Specific Phospholipid Association with Apolipoprotein A-I Stimulates Cholesterol Efflux from Human Fibroblasts

STUDIES WITH RECONSTITUTED SONICATED LIPOPROTEINS

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To understand how the lipid composition of high density lipoprotein mediates the efflux of cellular cholesterol, we have characterized the effects of variations in the lipid composition of well defined model sonicated apolipoprotein A-I (apoA-I)-containing lipoprotein (LpA-I) particle on cholesterol efflux from cultured human skin fibroblasts. LpA-I particles with varying content of phosphatidylcholine (POPC), phosphatidylsitol, sphingomyelin, cholesterol ester, and triolein were prepared by co-sonication. Association of as little as 5 mol of phosphatidylcholine with apoA-I is sufficient to transform lipid-free apoA-I into a distinct lipoprotein-like particle that is a significantly better acceptor of cellular cholesterol. Increasing the ratio of POPC/ apoA-I from 5/1 to 35/1 in the sonicated LpA-I is associated with a significant increase in the release of cellular cholesterol. At low POPC/apoA-I ratios, native gradient gel electrophoresis of the LpA-I shows these lipoproteins to be small complexes (around 5–6 nm), with only 1 molecule of apoA-I (Lp1A-I). At a POPC/ apoA-I ratio above 11/1, LpA-I form well defined complexes that contain 2 molecules of apoA-I (Lp2A-I) and range in size from 7.6 to 7.7 nm. Inclusion of sphingomyelin into an Lp1A-I further stimulates cholesterol efflux significantly. In contrast, inclusion of either sphingomyelin or phosphatidylsitol into a sonicated Lp2A-I has no effect on cholesterol efflux. Incorporation of cholesterol ester and/or triolein into an Lp2A-I particle is associated with a small reduction in cholesterol efflux to these lipoproteins. Therefore, cholesterol efflux from human fibroblasts is directly proportional to the amount and type of phospholipid in a sonicated LpA-I particle. Changes in the conformation and charge of apoA-I that result from changes in the lipid composition of a sonicated LpA-I particle appear to directly affect the ability of the lipoprotein to bind and retain cholesterol molecules. These data therefore suggest that the adsorption/desorption of cholesterol molecules to/from a sonicated LpA-I complex may be less sensitive to interfacial lipid-lipid interactions, but may depend on a conformation-dependent ability of apoA-I to bind cholesterol.

HDL\(^1\) are a highly heterogeneous class of lipoproteins of various origins formed during the catabolism of triglyceride-rich lipoproteins and the synthesis and secretion of nascent HDL particles by the liver or intestine (Banerjee and Redman, 1983; Eisenberg, 1984; McCall et al., 1988, 1989; Castle et al., 1991; Thrift et al., 1986). Recent studies have suggested that HDL may also be generated by the stepwise lipidation of apoA-I by acquisition of lipids from other lipoproteins (Hussain et al., 1989) or from extracellular (Hara and Yokoyama, 1991, 1992; Bielicki et al., 1991, 1992; Forte et al., 1993). Several lines of evidence suggest that HDL may be assembled extracellularly. First, apoA-I, the predominant apolipoprotein of HDL, appears to be secreted by the liver and the intestine mainly in the lipid free form; however, only about 3% of this apolipoprotein is present in plasma as lipid-free form (Neary and Gowland, 1987). Additionally, lipid-free apoA-I is able to release both phospholipid and cholesterol from extracellular HDLs (Bielicki et al., 1992; Hara and Yokoyama, 1991). Finally, the incubation of lipid-free apoA-I with Chinese hamster ovary cells in serum-free medium generates LpA-I with a gradual size increment (Forte et al., 1993). A subspecies of small particles (7.3 nm) composed of 94% apoA-I and 6% phospholipids has also been identified in these studies, which appears analogous both in composition and size to the human plasma pre-β-HDL reported earlier (Kunitake et al., 1985) and may be analogous to the particles reported to be highly active in cellular cholesterol efflux (Castro and Fielding, 1988).

