

Nascent HDL formation in hepatocytes and role of ABCA1, ABCG1, and SR-BI^S

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Abstract To study the mechanisms of hepatic HDL formation, we investigated the roles of ABCA1, ABCG1, and SR-BI in nascent HDL formation in primary hepatocytes isolated from mice deficient in ABCA1, ABCG1, or SR-BI and from wild-type (WT) mice. Under basal conditions, in WT hepatocytes, cholesterol efflux to exogenous apoA-I was accompanied by conversion of apoA-I to HDL-sized particles. LXR activation by T0901317 markedly enhanced the formation of larger HDL-sized particles as well as cellular cholesterol efflux to apoA-I. Glyburide treatment completely abolished the formation of 7.4 nm diameter and greater particles but led to the formation of novel 7.2 nm-sized particles. However, cells lacking ABCA1 failed to form such particles. ABCG1-deficient cells showed similar capacity to efflux cholesterol to apoA-I and to form nascent HDL particles compared with WT cells. Cholesterol efflux to apoA-I and nascent HDL formation were slightly but significantly enhanced in SR-BI-deficient cells compared with WT cells under basal but not LXR activated conditions. As in WT but not in ABCA1-deficient hepatocytes, 7.2 nm-sized particles generated by glyburide treatment were also detected in ABCG1-deficient and SR-BI-deficient hepatocytes. **Our data indicate that hepatic nascent HDL formation is highly dependent on ABCA1 but not on ABCG1 or SR-BI.**—Ji, A., J. M. Wroblewski, L. Cai, M. C. de Beer, N. R. Webb, and D. R. van der Westhuyzen. **Nascent HDL formation in hepatocytes and role of ABCA1, ABCG1, and SR-BI.** *J. Lipid Res.* 2012. 53: 446–455.

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HDL constitutes a heterogeneous group of particles differing in density, size, electrophoretic mobility, lipid

composition and apolipoprotein content. Numerous epidemiological studies indicate that HDL particles serve an anti-atherogenic function in that high levels of HDL cholesterol are associated with a decreased risk of atherosclerosis (1). An important atheroprotective effect of HDL is its ability to remove excess cholesterol from peripheral tissues and deliver it to the liver for biliary excretion by a process called reverse cholesterol transport (RCT) (2–4). Beyond promotion of RCT, other properties of HDL, including anti-inflammatory, anti-oxidative, anti-thrombotic and anti-apoptotic features of HDL, also contribute to its anti-atherogenic function (5). However, the fundamental mechanisms underlying the biogenesis and maintenance of plasma HDL levels are not well understood.

ABCA1 is recognized as the principal molecule involved in cholesterol efflux from macrophage foam cells (6). It is expressed in a variety of cell types, including hepatocytes and macrophages and is highly upregulated upon lipid loading through the activation of the nuclear liver X receptors (LXR α and/or LXR β) (7, 8). The absence of functional ABCA1 in Tangier disease patients results in severe HDL deficiency and deposition of cholesteryl esters (CE) in the reticulo-endothelial system (9–11). HDL deficiency and macrophage foam cell accumulation are also found in mice lacking ABCA1 in the liver (12). The observed HDL deficiency is a direct result of a severely impaired lipidation of apoA-I via the ABCA1 pathway; therefore, this pathway is not only important for lipid efflux from both peripheral and hepatic cells but also for the biogenesis of nascent HDL and maintenance of plasma HDL levels (13). The generation of nascent apoA-I-containing particles has been studied in various cell lines. Incubation of exogenous

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Abbreviations: BHK, Baby hamster kidney; CE, cholesteryl ester; CHO, Chinese hamster ovary; GGE, gradient gel electrophoresis; LXR α , liver X receptor α ; LXR β , liver X receptor β ; RCT, reverse cholesterol transport; rHDL, reconstituted HDL; RXR, retinoid X receptor; SR-BI, class B scavenger receptor type 1; WT, wild-type.

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apoA-I with fibroblasts, CaCo-2, or Chinese hamster ovary (CHO)-overexpressing ABCA1 cells generated a series of α -migrating apoA-I containing particles with diameters of 8–20 nm. The generation of such nascent HDL particles is dependent on ABCA1 because cells lacking ABCA1 or expressing an inactive ABCA1 mutant (Q597R) were unable to form such particles (14–17). Interestingly, incubation of exogenous apoA-I with either HepG2 or macrophages generated not only α -migrating but also pre β ₁-migrating particles, suggesting the presence of a link between specific cell types and the speciation of nascent HDL particles (15). The formation of nascent HDL particles has also been studied in primary hepatocytes. Analyzing culture medium of hepatocytes from ABCA1-deficient mice demonstrated a lack of nascent HDL production (16) or markedly reduced production of qualitatively similar particles (18). The mechanisms of the formation of nascent HDL particles in hepatocytes remain unclear.

In addition to ABCA1, another ABC transporter, ABCG1, has been shown to contribute to cholesterol efflux from macrophages (19). Newly formed nascent HDL particles generated through ABCA1 action were shown to function as efficient acceptors for ABCG1-mediated cholesterol efflux. A synergistic relationship between ABCA1 and ABCG1 in promoting cholesterol efflux has been proposed (20, 21). The role of ABCG1 in regulating HDL levels is uncertain. In chow-fed animals, ABCG1 did not influence HDL levels, possibly due to its low level of expression under these conditions (22). In contrast, ABCG1-deficient mice were shown to exhibit decreased plasma HDL cholesterol levels when fed a high-cholesterol diet (22). Other studies, however, failed to show altered HDL levels in ABCG1 deficient mice even when fed a high-fat diet, or in ABCG1 transgenic mice (23–25). These studies did provide evidence for a role of hepatic ABCG1 in regulating both biliary cholesterol secretion (22) and lipid accumulation (24). However, the role of ABCG1 in the process of hepatic HDL formation has not been examined.

