Published in final edited form as:
Arterioscler Thromb Vasc Biol. 2010 March ; 30(3): 526–532. doi:10.1161/ATVBAHA.109.196105.

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

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Abstract

Objectives—Reconstituted HDL (rHDL), is of interest as a potential novel therapy for atherosclerosis due to its ability to promote free cholesterol (FC) mobilization after intravenous administration. We performed studies to identify the underlying molecular mechanisms by which rHDL promote FC mobilization from whole body in vivo and macrophages in vitro.

Methods and results—Wild type (WT), SR-BI-KO, ABCA1-KO and ABCG1-KO mice received either rHDL or PBS intravenously. Blood was drawn before and at several time points after injection for apoA-I, phosphatidylcholine and FC measurement. In WT mice, serum FC peaked at 20 min and rapidly returned towards baseline levels by 24 h. Unexpectedly, ABCA1-KO and ABCG1-KO mice did not differ from WT mice regard to the kinetics of FC mobilization. In contrast, in SR-BI-KO mice the increase in FC level at 20 min was only 10% of that in control mice ($p<0.01$). Bone marrow-derived macrophages from WT, SR-BI-KO, ABCA1-KO and ABCG1-KO mice were incubated in vitro with rHDL and cholesterol efflux determined. Efflux from SR-BI KO and ABCA1 KO macrophages was not different from WT macrophages. In contrast, efflux from ABCG1-KO macrophages was ~ 50% lower as compared with WT macrophages ($p<0.001$).

Conclusions—The bulk mobilization of FC observed in circulation after rHDL administration is primarily mediated by SR-BI. However, cholesterol mobilization from macrophages to rHDL is primarily mediated by ABCG1.

Keywords

reconstituted HDL; cholesterol efflux; apolipoprotein A-I; SR-BI; ABCA1; ABCG1

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Disclosures: None.

Administration of reconstituted HDL (rHDL) is associated with increase in plasma free cholesterol, suggesting that rHDL promote cholesterol mobilization. We demonstrated that the bulk mobilization of free cholesterol observed in circulation after rHDL administration is primarily mediated by SR-BI. However, cholesterol mobilization from macrophages is primarily mediated by ABCG1.

Circulating high density lipoprotein (HDL) and its major protein apolipoprotein A-I (apoA-I) are generally considered to protect against atherosclerosis. The main mechanism by which HDL and apoA-I are thought to be protective is through the promotion of reverse cholesterol transport (RCT), the process by which excess cholesterol from the periphery is transported back to the liver to be further metabolized and excreted [1]. Novel therapeutic approaches that promote RCT and hold the potential to be a major tool to reduce cardiovascular disease risks are currently being tested. One promising approach is the use of syntheticdiscoidal particles containing either human apoA-I or one of its variants, apoA-I_{Milano} and phospholipids, and sometimes cholesterol[2], frequently referred as reconstituted HDL (rHDL). The intravenous administration in humans of such particles has been associated with regression of coronary atherosclerosis[3] and improvement in plaque characteristics[4, 5], endothelial function [6] and anti-inflammatory markers [7]. It is also associated with a transitory increase in plasma free cholesterol (FC) in both mouse models[8, 9] and humans[10, 11], as well as with increased cholesterol synthesis in peripheral tissues, consistent with cholesterol movement from tissues to plasma in mice [8], and increased fecal sterol excretion in humans[11, 12], suggesting that rHDL is able to promote RCT. However, the molecular mechanisms by which rHDL promote cholesterol mobilization from tissues are not understood.

The first step of the RCT pathway is the efflux of FC from peripheral cells to extracellular acceptor particles. Four different mechanisms are known to contribute to this step: ABCA1-mediated efflux to apoA-I [13], SR-BI and ABCG1-mediated efflux to mature HDL[14, 15], and aqueous diffusion to mature HDL [16]. In vitro data suggest that rHDL can be an excellent cholesterol acceptor in the context of SR-BI- and ABCG1-mediated efflux pathways, but not in the context of an ABCA1- mediated efflux pathway[17, 18]. The goal of this study was to investigate the molecular mechanism(s) underlying the FC mobilization observed after intravenous administration of rHDL and specifically to test the hypothesis that administration of rHDL in vivo promotes cholesterol mobilization via SR-BI-and ABCG1- mediated, but not ABCA1-mediated, pathways. As we were interested in investigating the mechanisms at both the systemic and macrophage levels, we conducted both in vivo studies in mice models and in vitro studies in primary macrophages.

Methods

A detailed description of animals, reagents and methods used in the studies reported in this manuscript are described in the supplemental materials appendix. Following is a brief description of the methods used.

Preparation and Characterization of Discoidal Reconstituted Lipid/Protein

rHDL containing human apoA-I (~3 mg) and egg phosphatidylcholine (PC; ~7 mg) were prepared by the cholate dialysis method as previously described [19]. The final rHDL particles had an average hydrodynamic diameter of approximately 10nm and a protein to phospholipid ratio of about 1:2 (w/w). The concentration of apoA-I in rHDL is similar to that used in other animal [8] and human [11, 12] studies.

