Enhanced Cellular Uptake of Remnant High-Density Lipoprotein Particles
A Mechanism for High-Density Lipoprotein Lowering in Insulin Resistance and Hypertriglyceridemia

Changting Xiao, Takehiko Watanabe, Yi Zhang, Bernardo Trigatti, Linda Szeto, Phil W. Connelly, Santica Marcovina, Tomas Vaisar, Jay W. Heinecke, Gary F. Lewis

Abstract—A low level of high-density lipoprotein (HDL) cholesterol is characteristic of insulin resistance and hypertriglyceridemia and likely contributes to the increased risk of cardiovascular disease associated with these conditions. One pathway involves enhanced clearance of lipolytically modified HDL particles, but the underlying mechanisms remain poorly understood. Here, we examine the effect of triglyceride enrichment and hepatic lipase hydrolysis on HDL binding, internalization, and degradation in cultured liver and kidney cells. Maximal binding of remnant HDL (HDL enriched with triglycerides followed by hepatic lipase hydrolysis), but not binding affinity, was markedly higher than native and triglyceride-rich HDL in both HepG2 cells and HEK293 cells. Compared with native and triglyceride-rich HDL, remnant HDL was internalized to a greater extent in both cell types and was more readily degraded in HEK293 cells. The increased binding of remnant HDL was not mediated by the low-density lipoprotein receptor or scavenger receptor class B type I (SR-BI), because enhanced remnant HDL binding was observed in low-density lipoprotein receptor–deficient cells with or without SR-BI overexpression. Disruption of cell surface heparan sulfate proteoglycans or blockage of apolipoprotein E–mediated lipoprotein binding also did not abolish the enhanced remnant HDL binding. Our observations indicate that remodeling of triglyceride-enriched HDL by hepatic lipase may result in enhanced binding, internalization, and degradation in tissues involved in HDL catabolism, contributing to rapid clearance and overall lowering of plasma HDL cholesterol in insulin resistance and hypertriglyceridemia. (Circ Res. 2008;103:159-166.)

Key Words: HDL • hepatic lipase • insulin resistance • hypertriglyceridemia

Population studies have shown a highly reproducible, inverse correlation between plasma concentrations of high-density lipoprotein cholesterol (HDL-c), apolipoprotein (apo)A-I and the risk of atherosclerotic cardiovascular diseases. A thorough understanding of the cellular/molecular mechanisms regulating HDL metabolism is important to the development of new therapeutic approaches to prevent cardiovascular diseases.

Low HDL is a characteristic feature of insulin resistance and hypertriglyceridemia, which is mainly attributable to enhanced clearance but not decreased production of HDL particles. The precise mechanisms underlying enhanced HDL catabolism are not clear. Intravascular remodeling of HDL by lipid transfer factors, lipases, and non-HDL lipoproteins plays a critical role in HDL metabolism. In insulin resistance and hypertriglyceridemia, HDL particles are enriched with triglycerides (TGs) by enhanced lipid exchange with the increased number and size of TG-rich lipoproteins, a process mediated by cholesteryl ester transfer protein. Increased hepatic lipase (HL) activity accompanies TG enrichment of HDL particles in these states. HL hydrolysis of TG-rich HDL induces the formation of pre–β1-HDL, leaving a residual α-migrating HDL particle referred to as remnant HDL. Remnant HDL particles have compositional and conformational features that are distinct from TG-rich HDL, and many of these features may affect their affinity and binding to cell surface binding sites. Shedding and rapid renal filtration and degradation of lipid-poor or lipid-free apoA-I that results from the HDL lipolysis may contribute to low HDL-c. We have previously demonstrated that TG enrichment in combination with HL lipolysis of HDL enhances HDL catabolism in vivo. The liver, kidney, and steroidogenic tissues are major sites of HDL catabolism. The liver has been recognized as the major site for selective

