ABCA1-dependent lipid efflux to apolipoprotein A-I mediates HDL particle formation and decreases VLDL secretion from murine hepatocytes

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Abstract **High levels of expression of the ATP binding cassette transporter A1 (ABCA1) in the liver and the need to over- or underexpress hepatic ABCA1 to impact plasma HDL levels in mice suggest a major role of the liver in HDL formation and in determining circulating HDL levels. Cultured murine hepatocytes were used to examine the role of hepatic ABCA1 in mediating the lipidation of apolipoprotein A-I (apoA-I) for HDL particle formation. Exogenous apoA-I stimulated cholesterol efflux to the medium from wild-type hepatocytes, but not from ABCA1-deficient (*abca1*$^{-/-}$) hepatocytes. ApoA-I induced the formation of new HDL particles and enhanced the lipidation of endogenously secreted murine apoA-I in ABCA1-expressing but not *abca1*$^{-/-}$ hepatocytes. ABCA1-dependent cholesterol mobilization to apoA-I increased new cholesterol synthesis, indicating depletion of the regulatory pool of hepatocyte cholesterol during HDL formation. Secretion of triacylglycerol and apoB was decreased following apoA-I incubation with ABCA1-expressing but not *abca1*$^{-/-}$ hepatocytes. These results support a major role for hepatocyte ABCA1 in generating a critical pool of HDL precursor particles that enhance further HDL generation and passive cholesterol mobilization in the periphery. The results also suggest that diversion of hepatocyte cholesterol into the "reverse" cholesterol transport pathway diminishes cholesterol availability for apoB-containing lipoprotein secretion by the liver.**—Sahoo, D., T. C. Trischuk, T. Chan, V. A. B. Drover, S. Ho, G. Chimini, L. B. Agellon, R. Agnihotri, G. A. Francis, and R. Lehner. **ABCA1-dependent lipid efflux to apolipoprotein A-I mediates HDL particle formation and decreases VLDL secretion from murine hepatocytes. *J. Lipid Res.* 2004.** 45: **1122–1131.**

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HDL particles are thought to protect against atherosclerosis primarily by mediating the return of excess tissue cholesterol to the liver for excretion in bile (1). HDLs are formed through the acquisition by apolipoprotein A-I (apoA-I) and other HDL apolipoproteins of phospholipids and cholesterol from cells and from the surface of VLDL and chylomicrons during their hydrolysis by lipoprotein lipase (2). The discovery that apolipoprotein-dependent phospholipid and cholesterol efflux from cells is almost abolished in a condition with extremely low HDL levels, Tangier disease (3, 4), suggested that the transport of cellular lipids to apoA-I is the rate-limiting step in HDL particle formation. The subsequent discovery by several groups of the mediator of apoA-I lipidation mutated in Tangier disease, the ATP binding cassette transporter A1 (ABCA1) [for review, see ref. (5)], uncovered a key player in this step and provided a new therapeutic target to increase the formation of HDL clinically.

The liver, in addition to its roles in lipoprotein secretion and reuptake and bile acid synthesis, secretes lipid-free or lipid-poor apoA-I (6) and expresses high levels of ABCA1 (7–10). Perfusates of isolated rat and monkey liver show the presence of small HDL particles (11–13), suggesting that the liver itself mediates some lipidation of HDL proteins. Some established lines of cultured hepatoma cells (e.g., HepG2) secrete nascent HDL particles (14, 15), but not all of those studied (e.g., Fu5AH) release cholesterol to apoA-I (16). In addition, the correlation between ABCA1 expression and efflux to apoA-I from hepatoma

Abbreviations: CE, cholesteryl ester; PC, phosphatidylcholine; SR-BI, scavenger receptor class B type I; TG, triacylglycerol.

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cells is not clear (16). Overexpression of ABCA1 in tissues, including the liver, results in an increase in plasma HDL levels (17, 18), whereas selective transgene- or agonist-induced overexpression or knockout of ABCA1 in tissue macrophages has little effect on plasma HDL concentrations (19–21). Adenoviral-directed expression of ABCA1 in the liver resulted in an increase in plasma HDL (22, 23) and an increase in cholesterol efflux to apoA-I from isolated ABCA1-transgenic mouse hepatocytes (22). ABCA1-deficient (*abca1*$^{-/-}$) mouse hepatocytes have also been shown to generate HDL particles, but released less phospholipid to endogenously secreted apoA-I or exogenously added apoA-I (24). Together, these results suggest that hepatic ABCA1 is a major contributor to HDL formation and a key determinant of circulating HDL levels (25, 26).

The depletion of hepatocyte cholesterol by ABCA1-dependent lipidation of apoA-I may also deplete the pool of cholesterol available for VLDL secretion. In support of this is the decrease in VLDL production in whole animals and in man (27–31), as well as in cultured primary hepatocytes (32), following treatment with HMG-CoA reductase inhibitors. These results suggest that the availability and/or distribution of hepatocyte cholesterol plays an important role in the assembly of nascent apoB particles within the endoplasmic reticulum and that perturbations of this tightly regulated system have major effects on VLDL secretion. In the present studies, we examined the dependence on ABCA1 of apoA-I-mediated cholesterol efflux and HDL particle formation in rat hepatoma and primary rat and mouse hepatocytes and the effects of ABCA1-dependent HDL formation on VLDL particle secretion by these cells.

MATERIALS AND METHODS

Apolipoprotein and HDL isolation

HDL_3 (d = 1.13–1.21 g/ml) was isolated from the pooled plasma of healthy male volunteers by density gradient ultracentrifugation (33). Human apoA-I was isolated by DEAE-cellulose chromatography of delipidated HDL (34).

Cell culture

Hepatocytes were isolated from adult male Sprague-Dawley rats (70–100 g), male and female DBA and *abca1*$^{-/-}$ DBA mice (30–40 g) (35), and male C57BL/6J and LDL receptor (LDLR)-deficient (*ldlr*$^{-/-}$) C57BL/6J mice (30–40 g) by collagenase perfusion of the liver (36). McArdle RH7777 rat hepatoma cells were cultured as previously described (37) and used at 70% confluence for all experiments. All cells were maintained at 37°C in humidified air containing 5% CO_2.

Lipid mobilization assays

Cells were washed in DMEM and incubated with [^{14}C]mevalonic acid lactone (0.5–1 μCi/ml; 50 mCi/mmol; Amersham Canada, Oakville, ON) in DMEM for 5–8 h. Cells were then rinsed twice with DMEM and incubated in the presence or absence of apoA-I (20 μg/ml) or HDL (100 μg/ml) in DMEM for 16–18 h. Triplicate control dishes were collected immediately following the mevalonic acid lactone labeling period to determine initial radioactivity incorporated into free and esterified cholesterol. For phosphatidylcholine (PC) and triacylglycerol (TG) synthesis and secretion analyses, the ± apoA-I medium also contained 1 mM glycerol and [^{3}H]glycerol (2.5 μCi/ml; 1 Ci/mmol; Amersham). Following incubations, media were collected and centrifuged at 1,500 rpm for 10 min to remove cellular debris. Cells were washed with ice-cold PBS, harvested in 2 ml PBS, and sonicated and analyzed for radiolabeled lipid content in cell homogenates and media by TLC (37).

Lipid mass analysis

Primary rat and mouse hepatocytes or McArdle RH7777 cells were incubated in the presence or absence of 20 μg/ml apoA-I in DMEM for 16 h. Primary hepatocytes were preincubated with DMEM containing 0.4 mM oleic acid complexed to BSA for 4 h to stimulate TG synthesis prior to incubation with apoA-I. Media and cells were collected for analyses. Lipids from 1 ml media or 1 ml cell sonicate were extracted (37) in the presence of tridecanoylglycerol as the internal standard, and phospholipids were digested by phospholipase C (38). Samples were derivatized with Sylon BFT (Supelco, Bellefonte, PA) and analyzed by gas chromatography (Agilent Technologies, 6890 Series equipped with a flame ionization detector; Palo Alto, CA). Samples were injected onto an Agilent high performance capillary column (HP-5, 15 m × 0.32 mm × 0.25 μm). The oven temperature was raised from 170 to 290°C at 20°C/min and then to 340°C at 10°C/min, with helium as a carrier gas (87 cm/s) with a constant flow rate of 4.5 ml/min. In some experiments, TG content in cells and media was determined using "Infinity" TG reagent (Sigma Diagnostics, Inc.) with authentic TG (triolein) as the internal standard.

