Removal of cellular cholesterol by pre-β-HDL involves plasma membrane microsolubilization

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Abstract **High density lipoprotein (HDL) is able to remove unesterified cholesterol from peripheral cells in the process of reverse cholesterol transport by an aqueous diffusion mechanism as well as by an apolipoprotein (apo)-mediated process. The aqueous diffusion mechanism is understood but the molecular mechanism of lipid-poor pre-β-HDL-(apo-) mediated cholesterol removal is not known. Measurements of the initial rates of efflux of unesterified cholesterol and phospholipid from human fibroblasts to lipid-free, human apoA-I showed that both lipids are released from the cells during a 10-min incubation with apoA-I. The concentration-dependence of efflux of the lipids is the same (K_m = 0.4 and 0.6 μg apoA-I/ml for cholesterol and phospholipid flux, respectively), suggesting a membrane microsolubilization process. A finite pool of about 1% of the plasma membrane cholesterol is accessible for release by solubilization; the limited size of this cholesterol pool is not due to a lack of availability of apoA-I, but rather to the restricted amount of phospholipid that is removed from the plasma membrane. Plasma membrane domains may be involved in membrane microsolubilization, but caveolar cholesterol seems not to be specifically accessed in this process. Membrane microsolubilization is the process by which pre-β1-HDL removes cell cholesterol in the first step of reverse cholesterol transport. When apoA-I is present in the extracellular space, the relative contributions of cholesterol efflux by membrane microsolubilization and by aqueous diffusion are determined by the degree of lipidation of the apoA-I molecules.—**Gillotte, K. L., W. S. Davidson, S. Lund-Katz, G. H. Rothblat, and M. C. Phillips. **Removal of cellular cholesterol by pre-β-HDL involves plasma membrane microsolubilization.** ***J. Lipid Res.*** **1998.** 39: **1918–1928.**

Supplementary key words apolipoprotein A-I • high density lipoprotein • cellular cholesterol efflux

High density lipoproteins (HDL) mediate the process of reverse cholesterol transport (RCT) in which excess peripheral cholesterol is carried to sites of metabolism (1, 2). This lipoprotein class is heterogeneous in nature and consists of subspecies that are defined on the basis of their apolipoprotein and lipid composition, charge, shape, and size (3, 4). For instance, HDL species containing only apolipoprotein (apo) A-I (Lp A-I) or both apoA-I and apoA-II (Lp A-I + A-II) behave differently as substrates for lecithin:cholesterol acyltransferase (LCAT) (5). In the last decade, attention has been drawn to a lipid-poor subspecies of HDL, pre-β1-HDL. Pre-β1-HDL contains apoA-I as its sole protein moiety and is understood to be an efficient initial acceptor of cellular unesterified (free) cholesterol (FC), in the first step of the RCT course (6–10). There is some suggestion that the lipid-poor pre-β1-HDL may act as a shuttle of FC from the plasma membrane to fully lipidated HDL species (10–12). However, at this point, the mechanisms giving rise to the proficiency of this HDL species in stimulating the efflux of plasma membrane FC in the initial stages of RCT are not known.

Cholesterol efflux from the cell plasma membrane to lipidated apoA-I, such as mature HDL_3 particles, occurs by the so-called aqueous diffusion mechanism (2, 13, 14). In this process, FC molecules desorb from the plasma membrane, diffuse through the extracellular aqueous phase, and incorporate into lipidated acceptors encountered by collision (for a review, see ref. 13). This diffusional mechanism is well defined and it can contribute significantly to FC efflux from cells. The presence of scavenger receptor type B class I (SR-BI) in the plasma membrane of cells can facilitate efflux of FC to HDL (15). Several studies have described that under particular conditions leading to a reduction in particle size, apoA-I molecules can dissociate

Abbreviations: apo, apolipoprotein; CETP, cholesteryl ester transfer protein; FBS, fetal bovine serum; FC, free (unesterified) cholesterol; GLC, gas–liquid chromatography; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; MEM, minimal essential medium; PBS, phosphate-buffered salt solution; PL, phospholipid; POPC, 1-palmitoyl, 2-oleoyl phosphatidylcholine; RCT, reverse cholesterol transport; rHDL, reconstituted HDL; TLC, thin-layer chromatography.

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from mature HDL particles (16–19) to create a pool of pre-β-HDL. When human apoA-I molecules are either lipid-free or complexed only with zwitterionic phospholipids (e.g., phosphatidylcholine) they exhibit pre-β mobility on agarose gel electrophoresis but, due to a change in conformation, apoA-I molecules exhibit α-mobility when present on spherical HDL particles containing a neutral lipid core (20). ApoA-I molecules in the incompletely lipidated pre-β-HDL state can acquire cellular lipid components to generate further lipoproteins (8, 21, 22). This process represents a second component of cellular FC efflux, particularly mediated by apoA-I in a lipid-free (poor) state (14, 22).

In contrast to the situation with efflux of cellular FC to fully lipidated apoA-I particles where the aqueous diffusion mechanism is largely understood, the mechanism of efflux mediated by incompletely lipidated apolipoprotein molecules is not well defined. It is known that lipid-free apolipoproteins access both cellular FC and phospholipid (PL) (23–27). Although various mechanisms of simultaneous or sequential removal of these lipids have been proposed, this aspect of the pathway has not been precisely investigated. Furthermore, the location of the cellular FC and PL available for efflux to apoA-I has not been defined. For instance, specific FC-rich regions of the human fibroblast membrane, termed caveolae, have been implicated as playing a role in cellular FC transport and efflux to mature HDL species and pre-β-HDL (28, 29). In addition, it has been suggested that transport of intracellular cholesterol is an important determinant in apoA-I-mediated FC efflux from cholesterol-enriched fibroblasts (30). These fundamental aspects of apoA-I-mediated FC efflux need to be understood so that the molecular mechanism of the overall process can be defined.

In the current study, the relative contributions of fully lipidated and incompletely lipidated apoA-I molecules in the first step of reverse cholesterol transport are compared. Experiments were directed at defining the mechanism by which lipid-free (poor) apoA-I molecules, which comprise the pre-β1-HDL pool, access human fibroblast cellular FC. Results indicate that apoA-I causes efflux of cellular FC by a "membrane microsolubilization" process in which interaction of the apolipoprotein with the plasma membrane removes cellular cholesterol and phospholipid simultaneously. ApoA-I solubilizes a limited kinetic pool of FC whose structural identity remains to be established.

