Detergent-Mediated Phospholipidation of Plasma Lipoproteins Increases HDL Cholesterophilicity and Cholesterol Efflux via SR-BI†

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ABSTRACT: Cellular cholesterol efflux is an early, obligatory step in reverse cholesterol transport, the putative antiatherogenic mechanism by which human plasma high-density lipoproteins (HDL) transport cholesterol from peripheral tissue to the liver for recycling or disposal. HDL−phospholipid content is the essential cholesterol-binding component of lipoproteins and therefore a major determinant of cholesterol efflux. Thus, increased phospholipidation of lipoproteins, particularly HDL, is one strategy for increasing cholesterol efflux. This study validates a simple, new detergent perturbation method for the phospholipidation of plasma lipoproteins; we have quantified the cholesterophilicity of human plasma lipoproteins and the effects of lipoprotein phospholipidation on cholesterophilicity and cellular cholesterol efflux mediated by the class B type I scavenger receptor (SR-BI). We determined that low-density lipoproteins (LDL) are more cholesterophilic than HDL and that LDL has a higher affinity for phospholipids than HDL whereas HDL has a higher phospholipid capacity than LDL. Phospholipidation of total human plasma lipoproteins enhances cholesterol efflux, an effect that occurs largely through the preferential phospholipidation of HDL. We conclude that increasing HDL phospholipid increases its cholesterophilicity, thereby making it a better acceptor of cellular cholesterol efflux. Phospholipidation of lipoproteins by detergent perturbation is a simple way to increase HDL cholesterophilicity and cholesterol efflux in a way that may be clinically useful.

Despite progress, management of low plasma high-density lipoprotein−cholesterol (HDL−C), a risk factor for cardiovascular disease (*1*−*5*), remains a challenge. Unlike the liver, extrahepatic tissues synthesize but do not degrade cholesterol. Thus, unless there is a mechanism for its disposal, cholesterol accumulates in arterial macrophages, a key cell type in atherosclerosis. That mechanism, reverse cholesterol transport (RCT),[1] comprises three steps: (1) cellular cholesterol efflux to HDL, (2) esterfication of HDL−cholesterol by lecithin: cholesterol acyltransferase (LCAT), and (3) selective HDL−lipid uptake by hepatic scavenger receptors, class B, type I (SR-BI). RCT requires cholesterophilic HDL and a mechanism for trapping cholesterol in HDL after efflux. Phosphatidylcholine (PC), the essential cholesterophilic component of HDL (*7*−*11*), and the acyl donor for the LCAT (*12*), converts cholesterol to its ester, which unlike free cholesterol (FC) does not transfer spontaneously between lipoproteins. Although the details of RCT have changed as new transporters, enzymes, and receptors have been identified (*13*−*20*), association of cellular FC with HDL has always been considered an obligatory first step. Thus, increasing plasma HDL−PC by phospholipidation should improve RCT.

Given that detergents can reconstitute membranes and lipoproteins, we applied detergent perturbation (DP) (*21*) to studies of lipoprotein stability. In DP, sodium cholate is combined with total human plasma lipoproteins (TLP) after which the cholate is removed by dialysis. These studies revealed the instability of HDL, which released ∼50% of its apolipoprotein (apo) A-I as a lipid-free species. Since sodium cholate and PC form mixed micelles, we hypothesized that DP would catalyze lipoprotein phospholipidation and thereby increase the cholesterophilicities of lipoproteins, particularly HDL (*18*). Given the positive correlation between lipoprotein−phospholipid content and cholesterol efflux (*4*−*6*), phospholipidation should make lipoproteins better acceptors of cellular cholesterol efflux than their untreated control analogues.

EXPERIMENTAL PROCEDURES

Materials. TLP were isolated by flotation of normal human plasma at $d = 1.21$ g/mL; HDL and low-density lipoproteins (LDL) were isolated by sequential flotation (*23*). Lipoproteins were pure according to SDS−PAGE and size exclusion chromatography (SEC). 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) was from Avanti Polar Lipids. [^{3}H]Cholesterol was purchased from Amersham Biosciences (Piscataway, NJ). Buffer salts were from Fisher Scientific, Inc. (Rockville, MD). Tris-buffered saline (TBS; 100 mM NaCl,

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[1] Abbreviations: apo, apolipoprotein; CDX, 2-hydroxypropyl-β-cyclodextrin; DP, detergent perturbation; HDL, LDL, and VLDL, respectively, high, low, and very low density lipoproteins; FC, free cholesterol; PC, phosphatidylcholine; LCAT, lecithin:cholesterol acyltransferase; RCT, reverse cholesterol transport; rHDL, reassembled HDL; SEC, size exclusion chromatography; TLP, total human plasma lipoproteins; TBS, Tris-buffered saline; SR-BI, class B type I scavenger receptor.

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10 mM Tris-HCl, 0.01% azide, 0.01% EDTA, pH = 7.4) was used throughout unless otherwise indicated. High-purity sodium cholate was from Anatrace, Inc. (Maumee, OH).

Compositional Analyses. Protein was determined according to Markwell et al. (*24*). FC and phosphatidylcholine were determined enzymatically (Wako Chemicals USA, Inc., Richmond, VA).