When lipid-free apoA-I is incubated with extracellular cells, the incorporation of phospholipids into apoA-I does not seem to parallel that of cholesterol as evidenced by the following. (i) The acquisition of phospholipid and cholesterol by human apoA-I from isolated microsomal membrane (Nunez and Swaney, 1984) as well as from intact human fibroblasts or mouse microphages (Li et al., 1993; Li and Yokoyama, 1995; Beliecki et al., 1992; Yancey et al., 1995) is non-stoichiometric; (ii) phospholipid efflux to lipid-free apoA-I precedes and facilitates the efflux of cholesterol (Yancey et al., 1995); (iii) association of phospholipids with apoHDL greatly increases its ability to release cellular cholesterol compared to the delipidated apo-HDL (Stein and Stein, 1973), while treatment of HDL with either phospholipase A\(_2\) or heparin-releasable rat hepatic lipase reduced cholesterol efflux (Johnson et al., 1986). Therefore, it appears that the formation of apoA-I-phospholipid complexes is an important preliminary step before apoA-I can

\(^1\) The abbreviations used are: HDL, high density lipoprotein; apoA-I, apolipoprotein A-I; LpA-I, apoA-I-containing lipoprotein; Lp2A-I, LpA-I containing 2 apoA-I-particle; PC, phosphatidylcholine; POPC, 1-palmitoyl 2-oleoyl phosphatidylcholine; PI, phosphatidylinositol; SM, sphingomyelin; UC, free cholesterol; CE, cholesteryl linoleate; TG, triolein; DMEM, Dulbecco’s modified Eagle’s medium; NDGGE, non-denaturing gradient gel electrophoresis.

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significantly promote the efflux of cellular cholesterol.

However, there is up to now no direct evidence for a relationship between the progressive lipidation of apoA-I and the ability of the corresponding complexes to release cellular cholesterol in short term incubations, and the corresponding changes in certain physical parameters assumed by the apoA-I-lipid complexes during their progressive lipidation. We have attempted to answer these questions by preparation of model complexes generated in vitro by co-sorption of phospholipid and apolipoprotein as described previously (Hirz and Scanu, 1986; Sparks et al., 1996a). We first prepared a series of reconstituted sonicated LpA-I complexes with varying 1-palmitoyl 2-oleoyl phosphatidylcholine (POPC)/apoA-I ratios in order to answer the above questions. In addition, two other series of POPC-apoA-I complexes have also been prepared to investigate the contribution of HDL lipid composition, specifically surface phospholipid components, sphingomyelin (SM) and phosphati-
dylinositol (PI), or core neutral lipids, cholesterol ester (CE) and triolein (TG), to cellular cholesterol efflux. These studies were carried out using reconstituted LpA-I designed to mimic the high affinity acceptor, pre-
β, LpA-I, which has been identified in whole plasma and lymph (Fielding and Fielding, 1995), and to investigate the effect of progressive incorporation of different lipids into apoA-I on its function in cellular cholesterol efflux.

EXPERIMENTAL PROCEDURES

Materials

POPC, bovine brain SM, and bovine liver PI were purchased from Avanti Polar Lipids Inc. (Birmingham, AL). TG, CE, free cholesterol (UC), and essentially fatty acid-free bovine serum albumin were obtained from Sigma. 1–2
m
HCholesterol, long-chain
H[i-]
PHphosphatidylinositol (1
H[PI]) and choline methyl-
H[4C]phosphatidylcholine (1
H[4C]SM) with specific specific activities of 52, 11, and 50 mCi/mmol, respectively, were obtained from Du Pont Canada Inc. (Mississauga, Canada). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, β-
glucamine, and penicillin-streptomycin used for cell culture were purchased from Life Technologies, Inc. All other reagents were analytical grade.

Methods

Isolation of HDL and Purification of Apolipoprotein A-I—HDL was isolated by standard sequential ultracentrifugation from fresh plasma obtained from normalplasmadonorbloodsmachmandPuppione,1986).The delipidation of HDL and purification of apoA-I was performed as described previously (Breuer et al., 1986).

