 0.5	54.1 ± 0.7	8.2 ± 1.0	636 ± 143

Fibroblasts labeled with [^{3}H]cholesterol were grown to near confluence then incubated with DMEM containing 1 mg ml BSA and 100 μg/ml LDL protein for 48 h then the same medium without LDL for an additional 24 h. Cells were subsequently incubated with DMEM containing 1 mg/ml BSA (DMEM) alone or with the indicated additions. After 20 h at 37°C, the medium was collected and the cells were pulse incubated with medium containing [^{14}H]oleate to assess relative ACAT activity. Cells and medium were analyzed as described in the legend to Fig. 1. Recovery of total ^{3}H was 48204 ± 2770 and 52627 ± 5012 cpm/dish and recovery of protein was 163 ± 4 and 170 ± 7 μg/dish for control and brefeldin A-treated cells, respectively. Results are the mean ± SD of three dishes and representative of two similar experiments.

mote cholesterol efflux from cholesterol-loaded cells, and suggest that efflux depends on a brefeldin A- and monensin-sensitive mechanism, consistent with a role for a Golgi apparatus-mediated pathway (23, 24) in these events.

To rule out that brefeldin A or monensin inhibited cholesterol efflux by interfering with the cholesteryl ester cycle, apoA-I-mediated efflux of cellular [^{3}H]cholesterol was measured in cells treated with an ACAT inhibitor. For these studies cells were treated as described in Fig. 2 except for the inclusion of the ACAT inhibitor, Sandoz compound 50-035 (2 μg/ml), during the cholesterol-loading and subsequent incubations. Under these conditions less than 1% of total [^{3}H]cholesterol was esterified compared with 20% in parallel incubations without the ACAT inhibitor. Although nearly all of the label was present as unesterified cholesterol, efflux from cells to medium containing apoA-I was inhibited by greater than 90% when brefeldin A or monensin were included during a 16-h incubation (not shown), comparable to the effects on apoA-I-mediated cholesterol efflux when ACAT was active. These results show that the effects of these drugs on inhibiting cholesterol efflux were not directly due to changes in ACAT activity.

The concentration dependence of apoA-I on cellular [^{3}H]cholesterol efflux was examined in control and brefeldin A-treated cells loaded with 30 μg/ml non-lipoprotein cholesterol. In control cells, apoA-I promoted cholesterol efflux in a dose-dependent and saturable manner over the range of 1 to 10 μg/ml (not shown). As above, efflux was accounted for by a decrease in both cellular free and esterified [^{3}H]cholesterol. Addition of brefeldin A during the incubations significantly inhibited cholesterol efflux at each dose of apoA-I (except 0) by an average of 85 ± 3% (mean ± SD of four concentrations of apoA-I) compared with control incubations. Thus, inhibition of cholesterol efflux by brefeldin A was not overcome by increasing the concentration of apoA-I in the medium.

Cholesterol esterification

As another index of intracellular cholesterol efflux, the ability of apoA-I to deplete cholesterol available for esterification was examined in control, brefeldin A or monensin-treated cells (**Fig. 3**). In control dishes, apoA-I decreased the substrate pool of cholesterol available for esterification in a dose-dependent fashion. When cells were treated with brefeldin A or monensin during incubation, apoA-I had no significant effect ($P > 0.5$) on cholesterol esterification at any dose tested compared with incubations without apoA-I. Brefeldin A or monensin had no significant effect on cholesterol esterification compared to control cells incubated in the absence of apoA-I, and treatment of cells with these compounds had no effect on [^{14}C]oleate incorporation into total phospholipids (not shown) or recovery of cell protein compared with controls.

Monensin treatment of cells resulted in a time-dependent decrease in cellular [^{3}H]cholesterol esters (Fig. 2); however, there was no apparent effect on ACAT activity when cholesterol esterification was measured (Fig. 3). Control studies were conducted to identify the cause of this apparent discrepancy. [^{3}H]cholesterol-labeled cells enriched with cholesterol were treated with or without monensin for 0 or 18 h then rinsed once with PBS and ACAT activity was measured after a 1-h pulse incubation with medium containing [^{14}C]oleate in the absence or presence of monensin (**Table 2**). At 0 time, addition of monensin to medium containing [^{14}C]oleate during the

