Efflux of Cellular Cholesterol and Phospholipid to Lipid-Free Apolipoproteins and Class A Amphipathic Peptides†

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ABSTRACT: The mechanism(s) by which lipid-free apolipoprotein (apo) AI is able to stimulate efflux of cholesterol and phospholipid from cells in cultures has (have) been examined. This process was found to be enhanced when macrophages were enriched with cholesterol. There were 12- and 4-fold increases in cholesterol and phospholipid efflux, respectively, from cholesterol-enriched mouse macrophages when compared to cells not loaded with cholesterol. This enhancement in cholesterol efflux to lipid-free apo AI from macrophages enriched with cholesterol was found to be controlled by the level of free cholesterol in the cells. When cholesterol-enriched mouse macrophages were exposed to lipid-free apo AI at 20 μg/mL (706 nM), there was significant efflux of [^{14}C]cholesterol and [^{3}H]phospholipid (20% ± 0.5%/24 h and 6% ± 0.3%/24 h, respectively). In comparison, HDL at equivalent protein concentrations only stimulated 11% and 4% efflux of cholesterol and phospholipid, respectively. Synthetic peptides containing amphipathic helical segments that mimic those present in apo AI were used to examine the structural features of the apoprotein which stimulate lipid efflux. Peptides containing only one (18A) or two (37pA) amphipathic helical segments stimulated as much cholesterol efflux from both mouse macrophages and L-cells as apo AI. The order of efficiency, as assessed by the mass concentration at which half-maximal efflux was reached (EC_{50}), was apo AI > 37pA > 18A, indicating that acceptor efficiency was dependent on the number of amphipathic helical segments per molecule. When the helical content of 18A was increased by neutralizing the charges at the ends of the peptide (Ac-18A-NH_2), there was a substantial increase in the efficiency for cholesterol efflux (EC_{50} 18A = 17 μg/mL vs Ac-18A-NH_2 = 6 μg/mL). In contrast, when the amphipathicity of the helix in 18A was decreased by scrambling the amino acid sequence, thereby reducing its lipid affinity, cholesterol and phospholipid efflux were not stimulated. The efficiency with which the peptides stimulated cholesterol efflux was in order of their lipid affinity (37pA > Ac-18A-NH_2 > 18A), and this order was similar for phospholipid efflux. The time courses of lipid release from mouse macrophages and L-cells indicated that phospholipid appeared in the extracellular medium before cholesterol. These results suggest that the apo AI or peptides first interacted with the cell to form protein/phospholipid complexes, that could then accept cholesterol.

The removal of cholesterol from peripheral cells by high-density lipoprotein (HDL)[1] is thought to initiate the process of reverse cholesterol transport (Glomset, 1968), and numerous studies have demonstrated that HDL is capable of stimulating the net release of cholesterol from cells (Ho et al., 1980; Bernard et al., 1990; Johnson et al., 1991). The HDL fraction of plasma is a heterogeneous group of particles that are rich in phospholipid and contain exchangeable apolipoproteins (apo) (Forte et al., 1990; Kawano et al., 1993; Johnson et al., 1991). It remains to be established which of these particles or precursors of these particles are the major acceptors of cellular cholesterol in the interstitium. The predominant protein component of HDL is the exchangeable apo AI molecule (Segrest et al., 1990). The importance of apo AI in reverse cholesterol transport has been established in studies showing that when apo AI-containing particles are removed from whole plasma the release of cholesterol from cells is greatly reduced (Fielding & Moser, 1982). Removal of apo AI from lipoprotein-deficient serum (LPDS) also results in a decrease in cholesterol efflux (Oram et al., 1981). It has been estimated that 3% of plasma apo AI is not associated with HDL, but rather is found in LPDS (Neary & Gowland, 1987). Likewise, a portion of the apo AI found in the interstitial fluid is in a lipid-free form (Roheim et al., 1990), and a fraction of the apo AI secreted by cells is lipid-deficient (Forte et al., 1990). In addition, studies have shown that under certain conditions apo AI can be displaced from HDL (Coetzee et al., 1986), and that this lipid-free apo AI can interact with cells (Blackburn et al., 1991). The function of lipid-free apo AI is unknown. However, recent studies from a number of laboratories have established that lipid-

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[1] Abbreviations: acLDL, acetylated low-density lipoprotein; ACAT, acyl-CoA:cholesterol acyltransferase; apo, apolipoprotein; BSA, bovine serum albumin; GLC, gas−liquid chromatography; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MEM, minimal essential medium; PBS, phosphate-buffered saline; PC, phosphatidylcholine.

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free apo AI and other apoproteins including apo E, AIV, and AII are all capable of stimulating significant release of phospholipid and cholesterol from cells (Hara & Yokoyama, 1991; Bielicki et al., 1992; Forte et al., 1993). This process results in the formation of nascent HDL particles, and occurs in many cell types including fibroblasts (Bielicki et al., 1992), Chinese hamster ovary cells (Forte et al., 1993), and mouse peritoneal macrophages (Hara & Yokoyama, 1991). Our previous studies demonstrated that phospholipid and cholesterol efflux from fibroblasts to apo AI is enhanced when the cells are enriched with cholesterol (Bielicki et al., 1992), and one goal of the present studies was to determine if this stimulation is also observed in mouse peritoneal macrophages and mouse L-cell fibroblasts. In addition, studies have been done to explore the relationship between the enhancement of cholesterol efflux to apo AI and the cellular free and esterified cholesterol contents.

The second goal of the present studies was to utilize synthetic peptides of defined structure to determine what structural properties of apo AI enable it to stimulate lipid efflux. Apo AI and the other exchangeable apoproteins are composed of a varying number of 22-residue-long amino acid repeats (Segrest et al., 1992). These repetitive segments are amphipathic α-helical domains that mediate interaction of the protein with lipid (Segrest et al., 1992). Peptide 18A is an 18 amino acid peptide with the amino acid sequence DWLKAFYDKVAEKLKEAF which forms a single amphipathic α-helix. This helix is similar to the class A domains present in apolipoproteins in that basic residues are located at the polar/nonpolar boundary of the helix and acidic residues are located in the center of the polar face (Segrest et al., 1992; Anantharamaiah et al., 1985). There is no sequence homology between this synthetic peptide and naturally occurring apolipoproteins (Segrest et al., 1992). Peptide 37pA contains two 18A peptides linked by a proline residue (Anantharamaiah et al., 1985; Anantharamaiah, 1986). The proline residue interrupts the formation of a single long α-helix so that this peptide consists of two amphipathic helical segments and has higher lipid binding affinity than 18A. Blocked 18A (Ac-18A-NH_2) is the 18A molecule modified to contain an acetyl group at the N-terminal and an amide group at the C-terminal. This neutralizes the charges at the ends of the molecule, and stabilizes and lengthens the amphipathic helical segment, giving this peptide higher lipid binding affinity than 18A (Segrest et al., 1992; Venkatachalapathi et al., 1993). Scrambled 18A (18S) contains the same amino acids as 18A, but the sequence, DWLAKDYFKKALVEEFAK, has been randomized to eliminate the amphipathic nature of the helix and the lipid binding affinity (Anantharamaiah, 1986). In studies using these peptides, we assess the role of lipid affinity, amino acid sequence, helix number, and helical length in the efflux of lipid from cells to lipid-free apolipoproteins in the extracellular medium.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA, essentially fatty acid free), heat-inactivated fetal bovine serum (FBS), phosphatidylcholine (PC), cholesteryl methyl ether, and unesterified cholesterol were purchased from Sigma (St. Louis, MO). The radioisotopes [^{14}C]cholesterol (51 mCi/mmol), [^{3}H]choline (87 Ci/mmol), cholesteryl [^{14}C]oleate (59.5 mCi/mmol), and [^{3}H]oleate (9.2 Ci/mmol) were obtained from New England Nuclear (Boston, MA). All organic solvents were purchased from Fisher Scientific (Pittsburgh, PA). Tissue culture flasks and plates were obtained from Falcon (Lincoln, NJ) and Corning Glass Works (Corning, NY). Culture media and trypsin were obtained from Gibco (Grand Island, NY). Sandoz compound 58-035 was a gift from Dr. John Heider.