Cholesterol Efflux Assays in Bone Marrow–Derived 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 [20]. Cellular cholesterol efflux assays were performed as previously described [20].

Mice study protocol

Wild-type (WT) control mice were obtained from Jackson Labs. SR-BI- ABCA1- and ABCG1-deficient mice were bred in house. Mice were fed a standard chow diet ad libitum. rHDL were administered by intravenous bolus injection. Serum was obtained by retro-orbital bleeding while the mice were under isofluorane anesthesia and collected in heparinized tubes. All mice experiments were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Lipid and Lipoprotein Analysis

Total cholesterol, HDL cholesterol, FC and phospholipid levels were measured 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 PC represent the majority of the phospholipids and report the results as such. Pooled plasma from each group was separated by fast protein liquid (FPLC) gel filtration and total cholesterol and PC in each fraction was measured.

Kinetic analysis

Transport and fractional catabolic rates (FCR) were calculated using the WinSAAM version 3.0.6[21]. Human apoA-I, PC and FC masses were calculated by multiplying their plasma concentrations for each mouse by the estimated plasma pool size (approximately 3.5% of body weight).

Statistical Analysis

All values are shown as the mean±SD. A 2-tailed Student *t* test was used to test for statistical significance. A probability value of <0.05 was considered significant.

Results

rHDL effectively mobilizes cholesterol in wild-type mice in vivo

We did not observe any changes in serum levels of either PC or FC following intravenous administration of PBS in WT mice (Figure 1). In contrast, following the iv administration of rHDL we observed the rapid appearance of human apoA-I (Figure 1A), and a rapid and significant increase in PC (Fig. 1B) in serum. In addition we observed a significant increase in serum FC (Figure 1C). At 20 min post injection, serum PC was increased more than 6 fold, and FC more than 5-fold as compared with the serum levels at baseline. PC and FC levels returned to baseline levels by 24 h post injection (Figure 1). HDL particles observed in the FPLC profile of serum obtained from wild type mice 20 min after injection of rHDL were larger and enriched in PC and cholesterol as compared with HDL particles observed in the FPLC profile of serum obtained before injection (Supplemental Figure 1A, 1B). Moreover, more than half of the cholesterol present in these particles was FC (Supplemental Figure 1C).

SR-BI is required for the rapid mobilization of free cholesterol observed in circulation after the administration of rHDL

We evaluated the effects of administration of rHDL in mouse models that lacked SR-BI, ABCA1 or ABCG1 to assess their role in mediating cholesterol efflux to rHDL. Baseline lipid levels for the knock-out (KO) mouse models and their respective controls are shown in Supplemental Table 1. Following administration of rHDL into SR-BI deficient mice, the human apoA-I peak at 20 min was lower than that observed in the control mice (85±17 vs. 113±13 mg/dl, p=0.051); however, by 24 h the levels in the two groups of animals were similar (19±9 vs. 25±16 mg/dl, p=0.58). Similarly, when expressed as change from baseline, the increase in

PC levels in SR-BI deficient mice (Figure 2B) were lower than that in control mice at the 20 min peak (201±53 vs. 338±43 mg/dl, p=0.01), but similar 24 h post-injection (10±10 vs. 4±54 mg/dl, p=0.93). The kinetic analysis of these data support the concept that the overall human apoA-I and PC clearance (FCR) and the PC transport is similar in SR-BI KO and control mice (Table 1). The most striking difference observed between SR-BI KO mice and their controls were the FC levels, as SR-BI deficient mice lacked the rapid increase from baseline seen in the control mice in response to the rHDL injection (Figure 2C). At 20 min post-injection, changes in FC levels from baseline in SR-BI KO mice were only 10% of the changes observed in control mice (6±3 vs. 61±13 mg/dl, p<0.01) and increased rather slowly up to 6 h post injection. The kinetic analysis suggests that the FCR of FC as well as the rapid input of FC into circulation were markedly reduced in the SR-BI deficient mice as compared with their controls (p< 0.01, Table 1).

ABCA1 and ABCG1 are not required for the rapid mobilization of free cholesterol observed in circulation after the administration of rHDL

Following administration of rHDL, the serum concentration of human apoA-I in ABCA1 deficient mice was similar to that in control mice 20 min post injection, but declined more rapidly over time (Figure 3A), consistent with the known increase in apoA-I catabolism in ABCA1 deficiency. Kinetic analysis showed that the human apoA-I FCR was significantly faster in ABCA1-KO mice as compared with wild type mice (p=0.002; Table 1), explaining the more rapid decline in concentration over time. Changes in the serum concentrations of PC in ABCA1 KO mice after rHDL injection were similar to those observed in control mice (Figure 3B). Kinetic analysis showed that the PC FCR observed in ABCA1 deficient mice was similar to that in control mice while PC transport into the circulation was significantly lower (p=0.03; Table 1). The increase in FC levels was similar to that observed in control mice at the earlier time points; however, a modest but significant decrease in FC concentrations was observed 6 h post-injection (Figure 3C). Kinetic analysis showed that, following rHDL injection, FC FCR and transport into circulation in ABCA1 deficient mice were similar to those observed in control mice (Table 1).

