Biogenesis and speciation of nascent apoA-I-containing particles in various cell lines

Larbi Krimbou, Houssein Hajj Hassan, Sacha Blain, Shirya Rashid, Maxime Denis, Michel Marcil, and Jacques Genest[1]

Cardiovascular Genetics Laboratory, Cardiology Division, McGill University Health Centre/Royal Victoria Hospital, Montréal, Québec H3A 1A1, Canada

Abstract **It is generally thought that the large heterogeneity of human HDL confers antiatherogenic properties; however, the mechanisms governing HDL biogenesis and speciation are complex and poorly understood. Here, we show that incubation of exogenous apolipoprotein A-I (apoA-I) with fibroblasts, CaCo-2, or CHO-overexpressing ABCA1 cells generates only α-nascent apolipoprotein A-I-containing particles (α-LpA-I) with diameters of 8–20 nm, whereas human umbilical vein endothelial cells and ABCA1 mutant (Q597R) cells were unable to form such particles. Interestingly, incubation of exogenous apoA-I with either HepG2 or macrophages generates both α-LpA-I and preβ$_1$-LpA-I. Furthermore, glyburide inhibits almost completely the formation of α-LpA-I but not preβ$_1$-LpA-I. Similarly, endogenously secreted HepG2 apoA-I was found to be associated with both preβ$_1$-LpA-I and α-LpA-I; by contrast, CaCo-2 cells secreted only α-LpA-I. To determine whether α-LpA-I generated by fibroblasts is a good substrate for LCAT, isolated α-LpA-I as well as reconstituted HDL [r(HDL)] was reacted with LCAT. Although both particles had similar V_{max} (8.4 vs. 8.2 nmol cholesteryl ester/h/μg LCAT, respectively), the K_m value was increased 2-fold for α-LpA-I compared with r(HDL) (1.2 vs. 0.7 μM apoA-I). These results demonstrate that *1*) ABCA1 is required for the formation of α-LpA-I but not preβ$_1$-LpA-I; and *2*) α-LpA-I interacts efficiently with LCAT. Thus, our study provides direct evidence for a new link between specific cell lines and the speciation of nascent HDL that occurs by both ABCA1-dependent and -independent pathways.**—Krimbou, L., H. Hajj Hassan, S. Blain, S. Rashid, M. Denis, M. Marcil, and J. Genest. **Biogenesis and speciation of nascent apoA-I-containing particles in various cell lines.** ***J. Lipid Res.*** **2005.** 46: **1668–1677.**

Supplementary key words ATP binding cassette transporter A1 • high density lipoprotein • α-nascent apolipoprotein A-I-containing particle • pre-β$_1$-nascent apolipoprotein A-I-containing particle

The molecular interaction of apolipoprotein A-I (apoA-I) with cell membranes has important implications in reverse cholesterol transport (RCT) because it provides a mechanism whereby excess cholesterol is removed from peripheral cells unable to catabolize cholesterol. This process is crucial for HDL biogenesis and is believed to be one of the major mechanisms by which HDL may protect against atherosclerotic vascular disease (1, 2). However, in spite of the importance of the HDL biogenesis pathway in RCT, very little is known about the mechanisms involved in determining the structural characteristics of nascent HDL particles.

It has been suggested that HDL heterogeneity involves not only intracellular factors that control the synthesis and cell uptake of HDL but also factors that promote the assembly and remodeling of HDL in the extracellular space. Indeed, several enzymes, such as lecithin:cholesterol acyltransferase, hepatic lipase, cholesteryl ester (CE) transfer protein, and phospholipid transfer protein, have been implicated in regulating apoA-I cycles between lipid-poor and lipid-associated forms as a part of a highly dynamic metabolism of HDL (3). HDL subspecies can be classified according to a variety of properties, including hydrated density (4), apolipoprotein composition, and charge characteristics (5). For example, an earlier study by Fielding and colleagues (6) documented that a minor subspecies of human HDL that migrates with preβ mobility on agarose gels can remove free cholesterol from cultured fibroblasts at a faster rate than α-migrating HDL, which constitutes the bulk of plasma HDL. Furthermore, it was documented that preβ-HDL particles were present in the peripheral lymph of dogs (7) and the interstitial space (8), suggesting a key role for these particles in the initial removal of cholesterol. Moreover, this concept is supported by studies demonstrating that preβ-HDL acts as

Manuscript received 1 February 2005 and in revised form 12 April 2005.

Published, JLR Papers in Press, May 16, 2005.
DOI 10.1194/jlr.M500038-JLR200

Abbreviations: apoA-I, apolipoprotein A-I; CE, cholesteryl ester; HUVEC, human umbilical vein endothelial cells; LpA-I, nascent apolipoprotein A-I-containing particle; MWCO, molecular weight cut off; RCT, reverse cholesterol transport; r(HDL), reconstituted high density lipoprotein; TD, Tangier disease; 2D-PAGGE, two-dimensional polyacrylamide nondenaturing gradient gel electrophoresis; 22OH/9CRA, 22(*R*)-hydroxycholesterol and 9-*cis*-retinoic acid.

[1] To whom correspondence should be addressed.
e-mail: jacques.genest@muhc.mcgill.ca

an initial acceptor of cellular cholesterol and shuttles it into a series of larger preβ particles and ultimately to α-migrating particles that contain LCAT for esterification (9, 10). A growing body of evidence indicates that ABCA1 is a critical cell surface protein required for the transfer of cellular lipids and the maintenance of HDL levels in plasma and is likely important for the first step of RCT from peripheral tissues, including macrophages in the vessel wall (11). Furthermore, Brewer and colleagues (12) have documented that hepatic ABCA1 is crucial for the formation and maintenance of plasma HDL levels. The importance of ABCA1 in the lipidation of apoA-I is highlighted by the finding that >50 mutations in the ABCA1 gene have been associated with a variety of clinically distinct HDL-deficiency diseases, including Tangier disease (TD) and familial HDL deficiency (13–15).

Although recent studies in a cell culture model suggest that the ABCA1 transporter plays a determinant role in the heterogeneity of nascent HDL particles (16, 17), the molecular mechanisms involved in the speciation of HDL particles are not well understood. Therefore, it was the aim of the present study to better define the role of ABCA1 in the biogenesis and speciation of nascent apolipoprotein A-I-containing particles (LpA-I) in various cell lines and to examine their maturation.

