Adipocyte Modulation of High-Density Lipoprotein Cholesterol

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Background—Adipose harbors a large depot of free cholesterol. However, a role for adipose in cholesterol lipidation of high-density lipoprotein (HDL) in vivo is not established. We present the first evidence that adipocytes support transfer of cholesterol to HDL in vivo as well as in vitro and implicate ATP-binding cassette subfamily A member 1 (ABCA1) and scavenger receptor class B type I (SR-BI), but not ATP-binding cassette subfamily G member 1 (ABCG1), cholesterol transports in this process.

Methods and Results—Cholesterol efflux from wild-type, ABCA1−/−, SR-BI−/−, and ABCG1−/− adipocytes to apolipoprotein A-I (apoA-I) and HDL3 were measured in vitro. 3T3L1 adipocytes, labeled with 3H-cholesterol, were injected intraperitoneally into wild-type, ABCA1−/−, or SR-BI−/− mouse embryonic fibroblast adipocytes. The effect of tumor necrosis factor-α on transporter expression and cholesterol efflux was monitored during adipocyte differentiation. Cholesterol efflux to apoA-I and HDL3 was impaired in ABCA1−/− and SR-BI−/− adipocytes, respectively, with no effect observed in ABCG1−/− adipocytes. Intraperitoneal injection of labeled 3T3L1 adipocytes resulted in increased HDL-associated 3H-cholesterol in apoA-I transgenic mice but reduced levels in apoA-I−/− animals. Intraperitoneal injection of labeled ABCA1−/− or SR-BI−/− adipocytes reduced plasma counts relative to their respective controls. Tumor necrosis factor-α reduced both ABCA1 and SR-BI expression and impaired cholesterol efflux from partially differentiated adipocytes.

Conclusions—These data suggest a novel metabolic function of adipocytes in promoting cholesterol transfer to HDL in vivo and implicate adipocyte SR-BI and ABCA1, but not ABCG1, in this process. Furthermore, adipocyte modulation of HDL may be impaired in adipose inflammatory disease states such as type 2 diabetes mellitus. (Circulation. 2010;121:1347-1355.)

Key Words: adipocyte • arteriosclerosis • cholesterol • inflammation • lipoproteins

Low plasma high-density lipoprotein (HDL) cholesterol (HDL-C) is a key feature of obesity and insulin resistance and has a strong inverse relationship with atherosclerotic cardiovascular disease. Reduced cholesterol lipidation of nascent HDL results in small, immature lipoprotein particles that are rapidly catabolized and excreted in the kidney. Thus, lipidation of HDL plays a role in supporting the atheroprotective functions of HDL in vivo.

Clinical Perspective on p 1355

Lipidation of HDL is determined via a number of cholesterol transporters in several cholesterol-rich tissues. Although macrophage cholesterol efflux to HDL plays a major role in attenuating atherosclerosis, macrophages play a minor role in regulation of HDL-C levels. In contrast, hepatic ATP-binding cassette subfamily A member 1 (ABCA1), through lipidation of apolipoprotein A-I (apoA-I), is required for formation of nascent HDL particles. Indeed, in cholesterol-rich tissues, both hepatic and extrahepatic, ABCA1 has a role in the maintenance of plasma HDL-C. ATP-binding cassette subfamily G member 1 (ABCG1) mediates cholesterol efflux from macrophages to mature HDL particles and may play a role in regulating plasma HDL-C levels. In contrast to ABC transporters, scavenger receptor class B type I (SR-BI) is a bidirectional transporter that plays a major role in hepatic uptake of HDL.
cholesterol. Although SR-BI is not believed to be important in macrophage cholesterol efflux to HDL, a role in lipidating HDL via other peripheral SR-BI–expressing tissues has not been examined. In fact, the relative role of tissues, beyond liver, in HDL lipidation requires further definition.

Adipose tissue contains a very large pool of free cholesterol. In fact, adipocytes are known to support cholesterol efflux to HDL and apoA-I in vitro. Recent work shows that ABCA1 and SR-BI are expressed in mature adipocytes, and adipocyte cholesterol homeostasis may be regulated in a cell-specific manner. Adipocyte cholesterol, therefore, represents a uniquely regulated and abundant depot for modulation of HDL-C levels. By extension, adipose inflammatory dysfunction in insulin-resistant states may impair adipocyte HDL lipidation and reduce circulating HDL-C levels in such settings.

In this study, we demonstrate that adipocytes are a regulated source of cholesterol transfer to HDL both in vitro and in vivo. In contrast to liver and macrophages, adipocyte cholesterol efflux is controlled by ABCA1 and SR-BI, but not ABCG1, and is suppressed, in a differentiation-dependent manner, by tumor necrosis factor-α (TNF-α), an inflammatory adipocytokine. In summary, we report a novel, adipocyte-dependent mechanism of cholesterol transfer to HDL that may uniquely contribute to reduced plasma HDL-C in adipose inflammatory settings.

Methods

Cell Culture

3T3L1 cells were differentiated to adipocytes as described previously (see online-only Data Supplement). Efflux studies were performed on days 0, 5, and 10 after differentiation. Mouse embryonic fibroblasts (MEFs) were isolated from 13.5- to 14.5-day embryos as described (online-only Data Supplement). MEFs were grown to confluence before addition of differentiation media (as for 3T3L1 except addition of 1 μmol/L of peroxisome proliferator-activated receptor-γ agonist GW347845). Human Simpson-Golabi-Behmel syndrome (SGBS) adipocytes were cultured as described previously (see online-only Data Supplement). Bone marrow macrophages (BMMs) were isolated from mouse femurs and tibias and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 30% L929-conditioned medium.