Lipoprotein analysis

Media from three identically treated dishes were pooled, concentrated, and separated by 3–10% gradient native polyacrylamide gel electrophoresis and transferred to nitrocellulose. ApoA-I was detected using anti-human apoA-I (Calbiochem, La Jolla, CA), anti-rat apoA-I (a kind gift from Dr. Norman Wong, Calgary, AB), or anti-mouse apoA-I (Biodesign, Saco, ME) polyclonal antibodies. Immunoblots were first probed with anti-human apoA-I, stripped, and then reprobed with the corresponding murine anti-apoA-I antibody. ApoB was detected using anti-human apoB polyclonal antibody (Roche Diagnostics, Inc., Indianapolis, IN).

Cholesterol synthesis

Hepatocytes were incubated in the presence or absence of 20 μg/ml apoA-I in serum-free DMEM for 16 h and washed and incubated for 4 h in DMEM containing 100 μM sodium acetate and 5 μCi/ml [^{3}H]acetic acid (2 Ci/mmol; Amersham Canada). Lipids were extracted and resolved by TLC, and radioactivity in cholesterol and cholesteryl ester (CE) was determined as indicated above.

Measurement of mRNA abundance

Total RNA was prepared from cultured hepatocytes with Trizol (Invitrogen Life Technologies, Burlington, ON). cDNA was synthesized from 10 μg of total RNA with Superscript II (Invitrogen Life Technologies). PCR was performed with intron-spanning, gene-specific oligonucleotides and Red *Taq* polymerase (Sigma Diagnostics, Inc.) in the presence of the fluorescent dye SYBR Green I (Sigma Diagnostics, Inc.). Amplicon production was monitored by fluorescence using a LightCycler (F. Hoffmann-La Roche Ltd., Switzerland). All reactions were optimized for linearity, and specificity of the products was confirmed by agarose gel electrophoresis. The primers used were: cyclophilin, 5′-TCC AAA GAC AGC AGA AAA CTT TCG (sense), 5′-TCT TCT TGC TGG TCT TGC CAT TCC (antisense); ABCA1, 5′-CCA TTA CAG GGG CAG TGC CT (sense), 5′-CTG GCA CAC TCA TTG CCA GC (antisense).

Immunoblot analyses

Cells were harvested in 50 mM Tris-HCl, pH 7.4, containing 500 ng/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF, and 2 mM EGTA; sonicated, and centrifuged at 700 rpm for 10 min to pellet unbroken cells; and the supernatant was used for analysis. The sample was adjusted to 0.45 M urea containing 0.1% Triton X-100 and 0.05% DTT, and SDS-PAGE dye was added to 50 μg protein prior to loading gels, without boiling the samples. Proteins were resolved by SDS-PAGE and immunoblotted with anti-ABCA1 polyclonal antibody [a gift from Dr. Shinji Yokoyama (39)], anti-scavenger receptor class B type I (SR-BI) polyclonal antibody (Novus Biologicals, Littleton, CO), or anti-protein disulfide isomerase polyclonal antibody (StressGen Biotechnologies, Victoria, BC).

ApoB analysis

Hepatocytes were incubated in DMEM containing 0.4 mM oleic acid complexed to BSA (37) for 4 h, and washed and incubated in the presence or absence of 20 μg/ml apoA-I in DMEM for 16–18 h. Cells were washed with leucine-free media and pulsed with [^{3}H]leucine (250 μCi/dish; 120 Ci/mmol, Amersham) for 20 min. The radiolabel was removed, and cells were incubated with DMEM for an additional 2 h. ApoB was immunoprecipitated from cell homogenates and media using anti-human apoB polyclonal antibodies (Boehringer-Mannheim, Laval, QC) (37). Nonlabeled apoB-100 and apoB-48 standards were added to the precipitates, and the samples were separated by SDS-5% PAGE. Gels were silver stained, the bands corresponding to apoB-48 and apoB-100 were excised, and radioactivity associated with the proteins was analyzed. To assess intracellular apoB degradation, 40 μg/ml acetyl-leucyl-leucyl-norleucinal (ALLN; Sigma) was added during some of the incubations (40). Alternatively, media apoB was precipitated using Cab-O-Sil (41), either from total media or from fractions obtained by density centrifugation (37), and apoB content was estimated by immunoblotting. The relative intensities of apoB bands were determined by densitometry using BioRad Quantity One software.

Statistics

Experiments were performed at least three times in triplicate, unless otherwise specified. Results are expressed as mean ± SD. *P* values were calculated using Student's *t*-test.

RESULTS

ABCA1 in hepatocytes

Whole liver (7, 8, 10) and hepatocytes (9) show high levels of ABCA1 expression. The expression of ABCA1 in cultured and primary hepatocytes used in our studies was determined by measurement of ABCA1 mRNA abundance (**Fig. 1A**). Immunoblots of whole-cell homogenates confirmed the presence of ABCA1 protein in both cell types (Fig. 1B). The presence of ABCA1 mRNA in the liver of DBA mice and the absence of ABCA1 mRNA and protein in *abca1*$^{-/-}$ DBA mice has been confirmed previously (35).

ApoA-I-mediated cholesterol efflux from murine hepatocytes

The ability of whole HDL particles to induce efflux of cholesterol from cultured HepG2 and Fu5AH rat hepatoma cells has been demonstrated previously (14, 42) and was

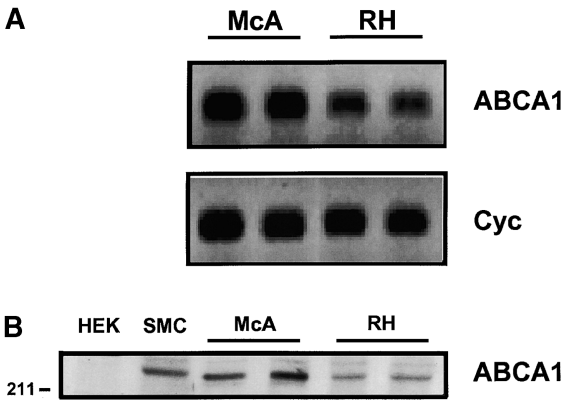

Fig. 1. Hepatocyte expression of ATP binding cassette transporter A1 (ABCA1). ABCA1 transcripts were detected by RT-PCR as described in Materials and Methods. ABCA1 amplicons were analyzed by 1.2% agarose gel electrophoresis. McArdle RH7777 cells (McA) and primary rat hepatocytes (RH) are shown. Cyclophilin (Cyc) amplification was used as a control. ABCA1 protein was detected by immunoblotting of 50 μg of cell homogenate protein with anti-ABCA1 antibody. Human embryonic kidney (HEK) and cholesterol-loaded human aortic smooth muscle cells (SMC) were used as negative and positive controls for *ABCA1* expression, respectively. The molecular weight marker is shown at left.

confirmed in our hands using cultured McArdle RH7777 rat hepatoma cells and primary rat hepatocytes incubated with HDL_3 (data not shown). ApoA-I-mediated cholesterol efflux is believed to require the prior or simultaneous efflux of cellular phospholipid (43). Incubation of [^{3}H]glycerol-labeled McArdle RH7777 cells with 20 μg/ml apoA-I for 16 h resulted in a 27 ± 4% increase in PC efflux to the medium compared with cells incubated in the absence of apoA-I (n = 3 experiments). The near absence of apoA-I-mediated PC efflux from *abca1*$^{-/-}$ mouse fibroblasts has been shown previously (35), as has a marked decrease in efflux of phospholipids to apoA-I from *abca1*$^{-/-}$ mouse hepatocytes (24). To assess ABCA1-dependent [i.e., apolipoprotein-specific or active, (44)] cholesterol efflux, primary rat, wild-type mouse, and *abca1*$^{-/-}$ mouse hepatocytes were enriched with radiolabeled mevalonate lactone and incubated in the presence or absence of lipid-free apoA-I. Efflux of [^{14}C]mevalonate lactone-derived cholesterol to apoA-I was 3- and 2.5-fold higher than in cells incubated without apoA-I for rat and mouse hepatocytes, respectively (**Fig. 2A**). Despite this increase in cholesterol efflux to apoA-I, labeled CE levels decreased significantly in the culture medium of ABCA1-expressing hepatocytes (Fig. 2B). This result suggested that there was a lack of lecithin:cholesterol acyltransferase (LCAT) activity in the hepatocyte medium and that the observed decrease in medium CE was perhaps due to decreased CE in apoB-containing lipoproteins. No change was seen in medium CE in *abca1*$^{-/-}$ hepatocytes. No significant changes were seen in the percent of total [^{14}C]sterols in cellular free cholesterol in any of the cell types following incubation with apoA-I (Fig. 2C). However, apoA-I induced a significant reduction in labeled cellular CE stores in primary rat and wild-type mouse hepatocytes but not in *abca1*$^{-/-}$