MATERIALS AND METHODS

Patient selection

For the present study, we selected fibroblasts from three normal control subjects and two patients with TD (TD1, homozygous for Q597R at the ABCA1 gene; and TD2, compound heterozygous carrying the mutations C1477R and the splice site G→C in exon 24), as described previously (13, 14). The protocol for the study was reviewed and accepted by the Research Ethics Board of the McGill University Health Centre. Separate consent forms for blood sampling, DNA isolation, and skin biopsy were provided.

Cell culture

Human skin fibroblasts were obtained from 3.0 mm punch biopsies of the forearms of patients and healthy control subjects and were cultured in DMEM supplemented with 0.1% nonessential amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FBS. HepG2, human umbilical vein endothelial cells (HUVEC), human monocyte-derived macrophages, THP-1, and CaCo-2 cells were cultured under standard conditions. ABCA1-expressing CHO cells were generously provided by Dr. Sean Davidson (Department of Pathology and Laboratory Medicine, University of Cincinnati) and were characterized and cultured as described previously (18).

Human plasma apoA-I

Purified plasma apoA-I (Biodesign) was resolubilized in 4 M guanidine-HCl and dialyzed extensively against PBS buffer. Freshly resolubilized apoA-I was iodinated with ^{125}I by Iodogen® (Pierce) to a specific activity of 800–1,500 cpm/ng apoA-I and used within 48 h.

Expression and purification of apoA-I mutants

The expression of wild-type apoA-I and its mutants in a bacterial system, and the isolation and purification of these proteins, have been described previously by Marcel and colleagues (19). Deletion mutant Δ122–165 was used as a negative control for LCAT-mediated α-LpA-I cholesterol esterification.

Removal of lipid-free apoA-I from LpA-I

Stimulated various cell lines in 100 mm diameter dishes were incubated with 10 μg/ml ^{125}I-apoA-I for the indicated times at 37°C. Lipid-free ^{125}I-apoA-I was removed from the medium by a size-exclusion centrifugal filter (spiral ultrafiltration cartridge, molecular weight cut off (MWCO) 50,000; Amicon) that discriminates between lipid-free apoA-I and other lipidated LpA-I particles with molecular mass ⩾ 50 kDa. Concentrated medium was washed three times in the same filter with 15 ml of PBS containing a protease inhibitor cocktail (Roche Diagnostics). LpA-I particles were further dialyzed for 16 h at 4°C using a dialysis membrane with a MWCO of 50,000 to remove any remaining lipid-free apoA-I. The same filtration system was used to remove lipid-free apoA-I from apoA-I-containing particles secreted by HepG2 and CaCo-2 cells. Lipid-free ^{125}I-apoA-I incubated in DMEM without cells was not retained by this filtration system. In separate experiments (see Results), we show that both the centrifugal filter and the dialysis membrane retained native plasma preβ$_1$-LpA-I having an apparent molecular mass of 67 kDa.

Isolation of α-LpA-I

α-LpA-I particles were isolated as described previously (20). Briefly, [^{14}C]cholesterol-labeled normal fibroblasts were stimulated with 2.5 μg/ml 22(*R*)-hydroxycholesterol and 5 μM 9-*cis*-retinoic acid (22OH/9CRA) and then incubated with 10 μg/ml ^{125}I-apoA-I for 24 h at 37°C. Media were recovered and concentrated with a size-exclusion centrifugal filter (spiral ultrafiltration cartridge, MWCO 50,000; Amicon), and then concentrated medium was washed three times in the same filter with 15 ml of PBS in the presence of a protease inhibitor cocktail (Roche Diagnostics). LpA-I particles were further dialyzed using a dialysis membrane with a MWCO of 50,000 to remove any remaining lipid-free apoA-I. The integrity of isolated α-LpA-I was verified by analysis with two-dimensional polyacrylamide nondenaturing gradient gel electrophoresis (2D-PAGGE).

Cellular cholesterol efflux and lipid labeling

Cholesterol efflux and lipid labeling were performed as described previously (17) with minor modifications. Briefly, 50 000 cells were seeded on 12-well plates. At mid-confluence, the cells were labeled with 3 μCi/ml [^{3}H]cholesterol (Perkin-Elmer) for 24 h. At confluence, cells were cholesterol-loaded (20 μg/ml) for 24 h. During a 24 h equilibration period, cells were stimulated with 22OH/9CRA for 20 h. Cholesterol efflux was determined for the indicated concentrations and times. Cholesterol efflux was determined as follows: ^{3}H cpm in medium/(^{3}H cpm in medium + ^{3}H cpm in cells); the results were expressed as percentages of total radiolabeled cholesterol. Cells from a normal and a TD subject were grown to confluence in 100 mm diameter dishes, labeled with 15 μCi/ml [^{14}C]cholesterol (Perkin-Elmer) for 48 h, cholesterol-loaded, equilibrated, and stimulated as described above. Cellular [^{14}C]cholesterol labeling was used for the characterization of LpA-I generated during the incubation of lipid-free apoA-I wild-type or apoA-I mutant (Δ122–165) with stimulated fibroblasts. Cell phospholipids were labeled with [^{32}P]orthophosphate as follows: fibroblasts from control and TD subjects were grown to confluence in 100 mm diameter dishes and then incubated for 48 h with 300 μCi of [^{32}P]orthophosphate mixed with DMEM. After the first 24 h incubation with [^{32}P]orthophosphate, cells were cholesterol-loaded (20 μg/ml) for 24 h; then during a 24 h equilibration period, the cells were

stimulated as described above before incubation with either wild-type or mutant (Δ122–165) apoA-I .

Preparation of reconstituted HDL particles

Complexes comprising apoA-I, POPC, and [^{14}C]cholesterol were prepared using the sodium cholate dialysis method as described by Jonas, Steinmetz, and Churgay (21). An apoA-I/POPC molar ratio of 1:100 was used in this experiment. Reconstituted high density lipoprotein [r(HDL)] particles were further concentrated by ultrafiltration (spiral ultrafiltration cartridge, MWCO 50,000; Amicon) to discard any lipid-free apoA-I or proteolytic peptides. ApoA-I-lipid complex formation was verified by analysis with 2D-PAGGE.

Separation of lipoproteins by 2D-PAGGE

ApoA-I-containing particles were separated by 2D-PAGGE as described previously (22). Briefly, samples (30–100 μl) were separated in the first dimension (according to their charge) by 0.75% agarose gel electrophoresis (100 V, 3 h, 4°C) and in the second dimension (according to the size) by 5–23% polyacrylamide concave gradient gel electrophoresis (125 V, 24 h, 4°C). Iodinated high molecular weight protein mixture (7.1–17.0 nm; Pharmacia) was run as a standard on each gel. Electrophoretically separated samples were electrotransferred (30 V, 24 h, 4°C) onto nitrocellulose membranes (Hybond ECL; Amersham). ApoA-I-containing particles were detected by incubating the membranes with immunopurified polyclonal anti-apoA-I antibody (Biodesign). The presence of labeled ^{125}I-apoA-I, [^{14}C]cholesterol, or [^{32}P]phospholipids was detected directly by autoradiography using Kodak XAR-2 film.