Preparation of Reconstituted Sonicated Lipid/ApoA-I Complexes—A series of POPC-apoA-I complexes were prepared by a method described previously (Sparks et al., 1996b). Briefly, POPC in chloroform was dried under nitrogen in glass tubes. 900 µl of reconstitution buffer containing 10 mM Tris, 150 mM NaCl, 0.01% EDTA, 1 mM NaF, (pH 8.0) was then added to the tubes and vortexed vigorously for 3 min to resuspend the POPC. The mixture was sonicated for 1 min at 100% duty cycle using a Branson 450 sonicator with a one-eighth-inch tapered microtip probe and an output control setting at 3 (manufacturer rated output of 40 watts), then incubated in a water bath at 37°C for 30 min, followed by sonication at 95% duty cycle for 5 min. All sonifications were performed in 12 × 75-mm test tubes in a 15°C water bath and under nitrogen. ApoA-I at a concentration of 1.4 mg/ml was added to the tubes, and sonicated again for 4 × 1 min at 90% duty cycle. The resulting mixtures were passed through a 0.22-µm filter, and then resolubilized by size exclusion chromatography on a Superose-6 column. For the preparation of sonicated spherical LpA-I with UC, SM, PI, CE, and/or TG, these components were mixed (at appropriate concentrations) with POPC and then processed as described for the POPC-apoA-I complexes. As control and for comparison with other model lipoproteins, reconstituted discoidal LpA-I were prepared in the presence of cholate as described previously by Sparks et al. (1992a) from an initial mixture of POPC-apoA-I at a molar ratio of 40/1.

Characterization of Reconstituted LpA-I Particles—Non-denaturing gradient gel electrophoresis (NDGGE) was carried out on precast gel (8–25%) using the Phast system (Pharmacia Biotech Inc.) to assess the homogeneity of LpA-I, and to estimate their Stoke’s diameters calculated from a quadratic equation, derived from polynomial regression of the Stoke’s diameters versus the migration distances of five standard proteins (high Mr standard, Pharmacia Biotech Inc.) (Nichols et al., 1986). The electrophoretic mobilities and surface potentials of LpA-I were determined by electrophoresis on 0.6% agarose gels (Beckman, Paragon Lipo kit) and calculated as described previously (Sparks and Phillips, 1992). The n-helix content of apoA-I in LpA-I was determined by circular dichroism (CD) spectroscopy at 222 nm (Sparks et al., 1992b). ApoA-I number in each reconstituted LpA-I particle was estimated by cross-linking of apolipoproteins with dimethyl suberimidate (Swaney, 1986). The protein concentration was determined by the Lowry method (Lowry et al., 1951) and free cholesterol, total cholesterol, and phospholipid concentrations were measured using commercial enzymatic test kits (Boehringer Mannheim GmbH, Mannheim, Germany). The PI and SM contents of LpA-I were determined by inclusion in representative preparations of [3H]PI or [3C]SM.

Cell Labeling and the Efflux of Cellular Cholesterol from Human Skin Fibroblasts—Normal human skin fibroblasts were purchased from Clonetics Inc. at the 9th passage, and maintained in a standard condition and used for efflux studies between the 16th and 22th passages. The conditions for seeding and labeling of the cells and for the study of cellular cholesterol efflux were described previously (Zhao and Marcel, 1996).

RESULTS

Characterization of LpA-I Complexes Prepared by Co-sorption—Co-sorption of POPC and apoA-I results in well defined POPC-apoA-I complexes, wherein the POPC content can be changed by varying the POPC/apoA-I ratio in the initial mixture. POPC-apoA-I complexes are separated from the lipid-free apoA-I and from the non-incorporated lipid by size exclusion chromatography. As shown in Fig. 1, when the initial molar ratio of POPC/apoA-I is below 20/1, the purified POPC-apoA-I complexes appear as well defined particles on gradient acryl-

FIG. 1. Non-denaturing gradient gel electrophoresis of sonicated POPC/apoA-I complexes with progressive increase of POPC content. POPC-apoA-I complexes were prepared by sonication from mixtures of POPC/apoA-I at molar ratios of 5:1, 10:1, 20:1, 30:1, and 60:1, and named, respectively, S1, S2, S3, S4, and SS. As a control and for comparison with other studies, we included a discoidal Lp2A-I made by dispersion in the presence of cholate (D1, initial POPC/apoA-I molar ratio of 40:1). After purification by gel filtration, the complexes were electrophoresed (1 µg of protein/lane) on precast NDGGE (8–25%) and stained with Coomasie Blue. The lane labeled as STD contains the standard proteins: thyroglobulin, apoferritin, catalase, lactate dehydrogenase, and albumin.
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Results are the averages of at least three preparations.

