Hyperhomocysteinemia Decreases Circulating High-Density Lipoprotein by Inhibiting Apolipoprotein A-I Protein Synthesis and Enhancing HDL Cholesterol Clearance

Dan Liao, Hongmei Tan, Rutai Hui, Zhaohui Li, Xiaohua Jiang, John Gaubatz, Fan Yang, William Durante, Lawrence Chan, Andrew I. Schafer, Henry J. Pownall, Xiaofeng Yang, Hong Wang

Abstract—We previously reported that hyperhomocysteinemia (HHcy), an independent risk factor of coronary artery disease (CAD), is associated with increased atherosclerosis and decreased plasma high-density lipoprotein cholesterol (HDL-C) in cystathionine β-synthase–apolipoprotein E–deficient (CBS–/–/apoE–/–) mice. We observed that plasma homocysteine (Hcy) concentrations are negatively correlated with HDL-C and apolipoprotein A1 (apoA-1) in patients with CAD. We found the loss of large HDL particles, increased HDL-free cholesterol, and decreased HDL protein in CBS–/–/apoE–/– mice, and attenuated cholesterol efflux from cholesterol-loaded macrophages to plasma in CBS–/–/apoE–/– mice. ApoA-1 protein was reduced in the plasma and liver, but hepatic apoA-1 mRNA was unchanged in CBS–/–/apoE–/– mice. Moreover, Hcy (0.5 to 2 mmol/L) reduced the levels of apoA-1 protein but not mRNA and inhibited apoA-1 protein synthesis in mouse primary hepatocytes. Further, plasma lecithin:cholesterol acyltransferase (LCAT) substrate reactivity was decreased, LCAT specific activity increased, and plasma LCAT protein levels unchanged in apoE–/–/CBS–/– mice. Finally, the clearance of plasma HDL cholesteryl ester, but not HDL protein, was faster in CBS–/–/apoE–/– mice, correlated with increased scavenger receptor B1, and unchanged ATP-binding cassette transporter A1 protein expression in the liver. These findings indicate that HHcy inhibits reverse cholesterol transport by reducing circulating HDL via inhibiting apoA-1 protein synthesis and enhancing HDL-C clearance. (Circ Res. 2006;99:598-606.)

Key Words: apoA-1 • coronary heart disease risk • HDL cholesterol • hyperhomocysteinemia

Hyperhomocysteinemia (HHcy) is a significant independent risk factor for coronary artery disease (CAD).1,2 Although the mechanistic link among HHcy, dyslipidemia, and atherosclerosis is unclear, it has been suggested that HHcy alters hepatic lipid metabolism, thereby contributing to fatty liver, a condition found in humans and animals with HHcy.3 In apoE-null mice, deletion of the gene for cystathionine β-synthase (CBS), which converts homocysteine (Hcy) to cystathionine, leads to severe HHcy and increased aortic lesions that are associated with increased plasma total cholesterol (TC) and decreased high-density lipoprotein cholesterol (HDL-C).4 Clinical studies of patients with CAD reveal a negative correlation between plasma levels of Hcy and HDL-C. A prospective study evaluating survival of patients with angiographically defined CAD found increased mortality and decreased HDL-C in patients with the highest Hcy levels. Thus, HHcy predicts all-cause mortality in CAD, independent of traditional risk factors.5 Case-control studies have provided supporting data, showing that plasma Hcy is negatively correlated with HDL-C levels in patients with myocardial infarction.6 Furthermore, we and others reported that elevated plasma Hcy concentrations in male CAD were associated with lower HDL-C.7,8,9,10 These studies suggest that altered HDL metabolism may be mechanistically linked to cardiovascular disease (CVD) in humans. Low plasma HDL-C (hypo–α-lipoproteinemia) is a risk factor for atherosclerosis11 and a component of the metabolic syndrome frequently found in patients with type 2 diabetes. As HDL metabolism plays a key role in the prevention of CVD, identifying the mechanistic links among HHcy, low HDL-C, and CVD could direct the design of new lifestyle and pharmacological interventions. Therefore, we studied the effect of Hcy on HDL-C in patients with CAD and explored the mechanism(s) by which HHcy alters HDL metabolism in CBS–/–/apoE–deficient (CBS–/–/apoE–/–) mice, a genetic

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model of both HHcy and atherosclerosis, and in mouse primary hepatocytes.

Materials and Methods

CBS and CBS/ApoE Mice

The creation, characterization, and care of the CBS/apoE double knockout mice previously has been as described. Mice were genotyped by PCR and fed a standard rodent diet. Age-matched littersmates (15 to 20 weeks old) with even sex distribution were selected for each experiment.