Original received November 21, 2007; revision received May 2, 2008; accepted June 2, 2008.
From the Departments of Medicine and Physiology (C.X., T.W., L.S., G.F.L.) and Department of Laboratory Medicine and Pathobiology (P.W.C.), University of Toronto, Ontario, Canada; Departments of Biochemistry and Biomedical Sciences (Y.Z., B.T.), McMaster University, Hamilton, Ontario, Canada; and Department of Medicine (S.M., T.V., J.W.H.), University of Washington School of Medicine, Seattle.
Correspondence to Dr. Gary F. Lewis, MD FRCP, Room EN12-218, The Toronto General Hospital, 200 Elizabeth St, Toronto, ON, M5G 2C4, Canada. E-mail gary.lewis@uhn.on.ca
© 2008 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org
DOI: 10.1161/CIRCRESAHA.108.178756

159
HDL lipid uptake mainly mediated through scavenger receptor class B type 1 (SR-BI) in the process of reverse cholesterol transport. The kidney is the principal tissue for the uptake of apoA-I, possibly through filtration at the glomerulus and catabolism by the renal tubular cells via the cubilin/megalin receptor complex. For these reasons, we chose to study the binding of HDL to human liver and kidney cell lines in the present study.

Clearance of HDL may be through receptor-mediated or nonreceptor-mediated pathways. Cholesteryl ester may be transferred to circulating apoB-containing lipoproteins and then delivered to the liver or may be selectively removed from the particle by the liver or extrahepatic tissues without catabolism of the whole particle. Alternatively, HDL may be removed by endocytic uptake and degradation of the whole particle, a process referred to as holoparticle uptake. Holoparticle HDL and apoA-I uptake and degradation are known to occur in both liver and kidney. A number of receptors have been identified that participate in either selective cholesteryl ester uptake or holoparticle uptake by tissues. SR-BI exhibits high-affinity binding to HDL and plays a major role in HDL selective lipid uptake. Other plasma membrane proteins such as cubulin, LDL receptor-related protein, HDL-binding proteins 1 and 2, HDL binding protein, apoA-I binding protein, and ectopic β-chain of ATP synthase have also been proposed as physiologically relevant HDL receptors, although their roles in HDL catabolism in vivo have not been fully elucidated.

We hypothesized that the more rapid HDL clearance from the circulation in insulin resistance and hypertriglyceridemia is associated with enhanced cellular uptake of HDL particles that are modified by TG enrichment and increased HL lipolysis. We prepared 3 species of HDL, ie, unmodified HDL, HDL enriched with TG from very-low-density lipoprotein (VLDL) ex vivo and TG-enriched HDL hydrolyzed by HL, and we examined their binding, internalization, and degradation in human liver and kidney cell lines. We further examined the potential roles of several factors in mediating their binding properties. We elected to use whole HDL, rather than 1 specific HDL subtraction (such as HDL2), firstly to mimic the conditions of our previous in vivo studies to relate the present in vitro findings to our previous in vivo observations and, secondly, to more closely mimic the real life situation in humans, in whom the HDL lipoprotein fraction is heterogeneous with respect to size, density, and composition.

Materials and Methods

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Cells

HepG2 (human hepatoma) and HEK293 (human embryonic kidney) cells were from the American Tissue Culture Collection and cultured in α-minimal essential medium and DMEM, respectively, both supplemented with 10% FBS. CHO cells and ldlA7 cells were maintained in Ham’s/F12 supplemented with 5% FBS and 2 mmol/L l-glutamine. ldlA7 cells with stable transfection of human SR-BI (ldlA[hsR-BI]) were selected and maintained in medium containing 400 μg/mL Geneticin (Invitrogen, Burlington, Ontario, Canada). One day before binding assays, CHO, ldlA7, and ldlA[hsR-BI] cells were washed and refed with medium containing 3% newborn calf lipoprotein-deficient serum.

Preparation and Analysis of HDL

Human fasting plasma was incubated with VLDL (final TG ~5 mmol/L) at 37°C for 6 hours to enrich HDL with TG. HDL (d = 1.063 to 1.25) was isolated by sequential ultracentrifugation and dialyzed. HDL from fasting plasma and from the plasma after TG enrichment are referred to as native and TG-rich HDL, respectively. HDL was purified from postheparin human plasma by heparin sepharose affinity chromatography. Half of the TG-rich HDL was incubated with HL at 34°C for 4 hours in the presence of 2.5% BSA and 60 U/mL heparin followed by isolation and dialysis. The lipolytically modified HDL is referred to as remnant HDL. HDL preparations were each iodinated by a modification of the iodium monochloride method, resolated, and dialyzed. Specific activities of the resolated HDL ranged from 9 to 36 cpm/ng protein.