Table I

| LpA-I  | POPC/apoA-I Molar Ratio (Initial) | POPC/apoA-I Molar Ratio (Final) | ApoA-I | POPC | Size | α-Helix | Surface Potential |
|--------|----------------------------------|----------------------------------|--------|------|------|---------|------------------|
|        | mol/LpA-I | nm | % | mV |        |        |        |                  |
| ApoA-I | 0 :1 | 47 | 8.3 |        |        |        |                  |
| S1     | 5:1 | 2.4 | 48 | 10.0 |        |        |                  |
| S2     | 10:1| 5.0 | 49 | 10.0 |        |        |                  |
| S3     | 20:1| 7.6 | 51 | 9.6  |        |        |                  |
| S4     | 30:1| 32.4| 53 | 9.4  |        |        |                  |
| S5     | 60:1| 71.0| 59 | 9.2  |        |        |                  |
| D1     | 40:1| 79.0| 55 | 7.8  |        |        |                  |

*The POPC/apoA-I molar ratios of the initial mixtures for the preparation of LpA-I.
*The POPC/apoA-I molar ratios of reconstituted LpA-I after re-isolation (S.D. < 5%).
*Estimated by protein cross-linking with dimethyl suberimidate and subsequent SDS-polyacrylamide gel electrophoresis.
*Total phospholipid content of each reconstituted LpA-I particle.
*Hydrodynamic diameters of LpA-I determined from NDGGE (± 0.5 nm S.D.). Particles for which size is not given are either heterogeneous or had a size outside of the standard range.
*Calculated from the electrophoretic migration of LpA-I on agarose gel (± 0.2 mV S.D.).
*A discoidal Lp2A-I prepared by cholate dialysis was included for comparison with results presented elsewhere.

The presence of cholate (Bergeron et al., 1988; Francone and Fielding, 1990) is required to form the homogeneous discoidal LpA-I prepared in the presence of cholate (discoidal LpA-I), which tends to decrease with an increase of POPC/apoA-I ratio (S9, Table II), which are Lp1A-I particles with a size similar to its phospholipid to apoA-I molar ratio (Fig. 2B), which can slightly affect the determination of particle size (Table II). We have also generated a species of SM-containing complexes with a low phospholipid/apoA-I ratio (S9, Table II), which are Lp1A-I particles with a size similar to its POPC/apoA-I counterpart (less than 6 nm, S3, Table I) and an electrophoretic mobility within the pre-β range (Fig. 2B).

Four subspecies of sonicated LpA-I containing CE and/or TG were made as indicated in Table II. Three moles of UC, 6 mol of CE, and/or 6 mol of TG were added to POPC-apoA-I mixtures with an initial molar ratio of 60/1, a ratio that we have shown to generate homogeneous LpA-I particles. Compared to the LpA-I particles containing only POPC and apoA-I, the presence of UC, CE, and/or TG did not affect the homogeneity of these sonicated LpA-I particles as demonstrated by NDGGE. The presence of UC slightly reduced the level of POPC that could be incorporated into apoA-I; however, at the molar ratios used here, the presence of TG and especially that of CE appeared to increase POPC incorporation into LpA-I. TG and CE alone could be very efficiently incorporated into LpA-I, with TG having an incorporation efficiency close to 100%. In addition, the presence of CE did not modify either the size of LpA-I particles (Table II), or their electrophoretic mobility on agarose gel (Fig. 2B). The presence of TG alone or TG and CE together did not alter the size of these particles (Table II); however, their electrophoretic mobility was slightly increased with both CE and TG (Fig. 2B).

**Cellular Cholesterol Efflux from Fibroblasts to LpA-I Complexes with Varying POPC/ApoA-I Ratios**—In contrast to Lp2A-I prepared in the presence of cholate (discoidal LpA-I), where variations in POPC content have no effect on the ability of these lipoproteins to accept cholesterol, an increase in the POPC content of sonicated POPC-apoA-I complexes significantly enhanced their ability to promote the efflux of cellular cholesterol (Fig. 3). Within the first 90 min of incubation, the efflux followed the biphasic pattern usually observed with fibroblasts (Zhao and Marcel, 1996). The stimulating effect of the phospholipid content of the LpA-I complexes could also be observed in both phases. The second phase of cellular cholesterol efflux to these sonicated LpA-I complexes was linear and positively related to the phospholipid levels incorporated into LpA-I. No saturation was observed up to 90 min of incubation. Cellular cholesterol efflux is very sensitive to the association of

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phospholipid with apoA-I, as demonstrated by the significant increase in efflux observed with as few as 2.4 mol of POPC/mol of apoA-I compared to lipid free apoA-I (results not shown). The efflux of cholesterol from fibroblasts to S2 or S4 was concentration-dependent up to 100 μg of apoA-I/ml, where both particles were close to saturation (Fig. 4). Again, the LpA-I complex containing more POPC showed higher ability to accept cell-derived cholesterol at all concentrations tested. The calculated V_{max} values were 16.9 and 12.6 μg/ml, respectively, for S2 and S4. At all concentrations tested, lipid free apoA-I was not efficient in promoting cholesterol efflux compared to the lipidated apoA-I.

