Factors controlling nascent high-density lipoprotein particle heterogeneity: ATP-binding cassette transporter A1 activity and cell lipid and apolipoprotein AI availability

Nicholas N. Lyssenko,* Margaret Nickel,* Chongren Tang,† and Michael C. Phillips*†

*Lipid Research Group, Division of Gastroenterology, Hepatology, and Nutrition, Children’s Hospital of Philadelphia, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, Pennsylvania, USA; and †Department of Medicine, Diabetes and Obesity Center of Excellence, University of Washington, Seattle, Washington, USA

ABSTRACT Nascent high-density lipoprotein (HDL) particles arise in different sizes. We have sought to uncover factors that control this size heterogeneity. Gel filtration, native PAGE, and protein cross-linking were used to analyze the size heterogeneity of nascent HDL produced by BHK-ABCA1, RAW 264.7, J774, and HepG2 cells under different levels of two factors considered as a ratio, the availability of apolipoprotein AI (apoAI) -accessible cell lipid, and concentration of extracellular lipid-free apoAI. Increases in the available cell lipid:apoAI ratio due to either elevated ATP-binding cassette transporter A1 (ABCA1) expression and activity or raised cell density (i.e., increasing numerator) shifted the production of nascent HDL from smaller particles with fewer apoAI molecules per particle and fewer molecules of choline-phospholipid and cholesterol per apoAI molecule to larger particles that contained more apoAI and more lipid per molecule of apoAI. A further shift to larger particles was observed in BHK-ABCA1 cells when the available cell lipid:apoAI ratio was raised still higher by decreasing the apoAI concentration (i.e., the denominator). These changes in nascent HDL biogenesis were reminiscent of the transition that occurs in the size composition of reconstituted HDL in response to an increasing initial lipid:apoAI molar ratio. Thus, the ratio of available cell lipid:apoAI is a fundamental cause of nascent HDL size heterogeneity, and rHDL formation is a good model of nascent HDL biogenesis.—Lyssenko, N. N., Nickel, M., Tang, C., Phillips, M. C. Factors controlling nascent high-density lipoprotein particle heterogeneity: ATP-binding cassette transporter A1 activity and cell lipid and apolipoprotein AI availability. FASEB J. 27, 2880–2892 (2013). www.fasebj.org

Key Words: macrophage reverse cholesterol transport · HDL quality · reconstituted HDL · discoidal HDL

High-density lipoprotein (HDL) is a collection of heterogeneous lipid-protein particles that contain amphipathic exchangeable apolipoproteins, primarily apolipoprotein AI (apoAI), as the major structural component (1, 2). Early functional studies revealed that HDL accepts free cholesterol from cells such as erythrocytes, fibroblasts, and macrophages and donates it in the form of ester to the liver and steroidogenic organs, and thus facilitates reverse cholesterol transport (RCT) from peripheral to cholesterol-catabolizing tissues (3, 4). At the same time, epidemiological investigations in human populations showed that higher levels of HDL-cholesterol (HDL-C) correlate with a lower risk of cardiovascular disease (CVD) (5, 6). This inverse association between HDL-C and CVD was proposed to reflect an atheroprotective effect that accrues from the macrophage branch of RCT (mRCT), i.e., cholesterol removal by HDL from macrophages residing in the arterial intima (5, 7). More recently, HDL was shown to possess a number of additional atheroprotective properties, such as anti-inflammatory and antithrombotic effects (1). A series of human clinical trials and investigations in transgenic murine...
models have demonstrated that interventions that potentially enhance mRCT also forestall atherogenesis (1, 8). However, it had been unclear whether HDL-C directly promotes mRCT, enhances other HDL atheroprotective properties, and reduces CVD risk, or merely marks a very active HDL-mediated macrophage cholesterol egress. Recently several clinical trials designed to pharmacologically elevate HDL-C levels in humans have been reported, but whether this increases aromatic 

HDL arises as nascent discoidal particles, which are remodelled to mature spherical HDL through the activity of lecithin:cholesterol acyltransferase (LCAT; ref. 1). Nascent HDL forms from cell lipid and lipid-free apoAI in a process mediated by ATP-binding cassette transporter AL (ABCA1) and may also appear as a by-product during lipolysis of apolipoprotein B (apoB)-containing lipoproteins (10). The complete absence of morphologically and biochemically normal HDL in the plasma of patients with Tangier disease (11), which is caused by a lack of active ABCA1, suggests that the ABCA1-mediated process is an essential source of the discoidal HDL precursors. The majority of nascent HDL particles, apoAI, and HDL phospholipid and cholesterol originates in the liver (12); the intestine is the second most important contributor to the HDL pool (13), while macrophages do not express apoAI and contribute relatively very little to the HDL lipid levels (14, 15). In hepatocytes, a significant fraction of endogenously expressed apoAI undergoes lipidation in subcellular compartments, while a majority acquires lipid from the plasma membrane after secretion (16, 17). However, in cells that do not express apoAI, including macrophages, lipidation of exogenous apoAI occurs primarily on the cell surface (18–20).

HDL particles exhibit substantial heterogeneity in terms of size and lipid and protein composition (2). Because certain HDL species may stimulate mRCT more than others (21), this heterogeneity is likely responsible for the considerable variability in the extent to which HDL samples from different individuals accept macrophage cholesterol (22). Nascent HDL includes a number of particle species that differs in size, the number of apoAI molecules per lipoprotein particle, and the number of lipid molecules per molecule of apoAI (23–28). The cause of nascent HDL heterogeneity is unknown; however, we noted that reconstituted HDL (rHDL) particles that arise from purified lipids and lipid-free apoAI in cell-free systems and resemble nascent HDL in structure are also similarly diverse in size and lipid and apoAI content (29–31). rHDL diversity comes about as a consequence of the intrinsic structural flexibility of apoAI, which can assume a variety of conformations to accommodate different amounts of lipid (32). Thus, as the starting lipid to apoAI molar ratio increases, i.e., lipid becomes more plentiful than apoAI, rHDL assembly shifts from the smaller to larger particles (29–31, 33). Given that ABCA1 appears to perform a facilitatory role during nascent HDL assembly (34, 35), we hypothesized that the availabilities of cell lipid and lipid-free apoAI also dictate nascent HDL particle heterogeneity. Here we show that increases in the ratio of available cell lipid to lipid-free apoAI lead to production of larger nascent HDL particles that contain more apoAI molecules per particle and more lipid molecules per molecule of apoAI. This finding identifies a fundamental cause of HDL particle heterogeneity.

**MATERIALS AND METHODS**

**Cell culture and biogenesis of nascent HDL**

BHK-ABCA1, BHK-ABCA1 (W5905), and BHK-ABCA1(C1477R) cells expressing human wild-type or mutant ABCA1 have been previously described (36, 37). RAW 264.7, J744, and HepG2 cells were from American Type Culture Collection (Manassas, VA, USA). The cell lines were routinely maintained in DMEM (BHK and RAW 264.7), RPMI (J744), or MEM (HepG2) medium supplemented with FBS to 10% and gentamicin to 50 μg/ml at 37°C in 5% CO₂. Nascent HDL particles were generated as described in the following for the BHK-ABCA1 line. BHK-ABCA1 cells (3 or 4 T75 flasks/condition) were plated at a 1:10 dilution (unless specified otherwise) from a confluent culture, allowed to attach overnight in DMEM/10% FBS/50 μg/ml gentamicin, and labeled in DMEM/2.5% FBS/30 μg/ml gentamicin overnight with one of the following: 1.3 μCi/ml [methyl-3H]choline chloride and 0.06 μCi/ml [4,14C]cholesterol, 0.5 μCi/ml [1,2-3H(N)]cholesterol, or 1 μCi/ml [methyl-3H]choline chloride (all radiochemicals were from PerkinElmer, Waltham, MA, USA). The cells were then treated with 0–10 nM mifepristone for 16–20 h in DMEM/0.2% BSA (fraction V, fatty acid free, EMD Millipore, Billerica, MA, USA)/50 μg/ml gentamicin and exposed to 0–40 μg/ml human apoAI, human [14C]apoAI (labeled to ~1 μCi/mg as described previously; ref. 27), or mouse recombinant apoAI (Q225, V226; recombinant mouse apoAI was expressed in Escherichia coli as described previously; ref. 38) in DMEM/50 μg/ml gentamicin for 8 h. 8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate sodium salt (8-CPT-cAMP; Sigma-Aldrich, St. Louis, MO, USA) was used at the indicated concentrations to induce ABCA1 expression in RAW 264.7 and J774 cells. HepG2 cells were plated at a 1:3 dilution from a confluent culture and allowed to grow for 4–5 d before labeling with radiochemicals and treatment with T0901317 (Sigma-Aldrich) at the indicated concentrations to up-regulate ABCA1 expression. Cell medium with nascent HDL was collected, filtered through a 0.45 μm PVDF membrane filter unit (EMD Millipore), reduced in volume 20× from 30 to 1.5 ml using an Amicon Ultracel-10K centrifugal filter (EMD Millipore) and stored at 4°C for further analysis.

