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Acylation of Lysine Residues in Human Plasma High Density Lipoprotein Increases Stability and Plasma Clearance In Vivo

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Abstract

Although human plasma high density lipoproteins (HDL) concentrations negatively correlate with atherosclerotic cardiovascular disease, underlying mechanisms are unknown. Thus, there is continued interest in HDL structure and functionality. Numerous plasma factors disrupt HDL structure while inducing the release of lipid free apolipoprotein (apo) AI. Given that HDL is an unstable particle residing in a kinetic trap, we tested whether HDL could be stabilized by acylation with acetyl and hexanoyl anhydrides, giving AcHDL and HexHDL respectively. Lysine analysis with fluorescamine showed that AcHDL and HexHDL respectively contained 11 acetyl and 19 hexanoyl groups. Tests with biological and physicochemical perturbants showed that HexHDL was more stable than HDL to perturbant-induced lipid free apo AI formation. Like the reaction of streptococcal serum opacity factor against HDL, the interaction of HDL with its receptor, scavenger receptor class B member 1 (SR-B1), removes CE from HDL. Thus, we tested and validated the hypothesis that selective uptake of HexHDL-[³H]CE by Chinese hamster ovary cells expressing SR-B1 is less than that of HDL-[³H]CE; thus, selective SR-B1 uptake of HDL-CE depends on HDL instability. However, in mice, plasma clearance, hepatic uptake and sterol secretion into bile were faster from HexHDL-[³H]CE than from HDL-[³H]CE. Collectively, our data show that acylation increases HDL stability and that the reaction of plasma factors with HDL

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Conflict of Interest

The authors have no relevant conflicts of interest to disclose.

Author Contributions

HJP developed the broad hypothesis and prepared the first draft of the paper. BKG designed and supervised the cell studies and their interpretation. YY conducted nearly all of the studies in vitro and in vivo. CR designed and supervised the mouse studies. AMG reviewed the paper as it was developed. All authors edited and approved the final submitted paper.

and SR-B1-mediated uptake are reduced by increased HDL stability. In vivo data suggest that HexHDL promotes charge-dependent reverse cholesterol transport, by a mechanism that increases hepatic sterol uptake via non SR-B1 receptors, thereby increasing bile acid output.

Keywords

Reverse cholesterol transport; high density lipoprotein stability; scavenger receptor class B member 1; high density lipoprotein acylation; atherogenesis

1. Introduction

1.1. Atherosclerosis

Atherosclerosis, which is characterized by the accumulation of lipids, mostly free and esterified cholesterol, in the subendothelial space of the arterial wall, can be ischemic, ultimately restricting blood flow, which leads to heart attacks and strokes. Reverse cholesterol transport (RCT), which reduces the burden of cholesterol in the arterial wall, comprises cholesterol efflux from arterial wall macrophages to apolipoprotein (apo) AI or high density lipoproteins (HDL), esterification in plasma, and selective lipid uptake by hepatic scavenger receptor class B member 1 (SR-B1). [1] Following hepatic uptake some cholesterol is converted to bile acids and ultimately excreted in the feces. HDL is central to RCT and although plasma HDL-cholesterol (HDL-C) concentration is a negative risk factor for atherosclerotic cardiovascular disease, most attempts to raise plasma HDL-C in a cardioprotective way have failed.[2–5] Several studies have shown that not all HDL are functionally equivalent and that one function of HDL, accepting cellular cholesterol, correlates better with reduced cardiovascular diseases (CVD) than HDL-C levels.[6–8] There is also a debate about the raising-HDL-C-is-better hypothesis and the regulatory mechanisms by which HDL-C is delivered to the liver. (Reviewed)[9] In mice, overexpression of SR-B1 in the liver reduces plasma HDL-C levels and is atheroprotective, [10–13] whereas genetic deletion of SR-B1 in mice increases plasma HDL-C levels, but not in an atheroprotective.[14–16] As a consequence, there is continuing interest in HDL functionality that correlates with its cardioprotective properties, which may include faster clearance from plasma.

1.2. HDL Stability

HDL stability is measured according to its dose-dependent resistance to changes in structure in response to a perturbation, which may be physico-chemical or biological. Using these criteria, others showed that HDL resides in a kinetic trap from which it escapes in response to chaotropic and thermal perturbations.[17] HDL structure is also readily disrupted by several plasma factors—lecithin: cholesterol acyltransferase (LCAT), cholesteryl ester and phospholipid transfer proteins (CETP, PLTP), endothelial and hepatic lipases, and Streptococcal serum opacity factor (SOF).[18–23] Concomitant with their disruptive activities, many of these factors, especially SOF, release lipid-free (LF) apo AI into the surrounding aqueous phase. Thus, HDL instability and apo AI exchangeability are intrinsic to HDL functionality. Given that apo AI but not other HDL apolipoproteins nor low density lipoprotein-apo B-100, is labile and readily released, we hypothesized that apo AI exchangeability is