**FIG. 2.** Electrophoretic mobilities of POPC-apoA-I complexes with or without sphingomyelin, phosphatidylinositol, and core neutral lipids. Re-isolated POPC-apoA-I complexes with a progressive increase in POPC content (panel A), and LpA-I complexes with SM, PI, or core neutral lipids (panel B) corresponding to those described in Table I and II were electrophoresed on 0.6% agarose gels for 30 min. In panel A the final molar ratio (POPC/apoA-I) is: S1, 2.4/1; S2, 5.0/1; S3, 11.1/1; S4, 16.2/1; S5, 35.5/1; D1 is a control discoidal Lp2A-I with a POPC/apoA-I ratio of 38.7/1. In panel B, the final molar ratio for S6–S9 is (POPC/apoA-I/UC/SM/PI): S6, 29/1/8/0/0; S7, 14/1/2/1/11.2/0; S8, 24/1/1.5/0.6/1 and S9, 4.7/1/0.5/4/0. For S10–S12 the final molar ratio (POPC/apoA-I/UC/CE/TG) is: S10, 46/1/1.2/2.9/0; S11, 38/1/1.3/0.6/4; S12, 43/1/2.6/6.5. The electrophoretic mobilities were calculated from the migration distance of the particles as previously described (Sparks et al., 1992a). The standard pre-β and α-migration positions were calculated from the migration distance of the corresponding lipoproteins isolated from fresh plasma and electrophoresed under the same conditions. The data are the average of at least four determinations.

**TABLE II**
Characterization of sonicated LpA-I complexes with sphingomyelin, phosphatidylinositol, or HDL core neutral lipids

| LpA-I Composition molar ratio | ApoA-I Size |
|--------------------------------|-------------|
| (Initial Final)                | nm          |

| Variation in sphingomyelin and phosphatidylinositol (POPC/apoA-I/UC/SM/PI) | mol/LpA-I | nm | mV |
|--------------------------------------------------------------------------|-----------|----|----|
| S6 60:1:4:0:0                                                             | 29:1:1:8:0:0 | 2  | 7.7 | 9.6 |
| S7 40:1:4:20:0                                                            | 14:1:2:11:2:0 | 2  | 7.8 | 9.1 |
| S8 50:1:4:0:10                                                            | 24:1:1:5:0:6.1 | 2  | 7.5 | 12.9 |
| S9 10:1:1:10:0                                                            | 47:1:0:5:4:0 | 1  | 8.3 |

| Variation in neutral core lipids (POPC/apoA-I/UC/CE/TG) | mol/LpA-I | nm | mV |
|---------------------------------------------------------|-----------|----|----|
| S10 60:1:3:6:0                                           | 46:1:2:2:9:0 | 2  | 7.3 | 9.3 |
| S11 60:1:3:6:0                                           | 38:1:1:3:0:6.4 | 2  | 7.7 | 10.1 |
| S12 60:1:3:6:8                                           | 43:1:1:2:6:6.5 | 2  | 7.65 | 10.2 |

FIG. 3. Effect of POPC content on the time-dependent cellular cholesterol efflux to POPC-apoA-I complexes. Human skin fibroblasts at 70% confluence were labeled with [3H]cholesterol in the presence of 5% fetal bovine serum and other supplements for 48 h. For the efflux study, the cells were washed twice with phosphate-buffered saline containing 0.2% bovine serum albumin and then twice with phosphate-buffered saline alone, and incubated with DMEM containing 45 μg of protein/ml of sonicated lipid-free apoA-I, or POPC-apoA-I sonicated complexes at a final POPC/apoA-I molar ratio of 5/1, 16.2/1, or 35.5/1 (S2, S4, S5, respectively), or of a control discoidal Lp2A-I (D1). These particles are described in Table I. DMEM containing no cholesterol acceptor was used as control. At the indicated time intervals, aliquots of medium were taken for radioactivity determination. Cholesterol efflux is expressed as medium radioactivity/μg of cell protein (n = 4).