Cholesterol Efflux Studies

Fully differentiated ABCA1−/−, ABCG1−/−, SR-BI−/−, and littermate, wild-type control MEFs as well 3T3L1 and SGBS adipocytes were labeled with 3H-cholesterol (5 μCi/mL) (Perkin-Elmer Analytic Sciences, Boston, Mass) for 24 hours. Wild-type BMMs were labeled with 5 μCi/mL 3H-cholesterol and loaded with 25 μg/mL acetylated low-density lipoprotein for 24 hours. After equilibration, 3H-cholesterol efflux from adipocytes and BMMs to apoA-I (20 μg/mL), HDL3 (50 μg/mL), 5% human (for SGBS cells) or 5% mouse (for 3T3L1, MEF, and BMM cells) serum, and MEM control was assessed over 4 hours as described previously for macrophages. In MEF adipocyte studies, cells were equilibrated overnight in MEM containing 0.2% bovine serum albumin plus liver receptor (LXR) agonist (10 μmol/L). Cells were subsequently cotreated with LXR agonist with or without probucol (20 μmol/L) for 2 hours before efflux. The effect of TNF-α (10 ng/mL overnight) on cholesterol efflux from 3T3L1 cells was measured at day 0 (preadipocyte), day 5 (∼50% differentiated), and day 10 (mature) of adipocyte differentiation. Cell lipid was extracted with isopropanol, and total cellular 3H-cholesterol was measured by liquid scintillation counting. Percent efflux to acceptors, minus MEM, is presented.

Adipocyte Transfer of Cholesterol to HDL In Vivo

We employed a modification of our published in vivo macrophage-to-HDL reverse cholesterol transport (RCT) model. Briefly, 3T3L1 adipocytes or MEF-adipocytes derived from SR-BI−/−, ABCA1−/−, or littermate wild-type mice were labeled with 5 μCi/mL 3H-cholesterol for 24 hours. Cells were washed, equilibrated in DMEM containing 0.2% bovine serum albumin for 6 hours, lifted off the plate by incubation with EDTA (10 mmol/L) for 10 minutes, centrifuged, and resuspended in MEM. Adipocyte levels of 3H-cholesterol were measured by liquid scintillation counting. Within individual studies, equal numbers of 3H-cholesterol counts (∼1.2×10⁶ cpm in MEFs and ∼3×10⁶ cpm in 3T3L1) and numbers of adipocytes (∼2×10⁶ MEFs and ∼7×10⁶ 3T3L1) were injected into each recipient C57BL/6 wild-type mouse. Movement of 3H-cholesterol from intraperitoneally injected adipocytes onto plasma HDL and through liver to bile/feces was monitored as described previously (see online-only Data Supplement). Animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Effects of Inflammation on Expression of Cholesterol Transporters in Adipose

Female C57BL/6 mice were injected intravenously with 10 μg/kg lipopolysaccharide, and after 6 hours, mice were euthanized by cervical dislocation. Adipose tissue was harvested. RNA and protein were isolated, and messenger RNA (mRNA) and protein expression of cholesterol transporters were assessed by real-time polymerase chain reaction and Western blotting, respectively (online-only Data Supplement).

General Laboratory Methods

A description of laboratory methods including lipoprotein analysis, quantitative real-time polymerase chain reaction, immunoblot analysis, and Oil red O staining is presented in the online-only Data Supplement.

Statistical Analysis

Data are reported as mean±SEM. For experiments with multiple treatments, ANOVA was used to test for differences in group means. For mouse experiments with multiple time points, we performed 2-way repeated-measures ANOVA to test for differences in means between groups. When ANOVA was significant, post hoc Bonferroni-corrected t tests were applied. For comparison of data between 2 groups at a single time point (liver, bile, feces, mRNA data), unpaired t tests were performed. GraphPad Prism 5 (GraphPad Software Inc, San Diego, Calif) and Stata 9.0 software (Stata Corp, College Station, Tex) were used for statistical analyses. Statistical significance is presented as *P<0.05, **P<0.01, and ***P<0.001 in all figures.

Results

Cholesterol Content and Cholesterol Transporter Expression Increase During Adipocyte Differentiation

During 3T3L1 differentiation from fibroblasts to adipocytes (Figure IA in the online-only Data Supplement), cellular cholesterol content increased ∼2-fold (Table IA in the online-only Data Supplement), whereas mRNA expression (reduced ΔCt values relative to β-actin) of ABCA1, ABCG1, and SR-BI all increased (Table IB in the online-only Data Supplement). In fully differentiated adipocytes, SR-BI was abundant, with expression similar to that of ABCA1 but with much lower levels of ABCG1. Immunoblotting showed increased SR-BI and ABCA1 protein during differentiation,
but ABCG1 protein was barely detectable in fully differentiated adipocytes (Figure IB and IC in the online-only Data Supplement).