Human CAD Subjects and Biochemical Tests

Male patients with angiographically confirmed CAD (at least 70% obstruction of 1 or more major coronary arteries; ages ranging from 53 to 73 years) were recruited from Fuwai Hospital (Beijing, China). A medical history and record review revealed CAD risk factors, including diabetes, hypertension, history of hypercholesterolemia, and cigarette smoking. Exclusion criteria included diabetes mellitus, hypothyroidism, renal dysfunction, and hepatic failure. All patients were treated with statin or other lipid-lowering drugs. Blood was drawn after an overnight fast and collected in EDTA-coated tubes for Hcy and lipid analysis using high-performance liquid chromatography (HPLC) and standard assays. This study protocol was approved by the institutional review board of the Ministry of Public Health in China. Informed consent was obtained from all subjects. Apolipoprotein A-I (apoA-I) protein was measured using a turbidimetric immunoassay (Wako Chemicals).

Plasma Hcy, Lipid, and ApoA Analysis

Mouse blood was collected into a 4 mmol/L EDTA-coated tube after a 4-hour fast. Hcy concentrations were measured by liquid chromatography/electrospray tandem mass spectrometry methods, and total cholesterol (TC) and triglycerides (TGs) were measured using identical method described. Free cholesterol (FC), phospholipid (PL), and HDL-C were measured by new approaches using commercial kits (Wako Chemicals).

HDL Isolation

Pooled mouse plasma was adjusted to a density of 1.063 g/mL with KBr and centrifuged in a TL100.2 rotor (95 000 rpm) at 15°C for 18 hours. The top one-third layer containing very-low-density lipoprotein (VLDL), intermediate density lipoprotein (IDL), and low-density lipoprotein (LDL) was removed. The remaining material was adjusted to d = 1.21 g/mL with KBr and centrifuged. The top third of the HDL layer was then collected and dialyzed against PBS containing 0.01% EDTA (EDTA-PBS) at 4°C overnight.

TABLE 1. Plasma Levels of Hcy and Lipid in CBS/ApoE Mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>Hcy (mmol/L)</th>
<th>TC (mg/dL)</th>
<th>FC (mg/dL)</th>
<th>TGs (mg/dL)</th>
<th>PL (mg/dL)</th>
<th>Non–HDL-C (mg/dL)</th>
<th>HDL-C (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBS+/+apoE−/−</td>
<td>14</td>
<td>3.8 ± 0.9</td>
<td>387.7 ± 111.3</td>
<td>109.9 ± 27.8</td>
<td>101.8 ± 56.6</td>
<td>283.3 ± 41.9</td>
<td>342.1 ± 118</td>
<td>31.8 ± 10.2</td>
</tr>
<tr>
<td>CBS−/−apoE−/−</td>
<td>14</td>
<td>7.4 ± 2.9*</td>
<td>465.9 ± 138.3</td>
<td>139.8 ± 32.7</td>
<td>135.8 ± 97.2</td>
<td>345.0 ± 66.6*</td>
<td>342.1 ± 118</td>
<td>27.8 ± 6.1</td>
</tr>
<tr>
<td>CBS−/−apoE−/−</td>
<td>12</td>
<td>210.4 ± 80.0*</td>
<td>561.1 ± 95.0*</td>
<td>188.7 ± 49.1*</td>
<td>77.2 ± 57.9</td>
<td>353.0 ± 55.5*</td>
<td>542.8 ± 191*</td>
<td>21.8 ± 5.3*</td>
</tr>
</tbody>
</table>

Plasma levels of Hcy and lipids in CBS/apoE mice. Plasma was collected for analysis from mice aged 15–20 weeks. Note that FC, TC, and non–HDL-C levels are increased and HDL-C is decreased in HHcy mice. Values are expressed as mean ± SD. *P < 0.05 vs CBS+/+apoE−/− mice, †P < 0.05 vs CBS−/−apoE−/− mice.

Human and Mouse Lipoproteins

Human and mouse lipoproteins were isolated by 1-step KBr gradient-sequential ultracentrifugation (95 000 rpm) at d = 1.21 g/mL for 12 hours at 15°C. The top one-third layer containing VLDL, IDL, LDL, and HDL was collected and dialyzed against EDTA-PBS at 4°C overnight. Total lipoproteins (200 µL) were analyzed by fast protein liquid chromatographic (FPLC) using an Amersham-Phararama Biotech AKTA-FPLC system equipped with 2 Superose 6 columns arranged in tandem. The column effluent was monitored by absorbance at 280 nm. The elution volumes for HDL, LDL, and LDL were determined by chromatography of authentic lipoprotein standards.