FIG. 4. Effect of POPC content on concentration-dependent cellular cholesterol efflux to POPC-apoA-I complexes. Human skin fibroblasts were seeded and labeled as described in Fig. 3. For the efflux study, the washed cells were incubated with DMEM containing increasing protein concentration of sonicated lipid-free apoA-I, or POPC-apoA-I complexes at the final POPC/apoA-I molar ratio of 5/1 or 16.2/1 (S2 and S4, respectively). Aliquots of medium were taken at 90 min of incubation for radioactivity determination. Efflux is expressed as medium radioactivity/μg of cell protein (n = 4).
To compare the abilities of sonicated LpA-I and discoidal LpA-I (prepared by cholate dispersion) to accept cellular cholesterol from cultured human skin fibroblasts, discoidal Lp2A-I and sonicated Lp2A-I with similar POPC/apoA-I ratios (38.7/1 and 35.5/1, respectively) were prepared from initial POPC/apoA-I molar ratios of 40/1 and 60/1 (Table I). In spite of similar final POPC and apoA-I composition, these two species of LpA-I differed significantly in size (9.2 versus 7.3 nm), α-helix content, and negative surface potential (Table I). Comparison of cellular cholesterol efflux to these two species of LpA-I particles indicated that discoidal LpA-I are more efficient cholesterol acceptors than sonicated LpA-I (Fig. 3).

Effect of Surface and Core Lipid Compositions of Sonicated LpA-I on Cellular Cholesterol Efflux—The cellular cholesterol efflux induced by sonicated Lp2A-I with different surface phospholipids or with neutral core lipids (POPC/apoA-I ratio of 29:1:1.8) did not affect cellular cholesterol from changes in the electrostatic properties of the LpA-I particle. To compare the abilities of sonicated LpA-I and discoidal LpA-I (prepared by cholate dispersion) to accept cellular cholesterol from cultured human skin fibroblasts, discoidal Lp2A-I and sonicated Lp2A-I with similar POPC/apoA-I ratios (38.7/1 and 35.5/1, respectively) were prepared from initial POPC/apoA-I molar ratios of 40/1 and 60/1 (Table I). In spite of similar final POPC and apoA-I composition, these two species of LpA-I differed significantly in size (9.2 versus 7.3 nm), α-helix content, and negative surface potential (Table I). Comparison of cellular cholesterol efflux to these two species of LpA-I particles indicated that discoidal LpA-I are more efficient cholesterol acceptors than sonicated LpA-I (Fig. 3).

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Most of the previous studies aiming to define how lipoprotein composition controls cellular cholesterol efflux have been done with model discoidal LpA-I particles (Johnson et al., 1986, 1991; Agnani and Marcel, 1993; Jonas et al., 1994), but these lipoproteins differ from plasma resident native HDL particles in both shape and composition (Sparks et al., 1992a, 1992b). Here we have studied sonicated LpA-I particles, which have been shown to have a spherical shape and to be similar to native LpA-I by a variety of structural criteria (Sparks et al., 1992a, 1992b, 1995b). In the present report, we have analyzed the effect of the progressive lipidation of apoA-I in model sonicated lipoproteins and observed a quantum jump in the ability of apoA-I to bind cellular cholesterol after complexing with POPC. Addition of only 5 mol of POPC to apoA-I is sufficient to nearly double the initial rate of efflux observed with lipid-free apoA-I (Fig. 3). The Lp1A-I complexes generated by sonication at low phospholipid/apoA-I ratio exhibit a composition analogous to that of pre-β1-LpA-I identified by Fielding and colleagues (Castro and Fielding, 1988; Miida et al., 1990, 1992; Francone et al., 1989, 1990; Fielding and Fielding, 1995). The apparent Stoke's diameters of sonicated Lp1A-I also compare well with that previously reported for pre-β1-LpA-I (Asztalos et al., 1993; Fielding and Fielding, 1995). Sonication can therefore generate complexes that, at low POPC/apoA-I ratio, mimic some of the structural and functional properties of pre-β1-LpA-I.