necessary or at least permitting for HDL functionality. We tested this hypothesis with an HDL particle containing apo AI modified in a way that increases its hydrophobicity thereby suppressing its exchangeability and release to the aqueous phase, and its negative charge, which may divert HDL to alternative receptors. Acylated HDL stability and function were tested by chemical and biological perturbation with guanidinium chloride (GdmCl), SOF and PLTP. Functionality was tested both in vitro as cellular cholesteryl ester (CE) uptake, and in vivo by plasma clearance, tissue uptake and CE metabolism. Earlier studies of acetylated low density lipoprotein (AcLDL) identified the LDL scavenger receptor on macrophages and other scavenger cells. [24, 25] SR-B1 is a promiscuous receptor that binds ligands other than HDL—AcLDL, OxLDL, apoptotic cells, and unmodified LDL and VLDL, maleylated serum albumin, and anionic phospholipids, (Reviewed).[26] Our studies probed the interaction of Acylated HDL with this receptor but also imply the existence of one or more receptors comparable to the LDL scavenger receptor that promotes in vivo plasma clearance and liver uptake of acylated HDL, which is more negatively charged than native HDL.

2. Materials and Methods

2.1. Acylation of HDL

HDL was isolated by flotation between $d = 1.063$ and 1.21 g/mL and further purified by size exclusion chromatography (SEC) over two $30\text{ cm} \times 1\text{ cm}$ Superose HR6 columns in tandem with a flow rate of 0.5 mL/min . [27] This SEC system was also used for analysis of the HDLs after treatment with GdmCl, SOF and PLTP. Reproducibility of peak elution volumes for this system is $SD = \pm 25\text{ }\mu\text{L}$. HDL was acylated with acetic or hexanoic anhydrides with minor modifications as described. [24] Briefly, $1 - 2\text{ }\mu\text{L}$ aliquots were added at 30 min intervals for a total of acetic anhydride 0.744 mg (100 mg/mL in ethanol) and hexanoic anhydride 1.56 mg (100 mg/mL in ethanol) with mixing to HDL (2.0 mg/mL in 2.9 mL) at an anhydride/lysine molar ratio of $1.6:1$. Incubations were continued overnight at room temperature (~ 18 hours). Addition of 20% more anhydride precipitated HDL. Commercial kits were used to determine the protein (DC protein assay, Bio-Rad, Hercules, CA) and lipid composition (total and free cholesterol, WAKO Life Sciences, Richmond, VA) of lipoproteins.

2.2. HDL Particle Analysis

The HDL, AcHDL and HexHDL were analyzed by electrophoresis in 0.78% and 1% agarose gels (90 mM Tris, 80 mM borate, pH 8.2). Lipoprotein samples ($5 - 13\text{ }\mu\text{g}$ of protein in $30\text{ }\mu\text{L}$) were loaded onto gels, and electrophoresed at 4°C at 90 V for 3 h, as described. [28] The HDLs were also analyzed by nondenaturing polyacrylamide gradient gel electrophoresis (PAGE) (Life Technologies, Novex, 4–20% Tris-Glycine Gel). Lipoproteins ($6 - 19\text{ }\mu\text{g}$ of protein in $20\text{ }\mu\text{L}$) were loaded onto gels, and electrophoresed at 4°C at 125 V for 2.5hr.

2.3. HDL Acylation Analysis

Fluorescamine was used to determine the extent of lysine acylation [29, 30]. A series of doses of HDL, AcHDL, and HexHDL between 0 and $500\text{ }\mu\text{g/mL}$ in PBS were made; $150\text{ }\mu\text{L}$ aliquots of samples and $50\text{ }\mu\text{L}$ of 10.8 mM (3 mg/mL) fluorescamine (Sigma-Aldrich)

dissolved in acetone were added to each well of 96-well black microplate with clear bottom (Costar). After the plate was shaken for one minute, the fluorescence was determined using a UV-Vis fluorescence and absorbance plate reader (TECAN, SAFIRE II) with a 400 nm, 20 nm bandwidth, excitation filter and a 460 nm, 20 nm bandwidth emission filter.[29, 30] The fluorescence vs. protein concentration curves were fitted to a first order polynomial in SigmaPlot and the number of free lysines in each HDL was calculated as the slope. The number acylated lysines was calculated as the difference between the number of lysines per particle (nonacylated HDL) and the number per particle labeled with fluorescamine.

2.4. Chaotropic Perturbation

Stability of HDL, AcHDL and HexHDL was compared by chaotropic perturbation with GdmCl.[17] Various aliquots of Tris-buffered saline (TBS) and 9 M GdmCl were combined with HDL, AcHDL, and HexHDL (0.27 mg/mL in 3 mL). After 18 hours, each reaction mixture was analyzed by SEC, which revealed shifts in the peaks for HDL, and the appearance of new peaks in the void volume and at an elution volume of ~34 mL, corresponding to fusion products and LF apo AI respectively.[31] The shift in the elution volume of the main HDL peak and the appearance of LF apo AI were correlated with the GdmCl concentration.

