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Apolipoproteins A-I, A-II and E are independently distributed among intracellular and newly secreted HDL of human hepatoma cells

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ABSTRACT

Whereas hepatocytes secrete the major human plasma high density lipoproteins (HDL)-protein, apo A-I, as lipid-free and lipidated species, the biogenic itineraries of apo A-II and apo E are unknown. Human plasma and HepG2 cell-derived apo A-II and apo E occur as monomers, homodimers and heterodimers. Dimerization of apo A-II, which is more lipophilic than apo A-I, is catalyzed by lipid surfaces. Thus, we hypothesized that lipidation of intracellular and secreted apo A-II exceeds that of apo A-I, and once lipidated, apo A-II dimerizes. Fractionation of HepG2 cell lysate and media by size exclusion chromatography showed that intracellular apo A-II and apo E are fully lipidated and occur on nascent HDL and VLDL respectively, while only 45% of intracellular apo A-I is lipidated. Secreted apo A-II and apo E occur on small HDL and on LDL and large HDL respectively. HDL particles containing both apo A-II and apo A-I form only after secretion from both HepG2 and Huh7 hepatoma cells. Apo A-II dimerizes intracellularly while intracellular apo E is monomeric but after secretion associates with HDL and subsequently dimerizes. Thus, HDL apolipoproteins A-I, A-II and E have distinct intracellular and post-secretory pathways of hepatic lipidation and dimerization in the process of HDL formation. These early forms of HDL are expected to follow different apolipoprotein-specific pathways through plasma remodeling and reverse cholesterol transport.

1. Introduction

Human plasma high density lipoprotein-cholesterol is a negative risk factor for cardiovascular disease for which current therapies are inadequate [1]. HDL promote reverse cholesterol transport (RCT) by carrying excess cellular cholesterol through the plasma compartment for disposal by the liver [2], inhibit LDL oxidation [3], and are anti-inflammatory [4]. Most plasma HDL are spherical particles with a neutral lipid core surrounded by a surface monolayer of apolipoproteins, cholesterol and phospholipids [5]. Apo A-I and apo A-II are 64% and 20% of HDL protein mass [5]. Some HDL particles contain only apo A-I (LpAI); most of the remainder contain both apo A-I and apo A-II (LpA-I/A-II) [6]. Formation of LpA-I/LpA-II in plasma occurs via LCAT-mediated fusion of nascent discoidal apo A-II HDL with small spherical LpA-I [7] or by displacement of apo A-I by the more lipophilic apo A-II [8–10]. Intracellular formation has not been fully delineated. Apo E, a minor HDL protein, preferentially associates with large HDL [11–13]. The distributions of HDL apolipoproteins, which are exchangeable, are modified by LCAT, CETP, PLTP and hepatic lipase [14]. Indeed, HDL structural remodeling and apo A-I dissociation are essential to RCT [2,15].

Apo A-I is antiatherogenic, promoting RCT, activating LCAT, and inducing regression of atherosclerotic lesions [16–18]. Whereas studies of mice over-expressing apo A-II reveal both anti- and pro-atherogenic activities [19,20], a recent study showed that apo A-II level is inversely associated with risk for atherosclerosis [21]. Human apo A-II is distinguished by Cys6, which forms disulfide-linked dimers [22,23]. Homodimeric apo A-II is more lipophilic than the monomer, giving it distinct properties [24]; >96% of human plasma apo A-II is dimeric. Apo A-II dimerization, which is slow in aqueous buffer [22], is catalyzed ~7500-fold by lipid surfaces [25]. Apo E, which occurs in three isoforms with different cysteine contents, mediates hepatic clearance of apo B-containing lipoproteins [26] and promotes RCT [27]. Apo E3 (Cys112, Arg158), the most common isoform, is synthesized by HepG2 cells, and in plasma of E3 homozygotes about 55% of the apo E3 is homodimeric or heterodimeric with apo A-II [28,29].

Hepatocytes synthesize and secrete HDL apolipoproteins [19,30]; ~20% of apo A-I is lipidated intracellularly with additional lipidation occurring after secretion [31–34]. The corresponding intracellular lipidation itineraries of apo A-II and apo E have not been reported [10]. Given the mechanistic link between apo A-II lipidation and dimerization, we investigated the synergism between these processes in lipoprotein biogenesis in HepG2 and Huh7 cells and found that apolipoproteins A-I, A-II and apo E are uniquely distributed among intracellular and newly secreted lipoproteins.