Following administration of rHDL into ABCG1 KO mice, the rapid appearance of human apoA-I and increase in PC and FC levels (Figure 4) was similar to those observed in control mice. Kinetic analysis showed that the human apoA-I and FC FCRs observed in ABCG1 deficient mice were similar, and that the slightly but significantly increased PC FCR (p=0.01) was balanced by a tendency of PC transfer to increase (p=0.08) as compared to control mice (Table 1).

ABCG1 contributes to the mobilization of free cholesterol from primary macrophages incubated in presence of rHDL

To directly assess the mechanism(s), if any, responsible for cholesterol mobilization from macrophages following the administration of rHDL we conducted in vitro studies using bone marrow-derived macrophages from SR-BI, ABCA1 and ABCG1 KO mice and appropriate controls. FC efflux from SR-BI KO-derived macrophages was not different than that from control cells when either HDL3 (20.70±0.80 vs 22.06±0.77 %FC/4h, p>0.1) or rHDL (16.92 ±0.99 vs 17.51±1.04 %FC/4h, p>0.5) were used as acceptors (Figure 5A). FC efflux from ABCA1 KO-derived macrophages to apoA-I, the acceptor for ABCA1, was as expected absent; while FC efflux to rHDL was present and slightly but significantly increased as compared to control cells (12.83±0.92 vs 14.97±0.39 %FC/4h, p<0.02, Figure 5B). Finally, FC efflux from ABCG1 KO-derived macrophages was significantly decreased as compared with controls when HDL3 was used as acceptor (25.37±1.23 vs 20.27±1.04 %FC/4h, p<0.005), and even more so when rHDL were used (18.18±1.37 vs 9.95±0.13 %FC/4h, p<0.001; Figure 5C).

Discussion

Administration of rHDL is associated with increased FC in plasma both in humans[10, 11] and mice[8], and with increased fecal sterol excretion [11, 12], increased ex-vivo cholesterol efflux ability [7] and improvement of atherosclerosis burden[4] in humans suggesting that administration of rHDL promotes RCT. The main objective of these studies was to evaluate the molecular mechanisms underlying the FC mobilization observed after the administration of rHDL at both systemic and macrophage levels.

Our in vivo results are consistent with the hypothesis that SR-BI, but not ABCA1 or ABCG1, is responsible for most of the increase in FC levels observed in circulation following the administration of rHDL. Relative to WT mice, we observed an altered response to rHDL in SR-BI-KO mice, with a significantly lower peak in FC levels soon after rHDL administration (Figure 2C). The kinetic analysis also supports the concept that SR-BI plays a critical role in the rapid early efflux of FC into circulation in response to rHDL injection, as demonstrated by the reduction of the rapid FC transport observed in the SR-BI KO mice, as well as a role in FC uptake, as demonstrated by the reduction in FC FCR observed in the SR-BI KO mice. These results are consistent with the known role of liver SR-BI in HDL metabolism in mice[22-24], the ability of SR-BI to mediate bi-directional cholesterol flux between cells and HDL[16] and the ability to promote cholesterol efflux from a variety of cell types to mature HDL[25] as well as to rHDL[26, 27].

Although we measured cholesterol concentrations in blood cells over time in animals injected with rHDL and excluded the possibility that blood cells are a major source of the increased serum FC observed in our studies (data not shown), we did not assess which tissues are the primary sources of the FC mobilization in response to rHDL infusion. SR-BI is abundantly expressed in the liver[28], and it is possible that a considerable proportion of the FC mobilized in response to rHDL is derived from this organ. Indeed, based on the data presented by Alam and colleagues[8], the liver is one of the biggest contributors to the flux of FC into the circulation following the intravenous administration of rHDL. Peripheral tissues also contribute to the net flux of cholesterol into circulation and to the liver in the hours following administration of rHDL[8].

The results of the in vivo studies suggest that an ABCA1- and ABCG1- mediated mechanism do not contribute substantially to the amount of FC mobilized following rHDL administration at least at the earlier time points. It is, however, interesting to notice that serum FC levels 6 h post-injection were significantly lower and human apoA-I was cleared from plasma more rapidly in ABCA1-deficient mice as compared with control mice. rHDL has been shown to undergo remodeling in circulation, including the generation of pre-beta particles [10, 11, 29]. Thus the observed faster apoA-I clearance in ABCA1 deficient mice could be explained by impaired lipidation of lipid-poor apoA-I created by remodeling of rHDL in circulation, consistent with the decreased PC transport into circulation and the tendency to decreased FC transport in the ABCA1-deficient mice as compared with control mice shown by the kinetics analysis. Taken together, these data suggest that ABCA1 may be important in maintaining FC efflux at later time points following rHDL injection and may contribute together with ABCG1 in promoting RCT from macrophages after administration of rHDL and to the improvement in the atherosclerotic burden that may be associated with the administration of rHDL in humans[3-5] and animals [9, 30].