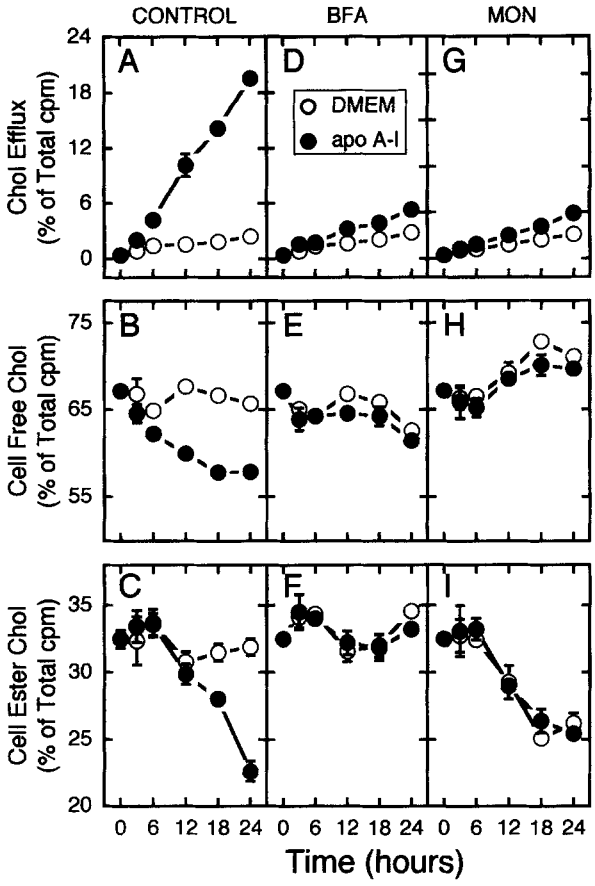

Fig. 2. Effects of brefeldin A and monensin on the time course of [^{3}H]cholesterol efflux from cholesterol-loaded cells to apoA-I. Human skin fibroblasts were labeled with [^{3}H]cholesterol then loaded with 30 μg/ml cholesterol as described in the legend to Fig. 1. Cells were incubated for the indicated times with DMEM containing 1 mg/ml BSA (DMEM) alone or containing 5 μg/ml apoA-I (panels A–C) or the same medium containing 4 μM brefeldin A (BFA; panels D–F) or 25 μM monensin (MON; panels G–I). Open and filled symbols represent incubations without or with apoA-I, respectively. Cholesterol efflux (Chol Efflux), cellular unesterified cholesterol (Cell Free Chol), and cellular cholesteryl esters (Cell Ester Chol) were determined as described under Methods and expressed as the percent of total [^{3}H] cholesterol. Results are the mean ± SD of three dishes at each time, and missing error bars are within limits of the symbols. Recovery of [^{3}H]cholesterol was 48365 ± 5175, 52677 ± 5020, and 48550 ± 6238 cpm/dish (mean ± SD, n = 36) and recovery of cellular protein after 24 h of incubation was 23.1 ± 0.6, 24.3 ± 0.4, and 21.4 ± 0.9 (n = 6) for control, brefeldin A- and monensin-treated cells, respectively.

1-h incubation had no effect on the cellular content of [^{3}H]cholesteryl esters compared to controls, but caused a 70% decrease in cholesterol esterification. In cells incubated with monensin for 18 h, there was a significant decrease in cellular [^{3}H]cholesteryl esters compared with controls as noted above. However, when monensin-treated cells were rinsed once with PBS prior to incubation with [^{14}C]oleate medium in the absence of monensin, cholesterol esterification was similar to controls.

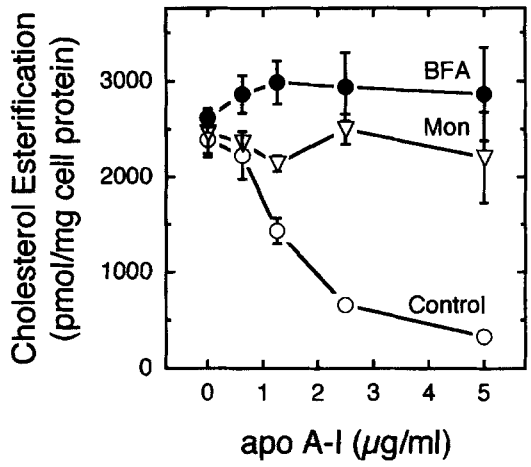

Fig. 3. Effects of brefeldin A and monensin on apoA-I-mediated inhibition of cholesterol esterification in cholesterol-loaded cells. Human skin fibroblasts were loaded with 30 μg/ml cholesterol as described in the legend to Fig. 1. Cells were then incubated for 18 h at 37°C with DMEM containing 1 mg/ml BSA alone (○, Control) or with 4 μM brefeldin A (●, BFA) or 25 μM monensin (▽, Mon) and the indicated concentrations of apoA-I. After incubation, cells were incubated with medium containing [^{14}C]oleate for 1 h and incorporation of oleate into cholesteryl esters was measured as described under Methods. Results are expressed as nmoles of [^{14}C]oleate incorporated into cholesteryl esters per mg of cell protein. Results are the mean ± SD of three dishes. Missing error bars are contained within the limits of the symbols. Protein recovery averaged 19.7 ± 1.7 μg/dish and was similar for all conditions.

When monensin was included during the final incubation, cholesterol esterification was again significantly inhibited. In contrast, incorporation of [^{14}C]oleate into triglycerides or phospholipids was slightly increased or not affected, respectively (Table 2), by monensin treatment of cells, suggesting that monensin does not directly affect oleate uptake or activation by cells. These results show that monensin causes an apparent inhibition of cellular ACAT activity after relatively short times and these effects are rapidly reversible.