Lipoproteins, Lipid Dispersions, Apolipoproteins, and Synthetic Peptides. Human LDL (d = 1.019−1.063 g/mL) and HDL_3 (d = 1.125−1.21 g/mL) were fractionated by sequential ultracentrifugation (Hatch & Lees, 1968). Lecithin:cholesterol acyltransferase was inhibited by the addition of *N*-ethylmaleimide to plasma (Johnson et al., 1986). To remove particles containing apo E, the HDL_3 fraction was chromatographed on a heparin−Sepharose column (Bamberger et al., 1985). Human LDL was acetylated by the procedure of Basu et al. (1976). Before use, all lipoproteins were dialyzed extensively against 0.15 M NaCl, and sterilized by filtration through a Millipore filter (0.45 μm). Dispersions containing free cholesterol and egg PC [(2−3):1, mol/mol] were prepared by sonication as described by Arbogast et al. (1976). Pure human apo AI was isolated as described before (Mahlberg & Rothblat, 1992). The lyophilized apo AI was solubilized in a 6 M guanidine hydrochloride solution at a concentration of about 1 mg/mL and dialyzed exhaustively against phosphate-buffered saline (PBS) containing 50 μg/mL gentamicin. The peptides 18A, scrambled 18A, Ac-18A-NH_2, and 37pA were synthesized and purified as described previously (Anantharamaiah, 1986; Venkatachalapathi et al., 1993). To solubilize the peptides, approximately 1 mg of the lyophilized preparation was added to 1 mL of water already containing 50 μg/mL gentamicin. The concentrations of peptide solutions were determined from the absorbance at 280 nm, and then the peptides or apoproteins were diluted with MEM containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and bicarbonate before addition to cells.

Cell Culture and Isolation of Mouse Peritoneal Macrophages. Mouse L-cells were grown in minimum essential medium (MEM) containing 10% FBS (v/v). Elicited mouse peritoneal macrophages were isolated as described previously (Yancey & St. Clair, 1991). Briefly, $B_6C_3F_1$ mice (Taconic Farms, Germantown, NY) were injected intraperitoneally with 0.5 mL of sterile 10% thioglycolate solution. On the fifth day after injection, the exudate cells were isolated by peritoneal lavage with two 5 mL washes of sterile PBS containing 10 IU/mL heparin. The cells were then pelleted by centrifugation for 15 min at 1000 rpm and resuspended in MEM containing HEPES (50 mM, pH 7.4). Exudate cells [(7−10) × 10^5] in 0.5 mL of HEPES-buffered MEM were added to 22-mm dishes already containing 1.5 mL of 10% FBS−MEM and allowed to attach to the tissue culture dishes by incubation at 37 °C. After 4 h, the cells were washed with HEPES-buffered MEM 1−5 times to remove nonadherent cells. At this point, the labeling phase of the experiments was initiated. Culture media for all the cell incubations were supplemented with 23 mM bicarbonate, 50 mM HEPES, and 50 μg of gentamicin/mL. All cells were incubated in a humid atmosphere at 37 °C with 95% air and 5% CO_2.

Radiolabeling of Cells. To label mouse macrophage phospholipid and cholesterol, the cells were incubated for 40 h in MEM containing 1% FBS, 3 μCi/mL [^{3}H]choline, and 1 μCi/mL [^{14}C]cholesterol. Ethanol was used to disperse

the cholesterol into the medium, and the final concentration of this solvent was 0.1% (v/v). For experiments where macrophages were enriched with cholesterol (unless otherwise indicated in the figure legends), 100 μg of protein/mL of acetylated LDL was included in the incubation medium. In experiments using mouse L-cells, cells from stock cultures were plated in 10% FBS−MEM onto 35 mm dishes at a density of 2×10^5 cells per dish. After 24 h, the cells were incubated for 4 days in MEM containing 5% FBS (v/v), 3 μCi/mL [^{3}H]choline, 1 μCi/mL [^{14}C]cholesterol, and a 1 μg/mL aliquot of the ACAT inhibitor Sandoz 58-035. In experiments where cells were enriched with cholesterol, 200 μg of FC/mL of FC/PC dispersions was added to the medium. At the end of the labeling period, mouse L-cells and macrophages were washed 3 times with MEM−HEPES, and then incubated for 1 h in MEM containing 2 mg/mL BSA. The monolayers were washed 3 more times with MEM−HEPES. Two milliliters of the media containing the apo AI or peptide at the desired concentration was added to the radiolabeled cell monolayer and incubated for varying periods.

Assay for Efflux of Cellular Cholesterol and Phospholipid. At the end of each incubation, the medium was aspirated and centrifuged at 2000 rpm for 15 min to pellet any floating cells. The supernatant was removed, and 10 μg of butylated hydroxytoluene was added to each sample to prevent the oxidation of phospholipids. The lipids were then extracted by the method of Bligh and Dyer (1959). The aqueous phase was aspirated, and to remove any remaining free [^{3}H]choline, the chloroform phase was washed 3 times with 10:9 (v/v) methanol/water. The chloroform phase was dried under a stream of nitrogen (N_2) and redissolved in 1 mL of chloroform/methanol (2:1, v/v). Then, 700 μL of each sample was transferred to a liquid scintillation vial and dried under a stream of N_2. Five milliliters of Scintverse (Fisher) was added to each vial, and the amounts of ^{3}H and ^{14}C were quantitated by liquid scintillation counting. To analyze cellular lipids, the cell monolayers were washed 3 times with PBS. Cellular lipids were recovered by the addition of 2 mL of 2-propanol containing 20 μg of butylated hydroxytoluene to each dish (Bielicki et al., 1992). The 2-propanol extracts were dried under a stream of N_2, and the free [^{3}H]-choline was extracted by the method of Bligh and Dyer (1959). The chloroform phase was washed 3 times with methanol and water (10:9, v/v). The chloroform phase containing the lipids was dried under N_2 and solubilized in chloroform and methanol (2:1, v/v). Aliquots were then taken for liquid scintillation counting. Total and free cholesterol contents of the cellular lipid extracts were quantified by the procedure of Ishikawa et al. (1974) and gas−liquid chromatography (Yancey & St. Clair, 1991) using cholesteryl methyl ether as an internal standard. Cellular phospholipid phosphorus was determined by the method of Sokoloff and Rothblat (1974). The different [^{3}H]choline-labeled phospholipid subclasses were separated by thin-layer chromatography and quantitated by liquid scintillation counting as described (Bielicki et al., 1991). Cellular proteins were measured using the method of Lowry (Lowry et al., 1951) as modified by Markwell and colleagues (Markwell et al., 1978). The cholesteryl ester bands were visualized with iodine; after evaporation of the iodine stain, the cholesteryl ester bands were cut and the radioactivity was measured by liquid scintillation counting. Cell permeability was measured by following the release of [^{14}C]adenine using the method of Shirhatti and Krishna (1985) as modified by Reid and colleagues (Reid et al., 1992).

Measurement of Oleic Acid Incorporation in Cholesteryl Oleate. Sodium [9,10-^{3}H]oleate was prepared essentially as described (Goldstein et al., 1983). During the last 2 h of the experiment, 31 nmol of the [^{3}H]oleate complex (23 000 dpm/nmol) was added to each dish. Cellular lipids were extracted with 2-propanol containing 50 000 dpm of cholesteryl [1-^{14}C]oleate as an internal standard to monitor recovery of cellular cholesteryl [^{3}H]oleate. The 2-propanol extracts were dried under N_2 and the lipids resolubilized in chloroform/methanol (1:1, v/v). The lipids were then separated on Silica Gel G thin-layer chromatography plates using hexane/ethyl ether/acetic acid (80:20:1, v/v/v) as the solvent system.