Abbreviations: βMSH, β-mercaptoethanol; LpA-I and LpA-I/A-II, HDL containing only apo A-I or both apo A-I and apo A-II respectively; NEM, N-ethyl maleimide; NP-40, nonidet P40; RCT, reverse cholesterol transport; SEC, size exclusion chromatography; TPS, thiopropyl sepharose

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2. Materials and methods

2.1. Cell culture

Human hepatoma HepG2 cells (American Type Culture Collection, Manassas, VA) and Huh7 cells (kindly provided by Dr. Yumin Xu in Dr. Boris Yoffe's laboratory, Baylor College of Medicine, Houston, TX) were cultured in MEM (InVitrogen, Carlsbad, CA) with 10% FBS, 1 mM sodium pyruvate and penicillin–streptomycin antibiotics (10 U/mL, 10 μg/mL respectively) (InVitrogen, Carlsbad, CA). Cells were plated and grown to about 80% confluency, washed twice with PBS, incubated with serum-free MEM, and then cells and media were harvested for analysis. Two time points were chosen, one relatively early (2 h) and one relatively late (24 h) after secretion. The early 2 h media is enriched in newly secreted lipoproteins, while by the time of collection of the late 24 h media extensive remodeling of the secreted lipoproteins has occurred [32,35–37]. The longer time has been used in many previous studies of HepG2 lipoprotein secretion [30,38–40]. Dimerization of apo A-II and E was blocked at the time of harvest by addition of 5 mM iodoacetamide or 10 mM N-ethyl maleimide (NEM) to alkylate any free cysteines. Medium was collected, centrifuged to pellet cell debris and the supernatant was transferred to fresh tubes. Cell dishes were placed on ice, cells were washed twice with ice-cold PBS, and lysed in NP-40 lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40 (Roche, Indianapolis, IN), pH 8.0) with 10 mM NEM and protease inhibitor cocktail (Roche, Indianapolis, IN). Control experiments showed that this lysis buffer did not affect HDL elution profiles by size exclusion chromatography (data not shown). Cell lysates were centrifuged to remove nuclear debris, and the supernatants transferred to fresh tubes. One-tenth volume 10× buffer (100 mM Tris–HCl pH 7.4, 1 M NaCl, 10 mM EDTA, 10 mM NaN_3) was added, and media and lysate samples were stored at 4 °C until further processing and analysis.

2.2. Ultracentrifugation

Lipidated and lipid-free apolipoproteins in cell media were separated essentially as described [31]. Media samples were adjusted to $d = 1.25$ g/mL with solid KBr and lipoproteins floated by centrifugation. Three fractions were collected: T: top 3 mL, containing the lipid-associated apolipoproteins; M: middle 3 mL (M); and B: bottom 6 mL, containing the lipid-free proteins (B). The middle fraction (M) contained less than 10% of the protein in the bottom (B) fraction and little if any of the lipidated proteins (data not shown). Buffer was exchanged for TBS and samples were concentrated with Centricon Plus-20 centrifugal filter devices, 5000 MWCO (Millipore, Bedford, MA).

2.3. Size exclusion chromatography (SEC)

Lipoproteins and lipid-free proteins in media and cell lysate samples were separated by SEC on tandem Superose 6 FPLC columns, as described [41,42]. Eluants (1 mL) were collected into tubes, which were pooled into 10 fractions each for media and lysate samples, as in Fig. 2. Peak elution volumes for human plasma lipoproteins were VLDL, Fraction 1, LDL, Fraction 3 and HDL, Fraction 6; lipid-free apo-A-I eluted in Fraction 8. Larger HDL eluted in Fraction 5, and lipid-poor HDL in Fraction 7 [42].