As administration of rHDL is being considered for the treatment of atherosclerosis, we were interested in assessing the ability of rHDL in promoting cholesterol efflux specifically from macrophages and the underlying mechanisms. We conducted in vitro studies using bone marrow-derived macrophages isolated from SR-BI-, ABCA1- and ABCG1-KO mice and their

respective controls, as described before[20], and showed that rHDL promotes FC efflux from primary macrophages by an ABCG1-mediated pathway, while SR-BI seems not to play a significant role. Although SR-BI is expressed in macrophages [14, 31] and a protective role of macrophage SR-BI has been suggested from studies done using bone marrow transplantation [32, 33], these results are consistent with our previously published data, in which we have shown that macrophage SR-BI does not play a dominant role in either FC efflux from macrophages[17, 34] or macrophage RCT[20], contrary to the key role played by macrophage ABCG1.

Our data in primary macrophages support the hypothesis that rHDL promote efflux from macrophages via an ABCG1-mediated mechanism (Figure 5C). ABCG1 is highly expressed in macrophages[35] and mediates macrophage cholesterol efflux to mature HDL as well as rHDL [18, 36]. Although its role in atherosclerosis is still debated [37-39], its role in controlling cholesterol homeostasis in macrophages is well supported by published data [15, 20, 34-36], and our results suggest that an ABCG1-mediated mechanism is at least in part responsible for the improved atherosclerotic burden observed in previous studies following administration of rHDL.

Other pathways, in particular aqueous diffusion [34], must also mediated cholesterol efflux in presence of rHDL, as efflux is preserved, albeit reduced, in ABCG1-deficient macrophages. It is also reasonable to think that, following remodeling in vivo with generation of lipid-poor apoA-I, rHDL may also promote ABCA1-mediated macrophage cholesterol efflux. Contrary to the role that ABCG1 has in mediating cholesterol efflux in macrophages, ABCG1-mediated cholesterol mobilization does not appear to contribute significantly to the bulk of FC mobilized soon after rHDL mobilization. This is probably due to the fact that the mass of cholesterol mobilized by macrophages via ABCG1 is very a very small part of the overall cholesterol tissue pool.

One possible limitation of our in vivo studies is the large difference in HDL levels in the different mouse models. The difference in pool size could potentially affect how the different mouse models responded to rHDL administration. However, we do not expect an effect of pool size on FC mobilization, since we believe there are sufficient phospholipids in the rHDL to allow for ample FC uptake.

In conclusion, we demonstrate that in mice SR-BI, and not ABCA1 or ABCG1, is responsible for the bulk of the rapid FC mobilization to the circulation (primarily non-macrophage) that is observed after intravenous administration of rHDL. In contrast, ABCG1, and not SR-BI or ABCA1, is primarily responsible for FC mobilization to rHDL from macrophages. Because macrophage cholesterol efflux is arguably more relevant to atherosclerosis than efflux from non-macrophage tissues, these results call into question the concept of using serum FC as a surrogate for the anti-atherosclerotic efficacy of rHDL infusion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Margaret Nickel, Vinh Nguyen, and Aisha Wilson for outstanding technical support.

Sources of Funding: This work was supported by a grant from NIH-NHLBI (HL22633). Dr Cuchel is supported by a K23 award from NIH-NHLBI (HL077146).