Data Analysis. The cholesterol and phospholipid efflux data are expressed as the percentage of cellular lipid released from cells: [(cpm in medium containing the protein at the end of the efflux phase minus cpm in control (protein-free) medium at the end of the efflux phase)/(cpm in cellular lipids at $t = 0$)] × 100. All values for each point are the mean ± SD for triplicate determinations. As mentioned above, after the cells were loaded with cholesterol, the cells were then incubated for 1 h in MEM containing 1 mg/mL BSA. This short equilibration phase was chosen to minimize the loss of labeled phospholipid resulting from the turnover of cellular phospholipid. When macrophages were incubated for 24 h with MEM alone, 50% of the cell-associated [^{3}H]phospholipid was degraded. No significant differences were found in the amount of total labeled phospholipid recovered per dish when macrophages were incubated for up to 24 h with either MEM alone or in the presence of apo AI, 37pA, and 18A (data not shown). This demonstrates that there are no significant differences in the turnover of choline-containing phospholipids when macrophages are incubated with the different acceptors. The influence of the turnover of labeled phospholipid on the quantitation of phospholipid efflux from cells in the presence of different acceptors has been discussed previously (Bielicki et al., 1991), and results in an underestimation of the mass of cell phospholipid released to the medium. However, the experimental protocol used in the present study has been shown previously to provide reliable comparative estimates of cellular phospholipid efflux (Bielicki et al., 1991, 1992). Due to the short equilibration phase, the specific activities of the cellular free and esterified cholesterol did not reach a constant level, and as a result, the percent cholesterol efflux when determined isotopically was found to underestimate the actual mass efflux by approximately 20%. Nonetheless, when parallel mass and isotopic measurements were made, the same patterns of cholesterol efflux were observed among the different acceptors, and the two different measurements of cholesterol efflux were correlated ($r = 0.78$). The GraphPAD Inplot software package (version 3.1, Graphpad Software Inc., CA) was used for the analysis of the lipid efflux concentration-dependence curves. Data were fitted via a four-parameter logistics equation which generated the best-line through the experimental data points and calculated the protein concentration (EC_{50}) at which half-maximal lipid efflux occurred.

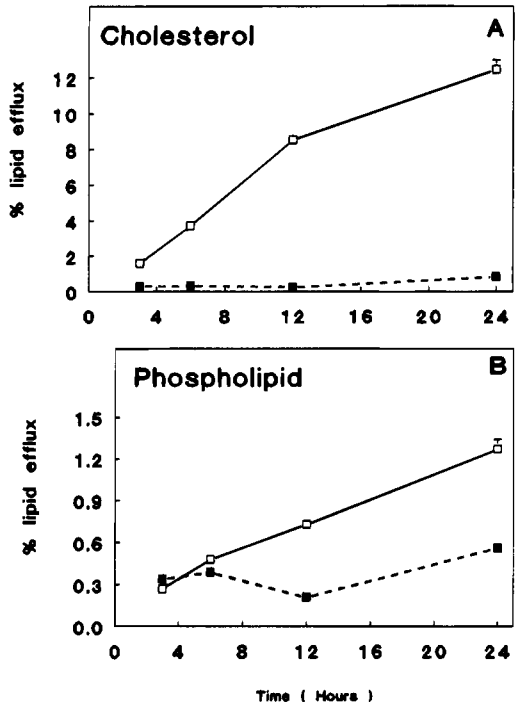

FIGURE 1: Time courses of the efflux of [^{14}C]cholesterol and [^{3}H]phospholipid from control and cholesterol-enriched mouse macrophages to apo AI. Mouse macrophages were labeled with [^{14}C]cholesterol and [^{3}H]choline, enriched with cholesterol, and the cells were washed as described under Materials and Methods. The macrophages were then incubated for up to 24 h at 37 °C with 20 μg of protein/mL of apo AI. Shown are the percent cholesterol (panel A) and phospholipid (panel B) efflux from control (■) or cholesterol-enriched (□) mouse macrophages. The free and esterified cholesterol contents of the control cells were 27 ± 1 and 0.6 ± 0.2 μg/mg of cell protein, respectively. The free and esterified cholesterol contents of the cholesterol-enriched cells were 34 ± 1 and 12 ± 3 μg/mg of cell protein, respectively. The initial counts present in the cholesterol-enriched cells as [^{14}C]cholesterol and [^{3}H]phospholipid were 273 770 ± 4903 and 327 499 ± 5492 cpm/dish, respectively. In the cells not loaded with cholesterol, there were 222 061 ± 7874 and 448 981 ± 14 641 cpm/dish present initially as [^{14}C]cholesterol and [^{3}H]phospholipid, respectively.

RESULTS

The Effects of Enriching Macrophages with Cholesterol on the Efflux of Lipid to Lipid-Free Apo AI. Previous results from our laboratory showed that phospholipid and cholesterol efflux to lipid-free apo AI was enhanced from cholesterol-enriched human skin fibroblasts when compared to cells not loaded with cholesterol (Bielicki et al., 1992). Initially, we determined if this process also was enhanced when mouse peritoneal macrophages were enriched with cholesterol. The time courses of both cholesterol and phospholipid efflux from control and cholesterol-enriched macrophages to lipid-free apo AI are shown in Figure 1A and Figure 1B. When mouse macrophages were enriched with free cholesterol, there was a 12-fold increase in the cholesterol efflux stimulated by apo AI in 24 h, when compared to control cells (Figure 1A). Phospholipid efflux was increased by 4-fold after 24 h of incubation (Figure 1B). Similarly, cholesterol and phospholipid efflux to apo AI were increased 3- and 4-fold, respectively, from cholesterol-enriched mouse L-cells in comparison to control cells (data not shown). On the basis of these results, we used macrophages and L-cells that had been enriched with cholesterol for subsequent experiments.

Next, the stimulation of cholesterol efflux from cholesterol-enriched mouse macrophages to lipid-free apo AI was further examined. To determine if cholesterol efflux to lipid-free apo AI was more enhanced when mouse macrophages were loaded with higher levels of cholesterol, the cells were first enriched with cholesterol by incubation for 40 h either with acLDL alone or with acLDL together with 200 μg of FC/mL of FC/PC dispersions, and then incubated for 24 h with 20 μg/mL apo AI. Also, to examine the effect of enriching cells with free cholesterol on this process, parallel incubations were done with macrophages which had been enriched with cholesterol by incubation with the same two cholesterol-loading media in the presence of the ACAT inhibitor 58-035. Shown in Table 1 are the total, free, and esterified cholesterol contents of cholesterol-enriched macrophages incubated for 24 h with MEM alone or with apo AI. Also, shown in parentheses are the percent decrease in cholesterol when the cells were incubated with apo AI relative to incubation with MEM alone. As observed previously, the macrophages that had not been cholesterol enriched only cleared 7% of their cholesterol mass. In comparison when macrophages were enriched with cholesterol by preincubation with acLDL, the cells cleared 19% of their total cholesterol mass in 24 h when incubated with apo AI when compared to the cells incubated with MEM alone. When the macrophages were enriched with a higher degree of cholesterol by incubation with FC/PC dispersions, the efflux of cholesterol to apo AI was further enhanced (33%/24 h) in comparison to the cells loaded with less cholesterol (19%/24 h). This decrease in total cholesterol mass was mainly accounted for by a clearance of esterified cholesterol from these macrophage foam cells (48%/24 h). While parallel incubations of the macrophages with compound 58-035 resulted in a lower total cholesterol content, the cells contained a higher fraction of free cholesterol than esterified cholesterol when compared to the cells not incubated with the ACAT inhibitor. The efflux of cholesterol was enhanced in the free cholesterol-enriched cells despite the lower level of total cellular cholesterol. The macrophages pretreated with acLDL and 58-035 cleared 31% of their total cholesterol, while cells that were not pretreated with 58-035 cleared 19%. In addition, when the macrophages were enriched with an even higher level of free cholesterol by pretreatment with acLDL, FC/PC dispersions, and 58-035, the cells released 60%, in comparison to the 33% by the cells not incubated with 58-035.