MATERIALS

Cholesterol, cholesteryl methyl ether, sodium cholate, and streptokinase were purchased from Sigma (St. Louis, MO). 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) was purchased from Avanti Polar Lipids (Birmingham, AL) (+99% grade). Okadaic acid (*Prorocentrum concavum*) was purchased from Calbiochem (La Jolla, CA). [1,2-^{3}H]cholesterol (43.5 Ci/mmol) and [methyl-^{3}H]choline chloride (81 Ci/mmol) were obtained from DuPont NEN (Boston, MA) and [8-^{3}H]adenine (54 mCi/mmol) was obtained from Amersham Life Science (Arlington Heights, IL). Minimal essential medium and phosphate-buffered saline (PBS) were purchased from BioWhittaker (Walkersville, MD). Bovine calf and fetal serum and trypsin/EDTA were supplied by Life Technologies, Inc. (Grand Island, NY). All media were supplemented with 50 μg/ml gentamycin (Sigma). All other reagents were analytical grade.

METHODS

Preparation of plasma

Blood for the preparation of plasma from fasting normolipidemic subjects was collected into ice-cooled plastic tubes containing 150 IU/ml streptokinase as an anticoagulant (9). The plasma was obtained by centrifugation (2000 *g*, 30 min, 0°C); samples were maintained on ice no longer than 30 min before use in a cellular experiment. For specific experiments, a portion of the plasma was preincubated for 90 min at 37°C to reduce the level of preβ-1-HDL present (10).

Preparation of lipoproteins

HDL_3 and LDL were isolated from fresh plasma obtained from normolipidemic donors by sequential ultracentrifugation as previously described (31). Prior to use, the isolated lipoproteins were dialyzed extensively against Tris buffer (10 mm Tris, 150 mm NaCl, 1.0 mm EDTA; pH 8.2).

Purification of apoA-I

Human HDL isolated from the fresh plasma of normolipidemic subjects was delipidated in ethanol–diethyl ether (32). Purified apoA-I was isolated by anion exchange chromatography on Q-Sepharose (33) and stored in lyophilized form at −70°C. Prior to use, the purified apolipoprotein was resolubilized in 6 m guanidine HCl and dialyzed extensively against Tris buffer, (10 mm Tris, 150 mm NaCl, 1.0 mm EDTA; pH 8.2).

Preparation of reconstituted HDL (rHDL)

Particles were prepared using the cholate dispersion/Bio-Bead removal technique as described in detail previously (34). A starting POPC/apoA-I ratio of 100:1 (mol:mol) was utilized and final compositions were determined after concentration (Centriprep 30, Amicon) under low speed centrifugation. The complexes were purified and characterized as described in detail previously (35).

Preparation of cell monolayers

Normal human skin GM3468A fibroblasts (passages <30) were plated in 22-mm, 12-well plates and grown to confluence in minimal essential medium/bicarbonate (MEM) supplemented with 10% fetal calf serum in a 37°C humidified incubator (95% air, 5% CO_2). Upon reaching confluence after 4 days, the cells were labeled with either 10–20 μCi/ml [^{3}H]cholesterol diluted in MEM supplemented with 1% fetal calf serum or 15 μCi/ml [^{3}H]choline chloride diluted in MEM with 1% fetal calf serum for 48–72 h. In the above preparations, the radioactive material dissolved in ethanol was dried under nitrogen and resolubilized in 50 μl ethanol before adding the media to ensure that the ethanol concentration was always less than 1% (v/v).

Efflux of cellular cholesterol (36)

After washing the cell monolayers with MEM containing HEPES (50 mm) (MEM-HEPES) (3×), unesterified (free) cholesterol (FC) efflux measurements were initiated by the application of 1.0 ml/well of the test medium, consisting of either 100% human plasma or the acceptor diluted to the desired protein concentration in MEM-HEPES. The experiments were con-

ducted in a covered 37°C water bath and all media were supplemented with HEPES. The radioactivity in an aliquot of the medium was determined by liquid scintillation counting at specific time intervals to estimate the fraction of FC released into the medium; any cellular material was removed prior to counting by filtration of the medium through a 0.45-μm filter. Upon completion of the timecourse, all cell wells were washed with phosphate-buffered salt solution (PBS) (BioWhittaker) three times and the cellular lipids were extracted with isopropanol (37). From the extracted lipids, the total amount of radioactive FC per well was measured by liquid scintillation counting and FC mass was also assayed.

For experiments investigating the effect of replating the radiolabeled fibroblasts on FC efflux, the procedure was slightly modified. Human fibroblasts were grown to confluence in T-75 tissue culture flasks and radiolabeled with 2 μCi/ml [^{3}H]cholesterol for 48 h. The cells were washed once with MEM, then removed by a 3-min incubation with 500 μl trypsin-EDTA (0.05% trypsin w/v, 0.53 mm EDTA in Hanks' balanced salt solution) at 37°C. The released cells were diluted with 6.5 ml MEM supplemented with 2% FBS; suspended cells from two T-75 flasks were combined and replated in a 22-mm tissue culture plate. After incubation at 37°C for an additional 24 h, measurement of cholesterol efflux was performed as described above.

In some experiments, the caveolar regions of the monolayers were selectively enriched with cholesterol by exchange of FC from low density lipoprotein (LDL) as described previously by Fielding and Fielding (29). After extensive dialysis of human LDL against Tris buffer (10 mm Tris, 150 mm NaCl, 1.0 mm EDTA; pH 8.2), the LDL FC was radiolabeled. The FC component of the LDL was radiolabeled by exchange of [^{3}H]FC from acid-washed Celite (38) during a 37°C overnight incubation. After this incubation period, the LDL was filtered to remove the Celite and aliquots were taken for both radioactivity and cholesterol mass determination (specific activity = 1.37 x 10^4 cpm/μg FC). The radiolabeled LDL present at 270 μg FC/ml in MEM-HEPES was incubated for 1 h in a covered 37°C water bath with human fibroblast monolayers grown to confluence in 6-well plates. After this incubation, the cells were washed 3× with 2 ml MEM-HEPES/well and 1 ml of unlabeled LDL was incubated with the cells at 270 μg FC/ml for 15 min to displace any surface-bound radioactive LDL particles (29). At this point, the monolayers were again washed 3× with 2 ml MEM-HEPES/well and 2 ml of a cholesterol acceptor was applied for FC efflux measurements.

To determine the activation energy of FC efflux to 50 μg/ml of either apoA-I or POPC:apoA-I rHDL complexes, FC efflux from human fibroblasts was measured at 4°, 15°, 25°, and 37°C. Care was taken to ensure that all washes and treatments were at the appropriate temperature. The activation energies were derived from the slope of the linear regression of the data, plotted according to the Arrhenius equation.

Efflux of cellular phospholipid

After washing the [^{3}H]choline-labeled monolayers with MEM-HEPES (3×), PL efflux measurements were initiated as described for FC efflux. An aliquot of the medium was removed at specific time intervals and filtered through a 0.45-μm filter; 10 μg of butylated hydroxytoluene was then added to prevent the oxidation of PL. The lipids were extracted from the sample by treatment with chloroform–methanol 1:1 (v/v) (39), then the aqueous phase was aspirated and the chloroform phase was washed 3× with methanol–water 10:9 (v/v). The chloroform phase was dried under nitrogen in liquid scintillation vials and radioactivity was quantitated by liquid scintillation counting. Upon completion of the timecourse, all cell wells were washed with PBS 3× and the cellular lipids were extracted with isopropanol containing 10 μg butylated hydroxytoluene/ml. The extracts were dried under nitrogen and the PL was separated from free [^{3}H]choline using the extraction protocol described above. From the extraction, the total amount of radioactive PL per well was determined as well as the mass of PL (40).

