ApoA-I secretion from HepG2 cells: evidence for the secretion of both lipid-poor apoA-I and intracellularly assembled nascent HDL

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Abstract The goal of this study was to determine whether apolipoprotein A-I (apoA-I) is lipidated before secretion by HepG2 cells. ApoA-I was extracted from microsomes after radiolabeling with [^{35}S]Met/Cys. After ultracentrifugal flotation, d $<$ 1.25 g/ml and d $>$ 1.25 g/ml fractions were immunoprecipitated and analyzed by SDS-PAGE. Under steady state radiolabeling conditions, 20% of extracted microsomal apoA-I floated at d $<$ 1.25 g/ml. Pulse-chase experiments demonstrated that the percentage of microsomal apoA-I associated with lipid peaked between 2 and 8 min postsynthesis. Density gradient ultracentrifugation, and nondenaturing gradient gel electrophoresis of HepG2 cell medium, indicated that 50% of secretory apoA-I existed as small HDL particles with a diameter of ~7.5 nm. These and additional data suggested that ~20% of newly secreted apoA-I is lipidated intracellularly and another 30% is secreted in lipid-free or lipid-poor form but acquires sufficient lipid to become small HDL within 1 h of secretion, with little further maturation over the time course of the incubation (2 h). These results indicate that a process exists for the presecretory intracellular assembly of apoA-I with lipid in HepG2 cells and that apoA-I is secreted in both lipid-poor and lipidated forms.—Chisholm, J. W., E. R. Burleson, G. S. Shelness, and J. S. Parks. **ApoA-I secretion from HepG2 cells: evidence for the secretion of both lipid-poor apoA-I and intracellularly assembled nascent HDL. *J. Lipid Res.* 2002.** 43: **36–44.**

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Apolipoprotein A-I (apoA-I) is a 28-kDa amphipathic plasma protein secreted by both the liver and intestine, and is the primary protein component of HDL (1, 2). HDL functions in the transport of cholesterol from extrahepatic tissues back to the liver through the reverse cholesterol transport pathway (3–6). Increased plasma HDL levels may reflect increased plasma reverse cholesterol transport conferring resistance to cholesterol deposition in extrahepatic tissues. This concept is consistent with studies demonstrating that plasma HDL levels are inversely correlated with the risk of developing atherosclerosis (7–9). Although considerable research has been directed toward understanding HDL metabolism, it currently remains unclear how HDL are assembled. Two possibilities have been proposed to explain the assembly of HDL particles: intracellular lipidation of apoA-I and extracellular lipidation after apoA-I secretion from either the liver or intestine. In the intestine, apoA-I appears to be secreted on chylomicron particles and, after lipolysis of core triacylglycerol, apoA-I and redundant surface lipid are either moved onto existing HDL and/or create new nascent HDL (10). In contrast, mammalian liver apoA-I is not secreted on triglyceride-rich apoB-containing lipoproteins and the extent of lipidation of hepatic apoA-I is unknown.

ApoA-I has been shown to undergo extracellular assembly with lipid when incubated with cells in culture (11, 12). This appears to occur through apoA-I interaction with the cell surface (13–16) and the ATP-binding cassette A1 (ABCA1) transporter (17–20). Gillotte et al. (15) have demonstrated in fibroblasts that the extracellular lipidation of apoA-I through cell surface interactions is rapid, but of limited capacity, suggesting perhaps that lipid availability or some other factor, such as ABCA1 expression (21), is important in determining the extracellular assembly of apoA-I with lipid.

Several studies using chicken (22, 23) and rat hepatocytes (24) have provided evidence for the secretion of intracellularly lipidated apoA-I [reviewed in ref. (25)]. Howell and Palade (24), using the HDL density fraction of rat liver Golgi extracts, found both HDL-sized particles and apoA-I, raising the possibility that apoA-I was assembled with lipid inside the cell and secreted as nascent HDL. In contrast, Hamilton, Moorehouse, and Havel (26) were un-

Abbreviation: apoA-I, apolipoprotein A-I.

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able to visualize discoidal particles that resembled nascent HDL using highly purified rat Golgi fractions and concluded that intracellular HDL does not exist. The reason for these opposing results remains unclear. Banerjee and Redman (22, 23) have performed more detailed studies of the intracellular lipidation of apoA-I, using chicken hepatocytes. They characterized a heterogeneous population of phospholipid-rich apoA-I-containing nascent HDL (22) that, on the basis of lipid radiolabeling studies, was assembled in the Golgi apparatus (23). However, the relevance of chicken hepatocytes to the study of mammalian apoA-I secretion and lipidation remains unclear, as chicken apoA-I, unlike mammalian apoA-I, has been shown to have a broad tissue distribution and is often found in circulation associated with apoB-containing lipoprotein particles. Because chickens lack apoE, it has been suggested that chicken apoA-I may function more like mammalian apoE (27).

Clearly, strong evidence demonstrating the intracellular assembly of mammalian apoA-I with lipid is lacking. The following studies, using HepG2 cells, were designed to investigate whether and to what extent mammalian apoA-I is associated with lipid inside the cell. The results of these studies suggest that apoA-I is secreted by the liver in both lipid-poor form and as nascent HDL.

MATERIALS AND METHODS

Cell culture

HepG2 cells were maintained in MEM (Mediatech, Ormond Beach, FL) that was supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml)-streptomycin (100 μg/ml), and 2× vitamins (Mediatech). For all experiments, cells were split into 150-mm dishes and experiments were initiated once the cells were near confluence. Cell monolayers were then washed three times with PBS (Mediatech) and used as indicated in the figure legends.