Lecithin:cholesterol acyltransferase assay

C-terminal histidine-tagged human recombinant lecithin:cholesterol acyltransferase (hrLCATH6) was a gift from Dr. John S. Parks (Wake Forest University School of Medicine, Winston-Salem, NC). Cholesterol esterification experiments were conducted as described previously (23). In these assays, two types of substrate were used: [^{14}C]cholesterol-labeled α-LpA-I and r(HDL) prepared with similar specific activity as described above. In separate experiments, α-LpA-I generated by either wild-type or mutant apoA-I were tested for their ability to stimulate the LCAT reaction. Equivalent amounts of either LpA-I or r(HDL) were reacted with 100 ng of LCAT for 1 h at 37°C. The LCAT reaction mixture consisted of varying amounts of either LpA-I or r(HDL), 1.5 mg of fatty acid-free BSA, 5 mM β-mercaptoethanol, and reaction buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, and 1 mM NaN_3, pH 8.0) to a final volume of 200 μl. ApoA-I concentrations of LpA-I and r(HDL) were estimated from the initial specific activity of ^{125}I-apoA-I. Conditions for the assay were generally as described previously. Under these conditions, initial rates were estimated with minimal substrate conversion.

RESULTS

Based on our previous studies demonstrating that the interaction of apoA-I with normal human fibroblasts generated only α-LpA-I (16, 20), the question was raised whether the formation of both α-LpA-I and preβ_1-LpA-I could be dependent on specific cell types. Because preβ_1-LpA-I are electrophoretically indistinguishable from lipid-free apoA-I, and because of the difficulty of separating small amounts of apoA-I-containing particle subspecies by fast-protein liquid chromatography, we developed a rapid controlled technique to remove lipid-free apoA-I from the medium using a size-exclusion centrifugal filter (MWCO 50,000) combined with a dialysis membrane with a MWCO of 50,000 to remove any remaining lipid-free apoA-I, as described in Materials and Methods.

The present centrifugal filtration system discriminates between lipid-free apoA-I and other lipidated LpA-I particles with molecular weight ⩾ 50 kDa. However, the incomplete removal of lipid-free apoA-I from the medium may result in an artifactual presence of preβ_1-LpA-I. To ensure the quality of the complete removal of lipid-free apoA-I, the filtration of the medium from either HepG2 cells or macrophages found to form preβ_1-LpA-I was carefully controlled for each experiment by the filtration of the medium from normal fibroblasts, ABCA1 mutant Q597R fibroblasts, or lipid-free ^{125}I-apoA-I incubated without cells. No detectable lipid-free ^{125}I-apoA-I remained in the medium after filtration followed by dialysis, as assessed by 2D-PAGGE (see below). In separate experiments (see below), we show that both the centrifugal filter and the dialysis membrane retained native plasma preβ_1-LpA-I having an apparent molecular mass of 67 kDa.

As shown in **Fig. 1** (upper panels), incubation of stimulated normal fibroblasts, CaCo-2 cells, or CHO-overexpressing ABCA1 (CHO-ABCA1) with 10 μg/ml exogenously added ^{125}I-apoA-I for 6 h at 37°C followed by the removal of lipid-free apoA-I as described above, and then separation of samples by 2D-PAGGE, generated only α-LpA-I with a particle size ranging from 8 to 20 nm, whereas lipid-free apoA-I incubated with either HUVEC or ABCA1 mutant (Q597R) was unable to form such particles. In separate experiments, we found that ABCA1 mutant C1477R also failed to form α-LpA-I (data not shown). Unexpectedly, incubation of stimulated HepG2, human monocyte-derived macrophages, or THP-1 cells with exogenously added ^{125}I-apoA-I generated both preβ_1-LpA-I and α-LpA-I (Fig. 1, upper panels).

Previous studies have documented that glyburide inhibited ABCA1-mediated cholesterol efflux from HEK[ABCA1] cells to apoA-I (10 μg/ml) with an IC_{50} of 300 μM and had little effect on the low background efflux in the absence of ABCA1 (24, 25). As shown in Fig. 1 (lower panels), the presence of 300 μM glyburide during the incubation period inhibited almost completely the formation of larger α-LpA-I in the majority of cell types tested but not the preβ_1-LpA-I generated specifically by HepG2, human monocyte-derived macrophages, and THP-1 cells. Furthermore, inactivation of ABCA1 with glyburide in fibroblasts, CaCo-2, or CHO-ABCA1 led to the formation of smaller α-LpA-I having a diameter of ~8 nm; these particles were not formed in the presence of ABCA1 mutant Q597R cells. Treatment of different cell lines with 300 μM glyburide for 1–12 h did not cause any significant morphological changes or toxicity, as measured by trypan blue exclusion.

A time-course analysis of nascent LpA-I generated by HepG2 and normal fibroblasts showed that ^{125}I-apoA-I was consistently associated with particles having both preβ and α electrophoretic mobility in HepG2 cells. In con-