Data analysis

The fractional release of FC was determined experimentally and analyzed as described previously for this system (36). Fractional PL efflux was assessed in the same manner. The kinetic analysis was based on the assumption that the system is closed and that all lipid therefore exists in either the cellular lipid pool or the acceptor pool. The cellular cholesterol pool exists primarily in the FC state as cholesterol esterification is negligible in the human fibroblast cells. The equilibration of FC or PL between the lipid and acceptor pools was fitted to the mono-exponential rate equation $Y = Ae^{-Bt} + E$ where Y represents the fraction of radiolabeled lipid remaining in the cells, t is the incubation time, A is a pre-exponential term that reflects the fraction of lipid that exists in the medium at equilibrium, B is a time constant characteristic of the release of FC or PL, and E is a constant that represents the fraction of labeled lipid that remains associated with the cells at equilibrium. These variables were derived by fitting the experimental data to the equation by nonlinear regression (Graph Pad Prism, Graph Pad Software Incorporated). The apparent rate constant for efflux (k_e) was derived from these parameters. The half time of efflux value in hours was then calculated as follows: $t_{1/2} = \ln 2/k_e$. The computed half time values were statistically compared by Student's *t*-test (Graph Pad Prism, Graph Pad Software Incorporated).

Modification of the state of caveolar plasma membrane domains

Radiolabeled human skin fibroblasts were treated with okadaic acid, a phosphatase inhibitor which has been shown to eliminate the presence of plasma membrane caveolae (41). The monolayers were treated with MEM-HEPES ± 1 μm of the inhibitor for 1 h at 37°C as described previously for this cell system (29). This treatment medium was then aspirated and the procedure for measurement of cholesterol efflux was carried out as described above.

Efflux of cellular adenine

The leakage of [^{3}H]adenine was used to monitor cell injury (42). Upon reaching confluence, the monolayers were incubated with MEM supplemented with 0.3% bovine serum albumin and containing 1 μCi/ml [8-^{3}H]adenine. After the uptake of [^{3}H]adenine into the cellular ATP pool for 4 h, the cells were washed with MEM-HEPES and the experiment was initiated by incubation of 1 ml of the treatment medium with the cells at 37°C. The radioactivity of an aliquot of the medium was determined at various time intervals to estimate adenine released into the medium. The total amount of cellular [^{3}H]adenine incorporated into the fibroblast monolayers was determined by lysing the cells with 5% Triton X-100 and measuring the radioactivity in an aliquot by liquid scintillation counting.

HPLC analysis of free cholesterol in the medium

The FC that appeared in the media exposed to the radiolabeled cell monolayers was analyzed on a Isco model 2360 HPLC using a reverse phase C18 column (23 cm) at a flow rate of 1.0 ml/min. One hundred μL of efflux medium was extracted (39) and the resulting lipids were reconstituted in an acetonitrile–isopropanol 3:1 (v/v) mixture, applied to the column, and eluted with a gradient of acetonitrile–isopropanol ranging from 3:1 to 2:3 (v/v) over 50 min. The FC mass was monitored by the absor-

bance at 210 nm and associated radioactivity was monitored by a flow-through scintillation counter. The retention times of the fibroblast [^{3}H]-FC appearing in the medium were compared to purified FC standards that had not been exposed to cells.

Analytical methods

Protein concentration was determined by a modification of the Lowry assay (43). Phospholipid mass was determined as inorganic phosphorus by the method of Sokoloff and Rothblat (40). The mass of choline-containing phospholipid was determined by an enzymatic assay (Wako). Total and free cholesterol contents of the cellular lipid extracts were quantified by gas–liquid chromatography using cholesteryl methyl ether as an internal standard as described previously (44).

RESULTS

Definition of rapidly released FC pools in human fibroblasts

Experiments investigating the pathway of pre-β-HDL-mediated cellular FC efflux have commonly involved incubation of 100% human plasma with human skin fibroblasts containing basal levels of FC (7, 9). As pre-β1-HDL is proposed to be an important initial acceptor of cellular FC (7), such experiments have been designed to focus on the flux occurring during the first few minutes of incubation. Data in **Fig. 1** and **Table 1** demonstrate the FC efflux measured during this timeframe to 100% plasma from several human normolipidemic subjects. Initial rates of efflux are 15 ± 5 ng/min and 7 ± 2 ng/min for fresh and preincubated plasma, respectively. Fitting the timecourses to a mono-exponential rate equation (see Methods) demonstrates that, during this timeframe, about 5% of the total cellular FC is released ($t_{1/2} \cong 2$ h) to fresh plasma. To determine the contribution of pre-β1-HDL to this process, plasma was preincubated for 90 min at 37°C, to deplete the plasma pre-β1-HDL pool as described by Miida et al. (10), before incubation with the fibroblast cells. As shown in Fig. 1, there is an approximately 50% reduction in FC efflux; this is consistent with the results published previously by Kawano and colleagues (9) and shows that the pre-β1-HDL species contributes significantly to the initial efflux of FC from fibroblasts.

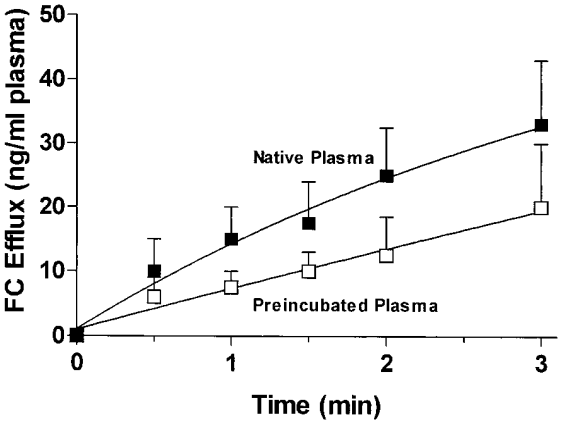

Fig. 1. Timecourses of free cholesterol efflux from human fibroblast monolayers to human native plasma and preincubated plasma. Human fibroblasts trace-labeled with [^{3}H]free cholesterol in 22-mm tissue culture wells as described in Methods were incubated with 1 ml test medium consisting of either 100% human plasma (■) or plasma preincubated (□) (90 min, 37°C) to reduce the pre-β1-HDL content. The experiment was conducted in a 37°C water bath, and at specific timepoints from 0–3 min an aliquot of plasma was removed, filtered, and analyzed by liquid scintillation counting. The FC efflux (ng FC/ml plasma) was calculated using the initial specific activity of the cellular FC (240 cpm/ng FC). Each point represents the mean FC efflux from triplicate measurements and the error bars represent 1 SD; curves were determined through fitting the data to a mono-exponential rate equation. All measurements have been corrected for cholesterol efflux to MEM-HEPES as control.