Lipid composition of HDL was assayed using commercial kits. Particle size was determined by 4% to 30% nondenaturing PAGE. A multiplex ELISA (Linco Research, St Charles, Mo) was used to quantify apolipoproteins. Lipophosphatidylcholine species were analyzed by electrospray tandem mass spectrometry using a Sciex API 4000 instrument. An immunoaffinity chromatography method was used to separate apoA-I only and apoA-I/A-II particles of labeled HDL. Labeled HDL were subjected to isoelectrofocusing and bands corresponding to specific apolipoproteins were excised and counted for radioactivity.

Cell Binding Assays

Cell binding assays were performed at 4°C (HepG2 and HEK293 cells) or 37°C (CHO, ldlA7, and ldlA[hsR-BI] cells) for 150 minutes, and radioactivity in cell lysate was counted. Nonspecific binding was determined using 100× unlabeled HDL and was always <40% of total binding.

Competitive Inhibition Assays

Cells were incubated with 100 μg/mL labeled remnant HDL and increasing concentrations of unlabeled ligand for 150 minutes at 4°C, and radioactivity in cell lysate was counted.

Internalization Assay

After incubation with ligands at 37°C for 150 minutes, cells were washed and further incubated with excess cold ligands at 4°C for 150 minutes, and radioactivity in cell lysate was counted. Non-specific internalization was determined by adding 100× unlabeled HDL and was always <40% of the total internalization.

Degradation Assay

Cellular degradation of HDL was assessed by measuring trichloracetic acid-nonprecipitable material present in the medium.

Western Blot Analysis

HEK293 cell lysate was analyzed by 6% SDS-PAGE under reducing conditions and immunoblotted with a primary antibody against human cubilin (1:200) (Santa Cruz Biotechnology; sc-23644) and a goat anti-rabbit secondary antibody (1:4000) conjugated with horseradish peroxidase. Detection was by enhanced chemiluminescence. Rat kidney extract (Santa Cruz Biotechnology; sc-2394) was used as positive control. Western blotting of human SR-BI was as previously described.

Statistics

Results are presented as means±SEM. Binding and competition assays were analyzed using Prism (v3.03, GraphPad, San Diego, Calif). F tests were performed in Prism to compare fitting of the binding data to 1 or 2 classes of binding sites. Time- or concentration-dependent data were analyzed by ANOVA with Tukey’s post hoc test. Student’s unpaired t test was used to compare
hydrolysis of rabbit HDL

Table. Particle Size and Composition of HDL Species

<table>
<thead>
<tr>
<th>HDL Species</th>
<th>Mean radius (nm)</th>
<th>TG-Rich</th>
<th>Remnant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>4.95±0.09</td>
<td>5.14±0.07*</td>
<td>5.06±0.07*</td>
</tr>
<tr>
<td>Percentage of mass</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGs</td>
<td>5.2±0.3</td>
<td>12.7±1.5†</td>
<td>9.8±1.3†</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>15.3±1.0</td>
<td>12.5±0.5†</td>
<td>13.2±0.7†</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>22.6±1.0</td>
<td>18.2±0.8†</td>
<td>17.9±1.5*</td>
</tr>
<tr>
<td>Protein</td>
<td>56.9±1.9</td>
<td>57.9±1.4</td>
<td>60.5±1.5</td>
</tr>
<tr>
<td>Apolipoprotein (percentage of protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-I</td>
<td>74.0±1.1</td>
<td>75.1±0.9</td>
<td>74.4±1.1</td>
</tr>
<tr>
<td>A-II</td>
<td>20.7±1.1</td>
<td>21.1±1.3</td>
<td>22.8±1.2</td>
</tr>
<tr>
<td>E</td>
<td>1.9±0.3</td>
<td>1.5±0.2</td>
<td>0.7±0.04†</td>
</tr>
<tr>
<td>C-II</td>
<td>0.7±0.05</td>
<td>0.4±0.1*</td>
<td>0.3±0.1*</td>
</tr>
<tr>
<td>C-III</td>
<td>2.6±0.2</td>
<td>1.8±0.2*</td>
<td>1.7±0.1*</td>
</tr>
</tbody>
</table>

Data are mean±SEM (n=11–13 for sizing, n=12–15 for compositional assays and n=6 for specific apolipoprotein assays). *P<0.05 vs native; †P<0.01 vs native; ‡P<0.05 vs TG-rich.