DP-Mediated Phospholipidation of Human Plasma Lipoproteins (*21*). Lipoproteins were phospholipidated with POPC, which is cholesterophilic (*21*), an LCAT substrate (*26*), and a natural phospholipid species of human plasma lipoproteins (*27*). TLP and 465 mM sodium cholate were mixed on wet ice with various amounts of POPC to achieve final concentrations of 65 or 90 mM cholate and the original plasma TLP concentration (~2.0 mg/mL); similarly, LDL and HDL (0.65 and 1.3 mg/mL protein, respectively) were phospholipidated with POPC in the presence of 90 mM cholate. The samples were dialyzed (Spectra/Por, $r = 7.3$ mm, molecular weight cutoff for retention ~6000−8000) for 48 h against a 1000-fold excess of TBS at 4 °C with a change of buffer at 24 h. The phospholipidated lipoprotein particles were analyzed by SEC (*21*).

Lipoprotein Cholesterophilicity. In triplicate, TLP (1 mL, 7.8 mg/mL TLP−protein) were mixed with 0, 0.165, 0.33, and 0.5 mL of POPC (20 mg/mL), 0.775 mL of sodium cholate (465 mM), and sufficient TBS to give a final concentration of 1.95 mg/mL TLP−protein. Control samples were prepared without detergent or POPC. The samples were dialyzed as described above. Cholesterophilicity was determined by measuring cholesterol partitioning between lipoproteins and 2-hydroxypropyl-β-cyclodextrin (CDX) (*25*). At room temperature, 400 μL of each sample was mixed with 200 mM CDX (75 μL) and 25 μL of TBS. The samples were incubated for 3 h and transferred to Microcon ultracentrifugal filters with an exclusion limit of 30 kDa and centrifuged for 10 min at 12000 rpm in an Eppendorf microfuge. The FC concentrations of the retentate (FC_r) and filtrate (FC_f) were determined; protein and phospholipid were also analyzed. The partition coefficient K_p for the distribution of cholesterol between TLP and CDX was calculated from

$$K_p = (FC_{TLP}[CDX])/(FC_{CDX}TLP_{Pro}) \quad (1)$$

where TLP_{Pro} and [CDX] are the TLP−protein (mg/mL) and CDX (molar) concentrations, FC_{TLP} and FC_{CDX}, respectively, are the concentrations of FC bound to TLP and CDX; $FC_{CDX} = FC_f$ and $FC_{TLP} = FC_r - FC_{CDX}$. The effects of DP-mediated phospholipidation of LDL and HDL on their respective cholesterophilicities were measured similarly.

Preparation of [^{3}H]Cholesterol−TLP. [^{3}H]Cholesterol (50 μCi) in toluene was reduced to dryness by a stream of nitrogen followed by drying under vacuum for 1 h. The residue was dissolved in ethanol (50 μL) and dispersed into 23.4 mg of TLP in 3 mL containing 2 mM DTNB to inhibit LCAT. The mixture was incubated at 37 °C for 7 h. An aliquot was analyzed by SEC, and the LDL and HDL fractions were collected and pooled. Measurements of radioactivity and FC showed that the specific radioactivities of LDL− and HDL−FC differed by less than 1%.

Lipoprotein Analysis and Separation. The effects of detergent perturbation on lipoprotein compositions and SEC profiles were determined essentially as described previously (*21*). Sodium cholate and lipoproteins were mixed on ice and transferred to dialysis sacks. Samples were twice dialyzed for 24 h against a >1000-fold excess of TBS at 4 °C and analyzed by SEC using an Amersham-Pharmacia ÄKTA chromatography system equipped with two Superose HR6 columns in tandem. On the basis of the measured residual cholate at the end of 48 h of dialysis, the concentrations of cholate remaining with TLP (1.3 mg/mL) without and with POPC (0.22 mg/mL) were 0.17 and 0.13 mM. The elution volumes for HDL, LDL, and very low density lipoproteins (VLDL) were determined by chromatography of lipoprotein standards isolated by sequential flotation at $d = 1.006$, 1.063, and 1.21 g/mL. Typically, a sample was filtered (0.2 μm), injected into the chromatograph using a 0.2 mL sample loop, and eluted with TBS. The column effluent was monitored by absorbance at 280 nm, and in some instances, 1 mL fractions were collected and analyzed for radioactivity by liquid scintillation counting. For preparative chromatography, a 0.5 mL sample loop was used.

Efflux via SR-BI. An LDL receptor-deficient Chinese hamster ovary cell mutant that expresses very little SR-BI protein or HDL binding/selective uptake activity and SR-BI overexpressing cells in the ldlA-7 cell line were gifts of Dr. Monty Krieger. Measurement of cholesterol efflux was essentially according to de la Llera Moya (*28*). Briefly, after purifying [1,2-$^3H_2$]cholesterol by silica gel chromatography to remove polar impurities, the pooled fractions were reduced to dryness under a stream of nitrogen and resolubilized in DMSO. The DMSO solution of [1,2-$^3H_2$]cholesterol (10 μCi/mL) was rapidly mixed with heat-inactivated (1 h, 55 °C) serum or serum-containing medium (F12), filtered (0.45 μm), and incubated in the presence of FR186054, an inhibitor of acyl-CoA:cholesterol acyltransferase (*29*), for 48 h at 37 °C. SR-BI$^{+/+}$ and ldlA-7 cells were grown to ~80% confluence in 35 mm dishes in medium containing 5% heat-inactivated calf serum. Cells were then incubated with the labeled serum-containing medium for 48 h and then for an additional 24 h in medium containing fatty acid-free bovine serum albumin to ensure equilibration of labeled cholesterol into all intracellular pools. Labeling with cholesterol was validated by liquid scintillation counting of the spots for cholesterol and cholesteryl ester, which were separated by thin-layer chromatography.