Phospholipid efflux

In addition to cholesterol efflux, apoA-I also promotes efflux of phospholipids from cholesterol-loaded cells (1, 4, 5). Studies were conducted to examine whether apoA-I-mediated [^{3}H]phosphatidylcholine and [^{3}H]sphingomyelin effluxes were sensitive to brefeldin A or monensin (**Fig. 4**). Brefeldin A had no effect on phospholipid efflux to medium in the absence of apoA-I. Monensin treatment also had no effect on phosphatidylcholine efflux but increased efflux of sphingomyelin to medium containing albumin (see figure legend). Neither compound affected efflux of lysophosphatidylcholine relative to controls (the only other phospholipid class with appreciable incorporation of label), which was unaffected by the presence of apoA-I and

TABLE 2. Effects of monensin on cholesterol esterification in cholesterol-loaded human skin fibroblasts

Pretreat	Time	Addition to [^{14}C]Oleate Medium	Cell [^{3}H]CE	[^{14}C]Oleate Incorporation		
				Cholesteryl Ester	Triglycerides	Phospholipids
	h		*% of total*	*nmol/mg protein*		
DMEM	0	none	37.0 ± 2.6	4.04 ± 0.15	1.74 ± 0.14	2.28 ± 0.23
DMEM	0	monensin, 25 μM	38.4 ± 1.7	1.13 ± 0.06[a]	2.78 ± 0.09[a]	2.80 ± 0.14
DMEM	18	none	38.0 ± 1.3	4.55 ± 0.08	2.95 ± 0.26	2.75 ± 0.47
Monensin, 25 μM	18	none	32.5 ± 0.4[a]	4.33 ± 0.27	2.54 ± 0.12	2.28 ± 0.26
Monensin, 25 μM	18	monensin, 25 μM	32.6 ± 0.9[a]	1.48 ± 0.06[a]	3.15 ± 0.16	2.86 ± 0.34

Cells were labeled with [^{3}H]cholesterol and cholesterol enriched by incubation with 30 μg/ml cholesterol as described in the legend to Fig. 1. Cells were incubated with DMEM containing 1 mg/ml BSA (DMEM) or with DMEM containing 25 μM monensin for the indicated times. Cells were then rinsed once with PBS and incubated with DMEM containing 9 μM [^{14}C]oleate and 3 μM BSA ([^{14}C]Oleate Medium) alone or with monensin as indicated and incubated for 1 h at 37°C. Cellular lipids and protein were analyzed as described in Methods. Results are expressed as the mean ± SD of three dishes.

[a] $P < 0.01$ compared to control incubations.