Results

Modification of HDL Particle Composition by Preincubation With VLDL and HL Hydrolysis

Ex vivo incubation of human plasma with VLDL significantly enriched HDL particles with TG by ≈2-fold, with concomitant reduction in both cholesterol and phospholipids (Table), as was the case previously with HDL isolated from hypertriglyceridemic individuals and from subjects who received an IV infusion of Intralipid and heparin to enrich HDL with TG in vivo. HL hydrolysis of TG-rich HDL reduced the TG content without significantly altering the mass percentage of other components, consistent with ex vivo hydrolysis of rabbit HDL, and HDL isolated from subjects following in vivo stimulation of lipolysis by IV heparin injection. TG enrichment after incubation with VLDL also yielded larger particles, whereas HL hydrolysis tended to decrease the particle size as compared to TG-rich HDL. ApoA-I and apoA-II accounted for the majority of protein and their relative contribution by mass to the total protein was not significantly affected by TG enrichment or HL hydrolysis. ApoCIII was reduced in TG-rich HDL, and their relative quantity in HDL was not further modified by HL. Remnant HDL had lower apoE compared to other HDL species. Among the lysophosphatidylcholine species, those with unsaturated fatty acids (18:2, 20:4, 22:5, and 22:6) were relatively increased in remnant HDL (Figure 1A). Lysophosphatidylcholines with 18:0 were also increased in both TG-rich and remnant HDL because of transfer of 18:0 and TG from VLDL during the TG enrichment (Figure 1B).

Binding of Native, TG-Rich, or Remnant HDL in HepG2 and HEK293 Cells

Binding of HDL in both cell lines exhibited concentration dependence with increased binding of remnant HDL compared to native and TG-rich HDL (Figure 2A and 2C). The maximal binding of remnant HDL was greater than that of either TG-rich or native HDL (P<0.001 in HepG2 cells and P<0.0001 in HEK293 cells). Binding affinity was similar for all ligands in HepG2 cells, whereas it was lower for remnant than for native or TG-rich HDL in HEK293 cells (P<0.05). Bmax and Kd were similar between native and TG-rich HDL in both cell types. F tests of the binding suggested no more than one binding site was needed to account for the binding kinetics. Scatchard analysis of the binding kinetics suggested a common binding site for all HDL ligands in both cell types (Figure 2B and 2D).

Competitive Inhibition of Remnant HDL Binding by Native, TG-Rich, or Remnant HDL in HepG2 and HEK293 Cells

Labeled remnant HDL binding was inhibited by unlabeled ligands in a concentration-dependent fashion (Figure 3A and 3B). Inhibition of labeled remnant HDL binding exhibited similar patterns by these 3 unlabeled ligands, ie, unlabeled native, TG-rich, or remnant HDL equally displaced labeled remnant bound to both cell types, demonstrating similar binding affinity and likely common binding site of all HDL species.

Figure 1. Lysophosphatidylcholine (LysoPC) concentrations in native, TG-rich, and remnant HDL. The y axis shows concentrations of LysoPC in nanomoles of choline per 20 μg cholesteryl-phospholipid. Data are presented in A and B separately with 2 different y scales for clarity. *P<0.05 vs native; †P<0.05 vs TG-rich.
Cellular Internalization of Native, TG-Rich, or Remnant HDL in HepG2 and HEK293 Cells

In both HepG2 and HEK293 cells, total internalization was greater for remnant HDL than for native and TG-rich HDL (Figure 4A and 4B). For instance, at the highest concentrations measured, compared with native and TG-rich HDL, internalization of remnant HDL was 1.7- and 2.1-fold higher in HepG2 cells and 2.3- and 1.6-fold higher in HEK293 cells.

Cellular Degradation of Native, TG-Rich, or Remnant HDL in HepG2 and HEK293 Cells

HDL degradation was considerably higher in HEK293 cells than in HepG2 cells. At 180 μg/mL, degradation of native or TG-rich HDL was approximately double their degradation in HepG2 cells, and remnant HDL degradation was more than 3-fold higher than in HepG2 cells. In HepG2 cells, degradation of all three HDL species was similar. In contrast, remnant HDL degradation in HEK293 cells was greater than native or TG-rich HDL (P<0.01) (Figure 5A and 5B).