Mature Mouse and Human Adipocytes Efflux Cholesterol to HDL Acceptors

Mature murine (3T3L1) and human (SGBS) adipocytes supported cholesterol efflux to lipid acceptors apoA-I, HDL3, and serum (Figure ID and IE in the online-only Data Supplement). Indeed, cholesterol efflux from mature adipocytes was comparable to that from BMMs in parallel efflux studies (Figure IF in the online-only Data Supplement). Efflux to MEM from 3T3L1 (0.77 ± 0.01%; n = 3) and SGBS (0.42 ± 0.044%; n = 3) in the absence of acceptor was minimal.

Adipocyte Cholesterol Efflux Is Mediated via ABCA1 and SR-BI Transporters

ABCA1+/−, ABCG1+/−, SR-BI−/−, and littermate wild-type control MEFs were differentiated into adipocytes (Figure IIA in the online-only Data Supplement), and cholesterol efflux studies were performed. Initially, the role of individual transporters was probed pharmacologically in wild-type MEF adipocytes. Probucol, an inhibitor of ABCA1-mediated efflux,27 reduced cholesterol efflux from MEF adipocytes to apoA-I and abolished LXR-enhanced cholesterol efflux to apoA-I (Figure 1A). Cholesterol efflux to HDL3 was reduced by BLT, an inhibitor of SR-BI,28 suggesting that adipocyte SR-BI mediates lipidation of mature HDL (Figure 1B).

Figure 1. MEF cells derived from wild-type, ABCA1+/−, or SR-BI−/− mice were differentiated and labeled overnight with 3H-cholesterol (5 μCi/mL). Cells were equilibrated and treated overnight ± LXR agonist (10 μmol/L) and cotreated ± BLT (10 μmol/L), or ± probucol (20 μmol/L) for 2 hours. Cholesterol efflux from wild-type (A and B), wild-type and ABCA1+/− (C and D), and wild-type and SR-BI−/− (E and F) MEF adipocytes to apoA-I (20 μg/mL) and HDL3 (50 μg/mL) over 4 hours is presented; background efflux to MEM was subtracted. Efflux is presented as percent total 3H-cholesterol loaded into cells (n = 3 to 4; *P < 0.05, **P < 0.01, ***P < 0.001 vs control).

Similar to probucol effects (Figure 1C, left panel), ABCA1 deficiency reduced efflux to apoA-I with complete attenuation of LXR-induced efflux to apoA-I (Figure 1C, right panel). Probucol had no incremental effect on efflux to apoA-I in ABCA1+/− adipocytes. In contrast, absence of ABCA1 had no impact on cholesterol efflux to HDL3 (Figure 1D). These data suggest that (1) ABCA1 plays a central role in adipocyte cholesterol efflux to apoA-I (consistent with macrophages13 and liver5,29); (2) LXR-induced adipocyte cholesterol efflux is mediated via ABCA1; and (3) adipocyte ABCA1 plays a minor role in efflux to mature HDL.

SR-BI deficiency had little effect on cholesterol efflux to apoA-I and did not influence inhibition of efflux to apoA-I by probucol (Figure 1E). Lack of adipocyte SR-BI, however, resulted in marked reduction in cholesterol efflux to HDL3 (Figure 1F, right panel); this inhibition was almost identical to the BLT effect on efflux to HDL in wild-type adipocytes (Figure 1F, left panel). BLT had no incremental effect on efflux to HDL in SR-BI−/− adipocytes. These data suggest that (1) SR-BI plays an important role in adipocyte cholesterol efflux to mature HDL particles (in contrast to findings in macrophages13) and (2) SR-BI does not mediate basal or LXR-induced adipocyte cholesterol efflux to apoA-I.

ABCG1 Does Not Regulate Adipocyte Cholesterol Efflux In Vitro

ABCG1 has been implicated in cholesterol efflux from macrophages8,13 and liver.10 Deficiency of ABCG1 in MEF adipocytes, however, had no effect on cholesterol efflux to
any lipid acceptor (Figure 2A). As expected, real-time polymerase chain reaction analysis revealed a marked reduction in ABCG1 mRNA in knockout cells (Figure 2B). However, ABCG1 protein expression was barely detectable in fully differentiated wild-type MEF adipocytes (Figure 2C). In fact, real-time analysis in MEFs, 3T3L1 adipocytes (Table I in the online-only Data Supplement), human-derived SGBS adipocytes, as well as mouse (Table II in the online-only Data Supplement) and human adipose (Table III in the online-only Data Supplement) revealed very low expression of ABCG1 compared with ABCA1 and SR-BI and suggests that ABCG1 plays little role in adipocyte cholesterol homeostasis.

Adipocytes Can Transfer Cholesterol to HDL In Vivo

We modified our macrophage-to-feces RCT model to examine adipocyte-to-HDL transport of cholesterol in vivo. We labeled fully differentiated adipocytes with $^3$H-cholesterol, injected labeled adipocytes intraperitoneally, and tracked movement of label onto plasma HDL and subsequently into liver and feces. First, we performed studies using 3T3L1 adipocytes injected into apoA-I transgenic, apoA-I deficient, or wild-type mice to establish proof of concept for this in vivo model. Movement of $^3$H-cholesterol from intraperitoneally injected adipocytes to plasma increased steadily in the first 24 hours (Figure 3A), tracked with the HDL fraction in each group (Figure 3C and 3D and Table 1), and resulted in detectable tracer in liver (not shown) and feces (Figure 3B). Overexpression of apoA-I increased plasma and HDL counts consistent with enhanced adipocyte-to-HDL cholesterol movement, whereas absence of apoA-I decreased plasma, HDL, and fecal counts, reflecting reduced movement of adipocyte label to HDL. Notably, the time course and extent of $^3$H-cholesterol movement from adipocytes to HDL and through liver to feces were similar to our published findings for intraperitoneal injection of macrophage foam cells in apoA-I transgenic and null mice. These findings suggest that cholesterol movement from adipocytes into plasma in vivo is modulated by the circulating levels of HDL acceptor particles.
Adipocyte ABCA1 and SR-BI Regulate Adipocyte Transfer of Cholesterol to HDL In Vivo