**Gel-filtration chromatography of nascent HDL**

A 1-ml aliquot of the 20× concentrated cell medium with nascent HDL was resolved into 1-ml fractions on a calibrated HiLoad 16/60 Superdex 200 gel-filtration column (GE Healthcare, Mickleton, NJ, USA). TBS (pH 7.4) was the mobile phase. Each fraction was combined with 5 ml of ScintiVerse BD cocktail (Fisher Scientific, Pittsburgh, PA, USA) and read in a scintillation counter; when dual labeling was used, [3H] and [14C] counts were adjusted for the energy emission spectra overlap. Alternatively, the fractions containing the larger (>8.6 nm) or the smaller (<8.6 nm) nascent HDL particles were combined together, concentrated to 0.8–1.0 ml using Amicon Ultracel-10K centrifugal filter units.
(EMD Millipore) and stored at 4°C for further analysis. The gel-filtration column was washed between runs with 30% isopropanol and 1 M NaOH, as recommended by the manufacturer. The following standards (Sigma-Aldrich) were used to calibrate the column: cytidine, \( V_t \); thymoglobulin, 17.0 nm; apolrerritin, 12.2 nm; lactate dehydrogenase, 8.16 nm; BSA, 7.1 nm; carbonic anhydrase, 4.4 nm; blue dextran, 2.0 nm.

Native polyacrylamide gel electrophoresis (PAGE) analysis of nascent HDL

Aliquots of the concentrated cell medium and the isolated larger and smaller nascent HDL particles were normalized to the same amount of cell medium or the same apoAI concentration and resolved on 4–20% Tris-glycine native PAGE (at 90 V for 17.3 h) and 3–12% Bis-Tris blue native PAGE gels (Invitrogen/Life Technologies, Grand Island, NY, USA), blotted, and probed with a goat polyclonal anti-apoAI antibody (NB400-147; Novus Biological, Littleton, CO, USA). Native-Mark unstained protein standards (Invitrogen/Life Technologies) were employed for estimating particle size. In Bis-Tris gels, the dark blue cathode buffer was used during the first third of the run and then replaced with the light blue cathode buffer.

ApoAI cross-linking

TBS suspensions of the isolated larger and smaller nascent HDL particles were extensively dialyzed in 10 mM sodium phosphate buffer, pH 7.4. ApoAI cross-linking was conducted at 2 concentrations of each particle preparation, undiluted, and 10× diluted with 10 mM sodium phosphate buffer (pH 7.4) in order to distinguish between intra- and interparticle cross-link products (39). Bis(sulfosuccinimidyl) suberate (BS3) was then added to the final concentration of 2.5 mM, and cross-linking reactions were allowed to proceed for 30 min at room temperature. To generate an apoAI oligomer ladder, lipid-free apoAI was dialyzed in 10 mM sodium phosphate buffer (pH 7.4), diluted with the same buffer to 0.5 mg/ml, and cross-linked with 0.25 mM BS3 at room temperature for 30 min (40). Cross-linking reactions were stopped by adding 0.5 M Tris base to the final concentration of 45 mM. Cross-linking products were re-dialyzed in 10 mM sodium phosphate buffer (pH 7.4) in order to distinguish between intra- and interparticle cross-link products. 

Lipid and apoAI content analysis of nascent HDL

\[^{[3]H}\]cholesterol- and \[^{[3]C}\]apoAI-containing larger and smaller nascent HDL particles were prepared as described above. The amount of apoAI in the preparations was calculated from the \[^{[3]C}\]apoAI-specific activity. The amount of cholesterol in the larger HDL particles generated at saturating mifepristone and apoAI concentrations (10 nM and 20 \( \mu \)g/ml, respectively) was measured using the cholesterol E kit (Waco Chemicals, Richmond, VA, USA) and then used to calculate the HDL particle \[^{[3]H}\]cholesterol-specific activity. This specific activity value was used to determine the amount of cholesterol in particle preparations that contained cholesterol amounts below the detection limit of the kit. \[^{[3]H}\]Cholesterol-specific activity was calculated for each independent experiment. Choline phospholipids were assayed using the phospholipids C kit (Waco Chemicals).

Cell cholesterol efflux assay

BHK-ABCA1 cells were seeded in 24-well plates at a 1:10 dilution from a confluent culture, allowed to attach overnight in DMEM/10% FBS/50 \( \mu \)g/ml gentamicin, labeled with 0.5 \( \mu \)Ci/ml [1,2-\(^{3}H\)]cholesterol in DMEM/2.5% FBS/50 \( \mu \)g/ml gentamicin overnight, treated with 0–10 nM mifepristone for 16–20 h in DMEM/0.2% BSA/50 \( \mu \)g/ml gentamicin, and exposed to 10 \( \mu \)g/ml human apoAI in DMEM/50 \( \mu \)g/ml gentamicin for 4 h. The medium was collected and filtered through a 0.45-\( \mu \)m filter plate (EMD Millipore); a 100-\( \mu \)l aliquot of each sample was read in a scintillation counter. Cell lipids were extracted with hexane-isopropanol (3:2, \( \nu/\nu \)); the solvent was evaporated, and the lipids were read in a scintillation counter. The percentage of cellular cholesterol released to apoAI was calculated by dividing the \(^{[3]H}\) counts in the medium by the sum of \(^{[3]H}\) counts in the medium and cells and multiplying by 100.

ABCA1 expression analysis

BHK-ABCA1 cells were plated from a confluent culture at a 1:10 dilution in 12-well plates in DMEM/10% FBS/50 \( \mu \)g/ml gentamicin, allowed to grow for 2 d, and then treated with 0–10 nM mifepristone in DMEM/0.2% BSA/50 \( \mu \)g/ml gentamicin for 16 h. Thereafter, the medium was removed, and the cells were lysed in radioimmunoprecipitation assay buffer (Sigma-Aldrich). Cell proteins were resolved on a Tris-glycine SDS-PAGE gel, blotted, and probed with a rabbit polyclonal ABCA1 antibody (NB 400-105) and a mouse monoclonal GAPDH antibody (NB300-221) (both from Novus Biological).

\[^{[3]H}\]Choline-phospholipid-specific activity in the plasma membrane

BHK-ABCA1 cells were plated in T75 flasks (4 flasks/treatment), labeled with 1 \( \mu \)Ci/ml [methyl-\(^{3}H\)]choline chloride, and treated with mifepristone at the indicated concentrations for production of nascent HDL particles. However, instead of adding apoAI, cells were washed twice with a HEPES buffer (10 mM HEPES/150 mM NaCl/2 mM CaCl, pH 7.4) and then incubated in 5 ml/flask of HEPES buffer/50 mM formaldehyde/2 mM DTT for 90 min at 37°C to allow giant plasma membrane vesicles to form (41, 42). Subsequently, the buffer with plasma membrane vehicles was collected and centrifuged at 100,000 \( g \) for 10 min in a swinging bucket rotor. The supernatant was transferred to an ultracentrifuge tube and further centrifuged at 20,000 \( g \) for 1 h at 4°C to pellet plasma membrane vesicles. The upper part of the supernatant was discarded, while the bottom 3 ml was pipetted up and down to resuspend the pellet, transferred to a borosilicate glass tube (Kimble Chase, Vineland, NJ, USA) and mixed by vortexing with 12 ml of chloroform/methanol (2:1, \( \nu/\nu \)). The mixture was centrifuged at 1000 g for 10 min in a swinging bucket rotor. The lower chloroform phase was collected into a fresh glass tube, and its volume was estimated. Two 100-\( \mu \)l fractions of the lower phase were transferred to glass scintillation vials, evaporated, and read in a scintillation counter. The remainder (~7.8 ml) was evaporated in a stream of nitrogen and assayed for phosphorus (43). Before reading at 797 nm, samples were centrifuged at 5000 \( g \) for 10 min to pellet colloidal particles. \[^{[3]H}\]Choline-phospholipid-specific activity was expressed as \[^{[3]H}\]choline-phospholipid counts per minute (cpm) per 1 \( \mu \)g of phospholipid.