2.5. SOF Kinetics

SOF was prepared as described.[32] The kinetics of the SOF reaction with HDL and acylated HDL were determined by right angle light scattering in real time and according to the distribution of reaction products by SEC as described.[23, 33] Briefly, HDL (0.27 mg/mL in 3 mL) was placed in a fluorescence cuvette, which was transferred to the cell compartment of an Aviv spectrofluorimeter. After equilibration to 37 °C, SOF (2.8 µg/2.5 µL) was added with constant stirring and the light scattering intensity (325 nm) monitored at 90° to the incident light. The light scattering intensity vs. time data were fitted to a two-parameter growing exponential function in SigmaPlot.

2.6. PLTP Reactivity of HDL and Acyl HDL

The disruptive effects of PLTP on HDL structure[34, 35] were also used to assess HDL stability. A His-tagged PLTP, prepared according to Albers,[36] was a gift from Xian-cheng Jiang, Ph.D. (SUNY Downstate Medical Center). HDL and acylated HDL (0.27 mg/mL) and rPLTP (4 µg/mL) were incubated at 37 °C overnight with stirring. Aliquots of the HDL and acyl HDL were then analyzed by SEC.

2.7. Biological Labeling of HDL with [³H]CE

HDL was labeled with [³H]cholesterol (Perkin Elmer, Boston MA), which was physiologically esterified using the LCAT activity of human lipoprotein-deficient serum; labeled free cholesterol was removed by multiple exchanges with low density lipoprotein (LDL), as described, [23] leaving HDL-[³H]CE that was further purified by SEC. Radiolabelled acylated products AcHDL-[³H]CE and HexHDL-[³H]CE were obtained by acylation of HDL-[³H]CE. Specific activities of lipoproteins were calculated as the ratio of

radioactivity to CE mass of aliquots. Lipoprotein stocks were dialyzed into phosphate-buffered saline (PBS) and sterile filtered prior to incubation with cells or injection into mice.

2.8. Cellular Uptake of HDL-CE

Cellular uptake of HDL-[³H]CE was determined in LDL-receptor null Chinese Hamster Ovary cells (CHO IdIA7), IdIA7 cells overexpressing SR-B1 (CHO SR-B1). Hepatocyte uptake via multiple receptors was determined using the human hepatoma cell line, Huh7, as described.[27, 37] Uptake was initiated by addition of 20 μ L aliquots of control HDL-[³H]CE, AcHDL-[³H]CE, or HexHDL-[³H]CE in PBS to cells in 1 mL of cell culture medium with 0.5% fatty-acid free bovine serum albumin (BSA), (Medium B, [37]). After incubation for 0–4 h (37 °C, 5% CO₂), the cells were transferred to ice, and HDL-[³H]CE uptake determined by β -counting 2-propanol extracts of the washed cells. Time courses for HDL-[³H]CE uptake by Huh7 and CHO cells respectively were fitted to a first order polynomial in SigmaPlot. Dose response curves for CE uptake as a function of HDL-[³H]CE (DPM) were determined after a 3 h incubation. Data were analyzed in SigmaPlot according to $U = V_{\max} \times CE / [K_m + CE]$, where U is the uptake (DPM), $V_{\max}$ = maximum CE uptake, K_m = CE at which 50% maximum CE (DPM) uptake is observed, and CE (DPM) is amount of CE incubated with the cells.

2.9. CE Metabolism in Vivo

All animal procedures were conducted in accordance to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the Houston Methodist Research Institute Institutional Animal Care and Use Committee. HDL-[³H]CE and HexHDL-[³H]CE purified by SEC were concentrated by Amicon Ultra Centrifugal Filter Units (EMD Millipore, 10K NMWL) and dialyzed into PBS. Traces of lipoproteins of high specific activity (0.35 million DPM/50 μ L) were injected into C57BL/6J mice (Jackson Laboratories) via the tail-vein. At various post injection times, mice were euthanized and their blood collected by heart puncture into EDTA vacutainer tubes; tissues were harvested for lipid analyses as described.[38, 39] Hepatic lipid extracts were separated by high performance thin layer chromatography (HPTLC) on HPTLC Silica Gel 60 plates (EMD Chemicals, Darmsted, Germany) as described.[28, 40] CE, free cholesterol (FC) and oxysterols were separated by developing the plate to 70% of plate length with hexane:ethyl acetate 8:2 v:v. The plate was dried and lipids visualized with iodine vapor. Radioactivity in the HPTLC bands was quantified by β -counting. Partitioning [39, 41] was used to determine the fraction of total sterol converted to bile salts. Cold carrier lipids—cholate, taurocholate (30 μ g each), cholesterol and CE (50 μ g each)—were added to mouse gallbladder bile to minimize non-specific loss of the radioactive sterols. Bile samples diluted in PBS (0.9 mL) were combined with 2.7 mL of chloroform:methanol (1:2 v:v), followed by addition of 1.8 mL chloroform and 0.9 mL of water with vortexing. Following low speed centrifugation, the solutions separate into upper and lower phases containing bile salts and neutral sterols respectively. The volumes of each phase were recorded; aliquots of each phase were evaporated and β -counted. Bile acid formation was expressed as the ratio of bile acid to total sterol radioactivity.