Class B scavenger receptor type 1 (SR-BI) is recognized primarily as an HDL receptor. SR-BI is abundantly expressed in the liver where it functions to deliver HDL CE into liver for cholesterol secretion (26). SR-BI can also promote cellular cholesterol efflux to HDL (27). However, SR-BI has been reported to inhibit ABCA1-mediated cholesterol efflux in macrophage possibly through its ability to mediate cellular cholesterol uptake (28). The possible effects of SR-BI on HDL biogenesis have not been studied. In this study, we investigated the roles of these cholesterol transporters in cholesterol efflux and nascent HDL biogenesis in primary hepatocytes.

MATERIALS AND METHODS

Animals and cells

Liver-specific ABCA1-knockout mice (albumin Cre⁺, ABCA1^{fllox/fllox}) were provided by J. S. Parks (29) with C57BL/6 background. ABCG1-knockout mice (ABCG1^{-/-}) from Deltagen with mixed C57BL/6 \times 129 backgrounds were backcrossed to a C57BL/6 background (>99.9%). SR-BI-knockout mice (SR-BI^{-/-}) were

obtained from M. Krieger (26). SR-BI homozygous (SR-BI^{-/-}) mutant mice and wild-type (WT) mice (both 1:1 mixed C57BL/6 \times 129 backgrounds) were bred from mice generated from a common mating pair of SR-BI heterozygous (SR-BI^{+/-}) mice. All animals were fed on a standard chow pellet diet with free access to water and maintained on a 12 h light-dark cycle. For all animal experiments, 8- to 12-week-old male mice, weighing 20–25 g, were used. All animal experiments were approved by the Veterans Affairs Medical Center Institutional Animal Care and Use Committee.

Baby hamster kidney (BHK) cells expressing human ABCA1 or human N-terminal FLAG-tagged ABCG1 were a generous gift from J. F. Oram (30, 31). They were generated using the mifepristone-inducible GeneSwitch system. Cells were grown in DMEM supplemented with 10% FBS, 1% penicillin and streptomycin, as well as 2 mM glutamine. ABCA1 and ABCG1 were induced with overnight incubation of cells with DMEM containing 0.2% fatty acid-free BSA and 10 nM mifepristone (Invitrogen). All cell culture incubations were performed at 37°C in a humidified 5% CO₂ incubator.

Preparation of human HDL₃ and rHDL

HDL (d = 1.063–1.21 g/ml) was isolated from human plasma by density gradient ultracentrifugation. Human HDL was subfractionated to obtain HDL₃ (d = 1.13–1.18 g/ml) as described (32). Reconstituted HDL (rHDL) was prepared by the sodium cholate dialysis method as described (33). Briefly, rHDL containing human apoA-I was prepared using molar ratios of 1/5/95, apoA-I/free cholesterol/palmitoylcholine. The purity and size of rHDL were examined on 4–20% nondenaturing gradient gel electrophoresis (GGE). The rHDL particles were \sim 100 Å in diameter and particles were >95% homogeneous in size.

Primary hepatocyte preparation

The procedure for isolation of mouse primary hepatocytes was based on Seglen's two-step perfusion method (34). Briefly, liver was perfused through the portal vein with Ca²⁺/Mg²⁺-free HBSS containing 10 mM glucose, 10 mM HEPES, and 0.3 mM EDTA followed by HBSS containing 0.05% collagenase type IV (Sigma C5138). The cell suspension was spun down at 50 *g* for 2 min. After three washes, hepatocytes were resuspended in Williams' Medium E (GIBCO) containing 10% FBS (GIBCO), 2% penicillin-streptomycin, 1% sodium pyruvate, 1% L-glutamine, and 1% insulin-transferrin-selenium (GIBCO). In line with previous reports (35, 36), hepatocytes comprised 98% (98% \pm 0.3 in six preparations) of the isolated cells as judged by cell morphology and size. Cell viability assessed by trypan blue exclusion was 94% \pm 0.7 from six preparations. Hepatocytes were plated into 12-well plates (2 \times 10⁵ cells/per well) precoated with rat tail collagen (BD Biosciences) and incubated at 37°C in 5% CO₂. After overnight cell culture, the LXR agonist T0901317 (Cayman) was used to stimulate ABCA1 and ABCG1 transporter expression.

Real time PCR

Total RNA was isolated from primary hepatocytes using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. RNA samples were treated with DNase I (Roche) for 30 min at 37°C and then purified with the RNeasy Mini Kit (QIAGEN). RNA (2 μ g) was reverse transcribed into cDNA using a reverse transcription system (Promega). After 4-fold dilution, 5 μ l was used as a template for real-time PCR. Amplification was done for 40 cycles using Power SYBR Green PCR master Mix Kit (Applied Biosystems). Quantification was performed using the standard curve method and normalizing to GAPDH. The primers used for ABCG1 are as follows: mABCG1, NM_009593: forward 5'-aggctctcagctcttaagttctcctc-3', reverse 5'-tctctcgaagtgaatgaaattatcg-3', 85bp.

Western blotting

Total cell proteins (10 µg) were separated on a 4-20% polyacrylamide gradient gel, transferred to polyvinylidene fluoride membranes and immunoblotted with anti-mouse SR-BI (1:1000) (Novus, NB400-104), anti-human/mouse ABCA1 (1:750) (from M. Hayden), anti-mouse ABCG1 (1:500) (Novus, NB400-132), or anti-β-actin (1:2000) (Sigma, A5441) overnight at 4°C. Membranes were washed and then incubated with anti-rabbit or anti-mouse IgG antibody conjugated with HRP for 2 h at room temperature. Immunoblots were visualized by the Amersham™ ECL™ Western Blotting Detection Reagents (GE Healthcare, Rockford, IL). Quantification was carried out by densitometric scanning.