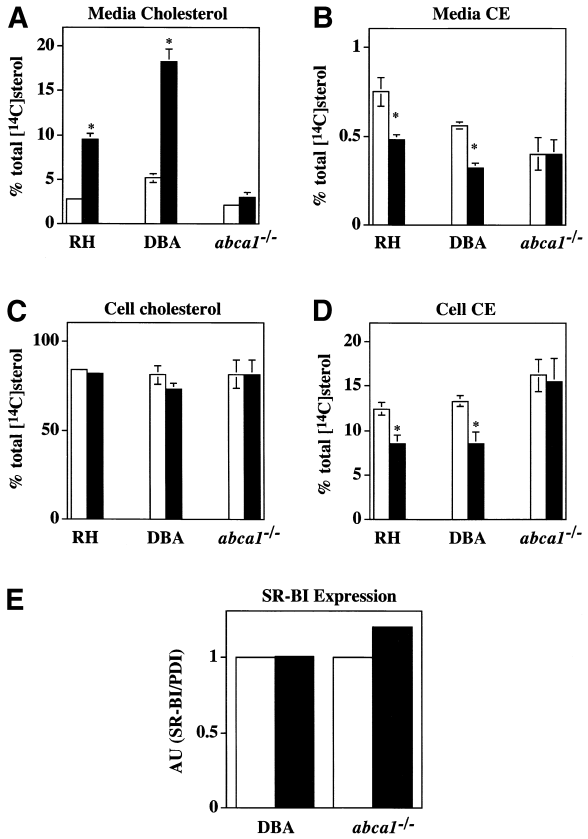

Fig. 2. Apolipoprotein A-I (apoA-I)-mediated cholesterol efflux from hepatocytes. Primary rat (RH) and control (DBA) and ABCA1-deficient (*abca1*$^{-/-}$) mouse hepatocytes were labeled with [^{14}C]mevalonate lactone and incubated in the absence (open bars) or presence (filled bars) of 20 μg/ml apoA-I for 16 h as described in Materials and Methods. Total incorporation of [^{14}C]mevalonate lactone into cholesterol plus cholesteryl esters (CEs) among the hepatocytes was in the range of 200–300 × 10^3 dpm/mg cell protein. Data are expressed as percentages of total [^{14}C]mevalonate-derived sterols migrating with cholesterol or CEs by TLC in media cholesterol (A), media CEs (B), cell cholesterol (C), and cell CEs (D). Values are the mean ± SD of triplicate determinations and are representative of three to five experiments. * $P < 0.05$ compared with cells incubated in the absence of apoA-I. E: Expression of scavenger receptor class B type I (SR-BI) as determined by arbitrary densitometry units (AU) of the ratio of SR-BI to protein disulfide isomerase protein in immunoblots of hepatocyte homogenates from DBA and *abca1*$^{-/-}$ mice in the absence (open bars) or presence (filled bars) of apoA-I.

hepatocytes (Fig. 2D). This suggested active removal of cholesterol by apoA-I, thus making less cholesterol available for esterification in ABCA1-expressing hepatocytes. Changes in radiolabeled medium and cellular cholesterol and CE showed patterns similar to changes of the mass of these sterols in apoA-I-treated DBA and *abca1*$^{-/-}$ mouse hepatocytes (**Table 1**). In particular, no significant changes were seen in the mass of any of these cholesterol compartments following incubation of *abca1*$^{-/-}$ cells with apoA-I. A trend toward decreased CE mass was observed in the DBA hepatocytes upon incubation with apoA-I, along with a concomitant significant increase in media free cholesterol mass. The significant decrease of radiolabeled cell CE

TABLE 1. Cholesterol and CE mass in DBA wild-type and ABCA1-deficient mouse hepatocytes following incubation with apoA-I

	DBA		*abca1*$^{-/-}$	
	−apoA-I	+apoA-I	−apoA-I	+apoA-I
	μg/mg cell protein			
Media				
Cholesterol	2.16 ± 0.09	3.42 ± 0.07*	1.08 ± 0.12	1.10 ± 0.03
CE	0.50 ± 0.10	0.21 ± 0.004*	0.88 ± 0.18	0.78 ± 0.12
Cells				
Cholesterol	7.44 ± 1.30	7.69 ± 1.40	9.23 ± 1.20	10.6 ± 1.90
CE	6.88 ± 0.07	6.05 ± 1.20	7.26 ± 1.30	8.35 ± 0.47

ABCA1, ATP binding cassette transporter A1; *abca1*$^{-/-}$, ABCA1-deficient; apoA-I, apolipoprotein A-I; CE, cholesteryl ester. Hepatocytes were incubated for 16 h in the presence or absence of exogenous human apoA-I, and lipids were extracted and analyzed by gas chromatography as described in Materials and Methods. The data are the average of three replicates and are representative of three independent experiments. * $P < 0.05$ compared with minus apoA-I condition.

(Fig. 2D) compared with the decrease in CE mass (Table 1) in DBA cells could be explained by increased removal of de novo synthesized cholesterol from the ACAT-accessible pool compared with mobilization of preformed CE.

Although the presence of SR-BI might provide another mechanism for cholesterol efflux from hepatocytes, lipid-free apoA-I does not mediate cholesterol efflux via SR-BI (45). In addition to the very low levels of cholesterol efflux from *abca1*$^{-/-}$ cells, we found no significant up-regulation of SR-BI in *abca1*$^{-/-}$ compared with wild-type mouse hepatocytes before or after incubation with apoA-I (Fig. 2E). These results demonstrate that endogenous ABCA1 plays a crucial role in the cholesterol efflux to apoA-I from primary hepatocytes, as previously demonstrated for other cell types [for review, see ref. (16)], and that this efflux depletes CE stores in these cells.

ABCA1-dependent HDL particle formation by hepatocytes

HDL particles formed by murine hepatocytes in the presence or absence of exogenous apoA-I were assessed by native lipoprotein gel electrophoresis. Concentrated media samples recovered from rat or mouse hepatocytes were separated on 3–10% gradient native polyacrylamide gels and analyzed by immunoblotting with anti-human apoA-I to assess particle formation with exogenous human apoA-I. The membrane was then stripped of anti-human apoA-I and reprobed with anti-murine apoA-I to assess HDL particle formation with endogenously secreted rat or mouse apoA-I. No cross-reactivity was seen between the human and murine apoA-I antibodies. Using primary rat hepatocytes, the addition of human apoA-I resulted in the formation of a series of HDL particles between 66 and 140 kDa (**Fig. 3A**). Over the 16 h time interval, an increase in endogenous rat apoA-I-containing HDL formation was seen in the absence of exogenous human apoA-I (Fig. 3B, minus apoA-I lanes). Addition of exogenous human apoA-I resulted in an increase in the number of larger rat apoA-I-containing HDL particles, in addition to inducing smaller HDL particle formation at 16 h (Fig. 3B, plus apoA-I