Dependence of Cholesterol and Phospholipid Efflux from Cholesterol-Enriched Mouse Macrophages and L-Cells on Apo AI and Peptide Concentrations. The dose-response curves for the efflux of cholesterol and phospholipid from cholesterol-enriched macrophages to apo AI, 37pA, Ac-18A-NH_2, and 18A are shown in Figure 2A,B. Saturation of phospholipid efflux was observed only when apo AI was used as an acceptor (Figure 2B). In addition, the amount of phospholipid released from the macrophages to the peptides was significantly greater than that to apo AI. Similar results were observed for the mouse L-cells (data not shown). For example, exposure to Ac-18A-NH_2 produced an extensive release of phospholipid (22%/4 h). After an 8 h incubation with 4.5×10^{-4} M Ac-18A-NH_2, the cells released 40% of their labeled phospholipid, but there was only a 20% decrease in the mass of cellular phospholipid, suggesting that the cells partially compensate for the efflux of phospholipid through

Table 1: Effect on the Efflux of Cholesterol to Lipid-Free Apo AI When Macrophages Are Enriched with either Excess Free or Esterified Cholesterol[a]

cholesterol loading treatment	58 035 + or −	cell cholesterol mass (μg/mg of protein) after 24 h incubation with MEM: cellular TC mass	cellular EC mass	cellular FC mass	decrease in cholesterol mass (μg/mg protein) after 24 h incubation with apo AI: cellular TC mass	cellular EC mass	cellular FC mass
none	−	27 ± 2	0	27 ± 2	2 ± 0.1 (−7)	0	2 ± 0.1 (−7)
acLDL	−	103 ± 3	34 ± 2	77 ± 1	19 ± 0 (−19)	9 ± 1 (−27)	10 ± 0 (−14)
acLDL	+	87 ± 4	10 ± 1	77 ± 4	27 ± 1 (−31)	4 ± 1 (−40)	23 ± 0 (−30)
acLDL + FC/PC dispersions	−	173 ± 3	97 ± 4	77 ± 1	56 ± 3 (−33)	47 ± 5 (−48)	10 ± 0 (−13)
acLDL + FC/PC dispersions	+	185 ± 4	11 ± 2	175 ± 5	95 ± 5 (−60)	3 ± 0 (−36)	92 ± 5 (−61)

[a] Mouse macrophages were incubated for 40 h at 37 °C in MEM containing 1% FBS alone or with one of the following: 100 μg of protein/mL of acLDL, 100 μg of protein/mL of acLDL + 1 μg/mL 58 035, 100 μg of protein/mL of acLDL + 200 μg of FC/mL of FC/PC dispersions, or 100 μg of protein/mL of acLDL + 200 μg of FC/mL of FC/PC dispersions + 1 μg/mL 58 035. The cells were then washed as described under Materials and Methods, and incubated for 24 h in MEM containing 20 μg of protein/mL of apo AI alone or with 1 μg/mL 58 035. Shown are the cell total, free, and esterified cholesterol contents when macrophages were incubated for 24 h with MEM alone. Also shown are the differences in the total (TC), esterified (EC), and free (FC) cholesterol contents when the cells were incubated for 24 h with apo AI when compared to incubation with MEM alone. These same data are expressed as the percent decrease in cholesterol content relative to incubation with MEM alone, and are shown in the parentheses. The values are the mean ± SD for triplicate dishes.

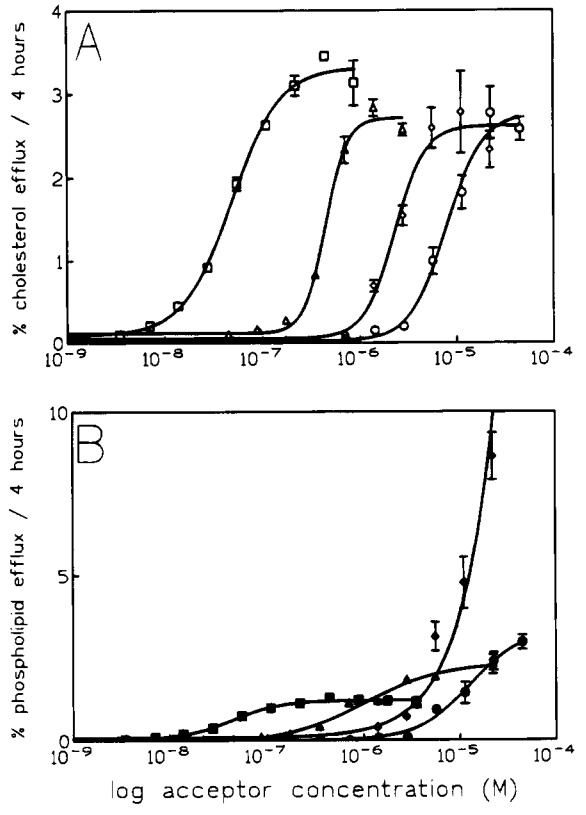

FIGURE 2: Dependence of [^{14}C]cholesterol and [^{3}H]phospholipid efflux from cholesterol-enriched mouse macrophages on apo AI and peptide concentrations. Mouse macrophages were labeled with [^{14}C]cholesterol and [^{3}H]choline, enriched with cholesterol, and the cells were washed as described under Materials and Methods. The cells were then incubated for 4 h at 37 °C in MEM−HEPES containing the indicated concentrations of apo AI or peptides. Shown are the percent cholesterol (open symbols) and percent phospholipid (closed symbols) efflux/4 h from macrophages to apo AI (□), 37 pA (△), Ac-18A-NH_2 (◇), and 18A (○). Values are the mean ± SD for triplicates. The initial cell counts present as [^{14}C]cholesterol and [^{3}H]phospholipid were 284 326 ± 22 179 and 925 825 ± 92 473 cpm/dish, respectively.

de novo synthesis. The distribution of choline-labeled phospholipids in the medium differed between macrophages and L-cells, but was not influenced by the type of acceptor (Table 2). In the case of macrophages, sphingomyelin was the predominant phospholipid released, whereas the L-cells phosphatidylcholine was the major type of choline-containing

Table 2: Percent Distribution of the Different Choline-Labeled Phospholipid Types Appearing in the Medium When Mouse Macrophages Were Incubated with either Apo AI or the Peptides[a]

acceptor	% distribution of [^{3}H]choline-labeled phospholipids: % phosphatidylcholine	% sphingomyelin	% lysophosphatidylcholine
	Mouse Macrophages		
apo AI	43 ± 2	51 ± 4	6 ± 1
Ad-18A-NH_2	55 ± 4	38 ± 1	7 ± 0
	Mouse L-Cells		
apo AI	78 ± 1	20 ± 1	1 ± 0
37pA	85 ± 1	14 ± 1	1 ± 0
Ac-18A-NH_2	85 ± 2	15 ± 2	1 ± 0

[a] Mouse macrophages and L-cells were labeled with [^{3}H]choline, cholesterol-enriched, and washed as described under Materials and Methods. Macrophages were incubated for 24 h in MEM containing 50 μg of protein/mL of either apo AI or Ac-18A-NH_2. Mouse L-cells were incubated for 24 h in MEM containing 15 μg of protein/mL of one of the following: apo AI, 37pA, or Ac-18A-NH_2. Shown is the percent of total choline-labeled phospholipid that was present as phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine.

phospholipid appearing in the medium (Table 2). The elevated release of phospholipid observed when macrophages were incubated with Ac-18A-NH_2 was not associated with microscopic signs of cell toxicity, and there was no decrease in cellular protein at the end of the 8 h incubation. However, quantitation of the release of cellular [^{14}C]adenine (Reid et al., 1992) demonstrated that the release of labeled adenine was 2-fold greater when the cells were incubated with 4.5 × 10^{-4} M Ac-18A-NH_2 compared to incubation with MEM alone where 9.6% was released in 4 h (data not shown). The increase in adenine leakage indicates that this large release of phospholipid did increase membrane permeability. No significant toxicity was observed when the cells were incubated with apo AI as judged by the release of [^{14}C]-adenine.

