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HDL SUPERPHOSPHOLIPIDATION ENHANCES KEY STEPS IN REVERSE CHOLESTEROL TRANSPORT

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Abstract

HDL-phospholipids (HDL-PL) play an important role in reverse cholesterol transport (RCT). Phosphatidylcholine (PC) is the most important phospholipid in RCT because it is the essential cholesterol-binding component of lipoproteins and is the acyl donor in the esterification of FC by lecithin:cholesterol acyltransferase (LCAT). FC efflux to sera is a positive anti-atherogenic function of HDL-PL. Although PC has long been recognized as an anti-atherogenic agent, development of new HDL therapies based on PC have been fraught with issues of efficacy, cost, and safety. Moreover, some methods to increase HDL-PC perturb HDL and release lipid-free apolipoproteins (apo) A-I. We developed a new method, HDL SPLn (SPLn) using a modified detergent removal method that obviates these concerns. SPLn can incorporate PC into HDL and increase HDL-PC >10 fold. This is achieved with no loss of apo A-I. According to size exclusion chromatography and native gradient gel electrophoresis, SPLn raises the HDL particle weight in a dose-dependent way, from ~120 kDa to ~350 kDa. Kinetic analysis of FC efflux to the resulting SPLn particles shows that K_m and V_{max} for SPLn HDL are lower and higher respectively than for native HDL. As a consequence, the catalytic efficiency, V_{max}/K_m , increases by more than 400%. Clinically, small increases in serum HDL-PL are associated with significant and profound increases in FC efflux to serum. Treatment of relatively small amounts of plasma by SPLn is a potential method of improving at least one step in RCT.

Keywords

Cholesterol transport; lipoproteins; lipid transport; HDL therapy; SR-BI

1. Introduction

Low plasma high density lipoprotein-cholesterol (HDL-C) concentrations are a risk factor for cardiovascular disease^{1–5} for which current therapies are inadequate. HDL elicits its cardioprotective effects through its role in reverse cholesterol transport (RCT). RCT comprises cellular FC efflux to HDL; esterification of HDL-FC by lecithin:cholesterol acyltransferase (LCAT); and selective HDL-lipid uptake by hepatic scavenger receptors class B, type I (SR-BI).⁶ Transfer of cellular FC to various forms of HDL is an obligatory first step for clearance of excess cholesterol from peripheral tissue. Cellular FC efflux occurs by one of three mechanisms: interaction of lipid-free apo A-I with ABCA1 followed by microsolvubilization,

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and lipid efflux, interaction of lipidated apos with ABCG1 with subsequent efflux and desorption of FC into the aqueous phase. Phosphatidylcholines (PCs) are the essential cholesterophilic component of HDL that determines the magnitude of efflux,^{7–11} and are the acyl donor for LCAT-catalyzed conversion of FC to CE,¹² which unlike FC does not transfer spontaneously between lipoproteins. Given that PCs are essential to RCT in both promoting HDL FC acceptor activity and FC conversion to CE, increasing HDL-PC is one approach to improving RCT.

We described a detergent-mediated method of HDL phospholipidation that increases its PC content and cholesterophilicity and makes it a better acceptor of cellular cholesterol efflux via SR-BI.¹³ However, in the context of HDL therapy, our method had limitations. At low PC concentrations, the detergent released lipid-free apo A-I,¹⁴ which in a physiological setting might be removed by renal filtration,^{15, 16} a process that would likely reduce the total RCT. Moreover, low phospholipid doses actually made HDL a poorer acceptor of cellular FC,¹³ and at best enhancement of efflux was modest. Therefore, we designed and tested a rapid, simple method of SPLn that increases HDL-PC >ten-fold, resulting in a product that better supports FC cholesterol efflux and esterification.

2. Experimental Procedures

2.1 Materials

HDL were isolated from normal human plasma by sequential flotation¹⁷ followed by size exclusion chromatography (SEC).^{15, 16} Lipoprotein purity was assessed by SDS-PAGE and SEC. 1-Palmitoyl-2-oleoyl (PO) PC was from Avanti Polar Lipids (Alabaster, AL) [³H] Cholesterol, was from Amersham Biosciences (Piscataway, NJ). Buffer salts were from Fisher Scientific, Inc. (Rockville, MD). Tris-buffered saline (TBS; 10 mM Tris HCl, 100 mM NaCl, 0.01% EDTA, pH = 7.4) was used throughout unless otherwise indicated. High purity sodium cholate was from Anatrace, Inc. (Maumee, OH).