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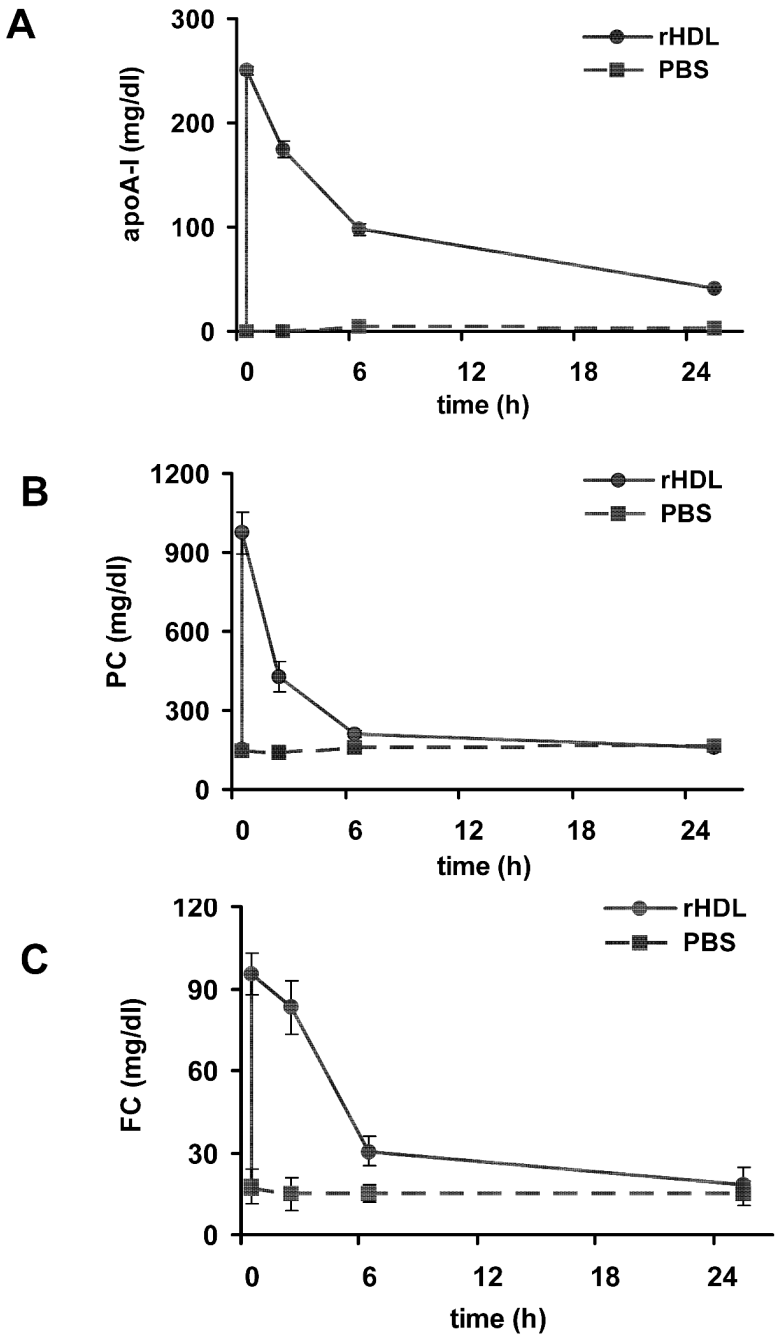

Figure 1.

Human apolipoprotein A-I (apoA-I, panel A), phosphatidylcholine (PC, panel B), and free cholesterol (FC, panel C) levels in serum from C56BL/6 female mice before and 20 minutes, 2, 6 and 24 hours after i.v administration of either PBS (n=4, dashed line) or rHDL (n=8, solid line). See methods section for details.