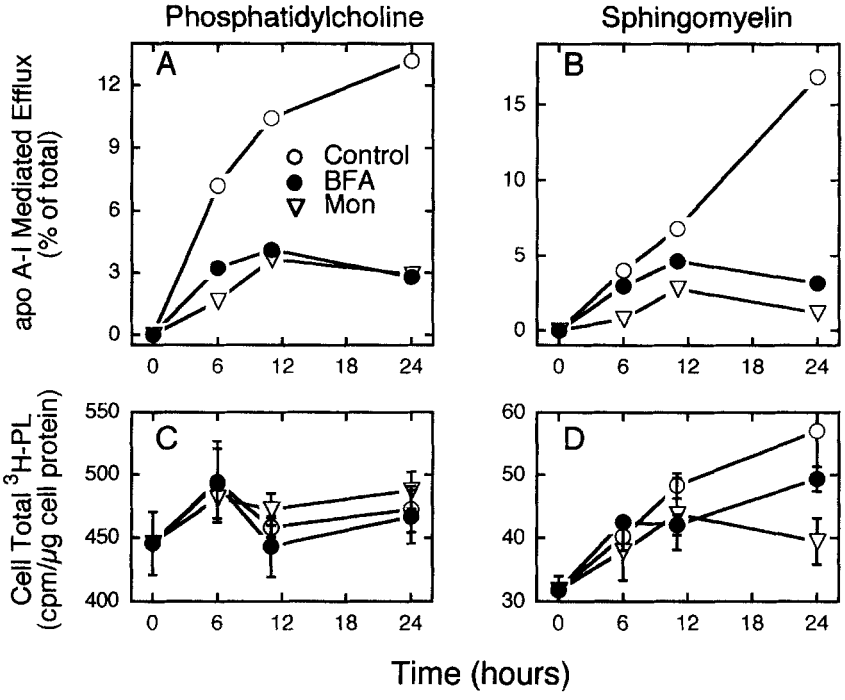

Fig. 4. Effects of brefeldin A and monensin on apoA-I-mediated phospholipid efflux. Cholesterol-loaded fibroblasts were incubated with [^{3}H]choline to label cell phosphatidylcholine and sphingomyelin as described under Methods. Cells were incubated at 37°C in DMEM containing 1 mg/ml BSA (○, Control) alone or with 4 μM brefeldin A (●, BFA), or 25 μM monensin (▽, Mon) and with or without 5 μg/ml apoA-I. After the indicated times, medium and cell phosphatidylcholine and sphingomyelin radioactivity were quantitated as described under Methods. Panel A: ApoA-I-mediated efflux of cellular [^{3}H]phosphatidylcholine calculated as the difference between incubations with and without apoA-I. Efflux to DMEM with BSA alone was 1.6 and 2% of total counts after 6 and 24 h of incubation, respectively, and similar for all conditions. Panel B: ApoA-I-mediated efflux of cellular [^{3}H]sphingomyelin calculated as the difference between incubations with and without apoA-I. Efflux to DMEM with BSA alone was 3.0 and 4.9% for control and BFA-treated cells and 5.2 and 6.8% of total counts in monensin-treated cells, after 6 and 24 h of incubation, respectively. For panels A and B results are the average of duplicate dishes that varied by less than 7 and 9% of the mean for phosphatidylcholine and sphingomyelin efflux, respectively. Panel C: total [^{3}H]phosphatidylcholine recovered at each time point expressed as cpm/mg of cell protein. Panel D: total [^{3}H]sphingomyelin recovered at each time point expressed as cpm/mg of cell protein. In panels C and D results are the mean ± SD of 4 dishes. Similar results were observed in three independent experiments.

presumed to be mediated by apoA-I-independent mechanisms (data not shown). In control cells, apoA-I promoted efflux of both [^{3}H]choline-labeled phosphatidylcholine and sphingomyelin in a time-dependent manner. In cells treated with brefeldin A, apoA-I-mediated phosphatidylcholine and sphingomyelin effluxes were inhibited at all times compared with controls. Decreased phospholipid efflux could not be attributed to differences in phosphatidylcholine or sphingomyelin radioactivity over the course of the experiments, which were similar to controls at all times (Fig. 4, panels C and D). Monensin treatment inhibited phosphatidylcholine efflux to apoA-I at all times compared with controls, similar to the effects of brefeldin A. Efflux of cellular sphingomyelin to apoA-I was also inhibited by monensin and paralleled inhibition due to brefeldin A, but showed a somewhat greater effect. By 24 h, inhibition of apoA-I-mediated phospholipid efflux by brefeldin A or monensin were similar, ~80% for phosphatidylcholine and sphingomyelin efflux. While monensin had no effect on the recovery of labeled phosphatidylcholine compared with controls, sphingomyelin-associated radioactivity was significantly decreased ($P < 0.05$) after 24 h of incubation compared with controls. However, this in itself was insufficient to account for the inhibition of sphingomyelin efflux to apoA-I, as inhibition was observed at times when levels of cellular sphingomyelin radioactivity were similar in control and monensin-treated cells.

Effects of brefeldin A and monensin on cellular lipid efflux to lipid-containing acceptors

ApoA-I, unlike lipoproteins, cannot readily participate in processes involving cholesterol desorption and diffusion events resulting in the uptake of cellular cholesterol onto the lipoprotein lipid surface as predicted by the aqueous diffusion model of cholesterol efflux (25). Thus, the ability of apoA-I to promote lipid efflux may occur by a unique process(es) distinct from those mediated by lipid-containing acceptors. We tested the effects of brefeldin A and monensin on cellular [^{3}H]cholesterol efflux to various lipid-containing acceptors and apoA-I to determine whether similar efflux mechanisms were involved for different acceptor types. HDL, containing lipids and apolipoproteins, can presumably promote efflux by apolipoprotein-dependent and -independent pathways. In contrast, HDL depleted of apolipoproteins by modification with trypsin (trypsin-HDL) is unable to participate in apolipoprotein-dependent efflux (16). Sonicated phospholipid vesicles were used as acceptors that by nature can only participate in efflux by apolipoprotein-independent mechanisms (e.g., ref. 5). [^{3}H]cholesterol efflux from cholesterol-loaded cells by each acceptor was compared in control, brefeldin A- or monensin-treated cells (**Table 3**). Cholesterol efflux to medium without acceptors was the same for all conditions. Similar to results shown above, cellular esterified [^{3}H]cholesterol was significantly decreased by monensin treatment and not affected by brefeldin A compared with controls. ApoA-I promoted efflux of [^{3}H]cholesterol to the medium and in cells treated with brefeldin A or monensin, efflux was again significantly inhibited (by 84 and 87%, respectively). The ability of HDL to promote cholesterol efflux in the presence of monensin or brefeldin A was also significantly inhibited (by 40% for both drugs) compared with controls, confirming our previous observations (12). The decrease in cholesterol efflux in cells treated with monensin or brefeldin A may be assumed to reflect changes in net efflux as these compounds also prevented HDL-mediated cholesterol mass efflux (Table 2 and ref 12) and neither compound affected influx of HDL-associated [^{3}H]cholesterol to cells under similar experimental conditions (not shown). Trypsin-HDL was less effective at promoting cholesterol efflux than HDL under control conditions as previously documented (16). However, unlike control HDL, there was no effect on the extent of [^{3}H]cholesterol efflux to trypsin-HDL in cells treated with brefeldin A or monensin. Phospholipid vesicles were also effective at promoting cholesterol efflux from cells. Brefeldin A had no effect on [^{3}H]cholesterol efflux, while monensin caused a small, but significant, 15% inhibition of efflux mediated by the vesicles. The ability of the various acceptors to deplete the substrate pool of cellular cholesterol available for esterification by ACAT was also examined (Table 3). Under control conditions, only HDL and apoA-I significantly decreased cell cholesterol esterification compared with cells incubated with medium containing 1 mg/ml BSA. Treating cells with brefeldin A or monensin completely prevented the decrease in cholesterol esterification by HDL and apoA-I, in agreement with previous results (12) and the above data. Trypsin-HDL and phospholipid vesicles were unable to decrease cholesterol esterification under the conditions studied and brefeldin A and monensin had no further effects, as expected. Comparable results were observed in three to five separate experiments for each acceptor.