Cellular Binding of Native, TG-Rich, or Remnant HDL in CHO, ldlA7, and ldlA[hSR-BI] Cells

Expression of human SR-BI in ldlA[hSR-BI] cells was confirmed by immunofluorescence and Western blot (Figure 6A and 6B). Binding of HDL, regardless of modifications, to SR-BI expressing cells was greater than to control cells, indicating the role of SR-BI as an HDL receptor (Figure 6C).

Binding of remnant as compared to native and TG-rich HDL was greater regardless of SR-BI expression, ie, in wild-type CHO, ldlA7, and ldlA[hSR-BI] cells, suggesting that SR-BI was not responsible for the selective increase in remnant HDL binding. Because both ldlA7 cells and ldlA[hSR-BI] lack the LDL receptor, the results also suggest that the different binding properties of the HDL species are not accounted for by mediation through the LDL receptor.

Effects of Heparin Treatment on Cellular Binding of Native, TG-Rich, or Remnant HDL in HepG2 Cells

At low concentration (100 μg/mL), heparin selectively blocks lipoprotein binding to heparin sulfate proteoglycan (HSPG), but not to the members of the LDL receptor family or other lipoprotein receptors. At high concentration (5 mg/mL), heparin blocks apoE receptor–mediated lipoprotein binding to cells. Treatment of cells with heparin did not abolish the enhanced binding of remnant HDL to HepG2 cells (Figure 6D). Disruption of HSPG using heparinase yielded similar results (not shown). These results suggest that enhanced binding of remnant HDL cannot be accounted for by a process mediated by cell surface HSPGs or apoE receptors.

Discussion

In the present study, we have shown that remnant HDL, ie, HDL particles that were first incubated with VLDL to allow
exchange of lipids, and subsequently exposed to lipolytically active HL, exhibited higher binding capacity and internalization as compared with TG-rich and native HDL in liver and kidney cells and increased degradation in kidney cells. In several highly prevalent clinical scenarios, including the metabolic syndrome and type 2 diabetes, plasma concentrations of HDL-c and apoA-I are low.4,44 This is in large part attributed to enhanced HDL clearance, rather than reduced production.5 Under such conditions, HDL particles undergo extensive intravascular remodeling, including enrichment with TG and enhanced lipolysis by HL, whose activity is also increased in these metabolic states.8 Endothelial lipase is also elevated in insulin resistance 45 and likely also plays an important role in HDL remodeling.46 In previous in vivo studies, we have demonstrated that both ex vivo14 and in vivo15,46 lipolysis of TG-rich HDL by HL enhances HDL clearance from the circulation. TG-rich HDL is cleared more rapidly than TG-poor HDL in healthy humans16 but not in those with functionally defective HL.47 In the New Zealand white rabbit, an animal model naturally deficient in HL, TG enrichment of HDL per se, in the absence of HL, is insufficient to enhance HDL clearance.13 Collectively, these findings suggest that TG enrichment of HDL, combined with HL hydrolysis, is associated with enhanced HDL clearance in vivo. The present study provides in vitro evidence that remodeling of TG-enriched HDL by HL may promote HDL catabolism via enhanced binding to hepatic and renal cells.