TABLE 1. Comparison of cholesterol efflux characteristics of various plasma samples

Donor[a]	Fractional Efflux[b] 1 min	Fractional Efflux[b] 3 min	Halftime of Efflux[c]	Pool Size[c]
			h	*% cell FC*
1	0.005	0.014	2.3	5.0
2	0.005	0.017	1.9	11.3
3	0.004	0.012	1.8	2.1
4	0.007	0.011	1.9	1.9
Avg ± SD	0.005 ± 0.001	0.014 ± 0.003	2.0 ± 0.2	5.0 ± 4.4

[a] Plasma was obtained from fasting normolipemic volunteers as described in Methods.

[b] Values represent the mean of three experiments measuring the fraction of cell [^{3}H]cholesterol in the plasma at 1 min or 3 min as described in detail in Methods. SD for each mean was approximately 10–20%.

[c] Halftimes and pool size were derived by fitting the experimental timecourse to a mono-exponential rate equation as described in Methods.

These experiments involve efflux of a very small pool of cellular FC and the potential for artifacts is high. Therefore, the following control experiments were conducted. Radiolabeled sterols in the plasma after cell exposure for 1 and 5 min were compared by high performance liquid chromatography to a purified FC standard. The distribution of radioactivity indicated that effectively 100% of the radiolabeled species transferred to the plasma was FC and not a more polar metabolite. Dislodged radiolabeled fibroblasts did not contribute to the rapid initial FC efflux, as the collected aliquots of media were passed through a 0.45-μm filter to remove any floating cells before analysis by liquid scintillation counting. In addition, to ensure that the rapid release of FC did not arise from leaky or damaged cells, [^{3}H]adenine release from the cell ATP pool was monitored (42). The results from these experiments indicated that the human fibroblast cell line is resistant to toxic effects imposed by plasma treatment for incubations of up to 1 h, and thus cell toxicity is not a factor in the observance of a rapidly released pool of FC. Table 1 demonstrates that the fast pool is not a result of an unusual component in certain plasma samples, because this FC pool was consistently identified with plasma samples from several individuals. Additionally, when radiolabeled cells were trypsinized and replated (see Methods), the kinetics of FC release were not affected (data not shown). This result confirms that the measurements followed cellular FC

movement and were not influenced by excess FC adhering to either the tissue culture plates or the cell surface.

Figure 2 demonstrates that intact plasma is not essential for efflux of the fast pool of FC in fibroblasts. Isolated HDL_3 at its concentration in normal plasma (45) gives rise to identical kinetics of FC efflux during these short incubation experiments. This result suggests that pre-β1-HDL is not the only HDL subspecies able to access the FC pool of interest; however, it should be noted that if some apoA-I dissociated from the mature HDL_3 (17, 46), then some pre-β1-HDL could have been generated. This ability of an HDL_3 incubation to release the fast pool of FC simplifies the system and allows the comparison of short versus long incubation FC efflux kinetics to further characterize the cell FC pool involved; 100% human plasma is toxic to the fibroblast cell monolayers when incubations of longer than 1 h are performed. In a timecourse ranging from 0 to 10 min, 4% of the cellular FC is rapidly released to either plasma or HDL_3 with a $t_{1/2}$ of 1.9 ± 1.3 h. The remainder of the FC is available when incubations are extended to 10 h, although the $t_{1/2}$ of 9.9 ± 0.5 h is longer. From these results it can be concluded that human fibroblast FC is organized into multiple kinetic pools.

The plasma experiments depicted in Fig. 1 indicate that the fast pool of FC is accessed by the HDL subspecies, pre-β1-HDL, while the experiments in Fig. 2 indicate that HDL_3 is also involved. To further investigate the mechanism of FC efflux to pre-β-HDL we have utilized apoA-I as an acceptor, as the pre-β1-HDL species in human plasma have been described as lipid-free (poor) apoA-I (8, 18). It is virtually impossible to investigate the efficiency of pre-β1-HDL in a plasma system such as that in Fig. 1, as other species such as mature HDL, LDL, and albumin contribute to the net efflux, even at early timepoints (data not shown). Furthermore, the added effects of multiple plasma components also complicate any comparison of the kinetics for pre-β1-HDL in plasma to those for "pure" pre-β1-HDL (apoA-I) in MEM. It follows that the mechanism by which pre-β1-HDL removes cellular FC can be studied more clearly by using lipid-free apoA-I as an acceptor.

ApoA-I-mediated membrane microsolubilization

It is recognized that lipid-free apoA-I removes cellular FC by a mechanism distinct from the aqueous diffusion pathway by which lipidated apoA-I accepts FC (for reviews, see refs. 8, 14). Further support for two separate processes is apparent upon investigation of the temperature-dependence of FC efflux mediated by either lipidated or lipid-free apoA-I (**Fig. 3**). The activation energies (E_a) are 12 kcal/mol and 5 kcal/mol for POPC:apoA-I reconstituted HDL (rHDL) and apoA-I, respectively. The E_a for efflux of FC from cell monolayers by the aqueous diffusion mechanism is reported to be 12 kcal/mol (13) which is similar to the value observed for the rHDL discoidal complex. Overall, the results clearly demonstrate that the rate-limiting step for efflux to lipidated and unlipidated apoA-I is different because the E_a values are different.

Additional investigation of the apoA-I-mediated pathway of cell FC efflux distinctly illustrates that a limited pool of FC is accessible in this process. **Figure 4** represents

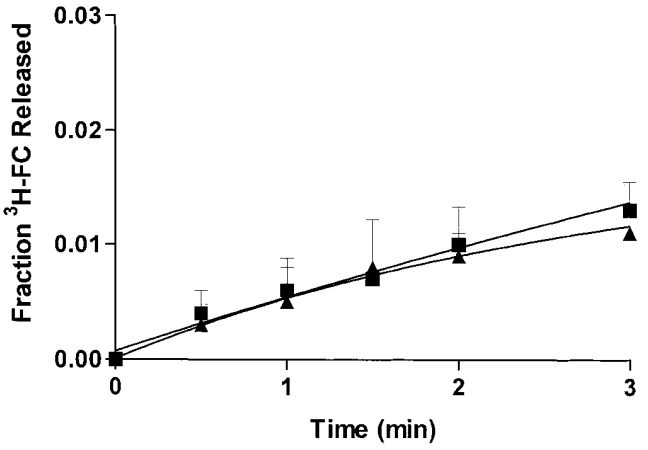

Fig. 2. Timecourses of free cholesterol efflux from human fibroblast monolayers to human plasma and HDL_3. Monolayers were prepared as described in the legend of Fig 1. The incubation treatments were 100% human plasma (■) or MEM-HEPES containing HDL_3 present at its physiological concentration in normal human plasma (▲) (1.6 mg apoA-I/ml). The vertical axis indicates the fraction of labeled free cholesterol released from the cells at specific times. Each point represents the mean cholesterol efflux determined from triplicate measurements of two separate experiments, while the error bars indicate 1 SD. Curves were obtained by fitting the data to a mono-exponential rate equation as described in Methods. All measurements have been corrected for cholesterol efflux to MEM-HEPES as control.