2.10. Statistical Analysis

All values are expressed as mean $\pm$ SD, unless noted otherwise. Data were compared using ANOVA followed by Student's t-test for pairwise comparison. A $p < 0.05$ was considered statistically significant. Statistical analyses and calculation of regression curves were performed using Sigma Plot 12.0 (SyStat Software, Inc.). Rate constants were calculated from regression fits of the data points vs. time.

3. Results

3.1. HDL Particle Analysis

According to native gradient gel electrophoresis, the sizes of HDL and acylated HDL were not very different (Figure 1 A). All three HDL species exhibit considerable size heterogeneity. In contrast, agarose gel electrophoresis shows that acylation alters the charge on HDL. The more rapid migration of acylated compared to native HDL corresponds to the increased negative charge due to acylation of the positively charged lysines (Figure 1 B).

3.2. HDL Acylation Analysis

The values of fluorescamine assay of HDL, AcHDL, and HexHDL regarding lysine numbers were well fitted to linear regression curves. (Supplementary Figure 1). The ratios of k_{HexHDL} and k_{AcHDL} to k_{HDL} are 0.68 and 0.82 respectively, indicating ~20 and ~10 of the total of 60 lysines in HDL were anchored with hexanoic and acetic residues after acylation.

3.3. Chaotropic Perturbation

HDL, AcHDL, and HexHDL were analyzed by SEC after incubation with various GdmCl concentrations. As previously shown, [17, 31] according to the attendant changes in the SEC profile (Supplementary Figure 2), as the concentrations of GdmCl increased the amount of LF apo AI, which elutes at ~34 mL, increased. Concurrently, the main HDL peak shifted to earlier elution volumes corresponding to a larger particle; between 3 and 6 M GdmCl, the absorbance due to aggregated lipid [17] in the void volume (~15 mL) also increased. AcHDL treated with increasing GdmCl gave similar changes in SEC profiles but at higher GdmCl, indicative of greater stability to chaotropic perturbation, and the amount of absorbance appearing in the void volume at higher GdmCl concentrations was lower (Supplementary Figure 1 B). Treatment of HexHDL with GdmCl yielded distinctly different SEC profiles (Supplementary Figure 1 C). HexHDL was most stable, with almost no change in SEC profile until >3M GdmCl. Like the AcHDL, only a small amount of mass appeared in the void volume. Little LF apo AI was released even at the highest GdmCl concentration. The concentration-dependent effects of GdmCl on two aspects of the SEC profiles—peak HDL elution volume and LF apo AI—are summarized in Figure 2. Figure 2 A reveals that the shifts in the main HDL peak that occur with increased GdmCl concentrations are similar for all three HDL species at 0 – 2 M GdmCl, while at higher concentrations, the fusion products of the acylated HDL are smaller than those of HDL. In contrast, Figure 2 B shows that the appearance of LF apo AI with increasing GdmCl concentrations is similar for HDL and AcHDL but ~80% lower for HexHDL.

3.4. SOF Activity on HDL and Acylated HDL

The kinetics of SOF action on the various HDL according to right-angle light scattering are shown in Figure 3. The data show that the rates of the SOF reaction decrease as HDL ~ AcHDL >> HexHDL with the rate of the SOF reaction on HexHDL being ~75% slower than that on HDL. SEC analysis shows that the product profiles for the SOF reaction products with HDL and AcHDL are similar, containing prominent peaks for CERM in the void volume, and peaks for neo HDL and LF apo AI with similar magnitudes and elution volumes (Figure 4 A, B). Likewise, according to its SEC profile, a similar amount of CERM is formed by the SOF reaction on HexHDL (Figure 4 C). However, in contrast to the HDL and AcHDL SEC profiles, that for HexHDL exhibits only a small peak for LF apo AI.

3.5. PLTP Reactivity

According to SEC, the effects of rPLTP on HDL and acetylated HDL were similar (Figure 4 D, E). Both profiles contained a small peak eluting at ~34 mL as LF apo AI and a second peak corresponding to a larger HDL. These effects were profoundly smaller with HexHDL (Figure 4 F); the main HDL peak was little shifted and the magnitude of the peak for LF apo AI was smaller than those for HDL and AcHDL. The respective ratios of the apo AI peaks to the main HDL peaks for HDL, AcHDL, and HexHDL were 0.40, 0.26, and 0.09. Thus HexHDL is more resistant than HDL and AcHDL to PLTP remodeling.