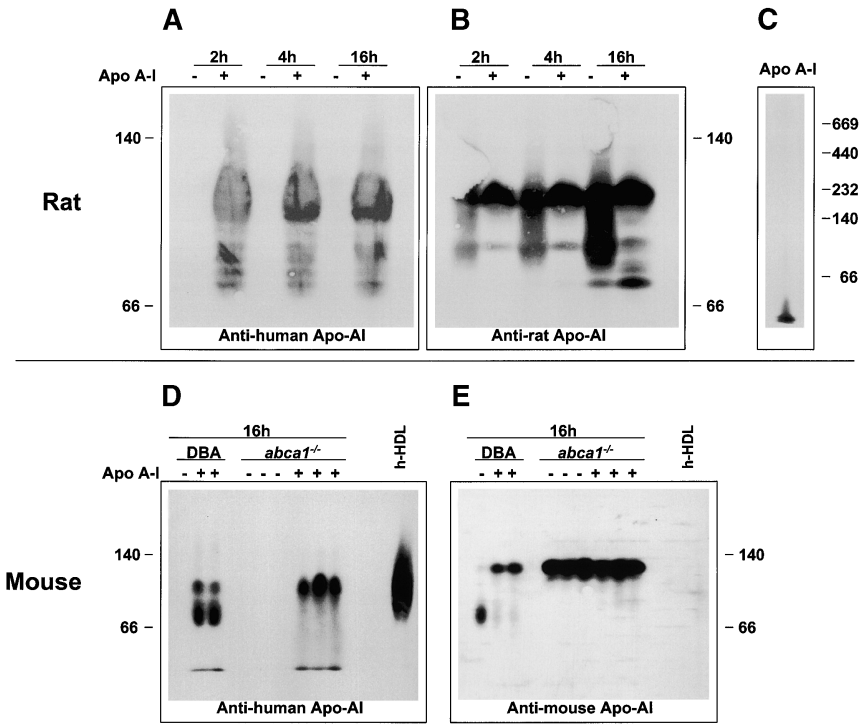

Fig. 3. Immunoblot analysis of apoA-I-containing lipoproteins. Media (10 ml) from primary rat (A, B) or mouse (D, E) hepatocytes treated in the absence (−) or presence (+) of 20 μg/ml human apoA-I were collected at the indicated time points, concentrated 20-fold, separated by native-PAGE on a 3–10% acrylamide gel (30 μg cell protein), transferred to a nitrocellulose membrane, and probed for human apoA-I (A, D). The membranes were then stripped of anti-human apoA-I antibody and reprobed for rat (B) or mouse (E) apoA-I. Data are representative of two independent experiments. C: The position of lipid-free apoA-I run on 3–10% native-PAGE and probed with anti-human apoA-I antibody, in relation to the indicated molecular weight markers. h-HDL (D, E) represents the position of migration of human HDL_3. Panels A and B and panels D and E gels are aligned in the vertical plane, as indicated by the position of molecular weight markers.

lanes). Although there may have been an increase in rat apoA-I secreted into the medium in the presence of exogenous human apoA-I, it appears that the main effect was a shift in rat apoA-I-containing HDL particle sizes. The larger rat apoA-I-containing particles formed upon addition of exogenous human apoA-I were within the size range of particles formed with human apoA-I (Fig. 3A).

HDL particle formation by mouse hepatocytes was assessed at 16 h (Figs. 3D, E). Addition of exogenous human apoA-I to wild-type DBA mouse hepatocytes resulted in the formation of two distinct HDL particles between 66 and 140 kDa in size and a predominant single larger particle following incubation with *abca1*$^{-/-}$ hepatocytes (Fig. 3D). The apoA-I band migrating below the 66 kDa marker seen in Fig. 3D represents unlipidated or poorly lipidated human apoA-I, as seen in the immunoblot of delipidated human apoA-I also run on native gel electrophoresis (Fig. 3C). Incubation of wild-type (DBA) mouse hepatocytes with exogenous human apoA-I resulted in an almost complete shift in the size of endogenous mouse HDL-containing particles from a lower (Fig. 3E, DBA, minus apoA-I lane) to a higher molecular weight (plus apoA-I lanes). In contrast, HDL particles containing endogenous mouse apoA-I secreted by *abca1*$^{-/-}$ hepatocytes were all of a larger size, which did not change upon addition of exogenous human apoA-I. There was no apparent increase in endogenous apoA-I secretion to the medium of DBA or *abca1*$^{-/-}$ mouse hepatocytes upon addition of exogenous human apoA-I (Fig. 3E). These combined results indicate that ABCA1 mediates lipid efflux to exogenous and endogenous apoA-I for the formation of new HDL particles by hepatocytes, and that addition of exogenous human apoA-I increases the number of larger HDLs containing endogenous rat or mouse apoA-I in the presence of functional ABCA1. Our results also demonstrate the formation of HDL particles even in the face of nonfunctional ABCA1 and very low cholesterol efflux (Fig. 2A). The absence of smaller HDL particles in the medium of *abca1*$^{-/-}$ hepatocytes at 16 h (Fig. 3E), as seen at earlier time points in the medium of rat hepatocytes (Fig. 3B) and reported previously for *abca1*$^{-/-}$ mouse hepatocytes after a 3.5 h incubation (24), may have been due to fusion of smaller HDL particles by 16 h in our experiments. The presence of larger HDL particles despite low levels of cholesterol efflux may be explained by the increased triacylglycerol content in HDL described previously in Tangier disease (46) and *abca1*$^{-/-}$ mice (47).

Depletion of the regulatory pool of hepatocyte cholesterol by apoA-I

ApoA-I readily depletes nonhepatic cells of their regulatory pool of cholesterol available for esterification by ACAT (3, 48), an effect also apparent in hepatocytes of the depletion of CE stores in these cells (Fig. 2D). To further assess depletion of the regulatory cholesterol pool, new cholesterol synthesis by hepatocytes was assessed following incubation with apoA-I. ApoA-I-treated McArdle RH7777 cells and primary rat and mouse hepatocytes showed a 15–20% increase in new cholesterol synthesis from ^{3}H-labeled acetate compared with cells preincubated in the absence of apoA-I (**Fig. 4**). No increase in new cholesterol synthesis was seen following incubation of *abca1*$^{-/-}$ hepatocytes with apoA-I. These results suggest that ABCA1-dependent cholesterol mobilization to apoA-I depletes the regulatory pool of cholesterol in hepatocytes, thereby increasing cholesterol synthesis by upregulation of HMG-CoA reductase.

ABCA1-dependent HDL formation decreases VLDL secretion by primary murine hepatocytes

Previous studies have shown inhibition of VLDL secretion from cultured hepatocytes incubated with HMG-CoA reductase inhibitors, suggesting that cholesterol availability is a key determinant of VLDL secretion (27, 32). Depletion of the regulatory pool of cholesterol by apoA-I, indicated in Figs. 2 and 4, suggests less cholesterol would be available for VLDL synthesis and secretion. Decreased VLDL secretion was also suggested by the diminished CE content of the media after incubation of primary hepatocytes with apoA-I (Fig. 2B). To assess the effects of hepatocyte HDL formation on VLDL secretion, the appearance of TG in hepatocyte medium was examined following incubation with apoA-I. Cells were again incubated in the presence or absence of 20 μg/ml apoA-I for 16 h. ApoA-I had no effect on synthesis of TG in any of the hepatocytes tested (data not shown). Primary rat hepatocyte medium showed a decreased proportion of total synthesized TG secreted into the medium after incubation with apoA-I (60% less compared with no apoA-I) (**Fig. 5A**). McArdle RH7777 rat hepatoma cells and DBA mouse hepatocytes showed an ~25% decrease in TG secretion to the medium. Consistent with the lack of cholesterol depletion by apoA-I, *abca1*$^{-/-}$ mouse hepatocytes showed no decrease in TG secretion at the end of the incubation period.

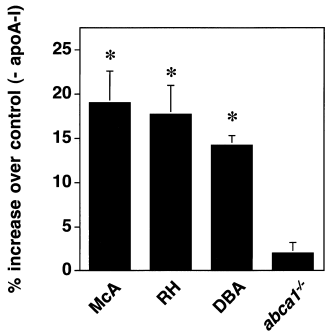

Fig. 4. Hepatocyte cholesterol synthesis following incubation with apoA-I. McArdle RH7777 (McA), primary rat (RH), control (DBA), and ABCA1-deficient (*abca1*$^{-/-}$) mouse hepatocytes were treated in the presence or absence of 20 μg/ml apoA-I for 16 h. Cells were washed and incubated with 100 μM [^{3}H]acetate as described in Materials and Methods. Data are expressed as percentage increase in total new cholesterol synthesis (cholesterol and CE in cells and media) in apoA-I treated cells compared with non-apoA-I-treated cells. Values are the mean ± SD of triplicate determinations and are representative of at least two experiments. * $P <$ 0.02 compared with cells incubated in the absence of apoA-I.