The effects of brefeldin A and monensin on the ability of HDL, trypsin-HDL, and phospholipid vesicles to promote phospholipid efflux from cells were compared (**Fig. 5**). Acceptors were incubated at equal concentration based on phospholipid content. Under control conditions, HDL was more effective at promoting efflux of both phosphatidylcholine and sphingomyelin than either of the acceptors deficient in apolipoproteins. More interestingly, only HDL-mediated efflux of cellular phospholipids was inhibited by brefeldin A or mo-

TABLE 3. Effects of brefeldin A and monensin on cellular [^{3}H]cholesterol efflux and cholesterol esterification by different acceptor types

	[^{3}H]Cholesterol Efflux	Cell [^{3}H]Cholesterol	Cell [^{3}H]Cholesteryl Esters	Cholesterol Esterification
		% of total [^{3}H]cholesterol		*nmol/mg cell protein*
DMEM	1.4 ± 0.0	56.1 ± 0.7	42.5 ± 0.7	4.58 ± 0.11
+ BFA	1.4 ± 0.1	55.5 ± 0.3	43.1 ± 0.3	4.59 ± 0.02
+ MON	1.5 ± 0.1	62.5 ± 0.9	36.0 ± 0.8[a]	4.67 ± 0.21
ApoA-I, 5 μg/ml	11.7 ± 0.5	52.6 ± 1.7	35.7 ± 2.0	3.00 ± 0.10[b]
+ BFA	3.1 ± 0.3[a]	53.5 ± 1.7	43.3 ± 1.1	4.99 ± 0.16
+ MON	2.8 ± 0.1[a]	61.0 ± 0.8	36.1 ± 1.0	4.44 ± 0.15
HDL, 25 μM	19.3 ± 0.4	46.0 ± 0.3	34.7 ± 0.5	3.82 ± 0.08[b]
+ BFA	12.1 ± 1.0[a]	48.3 ± 1.1	40.0 ± 2.1	4.44 ± 0.14
+ MON	12.3 ± 0.2[a]	52.2 ± 0.5	35.5 ± 0.2	4.56 ± 0.22
Trypsin-HDL, 25 μM	9.1 ± 0.3	50.8 ± 0.4	40.1 ± 0.1	4.65 ± 0.51
+ BFA	9.0 ± 1.8	49.8 ± 1.7	41.3 ± 3.6	4.59 ± 0.11
+ MON	9.6 ± 0.3	54.4 ± 0.4	36.0 ± 0.5	4.46 ± 0.04
PLV, 40 μM	10.3 ± 0.2	50.2 ± 1.5	37.7 ± 1.5	4.43 ± 0.14
+ BFA	10.1 ± 0.3	52.4 ± 0.6	37.5 ± 0.4	4.97 ± 0.24
+ MON	89.0 ± 0.5[a]	54.4 ± 0.4	36.0 ± 0.5	4.71 ± 0.35

Fibroblasts, labeled with [^{3}H]cholesterol for efflux studies, were loaded with non-lipoprotein cholesterol as described in the legend to Fig. 2. Cells were incubated with DMEM containing 1 mg/ml BSA alone (DMEM) or with 4 μM brefeldin A (+ BFA) or 25 μM monensin (+ MON) and the same media containing the indicated concentrations of apoA-I protein, HDL, trypsin-modified HDL (trypsin-HDL), or phospholipid vesicles (PLV) based on total phospholipid content at 37°C for 16 h (cholesterol efflux) or 6 h (cholesterol esterification). Medium and cellular [^{3}H]cholesterol were measured as described under Methods and Results are expressed as percent of total radioactivity. Recovery of cellular protein and radioactivity were 86 ± 9 μg/35 mm dish and 50900 ± 7500 cpm/dish, respectively, similar for all conditions. Cellular cholesterol esterification was measured by a 1-h incubation with [^{14}C]oleate after incubation with experimental medium as described under Methods and expressed as nmoles of [^{14}C]oleate incorporated into cholesterol esters per mg of cell protein. Recovery of cell protein was 22.3 ± 1.9 μg/16-mm well, similar for all conditions. Results are the mean ± SD of three dishes.