3.6. Cellular HDL-CE Uptake

To assess the effect of acylation on biological function of HDL, we measured the rate of HDL-CE uptake by Huh7 hepatocyte cells. The rate of CE uptake was AcHDL > HDL > HexHDL (Figure 5 A). Similarly, according to the dose-dependence for CE uptake, the maximum CE uptake (V_{max}) also was greatest for AcHDL, with AcHDL > HDL > HexHDL whereas the apparent binding constants for uptake, K_m , were not different (Figure 5 B; Table 1). Given that SR-B1 is the HDL receptor and mediates the selective uptake of HDL-CE, we repeated the uptake experiments with CHO IdIA7 and CHO SR-B1 cells, which respectively are LDLR-deficient and LDLR-deficient but overexpressing SR-B1 [37]. In CHO IdIA7 cells, the uptake rates and the dose-dependence for uptake were similar for AcHDL and HexHDL while the CE uptake rate from HDL was slightly but significantly less (Figure 5 C, D). In CHO SR-B1 cells the much greater CE uptake rates increased as HDL ~ AcHDL > HexHDL, as shown in both the time course and dose-response data (Figure 5 E, F). The kinetic data are summarized in Table 1. The data shows that CE uptake from HDL by hepatocytes and CHO SR-B1 cells is enhanced or not altered from AcHDL, but significantly diminished from HexHDL in both cell types.

3.7. In Vivo Metabolism

Given the profound differences in their biophysical properties and cell uptake in vitro, we compared the plasma kinetics and tissue distribution of HDL- and HexHDL- $[^3H]$ CE in vivo in C57BL/6J mice. In contrast to the in vitro results, our studies revealed a faster plasma clearance of HexHDL- $[^3H]$ CE compared to HDL (3.05 ± 0.55 and 0.83 ± 0.52 %DPM/h respectively; Figure 6 A). Concurrently, hepatic accumulation of HexHDL- $[^3H]$ CE was faster and more profound than that of HDL- $[^3H]$ CE (Figure 6 B). A comparison of the tissue

distribution revealed that the liver was the main site of CE uptake from both lipoproteins and that both liver and spleen accumulated more HexHDL- than HDL-[^3H]CE (Figure 6 C).

The rate of hepatic metabolism of HexHDL and HDL [^3H]CE to [^3H]cholesterol over time was similar (Figure 7 A, B) with more hepatic accumulation of oxysterol derived from HDL than from HexHDL at early time points (Figure 7 C). Concurrently, total HexHDL-derived [^3H]bile sterol was higher than that from HDL (Figure 8 A, C) while HDL-derived bile acids in bile was greater than that derived from HexHDL (Figure 8 B, D). This resulted in significantly more HexHDL-[^3H]CE- than HDL-[^3H]CE -derived bile acids in the bile at 2 h (DPM 774 ± 193 vs 238 ± 150 , $p=0.03$). Thus, the in vivo mouse data suggest that HexHDL is a vehicle that promotes RCT, by a mechanism that increases liver uptake of sterols via receptors other than SR-B1, which leads to an initial (2 h) increased bile acid output.

4. Discussion

4.1. Acylation alters HDL stability

HDL reside in a kinetic trap[17] from which they are displaced by many HDL-associating activities. [18–23] This process is characterized by the transfer of LF apo AI to the aqueous phase, a hallmark of HDL instability.[31, 42] Given that the amphipathic helical model of apos places the hydrophobic amino acid residues on one face of the helices where they collectively interact with lipid surfaces, we hypothesized that increasing the hydrophobicity of HDL by protein acylation would increase its stability. Furthermore, given that the free energy of activation for apo AI transfer from HDL[43] is identical to those for SOF activity on HDL[23] and HDL denaturation with GdmCl,[31] we tested whether increasing the lipophilicity of HDL by acylation of its apo lysines would stabilize it against these physicochemical and biological perturbations.

There is precedent for acylation altering apo properties. LDL and AcLDL cell uptake is mediated by two different receptors, LDLR and SR-A1, which have different tissue distribution. [24, 25, 44] Acylation of the apo mimetic, LAP-20, increased its lipophilicity and its plasma lifetime in rat; [45, 46] diacylation rendered LAP-20 nontransferable, i.e., highly stable.[47, 48] Here, native HDL were compared with HDL acylated with acetic and hexanoic anhydrides. The responses of native and acetyl HDL to physicochemical perturbation with GdmCl and biological perturbation with SOF and PLTP were similar, liberating similar amounts of LF apo AI, i.e., acetylation only modestly altered HDL stability. There are two likely reasons for the small effect of acetylation on stability. First, according to the amphipathic helical model of apos, the lysines, the main acylation target, are on the polar face of the helix and remote from sites of interaction with lipids. Second, the acetyl group is not very lipophilic, each methyl or methylene group contributes ~ 700 cal/mol to the free energy of transfer to a hydrocarbon-like environment[49, 50] that emulates the hydrophobic region on the surface HDL monolayer. Moreover, the acetyl moiety would be expected to be hydrophilic, e.g., acetic acid is miscible with water.