Lipoprotein Structural Analysis by NMR

Lipoprotein size and subclass profiles were measured in 150 µL of plasma by 400-MHz proton NMR spectroscopy at Liposcience (Raleigh, NC). HDL subclass particle concentrations, in units of micromoles per liter, were measured for 7 subclasses: H1 (7.3 to 7.7 nm), H2 (7.8 to 8.2 nm), H3 (8.3 to 8.9 nm), H4 (9.0 to 10.5 nm), H5 (10.6 to 12.5 nm), H6 (12.6 to 13.5), and H7 (13.5 to 14.5). HDL subclasses were grouped into small (diameter, 7.3 to 8.2 nm), intermediate (diameter, 8.3 to 8.9 nm), and large HDL (diameter, 9.0 to 14.5 nm). Average HDL and VLDL particle size was determined by weighing the relative percentage of each subclass against its diameter.

HDL Compositional Analysis by Biochemical Measurement

Isolated mouse HDL composition was based on concentrations of FC, CE, TG, and PL, as determined by commercial kits (Wako Chemicals).

Cholesterol Efflux Assay

The cholesterol efflux capacity of mouse plasma or HDL function was determined on [3H]-cholesterol–labeled RAW264.7 cells. Subconfluent RAW264.7 cells were cultured on 24-well plates and incubated for 16 hours with 0.5 Ci of [3H]-cholesterol plus 0.3 mmol/L [8-(4-chlorophenylthio)adenosine 3′:5′-cyclic monophosphate sodium salt (CPT-cAMP)] (Sigma-Aldrich), an ATP-binding cassette transporter A1 protein (ABCA1) activator, and 2 µg/mL compound SS035 (provided gratis by Novartis) to inhibit intracellular cholesterol esterification. After extensive washing with PBS containing 0.2% BSA, cells were equilibrated with fresh serum-free DMEM containing 2 mg/mL fatty acid–free albumin for 1 hour and replaced with fresh serum-free DMEM in the presence or absence of 0.5% plasma from CBS/apoE mice. Mouse plasma-

TABLE 2. Plasma Levels of Hcy and Lipid in Male CAD Patients

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Age (y)</th>
<th>Hcy (µmol/L)</th>
<th>TC (mg/dL)</th>
<th>TGs (mg/dL)</th>
<th>LDL-C (mg/dL)</th>
<th>HDL-C (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Hcy</td>
<td>8</td>
<td>61 ± 8</td>
<td>9.1 ± 1.0</td>
<td>187.3 ± 34.4</td>
<td>149.7 ± 54.9</td>
<td>102.1 ± 23.1</td>
<td>48.9 ± 10.0</td>
</tr>
<tr>
<td>Moderate HHcy</td>
<td>17</td>
<td>63 ± 10</td>
<td>24.2 ± 2.5*</td>
<td>189.6 ± 27.6</td>
<td>129.9 ± 78.9</td>
<td>108.7 ± 26.6</td>
<td>46.2 ± 8.1</td>
</tr>
<tr>
<td>Intermediate HHcy</td>
<td>15</td>
<td>63 ± 10</td>
<td>48.7 ± 18.4*</td>
<td>173.6 ± 40.7</td>
<td>133.6 ± 83.0</td>
<td>93.5 ± 25.5</td>
<td>36.9 ± 6.5*</td>
</tr>
</tbody>
</table>

Plasma levels of Hcy and lipids in CAD patients. Blood samples from fasted male CAD patients were collected and assayed for Hcy and lipid levels. Note that HDL-C levels are decreased in HHcy CAD patients. Values are mean ± SD. *P < 0.01 vs normal HHcy group, †P < 0.01 vs moderate HHcy.
deficient medium was a negative control for background cholesterol efflux. After 3 hours of incubation, medium and cell lysates (in 0.2 M NaOH) were collected and analyzed by liquid scintillation counting. Cholesterol efflux activity of mouse plasma was expressed as the ratio of medium radioactivity to the sum of medium and cellular radioactivity minus background cholesterol efflux.

**Lecithin:Cholesterol Acyltransferase Activity Assay**

The activity of lecithin:cholesterol acyltransferase (LCAT), a key enzyme for HDL maturation, was determined by measuring the conversion of [3H]-cholesterol to CE. Briefly, [3H]-cholesterol-proteoliposome (apoA-I/lecithin/cholesterol, 0.8/250/12.5 mol) was prepared as an artificial exogenous substrate.16 Mouse plasma (10 μL) was incubated with 100 μL of [3H]-cholesterol-proteoliposome (62.5 μCi/mL) for 30 minutes at 37°C. Alternatively, mouse plasma (20 μL) was used as the endogenous substrate and incubated with 10 μL of tracer [3H]-cholesterol-albumin emulsion (5 μCi/mL, 2% BSA) for 4 hours at 4°C and then for 30 minutes at 37°C. Lipids were extracted into hexane/ethyl acetate (8:2, vol/vol) and analyzed by thin layer chromatography.37 [3H]-FC and [3H]-CE were removed by scraping and analyzed by liquid scintillation counting. LCAT activity or endogenous cholesterol esterification was expressed as the fraction of total radioactivity converted to CE; each was corrected for cholesterol esterification in the absence of plasma.