[a] $P < 0.01$ compared to the same medium without BFA or MON.

[b] $P < 0.025$ compared to DMEM alone.

nensin, reducing efflux to levels comparable with trypsin-HDL and phospholipid vesicles. Treating cells with brefeldin A and monensin had no effect on phosphatidylcholine or sphingomyelin efflux to either trypsin-HDL or phospholipid vesicles.

Cell surface ^{125}I-labeled apoA-I and ^{125}I-labeled HDL binding

Several studies have suggested that apolipoprotein-mediated lipid efflux depends on the cell surface binding of HDL or apolipoproteins as an initiating event to promote intracellular cholesterol translocation to plasma membrane sites available for efflux (e.g., 26–28). The effects of incubating cells with of brefeldin A or monensin on ^{125}I-labeled apoA-I and ^{125}I-labeled HDL binding to cholesterol-enriched cells were examined (**Table 4**). Results show that these agents did not reduce total, nonspecific, or specific binding of apoA-I or HDL under the same conditions that inhibited lipid efflux. Monensin treatment actually increased binding of both HDL and apoA-I, although a consistent observation was that the increase varied between 5 and 30% in four different experiments. Also, worth noting is the significantly greater binding of apoA-I to cells compared with HDL. The cause of this difference is unknown but has been a consistent observation.

DISCUSSION

We have begun to address the cellular events involved in transport of excess cholesterol for eventual removal by extracellular acceptors. In the present study we have shown that apoA-I-mediated efflux of excess cellular cholesterol and cellular phospholipids depends on a brefeldin A- and monensin-sensitive pathway. Based on the known effects of these compounds on Golgi apparatus structure and function (23, 24) our results support the hypothesis that removal of cholesterol from cholesterol-enriched cells by apolipoprotein-dependent mechanisms is due to depletion of intracellular cholesterol that is transported to sites available for efflux by a Golgi apparatus-dependent mechanism(s). The same or related pathway appears to be involved in apolipoprotein-mediated efflux of cellular phospholipids as phosphatidylcholine and sphingomyelin efflux by apoA-I and HDL from cholesterol-enriched cells were

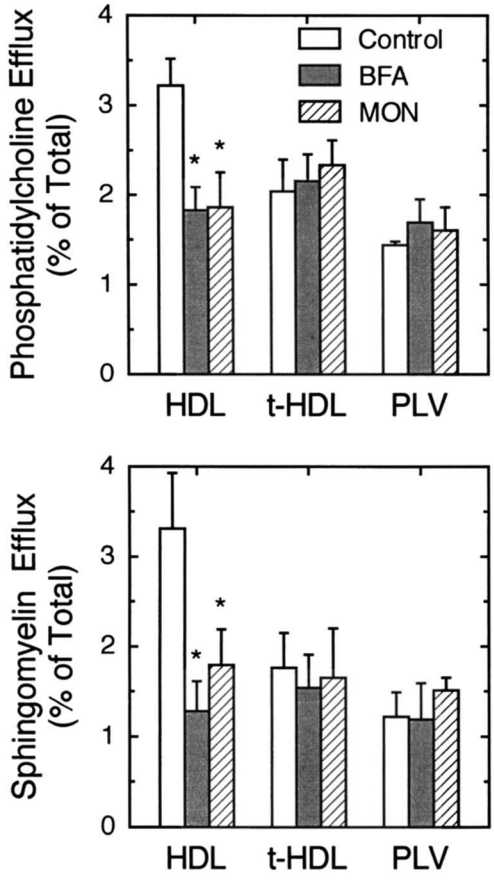

Fig. 5. Effects of brefeldin A and monensin on phospholipid efflux by different acceptors from cholesterol-loaded cells. Fibroblast cultures were treated and labeled with [^{3}H]choline as described in the legend to Fig. 4. Cells were incubated with DMEM containing 1 mg/ml BSA alone (Control, □) or with 4 μM brefeldin A (BFA, ■), or 25 μM monensin (Mon, ▨) and the same media containing 25 μM total phospholipid of HDL, trypsin-modified HDL (t-HDL) or phospholipid vesicles (PLV) for 16 h at 37°C. Labeled phosphatidylcholine and sphingomyelin efflux were measured as described in Fig. 4. Results are the mean ± SD of three dishes expressed as acceptor-mediated efflux after subtracting efflux to medium without acceptors (not shown, e.g., see Fig. 4). Recovery of cellular protein, [^{3}H]phosphatidylcholine, and [^{3}H]sphingomyelin were (mean ± SD, n = 36) 120 ± 5 μg/dish, 267 ± 25 cpm/μg protein, and 28 ± 4 cpm/μg protein, respectively, and similar for all conditions. *Indicates $P < 0.04$ compared to incubations without BFA or Mon.