SR-BI silencing in primary hepatocytes

Mouse SR-BI shRNAmir (RMM4431-98740265) and control nonsilencing-GIPZ lentiviral shRNAmir (RHS4346) were obtained from Open Biosystems (Thermo Scientific, Huntsville, AL). Mouse SR-BI shRNAmir sense sequence is gactcagcaagatcgatta; antisense sequence is taatcgatcttgctgagtc. Freshly isolated mouse hepatocytes (7×10^5 cells) were transfected with 4 µg plasmid using Amaxa™ Mouse/Rat Hepatocyte Nucleofector™ Kit (Lonza, VPL-1004) according to the manufacturer's instructions and seeded in 12-well culture plates at 3.5×10^5 cells per well. Gene silencing efficiency was analyzed by Western blotting following the incubations carried out to determine cholesterol efflux and nascent HDL formation as described below.

Cholesterol efflux determination

Cellular cholesterol efflux was determined as described (37). Briefly, hepatocytes were seeded into 12-well plates (2×10^5 cells/well). After overnight culture, cells were labeled with 0.2 µCi/ml [³H] cholesterol (35–50 Ci/mmol, Amersham Biosciences) for 48 h, washed, and equilibrated in serum-free medium containing 0.2% fatty acid-free BSA for 16 h. Cellular ABCA1 expression was stimulated by incubating with 5 µM T0901317 in medium containing 0.2% fatty acid-free BSA during the 16 h equilibration period. Cells were then pretreated in the presence or absence of glyburide (500 µM) in serum-free medium containing 0.2% fatty acid-free BSA for 1 h at 37°C and then incubated with the same concentration of glyburide in the presence or absence of 20 µg/ml lipid-free human apoA-I (Biodesign, Meridian Life Science, Saco, ME) for the indicated time period. Following incubation, radioactivity in the medium and cells was determined. Efflux was calculated as the percentage of counts in the medium relative to the total counts in the medium and cells together. Values shown were the mean ± SEM of triplicate determinations.

Nascent HDL particle formation

ApoA-I lipidation and nascent HDL particle formation was determined in the same cell culture experiments used to determine cholesterol efflux. Aliquots of medium from cholesterol efflux experiments were electrophoresed on 4-20% nondenaturing GGE for 3.5 h at 200 V, 4°C. They were then transferred to polyvinylidene fluoride membranes (40 min at 100 V at 4°C) for subsequent Western blotting with anti-human apoA-I (Calbiochem). Immunoblots were visualized by the Amersham™ ECL™ Western Blotting Detection Reagents (GE Healthcare, Rockford, IL). A protein standard mix containing 7.1, 8.2, 10.4, 12.2, and 17.0 nm proteins was obtained from GE healthcare.

Chemical cross-linking and immunoprecipitation analysis

Chemical cross-linking and immunoprecipitation analysis was performed essentially as previously described (38) with the following modifications. Hepatocytes were plated into 6-well plates (4×10^5 cells/well). After overnight culture, cells were treated

with 5 µM T0901317 in medium containing 0.2% fatty acid-free BSA for 16 h. Cells were then pretreated with 500 µM glyburide in serum-free medium containing 0.2% fatty acid-free BSA for 1 h at 37°C and then incubated with 3 µg/ml iodinated lipid-free human apoA-I in the presence or absence of 30-fold excess of unlabeled apoA-I for 1 h at 37°C in the presence of the same concentration of glyburide. Cells were then placed on ice for 15 min and washed three times with PBS. The cross-linking reaction was performed at room temperature for 30 min using 1.5 ml of 1 mM dithiobis(succinimidylpropionate) (DSP) (Thermo Scientific, Rockford, IL) per well. Medium was removed, and cells washed twice with PBS. Cells were lysed at 4°C in lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 0.25% sodium deoxycholate, 1% Nonidet P-40, protease inhibitor mixture (Roche) and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were centrifuged at 10,000 g for 10 min. Supernatant samples (200 µg protein) were incubated with 5 µg of an affinity-purified anti-ABCA1 antibody (ab7360, Abcam Inc. Cambridge, MA) for 20 h at 4°C, followed by the addition of 30 µl of resuspended volume of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) and incubated overnight at 4°C. Samples were centrifuged at 1,000 g for 5 min and the pellets washed twice with lysis buffer and twice with PBS. The amount of cross-linked (immunoprecipitate) or noncross-linked (supernatant) iodinated apoA-I was determined by γ-counting. Results are expressed as ng apoA-I/mg cell protein.

Statistical analysis

Statistics were calculated with Graphpad's Prism software. Data were expressed as means ± SEM. Results were analyzed by two-way ANOVA with a Bonferroni posttest. Significance was set at *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

RESULTS

Role of ABCA1 in cholesterol efflux and nascent HDL formation in primary hepatocytes

To investigate the role of ABCA1 in nascent HDL formation in hepatocytes, we examined cultures of primary mouse hepatocytes. The known LXR agonist T0901317 was used to stimulate the expression of the ABCA1 and ABCG1 transporters (39) and the lipidation of exogenously added apoA-I was then analyzed by nondenaturing gel electrophoresis. As expected, ABCA1 expression was markedly upregulated by T0901317 and high expression levels were maintained during the incubation period (Fig. 1B). As shown in Fig. 1A, incubation of cells with lipid-free apoA-I resulted in the formation of a series of discretely sized nascent HDL particles whose generation was markedly increased by T0901317. The nascent HDLs produced were similar in size to those produced by other cells, including macrophages and hepatocytes (15). The formation of small as well as large nascent HDL particles increased with time. However, it is not clear whether the smaller particles serve as precursors of larger particles.

To investigate the specific role of ABCA1 in both cholesterol efflux and nascent HDL formation, primary hepatocytes from liver-specific ABCA1 knockout mice and C57BL/6 control mice were used. Cells were pretreated with T0901317 for 16 h and then incubated with apoA-I (20 µg/ml) for 16 h in the presence or absence of glyburide, an inhibitor of ABCA1 transporter activity (15, 40).