2.2 HDL Particle Analyses

Protein was determined according to Markwell et al.¹⁸ FC, CE, TG, and PL as PC, were determined enzymatically (Wako Chemicals USA, Inc., Richmond, VA). Lipoprotein particle size was determined by gradient (4–20% acrylamide) gel electrophoresis (GGE) using Ready Cast gels from (BioRad, Hercules, CA).

2.3 SPLn of Human HDL

Lipoproteins were SPLd with POPC, which is cholesterophilic,^{15, 19} an LCAT substrate,²⁰ and an abundant molecular species of human plasma lipoprotein-PC.²¹ Dilution of cholate-lipoprotein solutions below the critical micelle concentration (CMC) of cholate (~15 mM) could achieve effects on HDL similar to that of dialysis. Thus, we used a modification (Figure 1) of our HDL phospholipidation procedure¹⁴ by substituting dilution for dialysis. Aliquots of mixed micelles of sodium cholate (52.6 mM) and [³H]POPC (26.3 mM) were added to HDL with sufficient TBS to give a final HDL concentration of 1.3 mg/mL protein while diluting the cholate below its CMC of ~15 mM. Under these conditions, the dissociated POPC with a CMC ~ 10⁻¹⁰ M cannot remain monomeric²² and diffuses to and associates with the HDL. In the experiments shown in Figure 2, equal amounts of [³H]POPC were added as a tracer.

SEC profiles were determined essentially as described,¹⁴ using an Amersham-Pharmacia ÄKTA chromatography system equipped with two Superose HR6 columns in tandem. Samples were 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; 1-mL fractions were

collected and analyzed by liquid scintillation counting. For preparative chromatography, a 0.5 mL sample loop was used.

2.4 Cholesterol Esterification via LCAT

HDL preparations were labeled with [^3H]cholesterol by injection of [^3H]cholesterol in ethanol (50 μL) into HDL (5 mg in 1 mL), SPLd HDL, SPLd HDL + cholesterol, and HDL + cholesterol. Cholesterol ester formation due to LCAT activity was measured as previously described²³ in a reaction volume of 500 μL in 50 mM Tris buffer, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5% bovine serum albumin and various amounts of substrate. The samples were equilibrated to 37 °C for 30 min, 50 μL of LCAT, obtained as the $d > 1.21$ g/mL fraction of human plasma was added, and the reaction was stopped after 1 h by adding 0.5 mL methanol. All reactions were in triplicate. FC and CE were extracted with 4 mL hexane and the phases separated by centrifugation at low speed for 5 min. Three mL aliquots of the upper hexane layer were evaporated under nitrogen. The extracts were dissolved in 30 μL chloroform and the cholesteryl esters resolved by thin layer chromatography (Whatman TLC silica gel 60A plates) in hexane:diethyl ether:acetic acid (75:35:1) solvent. Free cholesterol and cholesteryl ester bands were identified by comparison with migration of cold FC and CE standards after spraying the plates with Primuline dye (Sigma-Aldrich, St. Louis, MO). The bands for FC and CE were collected and radioactivity was quantified by γ -counting. The content in proteins, PL, and FC of the substrates were assayed for the determination of the rate of CE formed by LCAT activity.

2.5 Cellular Cholesterol Efflux

The variously SPLd HDL were exhaustively dialyzed at 4 °C against TBS and tested as acceptors of cholesterol from the human monocyte-derived macrophage cell line, THP-1 and from Chinese hamster ovary (CHO) cells over expressing SR-BI and the parent control CHO cells (IdlA-7) that are deficient in LDL receptor²⁴. THP-1 cells (ATCC) were maintained in RPMI 1640 medium with 10% FBS, 100 units/mL penicillin, 100 mg/mL streptomycin, 1 mmol/L sodium pyruvate, and 2 mmol/L glutamine. The CHO cell lines were gifts of Dr. Monty Krieger.²⁴ The LDL receptor-deficient CHO cell mutant IdlA-7 expresses a small amount of endogenous SR-BI protein, binds only small amounts of HDL and has minimal selective uptake activity.²⁴ The SR-BI over-expressing cells in the IdlA-7 background have high HDL binding and selective uptake activity.²⁴ The CHO lines were maintained in Ham's F-12 medium, supplemented with 5% FBS, 100 units/mL penicillin, 100 mg/mL streptomycin, 1 mmol/L sodium pyruvate, and 2 mmol/L glutamine. G418 was added to the medium for the SB-BI cells.