Gas-liquid chromatography

Lipids extracted from nascent HDL particles with chloroform/methanol following the Bligh-Dyer method were assayed for cholesterol and cholesterol ester using gas-liquid chromatography (44).
RESULTS

ABCA1-created abundance of cell lipid available for efflux favors production of larger nascent HDL particles in BHK-ABCA1 cells

Investigations of cell-free reassembly of nascent HDL from its purified constitutive components, lipids and lipid-free apoAI, have found that the starting lipid to apoAI molar ratio determines the size distribution of the resultant rHDL particles (33). The BHK-ABCA1 line was used to elucidate whether the lipid to apoAI ratio also controls the size heterogeneity of cell-generated nascent HDL. BHK-ABCA1 cells express human ABCA1 under the control of a mifepristone-inducible promoter (36). Although the manufacturer of the vectors used in the construction of the cell line recommends applying mifepristone at the concentration as high as 10 nM for the maximal expression of the transgene (36), a dose-response curve showed that percentages of cellular cholesterol efflux to a saturating amount of apoAI rose linearly with increasing mifepristone concentrations only in the 0- to 1-nM range (Fig. 1A). ABCA1 expression levels also increased continually in the same range of mifepristone concentrations (Fig. 1B). Elevation of cellular cholesterol efflux as a result of rising ABCA1 expression indicates that ABCA1 ensures availability of cell lipid for nascent HDL formation (45, 46).

BHK-ABCA1 cells were labeled with [3H]choline and [14C]cholesterol, treated with increasing mifepristone concentrations to induce progressively higher intensities of ABCA1 expression, and exposed to a saturating concentration of human lipid-free apoAI. After an 8-h time period to permit lipoprotein production, nascent HDL was collected, concentrated, and analyzed using gel-filtration chromatography. In line with previous observations in BHK-ABCA1 and other cell types (23, 24, 26–28, 38, 47), nascent HDL particles segregated on a gel-filtration column into 2 size species: a larger and a smaller, each represented by distinct [3H]choline-phospholipid and [14C]cholesterol elution peaks centered at the 9.9- to 10.9- and 7.7-nm, respectively, size marks (Fig. 2A, top and middle panels). At the lowest ABCA1 expression level, the larger (sizes from 8.6 to 14.7 nm) and smaller (sizes from 5.7 to 8.6 nm) particles formed in about equal amounts, judging from the peak magnitude. However, as ABCA1 expression and overall lipoprotein production increased, the peaks for the larger particle grew more than the peaks for the smaller species, leading to a change in the particle size distribution. To quantitate this change, the sum of cpm values in elution fractions containing the larger species was divided by the sum of cpm values in fractions containing the smaller species to derive the large/small particle ratio for each isotope. Both [3H]choline-phospholipid and [14C]cholesterol large/small particle ratios increased with increasing ABCA1 expression levels (Fig. 2B) and rising total amounts of [3H]choline-phospholipid and [14C]cholesterol in nascent HDL (Fig. 2C, D). With lipid-free apoAI at a saturating level, the quantity of lipid in newly formed nascent HDL particles is proportional to the amount of cell lipid available for HDL formation through ABCA1 activity (Fig. 1). Thus, the close correlation between high amounts of HDL [3H]choline-phospholipid and [14C]cholesterol and high large/small particle ratios (Fig. 2C, D) indicates that ABCA1-created abundance of apoAI-accessible cell lipid leads to preferential assembly of the larger particles at the expense of the smaller species.

Very limited production of nascent HDL particles at low mifepristone concentrations precluded usage of lipid mass analysis for determination of the large/small particle ratios. However, [3H]choline-phospholipid and [14C]cholesterol were good proxies for lipid mass in test experiments at 10 nM mifepristone treatments. The difference between mass/mass and cpm/cpm large/small particle ratios ranged from 5.3 to 5.8% for choline-phospholipid and from 4.4 to 7.2% for cholesterol (assuming the mass/mass values as 100%; n = 2). Furthermore, [3H]choline-phospholipid seemed to faithfully reflect any changes in unlabeled phospholipid levels induced in the plasma membrane, the main site of nascent HDL assembly in BHK-ABCA1 cells (ref. 18 and unpublished results), by variable levels of ABCA1 expression (48). The plasma membrane
[H]choline-phospholipid values for 0.05 and 10 nM mifepristone-treated cells deviated up or down no more than 7% from the corresponding values for untreated cells (assuming the [H]choline-phospholipid-specific activity for untreated cells as 100%). Actual values were 6.2 × 10^3, 5.9 × 10^3, and 6.1 × 10^3 [H]choline-phospholipid cpm/µg of phospholipid for 0, 0.05, and 10 nM mifepristone-treated cells, respectively, in experiment 1, and 6.8 × 10^3, 7.3 × 10^3, and 7.2 × 10^3 [H]choline-phospholipid cpm/µg of phospholipid for 0, 0.05, and 10 nM mifepristone-treated cells, respectively, in experiment 2). To rule out post-assembly interconversion of nascent HDL particles between the larger and smaller species as a contributing factor to the lipoprotein heterogeneity, filtered cell medium containing nascent HDL was divided into 2 equal aliquots: one was incubated at 37°C for 8 h before being stored at 4°C, and the other was immediately placed at 4°C. The large/small particle ratio did not significantly deviate between the aliquots (data not shown), indicating a lack of particle remodeling after assembly.

**Abundance of cell lipid available for efflux due to high cell density also favors production of the larger nascent HDL particles in BHK-ABCA1 cells**

ABCA1 activity has a significant effect on the cell lipid state (48). In a different approach that eliminates the level of ABCA1 expression and thus the lipid state of the cell as variables, the amount of lipid available for nascent HDL formation was manipulated by changing cell density in tissue culture flasks. Greater numbers of cells packed at higher densities would supply larger amounts of lipid for efflux even though the level of ABCA1 expression in each individual cell would be the same. BHK-ABCA1 cells were seeded at 0.2 × 1 × 2 × of normal plating density, labeled with [H]choline and [14C]cholesterol, treated with a high concentration of mifepristone to induce ABCA1 expression, and exposed to a saturating concentration of human lipoprotein particles. The [H]choline-phospholipid and [14C]cholesterol large/small particle ratios dramatically increased with increasing plating densities, rising total amounts of [H]choline-phospholipid and [14C]cholesterol in nascent HDL and, hence, growing availabilities of cell lipid for efflux (Fig. 3). This observation further shows that abundance of apoAI-accessible cell lipid promotes formation of the larger nascent HDL particles and suggests that the critical parameter for nascent HDL heterogeneity is the total amount of lipid available from the entire cell population, rather than the level of lipid availability in single cells.

To determine whether the abundance of available lipid also affects the cholesterol content of nascent HDL particles, [14C]cholesterol/[H]choline-phospholipid cpm/cpm ratios were calculated for every elution fraction within the particle peaks (Figs. 2A and 3A, bottom panels). These ratios did not exhibit substantial changes with increasing ABCA1 expression or cell plating density, but were significantly higher for the larger than for the smaller particles.

**Assembly of larger nascent HDL particles by BHK-ABCA1 cells is further promoted by scarcity of apoAI**

Increases in the available cell lipid:apoAI ratio due to a higher numerator, i.e., the amount of available for nascent HDL formation cell lipid, led to preferential
production of the larger particles (Figs. 2 and 3). Further increases in the ratio due to a lower denominator, i.e., the concentration of lipid-free apoAI, should stimulate assembly of the larger species even more at the expense of the smaller variety. To test this prediction, BHK-ABCA1 cells were labeled with either $[^3]$Hcholine or $[^3]$Hcholesterol, treated with a high concentration of mifepristone to induce ABCA1 expression to the maximum level and exposed to different amounts of human lipid-free apoAI. As the amount of the apolipoprotein applied to cells decreased and overall nascent HDL assembly waned, the $[^3]$Hcholine-phospholipid and $[^3]$Hcholesterol peaks for the smaller particles (<8.9 nm) diminished more steeply than the peaks for the larger species (>8.9 nm) (Fig. 4A). As a result, in line with the prediction, the $[^3]$Hcholine-phospholipid and $[^3]$Hcholesterol large/small particle ratios increased, indicating that the fraction of the smaller particles in the total nascent HDL declined (Fig. 4B). Thus, the available cell lipid:apoAI ratio controls heterogeneity of nascent HDL, with higher ratios favoring the larger particles.