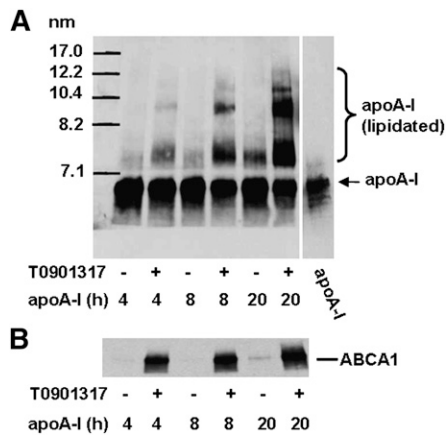


Fig. 1. LXR activation in primary hepatocytes stimulates nascent HDL formation. Primary hepatocytes from C57BL/6 mice were treated with 5 μ M T0901317 for 16 h and then incubated with lipid-free apoA-I (20 μ g/ml) for 4 h, 8 h, and 20 h. A: The conversion of apoA-I to HDL-sized particles assessed by nondenaturing GGE of cell culture medium (5 μ l), followed by immunoblotting using anti-human apoA-I. B: The expression of ABCA1 in hepatocytes assessed by Western blot analysis after 16 h treatment with T0901317 followed by 4 h, 8 h, and 20 h incubation with apoA-I.

Following the incubation, ABCA1 protein expression in ABCA1^{+/+} hepatocytes was markedly increased by LXR activation (Fig. 2A). Glyburide treatment did not affect ABCA1 levels. Cholesterol efflux from hepatocytes to apoA-I was determined (Fig. 2B). LXR stimulation led to an 8-fold increase in cholesterol efflux to apoA-I in ABCA1^{+/+} cells. Glyburide treatment markedly but not completely inhibited cholesterol efflux in ABCA1^{+/+} cells under both the basal and T0901317-treated conditions. Thus, efflux was greater in the T0901317-treated cells in the presence of glyburide than in cells in the absence of T0901317. Cholesterol efflux to apoA-I was very low in hepatocytes from ABCA1^{-/-} mice, both under normal and LXR stimulated conditions (Fig. 2B).

In line with cholesterol efflux to apoA-I, apoA-I lipidation was similarly increased to a marked degree by LXR activation in ABCA1^{+/+} hepatocytes as evident from the formation of the major nascent HDL species having sizes of 7.4, 9.2, and 10.6 nm diameter (Fig. 2C). In contrast to ABCA1^{+/+} hepatocytes, no apoA-I lipidation was detected in ABCA1^{-/-} hepatocytes, indicating that nascent HDL formation required ABCA1. As expected, glyburide treatment abolished the formation of the major nascent HDLs in ABCA1^{+/+} hepatocytes. Interestingly, treatment with this ABCA1 inhibitor led to the formation of a novel distinct 7.2 nm diameter species, which was generated by ABCA1^{+/+} hepatocytes under both LXR activated and nonactivated conditions. As in the case of nascent HDLs, LXR activation markedly increased the formation of the 7.2 nm particles in ABCA1^{+/+} cells. The accumulation of the 7.2 nm particles in the presence of glyburide suggested that their formation resulted from the lipidation of apoA-I that was independent of ABCA1 transporter activity. Unexpectedly, however, no 7.2 nm particles were detected in ABCA1^{-/-} hepatocytes (Fig. 2C), indicating that the formation of these

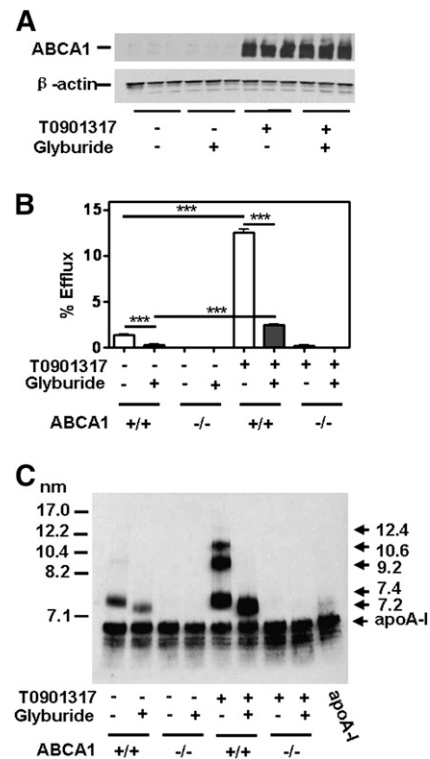


Fig. 2. Role of ABCA1 in nascent HDL particle formation and cholesterol efflux in primary hepatocytes. Primary hepatocytes from liver-specific ABCA1 knockout mice and C57BL/6 control mice were treated with or without LXR agonist T0901317 (5 μ M) for 16 h and then treated with or without glyburide (500 μ M) for 1 h. Cells were then incubated in medium with or without glyburide with 20 μ g/ml human apoA-I for 16 h. A: ABCA1 expression was determined by Western blot analysis using anti-human/mouse ABCA1, and anti-mouse β -actin as loading control. B: Hepatocytes were labeled with 0.2 μ Ci/ml [³H] cholesterol for 48 h. Cells were treated with T0901317 and glyburide as described in (A). Following incubation with 20 μ g/ml human apoA-I for 16 h, radioactivity in the medium and cells was determined. Cholesterol efflux was calculated as the percentage of counts in the medium relative to the total counts in the medium and cells together. Values shown were the mean \pm SEM of triplicate determinations. C: ApoA-I lipidation was assessed by separating cell culture medium (5 μ l) by nondenaturing GGE and immunoblotting with anti-human apoA-I. Sizes of nascent HDL particles, determined as set out in Materials and Methods are indicated on the right. Results are representative of two experiments. Significance at ***: $P < 0.001$.

particles required ABCA1 expression. This was in line with the observed increase in 7.2 nm particles in the case of ABCA1^{+/+} hepatocytes treated with T0901317 compared with that in basal conditions. Overall, the extent of cholesterol efflux to apoA-I was correlated with the levels of apoA-I lipidation in this experiment.

The mechanism by which glyburide affects the formation of the 7.2 nm particle is not known. Some reports have shown that glyburide decreases the level of apoA-I binding to ABCA1 in HEK and BHK cells (14, 38). The effect of glyburide on apoA-I binding to ABCA1 in hepatocytes was determined using chemical cross-linking and immunoprecipitation. Treatment with glyburide (500 μ M) did not significantly alter the ABCA1 dependent (cross-linked) level of radiolabeled apoA-I binding (Fig. 3).