also inhibited by brefeldin A and monensin. Lipid efflux mediated by aqueous diffusion mechanisms depends on desorption of lipids from the cell membrane and diffusion across the unstirred water layer surrounding the membrane for uptake by an appropriate acceptor and is assumed to be independent of active cellular processes (11, 25, 29, 30). Efflux by this pathway is not affected by cellular cholesterol content and predicted to be insensitive to agents affecting active cellular transport processes. Indeed, efflux of cellular cholesterol and phospholipids to apolipoprotein-depleted acceptors; trypsin-HDL, phospholipid vesicles, or albumin, was little affected by brefeldin A or monensin. In the case of the latter acceptors, efflux of cellular lipids most likely occurs by aqueous diffusion (5, 15), processes apparently not affected by Golgi apparatus disruption. These data suggest a dissociation between apolipoprotein-dependent and -independent lipid efflux mechanisms based on sensitivity to brefeldin A and monensin. As one target of brefeldin A action is ADP-ribosylation factor (31, 32) one possibility is that an ADP-ribosylation factor-dependent vesicular transport step is involved in intracellular cholesterol transport.

At least two models for brefeldin A- and monensin-sensitive (i.e., Golgi-dependent) lipid transport and efflux can be suggested based on the known effects of these drugs on Golgi apparatus structure and function. Cellular lipid transport may be a constitutive process involving 'lipid recycling' among various cellular compartments mediated by vesicular transport pathways and lipid efflux depends on the availability of appropriate extracellular acceptors. Rothblat et al. (33) proposed a model whereby lipid-poor nascent HDL-associated apolipoproteins interact directly with cell membrane lipids, thereby increasing cholesterol transfer rates from cells to the adjacent acceptor by obviating the need for cholesterol molecules to traverse an unstirred water layer. The current results may be described by extending this model to include steps whereby excess cellular cholesterol destined for efflux, together with phospholipids, are transported from sites of accumulation and storage through Golgi apparatus-dependent mechanisms to plasma membrane sites available for interaction with extracellular acceptors leading to lipid efflux. Alternatively, transport of excess cholesterol pools to efflux accessible sites may be stimulated by the interaction of apolipoproteins with cell surface binding sites. The latter mechanism is supported by data showing that apoA-I binds to cells in a saturable fashion (34) and that binding is up-regulated when cell cholesterol is increased (26). This interaction need not require binding to a specific cell surface protein, but such interactions may be involved (5, 26–28) and cannot be ruled out based on the available data. However, brefeldin A and monensin do not appear to decrease HDL or apoA-I binding sites in fibroblasts under conditions that significantly inhibited cholesterol efflux; thus, if apolipoprotein binding to specific cellular sites is necessary to promote cholesterol efflux, then these drugs may affect steps subsequent to cell surface binding. While several possible mechanisms exist for the observed effects of brefeldin A and monensin, the current data clearly suggest the participation of active cellular processes involved in apolipoprotein-mediated lipid efflux.

Our results suggest that brefeldin A- and monensin-

TABLE 4. Effects of brefeldin A and monensin on HDL and apoA-I binding to cholesterol-loaded fibroblasts

Treatment	^{125}I-Labeled ApoA-I Binding			^{125}I-Labeled HDL Binding		
	Total	Nonspecific	Specific	Total	Nonspecific	Specific
	ng/mg cell protein			*ng/mg cell protein*		
Control	110 ± 5	35 ± 2	75	18 ± 1	4 ± 0	14
Brefeldin A	109 ± 8	32 ± 3	77	19 ± 1	6 ± 1	13
Monensin	126 ± 8	26 ± 2	100	28 ± 2	6 ± 1	22

Cells were grown and loaded with cholesterol as described in the legend to Fig. 2. Cells received DMEM containing 1 mg/ml BSA alone (Control) or the same medium with 4 μM brefeldin A (Brefeldin A) or 25 μM monensin (Monensin) for 16 h at 37°C. Cells were chilled on ice and total binding was determined after a 3-h incubation at 2–4°C with DMEM containing 1 mg/ml BSA and 1 μg/ml ^{125}I-labeled apoA-I or 5 μg/ml ^{125}I-labeled HDL and with or without brefeldin A or monensin. Nonspecific binding was determined by incubation in the same medium with 40-fold excess unlabeled apoA-I or HDL as appropriate. Results are expressed as mean ± SD of four dishes expressed as ng of bound ^{125}I-labeled protein per mg cell protein. Specific binding is the difference between total and nonspecific binding. Recoveries of cell proteins were 53 ± 4, 45 ± 3, and 48 ± 2 μg/dish for control, brefeldin A- and monensin-treated cells, respectively.