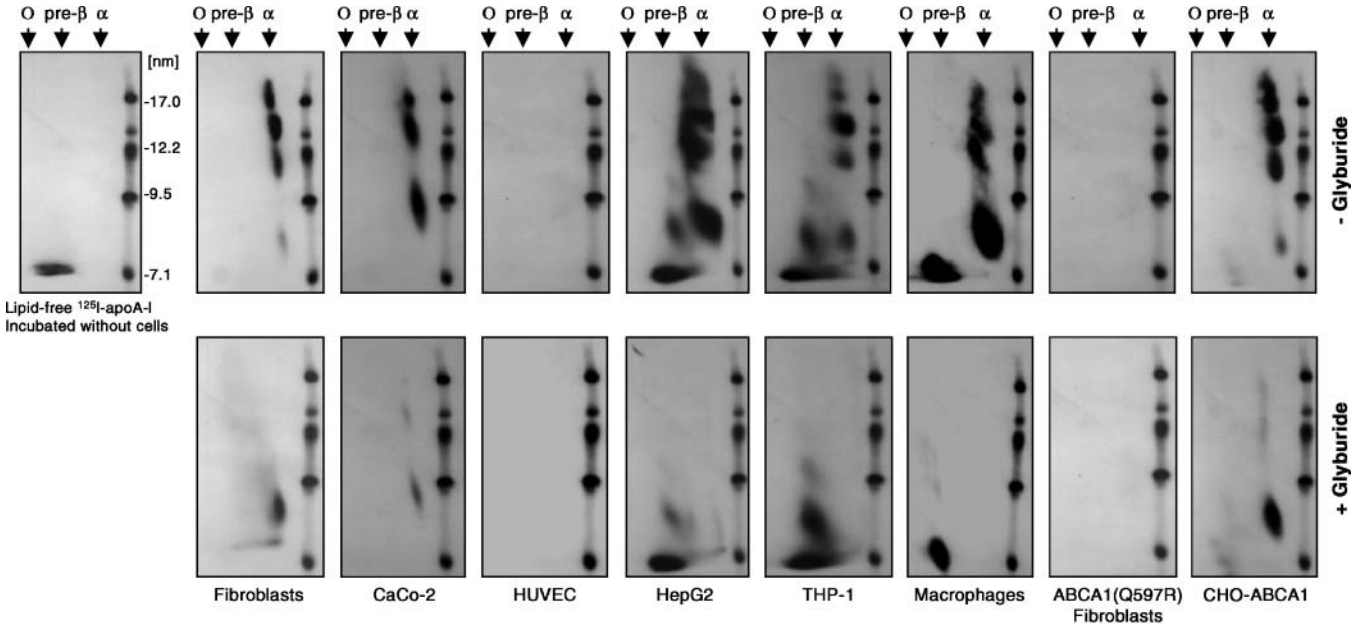


Fig. 1. Analysis of nascent apolipoprotein A-I-containing particles (LpA-I) generated by incubation of exogenously added lipid-free apolipoprotein A-I (apoA-I) with various cell lines in the presence or absence of glyburide. Normal fibroblasts, CaCo-2, human umbilical vein endothelial cells (HUVEC), HepG2, or ABCA1 mutant (Q597R) cells in 100 mm diameter dishes were loaded with 20 μg/ml cholesterol for 24 h and then stimulated with 2.5 μg/ml 22(*R*)-hydroxycholesterol and 10 μM 9-*cis*-retinoic acid (22OH/9CRA) for 20 h. THP-1 cells and human monocyte-derived macrophages in 100 mm diameter dishes were loaded with 20 μg/ml cholesterol for 24 h and then stimulated with 0.3 mM cAMP for 12 h. CHO-overexpressing ABCA1 cells (CHO-ABCA1) were used as a control. Different cell types were incubated with 10 μg/ml ^{125}I-apoA-I for 6 h at 37°C in the presence or absence of 300 μM glyburide. Lipid-free ^{125}I-apoA-I was removed from the medium by ultrafiltration followed by dialysis, as described in Materials and Methods. Lipid-free ^{125}I-apoA-I incubated for 6 h at 37°C in DMEM without cells was used as a control. Samples were separated by two-dimensional polyacrylamide nondenaturing gradient gel electrophoresis (2D-PAGGE), and ^{125}I-apoA-I was detected directly by autoradiography using Kodak XAR-2 film. Molecular size markers are shown.

trast, fibroblasts generated only α-LpA-I (**Fig. 2**, upper panels). To further investigate the contribution of ABCA1 to the formation of preβ$_1$-LpA-I and α-LpA-I, lipid-free ^{125}I-apoA-I was incubated with HepG2 cells or fibroblasts in the presence of 300 μM glyburide for the indicated times. As shown in Fig. 2 (lower panels), glyburide inhibited almost completely α-LpA-I subspecies in both HepG2 cells and fibroblasts, but not preβ$_1$-LpA-I generated by HepG2 over a 12 h incubation period. Moreover, both the charge and the size of preβ$_1$-LpA-I and α-LpA-I were stable over a 12 h incubation period. On the other hand, ABCA1 protein expression was highly responsive to stimulation with 22OH/9CRA in fibroblasts, CaCo-2, and HepG2 cells or to stimulation with cAMP of macrophages, whereas ABCA1 was not detected in HUVEC, as examined by gel electrophoresis of total cell lysate from different cell lines followed by immunoblotting with an anti-ABCA1 antibody. This is consistent with our findings that the majority of α-LpA-I subspecies were strongly increased by stimulation with 22OH/9CRA in normal fibroblasts, CaCo-2, HepG2, or cAMP-stimulated THP-1. In contrast, preβ$_1$-LpA-I formed by HepG2 or THP-1 were insensitive to stimulation with 22OH/9CRA or cAMP, respectively (data not shown).

Having determined that the incubation of exogenously added lipid-free apoA-I with HepG2 cells generates both preβ$_1$-LpA-I and α-LpA-I, the question was raised whether different preβ$_1$-LpA-I particles found in the circulation were produced by hepatocytes and/or intestine, or whether they were a product of plasma apoA-I-containing lipoprotein remodeling. An experiment was carried out in which the medium of endogenously secreted apoA-I-containing particles from either stimulated HepG2 or CaCo-2 cells was depleted or not from lipid-free apoA-I as described above and analyzed by 2D-PAGGE. As shown in **Fig. 3**, stimulated HepG2 cells secreted apoA-I-containing lipoproteins with a particle diameter ranging from 7.1 to 20 nm and have both preβ and α electrophoretic mobility (Fig. 3A, right panel), similar to native plasma preβ$_1$-LpA-I and α-LpA-I (Fig. 3C, right panel). In contrast, stimulated CaCo-2 cells secreted only α-LpA-I (Fig. 3B, right panel). Furthermore, a significant amount of lipid-free apoA-I was secreted in both HepG2 and CaCo-2 cells, as assessed by a 2D-PAGGE separation of the medium before the removal of lipid-free apoA-I (Fig. 3A, B, left panels). At the same time, glyburide inhibited almost completely the formation of endogenously secreted α-LpA-I in both HepG2 and CaCo-2 cells but not preβ$_1$-LpA-I particles specifically secreted by HepG2 cells (data not shown). In separate experiments, we verified that glyburide (300–500 μM) had no effect on apoA-I gene expression in both HepG2 and CaCo-2, as assessed by Northern blotting (data not shown).