In contrast, HexHDL was resistant to physicochemical and biological perturbations, releasing less LF apo AI and in the case of SOF, releasing less apo AI and reducing the reaction rate by $\sim 70\%$. Assuming two apo AI (each with 21 lysines) and one apo AII dimer

(18 lysines) per HDL, apo AI contains 42 (70 %) of the HDL lysines and with random and equivalent acylation of apo AI and noting that the fluorescamine titration showed that 30 % of the HDL lysines were acylated, the average number of hexanoylated apo AI-lysines is $0.3 \times 21 = \sim 6$ lysines/apo AI. Given that each methylene contributes 700 cal/mol to the free energy of binding,[50] and that each hexanoyl group contains five methylenes, the calculated increase in the free energy of apo AI binding by hexanoylation is $6 \text{ lysines/apo AI} \times 5(\text{methylenes})/\text{lysine} \times 700 \text{ cal/mol methylene} = \sim 21 \text{ kcal}$. The modest effect of acetylation on HDL stability is likely due to the short acyl chain that with the same calculation would increase binding by only $\sim 4 \text{ kcal/mol apo AI}$. There could also be differences in the identity of the lysines that are labeled by hexanoylation and acetylation but we aver that it is likely a random process although some lysines may be differentially protected from acylation.

4.2. Acylation alters HDL biological activity

Given that HDL hexanoylation inhibited SOF-mediated release of LF apo AI from HDL and that both the SOF reaction and selective HDL-CE uptake by SR-B1 release HDL protein, we tested whether CE uptake from HexHDL by CHO SR-B1 cells is less than that from control CHO cells. As hypothesized, CE uptake from each of the three HDL was greater by the CHO SR-B1 cells than by the CHO IdIA7 cells. However, CE uptake from HexHDL by the CHO SR-B1 cells was less than that from both HDL and AcHDL. For comparison to HDL, the difference could be due to differences in the charges on the acylated and native HDL or to differences in HDL stability and apo AI exchangeability. However, AcHDL and HexHDL have similar charges (Figure 1) but behave differently in the cell assays so we concluded that the differences are due to changes in HDL stability and/or apo AI exchangeability; these two effects are difficult to separate and it is likely that one is intrinsic to the other. These data support the hypothesis that HDL instability and apo AI exchangeability are permitting factors in selective CE uptake via SR-B1. Of note, while CE uptake from HDL by SR-B1 is known to be selective for the lipid, this may not be the case for HexHDL-CE, where the increased HexHDL stability may result in significant CE uptake by holoparticle uptake. Given that Huh7 cells express SR-B1 [39, 51], these data suggest but do not prove that the reduced CE uptake of HexHDL-CE by Huh7 cells is at least, in part, via reduced SR-B1 uptake.

4.3. Acylation effects on HDL Metabolism In Vivo

Given that the most prominent effects of HDL acylation were seen with hexanoylation, we compared HDL and HexHDL metabolism in mice. HexHDL-CE disappeared from plasma and appeared in liver more rapidly than HDL- ^3H CE (Figure 6); this was unexpected given the lower uptake of HexHDL- ^3H CE compared to HDL- ^3H CE in cells and that acylation of an apo mimetic that binds HDL increases the plasma lifetime of the mimetic.[46] However, unlike the mimetic, acylated HDL has a high negative charge which could redirect its uptake to other hepatic receptors. On the basis of the in vitro data, we conclude that the in vivo uptake is not primarily via SR-B1. Rapid disappearance of HexHDL- ^3H CE from plasma coincides with rapid hepatic uptake indicating that the liver is the major organ for plasma clearance. The only other organ exhibiting differential uptake of HexHDL- ^3H CE and HDL- ^3H CE is the spleen. Although the mechanisms and receptors mediating this process are not known, some can be excluded. In humans, the CE could transfer to another

lipoprotein, e.g., LDL, and enter the liver via the LDL receptor. This is unlikely because CE is nontransferable in the mouse, which lacks CETP. We speculate that this occurs via a whole particle uptake mechanism because selective CE uptake via SR-B1 was slower (Figure 5). Thus, as with LDL and AcLDL[25], HDL and HexHDL appear to be cleared from plasma by different receptors resulting in differences in in vivo metabolism.