Size distribution of nascent HDL particles generated at different cell lipid:apoAI ratios

Two different kinds of native PAGE were used to resolve nascent HDL particles that formed with human apoAI in BHK-ABCA1 cells at a low (0.04 nM mifepristone and 20 μg/ml apoAI), medium (10 nM mifepristone and 20 μg/ml apoAI), and high (10 nM mifepristone and 3 μg/ml apoAI) available cell lipid:apoAI ratios. There was a good correspondence between particle sizes determined from gel-filtration profiles and from native PAGE gels. The particles that eluted as the smaller (6.7- to 8.3-nm) species on a gel-filtration column migrated on Tris-glycine native and Bis-Tris blue native PAGE gels in the 7.1- to 8.2-nm size range (Fig. 5A, B); the particles that eluted as the larger (8.9- to 15-nm) species on the column migrated on the native PAGE gels in the 8.2- to 17.4-nm size range (Fig. 5A, B). Tris-glycine native PAGE gels

---

**Figure 3.** Effects of cell plating density on nascent HDL particle heterogeneity. A) Gel-filtration profiles (top and middle panels) of nascent HDL particles formed by BHK-ABCA1 cells seeded at the indicated plating densities. Bottom panel shows $[^1]$Ccholesterol/$[^3]$Hcholine-phospholipid ratio curves derived from data in the top and middle panels. B) $[^3]$Hcholine-phospholipid and $[^1]$Ccholesterol large/small particle ratios calculated from the gel-filtration profiles shown in panel A increased with higher plating densities and hence elevated cell lipid availabilities. C) Large/small particle ratios shown in panel B closely correlated with the total amount of $[^3]$Hcholine-phospholipid and $[^1]$Ccholesterol counts present in nascent HDL particles.

**Figure 4.** HDL particle heterogeneity as a function of apoAI availability. A) $[^3]$Hcholine-phospholipid (left panel) and $[^3]$Hcholesterol (right panel) gel-filtration profiles of nascent HDL particles generated by BHK-ABCA1 cells expressing ABCA1 at the maximum level with the indicated amounts of apoAI. Unlike in Fig. 2, in this case cells were labeled with only one of the radiochemicals per experiment. These profiles are representative of 4 independent experiments, 2 with each isotope. B) $[^3]$Hcholine-phospholipid and $[^1]$Ccholesterol large/small particle ratios calculated from the gel-filtration profiles shown in panel A increased with lower availabilities of apoAI and hence higher cell lipid:apoAI ratios.
revealed that the larger nascent HDL particles that formed at the low available cell lipid:apoAI ratio included 3 subspecies (9.1, 9.8, and 11.0 nm) of about equal prominence (Fig. 5C, D). The larger particles that arose at the medium and high ratios included 3 additional subspecies (12.8, 15.9, and 17.6 nm) and had the 11.0 nm subspecies as the most dominant particle. The 9.1- and 11.0-nm subspecies were completely absent, and only the 9.8-, 12.8-, 15.9-, and 17.6-nm particles were present in the larger nascent HDL generated at the high available cell lipid:apoAI ratio. On Bis-Tris blue native gels, the larger particles generated at the medium and high ratios separated into 3 broad bands 10.7, 12.8, and 15.5 nm in size (Fig. 5D). The larger HDL formed at the low ratio migrated as the 10.7-nm subspecies. On both native Tris-glycine and Bis-Tris blue native gels, the smaller particles (7.7–8.0 nm) were the dominant HDL fraction at the low available cell lipid:apoAI ratio and a minor fraction at the high value of the ratio (Fig. 5C, D). The above findings corroborate the observations in gel filtration and further demonstrate that the size distribution of nascent HDL subspecies reflects the available cell lipid:apoAI ratio at the time of particle formation.

**High cell lipid:apoAI ratios promote formation of nascent HDL with higher apoAI and lipid content**

Cross-linking analysis showed that the larger particles generated in BHK-ABCA1 cells with human apoAI at the low available cell lipid:apoAI ratio had no more than 3 apoAI molecules per lipoprotein particle (Fig. 6A). The larger particles generated at the medium and high ratios had up to 5. Furthermore, lipid mass analysis revealed sharp increases in the choline-phospholipid:apoAI and cholesterol:apoAI molar ratios in the larger particles with the transition from low to medium to high available cell lipid:apoAI ratios (Fig. 6B). Taken together, the cross-linking and lipid mass analyses indicate that a relative abundance of available cell lipid over apoAI leads to formation of larger nascent HDL particles that contain more apoAI molecules per particle and, possibly as a result of this, hold more phospholipid and cholesterol molecules per molecule of apoAI.

A recent report presented evidence for the existence of cholesteryl ester in nascent HDL particles (23). A gas-liquid chromatography analysis of nascent HDL generated by BHK-ABCA1 cells treated with 10 nM mifepristone and exposed to 20 μg/ml human apoAI did not detect esterified cholesterol (data not shown). The divergence between this and the previously reported finding may stem from the difference in cell types used to study nascent HDL biogenesis.

**Mutations in ABCA1 that compromise its functionality shift nascent HDL production to the smaller particle species**

W590S and C1477R are naturally occurring mutations in human ABCA1 that impair its functionality and cause Tangier disease (49) but do not affect its subcellular localization or expression levels (37). To determine how deficiency in ABCA1 functionality affects size heterogeneity of nascent HDL particles, BHK-ABCA1, BHK-ABCA1(W590S), and BHK-ABCA1(C1477R) cells were plated at the same density, labeled with [3H]cholesterol, induced to express ABCA1 at the maximal level, and exposed to a high concentration of human lipid-free apoAI, followed by the regular procedure to collect and analyze nascent HDL via gel filtration. The total amount of newly generated nascent HDL, the [3H]cholesterol large/small particle ratio, and the size of the larger particles were all the lowest for the C1477R mutant, intermediate for the W590S variant, and the highest for the wild-type ABCA1 (Fig. 7). These results suggest that ABCA1 mutagenesis has the same effect on nascent HDL particle heterogeneity as reductions in

---

**Figure 5.** Native PAGE analysis of nascent HDL generated at different available cell lipid:apoAI ratios. A) Gel-filtration profile of nascent HDL particles that were produced by BHK-ABCA1 cells at the maximum ABCA1 expression and 10 μg/ml apoAI. B) Elution fractions containing the larger and smaller particles (shaded portions of the profile) were combined, concentrated, and further resolved on 4–20% Tris-glycine native and 3–12% Bis-Tris blue native PAGE gels. C, D) Tris-glycine native and Bis-Tris blue native gels of nascent HDL particles generated in BHK-ABCA1 cells at the indicated available cell lipid:apoAI ratios. When loaded at the same volume of medium, the lanes with particles assembled at the low and medium available cell lipid:apoAI ratios have ~7 times more apoAI than the lane with HDL that formed at the high value of the ratio. Representative gels from 3 independent experiments are shown.
ABCA1 expression and decreases in cell plating density: the less cell lipid is available for nascent HDL formation, in the case of ABCA1 mutants, because of impaired activity of the transporter, the more nascent HDL production is skewed to the smaller particles. Cell lipid:apoAI ratio controls heterogeneity of nascent HDL particles produced by RAW 264.7, J774, and HepG2 cells

Murine macrophage cells RAW 264.7 express endogenous ABCA1 under the control of a cAMP-inducible promoter (53). To extend to other cell lines the findings in BHK-ABCA1 cells regarding the available cell lipid:apoAI ratio as a factor controlling nascent HDL heterogeneity, nascent HDL particles were generated in RAW 264.7 cells with human lipid-free apoAI at a low (0.04 mM 8-CPT-cAMP, 20 μg/ml apoAI) and high (0.3 mM 8-CPT-cAMP, 20 μg/ml apoAI) available cell lipid:apoAI ratio. Nascent HDL particles produced by RAW 264.7 cells segregated on a gel-filtration column into larger and smaller species, which were similar in size to the corresponding species of BHK-ABCA1 nascent HDL (Figs. 2A and 8A). The [3H]cholesterol-phospholipid:apoAI and [3H]cholesterol:apoAI molar ratios for the larger particle species of nascent HDL formed at the indicated cell lipid:apoAI ratios in BHK-ABCA1 cells. Error bars = so for 3 independent experiments.