Our data show that the ability of sonicated LpA-I to release cellular cholesterol is directly related to the amount of POPC associated with apoA-I. This is in marked contrast to what has been observed for discoidal Lp2A-I, where variations in POPC content had no effect on the ability of these lipoproteins to retain cholesterol molecules. This unique effect of phospholipid on efflux to sonicated LpA-I may simply reflect a requirement for a substantial lipid milieu to solubilize cholesterol molecules. Alternatively, the effect of phospholipids may reflect changes in the collisional properties of the lipoprotein resulting from changes in the electrostatic properties of the LpA-I particles. The increased ability of apoA-I to retain cellular cholesterol as the phospholipid/apoA-I ratio is increased in sonicated LpA-I correlates with the negative surface potentials of these complexes. This suggests that reducing the net negative charge

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**DISCUSSION**

Most of the previous studies aiming to define how lipoprotein composition controls cellular cholesterol efflux have been done with model discoidal LpA-I particles (Johnson et al., 1986, 1991; Agnani and Marcel, 1993; Jonas et al., 1994), but these lipoproteins differ from plasma resident native HDL particles in both shape and composition (Sparks et al., 1992a, 1992b). Here we have studied sonicated LpA-I particles, which have been shown to have a spherical shape and to be similar to native LpA-I by a variety of structural criteria (Sparks et al., 1992a, 1992b, 1995b). In the present report, we have analyzed the effect of the progressive lipidation of apoA-I in model sonicated lipoproteins and observed a quantum jump in the ability of apoA-I to bind cellular cholesterol after complexing with POPC. Addition of only 5 mol of POPC to apoA-I is sufficient to nearly double the initial rate of efflux observed with lipid-free apoA-I (Fig. 3). The Lp1A-I complexes generated by sonication at low phospholipid/apoA-I ratio exhibit a composition analogous to that of pre-β1-LpA-I identified by Fielding and colleagues (Castro and Fielding, 1988; Miida et al., 1990, 1992; Francone et al., 1989, 1990; Fielding and Fielding, 1995). The apparent Stoke's diameters of sonicated Lp1A-I also compare well with that previously reported for pre-β1-LpA-I (Asztalos et al., 1993; Fielding and Fielding, 1995). Sonication can therefore generate complexes that, at low POPC/apoA-I ratio, mimic some of the structural and functional properties of pre-β1-LpA-I.

Our data show that the ability of sonicated LpA-I to release cellular cholesterol is directly related to the amount of POPC associated with apoA-I. This is in marked contrast to what has been observed for discoidal Lp2A-I, where variations in POPC content had no effect on the ability of these lipoproteins to retain cholesterol molecules. This unique effect of phospholipid on efflux to sonicated LpA-I may simply reflect a requirement for a substantial lipid milieu to solubilize cholesterol molecules. Alternatively, the effect of phospholipids may reflect changes in the collisional properties of the lipoprotein resulting from changes in the electrostatic properties of the LpA-I particles. The increased ability of apoA-I to retain cellular cholesterol as the phospholipid/apoA-I ratio is increased in sonicated LpA-I correlates with the negative surface potentials of these complexes. This suggests that reducing the net negative charge
on an HDL particle may increase the collisional frequency and the rate of cholesterol transfer from a cell membrane. Changes in the phospholipid/apoA-I ratios of the sonicated LpA-I may also affect the ability of cholesterol to bind directly to apoA-I. Previous reports have shown that the apoA-I conformation changes substantially upon association with phospholipids (Jonas et al., 1989; Sparks et al., 1992a, 1992b, 1993; Bergeron et al., 1995). Epitope expression studies in this laboratory have further demonstrated that incorporation of few phospholipid molecules in sonicated LpA-I directly affects epitope exposure in a central region of apoA-I, a region that has been proposed to be a cholesterol binding domain (Sparks et al., 1993; Bergeron et al., 1995). This central region has also been shown by others to be involved in the process of cellular cholesterol efflux (Banka et al., 1994; Fielding et al., 1994; Sviridov et al., 1996). Fielding and colleagues (Fielding et al., 1994) have also observed that epitopes characteristic of this domain are specifically expressed in pre-β, HDL. The increased ability of apoA-I to retain cellular cholesterol upon association with phospholipids is also significantly correlated with the α-helix content of these complexes. This differential effect of phospholipids on cholesterol efflux to sonicated and discoidal LpA-I may be related to alterations in the apoA-I α-helix thermodynamic stability on the different complexes. On sonicated LpA-I particles, apoA-I exhibits a thermodynamic stability that is less than that observed for lipid-free apoA-I (ΔG°φ < 2.5 kcal/mol apoA-I; Sparks et al. (1995a)). On these lipoproteins, apoA-I would thermodynamically prefer to be in a lipid-free state. Addition of small amounts of cholesterol to these particles increases the stability of apoA-I (Sparks et al., 1993) and therefore cholesterol retention in these particles would be thermodynamically favored. In contrast, discoidal LpA-I are generally more stable (ΔG°φ > 2.5 kcal/mol apoA-I) than lipid-free apoA-I. This may render these lipoproteins less metastable and minimize the variability in the ability of discoidal LpA-I to retain cholesterol, while at the same time allowing a relatively high capacity for lipid storage. This is indeed what we observed (Fig. 3); the initial rates of cholesterol efflux measured during the first 5 min are similar for the sonicated LpA-I and the control discoidal LpA-I, but efflux to the latter increases more rapidly over time as compared to sonicated LpA-I. The higher ability of discoidal LpA-I to act as a cholesterol acceptor may also be due partially to a larger lipid interfacial surface area with a higher capacity to bind cholesterol molecules released from cell plasma membrane (Phillips et al., 1987; Davidson et al., 1995).