Cell monolayers were washed twice with medium containing 1% albumin after which medium (1 mL) containing FR186054 and acceptors (DP−LDL and −HDL fractions), prewarmed to 37 °C, were added to triplicate plates. After incubation for 150 min, the medium was collected, and the cells were extracted into 2-propanol−hexane (3:2) and analyzed by liquid scintillation counting. All efflux data were corrected for efflux from control ldlA cells. Efflux was compared on the basis of both protein and PC contents, which were measured after phospholipidation. Using Systat-Sigma Plot (Point Richmond, CA), efflux (E) was expressed as % $E = E_{max}[LP]/(K_m + [LP])$, where [LP] is the lipoprotein concentration as protein, E_{max} is the maximum efflux, and K_m is the efflux for the binding of lipoproteins to SR-BI.

RESULTS

Phospholipidation of Plasma. Association of POPC with lipoproteins in whole plasma was assessed with a radioactive

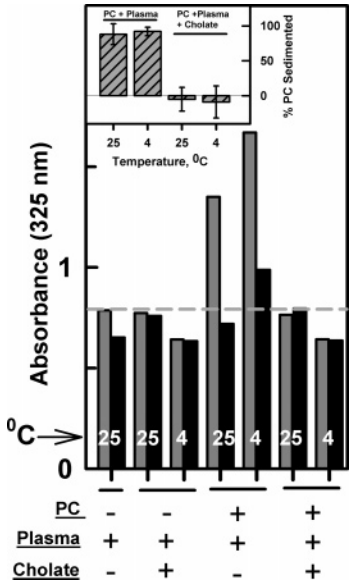

FIGURE 1: Incorporation of POPC into plasma by DP. Plasma (1 mL), [^{3}H]POPC liposomes (2 mg in 0.2 mL of TBS), and 465 mM sodium cholate (0.2 mL) were combined and exhaustively dialyzed at room temperature or 4 °C (arrow). At the end of dialysis, the turbidity of each sample was estimated from the absorbance at 325 nm (shaded bar); each sample was centrifuged to sediment uncombined liposomes and the turbidity measured again (solid bar). Insert: Results of a duplicate procedure conducted with [^{3}H]POPC showing the percent of radioactivity sedimented by centrifugation.

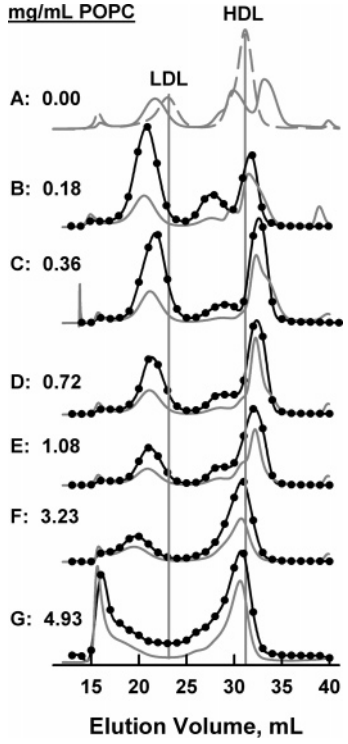

FIGURE 2: SEC of POPC-enriched TLP. Concentrated TLP (protein = 13.2 mg/mL; phospholipid = 8.2 mg/mL), 465 mM sodium cholate, TBS, and various amounts of [^{3}H]POPC were combined to give final concentrations of 1.95 mg/mL protein and 66 mM cholate and exhaustively dialyzed at 4 °C. Aliquots (0.2 mL) were analyzed by SEC; analysis is presented as absorbance (280 nm) for protein without (---) and with (−) DP and as radioactivity for [^{3}H]POPC (●). The elution volumes for LDL and HDL were 18−23 and 29−33 mL, respectively.

tracer. [^{3}H]POPC (10 mg/mL, 0.4 μCi/mg) was suspended in TBS (2.5 mL) at room temperature and vortexed. Human plasma, [^{3}H]POPC liposomes, and sodium cholate were mixed and dialyzed at room temperature or 4 °C (*17*), after which the turbidity of each sample was measured by absorbance at 325 nm. The samples were assayed for radioactivity before and after ultracentrifugation at 10000*g* for 10 min, which sediments the liposomes (>90%; data not shown). Plasma alone had an absorbance of ∼0.7 (Figure 1), which represents the sum of the absorbance and light scattering by macromolecules. Neither the addition of cholate nor sedimentation significantly affected the turbidity at 4 or 25 °C. However, at both 4 and 25 °C, addition of POPC liposomes to plasma increased the turbidity. Centrifugation, which sediments the liposomes, reduced the absorbance to a value similar to that of plasma alone. Those samples that were incubated with sodium cholate and then dialyzed showed no increase in turbidity when compared to plasma alone, and centrifugation had no additional clearing effect, suggesting that cholate "catalyzes" POPC incorporation into macromolecules that are too small for low-speed sedimentation. Analysis of the radiolabeled POPC confirmed this (Figure 1, insert); centrifugation sedimented ∼90% of the POPC that was incubated with plasma only. In contrast, little or no [^{3}H]POPC sedimented from samples that were incubated with cholate before dialysis. Thus, DP phospholipidates lipoprotein particles in whole plasma.

Phospholipidation of TLP. In the remainder of our studies, tests were conducted with the TLP fraction of whole plasma, which contains VLDL, LDL, and HDL. TLP was used because the proteins in the lipoprotein-deficient fraction of plasma elute with HDL and obscure its spectral analysis by SEC. TLP was adjusted to its original plasma concentration (TLP−protein = 2.0 mg/mL) and [^{3}H]cholate and graded amounts of POPC liposomes were added and dialyzed. [^{3}H]-Cholate disappeared exponentially during dialysis; the respective half-times for this process were 2.7, 3.0, and 3.5 h for cholate, TLP, and TLP + 1.6 mg/mL POPC (data not shown). A similar experiment conducted with [^{3}H]POPC, cholate, and TLP showed quantitative recovery (∼99%) of POPC (data not shown) in the TLP after DP and dialysis.