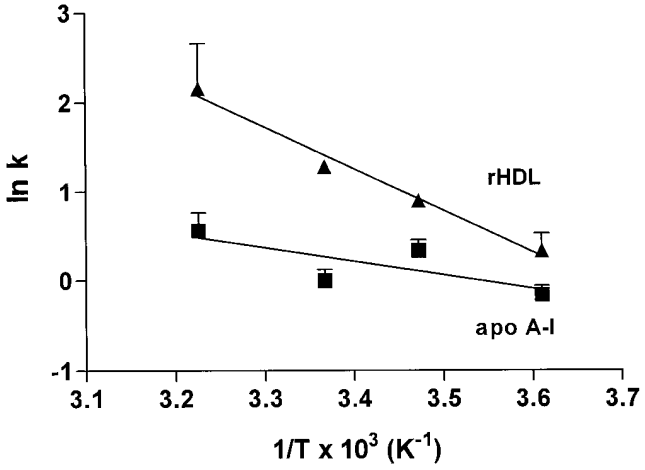

Fig. 3. Temperature-dependence of the efflux of cholesterol from human fibroblast monolayers to lipidated and lipid-free apoA-I. Monolayers grown to confluence in 22-mm tissue culture wells were trace-labeled with [^{3}H]cholesterol as described in Methods. Lipid-free apoA-I or apoA-I complexed with POPC as rHDL discoidal complexes (100:1 (mol:mol), PL:apoA-I) at concentrations of 50 μg protein/ml were incubated with the cell monolayers. The incubations were carried out at 4°, 15°, 25°, and 37°C; at 10 min, 2 h, and 4 h an aliquot was removed, filtered, and analyzed for [^{3}H]cholesterol content by liquid scintillation counting. Results were similar at all timepoints; the rate constants for efflux using the 4 h data are plotted according to the Arrhenius equation. Symbols are the mean of triplicate measurements ±1 SD. All measurements have been corrected for cholesterol efflux to MEM-HEPES as control.

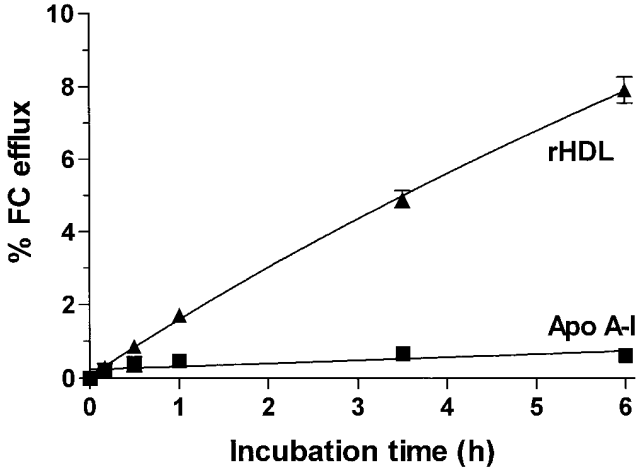

Fig. 4. Timecourses of free cholesterol efflux from human skin fibroblasts to lipidated and lipid-free apoA-I. Normal human skin fibroblasts were grown to confluence in 36-mm tissue culture wells and trace labeled with [^{3}H]free cholesterol in 1% fetal bovine serum. The radiolabeled monolayers were incubated at 37°C with 2 ml of MEM-HEPES containing 50 μg protein/ml of either human apoAI (■) or rHDL discoidal POPC:apoAI complexes (▲) for periods ranging from 0–6 h. At specific timepoints an aliquot was removed, filtered, and analyzed for [^{3}H]cholesterol content by liquid scintillation counting. Symbols represent the mean % FC released measured from triplicate wells ±1 SD; data are representative of that observed in two independent experiments. All values have been corrected for flux measured to MEM-HEPES alone.

a 6-h timecourse of FC efflux from the fibroblast monolayers to 50 μg/ml of apoA-I either in a lipid-free state or complexed with POPC as a discoidal rHDL particle. Fitting of the data to a mono-exponential rate equation indicates that 0.6 ± 0.5% of the cellular FC is accessible to apoA-I, while over 25% of the FC is available for release to the discoidal complexes at this concentration. ApoA-I very rapidly accesses this pool of FC as the halftime of efflux is 0.4 ± 0.2 h. The size of the pool accessed by lipid-free apoA-I over the course of experiments with several fibroblast monolayers ranged from 0.6 to 1.5% of the cellular FC. The contribution of the cell FC pool accessed by lipid-free apoA-I to discoidal rHDL-mediated FC efflux is not clear; the initial rates of efflux to either lipidated or lipid-free apoA-I are the same, yet the rHDL continues to remove a large percentage of the plasma membrane FC by the aqueous diffusion mechanism (13) with a $t_{1/2}$ of 11.3 ± 1 h.

Previous studies have demonstrated clearly that lipid-free apolipoproteins access both cellular FC and PL during incubations of 4–24 h (23, 24, 26). Furthermore, examination of apoA-I-mediated FC and PL efflux from cholesterol-enriched mouse macrophages indicated that both lipids are released during a 0.5-h incubation (25). These observations are consistent with apoA-I removing cellular FC by a "membrane microsolubilization" mechanism. This process involves apolipoprotein-mediated solubilization into the extracellular medium of a small amount of plasma membrane PL and FC; the mechanism is facilitated by the interaction of apolipoprotein with the plasma membrane and occurs without obvious cellular damage.

To address the question of whether PL is accessed prior to FC removal or whether the PL and FC are removed simultaneously, the monolayers were radiolabeled to high specific activity in parallel with either [^{3}H]cholesterol or [^{3}H]choline chloride to monitor the FC or PL movement at early timepoints. From the timecourses in **Fig. 5**, it is clear that efflux of both FC and PL occurs during a 10-min timecourse. Furthermore, the initial rates of release are similar (0.16 ± 0.03 pmol/min and 0.12 ± 0.03 pmol/min for FC and PL release, respectively), suggesting that the processes are linked. This apoA-I-induced simultaneous release of FC and PL was also observed upon incubation of apoA-I with monolayers dual-labeled with [^{14}C]FC and [^{3}H]PL for 10 min (data not shown). Investigation of the concentration-dependence of FC and PL efflux to apoA-I lends further support to the idea of coordinate release of the lipids (**Fig. 6**). The similar K_m values of 0.4 ± 0.2 μg/ml and 0.6 ± 0.3 μg/ml for FC and PL efflux, respectively, also support this conclusion.