To further investigate the relationship between the sizes of α-LpA-I particles and their lipids, fibroblasts were la-

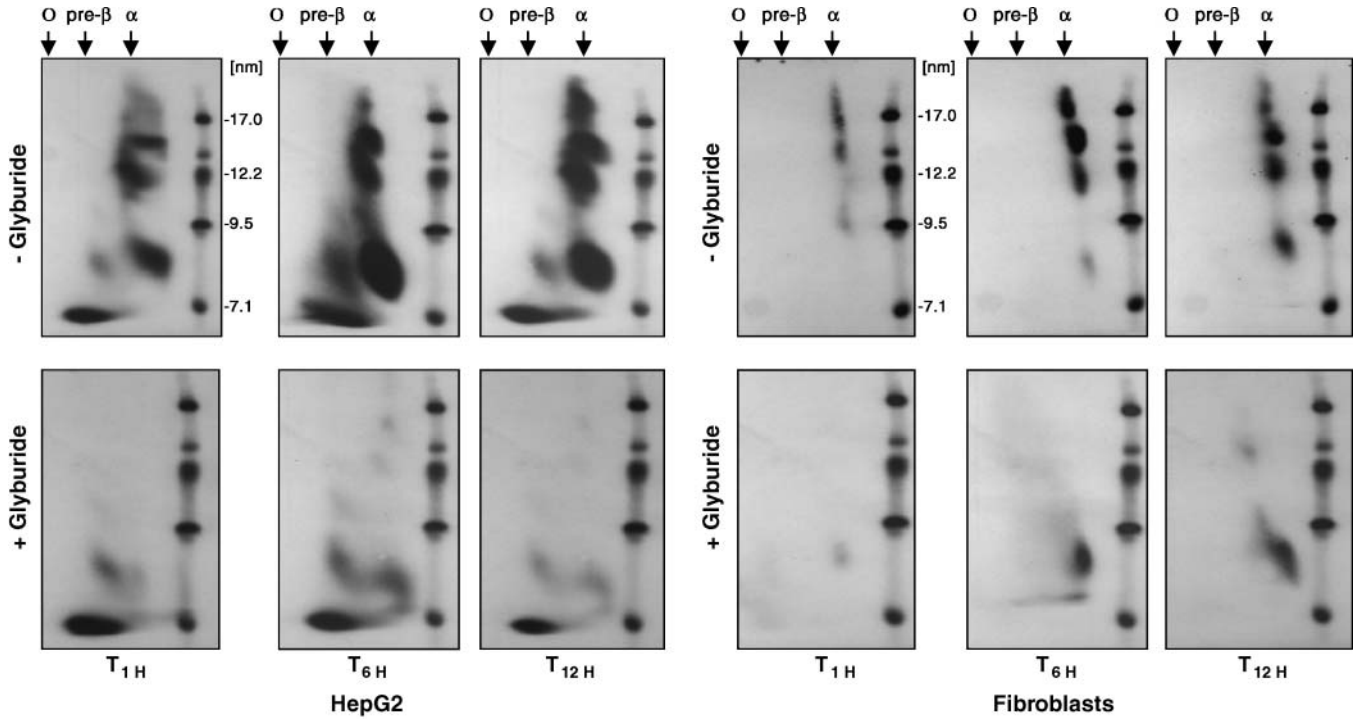


Fig. 2. Time-course analysis of the formation of LpA-I generated by the incubation of exogenously added lipid-free apoA-I with HepG2 cells and fibroblasts. Either HepG2 cells or normal fibroblasts in 100 mm diameter dishes were loaded with 20 μg/ml cholesterol for 24 h and then stimulated with 22OH/9CRA for 20 h. Cells were incubated with 10 μg/ml ^{125}I-apoA-I for 1, 6, or 12 h in the presence or absence of 300 μM glyburide. Lipid-free ^{125}I-apoA-I was removed from the medium by ultrafiltration followed by dialysis, as described in Materials and Methods. Samples were separated by 2D-PAGGE, and ^{125}I-apoA-I was detected directly by autoradiography using Kodak XAR-2 film. Molecular size markers are shown.

beled with [^{14}C]cholesterol or [^{32}P]orthophosphate as described in Materials and Methods and then incubated with 10 μg/ml lipid-free apoA-I for 24 h at 37°C. Radiolabeled ABCA1 mutant Q597R cells were used as a control in the present experiment. The medium was concentrated with a centrifugal filter (MWCO 10,000), and then both [^{14}C]apoA-I- and [^{32}P]apoA-I-containing particles were separated by 2D-PAGGE. As shown in **Fig. 4**, the majority of ^{14}C- and ^{32}P-lipidated apoA-I colocalized with apoA-I-containing particles revealed with an anti-apoA-I antibody. At the same time, we have not been able to detect any α-LpA-I formation during incubation with ABCA1 mutant Q597R (Fig. 4). Interestingly, α-LpA-I subspecies with a molecular diameter of ~9.5 nm seem to contain only phospholipids.

Having determined that α-LpA-I subspecies contain cholesterol and phospholipids (Fig. 4), the question was raised whether α-LpA-I is a good substrate for LCAT. [^{14}C]cholesterol-labeled normal fibroblasts were stimulated with 22OH/9CRA and then incubated with 10 μg/ml ^{125}I-apoA-I for 24 h at 37°C. The medium was recovered and concentrated, and lipid-free apoA-I was removed using both filtration (MWCO 50,000) and dialysis (MWCO 50,000). At the same time, [^{125}I]apoA-I, POPC, and [^{14}C]cholesterol were used to form r(HDL) with a specific activity similar to that of α-LpA-I. Equivalent amounts of either α-LpA-I or r(HDL) were reacted with purified recombinant human LCAT as described in Materials and Methods. After lipid extraction, both CE and unesterified cholesterol were separated by TLC. The initial velocities shown in **Fig. 5** were estimated with less than ~15% substrate conversion to avoid conditions in which the substrate concentration would become rate-limiting. There is no significant difference between the V_{max} of the LCAT reaction between α-LpA-I and r(HDL) (8.1 vs. 8.5 nmol CE/h/μg LCAT, respectively). In contrast, estimation of a half-maximal velocity (K_m) from double-reciprocal plots of initial velocities as a function of substrate concentration for both α-LpA-I and r(HDL) showed that the apparent K_m was 2-fold higher for α-LpA-I than for r(HDL) (1.2 vs. 0.7 μM, respectively). Furthermore, the catalytic efficiency of LCAT (V_{max}/K_m) was 2-fold lower for α-LpA-I compared with r(HDL) (6.8 vs. 12.5 nmol CE/h/μM apoA-I). No significant esterification was detected with α-LpA-I mutant Δ122–165 compared with wild-type α-LpA-I (0.21 ± 0.1% vs. 10.5 ± 0.5%, respectively). In separate experiments, we found that incubation of apoA-I (Δ122–165) with stimulated normal fibroblasts generated α-LpA-I particles similar to those formed with wild-type apoA-I, which contained both [^{14}C]cholesterol and [^{32}P]phospholipids, as examined by 2D-PAGGE (data not shown). All data presented are representative of results from two or more independent experiments.