Following phospholipidation, we determined the distribution of POPC among the lipoprotein fractions. TLP was mixed with sodium cholate and [^{3}H]POPC, dialyzed, and analyzed by SEC, which separates control TLP into VLDL, LDL, and HDL (Figure 2A, dashed gray curve). DP of TLP alone shifted the LDL peak to an earlier elution time and split HDL into two peaks, an early one corresponding to fused HDL and a late one, which is lipid-free apo A-I (*17*) (Figure 2A, solid gray curve). With POPC addition, the LDL particle size increased while the two peaks for HDL (Figure 2A) collapsed into a single peak that was relatively symmetrical at higher concentrations of POPC (Figure 2B−F); addition of ≥4.93 mg/mL POPC ablated the LDL peak, and most of the additional POPC appeared in the void volume (Figures 2G and 3A). The amounts of [^{3}H]POPC associated with LDL and HDL were dose-dependent. At low concentrations, most of the [^{3}H]POPC associated with LDL (Figures 2B and 3B), but as the [^{3}H]POPC concentration increased, the percent of POPC associated with LDL and HDL decreased and increased, respectively (Figures 2C−F and

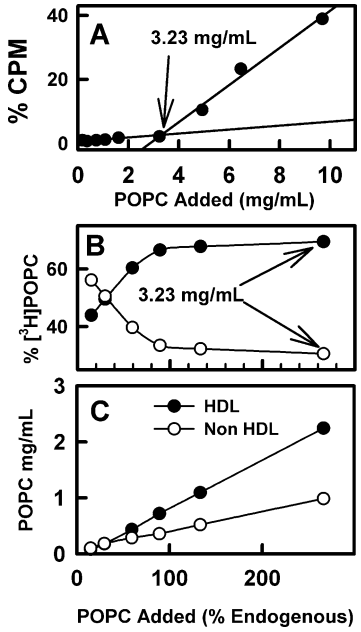

FIGURE 3: Analysis of SEC data of Figure 2. (A) Percent of [^{3}H]-POPC radioactivity appearing in the void volume. (B) Percent of lipoprotein-associated [^{3}H]POPC radioactivity in HDL (●) and non-HDL (○) fractions. (C) Total lipoprotein-associated POPC mass associated with HDL (●) and non-HDL (○) fractions. Non-HDL and HDL included elution volumes of 14−23 and 24−34, respectively, of Figure 2.

3B,C). Thus, LDL has a higher affinity for POPC than HDL, whereas HDL has a greater capacity.

The effects of enriching isolated LDL and HDL with POPC were also studied. Detergent treatment alone had no effect on isolated LDL but split HDL into two peaks (Figure 4A,B). Whereas all of the samples prepared from HDL were optically clear, at higher POPC concentrations, the LDL samples were cloudy. After filtration and centrifugation (15 min at 10000*g*), LDL samples corresponding to Figure 4A−D lost little or no phospholipid, whereas those corresponding to panels E and F of Figure 4, respectively, lost 25% and 70% of the phospholipid. As increasing amounts of phospholipid were added to LDL, the LDL peak disappeared, and an increasing fraction of material eluted in the void volume (Figure 4C−F). Again, this behavior is consistent with a low capacity of LDL for exogenous POPC. In contrast, with the addition of increasing amounts of POPC to HDL, the two peaks observed with detergent alone coalesced into one peak with an elution volume similar to that of HDL (Figure 4). Thus, the capacity of HDL for exogenous POPC is ∼2.5 times that of endogenous PL concentrations.

Lipoprotein Cholesterophilicity. According to SEC of control TLP labeled with [^{3}H]cholesterol, at equilibrium ∼70% and ∼30% of radiolabel were associated with LDL and HDL, respectively (Figure 5A). Following DP, the radiolabel was almost exclusively (∼95%) associated with LDL (Figure 5B); the HDL peak was bimodal, appearing as early and late eluting fractions. However, as the amount of added PC was increased, the fraction of [^{3}H]cholesterol associated with HDL increased (panels C and D of Figure 5, respectively). Qualitatively, this showed that DP-mediated phospholipidation of TLP increases the association of cholesterol with HDL, particularly at higher ratios of exogenous POPC to TLP.

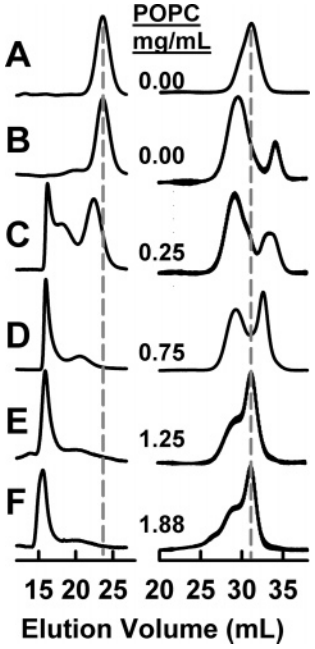

FIGURE 4: SEC analysis of LDL and HDL as a function of PC doses added by DP. (A) Isolated LDL and HDL before DP. Isolated LDL (left) and HDL (right) were modified by DP in the presence of various amounts of added POPC and analyzed by SEC. LDL (0.44 mL, 6.0 mg/mL) or HDL (0.50 mL, 10.4 mg/mL), cholate (0.80 mL, 465 mM), and 0, 50, 150, 250, and 375 μL of 26.3 mM POPC (B−F, respectively) were combined with enough TBS to give a final volume of 4 mL and LDL− and HDL−protein concentrations of 0.65 and 1.30 mg/mL, respectively.