The increased binding and internalization of remnant HDL was not attributable to a disproportionate enrichment of the particles with specific apolipoproteins after incubating with VLDL, because there was no difference between the HDL species used in our experiments with respect to their relative quantity of iodinated apolipoproteins (Figure I in the online data supplement). In addition, HL hydrolysis of the particle without prior TG enrichment and other compositional modifications that resulted from incubation with VLDL was insufficient to enhance binding and internalization, because hydrolysis of native HDL did not promote cell binding (supplemental Figure II). This latter experiment also demonstrates that any residual HL in the HDL fractions used in the binding experiments did not account for the enhanced binding of remnants through HL ligand binding activity. Thus, we have demonstrated in vitro, in accordance with our previous in vivo studies in rabbit,14,15 that both the prior exchange of lipids between HDL and VLDL and the subsequent lipolysis of HDL by HL are necessary to promote HDL cellular binding and clearance. Furthermore, all 3 HDL species used in our experiments contained a similar proportion of labeled lipoprotein AI/AII particles (66% in native, 62% in TG-rich, and 63% in remnant HDL), and the relative radioactivity of individual apolipoproteins did not differ between the three
HDL species (supplemental Figure I). Remnant HDL had relatively greater long-chain fatty acids in lysophosphatidylcholine, particularly polyunsaturated fatty acids. It has been proposed that HDL that contain a greater proportion of polyunsaturated fatty acids in lysophosphatidylcholine may have impaired capacity for reverse cholesterol transport. It would be informative if remnant HDL particles could be isolated directly from insulin resistant or hypertriglyceridemic subjects, to determine their precise composition and to test whether they behave in a pattern similar to that observed in our experimental system. Not only is it presently not technically possible to physically separate remnant HDL particles from nonremnant HDL species in human plasma but their more rapid clearance from the circulation in humans who have hypertriglyceridemia and higher HL activity would tend to relatively deplete the remnant HDL fraction, making them harder to isolate. In addition, endothelial lipase is also increased in these conditions, thus, HDL from these subjects would likely result from more complex vascular remodeling.

Remnant HDL was degraded to a greater extent in HEK293 cells than native and TG-rich HDL, which is in agreement with the finding that the kidney is the major site of HDL apoAI catabolism. The greater degradation of remnant HDL provides a possible explanation of HDL lowering in vivo. In monkeys made hypertriglyceridemic through inhibition of lipoprotein lipase, the increased catabolic rate of apoA-I was associated with increased renal catabolism. HDL isolated from humans with low HDL-c, or from humans with high HDL-c but enriched with TG and lipolytically modified, exhibited higher apoA-I accumulation in perfused rabbit kidney. In our experiments, HDL was degraded to a much lesser extent in HepG2 cells than in HEK293 cells. This may reflect the mechanism of retroendocytosis, where HDL holoparticle uptake is followed by recycling of HDL particles back to the cell surface and resecretion of cholesteryl ester–poor HDL, leading to net uptake of lipids.

We excluded several factors as playing a role in the enhanced binding of remnant HDL, including SR-BI, the LDL receptor, cell surface HSPGs, or apoE-mediated HDL binding. SR-BI plays a pivotal role in HDL cholesteryl ester selective uptake in the liver, but in our experiments in ldlA7 and ldlA[hSR-BI] cells indicate that remnant HDL binding is not preferentially increased in SR-BI overexpressing versus low SR-BI–expressing cells, suggesting that SR-BI does not account for the greater binding of HDL remnants compared to native or TG-rich HDL particles. HDL particles can acquire apoE and are ligands for the LDL receptor and other apoE receptors. Because the increased binding of remnant versus native and TG-rich HDL was observed in LDL receptor deficient cells, with a similar pattern of binding as was seen in HepG2 and HEK293 cells, it is unlikely that the increased binding of remnant HDL is mediated by the LDL receptor.
The enhanced remnant binding was also not abolished by blocking of apoE-mediated HDL binding or by disruption of cell surface HSPIgPs in HepG2 cells. Cubilin expression in HEK293 cells in the present culture conditions was not detected using Western blot (supplemental Figure III), and it is not expressed in HepG2 cells. Therefore, the enhanced binding of remnant HDL cannot be attributed to cubilin. We speculate that the enhanced binding of remnant HDL may not be attributable to a single factor; rather, it might be the amalgamated result of a series of events, such as modified lipid content and apolipoprotein composition, changes in particle size, surface charge, and HDL particle interaction with cell surface receptors. This is not surprising, considering the heterogeneous nature of the HDL particles and continuous remodeling of HDL particles in vivo. Precisely which receptor(s) plays a major role in binding and internalizing these lipolytically modified, TG-rich HDL particles will require further study.

In summary, the present studies have extended our previous in vivo observations to demonstrate that lipolytically modified TG-rich HDL have greater specific binding to and internalization in both liver cells and kidney cells and greater degradation in kidney cells, providing a biochemical mechanism for their more rapid clearance from the circulation. Further studies are needed to elucidate the nature of the HDL binding site and precisely which characteristics of the remnant HDL particles are responsible for the increased binding.