ABC1A mutagenesis and size heterogeneity of nascent HDL particles. BHK cells expressing wild-type ABCA1, ABCA1(W590S), or ABCA1(C1477R) were plated at the same density, labeled with [3H]cholesterol, treated with 10 nM mifepristone, and exposed to 10 μg/ml of human apoAI to generate nascent HDL. The decreased size of gel-filtration elution peaks indicates that W590S and especially C1477R mutations dramatically reduced the ability of ABCA1 to facilitate nascent HDL assembly. As nascent HDL production diminished, so did the size of the larger particles (>8.9 nm) and the [3H]cholesterol large/small particle ratio (inset).

Available cell lipid:apoAI ratio affects heterogeneity of nascent HDL particles produced by RAW 264.7 cells. A) Gel-filtration profiles of nascent HDL particles generated by RAW 264.7 cells at the indicated concentrations of cAMP and human apoAI. B) [3H]Choline-phospholipid and [14C]cholesterol large/small particle ratios calculated from the gel-filtration profiles shown in panel A. Representative results from 2 independent experiments are shown.
(Fig. 8B), as was also observed in the BHK-ABCA1 cell line (Fig. 2B). Similar results were obtained with another murine macrophage cell line J774 (data not shown) and with hepatocellular carcinoma cells HepG2 (Fig. 9). Thus, the available cell lipid:apoAI ratio controls the heterogeneity of nascent HDL regardless of cell type.

**Heterogeneity of nascent HDL particles formed with mouse apoAI also reflects the cell lipid:apoAI ratio**

Human and mouse apoAI differ substantially in primary structure and form nascent HDL particle populations significantly divergent in size distribution (38, 54). To determine whether mouse apoAI is also responsive to the available cell lipid:apoAI ratio, BHK-ABCA1 cells were processed as normal to label cellular lipid pools with the radio-lipids and then either treated with 0.05 nM mifepristone and exposed to 20 μg/ml mouse apoAI (C57 variant Q225, V226; ref. 55; low available cell lipid:apoAI ratio) or treated with 10 nM mifepristone and exposed to 5 μg/ml mouse apoAI (high cell lipid:apoAI ratio). In line with the previous observations (38), the larger mouse nascent HDL particles (>8.6 nm) were clearly bigger than the corresponding species of human apoAI HDL, while the smaller species (<8.6 nm) were about the same in size (compare Figs. 2A, 4A, and 10A). Also, at the same ABCA1 expression level and apoAI concentration, the smaller species of mouse nascent HDL comprised a noticeably diminished fraction of the total nascent HDL in comparison with the smaller species of human apoAI HDL. This was reflected in the generally higher mouse [3H]choline-phospholipid and [3H]cholesterol large/small particle ratios (Figs. 2B, 4B, and 10B, data not shown, and ref. 38). Nonetheless, the large/small particle ratios for the mouse nascent HDL increased with the transition from the low to high available cell lipid:apoAI ratio (Fig. 10B), indicating that structural features responsible for the genesis of nascent HDL particle heterogeneity are conserved to some degree between human and mouse apoAI.

**DISCUSSION**

**Ratio of available cell lipid to lipid-free apoAI controls heterogeneity of nascent HDL particles**

Studies in vivo with individuals deficient in LCAT and in vitro with cells of various types have consistently shown that nascent HDL particles form in different sizes (23–28, 38, 47, 56). Mechanisms that bring about this size heterogeneity have not been sufficiently elucidated. On a gel-filtration column, nascent HDL generated by ABCA1-expressing cells, BHK-ABCA1, RAW 264.7, J774, and HepG2, segregated into distinct larger and smaller size particle species (Figs. 2, 8, and 9). The ratios of radiolabeled choline-phospholipid and cholesterol in the larger over the smaller species provided a convenient measure of the particle size distribution. Toward identification of factors responsible for nascent...
HDL size heterogeneity, we determined how changes in the availability of cell lipid and the concentration of extracellular lipid-free human apoAI affect the relative production of the larger and smaller nascent HDL particles in BHK-ABCA1 cells, which inducibly express human ABCA1. When the amount of cell lipid available for nascent HDL formation increased because of rising ABCA1 expression levels, higher cell densities or an absence of functional impairment in ABCA1, while apoAI concentration remained constant, the $[^{3}H] \text{choline-phospholipid}$ and $[^{14}C]\text{cholesterol}$ large/small particle ratios also increased, indicating a shift in the particle production from the smaller to the larger particle species (Figs. 2, 3, and 7). When cell lipid availability was unchanged, while apoAI concentrations decreased, the ratios increased still further, reflecting greater predominance of the larger particles over the smaller (Fig. 4). These shifts in the particle size distribution are best understood if cell lipid availability and lipid-free apoAI concentration are considered not individually but as a ratio. As the available cell lipid:apoAI ratio increased, at first due to increases in the numerator, the amount of available for efflux cell lipid, and then due to decreases in the denominator, the concentration of lipid-free apoAI, the large/small particle ratios rose in concert with the share of the larger particle species in the total nascent HDL. The available cell lipid:apoAI ratio also affected the size distribution of nascent HDL particles formed in murine macrophage cell lines expressing endogenous murine ABCA1 (RAW 264.7 and J774) and in hepatocellular carcinoma cells expressing human endogenous ABCA1 (HepG2) (Figs. 8 and 9).

At present, it is unclear how nascent discoidal HDL heterogeneity translates into the heterogeneity of mature spherical HDL, which represents by far the greater portion of serum HDL. Human apoAI supports formation of two different size species of nascent HDL in vitro (Fig. 2) and at least two major size classes of mature HDL, HDL$_{2}$ and HDL$_{3}$, in vivo in the human body and in mice expressing the human apoAI gene (57). This implies a correspondence between the size distribution of nascent and mature HDL. Mouse apoAI, in contrast, drives production of more homogenous mature HDL particles comprising a single size species in vivo (55, 57), which would suggest, if the nascent-mature HDL correspondence were to hold, that it also generates a single species of nascent HDL in vitro. However, mouse apoAI nascent HDL synthesized by BHK-ABCA1 cells segregated into a larger and smaller species, just as human apoAI nascent HDL particles did (Fig. 10A). Furthermore, the available cell lipid:apoAI ratio also affected the size distribution of nascent mouse apoAI HDL particles (Fig. 10B). On the other hand, the smaller species was less prominent in the mouse than in the human apoAI nascent HDL at the same ABCA1 expression level and apoAI concentration (Figs. 2B, 4B, and 10B and data not shown). Furthermore, when mouse apoAI is overexpressed using adenovirus vectors in apoAI-null mice, it drives production of HDL with a more complex size distribution reminiscent of human HDL (58). Thus, mouse apoAI exhibits a greater tendency than human apoAI to form larger nascent HDL particles, but possesses the ability to produce two different size HDL species in proportion to the available cell lipid:apoAI ratio.

Native PAGE revealed that BHK-ABCA1-generated nascent HDL that eluted on a gel-filtration column as the larger particle species consisted of several discrete subspecies (Fig. 5). With the progressive transition from low to high values of the available cell lipid:apoAI ratio, new subspecies of the larger particle emerged and became prominent, while others completely disappeared (Fig. 5C, D). The particles that formed at the high available cell lipid:apoAI ratio had more apoAI molecules per particle and more choline-phospholipid and cholesterol molecules per apoAI molecule than those that arose at the low value of the ratio (Fig. 6). The capacity to form lipoprotein particles with different numbers of cholesterol molecules per molecule of apoAI may ensure that the available cell cholesterol and lipid-poor apoAI are both completely used up for assembly of nascent HDL regardless of their initial amounts. Given that lipid-poor apoAI is rapidly catabolized in the kidney (59), while an excess of cholesterol is damaging to the cell (60), this may be an important feature of nascent HDL formation. Overall, our findings show that the available cell lipid:apoAI ratio is a fundamental factor controlling nascent HDL heterogeneity.