**HDL Clearance in CBS/ApoE Mice**

Mouse HDL from apoE−/− mice was labeled with [3H]-cholesteryl-hexadecyl-ether (PerkinElmer Life and Analytical Sciences) or with 21, using the iodine monochloride method.19 [3H]-CE–HDL or [125I]-HDL were injected intravenously into mice, through the femoral vein (≈106 cpm/10 g body weight). Mouse tail blood (20 μL) was collected at 0.5, 5, 15, and 30 minutes and, subsequently, at 1, 3, 6, and 9 hours after injection and analyzed. Radioactivity at 0.5 minutes postinjection was defined as 100%, and the fractional catabolic rate (FCR) was calculated as the inverse of the area under the decay curves using an exponential curve fitting the data points through nine hours.20

**Hepatocyte Culture and Treatment**

Mouse primary hepatocytes were isolated from 8- to 10-week-old CBS+/+apoE−/− mice by collagenase liver perfusion and purified through a Percoll gradient.21 The cells (4×106) were plated on collagen I (100 μg/mL) coated 35-mm dishes and cultured in William’s E Media containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 10−8 mol/L insulin and 10−6 mol/L dexamethasone for 4 hours to allow cell attachment. Cells were changed to the Hepatocyte medium (11705-021, Invitrogen) and treated with DL-Hcy for another 24 hours. Culture medium were collected to determine the level of secreted apoA-I. Total RNA and cell lysate were collected for apoA-I immunoprecipitation using a goat anti-mouse apoA-I antibody (US Biological) and protein G-Sepharose (Amersham) and analyzed by 12% SDS-PAGE. Purified mouse apoA-I protein standard (2 μg/lane, Biodigest) was mixed with each sample before electrophoresis for visual identification of the apoA-I bands. The gels were stained with Coomassie blue, and the bands corresponding to apoA-I were excised and counted.

The results were normalized to cellular protein content. The t1/2 was calculated using the equation t1/2=(ln2)/λ, where λ=decay constant, based on the calculated best-fit exponential decay curves.

**Western Blot Analysis**

Mouse plasma (1 μL), hepatocyte culture medium (15 μL), liver extracts (100 μg), or primary hepatocyte lysate (60 μg) were analyzed by Western blot analysis with antibodies against mouse apolipoprotein A-I, apoA-II (Biodigest), SRB-I (Novus Biologicals), LCAT (provided gratis by John S. Parks, Wake Forest University), and ABCA1 (provided gratis by Michael Fitzgerald, Massachusetts Institute of Technology). Blots were rebotted with mouse α-tubulin, β-actin, or albumin antibodies (Invitrogen and Molecular Probes) for a loading control. Plasma blots were stained with 1% Ponceau S for loading controls.

**Northern Blot analysis and Real-Time PCR**

Northern blot analysis and real-time PCR were performed as described22,24 and as detailed in the expanded Materials and Methods section in the online data supplement, available at http://circres.ahajournals.org.

**Figure 1. Correlations of Hcy with HDL-C and apoA-I in CAD patients.** Plasma of fasted male CAD patients was analyzed for HDL-C, apoA-I protein, and Hcy concentrations. Regression analysis was performed. Each data point represents 1 patient. Inner bar graph shows HDL-C or apoA-I protein levels in grouped CAD patients. A, Correlation of HDL-C concentration vs plasma levels of Hcy. B, Correlation of apoA-I protein concentration vs plasma levels of Hcy. Note that both HDL-C and apoA-I concentrations are negatively correlated with Hcy levels. Values represent mean±SD. *P<0.01 vs normal HHCy, #P<0.01 vs moderate HHcy,
HHcy Is Associated With Increased TC, FC, PL, and Non–HDL-C and Decreased HDL-C Levels in CBS⁻/⁻/ApoE⁻/⁻ Mice