Metabolic radiolabeling

For steady state radiolabeling of intracellular apoA-I, washed cell monolayers were incubated for 1–2 h in 5 ml of Met/Cys-free MEM (ICN, Costa Mesa, CA) that was supplemented with [^{35}S]Met/Cys (0.1 mCi/ml) (EasyTag Express; New England Nuclear, Boston, MA). For pulse-chase experiments, intracellular Met and Cys pools were depleted by incubation in serum-free, Met/Cys-free medium for 20–30 min. The medium was then removed and 5 ml of radiolabeling medium containing radiolabel at 0.1 mCi/ml was added for a pulse of 2–10 min. After the pulse period, 5 ml of serum-free medium containing 1 mM Cys and 2.5 mM Met (chase medium) was added to the radiolabeling medium. In some experiments cycloheximide (Sigma, St. Louis, MO), a protein synthesis inhibitor, was included at a final concentration of 0.1 mM. The medium was then removed and replaced with 5 ml of fresh medium containing 1 mM Cys, 2.5 mM Met, and in some experiments 0.1 mM cycloheximide. All chase periods were stopped by placing the culture dishes on ice. For pulse-only samples, radiolabeling was stopped by adding ice-cold chase medium and placing the culture dishes on ice. For experiments in which lipid-poor apoA-I was added, the source of apoA-I was either delipidated (28) [^{35}S]Met/Cys-radiolabeled HepG2 cell medium (6 h of labeling in serum-free medium) or delipidated (28) ^{125}I-radiolabeled (29, 30) human plasma apoA-I (31, 32) that had been denatured by chemical (16) (6 M guanidine-HCl denaturation followed by refolding) or thermal (33) (60°C for 30 min) methods. Lipid-free human plasma apoA-I had less than one molecule of phospholipid per molecule of apoA-I as determined by phosphorus assay (34) of 1 mg of purified apoA-I.

Microsome preparation

Microsomes were prepared by a modification of the method described by Ingram and Shelness (35). The medium was removed from plates and the cells were washed with 5 ml of ice-cold PBS. The cells were recovered by scraping into a 15-ml centrifuge tube in a volume of 5 ml of ice-cold PBS. Cells were pelleted at 1,500 *g* for 10 min and the supernatant was removed by aspiration. The cells were gently resuspended in 1 ml of hypotonic buffer (10 mM HEPES, pH 7.4), containing protease inhibitors [PI; 2× complete EDTA-free PI cocktail (Roche, Nutley, NJ) supplemented with pepstatin (2 μg/ml; Sigma) and 0.1% EDTA]. Cells were pelleted again by low speed centrifugation at 1,500 *g* for 10 min and then resuspended in 1 ml of hypotonic buffer containing PI and incubated on ice for 15 min. The cells were then transferred to a 2-ml Dounce homogenizer and homogenized by 25 strokes, using the tight pestle. The homogenate was adjusted to 0.25 M sucrose and centrifuged for 10 min at 1,000 *g*. The supernatant was removed and centrifuged for an additional 10 min before being transferred to a thick-walled polycarbonate ultracentrifuge tube. Samples were centrifuged in a TL-100 centrifuge at 70,000 rpm for 15 min at 4°C, using a TLA 100.3 rotor (Beckman-Coulter, Fullerton, CA). After ultracentrifugation, the supernatant was aspirated and discarded and the microsomal pellet was used for carbonate extraction experiments.

Carbonate extraction

Microsomes were washed with 1 ml of ice-cold membrane buffer [10 mM HEPES (pH 7.4), 250 mM sucrose, 10 mM NaCl, 10 mM KCl, and PI], and the microsomes were resuspended in 300 μl of membrane buffer and transferred to a Dounce homogenizer. Thirty-three microliters of 1 M sodium carbonate, pH 11.5, was added and the microsomes were resuspended by 12 strokes of the Dounce homogenizer and placed in a thick-walled polycarbonate centrifuge tube. The Dounce homogenizer was washed with 1.33 ml of 0.1 M sodium carbonate, pH 11.5, and the wash was added to the resuspended microsomes. After a 1-h incubation on ice, the extracted membranes were separated from the microsomal lumenal contents by ultracentrifugation in a TL-100 centrifuge, using a TLA-100.3 rotor, for 25 min at 80,000 rpm at 4°C. The supernatant was removed and adjusted to 0.5% phenol red, 0.2 M NaCl, 0.05 M Tris, and 0.25% fatty acid-free BSA (Sigma). The final pH was adjusted to pH 7.4 with 20% HCl.