sensitive efflux of excess cellular cholesterol and phospholipids are closely related. Other studies have also suggested that cholesterol and phospholipid efflux by purified apolipoproteins are closely linked (1, 4, 5, 10) and we further suggest that efflux of these lipids depend on cellular events that require an intact and functional Golgi apparatus. Such a mechanism may transport phospholipid pools (possibly distinct from the bulk of phospholipids necessary for cellular membrane formation and/or maintenance) that increase in cells upon enrichment with free cholesterol (35) along with excess intracellular cholesterol to sites available for efflux. The (assumed) absence of these lipid pools in cells not enriched with cholesterol could explain why apoA-I has limited ability to promote cholesterol and phospholipid efflux from cholesterol-depleted cells (4). The latter observation and studies showing that certain cell types are resistant to apolipoprotein-mediated lipid efflux (36, 37) demonstrate that (purified) apoA-I-mediated lipid efflux is not a consequence of nonspecific interactions of apolipoproteins with cell membrane lipids leading to the formation of extracellular lipid:protein complexes, but involves participation of specific cell-mediated events.

Recently, Li and Yokoyama (38) showed that apoA-I- and HDL-mediated efflux of cellular cholesterol could be selectively enhanced relative to phosphatidylcholine efflux in growth factor-transformed, cholesterol-enriched smooth muscle cells after phorbol ester activation of protein kinase C. The phorbol ester effect was only observed in the transformed smooth muscle cells in which apoA-I-mediated efflux resulted in the formation of (relatively) cholesterol-poor, pre-beta-like particles in the medium under control conditions. However, protein kinase C activation had no effect on cholesterol efflux from macrophage cultures that produced cholesterol-rich, pre-beta-like particles upon incubation with apoA-I. These data were taken to suggest that protein kinase C activation induced translocation of intracellular cholesterol from a pool available for esterification to a pool accessible for apolipoprotein-dependent efflux (38). These results suggest that the formation of apolipoprotein:phospholipid complexes alone is insufficient to induce cholesterol efflux and involves other cellular events. Those results are not inconsistent with our proposal that intracellular cholesterol efflux is mediated by a Golgi-dependent process linked to phospholipid efflux, if for example, activation of protein kinase C increased the availability of cholesterol to a pool available for transport by a brefeldin A- or monensin-sensitive pathway. We cannot rule out the additional possibility that brefeldin A and monensin treatment of cells inhibits apolipoprotein-dependent lipid efflux by altering the expression or cellular distribution of yet undefined proteins involved in lipid transport leading to removal by extracellular apolipoprotein-containing acceptors. Future identification of proteins directly involved in cholesterol and phospholipid efflux events are needed to address this possibility and distinguish between common or distinct mechanisms involved in apolipoprotein-mediated lipid transport and efflux.

It has been suggested that purified apolipoproteins first interact with cell membrane phospholipids forming nascent pre-β-like HDL particles which then participate in promoting cholesterol efflux by mechanisms predicted by the aqueous diffusion mechanism (1, 4, 10). The current results cannot directly rule out such a possibility, as inhibition of phospholipid efflux by monensin or brefeldin A would result in inhibition of cholesterol efflux as a secondary event. However, if phospholipid association with apolipoproteins is necessary for efficient cholesterol efflux, this model cannot account for the observation that intracellular cholesterol efflux to HDL (particles that contain both apolipopro-

teins and phospholipids) was also blocked by treating cells with brefeldin A and monensin under conditions where depletion of plasma membrane cholesterol, presumably through diffusion and desorption mechanisms, was unaffected (12). Also worth noting, brefeldin A and monensin reduced HDL-mediated cholesterol and phospholipid efflux to levels comparable to those obtained by apolipoprotein-depleted acceptors suggesting that, in addition to brefeldin A and monensin sensitive efflux, HDL also promotes efflux by apolipoprotein-independent mechanisms insensitive to those drugs. This observation may also explain why inhibition of apoA-I-mediated efflux was always of greater magnitude than for HDL as A-I cannot participate in apolipoprotein-independent efflux, and further implies that the majority of apolipoprotein-mediated lipid efflux depends on a brefeldin A- and monensin-sensitive pathway. Therefore, the prediction that apolipoprotein and phospholipid complexes must be present to promote cholesterol efflux is not supported by these data.

In the present study we have shown that efflux of cholesterol, phosphatidylcholine, and sphingomyelin from cholesterol-loaded fibroblasts to purified apoA-I, but not apolipoprotein-depleted acceptors, occurs by a brefeldin A- and monensin-sensitive mechanism. These observations are consistent with a role for the Golgi apparatus in mediating transport of lipids from intracellular sites of accumulation and storage to sites available for efflux by apolipoprotein-dependent events. Further elucidation of the mechanisms that promote intracellular lipid transport are needed to establish the contribution of various pathways and molecular events active in ridding cells of excess cholesterol.

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