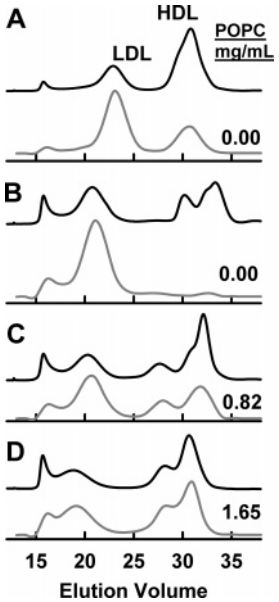

FIGURE 5: SEC analysis of [^{3}H]cholesterol-labeled TLP as a function of PC content. [^{3}H]Cholesterol-labeled TLP was modified by DP in the presence of various amounts of added POPC and analyzed by SEC. TLP (2 mg/mL) was combined with POPC to give final concentrations of 0.0, 0.82, and 1.65 mg/mL (B, C, and D respectively) and enough cholate (465 mM) to give a final concentration of 90 mM and dialyzed. (A) TLP (1.95 mg/mL) without added cholate or POPC. The data are plotted as column effluent absorbance (black line) and the radioactivity associated (gray line) with collected fractions.

The cholesterophilicity of TLP as measured by CDX partitioning increased as a positive function of the amount of POPC in the TLP (Figure 6A and insert). K_p for native

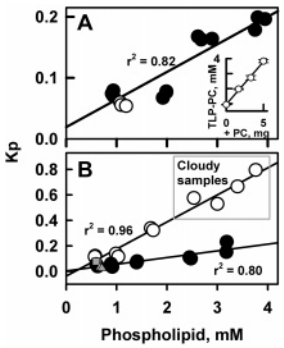

FIGURE 6: Effect of phospholipidation of TLP, LDL, and HDL on cholesterophilicity. (A) Cholesterophilicity of TLP was calculated as K_p, the coefficient for the partitioning of cholesterol between CDX and TLP, as a function of the TLP−PC molarity [POPC]. According to a first-order linear regression analysis, $K_p = 45$[POPC] + 0.019, $r^2 = 0.82$. (●) TLP plus various doses of POPC after DP; (○) TLP without added POPC or DP. The insert shows that the TLP−PC content is linear with the amount of added POPC. Data are presented as the means ± SD. (B) Cholesterophilicity of LDL and HDL after detergent-mediated enrichment of isolated lipoproteins with POPC. Data are plotted as a function of the amount of LDL- (○) or HDL-associated (●) phospholipid. For LDL, K_p = 212[POPC] − 0.036, $r^2 = 0.96$; for HDL, $K_p = 52$[POPC] + 0.0039, $r^2 = 0.80$. Triplicate values are also shown for untreated native LDL (gray squares) and HDL (gray triangles). The gray box denotes cloudy samples with material appearing in the void volume of SEC. Some error bars may be smaller than the symbols.

TLP was not changed significantly by DP. The linearity of the curve is consistent with each increment of added POPC contributing equally to cholesterophilicity. The *Y*-intercept for the plot (0.02) is nearly zero, suggesting similar contributions of endogenous and exogenous PC to cholesterophilicity.

The cholesterophilicities of isolated LDL and HDL enriched with POPC by DP were also measured by CDX partitioning. (This was done on the same samples shown in Figure 4 but was performed before filtration and centrifugation.) These data (Figure 6B) show a POPC dose-dependent increase in the cholesterophilicities of LDL and HDL with the effects of added POPC on K_p for LDL being greater than that of HDL. The cholesterophilicity of native LDL was higher than that of native HDL (0.075 ± 0.003 vs 0.027 ± 0.003), an effect that persisted with the incorporation of additional POPC up to ∼1.5 mM phospholipid. Meaningful comparisons cannot be made above this concentration because, according to SEC (Figure 4C−F), much of the increase in K_P for LDL with added POPC is due to non-LDL-associated POPC and to material eluting in the void volume. The slopes for cholesterophilicity vs TLP− and HDL−phospholipid were similar (compare solid circles in panels A and B of Figure 6; slopes = 0.042 and 0.052, respectively), suggesting that most of the POPC-dependent differences in TLP cholesterophilicity are due to increased phospholipidation of HDL.

Cholesterol Efflux via SR-BI. Cellular efflux of radiolabeled cholesterol from CHO cells expressing SR-BI to TLP, LDL, and HDL with various levels of DP-mediated phospholipidation was measured (Figure 7). DP-treated TLP was a poorer acceptor of cholesterol than native TLP. However, with increased phospholipidation the magnitude of efflux to TLP increased (Figure 7A). The contribution of LDL and HDL to this process was also assessed. Cholesterol efflux