Ultracentrifugation

Media or microsomal carbonate extracts were adjusted to d = 1.25 g/ml with solid KBr, transferred to quick-seal tubes, and floated by centrifugation in a TL-100 (TLA-100.3 rotor, 18 h, 80,000 rpm, 15°C). Samples floated by ultracentrifugation were recovered by tube cutting into ~1.0-ml tops and ~2.5-ml bottoms. In control experiments using either delipidated ^{35}S- or ^{125}I-radiolabeled apoA-I, 11.9 ± 1.1% (n = 6) of the apoA-I was found in the top fraction. This was the background for the assay, because a second ultracentrifugation spin of the bottom fraction resulted in a similar recovery of 12% in the top fraction. Because our data demonstrated that lipid-free apoA-I had less than one molecule of phospholipid per molecule of apoA-I and the recovery of lipid-free apoA-I in the top fraction was constant in all experiments, we subtracted this background from the percentage

of apoA-I floated in the d < 1.25 g/ml top fraction. In some experiments, samples were adjusted to d = 1.21 g/ml and ultracentrifuged in a density gradient using an SW-41 rotor (Beckman-Coulter) for 44 h at 40,000 rpm and 15°C (36). In one experiment the density gradient was modified (7-ml sample at d = 1.25 g/ml, 2-ml sample at d = 1.20 g/ml, and 3-ml sample at d = 1.05 g/ml) to increase the separation of particles in the density range of HDL. Gradients were recovered in 1-ml fractions, using a manual digital pipette (Oxford) or an automated gradient fractionator (Auto Densi-Flow; Labconco, Kansas City, MO). The density of each fraction was obtained by weighing known volumes of the fractions recovered from blank gradients. Immunoprecipitations were performed on samples after buffer exchanging the samples three times into TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.4), using a Centricon-10 centrifugal concentrator (Millipore, Bedford, MA), or in the case of density gradient fractions after diluting 4-fold with deionized H_2O. Samples were immunoprecipitated overnight at 4°C in TBS buffer containing 1% Triton X-100, 0.5% BSA, and 2× PI, using 5 μl of either polyclonal anti-human apoA-I (Roche) or monoclonal anti-human apoA-I antibody (37). Antibody-antigen complexes were recovered by adding 50 μl of protein G-Sepharose (50%, v/v) (Amersham-Pharmacia Biotech, Piscataway, NJ) and incubating for 2 h at 4°C. The protein G-Sepharose was pelleted in a microcentrifuge and washed three times with TBS containing 1% Triton X-100. Pellets were then boiled for 10 min in SDS-PAGE sample buffer and fractionated by 15% SDS-PAGE.

SDS-PAGE and autoradiography

SDS-PAGE analysis was performed with Tris-glycine gels in a Bio-Rad (Hercules, CA) PROTEAN II system as previously described (38). Gels containing radiolabeled samples were dried and exposed to film at −70°C, using a Kodak (Rochester, NY) Transcreen-LE and either Biomax MS or MR film.

Western blots

Samples and high molecular weight standards (Amersham-Pharmacia Biotech) were electrophoresed (1,400 V·h, 10°C), using a 4–30% nondenaturing gradient gel (39). Samples were transferred from gels to nitrocellulose (0.2 μm for 7 h at 35 V, 4°C) and incubated sequentially with anti-human apoA-I (diluted 1:1,000; Roche) and anti-sheep IgG-horseradish peroxidase conjugate (diluted 1:20,000; Sigma). Bands were visualized by chemiluminescence (Pierce, Rockford, IL) and captured on film. In some experiments, the nitrocellulose blots were developed with anti-goat IgG conjugated with alkaline phosphatase (diluted 1:2,000; Vector, Burlingame, CA) and NBT/BCIP reagent (Promega, Madison, WI).

Data analysis

Quantitation of sample bands after autoradiography was accomplished by scanning the film with a UMAX (Fremont, CA) Super-Vista S-12 scanner equipped with a transparency adapter. Images were imported into Scion Image (Scion, Frederick, MD) and densities were obtained from the grayscale image after calibrating the scanner with a density step table (Kodak). To validate the densitometry procedure, ^{125}I-labeled apoA-I or ^{35}S-labeled apoA-I bands were excised from the dried gels after autoradiography and counted either in a γ counter (^{125}I) or in a scintillation counter (^{35}S) after digestion with 30% hydrogen peroxide and addition of liquid scintillation cocktail. The correlation between densitometry and counting of the excised bands was r^2 = 0.97 ± 0.02 (n = 4 separate experiments). Statistical differences were determined by ANOVA followed by a Bonferroni/Dunn post hoc comparison to identify significant differences between individual time points.

RESULTS

Experiments were undertaken to examine the secretion and lipidation of apoA-I in HepG2 cells. Results shown in **Fig. 1** demonstrate that after a 10-min pulse, apoA-I secretion was linear between 15 and 60 min. Immunoprecipitation of apoA-I from the top (d < 1.25 g/ml) and bottom (d > 1.25 g/ml) fractions of chase medium subjected to ultracentrifugation demonstrated that ~50% of the radiolabeled apoA-I was associated with sufficient lipid to float (lipidated apoA-I) by 120 min (**Fig. 2**). Because ~25% of secreted apoA-I was lipidated at the earliest time point sampled (Fig. 2; 15 min), we hypothesized that some of the lipidation of apoA-I occurred in HepG2 cells before secretion and that the difference in lipidation between the early time points and 120 min was due to an extracellular lipidation process.