In contrast to phospholipid efflux, the release of cell cholesterol demonstrated pronounced saturation with all acceptors. This saturation of cholesterol efflux occurred when the cellular phospholipid release to the different acceptors was in a narrow range of 1.5−3% of the initial labeled phospholipid present in the mouse macrophages. Comparisons of the molar and mass acceptor concentrations which promoted half-maximal efflux (EC_{50}) from the mouse macrophages are shown in Table 3. Also shown are the EC_{50}

Table 3: Concentration of Apo AI or Peptide Yielding Half-Maximal Efflux of Cholesterol from Mouse Peritoneal Macrophages and L-Cells

	cholesterol efflux		phospholipid efflux	
acceptor	nM	μg/mL	nM	μg/mL
	Mouse Macrophages (EC_{50} ± SEM)			
apo AI	53 ± 1	1.3 ± 0.6	44 ± 1	1.3 ± 0.5
37pA	444 ± 5	2.0 ± 0.2	1011 ± 32	5.9 ± 1.2
Ac-18A-NH_2	2559 ± 22	5.7 ± 0.2	ND[a]	ND[a]
18A	7787 ± 74	17 ± 0.5	>10000[b]	>28[b]
	Mouse L-Cells (EC_{50} ± SEM)			
apo AI	79 ± 3	2.3 ± 0.5	53 ± 4	1.5 ± 1
37pA	409 ± 9	1.9 ± 0.4	7597 ± 532	38 ± 3
Ac-18A-NH_2	1613 ± 48	6.8 ± 2.1	ND[a]	ND[a]
18A	>20000[b]	>50	>50000[b]	>125[b]

[a] Not determined. [b] Minimal possible EC_{50} values are reported for cell peptide combinations for which clear saturation of efflux was not demonstrated. These minimal values are representative of the peptide concentration promoting half of the maximum observed efflux.

values obtained from similar experiments done with cholesterol-enriched L-cells. The order of efficiency (as reflected in the EC_{50} values) with which the acceptors stimulated cholesterol efflux from both cell types was apo AI > 37pA > Ac-18A-NH_2 > 18A. On a mass basis (Table 3), the relative order of efficiency for the peptides in both cell types was similar to that observed when the data were expressed on a molar basis (apo AI ≅ 37pA > Ac-18A-NH_2 > 18A), although 37pA and apo AI were equally effective on a mass basis. The order of efficiency with which the different acceptors stimulated phospholipid efflux is similar to that observed with cholesterol efflux in both cell types (Table 3). The EC_{50} values for cholesterol and phospholipid efflux to apo AI were in good agreement for both cell types (Table 3). Consistent with the lack of saturation of phospholipid efflux from the cells in the presence of the peptides, the estimated EC_{50} values for phospholipid efflux from mouse macrophages and L-cells to the peptides were 2−5-fold higher than those determined for cholesterol efflux.

Examination of the Necessity for Amphipathic Helical Segments in Cellular Lipid Efflux to Apo AI. To determine if amphipathic helical segments are required in order for apolipoproteins to stimulate lipid efflux, cholesterol-enriched mouse macrophages were incubated for 4 h in MEM alone or in the presence of 50 or 100 μg/mL of either the 18A or the scrambled 18A peptide. Even when present at these high concentrations, scrambled 18A was not able to stimulate significant efflux of either cholesterol or phospholipid in comparison to cells incubated with MEM alone (Figure 3A,B). In contrast, 18A was effective in promoting both cholesterol and phospholipid release from the mouse macrophages. Similar results were observed with mouse L-cells (data not shown).

Time Courses of the Efflux of Lipid from Mouse L-Cells and Macrophages to Lipid-Free Apo AI and Peptides. Figure 4 compares the relative rates of cholesterol and phospholipid release from cholesterol-enriched L-cells to apo AI, 37pA, and Ac-18A-NH_2. The initial specific activities of cellular cholesterol and phospholipid were used to estimate the mass of lipid which appeared in the medium (Bielicki et al., 1991, 1992). Shown are the estimated mass values for both cholesterol (Figure 4A) and phospholipid (Figure 4B) appearing in the medium for 30 min to 24 h incubations of the

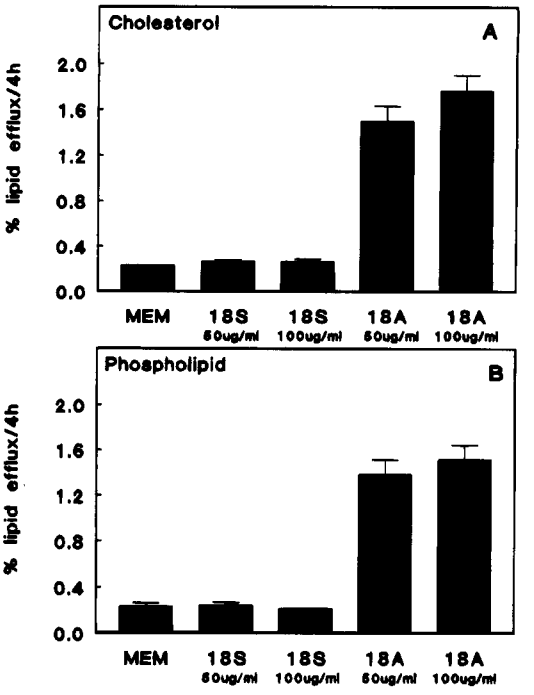

FIGURE 3: Efflux of cholesterol and phospholipid from cholesterol-enriched mouse macrophages to 18A and scrambled 18A. Mouse macrophages were labeled with [^{14}C]cholesterol and [^{3}H]choline, and enriched with cholesterol as described under Materials and Methods. The remaining conditions are those as described for Figure 1. Shown are the percent cholesterol (panel A) and phospholipid (panel B) efflux from macrophages that were incubated for 4 h at 37 °C in MEM−HEPES alone or in the presence of the indicated concentrations of either 18A or scrambled 18A. The efflux data are expressed as the percentage of the initial radiolabeled lipids released from the cells in the presence of the peptides. Values are the mean ± SD for triplicate dishes. The initial cell counts present as [^{14}C]cholesterol and [^{3}H]phospholipid were 19 323 ± 1429 and 1 340 784 ± 42 390 cpm/dish, respectively.

macrophages with the different acceptors. Also shown in Figure 4D,E are the corresponding data for the incubation period of 30 min to 3 h such that the early part of the time course is expanded for better detail. It is particularly important to note that at 30 min of incubation, there was no cholesterol detected in the medium for all the acceptors (Figure 4A,D), but there was significant release of phospholipid (Figure 4B,E). The efflux of cholesterol and phospholipid was greater in the presence of 37pA and Ac-18A-NH_2 than in the presence of apo AI. After 30 min, the release of cholesterol from the cells increased dramatically, and thereafter paralleled the phospholipid efflux. The molar ratio of cellular cholesterol to phospholipid appearing in the medium was dependent on the type of acceptor used (Figure 4C,F). Apo AI was a more potent stimulator of cholesterol efflux than the peptides when the data were normalized to the concentration of phospholipid in the incubation medium. Figure 5 compares the time courses of cholesterol and phospholipid efflux from mouse peritoneal macrophages to apo AI, 37pA, Ac-18A-NH_2, and HDL. In contrast to the L-cells, there was release of cholesterol into the medium as early as 15 min for all the acceptors used (Figure 5A). The cholesterol efflux to apo AI and the peptides was linear over 24 h and was significantly greater than to HDL. As was observed with the L-cells, there was greater release of phospholipid than cholesterol from macrophages to peptides at early time points (Figure 5B). The cholesterol to

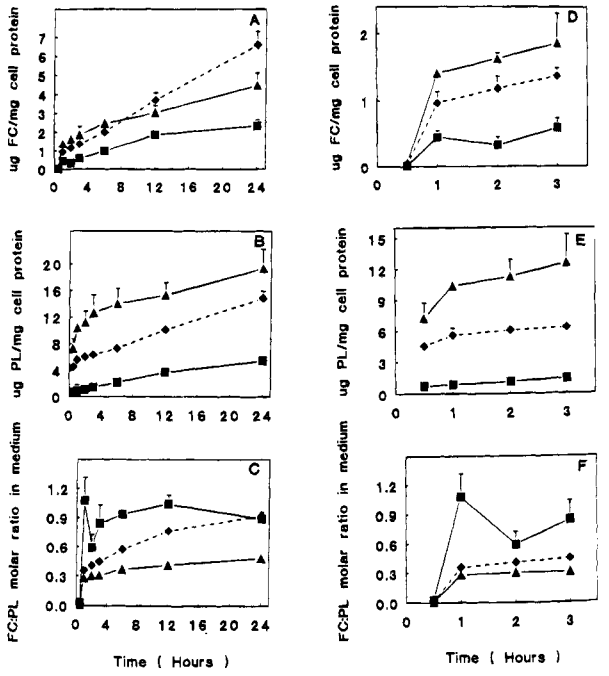

FIGURE 4: Efflux of [^{14}C]cholesterol and [^{3}H]phospholipid from cholesterol-enriched mouse L-cells to lipid-free apo AI, 37pA, and Ac-18A-NH_2. Mouse L-cells were labeled with [^{14}C]cholesterol and [^{3}H]choline, enriched with cholesterol, and washed as described under Materials and Methods. The cells were then incubated for up to 24 h at 37 °C in MEM−HEPES containing 15 μg of protein/mL of one of the following: apo AI (■); 37pA (▲); or Ac-18A-NH_2 (♦). The efflux of cholesterol (panel A) and phospholipid (panel B) mass were calculated using the specific activities of the initial cellular cholesterol and phospholipid. Shown in panel C are the results expressed as the cholesterol to phospholipid molar ratio of the lipids appearing in the medium. Shown in panels D, E, and F are the same data from the time courses shown in panels A, B, and C for the incubation period of 30 min to 3 h so that the early parts of time courses are shown in more detail. Values are the mean ± SD for triplicate dishes. The initial cholesterol and phospholipid contents of the cells were 27 and 167 μg/mg of cell protein, respectively. The initial specific activities of the cell cholesterol and phospholipid were 3925 cpm/μg of cholesterol and 22 367 cpm/μg of phospholipid.