Movement of \(^3\text{H}\)-cholesterol from intraperitoneally injected MEF adipocytes derived from ABCA1\(^{+/−}\) (Figure 4A and 4B) and SR-BI\(^{+/−}\) (Figure 4C and 4D) mice into plasma, onto HDL, and into feces was significantly reduced compared with intraperitoneal injection of adipocytes derived from their wild-type littermates. As expected, the majority of plasma \(^3\text{H}\)-cholesterol counts was associated with the HDL fraction (Table 1). These studies indicate that adipocytes, acting via functional ABCA1 and SR-BI, can transfer cholesterol to HDL in vivo.

Inflammation Impairs Cholesterol Efflux From 3T3L1 Adipocytes

We examined whether TNF-\(\alpha\), an inflammatory adipokine,\(^{31}\) modulated 3T3L1 adipocyte cholesterol efflux to HDL, and into feces was significantly reduced compared with intraperitoneal injection of adipocytes derived from their wild-type littermates. As expected, the majority of plasma \(^3\text{H}\)-cholesterol counts was associated with the HDL fraction (Table 1). These studies indicate that adipocytes, acting via functional ABCA1 and SR-BI, can transfer cholesterol to HDL in vivo.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Percent (^3\text{H})-Cholesterol Associated With HDL</th>
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<tbody>
<tr>
<td>ApoA-I modulation</td>
<td></td>
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<tr>
<td>Wild-type</td>
<td>70.8 ± 1.83</td>
</tr>
<tr>
<td>ApoA-I transgenic</td>
<td>70.29 ± 5.21</td>
</tr>
<tr>
<td>ApoA-I(^{−/−})</td>
<td>54.98 ± 7.17</td>
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<tr>
<td>Adipocyte ABCA1 deletion</td>
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<tr>
<td>Wild-type MEF (ABCA1 study)</td>
<td>76.21 ± 1.14</td>
</tr>
<tr>
<td>ABCA1(^{−/−}) MEF</td>
<td>81.63 ± 1.36</td>
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<tr>
<td>Adipocyte SR-BI deletion</td>
<td></td>
</tr>
<tr>
<td>Wild-type MEF (SR-BI study)</td>
<td>82.35 ± 4.06</td>
</tr>
<tr>
<td>SR-BI(^{−/−}) MEF</td>
<td>81.51 ± 5.83</td>
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Plasma was depleted of apolipoprotein B–containing lipoproteins by polyethylene glycol precipitation; counts in plasma before and after polyethylene glycol precipitation were determined, and the percent counts in HDL fraction were calculated. Data are presented as mean ± SEM; \(n = 3\) for in vivo studies of apoA-I modulation, adipocyte ABCA1 deletion, and adipocyte SR-BI deletion.
SR-BI, but not ABCG1, in adipocyte cholesterol efflux to apoA-I and mature HDL, respectively, and provide experimental evidence that both ABCA1 and SR-BI can regulate adipocyte cholesterol transfer to HDL in vivo. Finally, we show that adipocyte inflammation downregulates transporters and impairs adipocyte cholesterol efflux to HDL. Because adipose inflammation is a hallmark of central obesity and type 2 diabetes mellitus, loss of adipocyte lipidation of HDL may directly contribute to lower HDL-C in these adipose inflammatory states.

Lipidation of HDL particles in vivo involves the coordinated effect of several tissues\(^6,7\) likely involving cell-specific transporter functions. ABCA1 plays a major role in generation of nascent HDL particles\(^5,36\) and maintenance of plasma transporter functions. ABCA1 plays a major role in generation of HDL particles in vivo, primarily because of loss of hepatic lipidation of liver-secreted apoA-I. However, peripheral ABCA1 also contributes to HDL-C\(^6\) through intestinal\(^7\) and brain\(^7\) ABCA1. Although macrophage cholesterol efflux to HDL plays a major role in attenuating atherosclerosis, macrophages contain a very small pool of cholesterol and do not regulate circulating HDL-C in vivo.\(^4\) Because adipose tissue contains a large pool of free cholesterol,\(^15,16\) we hypothesized that adipocytes may play a unique role in cholesterol transfer to HDL both in vitro and in vivo. In fact, a role for adipose or involvement of non-ABCA1 transporters in peripheral lipidation of HDL has not been demonstrated.

Our findings support a model of adipocyte-specific regulation of cholesterol efflux to HDL acceptors. We identified a role for ABCA1 and SR-BI transporters in efflux to apoA-I and HDL, respectively, and demonstrated marked upregulation of these proteins during adipocyte differentiation. Although ABCG1 promotes macrophage cholesterol efflux to mature HDL,\(^8,9,38\) we found no evidence that ABCG1 protein is expressed in mature adipocytes or plays a role in adipocyte cholesterol efflux to HDL.