The increase in cholesterol synthesis indicated in Fig. 4 would also be expected to be associated with increased LDLR synthesis and activity. The decreased TG content in the medium of cultured hepatocytes following incubation with apoA-I was attributed to decreased VLDL secretion rather than increased uptake of apoB-containing particles from the medium for two reasons. First, Davis and colleagues (49) reported <10% reuptake of newly secreted apoB or apoE after an 18 h incubation with primary rat hepatocytes. Second, we previously reported lack of reuptake of [^{3}H]PC- and TG-labeled VLDL into McArdle RH7777 cells in a 6 h incubation (37), and <10% uptake of label when the incubation period was extended to 14 h (data not shown). To examine a potential role of the LDLR in intracellular degradation of apoB-containing lipoproteins (50) that might also explain decreased VLDL secretion in response to apoA-I, we utilized *ldlr*$^{-/-}$ and C57BL/6 control mouse hepatocytes. C57BL/6 mouse hepatocytes, like DBA mouse cells, showed an ~25% decrease in TG secretion during incubations with apoA-I. No difference in TG secretion was seen with *ldlr*$^{-/-}$ hepatocytes. Although this could be interpreted as indicating a major role of the LDLR in the intracellular degradation of VLDL and/or reuptake of apoB-containing particles in response to apoA-I treatment, alternate explanations are available. *ldlr*$^{-/-}$mice show a 1.6-fold increase in hepatic cholesterol mass, and also exhibit increased TG mass (51). Consistent with this, we found increases in TG mass in *ldlr*$^{-/-}$ hepatocytes and efflux media compared with their wild-type counterparts (Figs. 5B, C). Although we also found a 2.3-fold increase in efflux of cholesterol from *ldlr*$^{-/-}$ hepatocytes in the presence of apoA-I (compared with 3.1-fold increase from C57BL/6 mouse hepatocytes, data not shown), the increased synthesis of cholesterol by *ldlr*$^{-/-}$ cells may override any decreased availability of hepatocyte cholesterol for VLDL synthesis and secretion induced by apoA-I. These results do not allow us to rule out a role of the LDLR in decreasing VLDL secretion.

To further examine the effect of apoA-I-mediated lipid efflux on VLDL secretion and VLDL particle number, apoB secretion from hepatocytes into the efflux medium was determined. **Figure 6** shows that preincubation with apoA-I followed by pulse labeling with [^{3}H]leucine caused a 52% decrease in the secretion of labeled apoB-100 and a 28% decrease in apoB-48 secretion to the medium of primary rat hepatocytes. Decreases in apoB-100 and apoB-48 were noted across all densities upon fractionation of the media, most notably in the VLDL density range (data not shown). Furthermore, immunoprecipitation of cellular

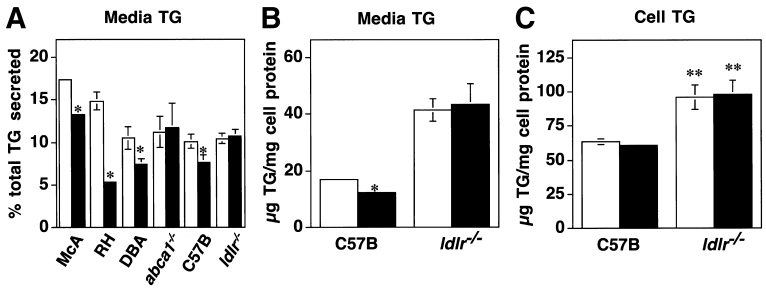

Fig. 5. Triacylglycerol (TG) synthesis and secretion by hepatocytes. A: TG secretion following incubation of hepatocytes with apoA-I. McArdle RH7777 (McA), rat (RH), DBA mouse (DBA), ABCA1-deficient mouse (*abca1*$^{-/-}$), C57BL/6J mouse (C57B), and LDL receptor (LDLR)-deficient (*ldlr*$^{-/-}$) mouse hepatocytes were incubated with 100 μM [^{3}H]glycerol in the absence (open bars) or presence (filled bars) of 20 μg/ml apoA-I for 16 h. Media and cellular lipids were extracted and TG analyzed by TLC as described in Materials and Methods. Values represent the percentage of total cell plus media TG that was secreted into the media. ApoA-I treatment had no effect on TG synthesis by all cell types. *, $P < 0.05$ for cells treated in the absence of apoA-I. B, C: Analysis of TG secretion (B) and TG levels (C) in C57BL/6J and *ldlr*$^{-/-}$ mouse hepatocytes. Hepatocytes were incubated in the absence (open bars) or presence (filled bars) of apoA-I as described above; TG content in media and cells was determined by using "Infinity" TG reagent (Sigma Diagnostics, Inc.) with authentic TG (triolein) as the internal standard. **, $P < 0.005$ for increased cellular TG levels in *ldlr*$^{-/-}$ mice.

apoB did not show accumulation of apoB but rather decreased apoB levels in primary rat hepatocytes treated with apoA-I, which was not prevented by addition of the proteosomal degradation inhibitor ALLN (data not shown). ApoA-I treatment of wild-type mouse hepatocytes decreased apoB-100 mass in the medium by 60% and apoB-48 mass by 35%, as assessed by densitometric measurements of Cab-O-Sil-precipitated medium. ApoA-I induced no significant decreases in apoB-100 or apoB-48 secretion from *abca1*$^{-/-}$ hepatocytes. Overall, these results suggest that ABCA1-dependent lipid efflux to apoA-I decreases cholesterol availability for VLDL synthesis and secretion by hepatocytes.

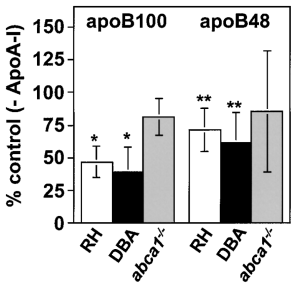

Fig. 6. ApoB secretion from primary rat hepatocytes. Rat hepatocytes (RH) were incubated in the presence or absence of 20 μg/ml apoA-I for 16 h, pulse labeled with [^{3}H]leucine for 20 min, and chased for 2 h in the absence of apoA-I. Total apoB was immunoprecipitated from media. A rat VLDL carrier was added, the samples were electrophoresed and silver stained, and the radioactivity associated with bands corresponding to apoB-100 and apoB-48 was analyzed as described in Materials and Methods. Hepatocytes from control (DBA) and ABCA1-deficient (*abca1*$^{-/-}$) mice were incubated in the presence or absence of 20 μg/ml apoA-I for 16 h. ApoB was precipitated from media by Cab-O-Sil and analyzed by immunoblotting as described in Materials and Methods. Data are expressed as percentage of apoB secreted by apoA-I-treated cells compared with cells incubated in the absence of apoA-I and are an average of the results of two experiments, each done in triplicate. $P < 0.05$ compared with cells incubated without apoA-I for apoB-100 (*) and apoB-48 (**).

DISCUSSION

High levels of ABCA1 expression in the liver (7–10), the requirement of hepatic expression of this transporter to raise HDL levels in ABCA1-transgenic animals (17–20), and increases in HDL levels in mice expressing adenoviral-directed ABCA1 in the liver (22, 23) have suggested a major role of hepatic ABCA1 in the formation of HDL particles and in determining plasma HDL levels. The aims of the present study were to directly define the role of endogenous hepatic ABCA1 in HDL particle formation and to investigate the relationship between primary hepatocyte HDL formation and VLDL secretion by these cells. The major findings of these studies are: *1*) hepatocyte ABCA1 is required for cholesterol efflux to apoA-I for new HDL particle formation by these cells; *2*) ABCA1-dependent cholesterol efflux actively depletes the regulatory pool of hepatocyte cholesterol, leading to increased new cholesterol synthesis by these cells; and *3*) ABCA1-dependent depletion of cellular cholesterol by apoA-I decreases secretion of VLDL by primary hepatocytes.

Several lines of evidence suggest a pivotal role of ABCA1 in hepatocyte lipoprotein metabolism. ApoA-I induced a marked increase in cholesterol efflux and decrease in cellular CE stores in primary rat and mouse hepatocytes but not in *abca1*$^{-/-}$ hepatocytes. Exogenous apoA-I-mediated lipid efflux resulted in new HDL particle formation and a shift in the size of HDL particles containing endogenous apoA-I secreted by both rat and mouse hepatocytes to larger particles. *abca1*$^{-/-}$ hepatocytes secreted a single species of HDL particles. These particles did not change in size upon addition of exogenous apoA-I, consistent with another recent report (24). Depletion of the regulatory pool of cholesterol was suggested by the increase in new cholesterol synthesis following incubation with apoA-I. This effect was absent in *abca1*$^{-/-}$ hepatocytes. A decrease in media CEs was seen following apoA-I incubation with rat and control mouse hepatocytes de-

spite a marked increase in cholesterol efflux to the medium. This suggested that there was an absence of significant LCAT activity in the efflux medium and that VLDL particle secretion was decreasing. Decreased VLDL secretion following apoA-I incubation was confirmed by the findings of markedly reduced TG and apoB in the medium compared with cells incubated in the absence of apoA-I. The dependence of this effect on ABCA1 was again confirmed using *abca1*$^{-/-}$ hepatocytes, which showed no decrease in TG or apoB secretion following incubation with apoA-I.