ApoA-I binding to ABCA1 reflects the total binding of apoA-I to ABCA1, including any ABCA1/apoA-I complexes that may have been internalized during the cross-linking period of 1 h at 37°C. Cross-linked apoA-I values were ABCA1-dependent because the values obtained in ABCA1^{-/-} hepatocytes were less than 10% of the values obtained in ABCA1^{+/+} cells (data not shown). The large bulk of cell-associated apoA-I was not cross-linked to ABCA1 and was 25% higher in the presence of glyburide.

ABCG1 does not contribute to nascent HDL particle formation in hepatocytes

ABCG1 has been reported to have synergistic effects with ABCA1 in promoting cellular cholesterol efflux in CHO cells and BHK cells (20, 21). To address whether ABCG1 contributes to nascent HDL formation in hepatocytes, we compared HDL formation and cholesterol efflux in primary hepatocytes from ABCG1^{+/+} and ABCG1^{-/-} mice. ABCG1 mRNA expression is detected under basal conditions and LXR activation increased ABCG1 expression by 8-fold in ABCG1^{+/+} hepatocytes (Fig. 4A). Western blotting failed to detect ABCG1 protein expression, consistent with earlier reports (23–25). The expression levels of ABCA1 in ABCG1^{+/+} and ABCG1^{-/-} cells showed no significant difference, either under basal or LXR stimulated conditions (Fig. 4B).

Compared with ABCG1^{+/+} hepatocytes, ABCG1^{-/-} cells showed a similar capacity to efflux cholesterol to apoA-I under basal conditions (Fig. 4D). LXR activation significantly increased cholesterol efflux to a similar extent in

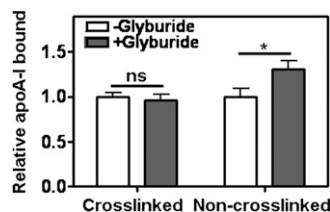


Fig. 3. Glyburide does not inhibit apoA-I binding to ABCA1. ApoA-I binding to ABCA1 and nonABCA1 binding sites determined by cross-linking and immunoprecipitation assay. Hepatocytes from ABCA1^{+/+} mice were treated with 5 μ M T0901317 for 16 h. Cells were then pretreated in the presence or absence of glyburide (500 μ M) for 1 h at 37°C and then incubated with 3 μ g/ml iodinated lipid-free human apoA-I in the presence or absence of 30-fold excess of unlabeled apoA-I for 1 h at 37°C in the presence of the same concentration of glyburide. Chemical cross-linking and immunoprecipitation were performed as described in Materials and Methods. The radioactivity found in pellets (cross-linked apoA-I/ABCA1) and in supernatants (noncross-linked apoA-I) was determined by γ -counting. Shown is the specific binding determined by subtracting nonspecific binding (binding in the presence of 30-fold excess of unlabeled ligands). Values are mean \pm SEM (n = 12) obtained in three independent experiments. Values in each experiment were normalized to the minus glyburide mean value, which was set at one. No statistical difference (ns) was observed in cross-linked apoA-I/ABCA1 between untreated and glyburide treated samples. Mean cross-linked apoA-I values in the absence or presence of glyburide were 0.79 and 0.7 ng/mg cell protein, respectively. Mean noncross-linked apoA-I values in the absence or presence of glyburide were 20.56 and 25.05 ng/mg cell protein, respectively. Significance at *: $P < 0.05$.

both ABCG1^{+/+} and ABCG1^{-/-} cells. Glyburide significantly decreased cholesterol efflux to apoA-I from both ABCG1^{+/+} and ABCG1^{-/-} hepatocytes under basal and LXR stimulated conditions.

ABCG1 has been shown in other cell types to act in concert with ABCA1 by facilitating efflux to preformed nascent HDL and mature HDL particles (20, 21). To further examine if ABCG1 contributes to cholesterol efflux from hepatocytes, rHDL and mature HDL were tested as cholesterol acceptors (Fig. 4C). As was the case of efflux to apoA-I, efflux from LXR-stimulated hepatocytes to rHDL as well as to human HDL₃ was similar in ABCG1^{+/+} and ABCG1^{-/-} cells. Together, these data fail to show that ABCG1 contributes to cholesterol efflux from hepatocytes.

SR-BI, like ABCG1, is able to facilitate cholesterol efflux to HDL particles (19, 27). Furthermore, SR-BI inhibited ABCG1-mediated cholesterol efflux to HDL when coexpressed with ABCG1 in a transfected cell line (41). To investigate whether SR-BI expression reduces ABCG1-dependent cholesterol efflux in hepatocytes, SR-BI expression was significantly reduced in ABCG1^{+/+} and ABCG1^{-/-} hepatocytes by SR-BI silencing. Under these conditions, cholesterol efflux to rHDL was tested and found to be similar in ABCG1^{+/+} and ABCG1^{-/-} hepatocytes (supplementary Fig. 1).

In line with cholesterol efflux to apoA-I, the formation of nascent HDL particles ranging in size from 7.4 to 14.0 nm in diameter was enhanced by LXR activation in both ABCG1^{+/+} and ABCG1^{-/-} hepatocytes (Fig. 4E). The lack of ABCG1 did not alter the particle levels or size compared with cells expressing ABCG1. Nascent HDL formation was inhibited by glyburide in a similar manner in ABCG1^{+/+} and ABCG1^{-/-} cells, indicating that ABCG1 does not play a significant role in nascent HDL particle formation. The accumulation of the 7.2 nm particle in the presence of glyburide was also similar for the two cell types, suggesting that its formation was also independent of ABCG1 expression.