To specifically assess the extent to which extracellular lipidation of apoA-I is responsible for HDL present in HepG2 cell medium, 1.5 μg of ^{35}S-labeled apoA-I from delipidated HepG2 cell medium or 1 μg of lipid-free ^{125}I-labeled human plasma apoA-I was incubated with HepG2 cells for the times indicated in **Fig. 3**. The quantity of lipid-free apoA-I added to medium was in the range of that secreted by HepG2 cells during a 1-h incubation, as determined by apoA-I enzyme-linked immunosorbent assay (40) (0.4–1.5 μg of apoA-I per 150-mm dish per h). After adjusting for the small background of apoA-I that floated in the absence of cells (see Materials and Methods), ~20% of the exogenous apoA-I acquired sufficient lipid to float at d < 1.25 g/ml by 120 min (Fig. 3). The lipidation event appeared to become saturated by 30 min, indicating that lipid or some other factor might be limiting. By contrast, at 120 min ~50% of newly secreted apoA-I

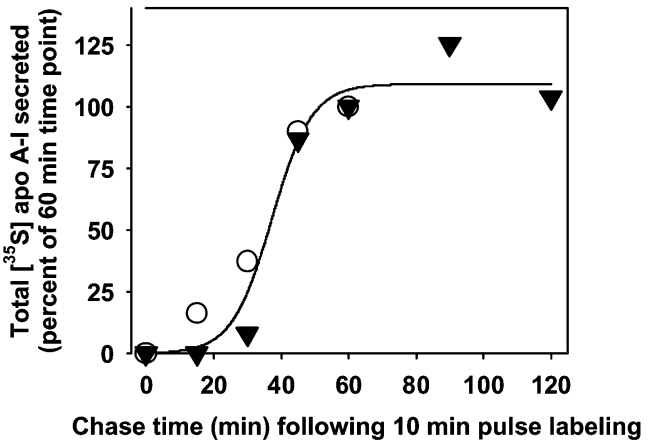

Fig. 1. Pulse-chase kinetic analysis of apoA-I secretion by HepG2 cells. HepG2 cells were pulse radiolabeled for 10 min and chased for the indicated times. Medium was ultracentrifuged at d = 1.25 g/ml and apoA-I in the top and bottom fractions was immunoprecipitated and analyzed by 15% SDS-PAGE and fluorography as described in Materials and Methods. The total apoA-I in medium during the chase at each time point was determined by densitometric analysis of the film. Each symbol type indicates an independent experiment. (Data are expressed as the percentage of the total radiolabeled apoA-I secreted at the 60-min time point.)

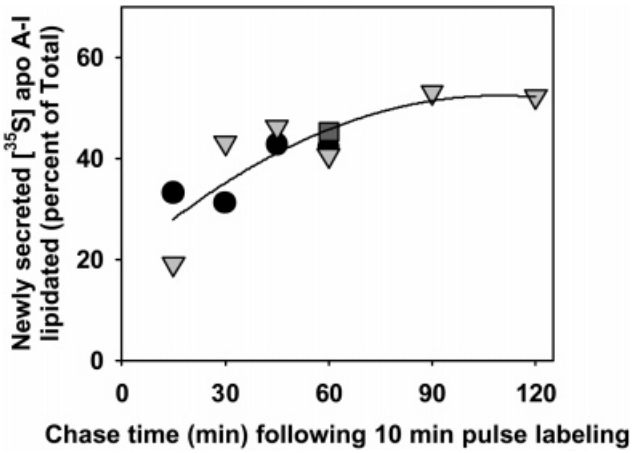

Fig. 2. Lipidation state of apoA-I secreted by HepG2 cells. HepG2 cells were pulse radiolabeled for 10 min and chased for the indicated times. Medium was ultracentrifuged at d = 1.25 g/ml and apoA-I in the top and bottom fractions was immunoprecipitated and analyzed by 15% SDS-PAGE and fluorography. The data are shown as the percentage of HepG2 cell medium apoA-I lipidated (i.e., percentage in d < 1.25 g/ml top fraction) during the chase as determined by densitometric analysis of the film. Different symbols indicate results from independent experiments. All data have been corrected for background in the separation method as described in Materials and Methods.

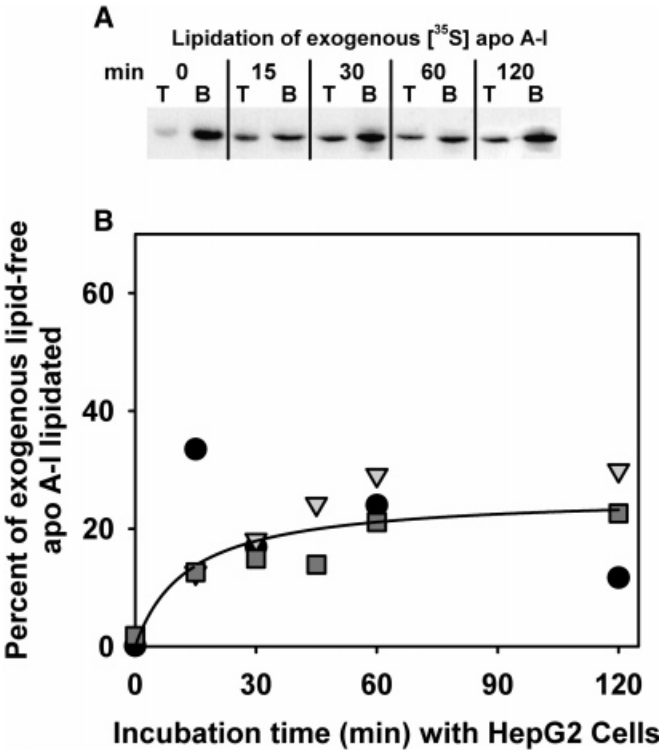

Fig. 3. Lipidation by HepG2 cells of exogenously added ^{35}S- or ^{125}I-labeled lipid-free apoA-I. HepG2 cells were incubated with ethanol-ether delipidated medium containing ^{35}S-radiolabeled apoA-I (~1.5 μg) or human plasma lipid-free ^{125}I-apoA-I (1 μg) for the indicated times and the proportion lipidated was determined as described in Fig. 2. A: A representative film showing the immunoprecipitated apoA-I recovered in the top (T) and bottom (B) at the indicated time points. B: Densitometric quantitation of apoA-I lipidation. Different symbols indicate results from independent experiments; circles represent data derived using ^{35}S-radiolabeled apoA-I, whereas triangles and squares represent data derived using human plasma lipid-free ^{125}I-apoA-I. All data have been corrected for background in the separation method as described in Materials and Methods.

was lipidated (Fig. 2), indicating that the lipidation of endogenous, newly secreted apoA-I exceeded that of lipid-free apoA-I added to the medium exogenously (Fig. 3). The difference (~20–30% depending on the time point) in the relative lipidation of exogenous and endogenous apoA-I was attributed to the presence of an intracellular lipidation mechanism.