Acknowledgments

We thank Dr Peixiang Wang for help with the cubilin Western blot.

Sources of Funding

This work was supported by Heat and Stroke Foundation of Ontario grant T4979 (to G.F.L.) and the NIH grant HL 086798 (to J.W.H.). G.F.L. is the recipient of a Career Investigator Award from the Heart and Stroke Foundation of Canada and a Canada Research Chair in Diabetes (http://www.chairs.gc.ca). C.X. is supported by postdoctoral fellowships from Canadian Diabetes Association. T.V. is supported by a Pilot and Feasibility Award from the Diabetes and Endocrinology Research Center, University of Washington.

Disclosures

None.

References

Enhanced Cellular Uptake of Remnant High-Density Lipoprotein Particles: A Mechanism for High-Density Lipoprotein Lowering in Insulin Resistance and Hypertriglyceridemia
Changting Xiao, Takehiko Watanabe, Yi Zhang, Bernardo Trigatti, Linda Szeto, Phil W. Connelly, Santica Marcovina, Tomas Vaisar, Jay W. Heinecke and Gary F. Lewis

Circ Res. 2008;103:159-166; originally published online June 12, 2008;
doi: 10.1161/CIRCRESAHA.108.178756

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/103/2/159

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2008/06/12/CIRCRESAHA.108.178756.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
ONLINE DATA SUPPLEMENT

MATERIALS AND METHODS

Cells

The human hepatoma cell line (HepG2) and human embryonic kidney 293 (HEK293) cells were from the American Tissue Culture Collection. HepG2 cells were cultured in Alpha minimum essential medium and HEK293 cells were cultured in Dulbecco’s modified Eagles medium, both supplemented with 10% FBS. Wild-type CHO cells and CHO cells lacking LDL receptor (ldlA7) were maintained in Ham’s/F12 supplemented with 5% FBS and 2 mM L-glutamine. ldlA7 cells with stable transfection of human SR-BI (ldlA[hSR-BI]) were selected and maintained in medium containing 400 μg/mL Geneticin® (Invitrogen, Burlington, ON, Canada). One day before binding assays, CHO, ldlA7 and ldlA[hSR-BI] cells were washed and re-fed with medium containing 3% newborn calf lipoprotein-deficient serum.

Preparation and analysis of HDL

VLDL was isolated from plasma of healthy, normolipidemic human donors 3 to 6h after ingesting a high fat meal. Human fasting plasma anticoagulated with 5 mM EDTA was incubated with VLDL (final TG ~5 mM) at 37°C for 6h to enrich HDL with TG. HDL (d=1.063-1.25) was isolated by sequential ultracentrifugation and dialyzed. HDL isolated from fasting plasma and from the plasma after TG enrichment are referred to as native and TG-rich HDL, respectively. Lipolytically active HL was purified from post-heparin human plasma by heparin sepharose affinity chromatography. Half of the TG-rich HDL was incubated with purified HL at 34°C for 4h in the presence of 2.5% BSA.
and 60 U/mL heparin\textsuperscript{3,4}, followed by isolation and dialysis. The lipolytically-modified HDL is referred to as remnant HDL. HDL preparations were each iodinated using \textsuperscript{125}I-sodium (Perkin Elmer, Woodbridge, ON, Canada) by a modification of the iodine monochloride method\textsuperscript{5}, re-isolated and dialyzed. Specific activities of the re-isolated HDL ranged from 9 to 36 cpm/ng protein.

Lipid composition of HDL was assayed using commercial kits. Particle size was determined by 4-30\% non-denaturing PAGE\textsuperscript{6,7}. Peak radii of small and large particles were determined by densitometric scanning (ImageQuant TL; GE Healthcare) based on relative migration distance of standard-molecular-weight proteins of known diameter (HMW Calibration Kit, Pharmacia, Piscataway, NJ). The mean (or weighted) particle radius was calculated by multiplying the size of each band by its fractional area. This mean particle radius therefore combines the size distribution as well as the relative concentration of each band\textsuperscript{6}. A multiplex ELISA (Linco Research, St Charles, MO) was used to quantify apolipoproteins. Lysophosphatidylcholine species were analyzed by electrospray tandem mass spectrometry using a Sciex API 4000 instrument\textsuperscript{8}. An immunoaffinity chromatography method developed in our lab was used to separate apoA-I only and apoA-I/A-II particles of labeled HDL in small quantities with a high degree of reproducibility\textsuperscript{6,7}. To assess the distribution of specific apolipoproteins iodinated, labeled HDL were subjected to isoelectrofocusing\textsuperscript{9} and bands corresponding to specific apolipoproteins were excised and counted for radioactivity.
Cell binding assays