Consistent with our previous observation, CBS⁻/⁻/apoE⁻/⁻ mice had a 2-fold increase in plasma Hcy levels (Hcy, 7.4 μmol/L). CBS⁻/⁻/apoE⁻/⁻ mice developed severe HHcy (plasma Hcy, 210.4 μmol/L), which was associated with significantly increased TC, FC, PL, and non–HDL-C and with decreased HDL-C levels (Table 1). The decrease in HDL-C in CBS⁻/⁻/apoE⁻/⁻ mice by chemical analysis is consistent with our previous observation by size fractionation of standard lipoprotein agarose electrophoresis analysis.⁴

HHcy Is Negatively Correlated with HDL-C and ApoA-I Levels in CAD Patients

After excluding 3 cases with combined diabetes mellitus and renal dysfunction, 40 male CAD subjects were enrolled and divided into 3 groups, based on plasma Hcy levels, in accordance with established standards,²⁵ as normal (≤10 μmol/L), moderate (11 to 30 μmol/L), and intermediate (31 to 100 μmol/L) HHcy (Table 2). Plasma TC, TG, and LDL-C concentrations in the 3 groups were not different. In contrast, HDL-C levels in CAD patients in the intermediate HHcy group were lower than those of the normal Hcy group (49 versus 37 mg/dL). Increased Hcy levels were negatively correlated with HDL-C concentration in CAD patients (Figure 1A). Similarly, apoA-I levels in intermediate HHcy CAD patients were lower than those in the normal Hcy group (126 mg/dL versus 85 mg/dL) and negatively correlated with Hcy levels (Figure 1B).

HHcy Is Associated with Hypo-α-Lipoproteinemia in CAD Patients and in CBS⁻/⁻/ApoE⁻/⁻ Mice

According to FPLC, which separates lipoproteins on the basis of size, HHcy is associated with decreased HDL protein in CAD patients and CBS⁻/⁻/apoE⁻/⁻ mice. A shoulder in HDL profile of normal Hcy subjects and a slightly left-shifting HDL profile in control mice suggest a bias toward larger HDL particle in normal Hcy and smaller HDL in the presence of HHcy in both human and mice. The fractions of IDL/LDL and VLDL were divergent, with reduced levels in HHcy CAD patients and increased levels in CBS⁻/⁻/apoE⁻/⁻ mice (Figure 2A).

HHcy Reduces HDL Size and Large HDL Particle Concentration and Increased VLDL Size in CBS⁻/⁻/ApoE⁻/⁻ Mice

NMR spectroscopy provides a direct measure of lipoprotein particle sizes and concentrations in plasma, with quantification based on the amplitudes of spectral signals that are size dependent.²⁶ NMR analysis showed that the total number of HDL particles was the same in all 3 groups...
Figure 3. HDL particle composition, plasma efflux ability and LCAT activity in CBS/apoE mice. A, HDL particle composition. HDL was isolated from pooled plasma for analysis of the concentrations of protein and lipids. Note that the HDL total protein content is decreased and that the percentage of HDL-FC is increased in CBS+/-/apoE-/- mice. B, Cholesterol efflux. Mouse plasma was applied in the culture medium of RAW264.7 cells loaded with [3H]-cholesterol. The percentage of cholesterol efflux was determined. C, Plasma LCAT substrate reactivity on endogenous substrates was measured using plasma lipoproteins as substrate. D, Plasma LCAT specific activity against an exogenous substrate, which provides a measure of LCAT specific activity, was higher in HHcy mice (1.0±0.1%, 1.3±0.2%, and 1.4±0.1%, respectively, for CBS+/-/apoE-/-, CBS+/-/apoE-/-, and CBS+/-/apoE-/- mice; Figure 3D).

HHcy Increases the Percentage of HDL-FC and Decreases HDL Total Protein in CBS+/-/apoE-/- Mice

A compositional analysis of HDL particles isolated from the pooled mouse plasma showed that the percentage of HDL-FC was twice as high in CBS+/-/apoE-/- mice (8%) compared with CBS+/-/apoE-/- mice (4%). In contrast, total HDL protein was lower in CBS+/-/apoE-/- mice (69 mg/dl) compared with CBS+/-/apoE-/- mice (116 mg/dl) (Figure 2D). The decrease in HDL particle size was attributable to a reduction in large HDL subclasses (H4–7) (Figure 2C). In contrast, VLDL mean size is increased in CBS+/-/apoE-/- mice (Figure in the online data supplement).