To further explore the lipidation state of newly secreted apoA-I, we analyzed medium obtained from HepG2 cells after a 2-h incubation as well as lipid-free apoA-I by equilibrium density gradient ultracentrifugation and size fractionation on 4–30% nondenaturing gradient gels. **Figure 4A** shows a density gradient profile of lipid-free ^{125}I-apoA-I. This preparation of apoA-I had less than one molecule of phospholipid per molecule of apoA-I, on the basis of chemical assay. The lipid-free apoA-I was distributed in the bottom four fractions of the density gradient (d > 1.223 g/ml), as expected.

Figure 4B shows the results of nondenaturing gradient gel electrophoresis of HepG2 cell medium. In lane 1, HepG2 medium demonstrated two size ranges of immunoreactive apoA-I particles, one in the size range of albumin (7.1 nm in diameter) and another above the 7.1-nm marker. The latter size range corresponds to that of small HDL particles in plasma that contain two molecules of apoA-I per particle (41). To characterize the nature of the 7.1-nm form of apoA-I present in HepG2 cell medium, lipid-free apoA-I was analyzed. As observed in lane 2 (Fig. 4B), lipid-free apoA-I shows two bands, which collapse to one band after thermal denaturation (60°C for 30 min) (Fig. 4B, lane 3). We hypothesize that the slower migrating apoA-I in lane 2 (Fig. 4B) represents aggregates, perhaps dimers, of apoA-I that are disrupted by thermal denaturation, whereas the lower band represents monomeric lipid-free apoA-I. The slower migrating (upper) band in lane 2 (Fig. 4B) cannot represent lipidated apoA-I, because chemically the preparation had less than one molecule of phospholipid per molecule of apoA-I and had a density >1.223 g/ml (Fig. 4A). Lanes 4 and 5 of Fig. 4B show the size distribution of top and bottom fractions, respectively, of HepG2 medium ultracentrifuged at d = 1.25 g/ml. The top fraction (d < 1.25 g/ml, lane 4; Fig. 4B) demonstrated an apoA-I band similar in size to that of the slow-migrating band in whole medium (lane 1, Fig. 4B), whereas the bottom fraction (d > 1.25 g/ml, lane 5; Fig. 4B) contained a band similar in size to the faster migrating band in whole medium. These results suggested that the d < 1.25 g/ml top fraction represents small HDL particles, whereas the d > 1.25 g/ml bottom fraction contains lipid-free or lipid-poor apoA-I. The difference in migration between the 7.1-nm band in HepG2 cell medium (lane 1, Fig. 4B) versus delipidated plasma apoA-I (lanes 2