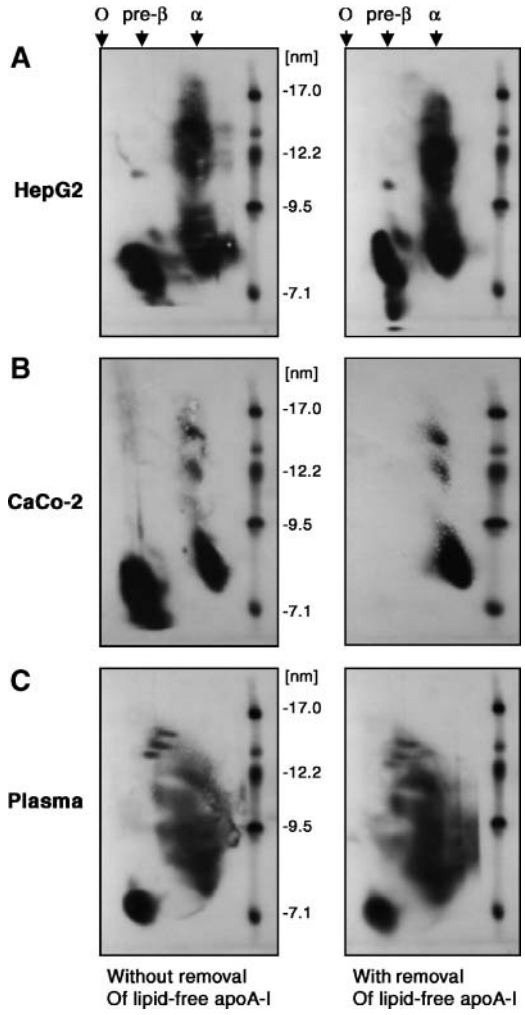


Fig. 3. Analysis of endogenously secreted LpA-I from HepG2 and CaCo-2 cells. HepG2 and CaCo-2 cells in 150 mm diameter dishes were loaded with 20 μg/ml cholesterol for 24 h and then stimulated with 22OH/9CRA for 20 h. Cells were incubated in DMEM alone for 24 h at 37°C. Lipid-free apoA-I was removed or not from the medium by ultrafiltration followed by dialysis, as described in Materials and Methods. Samples were separated by 2D-PAGGE, and apoA-I was detected by an anti-apoA-I antibody. A, B (left panels): apoA-I-containing particles secreted by either HepG2 or CaCo-2 cells without the removal of lipid-free apoA-I. A, B (right panels): apoA-I-containing particles secreted by either HepG2 or CaCo-2 cells after the removal of lipid-free apoA-I. C: Plasma apoA-I-containing particles before and after the removal of lipid-free apoA-I (left and right panels, respectively).

DISCUSSION

HDL is believed to be a potent physiological protective system against atherosclerotic vascular disease. A better understanding of the molecular basis for these protective mechanisms will be necessary for developing strategies for the prevention and treatment of atherosclerotic vascular disease. Although it has become generally accepted that this protective effect of HDL is attributable to its pivotal role in the RCT process, the structural basis for the biogenesis, speciation, and maturation of HDL particles remains complex and not well understood.

It is generally thought that the interaction of lipid-free apoA-I with phospholipids from cell membranes generates a particle with preβ mobility that releases cholesterol from cells (26, 27). Here, we present evidence that incubation of exogenously added lipid-free apoA-I with various cell lines, including human fibroblasts, CaCo-2, and CHO-ABCA1, generates only α-LpA-I. In contrast, lipid-free apoA-I was unable to form such particles in the presence of HUVEC and ABCA1 mutant Q597R cells (Fig. 1), consistent with previous studies showing that lipid-free apoA-I did not promote cholesterol efflux from endothelial cells in which the expression of ABCA1 protein is very low and seems not to be sensitive to treatment with cholesterol or 22-hydroxycholesterol (24, 28). Furthermore, the pivotal role of ABCA1 in the formation of α-LpA-I was supported by our results showing that glyburide, an inactivator of ABCA1 transporter activity, inhibited almost completely the formation of larger α-LpA-I particles in different cell types tested (Fig. 1, lower panels).

A major paradigm shift in the current concept of RCT comes from the proposition of Brewer and colleagues (12) that the liver is a major source of plasma HDL-cholesterol and that the level of expression of hepatic ABCA1 modulates intracellular cholesterol levels as well as plasma HDL-cholesterol concentrations. This concept is supported by our results showing that HepG2 cells incubated with exogenously added apoA-I generate both preβ$_1$-LpA-I and α-LpA-I (Figs. 1, 2). This result was further strengthened by our demonstration that endogenously secreted apoA-I-containing particles from stimulated HepG2 cells were associated with preβ$_1$-LpA-I and α-LpA-I with charge and size similar to native plasma preβ$_1$-LpA-I and α-LpA-I (Fig. 3). In contrast, CaCo-2 cells secreted only α-LpA-I. Thus, among various cell lines tested, hepatocytes and macrophages have the unique ability to form preβ$_1$-LpA-I. Although the structural requirements for the formation of preβ$_1$-LpA-I by hepatocytes and macrophages have not been determined, previous studies established that the lipid composition of preβ$_1$-LpA-I species as well as the conformation of apoA-I within these particles differ from those of spherical HDL (6, 29). Most importantly, the ABCA1 transporter seems to be not involved in the formation of preβ$_1$-LpA-I, consistent with our findings that *1*) glyburide did not significantly inhibit the formation of preβ$_1$-LpA-I in both HepG2 cells and macrophages (Figs. 1, 2, lower panels) and *2*) stimulation of HepG2 cells with 22OH/9CRA significantly increased the amount of endogenously secreted α-LpA-I, but not preβ$_1$-LpA-I, compared with unstimulated cells (data not shown). This is consistent with the observation that patients with homozygous TD do not have α-HDL in their plasma; rather, they only have preβ-migrating HDL (30, 31). This provides in vivo support for the concept that ABCA1 is not involved in the formation of preβ-migrating HDL.