Using a modified version of our published macrophage-to-HDL RCT model,\(^12,29\) we demonstrate that adipocytes are capable of transferring cholesterol to circulating HDL. Indeed, the time course and extent of cholesterol label movement onto HDL were similar to those of macrophages\(^26,39,40\).
Furthermore, cholesterol movement from intraperitoneally injected adipocytes to HDL was increased in apoA-I transgenic mice and reduced in apoA-I null mice. Adipocyte deficiency of ABCA1 or SR-BI reduced tracer movement onto HDL in vivo. Overall, our data provide indirect evidence for adipocyte regulation of HDL-C in vivo and suggest a role for SR-BI and ABCA1, but not ABCG1, in this process.

Recent studies suggest an underappreciated role for adipocyte cholesterol in adipose function and pathophysiologies. Zhao et al showed that primary adipocytes, isolated from rabbits fed a high-cholesterol diet or treated with statins, had altered cholesterol efflux to HDL that correlated with changes in SR-BI expression. They did not prove, however, that SR-BI was causal. Verghese and colleagues demonstrated that enhanced adipocyte cholesterol efflux to HDL occurs during lipolysis without change in SR-BI and ABCA1 expression. It is possible, however, that modulation of transporter function or membrane localization rather than change in protein level could mediate this efflux. Our studies provide novel data that go beyond prior correlative studies. We addressed directly the role of specific transporters and performed in vivo studies examining the potential for adipocytes and specific transporters to transfer cholesterol to HDL in vivo. Future work with adipose-specific, conditional modulation of ABCA1 and SR-BI in rodent models is required to confirm the importance of these transporters and adipose regulation of HDL-C mass in vivo.

Our in vivo experimental model has limitations, including its nonphysiological nature, use of exogenous cells, and reliance on cholesterol tracer rather than mass. The peritoneal space is a convenient experimental location in which cells are exposed to extracellular fluid that has many of the characteristics of extracellular fluid in other tissues. Importantly, this model has provided fundamental insights into the macrophage RCT process. Work by Sehayek and Hazen demonstrates that the subcutaneous administration of macrophages provides a pattern of RCT similar to that of the peritoneal cavity, arguing against any unique properties for the peritoneum. Although adipocytes do not occur as single cells in the peritoneum, intraperitoneal injection of labeled adipocytes resulted in cholesterol label movement to plasma HDL that was remarkably similar to that published for macrophages. Therefore, we doubt that there is a systematic difference between adipocytes and macrophages in the intraperitoneal model.

We examined the impact of an inflammatory adipocytkine on adipocyte cholesterol efflux to explore whether loss of adipocyte HDL lipidation is a possible mechanism for reduced HDL-C in adipose inflammatory settings. Because adipocyte susceptibility to inflammation depends on adipocyte maturity, we examined TNF-α effects during differentiation. TNF-α impaired cholesterol efflux most in partially differentiated adipocytes coincident with greatest suppression of ABCA1 and SR-BI. This is consistent with work by Chung et al, who reported that endotoxin impaired glucose transport maximally in partially differentiated adipocytes. We also found that endotoxemia downregulated adipose SR-BI and ABCA1 in vivo. Thus, despite increased adipose mass and adipose cholesterol in obesity, attenuation of adipocyte-mediated HDL lipidation may directly contribute to lower HDL-C in metabolic syndrome and type 2 diabetes mellitus (Figure 6).

In conclusion, adipocytes support transfer of cholesterol to HDL in vivo. This process is mediated by ABCA1 and SR-BI,
but not ABCG1, and is attenuated in inflamed adipocytes. Our findings suggest adipocyte-specific cholesterol transporter functions and a role for mature adipose in maintenance of HDL-C levels. Conversely, adipose inflammation may attenuate adipocyte lipidation of HDL, leading to lower HDL-C in metabolic syndrome and type 2 diabetes mellitus.

Acknowledgments

We would like to gratefully acknowledge Aisha Wilson, Maosen Sun, Edwige Edouard, and Leticia Pruscino for their technical expertise.

Sources of Funding

This work was supported by the Alternative Drug Discovery Initiative award to the University of Pennsylvania from GlaxoSmithKline, a Clinical and Translational Science Award (UL1RR024134) from the National Center for Research Resources, and a Diabetes and Endocrine Research Center (P20-DK 019525) award, both to the University of Pennsylvania, as well as P50 HL-083799-SCCOR (Dr Reilly).

Disclosures

Dr Reilly is the recipient of research grants from GlaxoSmithKline and Merck Research Laboratories. The other authors report no conflicts.

References


**CLINICAL PERSPECTIVE**

Adipose tissue harbors a major pool of free cholesterol, but its role in regulating circulating high-density lipoprotein (HDL) cholesterol is poorly understood. In this work, we present the first evidence that adipocytes transfer cholesterol to HDL in vivo as well as in vitro. We identified a differentiation-dependent role for the lipid transporters ATP-binding cassette subfamily A member 1 and scavenger receptor class B type I, but not ATP-binding cassette subfamily G member 1, in adipocyte cholesterol efflux to apolipoprotein A-I and mature HDL, respectively. We also provide experimental evidence that both ATP-binding cassette subfamily A member 1 and scavenger receptor class B type I can regulate adipocyte cholesterol transfer to HDL in vivo. Finally, we show that adipocyte inflammation downregulates transporters and impairs adipocyte cholesterol efflux to HDL. Our findings suggest a role for mature adipose in directly maintaining HDL cholesterol levels. Conversely, adipose inflammation may attenuate adipocyte lipidation of HDL and may directly contribute to lower HDL cholesterol in adipose inflammatory states such as central obesity and type 2 diabetes mellitus. Thus, adipose tissue cholesterol homeostasis may be a direct therapeutic target for modulation of HDL levels in vivo.
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Circulation. 2010;121:1347-1355; originally published online March 8, 2010;
doi: 10.1161/CIRCULATIONAHA.109.897330
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the
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SUPPLEMENTAL DATA