As was observed for apoA-I-mediated FC release, there appears to be a rather small pool of PL available for rapid efflux. Figure 6 shows that even at the highest concentration of 100 μg apoA-I/ml, only 0.8 ± 0.1% of the cellular PL is removed. The limited amount of FC and PL removed by apoA-I is apparently not a result of deficiency of acceptor, because a saturating concentration (50 μg/ml) of apolipoprotein was present in all incubations (Fig. 6).

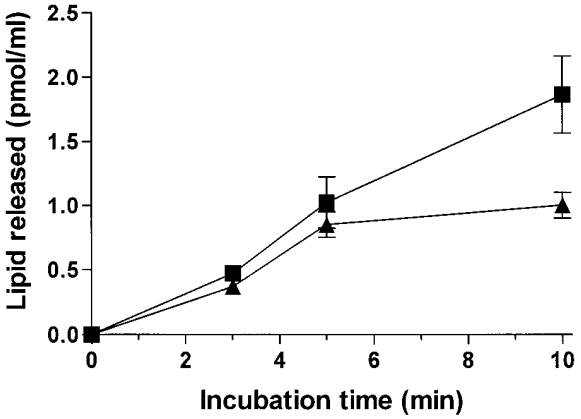

Fig. 5. Cholesterol and phospholipid efflux from human skin fibroblast monolayers to apoA-I. The cell monolayers were radiolabeled with either [^{3}H]cholesterol or [^{3}H]choline as described in Methods. One ml of MEM-HEPES containing 50 μg apoA-I/ml was incubated in parallel with cells radiolabeled with either cholesterol or choline. The plates were incubated in a 37°C water bath and, at specific timepoints from 0 to 10 min, an aliquot was removed and filtered. Media exposed to [^{3}H]cholesterol-labeled cells were directly analyzed for [^{3}H]cholesterol content by liquid scintillation counting, whereas the media exposed to [^{3}H]choline-labeled cells was first extracted as described in Methods. Lipid mass released was determined from the specific activities of the monolayers at time zero (FC = 4.33 × 10^3 cpm/pmol, PL = 2.16 × 10^3 cpm/pmol); FC mass was determined by GLC and choline-containing PL mass was determined by an enzymatic assay. Metabolism of PL was assumed not to alter the specific activity in the 10 min experiment. The symbols represent the mean mass (n = 3) of FC (■) or PL (▲) released from the monolayers ±1 SD and all data have been corrected for FC or PL release to MEM-HEPES in the absence of apoAI.

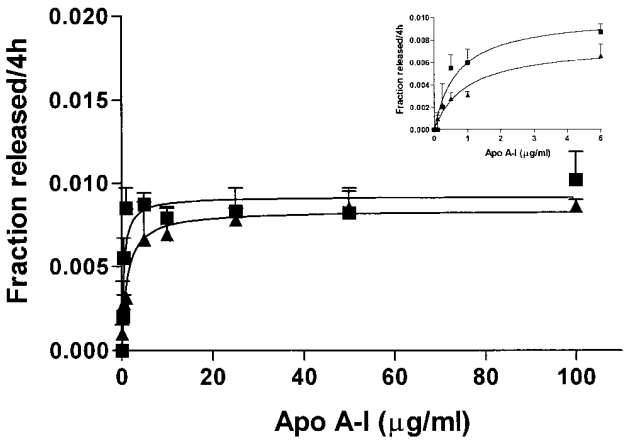

Fig. 6. Concentration-dependence of cholesterol and phospholipid efflux from human fibroblast cells to apoA-I. The experiments were conducted as described in the legend to Fig. 5 except that the concentrations of apoA-I in the MEM-HEPES ranged from 0.1 to 100 μg/ml. The symbols represent the mean ± 1 SD of triplicate measurements of the fraction of cholesterol (■) or phospholipid (▲) released from the monolayers at 4 h; all data have been corrected for cholesterol or phospholipid release to MEM-HEPES in the absence of apoAI. Curves were fitted by the Michaelis-Menten equation; K_m values of FC and PL efflux are 0.4 ± 0.2 μg/ml and 0.6 ± 0.3 μg/ml, respectively. The inset is an enlargement of the low concentration range.

Furthermore, replenishing the lipid-free apoA-I in the extracellular medium did not increase the limited FC pool released from the cells. When three sequential fresh apoA-I preparations were incubated with radiolabeled fibroblast monolayers for 4 h timecourses, a decrease in FC efflux was observed with each subsequent application of protein. During the first 4-h incubation, 0.9 ± 0.1% of the cellular FC was released. The following two 4-h incubations of apoA-I with the monolayers resulted in the release of 0.5 ± 0.1% and 0.1 ± 0.3%, respectively. Overall, only 1.5% of the cellular FC was accessed, identical to the percentage of FC obtained upon a 12-h incubation with no apoA-I replenishment (1.3 ± 0.3%). Furthermore, doubling the concentration of apoA-I by addition of 50 μg to 50 μg/ml of apoA-I that was incubated with the cells for 8 h, led to no further FC efflux (data not shown). These results taken together demonstrate that the factor limiting the amount of apoA-I-mediated membrane microsolubilization is a characteristic of the cell and not of the available apoA-I.

Role of caveolae in membrane microsolubilization

Caveolar domains within the plasma membrane have been proposed to contain 6–7% of the plasma membrane FC (47). Therefore, it is possible that the limited amount of FC accessed by apoA-I is located within these domains (29). Several experiments were performed to explore this possibility.

Okadaic acid has been demonstrated to eliminate the caveolar domains of the fibroblast membrane (29). Pretreatment of the monolayers with this phosphatase inhibitor does not affect the ability of apoA-I to access cellular FC (**Fig. 7**). The rate of FC efflux determined from the curve fitting is 0.18 ± 0.05 h^{-1} for microsolubilization of the membrane treated in the presence or absence of okadaic acid, indicating that caveolar FC is not solely accessed in this process. Treatment with okadaic acid also does not adversely affect HDL_3 or rHDL-mediated FC efflux (data not shown).

We attempted to use methods that have been proposed for specifically enriching the caveolae with FC (29), because a larger FC pool would ease the investigation of the role of this domain in membrane microsolubilization. We found that selective uptake of FC from LDL led to an encouraging increase to 22% in the size of the pool released to HDL_3 in a 2-h incubation under defined conditions; this is consistent with results published previously (29). To confirm that residual LDL stuck to the cells did not contribute to this pool, LDL dual-labeled with [^{125}]I-labeled apoprotein (to track the particle) and [^{3}H]FC (to monitor FC movement) was utilized to selectively increase the caveolar FC content. Despite an extensive washing procedure, about 0.08% of the dual-labeled LDL particles remained associated with the cells. During a 6-h incubation to monitor FC efflux to HDL, 34 ± 4% of the cell-associated ^{125}I label appeared in the media. This release of LDL particles contributes to the measured FC efflux as approximately 6% of the cell-associated FC is a component of these species. These results indicate that the use of LDL to selectively enrich caveolae with FC is prone to artifact because of the adherence of LDL particles to the cell surface.