Once having entered the liver, the rate of hydrolysis of HexHDL-derived [^3H]CE and HDL-[^3H]CE to free cholesterol are similar (Figure 7 A, B), although conversion to oxysterols is greater for HDL-[^3H]CE at 30 min (Figure 7 C). Output of sterol into bile for HexHDL-[^3H]CE is initially greater than that for HDL-[^3H]CE, reaching a plateau by 2 h (Figure 8). However the percent of sterol as bile acids at 2 h is greater for HDL-[^3H]CE than for Hex HDL-[^3H]CE, which is consistent with the greater hepatic conversion to oxysterols.

5. Conclusions

Acylation of HDL with hexanoic anhydride achieves the hypothesized goal of stabilization with the concomitant increase in the retention of apo AI on the HDL surface. We also hypothesized that apo AI exchangeability and HDL instability were permitting factors in selective CE uptake by SR-B1 and this was verified by comparing CE uptake in CHO cells expressing SR-B1 with those that do not. In vivo, HexHDL-[^3H]CE transferred from plasma to the liver more rapidly than HDL-[^3H]CE but converted to free cholesterol at similar rates with slightly more HDL-[^3H]CE converted to oxysterols but less HDL-[^3H]CE appearing as bile sterol. These findings suggest HDL stability is an important determinant of its metabolism and that other modifications, e.g., acylation with longer fatty acyl groups, can be used to further stabilize HDL. However, interactions with other proteins that participate in HDL metabolism— PLTP, CETP, LCAT, SR-B1 and, in particular, other scavenger receptors —could be factors in the alteration of HDL functionality by acylation. While it is not known whether these or similar modifications have any therapeutic value this work provides valuable information for understanding the parameters that affect HDL functionality in reverse cholesterol transport. The rapid uptake of acylated HDL in vivo might provide a therapeutic mechanism by which increasing the negative charge on HDL increases its rate of plasma clearance and hepatic uptake.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AcLDL	acetyl LDL
apo	apolipoprotein

BSA	bovine serum albumin
CVD	cardiovascular disease
CE	cholesteryl ester
CERM	cholesteryl ester-rich microemulsion
CETP	cholesteryl ester transfer protein
FC	free cholesterol
GdmCl	guanidinium chloride
HDL	high density lipoproteins
HDL-C	HDL-cholesterol
HPTLC	high-performance thin layer chromatography
LCAT, lecithin	cholesterol acyltransferase
LF apo AI	lipid-free apolipoprotein AI
LDL	low density lipoprotein
CHO IdIA7	LDL-receptor null Chinese Hamster Ovary cells
CHO SR-B1	LDL-receptor null Chinese Hamster Ovary cells overexpressing SR-B1
PBS	Phosphate-buffered saline
PLTP	phospholipid transfer protein
PAGE	polyacrylamide gradient gel electrophoresis
RCT	Reverse cholesterol transport
SR-B1	scavenger receptor class B member 1
SEC	size exclusion chromatography
SOF	streptococcal serum opacity factor
TBS	Tris-buffered saline

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Highlights

- HDL acylation increases its HDL stability to chemical and biological perturbants.
- HDL acylation alters its biological activity, decreasing its selective uptake by SR-B1.
- HDL acylation accelerates plasma clearance of its CE.
- Acylation of HDL increases the hepatic clearance of its sterols and their secretion as bile.
- Given that HDL acylation reduces its uptake by SR-B1, the increase in the in vivo clearance of HDL-CE must involve other hepatic receptors.

Fig. 1.

Characterization of HDL and acylated HDL according to mass and charge. A: Size analysis of various lipoprotein masses by 4–20% native PAGE as labeled; lipoproteins were visualized with Coomassie Blue. B: Mobility of lipoproteins according to 1% agarose gel electrophoresis. Samples were loaded into wells at the cathode (top) and electrophoresed (3 h, 90 V); given the absence of sieving, mobility reflects charge differences.

Fig. 2.

Summary of chaotropic perturbation of human HDL and acylated HDL by GdmCl. Lipoproteins, TBS, and GdmCl, combined to give a final HDL-protein concentration of 0.27 mg/mL and various concentrations of GdmCl, were incubated at room temperature for 22 h, after which the samples were analyzed by SEC. A: Elution Volumes of the GdmCl-mediated fusion product versus molar GdmCl concentration. B: Apo AI release from HDL versus molar GdmCl concentration. These plots summarize the SEC chromatograms in Supplementary Figure 2.

Fig. 3.

In vitro kinetics of the SOF reaction versus HDL. HDL (0.27 mg/mL protein) were pre-equilibrated at 37 °C in a fluorescence cell in the sample chamber of the fluorimeter for 10 min, after which SOF (0.9 µg/mL) was added and the right-angle light scattering at 325 nm monitored over time. Grey lines are data points; black lines are a 2-parameter fit to an exponential rise to maximum from which the $I_{\max}$ and k values were obtained. Rate constants for (A) HDL, (B) AcHDL, and (C) HexHDL were 4.4 ± 0.02 , 4.5 ± 0.01 , and $1.1 \pm 0.01 \text{ h}^{-1}$.