Cholesterol efflux was assayed as described previously.²⁵ In brief, cells were seeded for 24 h, then labeled in serum-containing medium with [$1\alpha,2\alpha(n)^3\text{H}$]cholesterol (specific radioactivity 1.81 TBq/mmol, final radioactivity 74 KBq/mL) for 48 h in a CO₂ incubator at which time they were ~ 80% confluent. After labeling, cells were washed twice to remove excess label, and incubated for an additional 18 h in serum-free medium to ensure equilibration of labeled cholesterol into all intracellular pools. During this phase, the SR-BI medium contained G418 which activate the transgene and THP-1 medium contained the LXR agonist T0901317 (1 $\mu\text{mol/L}$) to activate the macrophages in a way that increases the activities of ABCA1 and ABCG1 in cells that are not cholesterol-loaded. To start the efflux assay, cells were washed and incubated for 2 h at 37 °C in serum-free medium containing varied amount (10 to 200 $\mu\text{g/mL}$) of HDL or SPLd-HDL. After incubation, the medium was collected, centrifuged, and the supernatant counted for radioactivity. Cell lipids were extracted with isopropanol and analyzed by liquid scintillation counting. Cholesterol efflux (%E) was expressed as the percentage of total labeled cholesterol transferred from cells to the medium. For CHO-SR-BI cells, efflux data were corrected for efflux from control CHO-IdlA-7 cells. Efflux was compared on the basis of both protein and PC contents of the control HDL and SPLd HDL acceptors. The data

were fit to the equation $%E = E_{\max} \times (LP) / [K_m + (LP)]$, where (LP) is the lipoprotein concentration as protein, $E_{\max}$ is the maximum efflux and K_m is the lipoprotein concentration at half $E_{\max}$ using Systat-Sigma Plot (Point Richmond, CA),

3. Results

3.1. SPLn is linear, quantitative and does not destabilize HDL

According to chemical analysis, the HDL composition before SPLn was 46.8, 25.8, 1.7, 23.7, and 2.1% protein, phospholipid, free cholesterol, cholesteryl ester, and triglyceride respectively. Graded doses of SPLn produced a linear, quantitative increase in the HDL-PL content (Figure 2A, insert). SEC of the SPLdHDL in which the effluent was monitored for both PC radioactivity and protein absorbance showed that the phospholipid content of HDL could be increased by nearly more than 10 folds without producing lipid-free apo A-I, a measure of HDL stability²⁶ (Figure 2). As the PL content increased, the elution volume for both radioactivity and absorbance shifted to smaller elution volumes, i.e. larger particles. The profiles for absorbance and radioactivity were nearly coincident, and there were no peaks for free lipid or protein. Analysis of the variously phospholipidated HDL by non denaturing GGE showed a shift in HDL particle mass from ~150 to ~350 kDa (Figure 3). With increased SPLn, the HDL-PL increased and, as expected, the relative amounts of all other components decreased but the absolute amounts of HDL-protein do not change (Supplement Figure 1).

3.2. SPLn Increases LCAT HDL-cholesterol Esterification

The effects of HDL SPLn on LCAT reactivity were assessed. As shown in Figure 4 and Supplement Table 1, increasing the HDL-PL content 1.5-fold increased esterification by $861 \pm 183\%$ relative to HDL; a similar comparison with SPLd HDL in which both PL and free cholesterol contents were increased 1.4 and 1.2-fold respectively showed a smaller increase ($247 \pm 43\%$). In contrast, increasing HDL-cholesterol content 1.4-fold without added PC was inhibitory ($74 \pm 20\%$).