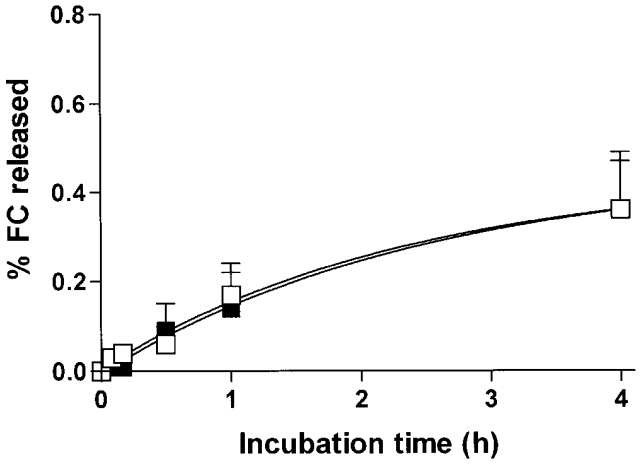

Fig. 7. Effect of okadaic acid treatment on apoA-I-mediated membrane microsolubilization. Monolayers of human skin fibroblasts grown to confluence in 22-mm tissue culture wells were trace-labeled with [^{3}H]cholesterol as described in Methods. The prepared monolayers were incubated at 37°C for 1 h with MEM in the presence or absence of 1 μm okadaic acid. ApoA-I was then incubated at a concentration of 50 μg/ml in MEM-HEPES with the monolayers; at specific timepoints ranging from 5 min to 4 h, aliquots were removed, filtered, and analyzed by liquid scintillation counting to determine the percentage of FC released. Symbols represent the mean % (±1 SD) FC released from triplicate wells from either okadaic acid-treated (□) or control (■) monolayers. Curves were obtained through fitting the data to a mono-exponential rate equation as described in Methods. All data have been corrected for FC release to MEM-HEPES alone.

DISCUSSION

Pre-β-HDL-mediated membrane microsolubilization

This study describes the phenomenon in which pre-β-HDL particles rapidly associate with cellular FC. It is known that the majority of apoA-I in vivo exists in a lipidated state, associated with mature HDL particles. However, roughly 5% of the apoA-I exists in a lipid-free (poor) form in human plasma as a result of dissociation from the lipoprotein (8, 16, 18); this temporarily lipid-depleted form of apoA-I has pre-β mobility on agarose gel electrophoresis (20). An incompletely lipidated apoA-I molecule, existing in a flexible conformation or molten globular state (48), can readily associate with lipids because of its amphipathic α-helical segments (49). The lipidation probably occurs by progressive interaction of 22-residue α-helical domains with PL (50). The abilities of apoA-I molecules in either the lipid-free or partially lipidated states (i.e., in pre-β1-HDL) to participate in membrane microsolubilization are presumably similar. In both cases, the amphipathic α-helices are in an unstable state (because of incomplete hydrophobic interaction with other nonpolar moieties) and have the capacity to interact with more PL and eventually form larger discoidal HDL particles. Through such assemblage with cellular PL and FC, the lipid-free (poor) apoA-I species comprising the pre-β1-HDL pool in plasma progressively expand in lipid content and size (51). This process explains the apparent transformation of apoA-I-containing pre-β1-HDL lipid-poor species to α-HDL fully lipidated species (11); it is apparent that apoA-I-mediated efflux initiates the flux of FC through this pathway (17, 22).

It is clear from prior work, that the interaction of incompletely lipidated apoA-I with cell membranes leads to the efflux of both FC and PL (24, 25, 51). However, the previous studies with human fibroblasts have involved lengthy incubations of apoA-I with the monolayers (4–24 h) and there has been some question as to whether the lipids are released simultaneously or sequentially, with the PL-apoA-I association preceding the FC incorporation. Through radiolabeling the FC or PL of the fibroblast monolayers to high specific activity, we have been able to measure initial rates of efflux and establish the sequence of events. From inspection of the efflux of lipids during the first 10 min of incubation of apoA-I with human skin fibroblast monolayers, as represented in Fig. 5, it is clear that FC and PL are released simultaneously. Furthermore, the identical concentration-dependence of PL and FC efflux indicates that the processes are linked (Fig. 6). Therefore, efflux mediated by incompletely lipidated apoA-I or pre-β1-HDL occurs by the simultaneous solubilization of FC and PL from the plasma membrane of cells. This membrane microsolubilization process is a consequence of the ability of apoA-I molecules to bind lipid (49) and explains how cellular FC rapidly associates with pre-β-migrating species of HDL. In this study it should be noted that the fibroblasts were not enriched in cholesterol so that the cell conditions were the same when membrane microsolubilization was caused by either plasma (Figs. 1, 2) or lipid-free apoA-I (Figs. 3–6). It is interesting that the rate of microsolubilization of plasma membrane FC and PL by apoA-I is enhanced when the cells are cholesterol-enriched (14, 24–26). However, the underlying mechanism of membrane microsolubilization is apparently independent of the level of cholesterol in fibroblasts (K. L. Gillotte, S. Lund-Katz, G. H. Rothblat, and M. C. Phillips, unpublished results).

Examination of the concentration-dependence of FC efflux mediated by membrane microsolubilization indicates that approximately 5 μg/ml apoA-I is necessary to achieve maximal FC release (Fig. 6). We have used a concentration several-fold in excess of this amount (50 μg/ml) as this concentration of apoA-I is similar to that expected to be present in a lipid-free (poor) state in vivo. Despite the excess of acceptor, only 1–2% of the FC present in the cell became associated with apoA-I by solubilization of the human fibroblast plasma membrane. Further addition of protein to the incubation mixture or refreshment of the treatment medium did not lead to additional efflux of FC. It is striking that when the same concentration of apoA-I was present in a lipidated state as rHDL much more cell FC was released by aqueous diffusion (Fig. 4) so the limitation is not apparent when PL is present in the extracellular medium to solubilize the FC (i.e., act as a sink (12, 52)). This requirement for PL is consistent with experiments examining FC efflux from skin fibroblasts from individuals with Tangier disease (14, 53, 54). ApoA-I exhibits a dramatic decrease in ability to remove PL from this cell type as compared to normal human skin fibroblasts, resulting in negligible FC efflux (53). These findings are compatible with a microsolubilization mechanism that is dependent upon the availability of a specific pool of plasma membrane PL and FC. Distinct membrane domains have been implicated as being involved in intracellular membrane sorting, signal transduction (55), and cellular FC efflux (29, 56) and influx (28); such a domain may be preferentially accessed in apoA-I-mediated membrane microsolubilization.