**rHDL formation as a model of nascent HDL biogenesis**

It has been well established that the starting lipid:apoAI molar ratio controls the size heterogeneity of the resultant rHDL particles (33). At very low lipid:apoAI molar ratios, only small rHDL particles that contain 2 apoAI molecules per particle form. At higher ratios, larger species containing 3 or more apoAI molecules per particle appear. With further increases in the ratio, the proportion of the smaller particles decreases, while the larger species become predominant. The exact sizes of the smaller and larger species depend on the molecular structure of the lipid, if a single kind of lipid is employed, and the molecular composition of the lipid mixture, if more than one kind of lipid is present. However, the progression from smaller to larger rHDL particles with increasing lipid:apoAI molar ratio holds regardless of either the lipid structural and compositional parameters or the method of rHDL particle assembly (spontaneous, cholate-mediated, or sonication-induced; refs. 29–31, 52, 61–63). The findings presented in this work show that the lipid:apoAI ratio rule also applies to the biogenesis of nascent HDL particles.

In addition to the relative availability of lipid and apoAI, a number of other factors bear on both rHDL and nascent HDL assembly. The same changes in the primary structure of apoAI that affect size distribution of rHDL particles also have an effect on size heteroge-
neity of nascent HDL (38). Another factor that influences rHDL and nascent HDL size heterogeneity is the cholesterol content of the starting lipid (27, 63, 64). Cholesterol is excluded from the 2-molecule-wide zone of phospholipid apposed to apoAI within the rHDL disc (63, 64). Given that the area of a circle increases exponentially to the power of 2, while its circumference expands linearly, this zone of apoAI contact occupies relatively less space in larger particles than in smaller. As a result, larger rHDL species contain more cholesterol than smaller, and as the cholesterol content of the starting lipid mixture increases, formation of the larger rHDL particles rises (63). Similarly, larger nascent HDL particles contain more cholesterol than smaller (Figs. 2A and 3A, bottom panels), and loading of cells with cholesterol likely increases the cholesterol content of the ABCA1-created exovesicle (see below), constrains apoAI to assume large-disc structural conformations, and thus promotes formation of progressively larger nascent HDL species (27, 63). Our recent observations further show that pharmaceutical compounds that reduce the cholesterol content of the plasma membrane (U18666A) or disrupt cell lipid metabolism (D609) shift nascent HDL formation from the larger to the smaller species (unpublished results). All in all, formation of rHDL appears to be an exceptionally good model of apoAI-apoAI, apoAI-lipid, and lipid-lipid interactions that occur during nascent HDL assembly.

Role of ABCA1 in nascent HDL formation

ABCA1 clearly and strongly affects size heterogeneity of nascent and spherical HDL to the extent that it controls the availability of cell lipid for nascent HDL biogenesis. In BHK-ABCA1 cells, ABCA1 expression levels, nascent HDL production and the share of the larger particle species in the total lipoprotein all rise in unison (Figs. 1 and 2). Furthermore, impairment of ABCA1 activity caused by mutagenesis of its primary sequence leads to reductions in both overall HDL formation and the share of the larger HDL particles. This occurs in vivo in terms of total HDL in heterozygous Tangier disease individuals, who carry one active and one inactive ABCA1 allele (65), and in vitro in terms of nascent HDL generated in BEK cells that express ABCA1 sequences with the same mutations found in patients with Tangier disease (Fig. 7). However, the conclusion that rHDL formation is a good model of nascent HDL biogenesis suggests that ABCA1 does not modulate apoAI-apoAI, apoAI-lipid, and lipid-lipid interactions that take place during lipoprotein particle assembly. This suggestion has ample empirical support. First, we have investigated whether the size heterogeneity of nascent HDL particles is a consequence of ABCA1 oligomerization or localization to different membrane domains or subcellular compartments and ruled out these possibilities (unpublished results). Second, in terms of nascent HDL heterogeneity, human and murine ABCA1, but not apoAI, are interchangeable. Under comparable assembly conditions, size profiles of nascent HDL par-

icles formed with either human or murine ABCA1 and human apoAI are very similar and unmistakably distinct from size profiles of nascent HDL assembled with either human or murine ABCA1 and murine apoAI (Figs. 2A, 8A, 9A, and 10A and ref. 38). Third, the same changes in the size heterogeneity of nascent HDL as those effected by increasing ABCA1 expression levels can be brought about by merely increasing cell density, while keeping apoAI and ABCA1 levels constant (Figs. 2 and 3).

Our model of nascent HDL biogenesis holds that ABCA1 facilitates extrusion of membrane exovesicles that are not much different from certain small tightly curved synthetically made liposomes used for cell-free generation of rHDL (34, 66). In the rate-limiting step, apoAI penetrates inside the exovesicle bilayer through the curvature-induced surface discontinuities and then microsolubilizes these exovesicles to nascent HDL independently of ABCA1 (34, 63, 67). ABCA1 expression likely correlates with the number of exovesicles and not the exovesicle size, because increases in size would lead to loss of both curvature and susceptibility to apoAI microsolubilization. From the perspective of apoAI activity, it matters little whether exovesicles are located on the same cell or different cells; hence, increases in the cell density can mimic rises in ABCA1 expression. On the other hand, an increase or a decrease in the number of exovesicles, regardless of its cause, would alter the ratio of available cell lipid:apoAI and shift the size composition of nascent HDL particles. The exovesicle model of nascent HDL formation accounts well for the role of the available cell lipid:apoAI ratio in nascent HDL heterogeneity.

CONCLUSIONS

Nascent HDL size heterogeneity reflects the ratio of available cell lipid to lipid-free apoAI at the time of nascent HDL particle assembly. High available cell lipid:apoAI ratios lead to preferential formation of larger nascent HDL species with more apoAI molecules per lipoprotein particle and more phospholipid and cholesterol molecules per molecule of apoAI. Because ABCA1 performs a facilitatory role and does not influence the interactions among apoAI and lipid molecules, rHDL assembly appears to be an excellent model of nascent HDL biogenesis. In future research, it would be critical to confirm our in vitro findings in an in vivo system, to delineate how nascent HDL heterogeneity translates into the heterogeneity of mature HDL and to test other factors known to control size distribution of rHDL particles. A better understanding of factors that instill and maintain HDL particle heterogeneity would be a valuable guide for development of novel antiatherogenic therapies that take advantage of HDL quality parameters.

The authors thank David Nguyen for radiolabeling apoAI and assistance with gas-liquid chromatography, Ginny Kellner-

Deu-

The FASEB Journal · www.fasebj.org
LYSSENKO ET AL.

Vol. 27 July 2013
2890
REFERENCES

1. Rothblat, G. H., and Phillips, M. C. (2010) High-density lipoprotein heterogeneity and function in reverse cholesterol transport. Curr. Opin. Lipidol. 21, 229–238

2. Rosenson, R. S., Brewer, H. B., Jr., Chapman, M. J., Fazio, S., Hussain, M. M., Kontush, A., Krauss, R. M., Otvos, J. D., Remaley, A. T., and Schaefer, E. J. (2011) HDL measures, particle heterogeneity, proposed nomenclature, and relation to atherosclerotic cardiovascular events. Clin. Chem. 57, 392–410

3. Scapin, A. M., Byrne, R. E., and Mihailovic, M. (1982) Functional roles of plasma high density lipoproteins. CRC Crit. Rev. Biochem. 13, 169–190

4. Glomset, J. A. (1970) Physiological role of lecithin-cholesterol acyltransferase. Am. J. Clin. Nutr. 23, 1129–1136

5. Miller, G. J. (1980) High density lipoproteins and atherosclerosis. Annu. Rev. Med. 31, 97–108

6. Negi, S., and Ballantyne, C. M. (2010) Insights from recent meta-analyses: role of high-density lipoprotein cholesterol in reducing cardiovascular events and rates of atherosclerotic disease progression. J. Clin. Lipidol. 4, 365–370

7. Lewis, G. F., and Rader, D. J. (2005) New insights into the regulation of HDL metabolism and reverse cholesterol transport. Circ. Res. 96, 1221–1232