Our interest in testing the effect of SM on the ability of LpA-I complexes to sustain cholesterol efflux stems from several different observations. A number of studies have shown that cholesterol has a greater affinity for SM than for PC (Fugler et al., 1985; Yeagle and Young, 1986; Lund-Katz et al., 1988). Pre-β, LpA-I complexes have been reported to be the most avid cholesterol acceptors and have also been shown to contain a high level of SM (Fielding and Fielding, 1995). Finally, a positive relation between the SM content of a series of sonicated SM-liposomes and their ability to release cholesterol from human skin fibroblasts has been reported (Stein et al., 1988). At low phospholipid/apoA-I ratios in Lp1A-I, we observed a significant increase in initial rates of efflux at the point when half of POPC was replaced with SM (Fig. 5B). This is consistent with the hypothesis that small pre-β, LpA-I complexes, which contain equimolar amounts of both PC and SM, are avid and efficient cholesterol acceptors (Fielding and Fielding, 1995). It is interesting to note that cholesterol efflux to these complexes is increased within 2 min of incubation. This may support the hypothesis proposed by Fielding and Fielding (1995), that cholesterol transferred to pre-β, LpA-I is retained because SM reduces its off-rate as it does in synthetic membranes (Kan et al., 1991). At high ratios of POPC/apoA-I in sonicated Lp2A-I, however, the inclusion of SM or PI does not have any effect on cholesterol efflux. Taken together, these data suggest that the effect of SM on cholesterol efflux is not likely to be due to a purely lipid affinity effect, but may instead result from unique changes in apoA-I conformation and stability. Studies have shown that changes in the lipid composition of model discoidal and plasma spherical LpA-I have unique effects on the conformation of apoA-I (Collet et al., 1991; Sparks et al., 1992a; Sparks and Phillips, 1992; Bergeron et al., 1995).

In conclusion, we have shown that cholesterol efflux from human skin fibroblasts to sonicated LpA-I particles is directly proportional to the amount and type of phospholipid. The incorporation of as little as 5 mol of phospholipid is critical to make lipid-free apoA-I an avid recipient of cellular cholesterol, and the presence of SM in Lp1A-I further stimulates efflux. In contrast to investigations with discoidal LpA-I, lipid efflux to sonicated LpA-I appears to be effected by specific changes in apoA-I conformation and charge. Changes in the conformation and charge of apoA-I that result from changes in the lipid composition of a sonicated LpA-I particle appear to directly affect the ability of the lipoprotein to bind and retain cholesterol molecules. While this may be directly associated with changes in interactions between apoA-I and cholesterol, further studies are needed to differentiate the importance of HDL surface lipid affinity versus apolipoprotein affinity for cholesterol molecules in order to fully understand the regulation of adsorption/desorption of this lipid from the surface of an HDL particle.

## Figure 6

### Effect of neutral core lipids on cholesterol efflux to sonicated LpA-I

All assay conditions were as described in Fig. 3. The labeled cells were incubated with DMEM containing 45 µg of protein/ml of sonicated LpA-I particles, S10–S12, where the final molar ratio (POPC/apoA-I/UCC/CE/TG) was: S10, 46/1/ 1.2/2.9/0; S11, 38/1/1.3/0.6/4; S12, 43/1/ 1.2/2.6/8.5, as indicated in Table II. Aliquots of medium were taken at different time intervals for determination of efflux.

### Graphs A, B, C

- **A**: Graph showing the medium cholesterol level over time with DMEM.
- **B**: Graph showing the medium cholesterol level over time with DMEM.
- **C**: Graph showing the medium cholesterol level over time with DMEM.
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