phospholipid molar ratio of the lipids in the medium increased over time when macrophages were incubated with either apo AI or the peptides (Figure 5C). In addition, incubation with apo AI resulted in a higher cholesterol to phospholipid molar ratio of the lipids in the media at all time points in comparison to incubation with the peptides. Shown in Table 4, for the same experiment described in Figure 5, are the total, free, and esterified cholesterol contents of the macrophages before and after the 24 h incubation with the different acceptors. The total cholesterol mass measurements of the cells after the 24 h incubation with the different acceptors follow the same pattern for cholesterol release from the cells that was observed when cholesterol efflux was measured isotopically.

The Effect of Apo AI and Peptides on the Mobilization of Intracellular Cholesterol. The study in Figure 6A,B was conducted to examine whether there are any differences in the abilities of apo AI and peptides to mobilize intracellular cholesterol. Cholesterol-enriched macrophages were incubated for 24 h with either 100 μg of protein/mL or a concentration approximating the EC_{50} for cholesterol efflux of one of the following: apo AI, 37pA, Ac-18A-NH_2, and 18A. The ability of the acceptors to mobilize intracellular

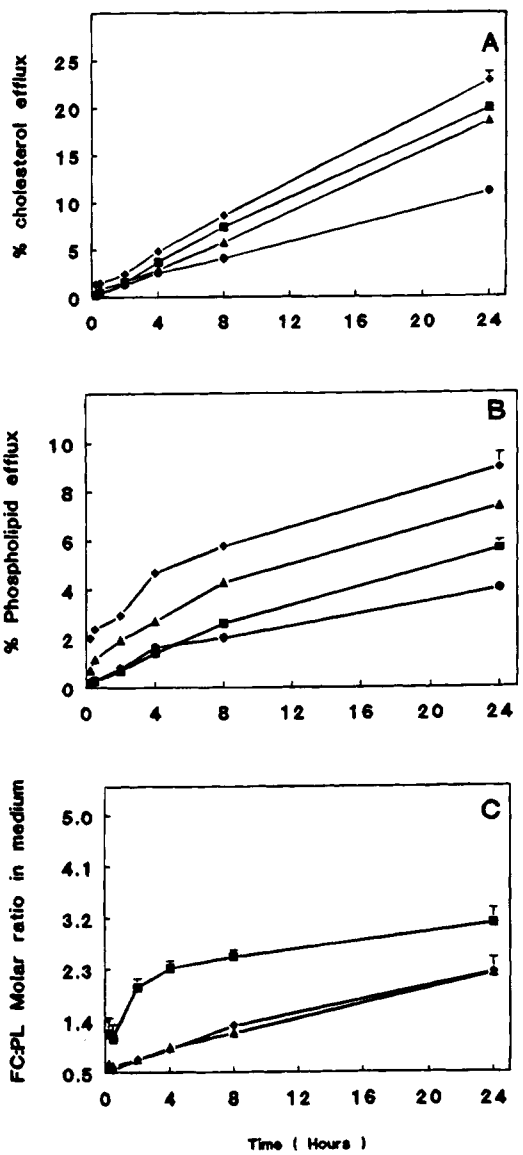

FIGURE 5: Efflux of [^{14}C]cholesterol and [^{3}H]phospholipid from cholesterol-enriched mouse macrophages to apo AI, 37pA, Ac-18A-NH_2, and HDL. Mouse macrophages were labeled with [^{14}C]-cholesterol and [^{3}H]choline, enriched with cholesterol, and the cells were washed as described under Materials and Methods. The cells were then incubated for up to 24 h at 37 °C in MEM−HEPES containing 20 μg of protein/mL of one of the following: apo AI (■); 37pA (▲); Ac-18A-NH_2 (♦); or HDL (●). Shown are the percent cholesterol (panel A) and phospholipid (panel B) efflux. The cholesterol and phospholipid masses were calculated using the specific activities of the initial cellular cholesterol and phospholipid, and shown are the results expressed as the cholesterol to phospholipid molar ratio (panel C) of the lipids appearing in the medium. The values are the mean ± SD for triplicate dishes. The initial free and esterified cholesterol contents were 54 and 33 μg/mg of cell protein, respectively. The phospholipid content of the cells at time zero was 196 μg/mg of cell protein. The cell initial specific activities for cholesterol and phospholipid were 15 554 cpm/μg of cholesterol and 10 182 cpm/μg of phospholipid.

cholesterol was determined by measuring the extent to which intracellular cholesterol was diverted from the cholesteryl ester synthesizing enzyme, ACAT, resulting in a depression of cholesteryl ester synthesis. To measure cholesteryl ester synthesis, [^{3}H]oleate was added to the incubation medium during the last 2 h of the incubation. The relationship between the level of ACAT inhibition and the percent cholesterol efflux in 24 h in the presence of the different

Table 4: Free, Esterified, and Total Cholesterol Mass Remaining in Mouse Macrophages after 24 h Incubation with Apo AI, Peptides, and HDL

	μg of TC/mg of protein	μg of EC/mg of protein	μg of FC/mg of protein
initial	87 ± 3	32.6 ± 2.4	54 ± 4
acceptor[a]			
apo AI	61 ± 3	9.2 ± 2.6	49 ± 2
37pA	62 ± 3	6.9 ± 0.7	56 ± 3
Ac-18A-NH_2	60 ± 2	7.9 ● 2.1	56 ± 4
HDL_3	70 ± 3	12.4 ± 2.4	57 ± 1

[a] Mouse macrophages were cholesterol-enriched and washed as described under Materials and Methods. The cells were then incubated for 24 h at 37 °C in MEM−HEPES containing 20 μg of protein/mL of the indicated acceptors. Shown are the cell total (TC), esterified (EC), and free (FC) cholesterol contents both initially and at the end of the 24 h incubation with the different acceptors.