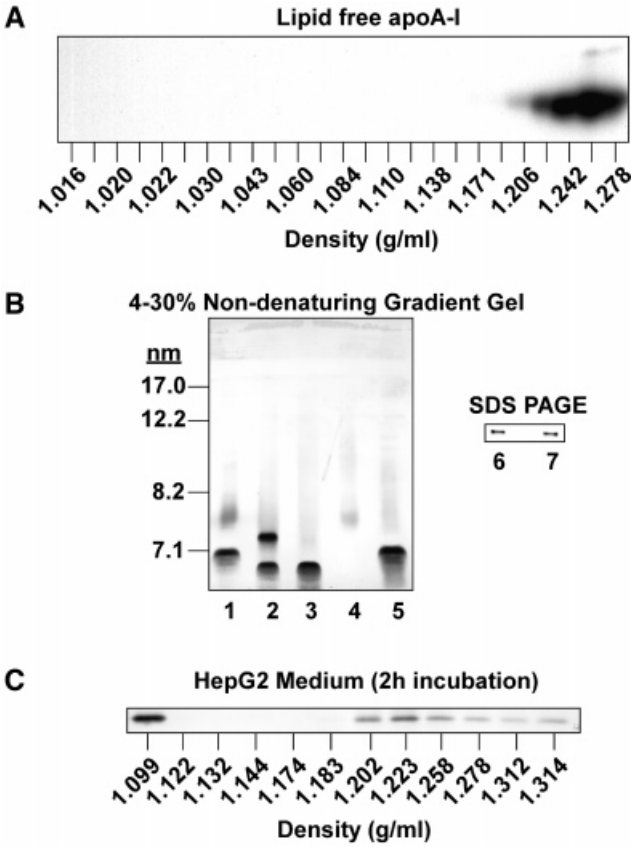

Fig. 4. Density and size distribution of apoA-I from HepG2 cell medium and lipid-free human plasma apoA-I. A: Lipid-free human plasma ^{125}I-apoA-I (2 μg) was fractionated, using a density gradient as described in Materials and Methods, and 0.5-ml fractions were collected. The fractions were diluted to 2 ml with deionized water and immunoprecipitated with anti-apoA-I antiserum. Samples were then analyzed by 15% SDS-PAGE and fluorography. B: 4–30% nondenaturing gradient gel electrophoresis of HepG2 cell medium and lipid-free human plasma apoA-I. Medium was collected after a 2-h incubation with HepG2 cells and concentrated before electrophoresis (lane 1) or fractionated by ultracentrifugation into top ($d < 1.25$ g/ml; lane 4) or bottom ($d > 1.25$ g/ml; lane 5) fractions. An aliquot of the concentrated top and bottom fractions was analyzed on 15% SDS-polyacrylamide gels (lanes 6 and 7, respectively). Lipid-free apoA-I (500 ng per lane) was analyzed before (lane 2) and after (lane 3) thermal denaturation (60°C for 30 min). C: Density distribution of medium apoA-I after a 2-h incubation with HepG2 cells. HepG2 cell incubation was performed as described in the caption to Fig. 2. The medium was fractionated on a modified Chapman gradient (36). One-milliliter fractions were collected from the gradient, buffer was exchanged, and fractions were concentrated before analysis by 15% SDS-PAGE and apoA-I Western blot.

and 3, Fig. 4B) may be due to differences in protein conformation. Alternatively, a small amount of lipid may be associated with the $d > 1.25$ g/ml apoA-I from HepG2 cell medium.

In contrast to density gradient ultracentrifugation results, which indicated that 50% of newly secreted apoA-I contained sufficient lipid to float at $d < 1.25$ g/ml, nondenaturing gradient gel electrophoresis indicated that only ~20% of apoA-I formed small HDL. To determine whether there was a difference in the ability to detect apoA-I in top and bottom fractions when using nondenaturing gradient gels compared with ultracentrifugation, we took aliquots of these fractions and analyzed them by SDS-PAGE (Fig. 4B). Lanes 6 (top fraction) and 7 (bottom fraction) contained similar amounts of apoA-I, in agreement with the data in Fig. 2, and supporting the conclusion that in the nondenaturing gradient gels, the apoA-I in small HDL particles is less immunoreactive or is transferred less readily to nitrocellulose than the lipid-free or lipid-poor apoA-I.

To further define density distribution of the apoA-I in HepG2 medium, we used a modified equilibrium density gradient designed to increase the separation of particles in the HDL density range. Figure 4C shows the results obtained for HepG2 cell medium, which demonstrated a bimodal distribution of apoA-I. Approximately 50% of the apoA-I was in the top fraction, which had a density of 1.099 g/ml or less, and the other half was distributed in the bottom half of the gradient from $d = 1.202$ g/ml to $d = 1.314$ g/ml. These experimental results suggested that half of the apoA-I in medium after 2 h of incubation had sufficient lipid to float in the $d < 1.21$ g/ml density gradient and agreed with results in Figs. 2 and 4B.

The preceding data are compatible with the hypothesis that some apoA-I was secreted from HepG2 cells in a lipidated form. Therefore, studies were undertaken to directly demonstrate the presence of intracellular lipidated apoA-I. HepG2 cell monolayers were radiolabeled with [^{35}S]Met/Cys for 1–2 h. Radiolabeled cell pellets were homogenized and microsomes were prepared and extracted with sodium carbonate, pH 11.5. The carbonate extracts were adjusted to $d = 1.25$ g/ml with KBr and subjected to ultracentrifugation. Immunoprecipitation of apoA-I in the top and bottom fractions revealed that approximately 27% ($n = 10$) of the recovered apoA-I was found in the $d < 1.25$ g/ml top fraction (**Fig. 5**, Endogenous). To assess whether any of the lipidated apoA-I was formed nonspecifically during the extraction procedure, delipidated ^{35}S-radiolabeled conditioned HepG2 cell medium or lipid-free ^{125}I-apoA-I was added to carbonate extraction buffer (Fig. 5, Control A) or carbonate extraction buffer containing nonradiolabeled HepG2 cell microsomes (Fig. 5, Control B). In contrast to the endogenous apoA-I that was intracellularly lipidated, only 8% ($n = 3$) of the exogenously added apoA-I was found to undergo nonspecific lipidation on incubation with unlabeled microsomes. This suggests that 19 ± 5% of carbonate-extractable lipidated apoA-I was formed by an intracellular lipidation mechanism, in agreement with data presented in Figs. 2 and 3.

To probe for possible contamination of the carbonate extracts by secreted apoA-I, delipidated ^{35}S-radiolabeled conditioned HepG2 cell medium was added to the cell pellet of nonradiolabeled HepG2 cells. After the cell wash, microsome preparation, and carbonate extraction procedure, none of the exogenously added apoA-I was detected in either the top or bottom ultracentrifuge fraction (data not shown).