8. Besler, C., Lüüscher, T. F., and Landmesser, U. (2012) Molecular mechanisms of vascular effects of high-density lipoprotein alterations in cardiovascular disease. EMBO Mol. Med. 4, 251–268

9. Rader, D. J., and Tall, A. R. (2011) The not-so-simple HDL story: Is it time to revise the HDL cholesterol hypothesis? Nat. Med. 18, 1344–1346

10. Eisenberg, S. (1984) High density lipoprotein metabolism. J. Lipid Res. 25, 1017–1058

11. Assmann, G., Herbert, P. N., Fredrickson, D. S., and Forte, T. (1977) Isolation and characterization of an abnormal high density lipoprotein in Tangier disease. J. Clin. Invest. 60, 242–252

12. Timmins, J. M., Lee, J. Y., Boudiquena, E., Kluckman, K. D., Brunham, A., Gebrue, A. K., Coutinho, J. M., Colvin, P. L., Smith, T. L., Haylen, M. R., Maeda, N., and Parks, J. S. (2005) Targeted inactivation of hepatic Aba1 causes profound hypoalphalipoproteinemia and kidney hypercatabolism of apoA-I. J. Clin. Investig. 115, 1333–1342

13. Brunham, L. R., Kuuti, J. K., Iqbal, J., Fievet, C., Timmins, J. M., Pape, T. D., Coburn, B. A., Bissada, N., Staels, B., Groen, A. K., Hussain, M. M., Parks, J. S., Kuipers, F., and Hayden, M. R. (2006) Intestinal ABCA1 directly contributes to HDL biogenesis in vivo. J. Clin. Investig. 116, 1052–1062

14. Miller, J. C., Barth, R. K., Shaw, P. H., Elliott, R. W., and Hastie, N. D. (1983) Identification of a cDNA clone for mouse apoprotein A-I (apo A-I) and its use in characterization of apo A-I mRNA expression in liver and small intestine. Proc. Natl. Acad. Sci. U. S. A. 80, 1511–1515

15. Haghpasand, M., Bourassa, P. A., Francone, O. L., and Aiello, R. J. (2001) Monocyte/macrophage expression of ABCA1 has minimal contribution to plasma HDL levels. J. Clin. Investig. 108, 1315–1320

16. Chisholm, J. W., Burleson, E. R., Shelniss, G. S., and Parks, J. S. (2002) ApoA1 secretion from HepG2 cells: evidence for the secretion of both lipid-poor apoA1 and intracellularly assembled nascent HDL. J. Lipid Res. 43, 36–44

17. Kiss, R. S., McManus, D. C., Franklin, V., Tan, W. L., McKenzie, A., Chimin, G., and Marcel, Y. L. (2003) The lipidation by hepatocytes of human apolipoprotein A-I occurs by both ABCA1-dependent and -independent pathways. J. Biol. Chem. 278, 10119–10127

18. Denis, M., Landry, Y. D., and Zha, X. (2008) ATP-binding cassette A1-mediated lipidation of apolipoprotein A-I occurs at the plasma membrane and not in the endocytic compartments. J. Biol. Chem. 283, 16178–16186

19. Faulkner, L. E., Panagotopulos, S. E., Johnson, J. D., Woollett, L. A., Hui, D. Y., Witting, S. R., Maiorano, J. N., and Davidson, W. S. (2008) An analysis of the role of a retroendocytosis pathway in ABCA1-mediated cholesterol efflux from macrophages. J. Lipid Res. 49, 1322–1332

20. Verghese, P. B., Arrese, E. L., Howard, A. D., and Soulages, J. L. (2008) Brefeldin A inhibits cholesterol efflux without affecting the rate of cellular uptake and re-secretion of apolipoprotein A-I in adipocytes. Arch. Biochem. Biophys. 478, 161–166

21. Camont, L., Chapman, M. J., and Kontush, A. (2011) Biological activities of HDL subpopulations and their relevance to cardiovascular disease. Trends Mol. Med. 17, 594–605

22. De la Llera-Moya, M., Drazul-Schrader, D., Asztalos, B. F., Cuchel, M., Rader, D. J., and Rothblat, G. H. (2010) The ability to promote efflux via ABCA1 determines the capacity of serum specimens with similar high-density lipoprotein cholesterol to remove cholesterol from macrophages. Arterioscler. Thromb. Vasc. Biol. 30, 796–801

23. Northcross, R. W., Ewen, J. S., Fulp, B., Bhat, S., Zhu, X., Parks, J. S., Shah, D., Jerome, W. G., Gerelus, M., Bababili, M., and Thomas, M. J. (2012) Nascent high density lipoproteins formed by ABCA1 resemble lipid rafts and are structurally organized by three apoA-I monomers. J. Lipid Res. 53, 1890–1900

24. Dunger, P. T., Collins, H. L., Nickel, M., Lund-Katz, S., Rothblat, G. H., and Phillips, M. C. (2006) Characterization of nascent HDL particles and microparticles by ABCA1-mediated efflux of cellular lipids to apoA-I. J. Lipid Res. 47, 832–843

25. Krinou, L., Hajj Hassan, H., Blain, S., Rashid, S., Denis, M., Marcell, M., and Genest, J. (2005) Biogenesis and specification of nascent apoA-I-containing particles in various cell lines. J. Lipid Res. 46, 1668–1677

26. Hayashi, M., Abe-Dohmae, S., Okazaki, M., Ueda, K., and Yokoyama, S. (2005) Heterogeneity of high density lipoprotein generated by ABCA1 and ABCA7. J. Lipid Res. 46, 1703–1711

27. Liu, L., Bortnick, A. E., Nickel, M., Dhanasekaran, P., Subbaiah, P. V., Lund-Katz, S., Rothblat, G. H., and Phillips, M. C. (2005) Effects of apolipoprotein A-I on ATP-binding cassette transporter A1-mediated efflux of macropathogenic phospholipid and cholesterol: formation of nascent high density lipoprotein particle. J. Biol. Chem. 278, 42976–42984

28. Forte, T. M., Goth-Goldstein, R., Nordhausen, R. W., and McCall, M. R. (1993) Apolipoprotein A-I-cell membrane interaction: extracellular assembly of heterogeneous nascent HDL particles. J. Lipid Res. 34, 317–324

29. Ruhr, J. P., and Merritt, D. (1977) Role of apolipoprotein A1 in the structure of human serum high density lipoproteins: reconstitution studies. J. Biol. Chem. 252, 1208–1216

30. Swaney, J. B. (1980) Properties of lipid-apolipoprotein association products: complexes of dimyristoyl phosphatidylcholine and human apo A-I. J. Biol. Chem. 255, 877–881

31. Matz, C. E., and Jonas, A. (1982) Micellar complexes of human apolipoprotein A1 with phosphatidylcholines and cholesterol prepared from cholate-lipid dispersions. J. Biol. Chem. 257, 4535–4540

32. Lund-Katz, S., and Phillips, M. C. (2010) High density lipoprotein structure-function and role in reverse cholesterol transport. Subcell. Biochem. 51, 183–227

33. Jonas, A. (1986) Reconstitution of high-density lipoproteins. Methods Enzymol. 128, 553–582

34. Vedhachalam, C., Dunger, P. T., Nickel, M., Nguyen, D., Dhanasekaran, P., Saito, H., Rothblat, G. H., Lund-Katz, S., and Phillips, M. C. (2007) Mechanism of ATP-Binding Cassette Transporter A1-mediated cellular lipid efflux to apolipoprotein A-I and formation of high density lipoprotein particles. J. Biol. Chem. 282, 25125–25131

35. Lysenko, N. N., Brukshaker, G., Smith, B. D., and Smith, J. D. (2011) A novel compound inhibits reconstituted high-density lipoprotein assembly and blocks nascent high density lipoprotein biogenesis downstream of apolipoprotein A-I binding to
ATP-binding cassette transporter A1-expressing cells. *Arterioscler. Thromb. Vasc. Biol.* **31**, 2700–2706.

36. Oram, J. F., Vaughan, A. M., and Stocker, R. (2001) ATP-binding cassette transporter A1 mediates cellular secretion of α-tocopherol. *J. Biol. Chem.* **276**, 39988–39992.