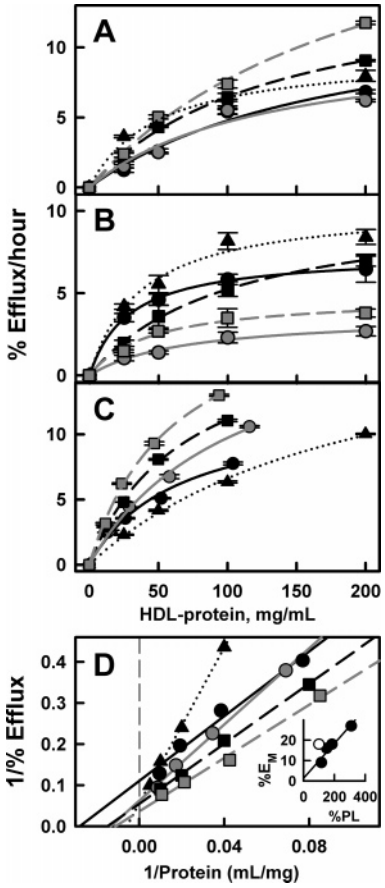

FIGURE 7: Effect of DP-mediated enrichment of TLP, LDL, and HDL with POPC on cholesterol efflux via SR-BI. (A) Efflux to TLP [phospholipid/protein = 0.69 (▲), 0.64 (●), 0.79 (gray circles), 1.04 (■), and 1.92 (gray squares)]. (B) Efflux to LDL [phospholipid/protein = 0.79 (▲), 0.73 (●), 1.12 (gray circles), 1.35 (■), and 2.04 (gray squares)]. (C) Efflux to HDL [phospholipid/protein = 0.52 (▲), 0.61 (●), 0.78 (gray circles), 0.96 (■), and 1.61 (gray squares)]. (D) Double reciprocal plot of efflux in terms of HDL−PC [legend same as (C)]. Insert: Percent maximum efflux (% E_M) as a function of the percent of endogenous HDL−PL for DP-modified samples: HDL (○); DP−HDL (●; $r^2 = 0.94$). Efflux from control cells to TLP, LDL, and HDL was 13−17%, 10−27%, and 11−14% of that from SR-BI expressing cells. Plotted efflux points are the difference between the efflux from SR-BI-expressing cells and the corresponding value for control cells.

to native and phospholipidated LDL was measured (Figure 7B); an increase in efflux with respect to LDL−protein was observed at all PC-to-protein ratios. However, unlike the increases seen with TLP, phospholipidation of LDL did not increase cholesterol efflux, and at most phospholipid-to-protein ratios, efflux was reduced by phospholipidation.

Cholesterol efflux to HDL and phospholipidated HDL was analyzed as a function of the HDL−protein concentration as assessed by SEC. Although DP of HDL in the absence of exogenous PC reduced cellular cholesterol efflux, efflux increased with increasing phospholipidation (Figure 7C). Analysis of these data according to Michaelis−Menten kinetics revealed that DP with and without phospholipidation profoundly altered efflux parameters (Figure 7D; Table 1). DP without PL, which reduces particle number while increasing particle size, decreased the maximum efflux velocity (E_{max}) and reduced K_m, the concentration at which efflux is 50% of E_{max}; DP also increased the catalytic efficiency expressed as E_{max}/K_m. In contrast, relative to DP−

Table 1: Kinetic Parameters for Cholesterol Efflux to Variously Phospholipidated HDL Species

HDL species (% endogenous PC)	E_{max} (% efflux/h)	K_m (μg/mL HDL−protein)	E_{max}/K_m
HDL (100)	18	171	0.11
DP−HDL (116)	9	38	0.24
DP−HDL (150)	16	75	0.21
DP−HDL (185)	18	67	0.27
DP−HDL (310)	27	89	0.30

HDL without phospholipid, increasing phospholipidation of HDL increased K_m and E_{max} (Table 1). Moreover, a plot of E_{max} vs PL content is linear with a *Y*-intercept that is nearly zero (Figure 7D, insert), so that E_{max} for cholesterol efflux is a positive function of HDL cholesterophilicity.

DISCUSSION

Cholesterol partitions among membranes and lipoproteins according to their respective cholesterophilicities and cholesterol-to-phospholipid ratio. HDL cholesterophilicity is modulated by removing cholesterol with a cyclodextrin or by the addition of phospholipids. One approach to HDL therapy has been to increase cellular cholesterol efflux, the first step of reverse cholesterol transport, by the addition of phospholipids to HDL (*3*, *6*). Though sound in principle, quantitative incorporation of phospholipids into lipoproteins is restricted to phospholipids exhibiting thermal phase transitions between ~20 and ~45 °C, a restriction that excludes most physiological phospholipids. In the present study we describe a method that quantitatively incorporates a physiologically abundant phospholipid, POPC, into plasma lipoproteins even in the presence of whole plasma (Figures 1−3). At the highest tested PC-to-TLP ratios, ~67% of the PC associated with HDL (Figures 2 and 3). Thus, DP increases plasma lipoprotein cholesterophilicity in a way that increases TLP-mediated cholesterol efflux (Figure 7A). This was confirmed by our studies showing that phospholipidation of TLP increased its cholesterophilicity, an effect that occurred mostly through phospholipidated HDL. Moreover, phospholipidation increased cellular cholesterol efflux to TLP, essentially through phospholipidated HDL. Thus, the DP method preferentially increases the phospholipid content of HDL in TLP in a way that increases TLP cholesterophilicity (Figure 6) and cholesterol efflux (Figure 7). Analysis of our data reveals a mechanism for TLP phospholipidation and differences in the lipophilicities of LDL and HDL that affect lipoprotein phospholipidation, cholesterophilicity, and cholesterol efflux.