To further characterize the intracellular lipidation of

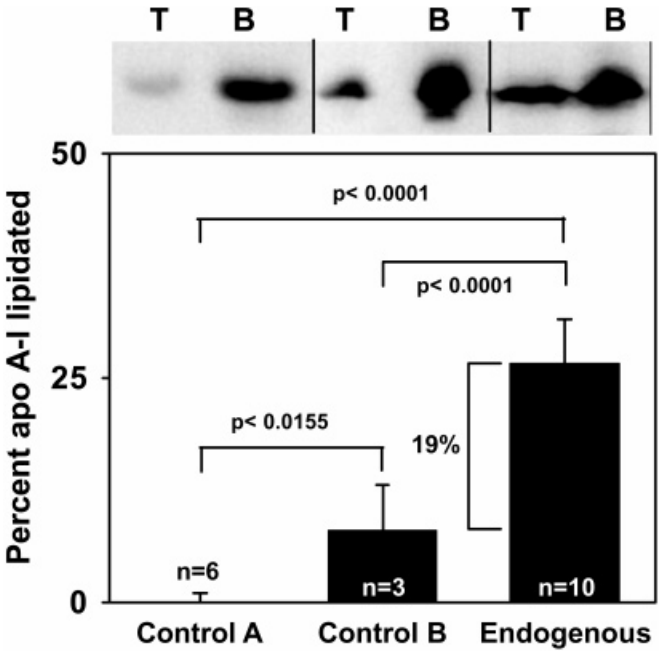

Fig. 5. Intracellular lipidation of HepG2 cell apoA-I. Delipidated medium containing ~2.5 μg of ^{35}S-labeled apoA-I or ~1 μg of ^{125}I-radiolabeled lipid-free human plasma apoA-I was added to either carbonate extraction buffer (Control A) or to nonradiolabeled microsomes during the carbonate extraction procedure (Control B). For analysis of apoA-I intracellular lipidation (Endogenous), HepG2 cells were labeled for 2 h and microsomes were isolated and extracted with sodium carbonate, pH 11.5. The samples were adjusted to d = 1.25 g/ml, ultracentrifuged, and immunoprecipitated with anti-human apoA-I. All data have been corrected for background in the separation method as described in Materials and Methods. Sample sizes are indicated and data are expressed as means ± SD. Significant differences between groups are indicated. The inset shows a representative autoradiograph of immunoprecipitated apoA-I recovered in the top (T) and bottom (B) fractions under each experimental condition

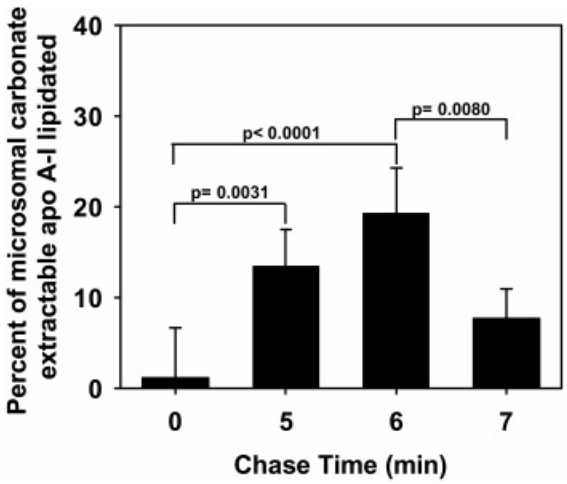

Fig. 6. Pulse-chase analysis of the intracellular lipidation of HepG2 cell apoA-I. HepG2 cells were pulse radiolabeled for 2 min and chased for the indicated times in the presence of 0.1 mM cycloheximide. The amount of apoA-I lipidated in microsomal carbonate extracts was determined as described in Fig. 5. The graph is a compilation of several independent pulse-chase experiments. Columns represent means and bars represent the SD (0 min, n = 9; 5 min, n = 3; 6 min, n = 4; 7 min, n = 3). Significant differences between groups are indicated. All data have been corrected for background in the separation method as described in Materials and Methods.

apoA-I, short-term pulse-chase studies were undertaken to demonstrate a precursor-product relationship between intracellular lipid-poor and lipidated forms of apoA-I (**Fig. 6**). HepG2 cell monolayers were pulse radiolabeled for 2 min and chased for up to 7 min in serum-free medium containing 0.1 mM cycloheximide to prevent the synthesis of additional radiolabeled apoA-I during the chase. Five and six minutes after the initiation of the chase, 13 and 19%, respectively, of the carbonate-extractable apoA-I was lipidated. Both of these time points were significantly increased over the 0-min time point. These data indicate that the intracellular lipidation of apoA-I occurs in a kinetically defined manner and is consistent with the rapid intracellular conversion of a portion of newly translated apoA-I from a lipid-free or lipid-poor form to nascent HDL. The extent of lipidation at 6 min was similar to that observed during steady state radiolabeling experiments (Fig. 5). The 7-min chase time point was not significantly different from either the 0- or 5-min time points, but was significantly lower than the peak 6-min time point. This appears to indicate that newly lipidated microsomal apoA-I, in contrast to lipid-free or lipid-poor apoA-I, is more rapidly secreted, and/or moved into a noncarbonate extractable cellular compartment.

DISCUSSION

The purpose of this study was to determine whether lipidation of apoA-I occurs before secretion from a mammalian hepatic cell line (HepG2). Historically, HDL assembly was thought to occur through the intracellular assembly of apoA-I with phospholipid and cholesterol followed by the secretion of discoidal HDL particles. The discoidal HDL were then acted on by LCAT and the free cholesterol was converted to cholesteryl ester, resulting in conversion of discoidal particles to spherical HDL. This concept of HDL assembly was supported by the work of Hamilton et al. (42), who demonstrated the accumulation of discoidal HDL particles in rat liver perfusate when an LCAT inhibitor was present. However, this view of HDL assembly was altered when Hamilton, Moorehouse, and Havel (26), using electron microscopy, were unable to demonstrate discoidal HDL inside the cell and an alternative extracellular HDL assembly pathway was reported (12, 13, 43–48). Our data demonstrate that approximately 20% of apoA-I isolated from microsomes of HepG2 cells has sufficient lipid to float at a density of 1.25 g/ml. The lipidation of apoA-I is rapid (<15 min), specific, and can be demonstrated kinetically.

