Hepatic overexpression of cholesteryl ester hydrolase enhances cholesterol elimination and in vivo reverse cholesterol transport

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Abstract Neutral cholesteryl ester hydrolase (CEH)-mediated hydrolysis of cellular cholesteryl esters (CEs) is required not only to generate free cholesterol (FC) for efflux from macrophages to feces, cholesterol-loaded and [3H]cholesterol-labeled J774 macrophages were injected intraperitoneally into mice and the appearance of [3H]cholesterol in gallbladder bile and feces over 48 h was quantified. Mice overexpressing CEH had significantly higher [3H]cholesterol radiolabel in bile and feces, and it was associated with bile acids. This CEH-mediated increased movement of [3H]cholesterol from macrophages to bile acids and feces was significantly attenuated in SR-BI/−/− mice. These studies demonstrate that similar to macrophage CEH that rate-limits the first step, hepatic CEH regulates the last step of RCT by promoting the flux of cholesterol entering the liver via SR-BI and increasing hepatic bile acid output. — Zhao, B., J. Song, and S. Ghosh. Hepatic overexpression of cholesteryl ester hydrolase enhances cholesterol elimination and in vivo reverse cholesterol transport. J. Lipid Res. 2008. 49: 2212–2217.

Supplementary key words liver • bile acid excretion • lipoprotein cholesterol removal • selective uptake • HDL cholesteryl esters

Liver plays a central role in maintaining whole-body cholesterol homeostasis (1). Dietary and endogenously synthesized lipids [triacylglycerols, (TGs) and cholesteryl esters (CEs)] are packaged in the liver and secreted as VLDL particles that carry and deliver lipids to the peripheral tissues (2). Following the delivery of TG and CE, cholesterol is either secreted into the bile or is secreted into the bile as FC. The initial experiments of Goodman and Lequire (9) showed that although lipoprotein-derived CE is rapidly taken up by the liver, within a short time, a significant amount of tracer is associated with FC, requiring an efficient hydrolysis. Shimada et al. (10) subsequently demonstrated that this hydrolysis of HDL-CE is extra lysosomal and catalyzed by a neutral CEH. Thus, neutral CEH catalyzes the first and the last step in RCT, underscoring its importance in regulating whole-body cholesterol homeostasis.

We have identified, cloned, and characterized human neutral CEH and demonstrated its expression in macrophages as well as liver (11, 12). In macrophages, overexpression of CEH resulted in increased mobilization of CE droplets and increased FC efflux, leading to decreased

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intracellular cholesterol (13). Macrophage-specific transgenic expression of CEH led to attenuation of diet-induced atherosclerosis in LDLR\(^{-/-}\) mice (14). In vivo RCT and elimination of cholesterol from the body into bile and feces was also significantly higher from macrophages with transgenic expression of CEH, establishing its role in regulating the first step of RCT. In hepatocytes, on the other hand, overexpression of neutral CEH leads to an increase in bile acid synthesis (12).

In the present study, we examined the hypothesis that increased expression of CEH in liver will increase elimination of cholesterol as bile acids. Because only a small portion (5–20%) of biliary cholesterol is derived from de novo synthesis (15, 16) and the bulk is supplied by the hepatic uptake of lipoproteins (17, 18), by enhancing the last step of RCT, hepatic CEH levels will also regulate the removal of HDL-derived CE into bile and feces. The data presented herein show that adenoviral-mediated overexpression of CEH in liver significantly increases bile acid output and also enhances the flux of FC from macrophages to bile and feces.

**METHODS**

**Adenoviral-mediated overexpression of CEH**

C57BL/6 mice were injected intravenously with recombinant adenovirus (10\(^6\) pfu/ml) encoding human CEH (Ad-CEH) or a control virus encoding β-galactosidase (Ad-β-Gal). Five days after injection, mice were euthanized, and nonfasting plasma, liver, and gallbladder were harvested for analyses. Total plasma cholesterol and cholesterol associated with HDL, LDL, and VLDL fractions (separated by FPLC) were measured as described previously (14). The expression of human CEH in total liver RNA was calculated using a standard curve (12). CEH activity in liver cytosolic fraction was determined using tissue per unit total RNA was calculated using a standard curve (14). The expression of human CEH in total liver RNA was determined by real-time RT-PCR as described previously (21). The level of expression for each gene in mice injected with Ad-β-Gal was normalized to the corresponding levels in mice injected with Ad-β-Gal.