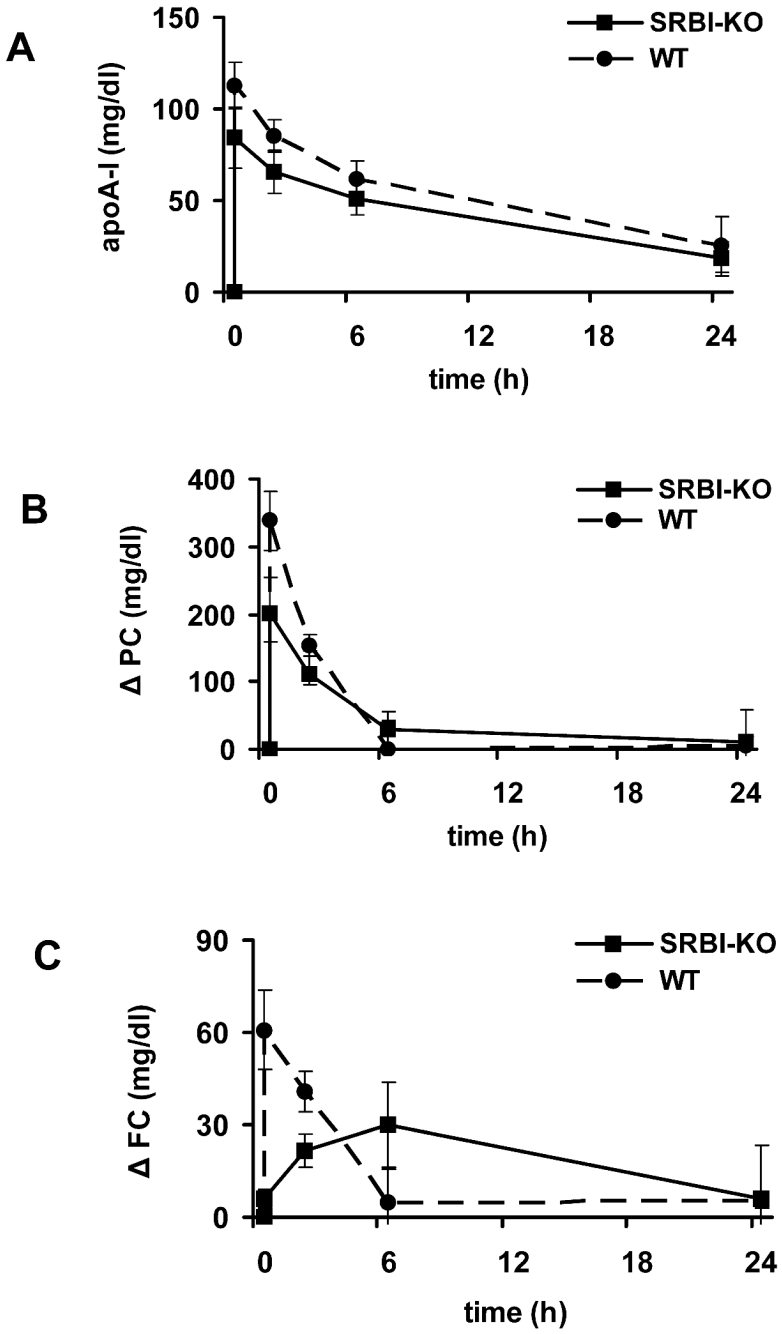

Figure 2.

Human apolipoprotein A-I (apoA-I, panel A), phosphatidylcholine (PC, panel B), and free cholesterol (FC, panel C) levels in serum from SR-BI KO (n=3) and control (n=4) male mice before and 20 minutes, 2, 6 and 24 hours after i.v. administration of rHDL. PC and FC levels are expressed as change from baseline. See methods section for details. Solid line: KO mice; dashed line: control mice.

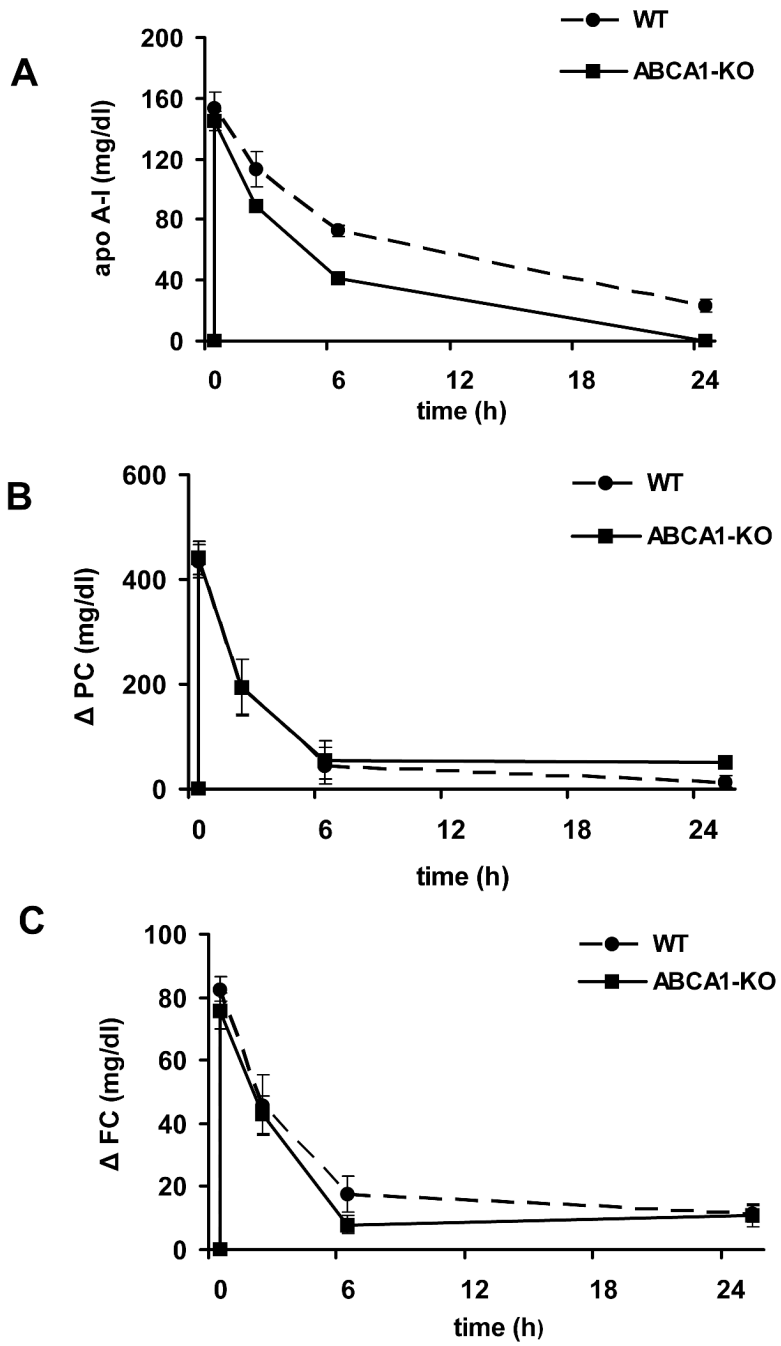

Figure 3.