Our native gel electrophoresis results with *abca1*$^{-/-}$ hepatocyte media suggest an ABCA1-independent mechanism for secretion of preformed HDL particles (Fig. 3), consistent with the recent report by Kiss et al. (24). We also found evidence for lipidation of exogenously added apoA-I by *abca1*$^{-/-}$ cells. This suggests that exogenous apoA-I is either associating with HDL particles secreted by these cells or that it is being endocytosed and resecreted in a partially lipidated form (52). However, *abca1*$^{-/-}$ hepatocytes show very low levels of cholesterol efflux (Fig. 2A), and Tangier disease patients as well as *abca1*$^{-/-}$ mice have extremely low levels of plasma HDL (53, 54). This suggests that HDL particles formed by the liver in the absence of ABCA1 are unable to act as effective precursors for further HDL particle formation or enlargement by the actions of LCAT and passive cholesterol efflux from peripheral or hepatic tissues. Francone et al. (47) have recently reported altered phospholipid content and impaired LCAT activity in plasma HDL of *abca1*$^{-/-}$ mice, which may help to explain this phenomenon. The ability of human apoA-I to increase the lipidation of endogenously secreted rat and wild-type mouse apoA-I provides support for the role of apoA-I in enhancing ABCA1 activity, possibly through inhibiting protease-mediated degradation of the transporter (55, 56).

Our results suggest decreased VLDL secretion in response to ABCA1-mediated HDL particle formation under experimental conditions in which there is little reuptake of secreted VLDL particles. Whether this would also occur in vivo is not yet known. However, this possibility is suggested by the finding of elevated fasting plasma TG in patients with Tangier disease and in *abca1*$^{-/-}$ mice (53, 54). Sniderman et al. (57) have recently reported decreased apoB-100 secretion by HepG2 cells in response to incubation with apoA-I. These results provide further support for a regulatory pool of cholesterol in hepatocytes shared for formation of both VLDL and HDL. These authors did not detect changes in TG secretion in response to apoA-I. However, inasmuch as HepG2 cells do not secrete true TG-rich VLDL particles, this result is difficult to interpret. Wellington et al. (23) found decreased plasma TG levels in mice expressing human ABCA1 delivered via a bacterial artificial chromosome and in mice expressing low levels of adenoviral-delivered human ABCA1. These results further support our conclusion that ABCA1-dependent HDL formation by the liver may decrease VLDL particle secretion in vivo.

Depletion of cellular cholesterol by apoA-I and the subsequent increase in new cholesterol synthesis by HMG-CoA reductase would also be accompanied by increased hepatocyte LDLR expression (which we also found following a 2 h incubation with apoA-I, data not shown). The net effect of this in vivo might be to replenish hepatocyte cholesterol stores by increasing clearance of remnant lipoproteins and LDL from plasma. The initial shunting of hepatocyte cholesterol to HDL formation by ABCA1, however, would increase the total amount of cholesterol in the reverse cholesterol transport pathway. Even if some of this cholesterol were then transferred back to apoB-100-containing lipoproteins by cholesteryl ester transfer protein, the larger proportion of hepatocyte cholesterol entering the circulation as HDL would increase the overall return of HDL-cholesterol to the liver. Studies in both humans and mice indicate preferential shunting of HDL-derived cholesterol into bile acid synthesis (58–60) and therefore out of the pool of lipids available for VLDL synthesis by the liver. The decreased LDL cholesterol levels seen in Tangier disease are not inconsistent with increased VLDL secretion in this disorder, inasmuch as a large proportion of LDL cholesterol is derived from HDL rather than VLDL. In addition, lipolysis of VLDL particles in Tangier disease is partially decreased (61), also likely contributing to the higher TG seen in this disorder and leading to a further decrease in LDL levels. Thus, although we cannot rule out the possibility that VLDL remnant and LDL particle uptake is also increased in response to depletion of hepatocyte cholesterol by ABCA1, we suggest that the overall effect of ABCA1 in the liver would be to remove cholesterol from atherogenic apoB-100-containing lipoprotein synthesis and increase cholesterol in the HDL/reverse cholesterol transport pathway.

The reasons for decreased VLDL secretion following hepatocyte incubation with apoA-I remain to be elucidated. It is possible that intracellular apoB degradation was increased in response to an increase in LDLR expression (50) and was partially responsible for the decreased intracellular apoB and VLDL secretion observed. The inhibition of LDLR function by Triton WR 1339, which might also block intracellular degradation of apoB by the LDLR (62), makes studies using the Triton method to assess apoB production in response to expression of the ABCA1 transgene difficult to interpret. One study in ABCA1 transgenic mice found an increase in apoB levels believed to be due to decreased clearance of apoB as assessed using the Triton method (18), while the same authors found a marked decrease in plasma apoB levels in the same animals fed an atherogenic diet (63). Future experiments are needed to address the effects of apoA-I incubation on apoB synthesis, degradation, and clearance by hepatocytes.

The likelihood that VLDL secretion was inhibited due to depletion of the regulatory pool of free cholesterol in the cell by apoA-I was suggested by the increase in new cholesterol synthesis following incubation of hepatocytes with apoA-I. This observation is consistent with the known ability of apoA-I to markedly deplete this regulatory ACAT-accessible pool of cholesterol in other cell types (3,

48, 64) and the impaired VLDL secretion in response to HMG-CoA reductase inhibitors. We can thus postulate that apoA-I-mediated removal of cholesterol results in increased synthesis of new cholesterol, used preferentially to restore membrane cholesterol in hepatocytes at the expense of VLDL particle assembly. Alternatively, it is possible that CE stores may be the source of VLDL cholesterol or CE, and hence, the decrease in intracellular CE levels following apoA-I incubation may be responsible for decreased VLDL secretion.

In conclusion, the results presented here suggest ABCA1-dependent cholesterol mobilization by hepatocytes to apoA-I mediates HDL particle formation and limits cholesterol availability for VLDL particle secretion. These results provide additional evidence that the liver plays a much greater role in HDL formation and reverse cholesterol transport than previously thought. We propose that hepatocyte ABCA1-generated HDL provide a critical pool of HDL precursor particles that circulate into the periphery as a source of apoA-I for further HDL formation via interaction with ABCA1 on cells such as macrophages or as a source of acceptor particles for passive cholesterol efflux from cells. Whether this pathway has significant effects on levels of apoB-containing lipoproteins in vivo remains to be seen. It would, however, be expected to divert more hepatic cholesterol into the reverse cholesterol transport pathway for eventual return to the liver and excretion out of the body in bile.