**Hepatic gene expression**

Total liver RNA was extracted using the SV total RNA Isolation System (Promega), and expression of genes involved in hepatic cholesterol homeostasis (HMG-CoA reductase, cholesterol 7α-hydroxylase, ACAT2) and biliary cholesterol, phospholipid, and bile acid secretion (ABCG5/G8, ABCB4, and ABCB11, respectively) were determined by real-time RT-PCR as described previously (21). The level of expression for each gene in mice injected with Ad-CEH was normalized to the corresponding levels in mice injected with Ad-β-Gal.

**Measurement of the flux of FC from macrophages to bile and feces in vivo**

**J774 cell culture, \([^{3}H]\)cholesterol labeling, and cholesterol loading.** J774 cells were grown in suspension in RPMI 1640 supplemented with 10% FBS and were radioactively labeled with \([^{3}H]\)cholesterol and cholesterol enriched with AcLDL as described by Zhang et al. (22).

**In vivo studies.** Three days after adenoviral injection, mice were injected intraperitoneally with cholesterol-loaded and \([^{3}H]\)cholesterol-labeled J774 foam cells and transferred to metabolic cages. Feces were collected from 0 to 48 h and stored at −20°C prior to lipid extraction. At 48 h, mice were euthanized and gallbladder bile was collected. SR-BI knockout mice and wild type littermates, generated by crossbreeding of SR-BI heterozygotes (Jackson Laboratory), were used for the experiments. The number of mice per group varied from four to seven, based on the genotypes obtained in any particular litter.

**Lipid extractions.** Biliary cholesterol and bile acids were extracted from gallbladder bile as described previously (20). Total feces were vacuum dried and powdered, and fecal cholesterol as well as bile acid were extracted as described by Batta et al. (23).

**Statistical analysis**

Results were analyzed by 2-tailed Student’s t-test with the use of GraphPad Prism Software version 4, which tests for normality before running the t-test. Although statistical significance for all comparisons was assigned at \(P < 0.05\), the individual \(P\) values are included in the text.

**RESULTS**

**Adenovirus-mediated CEH expression in liver**

In a pilot experiment, mice were injected with increasing levels of adenovirus (10\(^6\) to 10\(^11\) pfu/ml), and hepatic toxicity as well as CEH expression was monitored. The dose of 10\(^11\) pfu/ml was lethal, and significant hepatic toxicity, as measured by serum aspartate aminotransferase and alanine aminotransferase levels, was observed when mice were injected with 10\(^9\)–10\(^10\) pfu/ml (data not shown). The highest hepatic CEH expression with no hepatic toxicity was observed in mice injected with 10\(^8\) pfu/ml, and this dose was used for all subsequent experiments. Compared with mice injected with Ad-β-Gal, in which no human CEH mRNA was detected, high levels of CEH mRNA expression were seen in livers of mice injected with Ad-CEH (≥5 × 10\(^6\) copies/μg total RNA). This led to a significant increase in CEH activity (3.08 ± 0.2 vs. 9.47 ± 2.02 nmol/h/mg protein; \(P < 0.0339\)).

**Enhanced CEH expression in liver does not alter plasma lipoprotein profile**

Total plasma cholesterol and cholesterol associated with other lipoprotein fractions were determined, and the data are shown in Fig. 1. Hepatic CEH overexpression did not alter the distribution of cholesterol between the different lipoproteins. Although there was an increase in HDL-cholesterol (HDL-C), the difference did not reach statistical significance (\(P = 0.053\)). CEH overexpression also did not affect hepatic cholesterol levels; total cholesterol, FC, and esterified cholesterol levels were 1.29 ± 0.01, 1.08 ± 0.05, and 0.21 ± 0.05 mg/g wet weight, respectively, in mice injected with Ad-β-Gal compared with 1.26 ± 0.15, 1.07 ± 0.07, and 0.19 ± 0.08 mg/g wet weight in Ad-CEH-injected animals.

**Hepatic CEH overexpression increases bile acid content in the gallbladder bile**

Secretion of FC and bile acids was determined by analyzing the gallbladder bile. Compared with mice injected with...
control Ad-β-Gal virus, although there was no significant difference in the amount of FC in the bile, a significant 4-fold increase in total bile acid content was noted in Ad-CEH-injected mice (474.11 ± 77.35% control; \( P < 0.00018 \)), indicating that hepatic CEH regulates bile acid output (Fig. 2). These data are consistent with our earlier results demonstrating an increase in bile acid synthesis in primary hepatocytes transiently transfected with CEH cDNA (12).