Cholesterol Efflux to HHcy Plasma and Endogenous Cholesterol Esterification Is Lower in CBS+/-/apoE-/- Mice

HDL function was assessed by measuring cholesterol efflux from cholesterol-loaded RAW264.7 cells to mouse plasma. Cellular cholesterol efflux to plasma from CBS+/-/apoE-/- mice was ~26% lower than that of plasma from CBS+/-/apoE-/- mice (8.1% versus 10.9%; Figure 3B). Endogenous cholesterol esterification was lower in CBS+/-/apoE-/- (7.5±2.9%) and CBS+/-/apoE-/- mice (9.3±1.8%) than in CBS+/-/apoE-/- mice (12.3±2.6%; Figure 3C). In contrast, activity against an exogenous substrate, which provides a measure of LCAT specific activity, was higher in HHcy mice (1.0±0.1%, 1.3±0.2%, and 1.4±0.1%, respectively, for CBS+/-/apoE-/-, CBS+/-/apoE-/-, and CBS+/-/apoE-/- mice; Figure 3D).

Clearance of HDL-CE, but Not HDL Protein, Is Faster in CBS+/-/apoE-/- Mice

Because HDL-CE is cleared by a selective mechanism that is mediated by the hepatic receptor SRB-I, we studied HDL metabolism in vivo by comparing the rates of plasma clearance of HDL-CE and HDL protein by examining the rate of disappearance of intravenously injected mouse HDL-[1H]-CE and HDL-[13C]apolipoprotein. HDL-[1H]-CE clearance was faster in CBS+/-/apoE-/- mice, whereas the rates of HDL-[13C]-protein clearance were the same in all 3 groups of mice (Figure 4).

Decreased ApoA-I and Increased SRB-I Hepatic Protein Levels in CBS+/-/apoE-/- Mice

Western blot analyses showed that apoA-I was markedly lower in the plasma, purified HDL, and liver of CBS+/-/apoE-/- mice (Figure 5A through 5C). ApoA-I was present in
the fraction <1.21 g/mL (Figure 5B) and undetectable by Western Blot analysis in the bottom fraction during mouse HDL purification. ApoA-II, the second abundant HDL protein, was slightly decreased in CBS−/−/apoE−/− mice. In contrast, HHcy was associated with increased expression of hepatic SRB-I protein, whereas the expression of ABCA1 was unchanged. Hepatic mRNA levels of apoA-I, SRB-I, and ABCA1 were similar in mice of 3 genotypes by Northern blotting (Figure 5D). Unchanged mRNA levels of apoA-I and SRB-I in the liver was confirmed by real-time PCR quantification (Figure 5E and 5F).

**Hcy Reduces the Levels of Secreted and Intracellular ApoA-I Protein by Inhibiting Protein Synthesis in Mouse Primary Hepatocytes**

We examined the effect of Hcy on the expression of apoA-I and SRB-I in freshly isolated primary hepatocytes. ApoA-I mRNA levels were unchanged by real-time PCR quantification (Figure 6A). Both secreted and intracellular apoA-I protein were reduced by Hcy (0.5 to 2 mmol/L) in a dose-sensitive fashion (Figure 6B). Pulse-chase experiments demonstrated that Hcy significantly reduced the incorporation of [3H]-leucine into apoA-I to 32% and 51% in the medium and cell lysate after 1 hour of pulsing and to 50% and 48% after 2 hours of chasing compared with parallel controls (Figure 6C and 6D). The half-life ($t_{1/2}$) of newly synthesized apoA-I was unchanged in Hcy group (3.72 versus 3.28 hours in control group; Figure 6E).

**Discussion**

Although HHcy is a risk factor for CVD, the underlying mechanism remains unknown. Our earlier report of an association between HHcy with reduced plasma HDL-C and arterial lesions in mice pointed to HDL as a mediator of atherogenesis in HHcy. The major focus of this study was to study HDL metabolism in HHcy and to identify underlying metabolism.

Non–HDL-C was not changed in CAD patients with HHcy (Table 1), an effect that may be related to lipid-lowering medication used by all enrolled CAD patients. In contrast, plasma Hcy and HDL-C levels show a remarkable negative correlation in CAD patients (Figure 1A). Moreover, with increasing plasma Hcy, there is a concurrent decrease in the major HDL protein apoA-I (Figure 1B). The human studies were not initially designed to distinguish Hcy with lipid lowering therapy and were not the main part of this study. However, it is consistent with our animal studies. More clinical studies on large population are necessary to confirm the direct link of HHcy and HDL reduction in CVD.