Formation of nascent HDL in ABCA1- or ABCG1-overexpressing BHK cells

As another approach to assess the role of ABCA1 and ABCG1 in nascent HDL formation, BHK cells overexpressing ABCA1 or ABCG1 were studied. Significant amounts of ABCA1 or ABCG1 were expressed in mifepristone-treated ABCA1 or ABCG1 transfected BHK cells (Fig. 5A).

Similar to hepatocytes, a series of distinct nascent HDL particles were formed in BHK cells in an ABCA1-dependent manner and these nascent HDLs were of similar size to those produced by hepatocytes (Fig. 5B). The overexpression of ABCG1 exerted only a minor stimulating effect on nascent HDL formation. Together, these results demonstrate that ABCA1 is sufficient for the observed formation of nascent HDLs and that ABCG1 does not contribute to or influence this process.

Inhibitory effect of SR-BI on nascent HDL formation

SR-BI is a HDL receptor that promotes HDL CE selective uptake as well as cholesterol flux between cells and HDL (26). The role of SR-BI in cholesterol efflux and

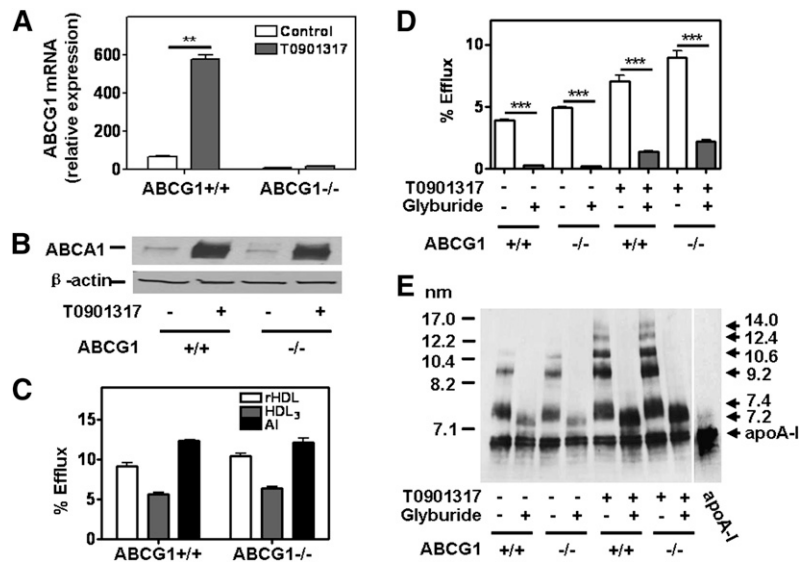


Fig. 4. Role of ABCG1 in nascent HDL particle formation and cholesterol efflux in primary hepatocytes. Primary hepatocytes from ABCG1^{+/+} and ABCG1^{-/-} mice were treated with and without LXR agonist T0901317 (5 μ M) for 16 h, and then with or without glyburide (500 μ M) for 1 h as described in Fig. 2. Cells were then incubated in the presence or absence of glyburide with 20 μ g/ml human apoA-I for 16 h. **A:** ABCG1 mRNA expression was determined by quantitative RT-PCR. The ct values for ABCG1 expression under basal and activated conditions were 26.64 ± 0.23 and 23.08 ± 0.09 , respectively, and **(B)** ABCA1 expression was determined by Western blot analysis. **C:** Hepatocytes were labeled with 0.2 μ Ci/ml [³H] cholesterol for 48 h. Cells were treated with T0901317 for 16 h. Following incubation with 20 μ g/ml rHDL, human HDL₃, and human apoA-I for 16 h, radioactivity in the medium and cells was determined. Cholesterol efflux was calculated as the percentage of counts in the medium relative to the total counts in the medium and cells together. Values shown were the mean \pm SEM of triplicate determinations. **D:** Hepatocytes were labeled with 0.2 μ Ci/ml [³H] cholesterol for 48 h. Cells were treated with T0901317 and glyburide as indicated. Following incubation with 20 μ g/ml human apoA-I for 16 h, radioactivity in the medium and cells was determined. Cholesterol efflux was calculated as the percentage of counts in the medium relative to the total counts in the medium and cells together. Values shown were the mean \pm SEM of triplicate determinations. **E:** ApoA-I lipidation was assessed by separating cell culture medium (5 μ l) by nondenaturing GGE and immunoblotting with anti-human apoA-I. Results are representative of three experiments. Significance at **: $P < 0.01$; ***: $P < 0.001$.

nascent HDL formation was investigated by assessing these two processes in primary hepatocytes from SR-BI^{+/+} and SR-BI^{-/-} mice. LXR activation did not significantly alter the expression of SR-BI in hepatocytes. ABCA1 protein expression was detected in both SR-BI^{+/+} and SR-BI^{-/-} cells, however, the expression level of ABCA1 relative to β -actin expression in SR-BI^{-/-} cells was significantly lower than in SR-BI^{+/+} cells (relative levels of 2.4 ± 0.2 , $n = 3$, vs. 3.8 ± 0.01 , $n = 3$) under LXR-stimulated conditions (**Fig. 6A**). Interestingly, compared with SR-BI^{+/+} hepatocytes, SR-BI^{-/-} cells showed enhanced capacity to efflux cholesterol to apoA-I under basal although not LXR stimulated conditions. LXR activation significantly increased cholesterol efflux in both SR-BI^{+/+} and SR-BI^{-/-} cells to levels that were similar in the two cell types. Glyburide significantly decreased cholesterol efflux to apoA-I from both SR-BI^{+/+} and SR-BI^{-/-} hepatocytes under basal and LXR-stimulated conditions (**Fig. 6B**).

As in the case of cholesterol efflux, the formation of HDL particles was also increased in SR-BI^{-/-} cells compared with that in SR-BI^{+/+} cells under basal conditions (**Fig. 6C**), suggesting that SR-BI may exert an inhibitory effect on nascent HDL particle formation. The formation of nascent HDL particles ranging in size from 7.4 to 14.0 nm in diameter was enhanced by LXR activation in both

SR-BI^{+/+} and SR-BI^{-/-} hepatocytes and under these conditions SR-BI did not alter nascent particle levels or size. Nascent HDL formation was inhibited by glyburide in SR-BI^{+/+} and SR-BI^{-/-} cells such that the formation of 7.4 nm or greater particles was blocked while 7.2 nm particles were formed. These results suggest that under basal conditions, SR-BI plays a role in reducing cholesterol efflux to apoA-I as well as HDL particle formation. When LXR is activated, the effects of SR-BI may be masked both by the high level of ABCA1 expression and by the reduced level of ABCA1 in the SR-BI^{-/-} cells.