**Hepatic gene expression**

Hepatic cholesterol homeostasis is maintained by coordinated regulation of four enzymes, namely, HMG-CoA reductase, cholesterol 7α hydroxylase, ACAT2, and CEH, which regulate cholesterol biosynthesis, bile acid synthesis, and reversible conversion of FC to CE, respectively. Secre-

tion of cholesterol, bile acids, and phospholipid into the bile is regulated by the respective transporters (ABCG5/G8, ABCB11, and ABCB4). The effect of CEH on the expression of these genes was determined, and the results are shown in Fig. 3. CEH overexpression affected neither the expression of genes involved in hepatic cholesterol homeostasis nor that of the genes involved in FC and bile acid transport. However, a significant increase was observed in the expression of the phospholipid transporter ABCG4.

**Increased flux of cholesterol from macrophages to bile and feces**

To evaluate the role of hepatic CEH in regulating the flux of cholesterol from macrophages to bile and feces, in vivo RCT was monitored. There was no significant difference in the radioactivity associated with the plasma in mice injected with Ad-β-Gal (control) and those injected with Ad-CEH (98.74 ± 0.48% control), suggesting that hepatic CEH overexpression did not affect the efflux of cholesterol from the macrophages. Compared with controls, hepatic overexpression of CEH significantly increased the secretion of \(^{3}H\)cholesterol tracer in gallbladder bile (176.67 ± 12.63% control; \( P = 0.005 \)) and feces (306.68 ± 48.02% control; \( P = 0.003 \)). The increased tracer was associated with the bile acid fraction, and no difference was noted in the excretion of tracer in the FC fraction (Fig. 4). These data are consistent with the observed increase in bile acid secretion, with no change in FC following CEH overexpression (Fig. 2).

In this in vivo RCT model, \(^{3}H\)cholesterol tracer is carried from the macrophage to the liver associated with HDL, and Zhang et al. (24) have demonstrated the obliga-
C57BL/6 mice (271.99 ± 12.68 vs. 90.48 ± 11.8% control; P = 0.006) or feces (306.68 ± 48.2 vs. 144 ± 16.11% control; P = 0.027), indicating that in the absence of SR-BI-mediated delivery of HDL-CE, CEH overexpression is unable to enhance the flux of cholesterol from macrophages to bile or feces (Fig. 4). These data suggest that CEH mobilizes [3H]cholesterol tracer delivered to the liver via SR-BI.

**DISCUSSION**

In this study, we demonstrate that hepatic CEH expression has a direct and substantial effect on bile acid secretion and elimination of cholesterol from the body as bile acids. Mice overexpressing CEH in the liver had markedly increased bile acid secretion into the bile and increased macrophage-derived [3H]cholesterol tracer in gallbladder bile as well as feces. These data indicate that hepatic CEH regulates the elimination of cholesterol from the body by increasing the secretion of bile acids and promoting RCT.

CEH catalyzes the obligatory first and rate-limiting step of RCT, namely, the hydrolysis of stored CE in peripheral tissues, including artery wall-associated macrophages, making FC available for HDL-mediated removal and transport to the liver. We have previously demonstrated that macrophage-specific transgenic expression of CEH attenuated diet-induced atherosclerosis in ldlr<sup>−/−</sup> mice (14). Once HDL-associated CEs are delivered to the liver, hydrolysis of CE is once again obligatory to generate FC for either direct secretion into bile or for conversion into bile acids, the two major pathways for cholesterol elimination from the body. The data presented here demonstrate that enhanced expression of CEH in the liver results in increased bile acid secretion as well as the flux of cholesterol from macrophages to feces, suggesting that hepatic CEH levels regulate the last step of RCT just as macrophage CEH levels regulate the first step.

