Pathways by which reconstituted HDL mobilizes free cholesterol from whole body and from macrophages

M. Cuchel: rHDL mobilizes cholesterol via different pathways

M. Cuchel[1], S. Lund-Katz[2], M. de la Llera-Moya[2], J. S. Millar[1], D. Chang[1], I.Fuki[1], G.H. Rothblat[2], M. C. Phillips[2], D. J. Rader[1]

[1]Institute of Translational Medicine and Therapeutics, University of Pennsylvania School of Medicine, [2]Division of Gastroenterology, Hepatology and Nutrition, Children's Hospital of Philadelphia, Philadelphia, PA

Materials. Egg phosphatidylcholine (PC) was purchased from Avanti Polar Lipids. ApoA-I was isolated from human plasma HDL as described before[1]. [1,2-^{3}H]-cholesterol was purchased from NEN Life Science Products. Bovine serum albumin (BSA), HEPES, and all reagents, solvents and chemicals were obtained from Fisher Scientific or as specified in the text. Phosphate-buffered saline (PBS), Eagle's minimal essential medium (MEM) and RPMI were purchased from Mediatech. Fetal bovine serum (FBS), bovine calf serum (CS), enzymes and antibiotics for cell culture were obtained from Sigma. Compound CP113, 818 was a gift from Pfizer. Tissue culture flasks were obtained from Corning. All filtration products were obtained from Millipore. Other materials and reagents were obtained as noted.

***Preparation and Characterization of Discoidal Reconstituted Lipid/Protein*.** rHDL containing human apoA-I and egg PC were prepared by the cholate dialysis method [2]. Briefly, the desired amount of egg PC in $CHCl_3$ was added to a 15 ml glass tube and dried under nitrogen, then under high vacuum. Subsequently, the egg PC was hydrated in TRIS-buffered saline (TBS), pH 7.4, vortexed to generate multilamellar vesicles, and incubated with the desired amount of sodium cholate at 37 °C for 1.5 h to generate mixed detergent-PC micelles. The solution was mixed every 15 min during the incubation until completely clear. Next, apoA-I freshly dialyzed from 6 M guanidine HCl in TBS was added to the lipid-detergent mixture at an egg PC to apoA-I ratio of 2.5:1 (w/w; equivalent to a molar ratio of 100:1). The lipid-detergent-apo A-I complexes were then further incubated for 1 h at 37° C. Sodium cholate was removed from the dispersion by extensive dialysis at 4° C against TBS. To obtain a homogenous

population of rHDL particles the preparation was fractionated on a gel filtration column (60 X 1.6-cm Superdex 200 HR column, Amersham Pharmacia Biotech)[3] as necessary. rHDL complexes were isolated, concentrated, dialyzed into phosphate-buffered saline and used within 2 days of preparation. The final particle compositions were determined by analyzing for PC, using an enzymatic assay kit (Wako Chemicals) and protein, using either the Lowry procedure[4] or the absorbance coefficient at 280 nm. The final rHDL particles had an average hydrodynamic diameter of approximately 10nm and a protein to phospholipid to protein ratio of about 2:1(w/w).

Cholesterol efflux assays in primary macrophages. Bone marrow-derived macrophages (BMM) were isolated from femurs and tibias of SR-BI-, ABCA1- and ABCG1-KO mice and respective controls, and cultured in DMEM supplemented with 10% FBS and 30% L929 conditioned media as described before [5].

Cells were labeled with [^{3}H]-cholesterol (2 µCi/mL, Amersham). In the experiments with BMMs from ABCA1 KO, ABCG1 KO animals or corresponding controls, BMMs were loaded with cholesterol using acLDL (25 ug/ml) during labeling. In the experiments with BMMs from SR-BI KO or corresponding control animals, cells were labeled in the absence of acLDL to avoid downregulation of SR-BI expression. After labeling step, all cells were washed and equilibrated overnight either in DMEM containing BSA (2mg/ml). For the cholesterol efflux, medium containing 25 µg/mL rHDL, 25 µg/mL HDL3 or 10 µg/ml free apoA-I was added to BMM cells. After 4 hours, aliquots of the medium were removed and the [^{3}H]-cholesterol released was measured by liquid scintillation counting.

The [^{3}H]-cholesterol present in the cells was determined by extracting the cell lipids with isopropanol and measured by liquid scintillation counting.