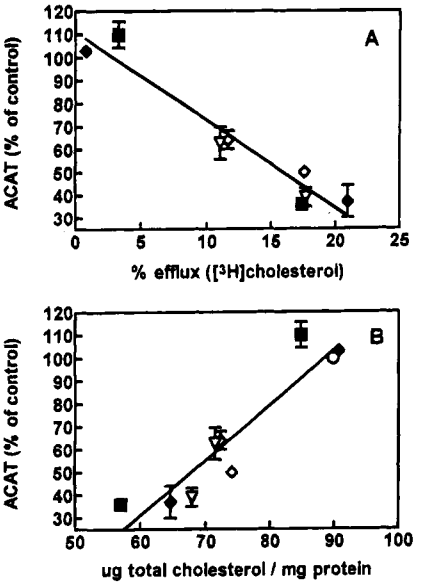

FIGURE 6: Relationship between cellular ACAT activity and cholesterol efflux in cholesterol-enriched macrophages incubated with apo AI, 37pA, Ac-18A-NH_2, and 18A. Mouse macrophages were labeled with [^{14}C]cholesterol and [^{3}H]choline, enriched with cholesterol, and the cells were washed as described under Materials and Methods. The cells were then incubated for 24 h at 37 °C with MEM alone (○) or with high or low concentrations of one of the following: apo AI (1 or 100 μg of protein/mL) (■), 37pA (2 or 100 μg of protein/mL) (△), Ac-18A-NH_2 (5 or 100 μg of protein/mL) (▽), and 18A (20 or 100 μg of protein/mL) (♦). During the last 2 h of the efflux incubation, 31 nmol of [^{3}H]oleate was added to the medium to measure cholesteryl ester synthesis. Shown on the *y*-axis are the cellular cholesteryl [^{3}H]oleate contents expressed as percent of control, where the cells were incubated with MEM alone, plotted against the percent [^{3}H]cholesterol efflux/24 h on the *x*-axis (panel A). There were 7917 ± 1107 [^{3}H]oleate cpm found in the cholesteryl esters of the control cells. Also shown are the cellular cholesteryl [^{3}H]oleate contents expressed as percent of control plotted against the micrograms of total cholesterol per milligram of cell protein remaining in the cells after the 24 h incubation with the different acceptors (panel B). The free and esterified cholesterol contents of the cells incubated with MEM alone were 50 ± 2 and 40 ± 1 μg/mg of cell protein, respectively.

acceptors is shown in Figure 6A. The extent of ACAT inhibition was found to be highly correlated with the amount of cholesterol that was released from the cells ($r = 0.94$). A high correlation ($r = 0.87$) was also observed between the level of ACAT inhibition and the mass of total cholesterol remaining in the cells (Figure 6B). In addition, a high correlation ($r = 0.85$) was observed between the degree of ACAT inhibition and the amount of cholesteryl ester remaining in the cells (data not shown). These observations are consistent with the ACAT activity being controlled by the availability of substrate cholesterol molecules, and indicate that the mobilization of intracellular cholesterol is independent of the apoprotein/peptide-acceptor molecule present in the extracellular medium.

DISCUSSION

The present investigation extends previous studies from this laboratory (Bielicki et al., 1991, 1992) and by Yokoyama and colleagues (Hara & Yokoyama, 1991, 1992; Hara et al., 1992; Komaba et al., 1992; Akira et al., 1993) on the flux of lipids from tissue culture cells exposed to lipid-free apoproteins. These studies have demonstrated that the kinetics and extent of cholesterol and phospholipid efflux are influenced by both acceptor and cell factors.

Factors Affecting the Efficiency of Acceptors. The use of peptides containing amphipathic helical segments that mimic those present in apo AI has given us insight into what structural features of apoproteins mediate phospholipid and cholesterol efflux from cells. First, comparative studies using 18A and scrambled 18A have shown that it is critical for the proteins to contain helices which are amphipathic and thus have the lipid binding characteristics of the natural apoproteins. Second, these studies have shown that peptides that contain only a single amphipathic helix are capable of promoting lipid efflux. This is consistent with our previous studies showing that a mixture of apo C molecules, which are thought to contain two amphipathic helical segments, stimulates lipid efflux from cholesterol-enriched fibroblasts (Bielicki et al., 1992). This is contrary to studies of Hara et al. (1992), who concluded that a minimum of four helical segments were needed for this process to occur based on studies showing that apo CIII and the carboxymethylated monomer of apo AII were not able to stimulate lipid efflux from mouse macrophages. These results are also contrary to the recently published data of Mendez et al. (1994) whose studies indicated that neither 18A nor Ac-18A-NH_2 was able to promote cholesterol and phospholipid efflux from resident mouse macrophages or human skin fibroblasts. The reasons for these discrepancies are not clear at this time.

We can also conclude that the efficiency of the peptides in removing lipid from cells is influenced by the number of amphipathic helical segments per molecule since the efficiency with which the proteins stimulated lipid efflux was 37pA > 18A (see EC_{50} values in Table 3). The fact that apo AI (six or more α-helices) and 37pA (two α-helices) are roughly equally effective in removing cholesterol in this process when compared on a mass basis suggests that helix−helix cooperativity has the most effect on cholesterol uptake when there are just two helices per molecule. It is interesting that per unit mass, apo AI is more effective than 37pA in removing cellular phospholipid; this implies that the interactions of several helices in the apo AI molecule are involved in the removal of phospholipid from the plasma membrane. However, further studies using synthetic peptides containing more than two helical segments of the same type and lipid affinity per molecule will be needed to more accurately examine the role of helix−helix cooperativity. Increasing the length of the amphipathic helix within a molecule also increased the efficiency with which the peptides stimulated

lipid efflux (Ac-18A-NH_2 vs 18A). Together these results suggest that the stimulation of lipid efflux by apoproteins is mediated by protein−lipid interactions. However, the efficiency with which the peptides and apo AI stimulated lipid efflux was not strictly correlated with their lipid binding affinity. The surface activities of the peptides ranking in order from highest to lowest are 37pA > Ac-18A-NH_2 > apo AI > 18A (Anantharamaiah, 1986; Venkatachalapathi et al., 1993). Therefore, other factors besides lipid affinity must contribute to this process.

Cell Factors Influencing Lipid Efflux. Consistent with our previous results using human skin fibroblasts (Bielicki et al., 1992), we observed an enhancement of both phospholipid and cholesterol efflux to lipid-free apo AI from mouse macrophages and L-cells enriched with cholesterol, when compared to control cells (Figure 1). When macrophages were loaded with cholesteryl ester, lipid-free apo AI was effective in stimulating the mobilization of intracellular cholesterol as evidenced by the decreases in cellular cholesteryl ester content and cholesteryl ester synthesis (Table 1, Figure 6). When an ACAT inhibitor was included in the loading medium to enrich macrophages with free cholesterol, the stimulation of cholesterol efflux by apo AI was even more enhanced in comparison to the control cells enriched with cholesteryl ester (Table 1). In addition, the amount of cholesterol efflux was found to be influenced by the degree of free cholesterol loading (Table 1). Taken together, these data suggest that the process of cholesterol efflux to lipid-free apo AI is not directly linked to cellular cholesteryl ester, but rather is free cholesterol-driven and controlled by the level of free cholesterol in the cells. Presumably, the majority of this excess free cholesterol is located in the plasma membrane (Lange & Ramos, 1983), and this enhancement in efflux may be linked to the formation of membrane lipid domains (Schroeder et al., 1991) or defects (Oram et al., 1983) which form upon cholesterol enrichment and cause increased interaction of acceptors with the plasma membrane. Li et al. (1993) have observed differences in the efficiency of cholesterol efflux to apo AI among cell types, with smooth muscle cells being much slower than macrophages, and have speculated that this is due either to differences in plasma membrane free cholesterol content or to efflux occurring from specific pools that are enriched with cholesterol in the plasma membrane. Our studies show that like smooth muscle cells, mouse L-cells do not release cholesterol to apo AI or the peptides as efficiently as the mouse macrophages (Table 3, Figures 4 and 5). Any of these mechanisms could explain differences in the release of cholesterol from cells. Since changes in phospholipid release parallel those of cholesterol efflux for both control cells and cells loaded with cholesterol, proposed mechanisms for the release of cholesterol to apoproteins (see below) need also explain the relationship between cholesterol and phospholipid efflux.

Mechanisms of Lipid Efflux. Two general models can be proposed for the mechanism for the release of cholesterol and phospholipid. First, the efflux of both lipids could occur simultaneously and be mediated by a single event. An example of such a mechanism would be the shedding of a membrane component containing both cholesterol and phospholipid. The second mechanism would involve an independent, but linked, release of cholesterol and phospholipid. Our data are not consistent with a single metabolic event being solely responsible for the release of lipid to apoproteins and peptides for a number of reasons: (1) The cholesterol to phospholipid molar ratio in the medium changes during the incubation of macrophages with the acceptors (Figure 5); (2) the cholesterol to phospholipid ratio is not the same among the different acceptors (Figures 4 and 5); and (3) in the case of the L-cells, there is a rapid initial release of phospholipid without efflux of cholesterol (Figure 4).