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REFERENCES

1. Fielding, C. J., and P. E. Fielding. 1995. Molecular physiology of reverse cholesterol transport. *J. Lipid Res.* **36:** 211–228.
2. Havel, R. J., and J. P. Kane. 2001. Structure and metabolism of plasma lipoproteins. *In* The Metabolic and Molecular Bases of Inherited Disease. 8th edition. C. R. Scriver, A .L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw Hill, Inc., New York. 2705–2716.
3. Francis, G. A., R. H. Knopp, and J. F. Oram. 1995. Defective removal of cellular cholesterol and phospholipids by apolipoprotein A-I in Tangier Disease. *J. Clin. Invest.* **96:** 78–87.
4. Remaley, A. T., U. K. Schumacher, J. A. Stonik, B. D. Farsi, H. Nazih, and H. B. Brewer, Jr. 1997. Decreased reverse cholesterol transport from Tangier disease fibroblasts. Acceptor specificity and effect of brefeldin on lipid efflux. *Arterioscler. Thromb. Vasc. Biol.* **17:** 1813–1821.
5. Oram, J. F., and R. M. Lawn. 2001. ABCA1. The gatekeeper for eliminating excess tissue cholesterol. *J. Lipid Res.* **42:** 1173–1179.
6. Windmueller, H. G., P. N. Herbert, and R. I. Levy. 1973. Biosynthesis of lymph and plasma lipoprotein apoproteins by isolated perfused rat liver and intestine. *J. Lipid Res.* **14:** 215–223.
7. Luciani, M. F., F. Denizot, S. Savary, M. G. Mattei, and G. Chimini. 1994. Cloning of two novel ABC transporters mapping on human chromosome 9. *Genomics.* **21:** 150–159.
8. Langmann, T., J. Klucken, M. Reil, G. Liebisch, M. F. Luciani, G. Chimini, W. E. Kaminski, and G. Schmitz. 1999. Molecular cloning of the human ATP-binding cassette transporter 1 (hABC1): evidence for sterol-dependent regulation in macrophages. *Biochem. Biophys. Res. Commun.* **257:** 29–33.
9. Lawn, R. M., D. P. Wade, T. L. Couse, and J. N. Wilcox. 2001. Localization of human ATP-binding cassette transporter 1 (ABC1) in normal and atherosclerotic tissues. *Arterioscler. Thromb. Vasc. Biol.* **21:** 378–385.
10. Wellington, C. L., E. K. Walker, A. Suarez, A. Kwok, N. Bissada, R. Singaraja, Y. Z. Yang, L. H. Zhang, E. James, J. E. Wilson, O. Francone, B. M. McManus, and M. R. Hayden. 2002. ABCA1 mRNA and protein distribution patterns predict multiple different roles and levels of regulation. *Lab. Invest.* **82:** 273–283.
11. Marsh, J. B. 1974. Lipoproteins in a nonrecirculating perfusate of rat liver. *J. Lipid Res.* **15:** 544–550.
12. Hamilton, R. L., M. C. Williams, C. J. Fielding, and R. J. Havel. 1976. Discoidal bilayer structure of nascent high density lipoproteins from perfused rat liver. *J. Clin. Invest.* **58:** 667–680.
13. Jones, L. A., T. Teramoto, D. J. Juhn, R. B. Goldberg, A. H. Rubenstein, and G. S. Getz. 1984. Characterization of lipoprotein produced by the perfused rhesus monkey liver. *J. Lipid Res.* **25:** 319–335.
14. Sviridov, D., and N. Fidge. 1995. Pathway of cholesterol efflux from human hepatoma cells. *Biochim. Biophys. Acta.* **1256:** 210–220.
15. Chisholm, J. W., E. R. Burleson, G. S. Shelness, and J. S. Parks. 2002. ApoA-I secretion from HepG2 cells: evidence for the secretion of both lipid-poor apoA-I and intracellularly assembled nascent HDL. *J. Lipid Res.* **43:** 36–44.
16. Bortnick, A. E., G. H. Rothblat, G. Stoudt, K. L. Hoppe, L. J. Royer, J. McNeish, and O. L. Francone. 2000. The correlation of ATP-binding cassette 1 mRNA levels with cholesterol efflux from various cell lines. *J. Biol. Chem.* **275:** 28634–28640.
17. Singaraja, R. R., V. Bocher, E. R. James, S. M. Clee, L. Zhang, B. R. Leavitt, B. Tan, A. Brooks-Wilson, A. Kwok, N. Bissada, Y. Yang, G. Liu, S. R. Tafuri, C. Fievet, C. L. Wellington, B. Staels, and M. R. Hayden. 2001. Human ABCA1 BAC transgenic mice show increased HDL-C and ApoAI-dependent efflux stimulated by an internal promoter containing LXREs in intron 1. *J. Biol. Chem.* **276:** 33969–33979.
18. Vaisman, B. L., G. Lambert, M. Amar, C. Joyce, T. Ito, R. D. Shamburek, W. J. Cain, J. Fruchart-Najib, E. D. Neufeld, A. T. Remaley, H. B. Brewer, Jr., and S. Santamarina-Fojo. 2001. ABCA1 overexpression leads to hyperalphalipoproteinemia and increased biliary cholesterol excretion in transgenic mice. *J. Clin. Invest.* **108:** 303–309.
19. Haghpassand, M., P. A. Bourassa, O. L. Francone, and R. J. Aiello. 2001. Monocyte/macrophage expression of ABCA1 has minimal contribution to plasma HDL levels. *J. Clin. Invest.* **108:** 1315–1320.
20. van Eck, M., I. S. Bos, W. E. Kaminski, E. Orso, G. Rothe, J. Twisk, A. Bottcher, E. S. Van Amersfoort, T. A. Christiansen-Weber, W. P. Fung-Leung, T. J. Van Berkel, and G. Schmitz. 2002. Leukocyte ABCA1 controls susceptibility to atherosclerosis and macrophage recruitment into tissues. *Proc. Natl. Acad. Sci. USA.* **99:** 6298–6303.
21. Joseph, S. B., E. McKilligin, L. Pei, M. A. Watson, A. R. Collins, B. A. Laffitte, M. Chen, G. Noh, J. Goodman, G. N. Hagger, J. Tran, T. K. Tippin, X. Wang, A. J. Lusis, W. A. Hsueh, R. E. Law, J. L. Collins, T. M. Willson, and P. Tontonoz. 2002. Synthetic LXR ligand inhibits the development of atherosclerosis in mice. *Proc. Natl. Acad. Sci. USA.* **99:** 7604–7609.
22. Basso, F., L. Freeman, C. L. Knapper, A. Remaley, J. Stonik, E. B. Neufeld, T. Tansey, M. J. Amar, J. Fruchart-Najib, N. Duverger, S. Santamarina-Fojo, and H. B. Brewer, Jr. 2003. Role of the hepatic ABCA1 transporter in modulating intrahepatic cholesterol and plasma HDL cholesterol concentrations. *J. Lipid Res.* **44:** 296–302.
23. Wellington, C. L., L. R. Brunham, S. Zhou, R. R. Singaraja, H. Visscher, A. Gelfer, C. Ross, E. James, G. Liu, M. T. Huber, Y. Z. Yang, R. J. Parks, A. Groen, J. Fruchart-Najib, and M. R. Hayden. 2003. Alterations of plasma lipids in mice via adenoviral-mediated hepatic overexpression of human ABCA1. *J. Lipid Res.* **44:** 1470–1480.
24. Kiss, R. S., D. C. McManus, V. Franklin, W. L. Tan, A. McKenzie, G. Chimini, and Y. L. Marcel. 2003. The lipidation by hepatocytes of human apolipoprotein A-I occurs by both ABCA1 dependent and independent pathways. *J. Biol. Chem.* **278:** 10119–10127.
25. Oram, J. F. 2002. ATP-binding cassette transporter A1 and cholesterol trafficking. *Curr. Opin. Lipidol.* **13:** 373–381.
26. Mulligan, J. D., M. T. Flowers, A. Tebon, J. J. Bitgood, C. Wellington, M. R. Hayden, and A. D. Attie. 2003. ABCA1 is essential for effi-