DISCUSSION

The major findings of the present study are that ABCG1 and SR-BI in hepatocytes do not contribute to cholesterol efflux to apoA-I or to the accompanying nascent HDL formation. In contrast, ABCA1 in hepatocytes is essential for nascent HDL formation and cholesterol efflux to apoA-I. A small novel lipidated apoA-I species (7.2 nm diameter) was identified whose accumulation, although completely dependent on ABCA1, was actually promoted by glyburide, an inhibitor of ABC transporters. Cholesterol efflux to apoA-I and nascent HDL formation were slightly but

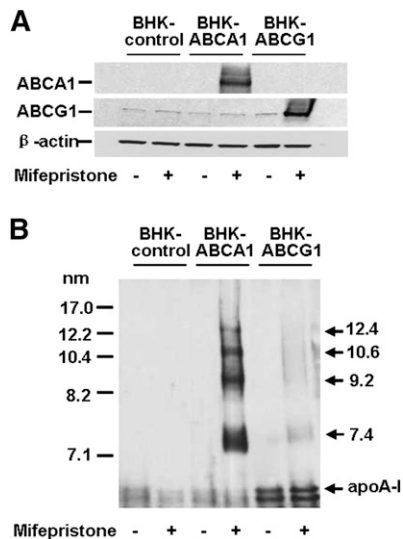


Fig. 5. Formation of HDL-sized particles in ABCA1 or ABCG1 overexpressing BHK cells. A: The expression of ABCA1 or ABCG1 stimulated by 10 nM mifepristone in human ABCA1, human ABCG1, and mock transfected BHK cells (control cells) was determined by Western blot analysis. B: Human ABCA1, human ABCG1 and mock-transfected BHK cells (control cells) were incubated with 20 $\mu\text{g}/\text{ml}$ lipid-free human apoA-I for 16 h. ApoA-I lipidation was assessed by separating cell culture medium (5 μl) by nondenaturing GGE and immunoblotting with anti-human apoA-I as described in Materials and Methods.

significantly enhanced in SR-BI^{-/-} cells compared with SR-BI^{+/+} cells.

Our studies using primary hepatocytes from liver-specific ABCA1 knockout mice demonstrated the key role of ABCA1 in nascent HDL formation and cholesterol efflux from hepatocytes. Cholesterol effluxed from cells may correlate to membrane cholesterol that is solubilized together with phospholipid during the formation of nascent HDLs (17). The lipidation of apoA-I and cholesterol efflux were markedly upregulated by LXR activation and resulted in the formation of a number of major nascent HDL species having sizes of 7.4, 9.2, and 10.6 nm in diameter. These findings are in line with previous studies showing that hepatocytes from ABCA1-deficient mice produced reduced amounts of nascent HDLs (16, 18) but qualitatively similar sized nascent HDL particles (18). In another report, the role of ABCA1 in HDL formation in hepatocytes was unclear (42). Two distinct-sized nascent HDLs were observed in ABCA1^{+/+} cells whereas in ABCA1^{-/-} cells, total HDL formation appeared similar to ABCA1^{+/+} cells except that in ABCA1^{-/-} cells, the larger nascent HDL was the predominant species (42). The reasons for the different results in the latter study are unclear.

In the present study, glyburide, a nonselective ABC transporter inhibitor (40), was used to further examine the contribution of ABC transporters in HDL biogenesis. Cholesterol efflux to lipid-free apoA-I was markedly, but not completely inhibited by glyburide treatment under both basal and LXR-stimulated conditions. In the presence of glyburide, cholesterol efflux was greater in the T0901317-treated cells than in cells not treated with T0901317. As expected,

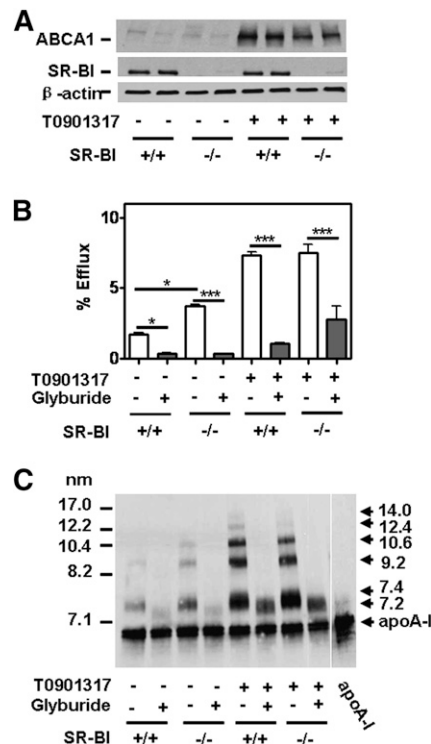


Fig. 6. Role of SR-BI in nascent HDL particle formation and cholesterol efflux in primary hepatocytes. Primary hepatocytes from SR-BI^{+/+} and SR-BI^{-/-} mice were treated with or without LXR agonist T0901317 (5 μM) for 16 h, and then with or without glyburide (500 μM) for 1 h. Cells were then incubated in the presence or absence of glyburide with 20 $\mu\text{g}/\text{ml}$ lipid-free human apoA-I for 16 h. A: ABCA1 and SR-BI expression were determined by Western blot analysis. Hepatocytes were labeled with 0.2 $\mu\text{Ci}/\text{ml}$ [³H] cholesterol for 48 h. Cells were treated with T0901317 and glyburide as indicated. B: Following incubation with 20 $\mu\text{g}/\text{ml}$ lipid-free human apoA-I for 16 h, radioactivity in the medium and cells was determined. Cholesterol efflux was calculated as the percentage of counts in the medium relative to the total counts in the medium and cells together. Values shown were the mean \pm SEM of triplicate determinations. C: ApoA-I lipidation was assessed by separating cell culture medium (5 μl) by nondenaturing GGE and immunoblotting with anti-human apoA-I. Results are representative of two experiments. Significance at *: $P < 0.05$; ***: $P < 0.001$.