It has been suggested that caveolae are involved in the cellular transport of FC (57). As these FC-enriched domains comprise approximately 6% of the total plasma membrane FC (47), specific interaction of apoA-I with the caveolar regions was considered. The data in Fig. 7 do not suggest any distinct preference of apoA-I for caveolar lipids as compared to lipids from the plasma membrane in general. This result may not be unexpected because FC molecules in caveolae can partition into other regions of the plasma membrane on the timescale of 10 min (58). The role of other detergent-resistant membrane domains (55, 59) in apoA-I-mediated FC efflux is uncertain at this point.

The role of membrane proteins (especially those that can interact with apoA-I) in the membrane microsolubilization process is not clear at this time. However, although SR-B1 binds HDL, the presence of this receptor does not seem to affect the removal of plasma membrane lipids by lipid-free apoA-I (15). The solubilization of membrane FC and PL may occur through an interaction of the amphi-

pathic α-helices of apoA-I with either a specific lipid domain or with a cell surface protein/receptor or both. Thus, the location of the limited pool of FC and PL available for efflux by membrane microsolubilization and the role of additional plasma membrane components remain to be elucidated.

Physiological relevance of membrane microsolubilization

It is clear from recent studies that apoA-I plays a central role in the process of RCT (for reviews see refs. 8, 14). In the initial stages of the pathway, apoA-I mediates cellular FC efflux by one of two distinct mechanisms. The first involves cholesterol diffusion from the plasma membrane to phospholipid-containing acceptor particles, while the second requires direct interaction of apolipoproteins with the plasma membrane. When the apoA-I is present in a fully lipidated state, such as in an HDL_3 particle, FC efflux occurs by the aqueous diffusion process (13). In this process of cholesterol removal from cells, the function of apoA-I is primarily as a stabilizing component of the HDL species, with minimal interaction with the plasma membrane. It should be noted that when the apoA-I molecules are associated with a full complement of PL molecules, there cannot be net removal of cell PL mass but rather there can be exchange of PL molecules between the plasma membrane and acceptor pools. In contrast, as described in the current work, when apoA-I is in a lipid-free (poor) state, such as the pre-β1-HDL species, FC and PL associate with the protein by means of microsolubilization of plasma membrane lipids. These two components of cellular FC efflux are schematically represented in **Fig. 8**.

The relative contributions of the two pathways of cellular FC efflux depicted in Fig. 8 to the overall RCT process are likely to vary with the local extracellular conditions. Clearly, the contribution of aqueous diffusion versus membrane microsolubilization relies on the proportion of apoA-I present in a lipid-free (poor) form. When LCAT activity is high, esterification of the HDL FC results in the overall enlargement of HDL particle size (60). These effects stimulate the direct incorporation of any incompletely lipidated apoA-I molecules into the HDL particles to provide surface coverage (61). In addition, a gradient of FC concentration between the plasma membrane and HDL is maintained, thereby promoting net FC efflux by diffusion (62, 63). Therefore, as summarized in Fig. 8, when local LCAT activity is high, the apoA-I exists primarily in the fully lipidated state and the aqueous diffusion mechanism mediates FC efflux. Enzymatic modification of the lipidated species by cholesteryl ester transfer protein (CETP) or hepatic lipase leads to a decrease in particle size and the shedding of lipid-free apoA-I molecules (17, 64). In this situation, the contribution of efflux by aqueous diffusion will be diminished in favor of increased membrane microsolubilization by the incompletely lipidated apoA-I.

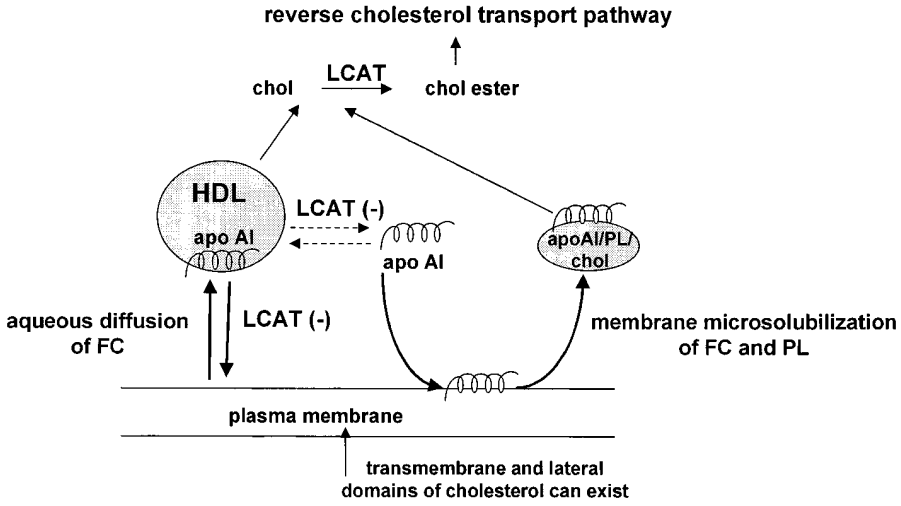

Fig. 8. The molecular mechanisms of apoA-I-mediated cellular cholesterol efflux. This model depicts the two possible mechanisms of efflux of cellular FC to apoA-I. When apoA-I exists in a fully lipidated state, movement of FC molecules between the plasma membrane and spherical HDL particles (α-migrating on agarose gel electrophoresis) occurs by the aqueous diffusion mechanism. In contrast, when apoA-I dissociates from the mature HDL particles to a lipid-free form to create a pool of pre-β1-migrating HDL on agarose gel electrophoresis, FC is incorporated into pre-β1-HDL by the simultaneous solubilization of plasma membrane FC and PL. Both of these mechanisms of cholesterol movement contribute to the reverse cholesterol transport pathway; the relative contribution of the pathways depends upon the degree of lipidation of apoA-I. Various enzymes influence this process and the effect of LCAT is indicated in the cartoon; the arrows marked LCAT(−) indicate the directions of the equilibria that are promoted by reduced LCAT activity. A low local activity of LCAT does not enhance the aqueous diffusion pathway because the concentration gradient of FC between the plasma membrane and HDL is not maintained. In contrast, low LCAT activity favors the membrane microsolubilization pathway by increasing the size of the free apoA-I pool. Refer to the text for further detail.

In summary, as Fig. 8 demonstrates, both the aqueous diffusion and membrane microsolubilization pathways contribute to the RCT pathway. The relative contribution of either apoA-I-mediated route is a result of the local environment and is subject to constant change. Outstanding questions include determination of the nature of the interaction of lipid-free (poor) apoA-I with the plasma membrane as well as the structural requirements of the apolipoprotein in this process.

This work was supported by NIH Program Project HL22633 and Training Grant HL07443, and pre-doctoral fellowships to W. S. Davidson and K. L. Gillotte from the American Heart Association, Southeastern Pennsylvania Affiliate. We thank Faye Baldwin, Sheila Benowitz, and Margaret Nickel for expert technical assistance.

Manuscript received 19 February 1998, in revised form 27 April 1998, and in re-revised form 22 June 1998.

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