Fig. 4. SEC of HDL following biochemical perturbation with SOF (A—C) and PLTP (D—F). (A, D) HDL, (B, E) AcHDL, and (C, F) HexHDL. HDL before (filled curve) and after (open curves) reaction with SOF and PLTP as labeled. Dashed lines denote elution volumes of neo HDL and LF apo AI as labeled

Fig. 5.

[^3H]CE uptake from HDL, AcHDL and HexHDL by human hepatoma Huh7 and CHO cell variants. A, B: Huh7 cells; C, D: CHO IdIA7 cells; E, F: CHO SR-BI cells. Time courses (A, C, E) for [^3H]CE uptake were conducted in quadruplicate. Rate constant k values were obtained from linear fit to the data. The rates of CE uptake (k , h^{-1}) in each of the cell lines were different as follows: (A) HDL > HexHDL, $p < 0.00002$; HDL < AcHDL, $p < 0.002$. (C) HDL < HexHDL, $p = 0.03$; HDL < AcHDL, $p = 0.001$. (E) AcHDL ~ HDL > HexHDL, $p < 0.00001$. Other rate constants were not significantly different. Dose-response curves for [^3H]CE uptake (B, D, F) were done in triplicate and fitted to a 2-parameter single rectangular hyperbola, from which the maximum uptake V_{max} and the added CE concentration at which uptake was 50% of maximum (K_m) values were obtained. Statistically significant differences at each dose are indicated as * HDL vs. AcHDL ($p < 0.05$); # HDL vs. HexHDL ($p < 0.05$). Statistical comparisons of rate constants from the regression fits are given in Table 1.

Fig. 6.

In vivo metabolism of HDL- $[^3\text{H}]$ CE and HexHDL- $[^3\text{H}]$ CE in C57BL/6J mice. A. Plasma $[^3\text{H}]$ CE clearance after injection of HDL as labeled. B. $[^3\text{H}]$ CE uptake by liver at 0, 0.5, 2, 4, 5, 8 and 24 h. C. Tissue uptake of sterols at 30 min. Asterisks indicate that HexHDL- $[^3\text{H}]$ CE uptake or clearance is significantly different from that of HDL-CE ($p < 0.05$) in liver, spleen and plasma. The data are the average of two independent experiments, each conducted in triplicate.

Fig. 7. Hepatic $[^3\text{H}]$ CE metabolism. Kinetics for hepatic (A) hydrolysis of $[^3\text{H}]$ CE and (B) formation of $[^3\text{H}]$ FC. Data were fitted to a 3-parameter exponential decay and rise to maximum respectively from which rate constants (k) were calculated as shown. C. Kinetics of hepatic $[^3\text{H}]$ oxysterol accretion. * Hepatic $[^3\text{H}]$ oxysterol formation from HexHDL is less than that from HDL at 30 min ($p < 0.05$).

Fig. 8.

³H-Sterol accretion in gallbladder bile. (A) Total bile sterol in gallbladder and (B) bile acids as a percent of total bile sterols over 5 h. (C) Total sterol in gallbladder bile and (D) bile acids as a portion of total bile sterols at 2 h. * Values for HexHDL and HDL are different ($p < 0.05$).

Table 1Kinetic Constants for Cellular [³H]CE Uptake

Cell Type	Kinetic Constants	HDL	AcHDL	HexHDL
Huh7	$k \text{ (h}^{-1}\text{)}^a$	0.57 ± 0.02	0.67 ± 0.02^b	$0.39 \pm 0.03^{b,c}$
ldlA7		0.25 ± 0.01	0.30 ± 0.01^b	0.30 ± 0.02^b
SR-BI		3.72 ± 0.20	3.89 ± 0.26	$2.30 \pm 0.15^{b,c}$
Huh7	$V_{\max}$ (nmol CE/mg protein)	4.6 ± 0.01	5.5 ± 0.2^b	$3.2 \pm 0.6^{b,c}$
ldlA7		4.9 ± 0.1	3.7 ± 0.2^b	3.9 ± 0.3^b
SR-BI		27.6 ± 2.5	22.5 ± 0.9	20.2 ± 1.8
Huh7	K_m (nmol CE/mL)	42 ± 2	43 ± 4	46 ± 2^b
ldlA7		93 ± 4	41 ± 4^b	45 ± 9^b
SR-BI		25 ± 7	12 ± 2	23 ± 6

^ak values were calculated from raw data in quadruplicate.^bDifferent from HDL, $p < 0.05$; additional statistical comparisons are in Figure 5 legend.^cDifferent from AcHDL, $p < 0.05$.