cient basolateral cholesterol efflux during the absorption of dietary cholesterol in chickens. *J. Biol. Chem.* **278:** 13356–13366.
27. Khan, B., H. G. Wilcox, and M. Heimberg. 1989. Cholesterol is required for secretion of very-low-density lipoprotein by rat liver. *Biochem. J.* **258:** 807–816.
28. Khan, B. V., T. V. Fungwe, H. G. Wilcox, and M. Heimberg. 1990. Cholesterol is required for the secretion of the very-low-density lipoprotein: in vivo studies. *Biochim. Biophys. Acta.* **1044:** 297–304.
29. Burnett, J. R., L. J. Wilcox, D. E. Telford, S. J. Kleinstiver, P. H. Barrett, R. S. Newton, and M. W. Huff. 1997. Inhibition of HMG-CoA reductase by atorvastatin decreases both VLDL and LDL apolipoprotein B production in miniature pigs. *Arterioscler. Thromb. Vasc. Biol.* **17:** 2589–2600.
30. Burnett, J. R., L. J. Wilcox, D. E. Telford, S. J. Kleinstiver, P. H. Barrett, R. S. Newton, and M. W. Huff. 1999. The magnitude of decrease in hepatic very low density lipoprotein apolipoprotein B secretion is determined by the extent of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibition in miniature pigs. *Endocrinology.* **140:** 5293–5302.
31. Ginsberg, H. N., N. A. Le, M. P. Short, R. Ramakrishnan, and R. J. Desnick. 1987. Suppression of apolipoprotein B production during treatment of cholesteryl ester storage disease with lovastatin. Implications for regulation of apolipoprotein B synthesis. *J. Clin. Invest.* **80:** 1692–1697.
32. Brown, A., D. Wiggins, and G. F. Gibbons. 1999. Manipulation of cholesterol and cholesteryl ester synthesis has multiple effects on the metabolism of apolipoprotein B and the secretion of very-low-density lipoprotein by primary hepatocyte cultures. *Biochim. Biophys. Acta.* **1440:** 253–265.
33. Chung, B. H., T. Wilkinson, J. C. Greer, and J. P. Segrest. 1980. Preparative and quantitative isolation of plasma lipoproteins: rapid, single discontinuous density gradient ultracentrifugation in a vertical rotor. *J. Lipid Res.* **21:** 284–291.
34. Yokoyama, S., S. Tajima, and A. Yamamoto. 1982. The process of dissolving apolipoprotein A-I in an aqueous buffer. *J. Biochem. (Tokyo).* **91:** 1267–1272.
35. Hamon, Y., C. Broccardo, O. Chambenoit, M. F. Luciani, F. Toti, S. Chaslin, J. M. Freyssinet, P. F. Devaux, J. McNeish, D. Marguet, and G. Chimini. 2000. ABC1 promotes engulfment of apoptotic cells and transbilayer redistribution of phosphatidylserine. *Nat. Cell Biol.* **2:** 399–406.
36. Yao, Z. M., and D. E. Vance. 1988. The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. *J. Biol. Chem.* **263:** 2998–3004.
37. Lehner, R., and D. E. Vance. 1999. Cloning and expression of a cDNA encoding a hepatic microsomal lipase that mobilizes stored triacylglycerol. *Biochem. J.* **343:** 1–10.
38. Myher, J. J., A. Kuksis, and S. Pind. 1989. Molecular species of glycerophospholipids and sphingomyelins of human plasma: comparison to red blood cells. *Lipids.* **24:** 408–418.
39. Kojima, K., S. Abe-Dohmae, R. Arakawa, I. Murakami, K. Suzumori, and S. Yokoyama. 2001. Progesterone inhibits apolipoprotein-mediated cellular lipid release: a putative mechanism for the decrease of high-density lipoprotein. *Biochim. Biophys. Acta.* **1532:** 173–184.
40. Cartwright, I. J., and J. A. Higgins. 1996. Intracellular degradation in the regulation of secretion of apolipoprotein B-100 by rabbit hepatocytes. *Biochem. J.* **314:** 977–984.
41. Vance, D. E., D. B. Weinstein, and D. Steinberg. 1984. Isolation and analysis of lipoproteins secreted by rat liver hepatocytes. *Biochim. Biophys. Acta.* **792:** 39–47.
42. Johnson, W. J., E. P. Kilsdonk, A. van Tol, M. C. Phillips, and G. H. Rothblat. 1991. Cholesterol efflux from cells to immunopurified subfractions of human high density lipoprotein: LP-AI and LP-AI/AII. *J. Lipid Res.* **32:** 1993–2000.
43. Oram, J. F., and S. Yokoyama. 1996. Apolipoprotein-mediated removal of cellular cholesterol and phospholipids. *J. Lipid Res.* **37:** 2473–2491.
44. Mendez, A. J. 1997. Cholesterol efflux mediated by apolipoproteins is an active cellular process distinct from efflux mediated by passive diffusion. *J. Lipid Res.* **38:** 1807–1821.
45. Ji, Y., B. Jian, N. Wang, Y. Sun, M. de la Llera Moya, M. C. Phillips, G. H. Rothblat, J. B. Swaney, and A. R. Tall. 1997. Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux. *J. Biol. Chem.* **272:** 20982–20985.
46. Cheung, M. C., A. J. Mendez, A. C. Wolf, and R. H. Knopp. 1993. Characterization of apolipoprotein A-I- and A-II-containing lipoproteins in a new case of high density lipoprotein deficiency resembling Tangier disease and their effects on intracellular cholesterol efflux. *J. Clin. Invest.* **91:** 522–529.
47. Francone, O. L., P. V. Subbaiah, A. van Tol, L. Royer, and M. Haghpassand. 2003. Abnormal phospholipid composition impairs HDL biogenesis and maturation in mice lacking Abca1. *Biochemistry.* **42:** 8569–8578.
48. Francis, G. A., M. Tsujita, and T. L. Terry. 1999. Apolipoprotein A-I efficiently binds and mediates cholesterol and phospholipid efflux from human but not rat aortic smooth muscle cells. *Biochemistry.* **38:** 16315–16322.
49. Davis, R. A., J. R. Boogaerts, R. A. Borchardt, M. Malone-McNeal, and J. Archambault-Schexnayder. 1985. Intrahepatic assembly of very low density lipoproteins. Varied synthetic response of individual apolipoproteins to fasting. *J. Biol. Chem.* **260:** 14137–14144.
50. Twisk, J., D. L. Gillian-Daniel, A. Tebon, L. Wang, P. H. Barrett, and A. D. Attie. 2000. The role of the LDLR in apolipoprotein B secretion. *J. Clin. Invest.* **105:** 521–532.
51. Horton, J. D., H. Shimano, R. L. Hamilton, M. S. Brown, and J. L. Goldstein. 1999. Disruption of LDLR gene in transgenic SREBP-1a mice unmasks hyperlipidemia resulting from production of lipid-rich VLDL. *J. Clin. Invest.* **103:** 1067–1076.
52. Takahashi, Y., and J. D. Smith. 1999. Cholesterol efflux to apolipoprotein A1 involves endocytosis and resecretion in a calcium-dependent pathway. *Proc. Natl. Acad. Sci. USA.* **96:** 11358–11363.
53. Assmann, G., A. Von Eckardstein, and H. B. Brewer. 2001. Familial analphalipoproteinemia: Tangier disease. *In* The Metabolic and Molecular Bases of Inherited Disease. 8th edition. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw Hill Inc., New York. 2937–2980.
54. Orso, E., C. Broccardo, W. E. Kaminski, A. Bottcher, G. Liebisch, W. Drobnik, A. Gotz, O. Chambenoit, W. Diederich, T. Langmann, T. Spruss, M. F. Luciani, G. Rothe, K. J. Lackner, G. Chimini, and G. Schmitz. 2000. Transport of lipids from golgi to plasma membrane is defective in tangier disease patients and Abc1-deficient mice. *Nat. Genet.* **24:** 192–196.
55. Arakawa, R., and S. Yokoyama. 2002. Helical apolipoproteins stabilize ATP-binding cassette transporter A1 by protecting it from thiol protease-mediated degradation. *J. Biol. Chem.* **277:** 22426–22429.
56. Wang, N., W. Chen, P. Linsel-Nitschke, L. O. Martinez, B. Agerholm-Larsen, D. L. Silver, and A. R. Tall. 2003. A PEST sequence in ABCA1 regulates degradation by calpain protease and stabilization of ABCA1 by apoA-I. *J. Clin. Invest.* **111:** 99–107.
57. Sniderman, A. D., Z. Zhang, J. Genest, and K. Cianflone. 2003. Effects on apoB-100 secretion and bile acid synthesis by redirecting cholesterol efflux from HepG2 cells. *J. Lipid Res.* **44:** 527–532.
58. Schwartz, C. C., L. G. Halloran, Z. R. Vlahcevic, D. H. Gregory, and L. Swell. 1978. Preferential utilization of free cholesterol from high-density lipoproteins for biliary cholesterol secretion in man. *Science.* **200:** 62–64.
59. Kozarsky, K. F., M. H. Donahee, A. Rigotti, S. N. Iqbal, E. R. Edelman, and M. Krieger. 1997. Overexpression of the HDL receptor SR-BI alters plasma HDL and bile cholesterol levels. *Nature.* **387:** 414–417.
60. Silver, D. L., and A. R. Tall. 2001. The cellular biology of scavenger receptor class B type I. *Curr. Opin. Lipidol.* **12:** 497–504.
61. Wang, C. S., P. Alaupovic, R. E. Gregg, and H. B. Brewer, Jr. 1987. Studies on the mechanism of hypertriglyceridemia in Tangier disease. Determination of plasma lipolytic activities, k1 values and apolipoprotein composition of the major lipoprotein density classes. *Biochim. Biophys. Acta.* **920:** 9–19.
62. Millar, J. S., C. Maugeais, I. V. Fuki, and D. J. Rader. 2002. Normal production rate of apolipoprotein B in LDLR-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **22:** 989–994.
63. Joyce, C. W., M. J. Amar, G. Lambert, B. L. Vaisman, B. Paigen, J. Najib-Fruchart, R. F. Hoyt, Jr., E. D. Neufeld, A. T. Remaley, D. S. Fredrickson, H. B. Brewer, Jr., and S. Santamarina-Fojo. 2002. The ATP binding cassette transporter A1 (ABCA1) modulates the development of aortic atherosclerosis in C57BL/6 and apoE-knockout mice. *Proc. Natl. Acad. Sci. USA.* **99:** 407–412.
64. Oram, J. F., A. J. Mendez, J. P. Slotte, and T. F. Johnson. 1991. High density lipoprotein apolipoproteins mediate removal of sterol from intracellular pools but not plasma membranes of cholesterol-loaded fibroblasts. *Arterioscler. Thromb.* **11:** 403–414.