Supplemental Methods:

Materials

3T3L1 cells were obtained from American Type Culture Collection (ATCC, Manassas, Ca). Simpson–Golabi–Behmel syndrome (SGBS) human pre-adipocytes were a gift from Dr Martin Wabitsch, University of Ulm, Germany. GIBCO Dulbecco’s Modified Eagle medium (DMEM) and minimum essential medium (MEM) were purchased from Life Technologies (Carlsbad, CA). SR-BI/- and ABCA1/- mice were purchased from Jackson Laboratories (Bar Harbour, Maine) and bred using heterozygous pairs. Wild-type litter-mates were used as age-matched controls. ABCG1/- mice were purchased from Deltagen and then backcrossed 5 generations into the Wild-Type (WT) C57BL/6 background as previously described. ApoA-I transgenic, apoA-I deficient (-/-), and wild-type C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbour, Maine). Male mice were used in all in vivo studies. The LXR agonist (GW3965) and PPARγ agonist (GW347845) were kindly provided by GlaxoSmithKline (King of Prussia, PA). The LXR agonist, 22r-hydroxycholesterol, and probucol were from Sigma Aldrich, BLT-1 was from ChemBridge (San Diego, CA), and recombinant murine TNFα was from R&D biosystems (Minneapolis, MN). Antibodies to ABCA1, ABCG1, SR-BI and β-actin were purchased from Novus Biologicals (Littleton, CO). Human apoA-I and HDL3 were generously donated by Dr. George Rothblat. All other reagents, unless otherwise stated were purchased from Sigma Aldrich (St Louis, MO).
Cell culture

3T3L1 cells were differentiated to adipocytes as described previously\textsuperscript{2}. Briefly, confluent cells were incubated in differentiation media (DMEM, 10% fetal bovine serum, insulin (1.7µM), dexamethasone (1µM), isobutylmethylxanthine (IBMX) (500µM)) for 7 days. Efflux studies were performed on days 0, 5 and 10 post-differentiation. Mouse embryonic fibroblasts (MEF) were isolated from 13.5 -14.5 day embryos. The head and soft tissues were removed and embryonic fibroblast tissue cultured as previously described\textsuperscript{3}. The embryonic tissue was minced, digested with trypsin for 10min prior to addition of 10mL of maintenance media (DMEM, 10% FBS, 1% glutamine, 1% non-essential amino acid and penicillin/streptomycin). Cells were filtered through a 100µm nylon mesh and seeded in either 24-well plates for efflux studies or 150mm dishes for \textit{in vivo} adipocyte-reverse cholesterol transport studies. MEFs were grown to confluence prior to addition of differentiation media (as for 3T3L1 except addition of 1µM of GW347845). Human SGBS adipocytes were cultured as previously described\textsuperscript{4}. Briefly, confluent cells were incubated in differentiation media (DMEM/F12, panthothenate (4mg/L), biotin (8mg/L), insulin (20nM), hydrocortisone (1µM), dexamethasone (250nM), isobutylmethylxanthine (IBMX) (500µM), GW347845 (1µM), T3 (0.2nM), human transferrin (10mg/L), and penicillin/streptomycin) for 7 days. Differentiation media was replaced with 3FC media (differentiation media excluding PPAR\textsubscript{γ} agonist and dexamethasone) for a further 7 days. Bone-marrow macrophages (BMM) were isolated from mouse femurs and tibias and cultured in DMEM supplemented with 10% FBS and 30% L929 conditioned medium\textsuperscript{1,5}.
Adipocyte-dependent HDL lipidation in vivo

We employed a modification of our published in vivo macrophage to HDL reverse cholesterol transport (RCT) model\textsuperscript{15, 42}. Briefly, fully differentiated 3T3L1 adipocytes, or MEF-adipocytes derived from SR-BI/-, ABCA1/- or litter mate wild-type mice, were labeled with 5µCi/mL \textsuperscript{3}H-cholesterol for 24h. Cells were washed, equilibrated in DMEM containing 0.2% BSA for 6h, lifted off the plate by incubation with EDTA (10mM) for 10min, centrifuged and resuspended in MEM. Adipocyte levels of \textsuperscript{3}H-cholesterol were measured by liquid scintillation counting. Within individual studies, equal numbers of \textsuperscript{3}H-cholesterol counts (~1.2x10\textsuperscript{6} cpm in MEF and ~3x10\textsuperscript{6} cpm in 3T3L1) and numbers of adipocytes (~2x10\textsuperscript{6} MEFs and ~7x10\textsuperscript{6} 3T3L1) were injected into each recipient mouse. Movement of \textsuperscript{3}H-cholesterol from intra-peritoneal injected adipocytes onto plasma HDL and through liver to bile/feces was monitored. Blood, collected via the retro-orbital plexus from anesthetized mice, was centrifuged for plasma. At 48h mice were sacrificed by cervical dislocation\textsuperscript{43} and \textsuperscript{3}H-cholesterol levels in plasma and extracted lipids were counted. Feces (0-48h) were collected and soaked overnight in ddH\textsubscript{2}O (100mg feces/1mL), ethanol added, samples homogenized and free cholesterol isolated by hexane extraction\textsuperscript{15, 42}. \textsuperscript{3}H-label counts in plasma and feces are expressed as a percentage of total \textsuperscript{3}H-cholesterol injected. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