37. Vaughan, A. M., Tang, C., and Oram, J. F. (2009) ABCA1 mutants reveal an interdependency between lipid export function, apoA1 binding activity, and Janus kinase 2 activation. *J. Lipid Res.* **50**, 285–292.

38. Vedhachalam, C., Chetty, P. S., Nickel, M., Dhanasekaran, P., Lund-Katz, S., Rothblat, G. H., and Phillips, M. C. (2010) Influence of apolipoprotein (Apo) A1 structure on nascent high density lipoprotein (HDL) particle size distribution. *J. Biol. Chem.* **285**, 31965–31973.

39. Davies, G. E., and Stark, G. R. (1970) Use of dimethyl suberimidate, a cross-linking reagent, in studying the subunit structure of oligomeric proteins. *Proc. Natl. Acad. Sci. U. S. A.* **66**, 651–656.

40. Swaney, J. B. (1986) Use of cross-linking reagents to study lipoprotein structure. *Methods Enzymol.* **128**, 613–626.

41. Sezgin, E., Kaiser, H.J., Baumgart, T., Schwille, P., Simons, K., and Levental, I. (2012) Elucidating membrane structure and protein behavior using giant plasma membrane vesicles. *Nat. Protoc.* **7**, 1042–1051.

42. Bellini, F., Phillips, M. C., Pickell, C., and Rothblat, G. H. (1984) Role of the plasma membrane in the mechanism of cholesterol efflux from cells. *Biochim. Biophys. Acta* **777**, 209–215.

43. Rouser, G., Fleischer, S., and Yamamoto, A. (1970) Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* **5**, 494–496.

44. Kłusek, J. J., Tancey, P., St Clair, R. W., Fischer, R. T., Johnson, W. J., and Glick, J. M. (1995) Cholesterol quantitation by GLC: artificial formation of short-chain steryl esters. *J. Lipid Res.* **36**, 2261–2266.

45. Zheng, P., Horwitz, A., Waele, C. A., and Smith, J. D. (2001) Stably transfected ABCA1 antisense cell line has decreased ABCA1 mRNA and cAMP-induced cholesterol efflux to apolipoprotein A1 and HDL. *Biochim. Biophys. Acta* **1534**, 121–128.

46. Costet, P., Lalanne, F., Gerbod-Giannone, M. C., Molina, J. R., Fu, X., Lund, E. G., Gudas, L. J., and Tall, A. R. (2003) Retinoic acid receptor-mediated induction of ABCA1 in macrophages. *Mol. Cell. Biol.* **23**, 7756–7766.

47. Vaughan, A. M., and Oram, J. F. (2006) ABCA1 and ABCG1 or ABCG4 act sequentially to remove cellular cholesterol and generate cholesterol-rich HDL. *Arterioscler. Thromb. Vasc. Biol.* **26**, 4652–4665.

48. Vaughan, A. M., and Oram, J. F. (2003) ABCA1 redistributes membrane cholesterol independent of apolipoprotein interaction. *J. Lipid Res.* **44**, 1373–1380.

49. Sinagara, R. R., Brunham, L. R., Visscher, H., Kastelein, J. J., and Hayden, M. R. (2003) Efflux and atherosclerosis: the clinical and biochemical impact of variations in the ABCA1 gene. *Arterioscler. Thromb. Vasc. Biol.* **23**, 1322–1329.

50. Bhat, S., Sorci-Thomas, M. G., Alexander, E. T., Samuel, M. P., and Thomas, M. J. (2005) Intermolecular contact between globular N-terminal fold and C-terminal domain of ApoA1 stabilizes its lipid-bound conformation: studies employing chemical cross-linking and mass spectrometry. *J. Biol. Chem.* **280**, 33015–33025.

51. Tian, S., and Jonas, A. (2002) Structural and functional properties of apolipoprotein A1 mutants containing disulfide-linked cysteines at positions 124 or 232. *Biochim. Biophys. Acta* **1599**, 56–64.

52. Jonas, A., Kézdy, K. E., and Wald, J. H. (1989) Defined apolipoprotein A1 conformations in reconstituted high density lipoprotein discs. *J. Biol. Chem.* **264**, 4818–4824.

53. Le Goff, W., Zheng, P., Brubaker, G., and Smith, J. D. (2006) Identification of the cAMP-responsive enhancer of the murine ABCA1 gene: requirement for CREB1 and STAT3/4 elements. *Arterioscler. Thromb. Vasc. Biol.* **26**, 527–533.

54. Lysenko, N. N., Hata, M., Dhanasekaran, P., Nickel, M., Nguyen, D. C., McGrath, J. P. S., Saito, H., Lund-Katz, S., and Phillips, M. C. (2012) Influence of C-terminal α-helix hydrophobicity and aromatic amino acid content on apolipoprotein A1 functionality. *Biochim. Biophys. Acta* **1821**, 456–463.

55. Sonntag, T. J., Carnevallo, R., Vaisar, T., Reardon, C. A., and Getz, G. S. (2012) Naturally occurring variant of mouse apolipoprotein A1 alters the lipid and HDL association properties of the protein. *J. Lipid Res.* **53**, 592–599.

56. Asztalos, B. F., Schaefer, E. J., Horvath, K. V., Yamashita, S., Miller, M., Franceschini, G., and Calabresi, L. (2007) Role of LCAT in HDL remodeling: investigation of LCAT deficiency states. *J. Lipid Res.* **48**, 592–599.

57. Rubin, E. M., Ishida, B. Y., Clift, S. M., and Krauss, R. M. (1991) Expression of human apolipoprotein A1 in transgenic mice results in reduced plasma levels of murine apolipoprotein A1 and the appearance of two new high density lipoprotein size subclasses. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 434–438.

58. Alexander, E. T., Vedhachalam, C., Sankaranarayanan, S., de la Llera-Moya, M., Rothblat, G. H., Rader, D. J., and Phillips, M. C. (2011) Influence of apolipoprotein A1 domain structure on macropage reverse cholesterol transport in mice. *Arterioscler. Thromb. Vasc. Biol.* **31**, 320–327.

59. Cravens, J. H., Capron, P., Kandoussi, A., Nielsen, H., Christensen, E. I., Norden, A., and Moestrup, S. K. (2008) A pivotal role of the human kidney in catabolism of HDL protein components apolipoprotein A1 and AIV but not of AII. *Lipids* **43**, 467–470.

60. Tabas, I. (2002) Consequences of cellular cholesterol accumulation: basic concepts and physiological implications. *J. Clin. Invest.* **110**, 905–911.

61. Gu, F., Jones, M. K., Chen, J., Patterson, J. C., Catte, A., Jerome, W. G., Li, L., and Segrest, J. P. (2010) Structures of discoidal high density lipoproteins: a combined computational-experimental approach. *J. Biol. Chem.* **285**, 4652–4665.

62. Zorich, N. L., Kézdy, K. E., and Jonas, A. (1987) Properties of discoidal complexes of human apolipoprotein A1 with phosphatidylcholines containing various fatty acid chains. *Biochim. Biophys. Acta* **919**, 181–188.

63. Massey, J. B., and Pownall, H. J. (2008) Cholesterol is a determinant of the structures of discoidal high density lipoproteins formed by the solubilization of phospholipid membranes by apolipoprotein A1. *Biochim. Biophys. Acta* **1781**, 245–253.

64. Tall, A. R., and Lange, Y. (1978) Interaction of cholesterol, phospholipid and apoprotein in high density lipoprotein recombaints. *Biochim. Biophys. Acta* **513**, 185–197.

65. Asztalos, B. F., Brousseau, M. E., McNamara, J. R., Horvath, K. V., Roheim, P. S., and Schaefer, E. J. (2001) Subpopulations of high density lipoproteins in homozygous and heterozygous Tangier disease. *Atherosclerosis* **156**, 217–225.

66. Lin, G., and Oram, J. F. (2000) Apolipoprotein binding to protruding membrane domains during removal of excess cellular cholesterol. *Atherosclerosis* **149**, 359–370.

67. Gillotte, K. L., Davidson, W. S., Lund-Katz, S., Rothblat, G. H., and Phillips, M. C. (1998) Role of the human kidney in catabolism of HDL protein components apolipoprotein A1 and AIV but not of AII. *Lipids* **43**, 467–470.

Accepted for publication March 25, 2013.