It is possible that lipid-free apoA-I interacts with specific microdomains of plasma membranes or endosomal compartments present in HepG2 cells and macrophages but not other cells, which results in the unusual phospholipid composition of these particles, causing its preβ electro-

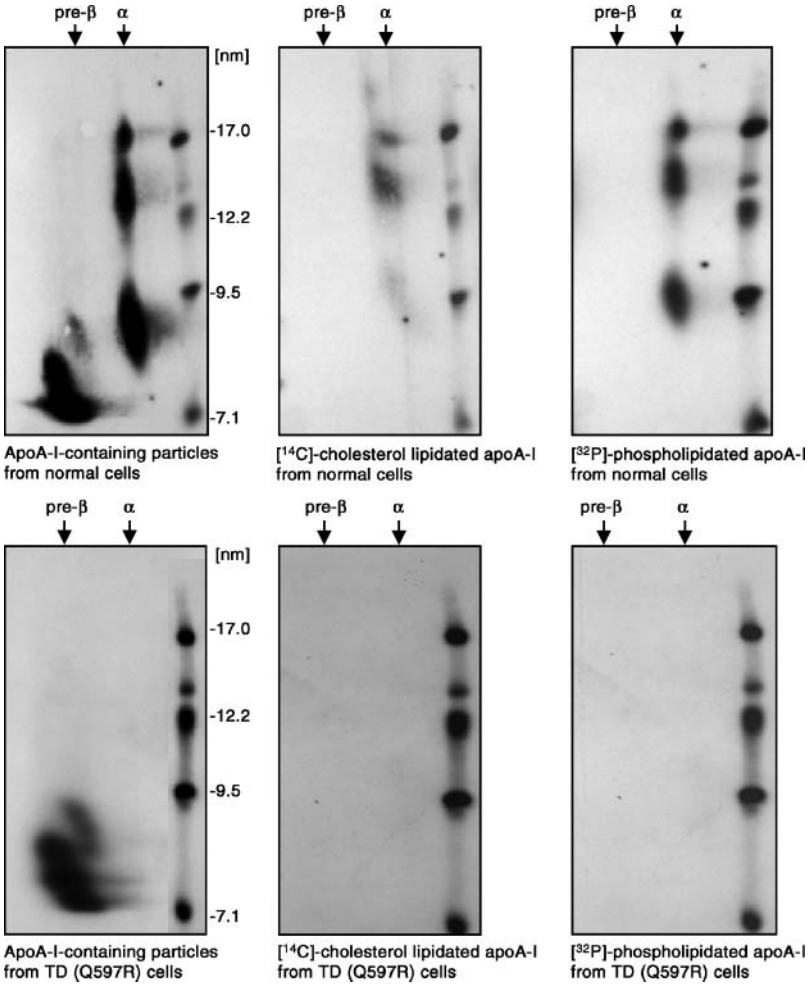
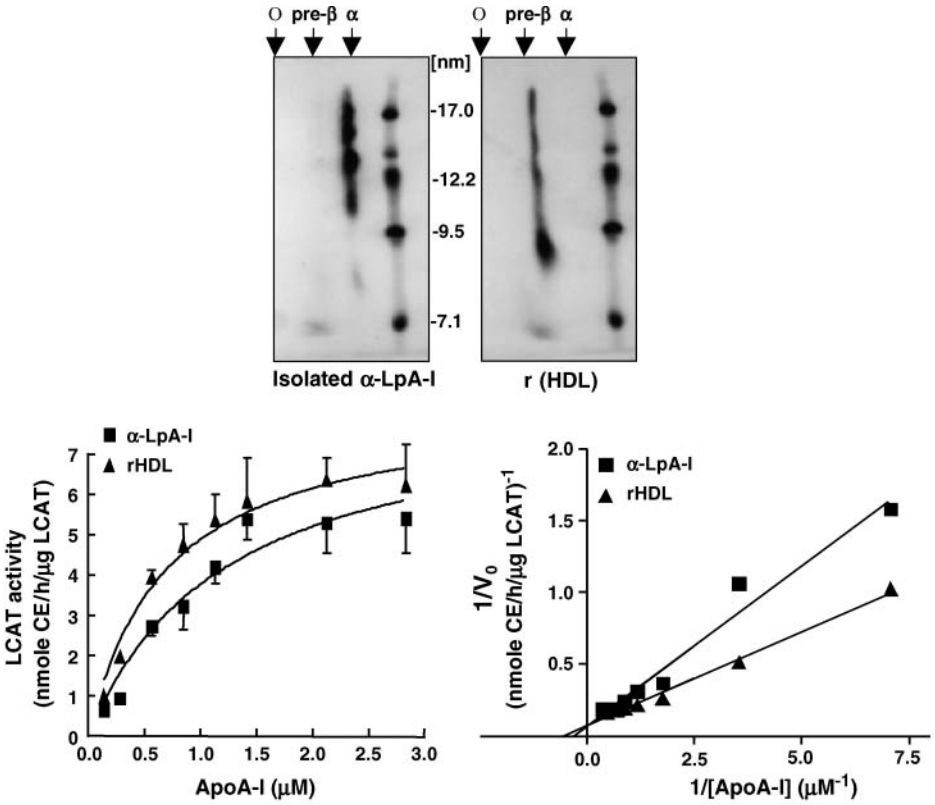


Fig. 4. Characterization of lipidated apoA-I-containing particles generated during lipid-free apoA-I incubation with normal fibroblasts and ABCA1 mutant (Q597R). Normal fibroblasts or ABCA1 mutant (Q597R) were labeled with either [^{14}C]cholesterol or [^{32}P]orthophosphate as described in Materials and Methods and incubated with 10 μg/ml apoA-I for 24 h. Media were recovered and concentrated with a size-exclusion filter with a MWCO of 10,000 (without the removal of lipid-free apoA-I). Samples were separated by 2D-PAGGE, and apoA-I was detected by an anti-apoA-I antibody. [^{14}C]cholesterol and [^{32}P]phospholipids associated with apoA-I-containing particles were detected directly by autoradiography using Kodak XAR-2 film. Molecular size markers are shown. TD, Tangier disease.

phoretic mobility. These conclusions are supported by a recent study by Marcel and colleagues (32) demonstrating that expression of ABCA1 in primary mouse hepatocytes is central to the lipidation of newly synthesized apoA-I; however, a significant basal level of apoA-I phospholipidation occurs in primary hepatocytes from ABCA1-deficient mice. Although the mechanism of the lipidation of newly synthesized apoA-I by ABCA1 transporter remains to be determined in hepatocytes, the present study shows that >60% of endogenously secreted apoA-I by HepG2 cells was associated with α-LpA-I (Fig. 3A); thus, it is more likely that these particles were formed by the translocase activity of ABCA1. This is consistent with a previous study by Parks and colleagues (33) demonstrating that ~20% of newly secreted apoA-I from HepG2 cells is lipidated intracellularly and another ~30% is minimally lipidated soon thereafter extracellularly.