Cholesterol synthesized de novo contributes only about 5–20% of biliary cholesterol (15, 16), and the bulk is supplied by the hepatic uptake of lipoproteins (17, 18). LDL is taken up as a holoparticle, and LDL-associated CEs are hydrolyzed within the lysosomes. Using [3H]cholesterol olate-labeled LDL, Pieters et al. (25) demonstrated the rapid appearance of tracer into bile, primarily as bile acids, suggesting that FC released from LDL-CE is utilized for bile acid synthesis. HDL-associated CE and FC, on the other hand, are selectively taken up via SR-BI. Using differentially labeled HDL-[14C]FC and HDL-[3H]CE, Ji et al. (26) demonstrated an increase in the appearance of the tracer in bile and also noted a delay in the appearance of HDL-CE-associated tracer, suggesting that although HDL-FCs may be secreted directly into the bile, HDL-CEs enter the hepatic metabolic pool, delaying the appearance into bile. Sampson et al. (27) showed that cholesterol entering the hepatocytes from HDL undergoes esterification and that subsequent hydrolysis is required for its secretion into the bile or conversion into bile acids, and Pieters et al. (28) demonstrated that selective uptake of CE from HDL is efficiently coupled to bile acid synthesis. To enter the hepatic cholesterol pool, HDL-CE needs to be hydrolyzed, but the identity of the enzyme responsible for this hydrolysis in the liver remains uncertain (29). The observed attenuation of [3H]cholesterol tracer in gallbladder bile and feces in SR-BI<sup>−/−</sup> mice despite CEH overexpression in the liver suggests that CEH metabolizes the CE delivered via selective uptake through SR-BI and makes FC available for bile acid synthesis. These data are consistent with our earlier results demonstrating an increase in bile acid synthesis by human hepatocytes transiently transfected with CEH cDNA (12). However, these data do not rule out the possibility that FC released by CEH-mediated

![Figure 4](image-url)
hydrolysis of SR-BI-delivered CE is reesterified by ACAT-2 and rehydrolyzed by CEH, i.e., enters the hepatic CE cycle before being utilized for bile acid synthesis.

The majority of bile acid molecules return to the liver via efficient entero-hepatic circulation after participating in cholesterol transport and fat digestion and are not lost in the feces. However, bile acids are secreted in such large amounts that even a small fractional loss of bile acid amounts to about 0.4 g/day in humans (30) and represents a major pathway for the elimination of cholesterol from the body. In our study, CEH overexpression increased bile acid output by 4-fold and also enhanced the flux of [3H]cholesterol tracer from macrophages to fecal bile acids by 3-fold. Thus, continued 3-fold higher removal of cholesterol in the form of fecal bile acids by overexpression of CEH in the liver would therefore enhance the elimination of cholesterol from the body and could potentially be anti-atherogenic. Transient adenoviral-mediated expression precludes long-term atherosclerosis studies, and liver-specific CEH-transgenic mice are currently being developed to directly demonstrate the effects of hepatic CEH on the attenuation of atherosclerosis.

CEH-mediated hydrolysis results in the release of FC that can potentially modulate the expression of other hepatic genes regulated by cellular FC levels via SREBP. We did not observe any significant difference in the expression of HMG-CoAR or LDLR, suggesting that CEH overexpression probably does not affect cellular FC homeostasis. This would be consistent with our earlier data, in which no cellular accumulation of FC was observed in macrophages isolated from CEH-transgenic mice (14). Based on the data described by Pieters et al. (25), that HDL-derived CE is efficiently coupled to bile acid synthesis, it is tempting to speculate that FC released by CEH-mediated hydrolysis of HDL-CE is effectively utilized for bile acid synthesis and does not change the regulatory FC within the hepatocyte. It is also important to note that FC released by CEH-mediated hydrolysis can be readily reesterified by ACAT2, preventing any change in cellular FC levels. Bile acids are ligands for nuclear receptor FXR, and we did not observe any change in FXR target genes, though no change in the expression of bile acid transporters ABCB11 was noted, there could still be increased transport of bile acids via this transporter. Because no significant difference was noted in the association of cholesterol with various plasma lipoproteins, it appears that CEH overexpression did not have any major effect on hepatic lipoprotein secretion. However, these studies used an adenovirus that leads to only a transient overexpression, and long-term effects of CEH overexpression on plasma lipoprotein profiles can only be evaluated in the liver-specific CEH-transgenic mice that are currently being developed.

In conclusion, this study provides further evidence for a major role of CEH in RCT. Our earlier work demonstrated that CEH regulates HDL-dependent cellular cholesterol efflux from macrophages and facilitates cholesterol flux to the liver, thereby reducing macrophage lipid burden and development of atherosclerosis (14). The current results indicate that CEH also stimulates hepatic bile acid secretion and its transport into bile. Further, hepatic CEH also increases the movement of cholesterol from macrophages to feces and thus enhances the elimination of cholesterol from the body. Thus, CEH promotes RCT by facilitating both the initial and final steps in the process.

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REFERENCES