glyburide treatment virtually abolished the formation of the major nascent HDLs in ABCA1^{+/+} hepatocytes. Most interestingly, a small novel lipidated apoA-I species (7.2 nm diameter) was identified whose pronounced accumulation was actually promoted by glyburide (Fig. 2C). The production of a similar-sized 7.2 nm particle has been reported by Krimbou et al. (15) as a pre β_1 -migrating apoA-I-containing particle produced by HepG2 cells and macrophages. Based on the inhibitory effect of glyburide on ABCA1, the generation of these small particles in the presence of glyburide was considered to be independent of ABCA1. However, we showed that the 7.2 nm species was not detected in ABCA1^{-/-} hepatocytes (Fig. 2C), indicating that the formation of these particles is dependent on ABCA1. This is consistent with the observation that 7.2 nm particle formation was increased by LXR activation. Whereas ABCA1 is required for the 7.2 nm particle production, ABCG1 and SR-BI did not affect this process.

Current models of the mechanism of ABCA1-dependent cholesterol efflux and nascent HDL formation (17, 43) indicate that ABCA1-dependent apolipoprotein lipidation involves a multistep process that is initiated by the high-affinity binding of apoA-I or other apolipoproteins to ABCA1 in the plasma membrane (17). Such binding stabilizes as well as activates ABCA1 phospholipid translocase activity (44). We suggest that the binding of apoA-I to ABCA1 is required to form the 7.2 nm particle. Earlier studies showed that apoA-I binding to ABCA1 in HEK cells was reduced in the presence of glyburide (40) although inhibition was only partial at the concentration used in our studies. Our results showed that glyburide treatment did not reduce the binding of apoA-I to ABCA1 in hepatocytes. Thus, binding of apoA-I to ABCA1 in the presence of glyburide may be sufficient to allow for limited apoA-I lipidation and the generation of the 7.2 nm particle through a process that is independent of ABCA1 functional activity. Understanding the mechanism by which this novel particle is generated may provide new insight into the initial steps of apoA-I lipidation. It is possible that the 7.2 nm particle is not a normal component of the HDL biogenesis pathway. Glyburide is a nonspecific inhibitor known to affect the activity of various ABC transporters and it may promote the generation of 7.2 nm particles through cellular effects unrelated to nascent HDL formation.

In the current model of cellular cholesterol efflux, ABCA1 converts lipid poor apoA-I to HDL particles that can serve as an acceptor for ABCG1-mediated cholesterol efflux (20). Because ABCG1 has a synergistic effect with ABCA1 in promoting cellular cholesterol efflux, ABCG1 may also play a role in HDL biogenesis. Studies on ABCG1 in promoting cholesterol efflux have been largely investigated in macrophages (19, 45) and endothelial cells (46, 47). In hepatocytes, we showed ABCG1 gene expression which is highly upregulated by LXR. This confirms previous studies that in chow-fed mice the hepatocyte ABCG1 gene is expressed and highly upregulated by LXR (24) or by a high-cholesterol diet (22). It has been reported that a lack of ABCG1 results in decreased plasma HDL cholesterol levels in mice fed a high-cholesterol diet (22). However, this remains controversial as other studies failed to demonstrate altered HDL levels in high-fat-fed ABCG1 deficient mice or in ABCG1 transgenic mice (23–25). Our studies addressed the possibility that ABCG1 contributes to hepatic HDL biogenesis. Our findings, however, demonstrated that an absence of ABCG1 did not affect cholesterol efflux to apoA-I or nascent HDL particle formation, either under basal or LXR-activated conditions. These results indicate that ABCG1 does not contribute to cholesterol efflux to apoA-I and nascent HDL formation, at least in hepatocytes.

Hepatic SR-BI plays a pivotal role in HDL cholesterol clearance from plasma and consequently, plasma HDL cholesterol levels (48). SR-BI can also promote cellular cholesterol efflux to HDL (27). However, SR-BI has been reported to inhibit ABCA1-mediated cholesterol efflux in macrophages (28). In the present study, we demonstrated

that the absence of SR-BI did not inhibit nascent HDL particle formation or cholesterol efflux to apoA-I. To the contrary, cells lacking SR-BI showed enhanced capacity both to efflux cholesterol to apoA-I and to form nascent HDLs under basal conditions. This suggests that SR-BI may exert effects by mediating reuptake of nascent HDLs or their lipid components from newly synthesized HDL particles. This is in line with the well-known function of SR-BI in mediating selective lipid uptake from HDL particles. In contrast to our finding that SR-BI decreases cholesterol efflux to apoA-I, a recent study demonstrated that SR-BI in hepatocytes can promote cholesterol efflux to human HDL₃ (49). The different effects of SR-BI on cholesterol efflux in the two studies are likely due to differences in the abilities of lipid-poor apoA-I and HDL₃ in promoting cholesterol efflux. The inhibitory effect of SR-BI on cholesterol efflux and nascent HDL particle formation in our studies could not be detected in LXR-stimulated conditions. This may be explained by the lower level of ABCA1 in SR-BI^{-/-} cells compared with SR-BI^{+/+} cells upon LXR activation.

In summary, the present study demonstrates that the extent of cholesterol efflux to lipid-poor apoA-I is correlated with the formation of nascent HDL particles. A small novel 7.2 nm particle accumulates under conditions in which ABCA1 activity is inhibited. ABCA1 is required for generating nascent HDL particles, whereas ABCG1 does not affect nascent HDL particle formation. SR-BI does not promote but can rather decrease nascent HDL formation. 

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