(A) Possible Mechanisms of Phospholipid Efflux. If the release of phospholipid and cholesterol is a sequential process, the primary event would be the initial release of the phospholipid with the associated formation of an apolipoprotein/phospholipid particle capable of accepting the cholesterol. There are at least three possible mechanisms by which phospholipid efflux could occur. First, phospholipid efflux could occur by an aqueous diffusion process where phospholipid molecules that desorb from the plasma membrane and diffuse through the aqueous phase are trapped by the lipid-free apoprotein, resulting in the formation of an apoprotein/phospholipid complex; these particles could then function as acceptors of cholesterol. Assuming that 10% of the total cellular phospholipid is present in the plasma membrane (Weinstein & Marsh, 1969), the estimated half-time of phospholipid efflux from the macrophages at high concentrations ($>10^{-6}$ M) of apo AI is 23 h. This rate of release is in the range observed for desorption of phospholipid molecules from model membranes (Phillips et al., 1987), and is consistent with an aqueous diffusion mechanism. However, the aqueous diffusion model predicts that the maximal rate of efflux should be relatively independent of the type of acceptor (Rothblat & Phillips, 1982), and this is not the case in our studies (Figure 2). Also, the half-time of 1.3 h for the efflux of phospholipid when macrophages are exposed to high concentrations of Ac-18A-NH_2 exceeds that expected for an aqueous diffusion process (Phillips et al., 1987). A second possible mechanism for phospholipid efflux is by the transient interaction of the apoprotein/peptide molecules with the plasma membrane, resulting in the formation of a particle. Consistent with this are the observations that both the binding of lipid-free apo AI to cells (Oram et al., 1983) and cellular phospholipid efflux to apo AI are increased (Figure 1) when cells are enriched with cholesterol. Likewise, the apo AI concentration at which binding saturates (Savion & Gamliel, 1988) is in the range of concentrations where a maximal rate of phospholipid efflux occurs. A binding mechanism would also be consistent with the observations that there is a close correlation between efflux efficiency of the peptides and their lipid binding characteristics. It is unlikely that such an interaction would be mediated by binding to a specific apo AI or HDL receptor since the peptides also mediate phospholipid efflux, although they have no amino acid sequence homology to apo AI (Segrest et al., 1992). The third potential mechanism for phospholipid efflux is that the interaction of the apolipoprotein with the cell stimulates the shedding of phospholipid vesicles from phospholipid-rich, cholesterol-poor domains of the plasma membrane (Schroeder et al., 1991). Such a mechanism could explain the very high amount of phospholipid efflux (Figure 2) observed when macrophages are exposed to high concentrations of Ac-18A-NH_2. As discussed below, this excess phospholipid release is not accompanied by an increase in cholesterol efflux. Studies have shown both the presence of multilamellar bodies

in the atherosclerotic lesions (Chao et al., 1988) and the secretion of multilamellar structures by macrophages upon loading with cholesterol by incubation either with acetylated LDL (Robenek & Schmitz, 1988) or with cholesterol crystals (Kruth et al., 1994). A membrane shedding mechanism may only operate with some types of acceptor peptides, and an increased release of adenine from macrophages upon incubation with Ac-18A-NH_2 is consistent with the peptide promoting membrane destabilization.

(B) Cholesterol Efflux. If, as proposed above, there is formation of nascent particles containing apoprotein/peptides and phospholipid that promote the efflux of cholesterol, then the characteristics of cholesterol efflux should be similar to those observed when native lipoproteins or reconstituted acceptor particles are added to the incubation medium. It has been established that an important mechanism for cholesterol efflux to these acceptors involves aqueous diffusion (Phillips et al., 1980). Both the lack of a dependence of the cholesterol efflux rate on acceptor type at high concentrations of acceptor (Figure 2) and the rates of cholesterol efflux observed from macrophages exposed to apo AI or peptides are consistent with an aqueous diffusion process (Phillips et al., 1987). However, there are data in the present studies that are not reconciled by a purely aqueous diffusion process. (1) The rate constant of cholesterol efflux to acceptors via an aqueous diffusion mechanism is not affected by the concentration of cholesterol in the donor cell (Johnson et al., 1988, 1991), but the fractional efflux of cholesterol from mouse macrophages to apo AI is enhanced when the cells are enriched with cholesterol (Figure 1, Table 1). (2) There is saturation of cholesterol release under conditions where a very large pool of peptide and phospholipid is present in the incubation medium (see particularly 18A and Ac-18A-NH_2; Figure 2). Taken together, these findings suggest that the mechanisms for cholesterol efflux to preformed apoprotein/phospholipid acceptors and apolipoproteins are not entirely similar. These differences could be explained if the cholesterol efflux was dependent on the type of phospholipid incorporated into the nascent HDL particles. No differences were found in the distribution of the different choline-labeled phospholipid types appearing in the medium when macrophages and L-cells were incubated for 24 h with either apo AI or Ac-18A-NH_2 (Table 2). Because of the low amount of cellular phospholipid released into the medium at early times, it is difficult to measure the contribution of the different phospholipid subclasses, and thus it is still conceivable that the type of phospholipid released from the cells at early times could influence cholesterol efflux. Likewise, the major type of phospholipid released at early times could differ depending upon the acceptor. These differences could also be reconciled if there was production of inefficient acceptors such as large phospholipid vesicles (Stein & Luzio, 1991) or multilamellar bodies (Schmitz & Muller, 1991) at high concentrations of 18A and Ac-18A-NH_2. Alternatively, as suggested by Li et al. (1993), these results may indicate that the cholesterol being released to apoproteins is originating from a pool of cholesterol distinct from that participating in efflux to HDL or reconstituted particles.

Oram and colleagues (Slotte et al., 1987; Mendez et al., 1994) have proposed that the binding of various acceptors including HDL, apo AI, and the peptide 37pA to a putative HDL receptor protein results in the facilitation of cholesterol efflux via the aqueous diffusion mechanism by effectively mobilizing stores of intracellular cholesterol to the plasma membrane. It is unlikely that an HDL receptor plays a role in cholesterol efflux to lipid-free acceptors. First, as discussed with phospholipid efflux, the peptides, although having no sequence homology to apo AI, were equally effective as apo AI in stimulating cholesterol efflux (Figures 5 and 6). In addition, the ability of the apo AI and peptides to mobilize intracellular cholesterol as judged by the level of ACAT activity was highly correlated with the degree of cholesterol efflux promoted by the acceptors (Figure 6) which in turn corresponds to their lipid binding affinity. Thus, we observe no selective ability of any one peptide to mobilize intracellular cholesterol.

Physiological Significance. It has been proposed by a number of investigators that the availability of lipid-free or lipid-poor apoproteins, particularly apo AI and apo AIV, in the interstitial fluid could play an important role in the initial steps of reverse cholesterol transport (Hara & Yokoyama, 1991). This process could be particularly important if the donor cells were the cholesterol-rich foam cells present in atherosclerotic lesions. In the present study, we have demonstrated, by both mass and isotopic analysis (Table 1, Figure 6), that lipid-free apo AI can participate in the clearance of substantial amounts of cholesterol from cholesterol-loaded macrophages. At equivalent concentrations, the lipid-free acceptors were even more efficient than native HDL in clearing cholesterol from the cells. At higher concentrations (>100 μg of protein/mL), the HDL particle becomes a more efficient acceptor of cholesterol than lipid-free proteins/peptides (Hara & Yokoyama, 1991). This is a reflection of the fact that cholesterol efflux to the lipid-free acceptor saturates, whereas efflux continues to increase as HDL concentrations increase (Brown et al., 1980). However, at the concentrations of HDL and unassociated apoproteins estimated to be present in the interstitial fluid (Roheim et al., 1990), the efflux of cholesterol to the unassociated apoproteins could be as great as that mediated by HDL particles. The EC_{50} (1.3 μg/mL) for cholesterol efflux to apo AI from macrophages is in the concentration range of apo AI reported to be in the interstitial fluid (Roheim et al., 1990). Also, in contrast to what is observed for the efflux of cholesterol to HDL (Johnson et al., 1988, 1991), the fractional efflux of cholesterol to lipid-free apo AI is controlled by the level of cholesterol in the cells. This provides a protective mechanism for the cells to maintain their own basal level of cholesterol. Additional studies, focusing on the spectrum of nascent particles present in the culture medium, will help to resolve both the mechanisms and the physiological significance of apoprotein-stimulated cellular cholesterol and phospholipid efflux.

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