DP without Added PC. DP has little effect on the size and composition of LDL (Figure 4A,B) but converts HDL to a fused "HDL-like" particle while releasing lipid-free apo A-I (Figure 4B); this effect is enhanced by the presence of LDL (Figure 2A) (*17*). In the presence of LDL, DP catalyzes cholesterol transfer from HDL to LDL (Figure 5B), an effect that may be due to increased cholesterophilicity of LDL that is produced by the transfer of PC from HDL to LDL (*17*); thus, "FC follows the phospholipid". The DP-mediated transfer of cholesterol and PC to LDL suggests that plasma LDL is a kinetic product and that when a mechanism is provided, in this case DP, lipids transfer from the less lipophilic HDL to LDL. The higher LDL lipophilicity is consistent with lipid transfer data; spontaneous transfer from LDL is slower than that from HDL (*30*, *31*). The cholesterophilicities of TLP, LDL, and HDL and cellular cholesterol efflux to these particles are reduced by DP (Figures 6 and 7). This correlation supports the hypothesis that lipoprotein cholesterophilicity drives cholesterol efflux. However, the reduction in cholesterol efflux by DP of TLP and HDL is greater than that of LDL, an effect that may be due to the reduction in the number of particles through DP-mediated HDL fusion (*21*).

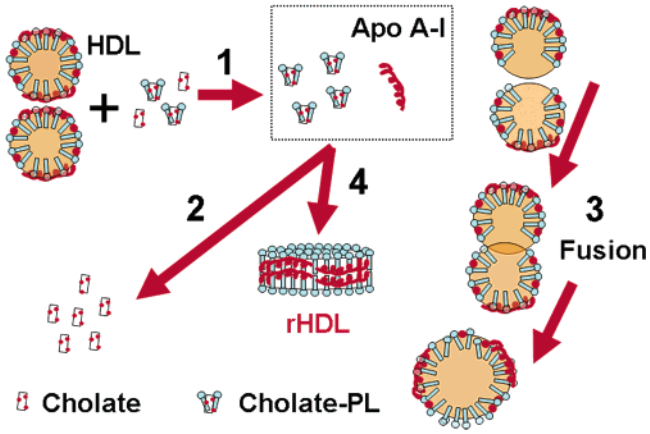

FIGURE 8: Model for the detergent-mediated enrichment of HDL with POPC. (1) Addition of detergent−POPC mixed micelles to HDL transfers HDL−PL to the micelles comprising cholate and PL. With the loss of HDL−PL, the affinity of apo A-I is reduced to the extent that a major fraction is released into the aqueous phase in a lipid-free form, leaving an HDL with part of its neutral lipid core exposed. Removal of the cholate by dialysis (2) permits two PL-poor HDL particles to fuse at sites of exposed neutral lipid (3), while the remaining PL and lipid-free apo A-I combine to give rHDL (4).

DP with Added PC. Cholate and its removal induce release of lipid-free apo A-I, HDL fusion, and transfer of some HDL phospholipids to LDL, giving a larger particle (*21*). Superimposing excess phospholipids on that model leads to a new model (Figure 8) in which additional POPC that is released by dialysis of mixed micelles of PC and cholate distributes between LDL and HDL. The preferential association of POPC with LDL at low concentrations reflects its higher lipophilicity (Figure 3). However, as LDL becomes saturated, excess POPC appears in HDL. The higher capacity of HDL for POPC can be explained almost entirely by the association of POPC with lipid-free apo A-I, which disappears as the HDL peak grows (Figures 2 and 4). The higher phospholipidation capacity of HDL in TLP likely prevents the precipitation and turbidity that are observed with isolated LDL (Figure 4C−F).

LDL has a higher affinity for PC than HDL, an effect that is paralleled by its higher cholesterophilicity. However, the cholesterophilicity of TLP is similar to that of HDL, which has the highest capacity (Figures 5 and 6). Thus, the high capacity of HDL for PC dominates the phospholipidation pattern for TLP (compare Figures 2 and 4). Given the importance of cholesterophilicity and particle number in cholesterol efflux, one might also expect that phospholipidation of HDL, which is present at a molar concentration that is ~10 times that of LDL (*33*), would dominate the pattern of efflux for TLP. This is confirmed by comparison of the effects of phospholipidation on cholesterol efflux to TLP and HDL (Figure 7A,C).

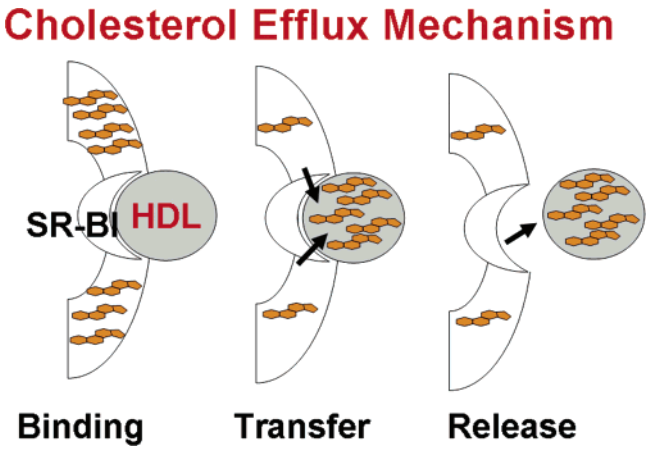

FIGURE 9: Hypothetical model of cholesterol efflux via SR-BI. Lipoproteins are transiently associated with cell surface SR-BI. Following binding of HDL to the receptor (A), cholesterol moves from the cell surface to the lipoprotein via SR-BI, giving a cholesterol-rich particle (B) after the particle is released into the aqueous phase (C). Phospholipidation, which increases its phospholipid-to-cholesterol ratio thereby increasing its cholesterophilicity, enhances the net transfer of cholesterol from the cell to the lipoprotein.