3.3. SPLn Improves Kinetic Parameters of Cholesterol Efflux

Cellular cholesterol efflux was evaluated in THP-1 macrophages and in CHO cells overexpressing SR-BI and the parent CHO IdIA-7 cells. The impact of SPLn on the kinetic parameters $E_{\max}$ and K_m of cholesterol efflux were evaluated on the basis of their dependence on the HDL-PL in the assays.

The effects of SPLn on efflux from THP-1 cells were modest but very reproducible (Figure 5). Increasing the HDL-PL content from 100% to $690 \pm 33\%$ increased $E_{\max}$ by ~12% and reduced K_m by ~40%. Over the same range, $Cat_{\text{eff}} = E_{\max} / K_m$ increased by ~90%. According to the slope of Figure 5D, increasing HDL-PL by 600% doubles Cat_{eff} for efflux. In contrast, SPLn greatly increases cellular efflux from SR-BI expressing cells in a way that is dose-dependent with respect to phospholipid content (Figure 6A). In addition, HDL SPLn also had a more profound effect on the kinetic parameters for efflux via SR-BI. According to this analysis, $E_{\max}$ and K_m respectively increase and decrease (Figure 6B, C). Moreover when expressed in terms of Cat_{eff} , SPLn increases efflux efficiency to 400% of control values (Figure 6D). Comparing the slopes of Figures 5D and 6D, the Cat_{eff} for cholesterol efflux from the CHO-SR-BI cells is ten times more sensitive to SPLn than efflux from THP-1 macrophages.

Our experiments were conducted using a radio tracer that can sometimes mislead because of differences in specific activity. However, when similar studies were conducted on selected efflux studies in which mass was measured, similar results were obtained (Supplementary Figure 2). Moreover, the effects of cholesterol-loading with AcLDL on cholesterol efflux were tested in the presence and absence of the LXR agonist, TO-901317 (Supplementary Figure 3).

These data show that SPLn produces similar increases in cholesterol efflux irrespective of cholesterol-loading and macrophage activation, an effect that was most profound for cholesterol efflux from activated, cholesterol loaded cells.

4. Discussion

4.1. Limits of SPLn

SPLn by our detergent dilution method catalyzes the association of POPC with HDL thereby forming particles with increased PL content and size. The association of POPC with HDL is linear with respect to POPC dose and neither free lipid nor apo are formed. In these studies, the amount of PL incorporated into HDL, as much as eleven times that of native HDL-PL, was limited by the requirement that the samples be diluted below the critical micelle concentration of sodium cholate, ~15mM. Beginning with 52.6 mM cholate, a ~3-fold dilution was required to satisfy this requirement thereby limiting the amount of POPC added to $26.3 \text{ mM}/3 = \sim 8.8 \text{ mM}$ or 6.6 mg/mL . In the presence of excess cholate, lipid-free apo A-I is formed^{13, 14}. When added to HDL (1.3 mg/mL protein, ~0.6 mg/mL PL), an 12-fold increase in HDL-PL is observed (Figure 2I). Other changes such as starting with more concentrated HDL or reducing the POPC/cholate ratio could have permitted the addition of only slightly more PC incorporation. The advantage of our new method is that it is rapid and produces very high levels of phospholipidation, higher by far (>1000%) than has been reported to date. This would be an important concept to consider for any experiment in which one wants to add amphiphilic molecules to lipoproteins or cells with minimal structural perturbation.

4.2. LCAT Reactivity

Our tests showed that SPLn potentiates LCAT activity (Figure 4). There are several explanations for this. The first is that native HDL is the mature CE-rich product of remodeling by plasma proteins, especially LCAT. CE might reduce activity via a product inhibition mechanism. Although this effect is expected to be small, high levels of POPC would be expected to solubilize small amounts of CE and relieve some inhibition. The second explanation is that high cholesterol concentrations are inhibitory. According to Aron and Fielding,¹² the optimal cholesterol/PL ratio for LCAT activity is 12.5 mol%, above which, activity declines. This optimum is considerably lower than the FC/PL ratio of native HDL, ~25 mol%,²⁶ so that dilution of free cholesterol by POPC would be expected to increase activity. Consistent with this, we observed that cholesterol addition to HDL without additional PC was inhibitory (Figure 4). Both of these explanations are supported by the observation that the LCAT catalyzed rate of CE formation from rHDL is ~10 times more than that formed from HDL.^{20,32} The third possibility is that the added POPC satisfies the molecular and matrix specificities of LCAT, which is more reactive with saturated or monounsaturated acyl PC species in a fluid environment.²⁰