To our knowledge, we have demonstrated for the first time that plasma Hcy level has a significant, negative correlation with apoA-I concentration in human CAD. A similar correlation was reported recently by Rozen and colleagues. Our and Rozen’s observations were confirmed in CBS/apoE-deficient mice, a disease model of HHcy and atherosclerosis. Whereas HDL-C levels in patients with intermediate HHcy were 25% lower than those in normal patients, CBS−/−/apoE−/− mice, which develop spontaneous atherosclerosis that correlates with plasma Hcy levels, had HDL-C levels that were 31% lower than those of control mice; this is consistent with FPLC data showing decreased HDL fractions in HHcy human CAD and CBS−/−/apoE−/− mice (Figure 2A). Most likely, Hcy-related HDL reduction is independent of apoE deficiency, because HHcy CAD subjects do not share
the apoE-deficiency background. Therefore, our data reveal a clear connection between plasma Hcy levels and dysregulated HDL metabolism. The association between plasma Hcy and HDL-C/HDL protein in both mouse and human suggest that the CBS\(^{-/-}\)/apoE\(^{-/-}\) mouse is a valid disease model for subsequent mechanistic studies of HDL metabolism in HHcy.

CBS\(^{-/-}\)/apoE\(^{-/-}\) mice have higher levels of circulating TC, FC, PL, and non–HDL-C and lower HDL-C (Table 2). However, recent studies have reported that TC and PL are increased in only the liver and not in the circulation of neonatal CBS\(^{-/-}\)/apoE\(^{-/-}\) mice,\(^{27}\) nor in adult CBS\(^{-/-}\)/apoE\(^{-/-}\) mice fed a high methionine (HM) diet (Hcy, 27 \(\mu\)mol/L) or a HM/low-folate diet (Hcy, 48 \(\mu\)mol/L).\(^{3}\) The increase in TC and PL in the CBS\(^{-/-}\)/apoE\(^{-/-}\) mice in our study may be related to the combination of hyperlipidemia and severe HHcy in adult mice. The larger VLDL peak in the chromatograph of FPLC and increased VLDL particle mean size by NMR (supplemental Figure) suggest an increase in larger VLDL particles in the CBS\(^{-/-}\)/apoE\(^{-/-}\) mice. These may be explained by increased cholesterol content in the VLDL particle in these mice, as shown in our earlier report.\(^{4}\)

HDL is central to reverse cholesterol transport (RCT), the cardioprotective mechanism by which cholesterol synthesized in peripheral tissue is transported to the liver for degradation or recycling. Within the RCT pathway, cellular cholesterol efflux transfers FC to early forms of HDL, and LCAT-mediated esterification converts FC to its ester leading to HDL maturation. Our data show a reduction in both cholesterol efflux to plasma of HHcy mice (Figure 3B) and LCAT substrate reactivity (Figure 3C). The increase in LCAT specific activity (Figure 3D) may result from a compensatory response to HDL reduction in HHcy. Reduced reactivity of the substrate HDL-apoA-I, which is a cofactor of LCAT, may contribute to decreased LCAT-mediated cholesterol esterification in HHcy. Cumulatively, our data reveal 2 potentially atherogenic effects of HHcy on RCT: reduced LCAT-mediated cholesterol esterification and impaired cholesterol efflux.

In the absence of other processes, reduced cholesterol efflux to the plasma would be expected to lower the FC content of plasma and HDL. However, in spite of lower cholesterol efflux, HDL-FC is twice as high in the plasma of CBS\(^{-/-}\)/apoE\(^{-/-}\) mice as in CBS\(^{+/+}\)/apoE\(^{-/-}\) mice (Figure 3). In normal lipid metabolism, the size of HDL increases with repeated cycles of cholesterol uptake and esterification. Impairment of this process in the CBS\(^{-/-}\)/apoE\(^{-/-}\) mouse is reflected in the absence of large HDL particles, as measured by NMR (Figure 2B through 2D) and by a left-shift tendency in HDL FPLC profile (Figure 2A). Consistently, a shoulder in HDL profile of human subjects suggests that large HDL particle is reduced by HHcy in CHD as well. Thus, the accumulation of FC and the smaller HDL size must be
attributable to the impairment of the major reaction that consumes FC and increases the size of HDL-C esterification via LCAT. Our studies support the hypothesis that Hcy impairs cholesterol esterification and HDL maturation via an apoA-I inhibition–related mechanism. This conclusion is consistent with the report that apoA-I transgenic mice exhibit increased HDL-C and greatly diminished vascular lesions.28

In addition to the profound reduction in hepatic apoA-I production and attendant decreases in plasma and HDL apoA-I in CBS/H11002/H11002/a apoE/H11002/H11002 mice (Figure 5), we discovered that Hcy, in concentrations (0.5 to 2 mmol/L) close to that in human severe homocystinuria (200 to 500 μmol/L), significantly reduced intracellular and secreted apoA-I protein levels, but not that of mRNA, in mouse primary hepatocytes (Figure 6A and 6B). These data are highly consistent with the pulse-chase studies showing that Hcy reduced new apoA-I synthesis and had no effect on apoA-I degradation in mouse primary hepatocytes (Figure 6C through 6E) and support the hypothesis that Hcy inhibits apoA-I protein synthesis, leading to HDL reduction in HHcy. Our findings are in contrast with a recent report using superphysiological levels of Hcy (5 mmol/L) in a hepatic tumor cell line, where transcriptional regulation of apoA-I by Hcy was identified.10