Human apolipoprotein A-I (apoA-I, panel A), phosphatidylcholine (PC, panel B), and free cholesterol (FC, panel C) levels in serum from ABCA1 KO (n=4) and control (n=4) female mice before and 20 minutes, 2, 6 and 24 hours after i.v. administration of rHDL. PC and FC levels are expressed as change from baseline. See methods section for details. Solid line: KO mice; dashed line: control mice.

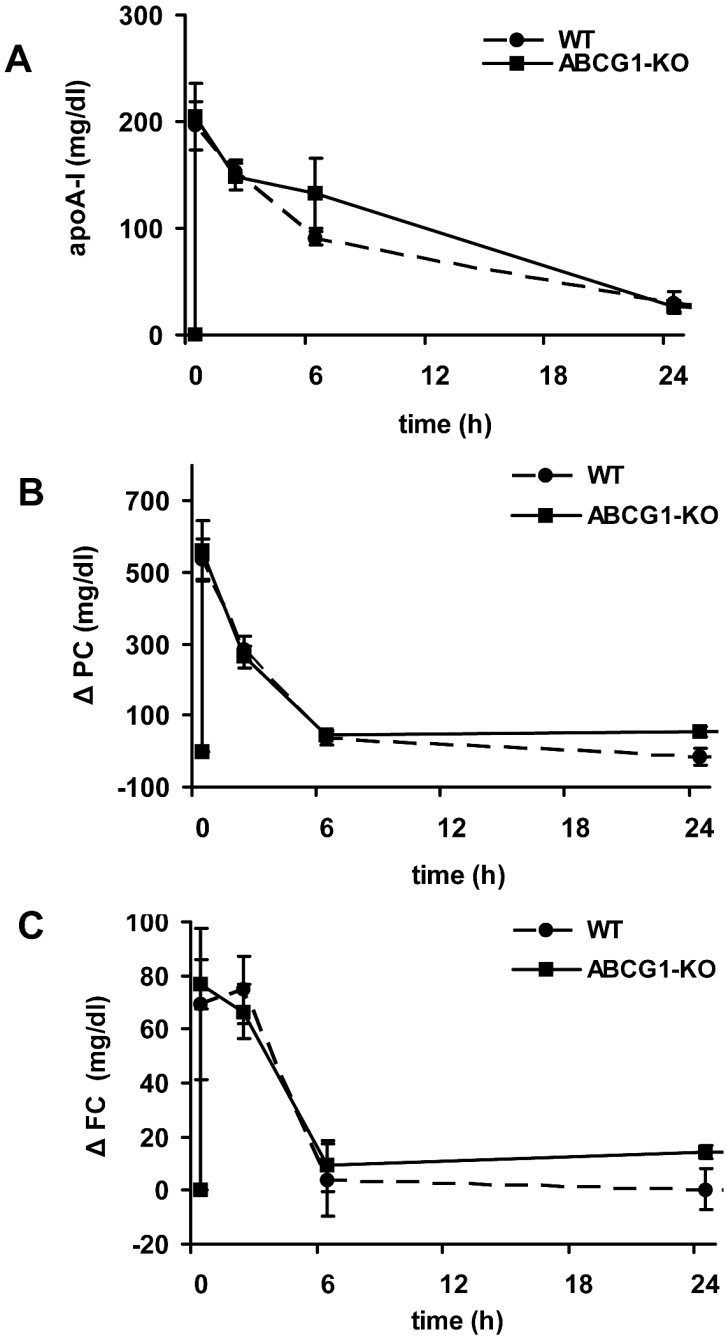

Figure 4.

Human apolipoprotein A-I (apoA-I, panel A), phosphatidylcholine (PC, panel B), and free cholesterol (FC, panel C) levels in serum from ABCG1 KO (n=5) and control (n=5) male mice before and 20 minutes, 2, 6 and 24 hours after i.v. administration of rHDL. PC and FC levels are expressed as change from baseline. See methods section for details. Solid line: KO mice; dashed line: control mice.