Mouse protocol. Wild type (WT) C57BL/6, DBA/J and B6;129S2/J mice were obtained from The Jackson Laboratories (Bar Harbor, ME). ABCA1 (DBA/1-Abca1/J) knock out (KO), SR-BI (B6;129S2-Scarb1^{tm1Kri}/J) KO and ABCG1KO (kindly donated by Dr. Marcelo Amar of NIH/NHLBI) mice were bred in house. Mice were fed a standard chow diet ad libitum. Mice (~3-6 months old) received 200 μl of either PBS containing rHDL or PBS alone as a bolus intravenous injection via tail vein. The dose of apoA-I was approximately 3 mg (range 3.0-3.5 mg/200 μl) and the dose of egg PC was approximately 5 mg (range 4.9-5.8 mg/200 μl). Blood was sampled via retro-orbital bleeding using heparinized glass tubes before and 20 min, 2h, 6h, 24h after injection. Experiments were performed at least twice for each strain of mice. All procedures were approved by the University of Pennsylvania Animal Care Committee.

Lipid and lipoproteins assays. Plasma total cholesterol, HDL cholesterol, FC, phospholipids and apoA-I levels were measured on a Cobas Fara (Roche Diagnostics System, Inc) using Wako Pure Chemical Industries reagents. The enzymatic method used to measure phospholipids selectively measures choline-containing phospholipids, i.e. PC and sphingomyelin. In the context of this paper, where high doses of PC are administered into mice in the form of rHDL, we assume that the contribution of sphingomyelin to the total phospholipids levels in serum is minor, and report the phospholipids levels as PC. Pooled plasma from each time point was separated using

FPLC gel filtration (Amersham Pharmacia Biotech) on 2 Superose 6 columns as described previously[6] for determination of cholesterol and PC profiles.

Kinetic analysis. Transport and fractional catabolic rates (FCR) were calculated using the WinSAAM version 3.0.6[7]. Human apoA-I mass (in mg) was calculated by multiplying the plasma concentration of apoA-I (in mg/L) for each mouse by the estimated plasma pool size (in L, approximately 3.5% of body weight). The human apoA-I FCR was calculated by fitting a monoexpoential curve to the human apoA-I mass in plasma at each timepoint. PC mass (in mg) in plasma was calculated by multiplying the plasma concentration of PC (in mg/L) for each mouse by the estimated plasma pool size (in L, approximately 3.5% of body weight). The PC FCR was calculated by fitting the total PC mass in plasma at each timepoint to a compartmental model consisting of a single compartment. The endogenous PC transport rate through plasma was calculated by multiplying the baseline PC mass, determined prior to injection of rHDL, by the PC FCR. FC mass (in mg) in plasma was calculated by multiplying the plasma concentration of FC (in mg/L) of each mouse by the estimated plasma pool size (in L, approximately 3.5% of body weight). The FCR for FC was calculated by fitting the total FC mass in plasma at each timepoint to a compartmental model consisting of a single compartment. The FC transport rate, which varied with time, consisted of two components. The major component was directly proportional to the plasma PC concentration at the corresponding time. This constant was very similar in all mouse strains studied, ranging between 0.11 to 0.15 mg FC/mg PC/h. The second component, representing the initial rapid transport of FC into plasma, was described by a function that rapidly decreased

over time. The area under the curve (AUC) was determined by integration of the equations describing FC transport between 0 and 24 hours.

Statistical analysis. Data are presented as mean ± SD. The t-test was used to test the difference between 2 groups and a p value of <0.05 was considered significant.

References

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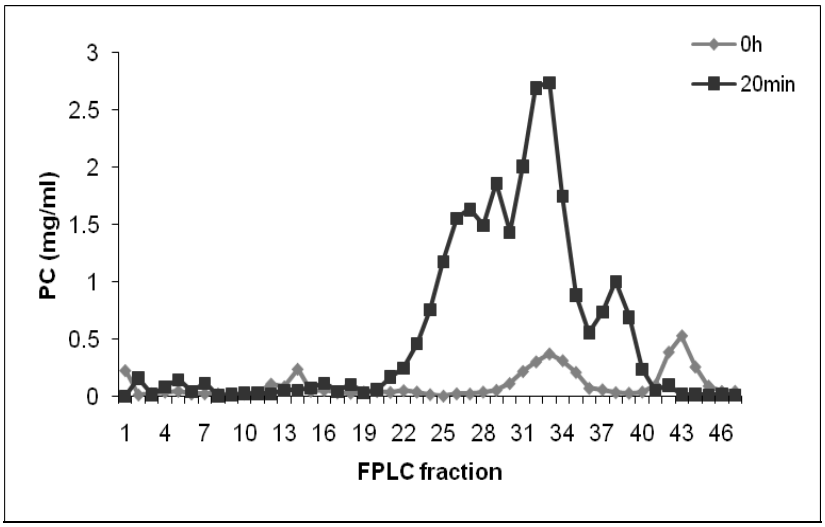
Supplemental Table 1. Serum lipid levels for mice at baseline