Cell binding assays were performed at 4°C (HepG2 and HEK293 cells) or 37°C (CHO, ldlA7 and ldlA[hSR-BI] cells). Cells were pre-incubated in medium with 4% BSA at 37°C for 20 min, washed twice with Hepes-HBSS (20 mM, pH 7.4) with 0.2% FFA-free BSA, then incubated in Hepes-HBSS with 0.2% FFA-free BSA for 30 min. Upon initiation of binding assays, cells were incubated with labeled HDL ligands in Hepes-HBSS with 0.2% FFA-free BSA. Where applicable, heparin sodium was added to the medium at 100 μg/mL or 5 mg/mL. After incubation for 150 min 200 μL of the medium was taken for radioactivity measurement. Cells were washed, lysed and radioactivity counted. Cell protein content was measured using the Lowry method. Nonspecific binding was determined using 100× unlabeled HDL and was always <40% of total binding.

Competitive inhibition assays

Cell were incubated and washed as above. After washing, 100 μg/mL labeled remnant HDL was added to each well with increasing concentrations of unlabeled ligand and incubated for 150 min at 4°C. After incubation, 200 μL of media was taken for radioactivity measurement. Cells were washed and lysed followed by determination of radioactivity and protein content.

Internalization assay

After incubation with ligands at 37°C for 150 min, cells were washed and further incubated with excess cold ligands at 4°C for 150 min to dissociate bound ligand.
Cells were lysed and radioactivity counted. Non-specific internalization was determined by adding 100× unlabeled HDL and was always <40% of the total internalization.

**Degradation assay**

Cellular degradation of HDL was assessed by measuring TCA-non-precipitable material present in the medium\(^{11}\).

**Western blot analysis**

HEK293 cell lysate was analyzed by 6% SDS-PAGE under reducing conditions and immunoblotted with a primary antibody against human cubilin (1:200) (Santa Cruz, sc-23644) and a goat anti-rabbit secondary antibody (1:4000) conjugated with horseradish peroxidase. Detection was by enhanced chemiluminescence. Rat kidney extract (Santa Cruz, sc-2394) was used as positive control. Western blotting of human SR-BI was as previously described\(^{12}\).

**Statistics**

Results are presented as mean ± SEM. Binding and competition assays were analyzed using a weighted, nonlinear, curve-fitting program Prism (v3.03, GraphPad, San Diego, CA). F-tests were performed in Prism to compare fitting of the binding data to one or two classes of binding sites. Time- or concentration-dependent data were analyzed by ANOVA with Tukey’s post-hoc test. Student’s unpaired t-test was used to compare kinetic parameters or effects between different HDL preparations. A p value of <0.05 was considered significant.
ONLINE FIGURE LEGENDS

Figure S1. Distribution of radioactivity in specific apolipoproteins in HDL preparations. 125I-labeled HDLs were subjected to IEF and bands on gels corresponding to specific apolipoproteins were cut and counted for radioactivities. E+ denotes apoE and other unidentifiable proteins above apoA-I band on the gel. Bands for apoCII and CIII-1 were not cut separately due to their close vicinity on the gel.

Figure S2. Binding of native HDL (i.e. HDL without prior incubation with VLDL) and native HDL subjected to HL hydrolysis in ldlA7 and ldlA[hSR-B1] cells. Cells were incubated with 50 μg/mL HDL for 2h at 4 C.

Figure S3. Western blot of human cubilin in HEK293 cell lysates, showing no expression of cubilin in HEK293 cells. Cell lysate was analyzed by 6% SDS-PAGE under reducing conditions and immunoblotted with an antibody against human cubilin and a goat anti-rabbit secondary antibody conjugated with horseradish peroxidase. Detection was by enhanced chemiluminescence. Rat kidney extract was used as positive control.
Reference List


Figure S1.

Figure S2.

Figure S3.

Supplementary Figures