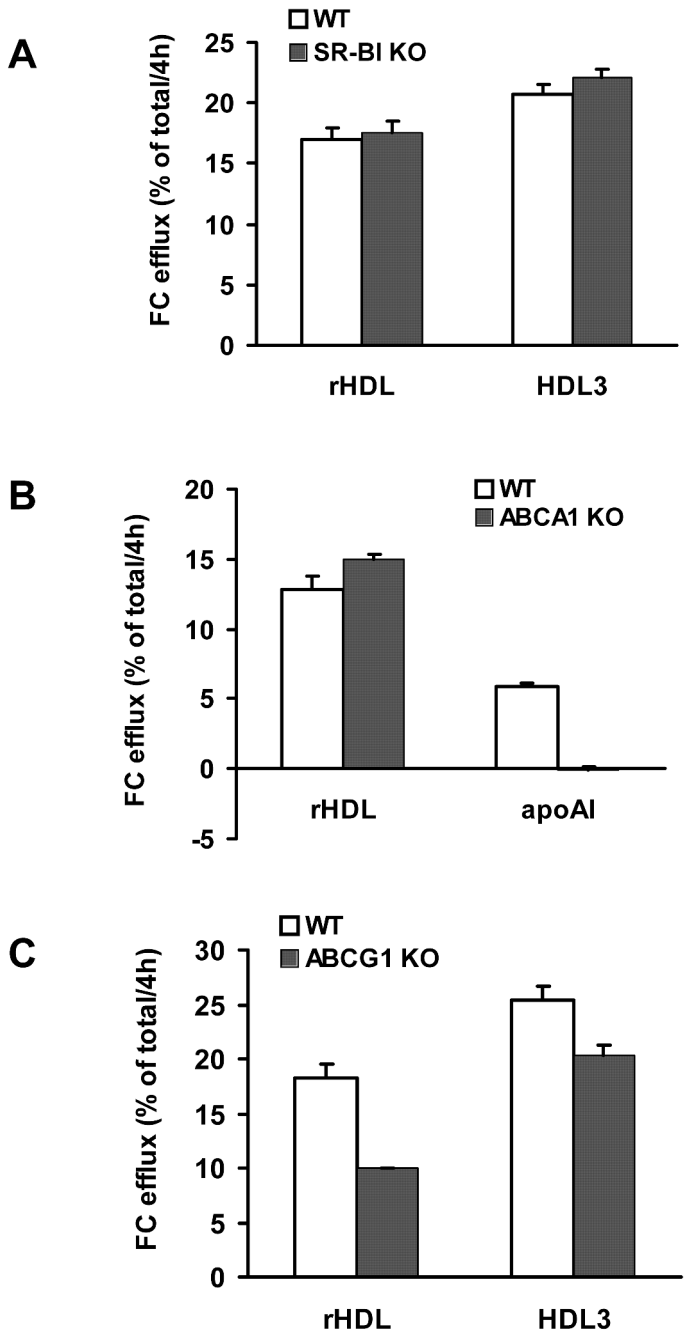

Figure 5.

In vitro cholesterol efflux from bone marrow macrophages obtained from SR-BI (panel A), ABCA1 (panel B) and ABCG1 (panel C) deficient mice and respective controls in the presence of rHDL as acceptor. Cells were labeled with [^{3}H]-cholesterol as described in the methods and incubated for 4 h with rHDL (25 μg/ml) and either free human apoA-I (10 μg/ml) or HDL3 (25 μg/ml) as controls. See methods section for details.

Arterioscler Thromb Vasc Biol. Author manuscript; available in PMC 2011 March 1.

Table 1

Kinetic parameters following administration of rHDL in mice

	apoA-I FCR pool/h	PC FCR pool/h	PC trans. mg/kg/h	FC FCR pool/h	FC trans. AUC (mg/kg)*h	Rapid FC trans. AUC (mg/kg)*h
C57BL/6 (n=8)	0.09 ± 0.01	0.67 ± 0.19	26.16 ± 5.89	0.95 ± 0.22	182.28 ± 50.86	23.11 ± 5.47
SRBI-WT (n=4)	0.04 ± 0.03	0.50 ± 0.07	30.39 ± 4.88	1.01 ± 0.06	241.00 ± 49.64	27.96 ± 9.99
SRBI-KO (n=3)	0.04 ± 0.01	0.37 ± 0.20	33.51 ± 19.60	0.18 ± 0.01	274.59 ± 15.25	1.01 ± 1.75
p value	0.88	0.27	0.76	<0.001	0.33	0.006
ABCA1-WT (n=4)	0.07 ± 0.01	0.51 ± 0.14	25.96 ± 6.89	1.25 ± 0.14	267.35 ± 39.93	53.86 ± 4.19
ABCA1-KO (n=4)	0.18 ± 0.04	0.54 ± 0.08	14.94 ± 2.96	1.36 ± 0.16	230.36 ± 35.36	63.95 ± 12.72
p value	0.002	0.73	0.03	0.36	0.21	0.18
ABCG1-WT (n=5)	0.08 ± 0.01	0.40 ± 0.04	16.05 ± 5.31	1.09 ± 0.12	311.74 ± 28.43	17.73 ± 10.06
ABCG1-KO (n=5)	0.07 ± 0.01	0.48 ± 0.04	21.21 ± 2.42	1.16 ± 0.15	311.02 ± 36.73	21.83 ± 2.75
p value	0.13	0.01	0.08	0.45	0.97	0.41

ApoA-I, human apolipoprotein A-I; FCR, fractional catabolic rate; PC, phosphatidylcholine; trans, transport; FC, free cholesterol; AUC, area under the curve; WT, control wild type mice; KO, knockout mice.

Values for C57BL/6 mice are shown for comparison.

p values refer to comparison between the wild type control mice (WT) and the knockout (KO) mice.