2.4. Thiopropyl sepharose (TPS) chromatography

Columns of thiopropyl sepharose 6B (GE Healthcare, Piscataway, NJ), 1 mL bed volume, were used to bind the cysteine-containing apo A-II to separate LpA-I from LpA-I/A-II. For study of cell lysates, HepG2 and Huh7 cells, incubated for 2 h in serum-free MEM, were lysed as above, except that NEM was omitted, and the lysis buffer pH was adjusted to 7.4, to maintain cytosolic binding conditions. Binding of dimeric apo A-II to TPS requires its prior reduction with DTT to form the free −SH groups to which TPS binds. Thus, lysates were treated with 20 mM DTT to reduce all disulfide bonds and then dialyzed against TBS containing 0.5 mM DTT to maintain the reduced −SH groups. The 1 mL bed volume minicolumns had a calculated −SH capacity of ~15 μmol, corresponding to a 5-fold excess for the amount of DTT to be loaded. The contribution of cell lysate proteins to the total −SH concentration was estimated to be at least an order of magnitude less than that from the 0.5 mM DTT. Human plasma HDL samples were reduced with 20 mM DTT, dialyzed and processed through TPS columns in parallel with the cell lysates as controls for separation of LpA-I from LpA-I/A-II (Fig. 4 and Table 2). Four to 6 mL of the reduced and dialyzed cell lysates or HDL was loaded onto the TPS columns. The initial flow-through was recycled through the column for 1 h. The subsequent flow-through was collected (free fraction), the columns were washed with 5 bed volumes (5 mL) TBS (wash fraction) and eluted with 5 bed volumes 20 mM DTT in TBS (bound fraction). Aliquots of each fraction were delipidated, solubilized in SDS-PAGE sample buffer, and analyzed by SDS-PAGE and Western blotting.

To determine the kinetics of LpAI/AII formation in hepatoma media, 60 mL media per time point was collected as above at 0.5, 1.0, 2.0 and 20 h. At the time of harvest, 2 mM paraoxon-ethyl (Sigma, St. Louis) was added to inhibit LCAT to stop further formation of LpAI/AII [43]. After 30 min, 1 mM DTT was added to reduce disulfide bonds, and after another 30 min, samples were loaded onto 7 mL bed volume TPS columns. After two passes through the column, the flow-through (free fraction) was collected, the column was washed with 10 bed volumes TBS, and bound fraction was eluted with 5 bed volumes 20 mM DTT in TBS. Free and bound fractions were concentrated and aliquots analyzed by SDS-PAGE and Western blotting.

2.5. SDS-PAGE and Western blots

Lipoproteins and apolipoproteins in media and cell lysates were separated according to density by ultracentrifugation, SEC or TPS and analyzed for apolipoproteins A-I, A-II, B and E by SDS-PAGE (15% Tris-Glycine Ready Gels; BioRad, Hercules, CA) and Western blotting. Samples were loaded in non-reducing or reducing (plus β-mercaptoethanol, (+βMSH)) sample buffer, as indicated in the figure legends. After transfer to nitrocellulose, the Western blotting method was essentially that of the Amersham ECL-Plus Western Blotting Manual (Amersham Biosciences/GE Healthcare, Piscataway, NJ). Band identification is based on molecular weight and reaction by Western blot with highly specific, high affinity antibodies. All SDS/PAGE gels and Western blots were done with both molecular weight standards and apolipoprotein standards on all the gels/blots. Intensity of the bands detected by Western blotting was quantitated either by densitometry of X-ray exposures using Kodak 1D image analysis software, or directly on a Storm 840 imaging system (GE Healthcare, Piscataway, NJ) with ImageQuant TL software. Antibodies for immunoblots were HRP-conjugated goat anti-human apo A-I, apo A-II, apo B and apo E (Academy Biomedical, Houston, TX), or goat polyclonal (Academy Biomedical, Houston, TX) or mouse monoclonal (BioDesign/Meridian Life Sciences, Cincinnati, OH) antibodies with appropriate HRP-conjugated second antibodies (Chemicon/Millipore, Billerica, MA or Jackson Immunoresearch, West Grove, PA).

3. Results

3.1. Apolipoprotein lipidation and dimerization in secreted lipoproteins

In our first experiments ultracentrifugation was used to separate lipidated and lipid-free apolipoproteins as previously reported [31]. Immunoblot analysis of fractions from HepG2 cell media harvested after 2 and 24 h (Fig. 1; Table 1) demonstrated that at 2 h, apo A-I was partially lipidated (85%) while apo A-II and apo E were essentially