General laboratory methods

**Lipoprotein profile analysis** - Plasma levels of total and HDL cholesterol of wild-type, apoA-I transgenic and apoA-I -/- mice were measured enzymatically (Wako Diagnostics, Richmond, VA) on a Cobas Fara analyzer (Roche Diagnostics Systems Inc). Pooled plasma (150µl) was separated by fast protein liquid chromatography (FPLC) gel filtration (Amersham Pharmacia
Biotech, Uppsala, Sweden). Cholesterol mass in FPLC fractions was measured enzymatically (Wako Pure Chemical Industries Ltd, Osaka, Japan) and $^3$H-tracer distribution across lipoprotein fractions was determined by liquid scintillation counting. Plasma from C57BL/6 mice injected with wild-type, ABCA1-/- or SR-BI-/- adipocytes was depleted of apoB-containing lipoproteins by polyethylene glycol (PEG) precipitation. Levels of $^3$H-tracer in this apoB-depleted fraction vs. total plasma were measured to estimate the percentage of $^3$H-cholesterol associated with HDL (Table 1).

**RNA isolation and quantitative Real-time PCR analysis** - Adipocytes ± indicated treatments were harvested in Trizol (Invitrogen, CA) and RNA isolated according to manufacturer instructions and quantified spectrophotometrically. RNA (500ng) was reverse transcribed using an Applied Biosystems High Capacity cDNA archive kit. Expression levels of various genes were assessed by quantitative Real-Time PCR using an Applied Biosystems 7300 sequence detector. Primers, probes and TaqMan Universal Mastermix were purchased from Applied Biosystems (ABI, CA). To control for between-sample variability, mRNA levels were normalized to β-actin for each sample by subtracting the C$_t$ for β-actin from the C$_t$ for the gene of interest, producing a ΔC$_t$ value. The ΔC$_t$ for each treatment sample was compared to the mean ΔC$_t$ for control samples using the relative quantitation $2^{-(ΔΔC)}$ method to determine fold-change.

**Immunoblotting** - Adipocytes were incubated ± treatments for indicated time-points prior to lysing in RIPA buffer. **Adipose tissue was harvested from mice after 24h treatment ± LPS. Adipose tissue was homogenized in RIPA buffer (100mg/ml).** Protein concentration was determined by the Pierce bicinechoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, IL). Equal concentration of protein (10μg/lane) were reduced, separated by SDS-PAGE electrophoresis, transferred to nitrocellulose membranes and blocked in 5% non-fat dry
milk in TBS-tween for 1h prior to overnight incubation at 4°C in primary antibody. Blots were probed with ABCA1, ABCG1, SR-BI (Novus Biologicals, Littleton, CO) or β-actin control (Abcam, MA). Blots were washed, incubated in secondary antibody and visualized by chemiluminescence.

**Fibroblasts and Adipocyte Lipid Analysis** - At indicated times during fibroblast differentiation to adipocytes, cellular cholesterol and triglycerides levels were measured enzymatically using the affinity triglyceride assay kit (Thermo Electron, CO) and the Cholesterol E assay kit (Wako Diagnostics, Richmond, VA) respectively. Cholesterol and triglyceride levels were expressed per mg of cellular protein. Protein concentration was quantified by Pierce bixinchonic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, IL). At indicated days after initiation of adipocyte differentiation cells were fixed in formalin prior to staining with oil red stain for 1h at room temperature. Cells were washed and visualized under at x100 magnification using a Nikon diaphot 300 fluorescent microscope.

**Human Subjects Study Protocol**
Healthy volunteers (N=20, 50% female, aged 18-40), without medical history, tobacco or prescription medication use, or laboratory abnormality were recruited from the Delaware Valley region as previously described. Adipose tissue biopsies were sampled from the volunteers, RNA extracted and subjected to RT-PCR and subsequent quantitative PCR (qPCR) on an Applied Biosystems 7300 Real-Time PCR System (ABI, Foster City, CA) for measurement of ABCA1, ABCG1 and SR-BI and house-keeping gene β-actin. PCR reactions used TaqMan Universal PCR Master Mix (ABI, Foster City, CA) under standard conditions. To control for
between-sample differences in total mRNA, mRNA levels were normalized to β-actin for each sample by subtracting the C_t for β-actin from the C_t for the gene of interest, producing a ΔC_t value presented in Supplement Table 2B. The Penn Institutional Review Board (IRB) approved the study and written informed consent was provided.
SUPPLEMENT FIGURES

Supplement Figure 1: (A) Oil Red staining of 3T3L1 adipocytes during differentiation. (B) Protein levels of ABCA1 and SR-BI in 3T3L1 adipocytes measured over time. (C) Protein levels of ABCG1 in mature 3T3L1 adipocytes ± overnight treatment with LXR agonist (10µM). Positive control lysates from ABCG1 over-expressing BHK cells and macrophage were run alongside. (D) 3T3L1 adipocytes (E) SGBS human adipocytes and (F) acLDL-loaded (25µg/ml) bone marrow derived macrophages (BMM) were labeled with \(^{3}H\)-cholesterol (5µCi/ml) overnight, equilibrated and efflux to apoA-I (20µg/ml), HDL3 (50µg/ml) and 5% serum monitored over a 4h period. Efflux to MEM was calculated and subtracted from all values to account for background efflux (n=3-4, ***p<0.001 vs. efflux to MEM).