	TC (mg/dl)	FC (mg/dl)	PC (mg/dl)
C57BL6 (n=12)	74 ± 9	17 ± 3	150 ± 19
SRBI-control (n=4)	119 ± 4	20 ± 4	208 ± 36
SRBI-KO (n=3)	234 ± 32	155 ± 9	248 ± 29
ABCA1-control (n=4)	67 ± 3	9 ± 1	140 ± 5
ABCA1-KO (n=4)	5 ± 3	2 ± 1	34 ± 9
ABCG1-control (n=5)	123 ± 22	41 ± 8	233 ± 29
ABCG1-KO (n=5)	83 ± 4	29 ± 3	155 ± 9

TC, total cholesterol; FC, free cholesterol; PC, phosphatidylcholine; control, wild type control mice on same background as knock out mice; KO, knock out

Supplemental Figure 1

A



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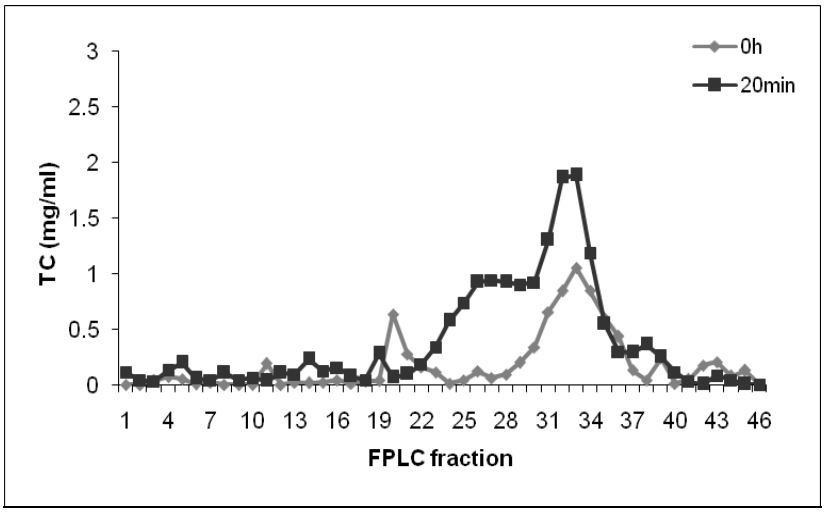

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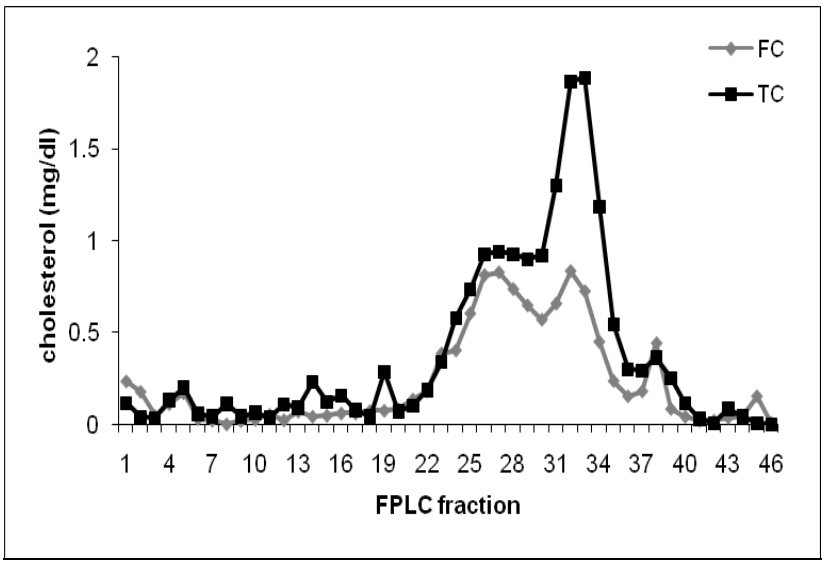

Supplemental Figure 1. FPLC profiles of serum of C57BL/6 female mice at baseline and after administration of rHDL. See methods section for details. A: Phosphatidylcholine (PC) concentrations at 0 and 20 min. B: Total cholesterol concentrations at 0 (gray line, solid diamonds) and 20 min (black solid line, square symbols). C: Total cholesterol (black solid line and symbols) and free cholesterol (gray solid line and symbols) concentration 20 minutes after rHDL injection.