According to the kinetic parameters of Table 1, DP in the absence of PC reduces E_{max}. This could be due to the effect of decreased particle number as a result of fusogenic effects of DP; i.e., a given amount of protein in DP−HDL represents fewer but larger particles (Figure 2A) (*21*). These data are qualitatively similar to those of Thuanhai et al. (*34*), who reported that when normalized to the number of particles, FC efflux efficiency is similar for large and small rHDL particles. A quantitative comparison cannot be made because of differences in the PC-to-protein ratio of rHDL, HDL, and DP−HDL. In contrast, E_{max} increases linearly with phospholipid content. This observation is the converse of the decreased cholesterol efflux found with phospholipid depletion by endothelial lipase (*32*). Among the DP-derived HDL, E_{max} increases linearly with phospholipid content, and the line of regression extrapolates to near zero. This correlation supports a model in which each increment of added phospholipid contributes equally to the increase in E_{max} and in which the contributions of exogenous and endogenous phospholipid to E_{max} are similar. Moreover, in this system, the major determinant of E_{max} is the phospholipid content.

We propose a model of cholesterol efflux via SR-BI in which the E_{max} is determined by the partitioning of cholesterol between phospholipid pools in the plasma membrane and in HDL (Figure 9). According to this model, phospholipid enrichment (or depletion) of HDL would provide a larger (or smaller) pool into which cholesterol can partition. Thus, while HDL is associated with SR-BI, cholesterol partitions between the plasma membrane and the HDL−PL according to the cholesterophilicity as determined by the phospholipid content. This mechanism would have one consequence that would distinguish it from efflux via spontaneous transfer. In the latter case, cholesterol desorbs into the surrounding aqueous phase where it randomly diffuses to many HDL particles, adding a similar number of cholesterol molecules to each. In contrast, during transient association of a particle with SR-BI, many cholesterol molecules can transfer to one HDL particle before it is released into the aqueous phase. This would, in the absence of cholesterol exchange between particles following their release by SR-BI, produce a population of HDL particles that is much more cholesterol-rich than the remainder of the particles.

As hypothesized, cholesterophilicity and cholesterol efflux are positively correlated. DP catalyzes the incorporation of POPC into total human plasma lipoproteins (Figure 2). LDL has a higher affinity for PC than HDL. This is clear at low concentrations of added PC where the majority of PC associates with LDL; as the "PC binding sites" of LDL are saturated, the excess PC associates with HDL (Figures 2 and 3). This is seen in the SEC of LDL and HDL after phospholipidation (Figure 4); there is little incorporation of PC into LDL, which turns cloudy due to the formation of multilamellar liposomes. In contrast, DP-mediated incorporation of PC into HDL is quantitative up to very high concentrations (Figure 4). It is notable that the changes in the HDL profiles with the phospholipidation of HDL and TLP are similar, suggesting that HDL−phospholipid is the predominant acceptor of cholesterol efflux to TLP. Our data showing that cholesterol efflux is associated with similar binding constants for variously phospholipidated HDL species (Figure 7D, insert) complements the report that reduction of HDL−phospholipid with endothelial lipase does not affect binding to SR-BI even though cholesterol efflux is reduced (*32*).

Therapeutic Phospholipidation. Charles Day wrote, "Only one agent has been proven to reverse experimental atherosclerosis in more than one animal species—lecithin (phosphatidylcholine) (*35*)." Subsequent models of RCT and the physical and biological properties of PC validate that statement. PC is sparingly soluble in water, forms a barrier between the neutral lipid core of lipoproteins and the aqueous phase, and is highly cholesterophilic. Although lipid-free apo A-I induces cholesterol and phospholipid efflux via the ABCA1 transporter (*15*, *37*), the PC component of HDL is directly involved in efflux via SR-BI (*10*, *38*), ABCG1 (*39*), and spontaneous transfer (*40*−*42*). Although short-chained PCs spontaneously associate with and enrich plasma lipoproteins (*8*−*10*, *43*−*45*), naturally occurring PCs are resistant to spontaneous incorporation into lipoproteins (Figure 1). Moreover, the short plasma lifetime of short-chained PCs (*46*), such as dimyristoyl-PC, which is frequently used to prepare reassembled (r)HDL, makes them unlikely candidates for therapeutic use.

In the absence of added PC, DP liberates apo A-I from HDL, which could improve cholesterol efflux and RCT via ABCA1. The preferential incorporation of exogenous PC into HDL by DP increases HDL cholesterophilicity and cellular cholesterol efflux via SR-BI and would also be expected to increase efflux via ABCG1/4 and enhance the rate of CE formation via LCAT. Recent studies have revealed the promise of infusion therapy using HDL mimetics (*47*); reconstituted (r)HDL has high cholesterophilicity (*48*, *49*); infusion of rHDL into men increases plasma PL concentrations and efflux of tissue cholesterol to small preβ HDL, where it is esterified (*50*). Small preβ HDL cross endothelium into tissue fluid, collect FC, and transfer it to the liver, where it is converted to bile acids (*51*). Finally, infusion of POPC and apo A-I_{Milano} induces lesion regression (*52*). These approaches have practical limitations. Formulations contain-

ing apo A-I or its variants raise concerns about safety and cost; the lifetime of rHDL is shorter than that of native HDL (*50*, *51*) so that its efficacy may be limited. Finally, the active cholesterophilic agent in RCT is PC, not apo A-I or its variants. Thus, DP with POPC addition is a therapeutic alternative that might provide some cardioprotective effects through it effects on cholesterol efflux via ABCG1, SR-BI, and spontaneous transfer. Additional studies of cardioprotection in other cell models of cholesterol transport and mouse models of atherosclerosis are needed to determine whether DP is a viable therapeutic approach.

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