Supplement Figure 2: (A) Oil Red staining of WT MEF adipocytes during differentiation. (B) Genotyping analysis of wild-type, ABCA1 +/- and ABCA1-/- animals according to Jackson Laboratories protocol. (C) Hepatic protein lysates from wild-type and SR-BI-/- animals were separated by SDS-page and immunoblotted for SR-BI protein expression. (D) C57BL/6 mice were injected with LPS (10µg/kg, IV) or saline and were sacrificed after 24h. Adipose tissue was harvested and protein isolated. Immunoblot analysis of ABCA1, SR-BI and β-actin expression.
Supplement Table 1. (A) Cellular triglyceride and cholesterol content and (B) ABCA1, ABCG1 and SR-BI mRNA levels during 3T3L1 differentiation. The ΔCt value between gene of interest and house-keeping β-actin gene is presented. Data presented as mean ± SEM, n=4.

(A)  

<table>
<thead>
<tr>
<th></th>
<th>Pre-adipocyte</th>
<th>Mature adipocyte</th>
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<tr>
<td>Triglyceride (µg/mg protein)</td>
<td>8.64 ± 2.78</td>
<td>148.19 ± 48.95</td>
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<tr>
<td>Cholesterol (µg/mg protein)</td>
<td>20.77 ± 2.23</td>
<td>47.76 ± 11.48</td>
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(B)  

<table>
<thead>
<tr>
<th>Differentiation state</th>
<th>ABCA1</th>
<th>SR-BI</th>
<th>ABCG1</th>
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<tr>
<td>Pre-adipocyte</td>
<td>4.72 ± 0.14</td>
<td>5.16 ± 0.18</td>
<td>9.02 ± 0.26</td>
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<td>50% differentiated</td>
<td>2.32 ± 0.26</td>
<td>0.93 ± 0.11</td>
<td>5.44 ± 0.23</td>
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<tr>
<td>Mature adipocyte</td>
<td>1.47 ± 0.19</td>
<td>0.37 ± 0.16</td>
<td>4.01 ± 0.11</td>
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</table>
**Supplement Table 2:** C57BL/6 mice were injected with LPS (10µg/kg, IV) or saline and were sacrificed after 6h. Adipose tissue was harvested and RNA isolated, cDNA synthesized and real-type PCR analysis of cholesterol transporter expression examined. The ΔCt value between gene of interest and house-keeping β-actin gene is presented ± LPS treatment. Data presented as mean ± SEM, **p<0.01, n=5.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>LPS</th>
<th>P-value</th>
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<tr>
<td>ABCA1</td>
<td>3.36 ± 0.47</td>
<td>5.29 ± 0.19</td>
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</tr>
<tr>
<td>SR-BI</td>
<td>3.49 ± 0.32</td>
<td>5.29 ± 0.38</td>
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<tr>
<td>ABCG1</td>
<td>6.22 ± 0.32</td>
<td>6.31 ± 0.19</td>
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</table>
**Supplement Table 3:** Cholesterol transporter mRNA expression in human subcutaneous adipose tissue and human SGBS adipocytes. The ΔCt value between gene of interest and house-keeping β-actin gene is presented, mean ± SEM, n=14.

<table>
<thead>
<tr>
<th>Delta Ct for gene of interest</th>
<th>ABCA1</th>
<th>SR-BI</th>
<th>ABCG1</th>
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</thead>
<tbody>
<tr>
<td>Human Adipose</td>
<td>5.16 ± 0.16</td>
<td>5.9 ± 0.16</td>
<td>8.66 ± 0.24</td>
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<tr>
<td>SGBS Adipocytes</td>
<td>3.32 ± 0.09</td>
<td>3.79 ± 0.17</td>
<td>7.01 ± 0.49</td>
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</tbody>
</table>
References


Supplement Figure 1

A. Time course of 3T3L1 differentiation: day 2, day 4, day 6, day 10

B. ABCA1 (254kDa), SR-BI (82kDa), β-actin (42kDa)

C. ABCG1 (90kDa)

D. 3T3L1 (% efflux/4h) for ApoA1, HDL3, Serum

E. SGBS (% efflux/4h) for ApoA1, HDL3, Serum

F. Bone-marrow macrophages (% efflux/4h) for ApoA1, HDL3, Serum
Supplement Figure 2

A. Time-course of MEF differentiation:

Day 0                               Day 5                              Day 10

A. Wild-type SR-BI-/-

B. PCR Products

HET = 750 bp and 540 bp
MUT = 540 bp
WT = 750 bp

ABCA1+/-  ABCA1+/+  ABCA1-/-

B. 750bp  540bp

C. SR-BI (82kDa)

Wild-type SR-BI/-

D. ABCA1 (254kDa)

SR-BI (82kDa)

β-actin (42kDa)

LPS (10µg/kg):  -  -  +  +