4.3. SPLn Enhances Cholesterol Efflux

We tested the effects of HDL SPLn on the kinetic parameters of efflux with THP-1 macrophages, and SR-BI-expressing CHO cells. In both cells types, HDL SPLn increased E_{max} , decreased K_m , and increased Cat_{eff} . Thus it appears that both the SR-BI and the ABCA1/G1 transporters are sensitive to SPLd HDL acceptors. While the predominant transporter for cholesterol efflux in the transfected CHO-SR-BI cells is SR-BI, cholesterol efflux from THP-1 cells is primarily via ABCA1 and ABCG1.²⁹ Given that cholesterol efflux via ABCA1 requires lipid-free apo A-I, efflux from THP-1 cells is expected to occur primarily via ABCG1 and by spontaneous transfer.³⁰ Although up-regulation of macrophage-ABCG1 increases cholesterol efflux to HDL,³⁰ in cholesterol-loaded cells, spontaneous transfer can contribute substantively to efflux.²⁹ In patients with impaired RCT due to low HDL-C, the reduced cholesterol efflux capacity is due to abnormalities in plasma acceptors and not monocytes or macrophages³¹.

Thus, it is important in the context of improving RCT to make plasma a better cholesterol acceptor. Cross sectional data have shown that in patients with low HDL-C, cholesterol efflux to plasma increases 30% as plasma HDL-PL increases from 0.8 to 1.4 mM (+75%).³² By comparison, SPLn can increase HDL-PL by 1000% (Figure 2), and these SPLd HDL at 20 ug/mL increase efflux from THP-1 cells by ~60%, and from CHO-SR-BI by ~200% as estimated from Figures 5 and 6.

4.4. Physiological Relevance

Our data shows that SPLn of HDL improves two important determinants of RCT: cellular cholesterol efflux and LCAT catalyzed HDL cholesterol esterification. In 1975 Day wrote, "Only one agent has demonstrated the ability to reverse experimental atherosclerosis in one or more animal species: lecithin (PC)."³³ Infusion therapies are now being considered and tested³⁴ because rHDL have high cholesterophilicity and are superior LCAT substrates.²⁸ Infusion of rHDL into healthy men increases plasma PL concentrations and the efflux of tissue cholesterol to small pre β -HDL where it is esterified.³⁵ Small pre β -HDL cross the endothelium into tissue fluid, collect free cholesterol, re-enter the circulation and transfer the esterified cholesterol to the liver where it is largely converted to bile acids.³⁶ Lesion regression follows infusion of a microemulsion of POPC and apo A-I_{Milano}.³⁷

We conducted these studies to provide validation for development of PC therapy as a way to enhance RCT. Previous work towards this goal has encountered roadblocks. For example, by several criteria, rHDL and formulations containing apo A-I or its variants will not be used therapeutically because of cost, safety and efficacy concerns. Any procedure that uses a heterologous protein will present immunological problems. Gram quantities of apo A-I are expensive and the cost of a recombinant variant would be prohibitive. Other steps that would reduce toxicity would only add to the cost. The lifetime of rHDL is much shorter than that of native HDL³⁵ so that relative to native HDL, rHDL has less time to elicit its therapeutic effect. According to our broad hypotheses about RCT and HDL therapy, the active agent is PC, not apo A-I or its variants. Our protocol for preparation of SPLd HDL specifically increases HDL-PC. Many questions must be addressed before this approach can move to human trials. To identify a practical PC therapy, we need a better understanding of how PC-rich HDL as well as its plasma-modified products effect cholesterol transfer from peripheral tissue to the liver. One drawback to SPLn and any other method that includes phospholipid infusion is the possibility that cholesterol may be removed from any tissue, including the liver, that is in communication with the plasma compartment. In vivo tests are needed to determine whether SPLn is a valid HDL therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Apo	apolipoprotein
DP	detergent perturbation
HDL	high density lipoproteins

FC	free cholesterol
CE	cholesteryl ester
POPC	1-palmitoyl-2-oleoyl phosphatidylcholine
LCAT, lecithin	cholesterol acyltransferase
RCT	reverse cholesterol transport
SEC	size exclusion chromatography
SPLd	superphospholipidated
SPLn	superphospholipidation
TBS	tris-buffered saline
ABC	ATP-binding cassette transporter
SR-BI	scavenger receptor, classB, typeI

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Figure 1.