Interestingly, we observed a decrease in HDL-apoA-II, a secondary abundant protein of HDL, in CBS/H11002/a apoE/H11002 mice (Figure 5B). ApoA-I is cardioprotective in both humans and mice. However, apoA-II has been associated with higher cardiovascular risk in humans, but rather cardioprotective in animal models. Therefore, the role of apoA-II reduction in HHcy-related atherosclerosis remains to be determined.

Whereas no change in the clearance of the [125I]-HDL protein, clearance of [1H]-CE–HDL was faster in CBS/H11002/a apoE/H11002 mice (Figure 4) and was correlated with increased hepatic SRB-I protein, the major receptor for HDL-CE via selective uptake. Increased hepatic SRB-I may determine a faster clearance of HDL-CE and hypo–α-lipoproteinemia in HHcy mice. SRB-1 protein induction was not observed in primary hepatocytes. This may be related with the limited culture time of these cells. Moreover, given the much smaller HDL pool sizes in HHcy mice, the actual uptake of HDL-CE is lower.

Figure 6. Gene expression in mouse primary hepatocytes. Mouse primary hepatocytes were treated with DL-Hcy at indicated concentrations for 24 hours. A, Real-time PCR of apoA-I. ApoA-I mRNA level was analyzed by real-time PCR quantification. Results are normalized to the amount of 18S ribosomal RNA and expressed relative to the value in control group. B, Protein levels of apoA-I in the culture media, or apoA-I and SRB-I in cell lysate of Hcy-treated hepatocytes, were examined by Western blot analysis. C through E, Pulse-chase analysis of apoA-I. Mouse primary hepatocytes were treated with DL-Hcy for 24 hours, pulsed with [3H]-leucine (200μCi/mL), and chased for indicated times. Radiolabeled apoA-I were purified, counted for radioactivity, and normalized to cellular protein content. Values in the control group were defined as 100%. The t1/2 was calculated based on the exponential decay curves indicated by dash lines. Values represent mean±SD (n=6). *P<0.05 vs control group at the same time point.
In conclusion, the primary effect of HHcy in the CBS \(^{-/-}\) /apoE\(^{-/-}\) mice appears to occur through inhibited hepatic apoA-I protein synthesis, which may contribute to resistance to LCAT-mediated cholesterol esterification. In addition, HHcy results in a faster HDL-CE clearance, probably because of hepatic induction of SRB-I. Our data suggest that hypo-\(\alpha\)-lipoproteinemia may contribute to accelerated atherosclerosis in HHcy CBS/apoE mice. Importantly, this may also contribute to higher mortality in CAD patients with HHcy. Identification of the molecular basis of Hcy-mediated alteration in HDL metabolism may provide important insight into the role of Hcy in CVD, thereby leading to new therapeutic strategies.

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**Disclosures**

None.

**References**


Hyperhomocysteinemia Decreases Circulating High-Density Lipoprotein by Inhibiting Apolipoprotein A-I Protein Synthesis and Enhancing HDL Cholesterol Clearance
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**Online Methods:**

**Northern blot analysis.** Total RNA (15 µg) was isolated from the mouse liver and analyzed by Northern blotting. The filters were then hybridized with different probes, including a final probe for 18S ribosomal RNA for loading control.

**Real-time PCR.** Total RNA (300 ng) was reverse transcribed and cDNA was subjected to real-time quantitative PCR by using TaqMan Universal PCR Master Mix without AmpErase UNG and TaqMan Gene Expression Assay kits for apoA-I, SRB-I or 18S (Applied Biosystems) as described. We used the cDNA amount giving the linear range of response for each gene (cDNA derived from 7.5ng RNA/reaction), and manufacture recommended thermal cycler conditions. Results were normalized to the amount of 18S rRNA in the samples, and expressed relative to the value in CBS+/+apoE−/− mice or control group.

**References**


Online Figure Legends:

Online Figure 1. VLDL particle size in CBS/apoE mice by nuclear magnetic resonance analysis (NMR). HDL/VLDL particle size and HDL concentration were measured by NMR analyzer. Note that VLDL particle size mean size are increased in CBS^{−/−}/apoE^{−/−} mice. Values represent mean±SD. n=8. * P< 0.05 vs. CBS^{+/+}/apoE^{−/−}.
Online Fig 1. Liao, NMR