Previous studies using chicken hepatocytes documented the intracellular association of apoA-I with lipid in the Golgi, a process that occurred within 2–8 min after synthesis of apoA-I (23). These results are consistent with

our kinetic observations (Fig. 6) and suggest the possibility that mammalian apoA-I lipidation may also occur in the Golgi compartment. However, application of the data in chicken hepatocytes to mammalian systems may not be valid because of differences in mammalian versus chicken apoA-I structure and function. In particular, chicken apoA-I is found in association with apoB lipoproteins in plasma, raising the possibility that presecretory chicken apoA-I may also associate with VLDL precursors intracellularly. Furthermore, chickens do not synthesize apoE and, because of the similar tissue distribution of chicken apoA-I and mammalian apoE, it has been suggested that chicken apoA-I may be the physiological equivalent of mammalian apoE (27).

The present study along with previous studies of presecretory apoA-I lipidation in both chicken (22, 23) and rat hepatocytes (24) all used carbonate extraction to isolate the contents of microsomes and isolated endoplasmic reticulum and Golgi fractions. Nunez and Swaney (49) have shown that under some experimental conditions, apoA-I will form complexes with microsomal lipid in vitro that resemble nascent HDL. Hence, it is possible that carbonate extraction, used in these studies, might contribute to an artifactual association of apoA-I with lipid (22, 23, 50). However, our results demonstrated that intracellular lipidation (27%) was in excess of nonspecific lipidation of exogenous ^{35}S-labeled apoA-I (8%) added during the carbonate extraction procedure (Fig. 5). The specificity of the apoA-I-lipid interaction was further supported by kinetic studies demonstrating a rapid posttranslational association of apoA-I with lipid (Fig. 6). Artifactual lipid association cannot explain these findings because all time points were subjected to the same experimental procedures. Thus, our results indicate that in HepG2 cells, newly synthesized apoA-I is secreted as lipid-free or lipid-poor apoA-I and in a form associated with sufficient lipid to float as nascent HDL.

Although we have documented presecretory lipidation of apoA-I, it is also well established that apoA-I can associate with lipid extracellularly (11–13, 15, 51). This process, although poorly understood, appears to be mediated by the ABCA1 transporter protein (17–20). The ABCA1 transporter is defective in Tangier disease, resulting in extremely low plasma HDL concentrations (52–54). In contrast to normal fibroblasts, cultured fibroblasts from Tangier patients fail to assemble exogenously added apoA-I into phospholipid and cholesterol complexes (55). Thus, there is abundant evidence that extracellular lipid-free apoA-I assembles with lipid, but the available data suggest that this lipidation pathway is relatively inefficient and dependent on ABCA1 expression (21). One group (15) has suggested that the inefficient assembly of extracellular lipid-free apoA-I into HDL particles is due to the restricted amount of available lipid for the pathway. In addition, this pathway can be stimulated in certain cell types by treatments that increase ABCA1 expression, such as loading cells with cholesterol or stimulating cells with cAMP (56–59). The results from our study show that in HepG2 cells the extracellular lipidation of apoA-I is rapid (<15 min) but relatively inefficient. For example, ~20% of delipidated apoA-I added to medium was lipidated between 15 and 120 min of incubation with HepG2 cells (Fig. 3). However, ~50% of secreted HepG2 apoA-I was lipidated over the same time course (Fig. 2), reflecting the ~20% of apoA-I that was lipidated intracellularly as well as the additional 20–30% that was secreted and rapidly acquired lipid extracellularly. The lipidated apoA-I that accumulated in HepG2 medium appeared to have a size range of 7.5–8.0 nm in diameter, similar to that of small HDL particles in plasma (see below). Our data agree with those of Gillotte et al. (15, 16), who showed a rapid but limited association of lipid-free apoA-I with phospholipid and cholesterol. Most studies have missed the rapid component of lipid-free apoA-I association with lipid because medium was not sampled at early time points. Thus, our data agree well with other studies (12, 15, 16, 51, 60) of extracellular lipidation of apoA-I, but also document an additional intracellular lipidation pathway in HepG2 cells that is more efficient and rapid.

Gradient gel electrophoretic characterization of the HDL particles that accumulated in HepG-2 medium from 2 h of incubation revealed predominantly small HDL particles (7.5–8.0 nm), with little change in particle size distribution over the time course of incubation (data not shown). On the basis of our studies of homogeneously sized HDL particles from plasma, the small HDL in HepG2 medium likely contain two apoA-I molecules per particle, whereas the medium-sized particles contain three apoA-I molecules (41). Results from our in vivo metabolic studies (41, 61) indicate that small HDL are precursor particles that are converted in a unidirectional pathway to medium and large HDL. We have also shown that the large HDL are terminal particles that are catabolized primarily by the liver (62).

Bringing these experimental observations together, we hypothesize that apoA-I can be secreted by the liver in lipid-free or lipid-poor and lipidated forms. The small HDL particles that are formed intracellularly or at the hepatocyte surface circulate in plasma and in extracellular spaces, gaining more lipid and apoA-I to become medium and large HDL (41, 61). The large, and perhaps medium, HDL particles are then removed and catabolized by the liver (62). This metabolic process would result in the net movement of cholesterol from peripheral tissues to the liver, resulting in reverse cholesterol transport.

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