Previous studies have suggested that preβ-HDL could be an α-HDL precursor (24, 26, 34); however the interrelationship between newly formed LpA-I particles in our cell culture model was unclear. It is possible that the interaction of lipid-free apoA-I with ABCA1 rapidly generates preβ-LpA-I that may be a precursor for α-LpA-I in our cell culture model. However, a time-course analysis showed that both preβ_1-LpA-I and α-LpA-I generated by HepG2 cells or α-LpA-I generated by fibroblasts appeared in the medium as soon as 1 h into the incubation period, without any important change in each subspecies over a 12 h incubation period (Fig. 2). Furthermore, the stability of the molecular diameter and charge of α-LpA-I and preβ_1-LpA-I subspecies over a 12 h incubation period did not support the existence of a clear precursor-product relationship between nascent LpA-I subspecies. This is also strongly supported by our results showing that glyburide did not affect the formation of preβ_1-LpA-I but had a dramatic effect on α-LpA-I, providing strong support for the



kinetic parameters	App V_{max}	App K_m	V_{max}/K_m
	nmol CE/h	μM	nmol CE/h/μM
α-LpA-I	8.1 ± 0.6	1.2 ± 0.3	6.8
r (HDL)	8.5 ± 1.8	0.7 ± 0.1	12.5

Fig. 5. LCAT-mediated cholesterol esterification of α-LpA-I labeled with cell-derived cholesterol. [^{14}C]cholesterol-labeled normal fibroblasts were stimulated with 22OH/9CRA and then incubated with 10 μg/ml ^{125}I-apoA-I for 24 h at 37°C. α-LpA-I were isolated as described in Materials and Methods. At the same time, ^{125}I-apoA-I, POPC, and [^{14}C]cholesterol were used to form reconstituted HDL particles [r(HDL)] with similar specific activity to α-LpA-I. A 2D-PAGGE separation of isolated α-LpA-I and r(HDL) is shown in the upper panels. Equivalent amounts of either LpA-I or r(HDL) were reacted with 100 ng of purified recombinant human LCAT for 1 h at 37°C. After lipid extraction, both cholesteryl ester (CE) and unesterified cholesterol were separated by TLC. Plotted values are means ± SD of triplicate measures. Reciprocals of initial velocities are plotted against the reciprocals of apoA-I concentrations. ApoA-I concentrations of LpA-I and r(HDL) were estimated from the initial specific activity of ^{125}I-apoA-I. Kinetic data were obtained using GraphPad Prism 4.0 software.

concept that preβ$_1$-LpA-I and α-LpA-I have different origins.

Previous work by Forte and others has demonstrated that apoA-I incubated with cells including CHO cells (35, 36), fibroblasts (37), and macrophages (26, 38) was able to recruit phospholipid and cholesterol from the cells to form protein-lipid complexes with distinct particle diameters. We reported here that the newly formed α-LpA-I in different cell lines had distinctly different sizes (Figs. 1, 2), suggesting that the heterogeneity of those particles may be attributable to the phospholipid-cholesterol ratio and/or the number of apoA-I molecules within each subspecies. This is consistent with our finding that α-LpA-I subspecies generated by fibroblasts having a molecular diameter of ~9.5 nm contained only phospholipids (Fig. 4). Although the structural characteristics that cause the α electrophoretic mobility of nascent LpA-I has not been determined, we have suggested previously that the high content in phosphatidylinositol (16) or the high number of apoA-I molecules per particle (20) may increase the net negative charge of nascent LpA-I and consequently cause their α electrophoretic mobility. Moreover, because of the absence of LCAT activity in the medium to convert unesterified cholesterol to CE, especially for α-LpA-I generated by normal fibroblasts, it is most likely that α-LpA-I particles are discoidal. However, our results contradict the traditional concept that the α electrophoretic mobility of HDL is attributable to the LCAT reaction (24, 29, 39).

Although the origin of plasma preβ$_1$-LpA-I has remained enigmatic and its role in preventing atherosclerosis is con-

sidered uncertain at this time (39–41), a recent study by Parks and colleagues (42) documented that preβ-LpA-I injected into human apoA-I transgenic mice has two metabolic fates: rapid removal from plasma and catabolism by kidney or remodeling to medium-sized HDL, which occurs independently of both LCAT and ABCA1. Furthermore, it is well accepted that remodeling of mature HDL by several enzymes, such as hepatic lipase, CE transfer protein, and phospholipid transfer protein, generates $preβ_1$-LpA-I species (3, 39). We are currently investigating the phospholipid composition and metabolic fates of $preβ_1$-LpA-I species generated by hepatocytes and macrophages.

Although the structural features of nascent LpA-I required to form mature HDL are as yet unknown, we have obtained evidence that α-LpA-I had distinctly different sizes: the larger particles contained both phospholipids and cholesterol, whereas the smallest particles contained only phospholipids and apoA-I (Fig. 4). Most importantly, kinetic determinations in this study have shown that the maximum velocities of cholesterol esterification by LCAT are similar for α-LpA-I subspecies and r(HDL) (Fig. 5). In contrast, LCAT was found to have a 2-fold greater catalytic efficiency (V_{max}/K_m) for r(HDL) compared with α-LpA-I. This suggests that the sensitivity that LCAT displays to α-LpA-I cholesterol content is likely physiologically relevant to the in vivo maturation of these nascent particles. It is possible that LCAT activity was affected by factors such as phospholipid composition, apoA-I conformation, size, and surface charge of α-LpA-I. Indeed, it was documented that sphingomyelin molecules inhibited the unfolding of apoA-I in discoidal and spherical r(HDL) and impaired the LCAT reaction (43), consistent with our previous results showing that a significant amount of sphingomyelin was found in α-LpA-I generated by stimulated normal fibroblasts (16). Interestingly, we found that secretory sphingomyelinase was able to hydrolyze [^{3}H]sphingomyelin-labeled α-LpA-I. This suggests that both secretory sphingomyelinase and LCAT may act in concert for an efficient maturation of nascent LpA-I particles (M. Marcil, personal communication).

Although the lipid composition and metabolic fates of nascent LpA-I species remain to be determined, the present study has provided evidence for a new link between specific cell lines and the speciation of nascent HDL that occurs by both ABCA1-dependent and -independent pathways.

The authors thank Dr. John S. Parks and Dr. Yves L. Marcel for kindly providing human recombinant lecithin:cholesterol acyltransferase and apoA-I (Δ122–165). The helpful advice of Dr. Marcel is also gratefully acknowledged. This work was supported by Grant MOP 15042 from the Canadian Institutes of Health Research and by the Heart and Stroke Foundation of Canada. J.G. holds the McGill University-Novartis Chair in Cardiology.

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