SPLn Principal. A solution of sodium cholate and POPC (2:1 M/M) is diluted into HDL so that the final cholate concentration is below its CMC. With the transfer of cholate from micelle to monomer, released POPC molecules rapidly diffuse to HDL, with which they associate.

Figure 2.

SEC Analysis of SPLd HDL. A. Concentrated HDL (10–30 mg/mL protein) was fractionated by SEC and the fractions corresponding to the middle of the HDL peak were pooled (A, bold line). HDL isolated by SEC (A) were SPLd with various amounts of POPC containing a constant amount of tracer [^3H]POPC. Aliquots of sodium cholate (52.6 mM) and [^3H]POPC (26.3 mM) were added to HDL with sufficient TBS to give a final HDL concentration of 1.3 mg/ml protein. B–I. Analytical SEC of variously SPLd species. The column effluent was monitored for protein absorbance at 280nm (—); and fractions were collected and analyzed for radiolabeled PC (—●—). The vertical dashed gray line denotes the elution volume of lipid-free apo A-I. The values on the right are the %PL in each sample relative to HDL (=100%), as

assessed by PC analysis. A, Insert. Correlation between the amount of phospholipid added to HDL and the amount recovered according to chemical analysis.

Figure 3. GGE of SPLd HDL. The numbers at the top represent the percent PC relative to native HDL. Control and SPLd HDL were applied to gels and visualized as shown. These were the same samples as in Figure 2 before SEC.

Figure 4.

SPLn increases LCAT Activity. Assays of LCAT activity on control HDL, SPLd HDL and HDL with added cholesterol were performed as described in Materials and Methods. The respective protein, PL, and cholesterol compositions of the substrates were as indicated in the Supplementary Table 1. Data shown are the mean $\pm$ SD of two experiments done in triplicate for each substrate. * $p < 0.005$ vs. HDL; # $p < 0.006$ vs. HDL + PC and ‡ $p < 0.005$ vs. HDL + PC + FC.

Figure 5.

Cholesterol Efflux from THP-1 Cells to SPLd-HDL. Cells were labeled with [3 H]cholesterol, and activated with 1 μ M TO-901317. Cholesterol efflux to various amounts of SPLd-HDL was assayed as described in Experimental Procedures. Cholesterol efflux is expressed as a % cellular [3 H]cholesterol transferred to medium. Data are means $\pm$ SD of two experiments in triplicate. HDL-PL for the acceptors was determined chemically. Cholesterol efflux parameters as a function of %HDL-PL were plotted as E_{max} (B), K_m (C) and catalytic efficiency E_{max} / K_m (D). $r^2 > 0.35, 0.84$, and 0.95 for B–D respectively. According to a paired t-test efflux via native HDL (100%) vs all other SPLd species, $P \leq 0.05$ except for comparison of HDL (100%) with HDL (387%) at 25 and 50 μ g/mL and at 200 mg/mL irrespective of level of SPLn.

Figure 6.

Effect of HDL SPLn on cholesterol efflux via SR-BI. (A) Cells were labelled with [3 H] cholesterol, and cholesterol efflux from SR-BI transfected cells to HDL with various levels of SPLn was assayed. (A). Dose response plots of specific SR-BI efflux. Data shown are the difference between the efflux from SR-BI-expressing cells and the corresponding values for CHO-IdIA-7 cells. Data are means $\pm$ SD of two experiments in triplicate. See Figure 5 for details. $r^2 > 0.58, 0.70$, and 0.99 for B–D respectively. According to a paired t-test efflux via native HDL (100%) vs all other SPLd species, $P < 0.04$.