Liver-specific transgenic expression of cholesteryl ester hydrolase reduces atherosclerosis in Ldlr<sup>−/−</sup> mice

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Abstract The liver plays a central role in the final elimination of cholesterol from the body either as bile acids or as free cholesterol, and lipoprotein-derived cholesterol is the major source of total biliary cholesterol. HDL is the major lipoprotein responsible for removal and transport of cholesterol, mainly as cholesteryl esters (CEs), from the peripheral tissues to the liver. While HDL-FC is rapidly secreted into bile, the fate of HDL-CE remains unclear. We have earlier demonstrated the role of human CE hydrolase (CEH, CES1) in hepatic hydrolysis of HDL-CE and increasing bile acid synthesis, a process dependent on scavenger receptor BI expression. In the present study, we examined the hypothesis that by enhancing the elimination of HDL-CE into bile/ feces, liver-specific transgenic expression of CEH will be anti-atherogenic. Increased CEH expression in the liver significantly increased the flux of HDL-CE to bile acids. In the LDLR<sup>−/−</sup> background, this enhanced elimination of cholesterol led to attenuation of diet-induced atherosclerosis with a consistent increase in fecal sterol secretion primarily as bile acids. Taken together with the observed reduction in atherosclerosis by increasing macrophage-mediated cholesterol efflux, these studies establish CEH as an important regulator in enhancing cholesterol elimination and also as an anti-atherogenic target.—Bie, J., J. Wang, Q. Yuan, G. Kakiyama, S. S. Ghosh, and S. Ghosh. Liver-specific transgenic expression of cholesteryl ester hydrolase reduces atherosclerosis in Ldlr<sup>−/−</sup> mice. J. Lipid Res. 2014. 55: 729–738.

Supplementary key words cholesterol elimination • bile acid excretion • high density lipoprotein-cholesterol ester hydrolysis • fecal bile acids • low density lipoprotein receptor

Homeostatic balance between dietary intake, endogenous synthesis, and fecal elimination of cholesterol is essential to prevent pathological accumulation of cholesterol in macrophage foam cells that leads to the development of atherosclerosis. Unlike other macromolecules such as carbohydrates, proteins, or nucleic acids, once synthesized the steroid nucleus of cholesterol cannot be degraded within the human body and excess cholesterol can only be removed following biotransformation to more water soluble bile acids or as free cholesterol (FC) solubilized in bile acids. Because bile acid synthesis and secretion of cholesterol into the bile is largely restricted to the liver, the liver plays a central role in regulating the elimination of cholesterol from the body. Furthermore, as only a small portion (5–20%) of biliary cholesterol is derived from de novo synthesis (1, 2) and the bulk is supplied by the hepatic uptake of lipoproteins (3, 4), the liver also plays a key role in the flux of cholesterol returning to the liver from the peripheral tissues via lipoproteins. Chylomicrons carrying the dietary cholesterol return to the liver as remnants, and following delivery of associated TGs and cholesterol to the peripheral tissues, liver-derived VLDLs return to the liver as LDLs (5). This hepatic uptake of remnant or LDL-associated cholesterol is thought to regulate hepatic cholesterol synthesis or VLDL secretion (6), and recently Sniderman et al. (7) have demonstrated that while chylomicron remnant-associated cholesterol enters the regulatory pool and modulates hepatic de novo synthesis, LDL cholesterol is resecreted as VLDL. In contrast to LDL, HDL removes cholesterol from peripheral tissues, including artery wall-associated macrophage foam cells, delivers it to the liver and represents the major mechanism for the flux of cholesterol from the peripheral tissues to the liver by the process of reverse cholesterol transport (RCT). Because cholesterol is carried as cholesteryl esters (CEs) in all lipoproteins, intracellular hydrolysis of CEs is obligatory for the release of FC within the hepatocyte. While the remnants

Abbreviations: CAD, coronary artery disease; CE, cholesteryl ester; CEH, cholesteryl ester hydrolase; ER, endoplasmic reticulum; FC, free cholesterol; hCEH, human cholesteryl ester hydrolase; LCEH2, liver-specific human CEH transgenic; LDLR, LDL receptor; LXR, liver X receptor; RCT, reverse cholesterol transport; SCP-2, sterol carrier protein-2; SR-BI, scavenger receptor BI; TC, total cholesterol; Tgh, TG hydrolase; TPC, total plasma cholesterol.

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The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of nine figures.
and LDLs are taken up by the endocytic pathway and the associated CEs are hydrolyzed within the acidic lysosomal compartment by acid CE hydrolase (CEH). CEs associated with HDL enter the hepatocyte by selective uptake pathway via scavenger receptor BI (SR-BI) and hydrolysis of HDL-CEs is extra lysosomal and catalyzed by a neutral CEH (8).

We purified (9), characterized, and cloned rat liver neutral CEH (10), a member of the carboxylesterase family, and established its role in hepatic cholesterol homeostasis (11). Recently, we also cloned and characterized human liver CEH and demonstrated that transient over-expression of CEH increased bile acid synthesis and secretion from primary human hepatocytes (12). Furthermore, adenosine-ribose-mediated over-expression of this enzyme enhanced cholesterol elimination by increasing the flux of cholesterol from macrophages to feces (in vivo RCT) (13). More importantly, SR-BI deficiency completely abolished CEH-mediated increase in in vivo RCT, demonstrating the requirement of functional SR-BI for CEH to channel HDL-CEs to bile and feces (13). Direct association of CEH with SR-BI delivered HDL-CEs, and conversion of HDL-CEs to bile acids further confirmed the role of hepatic CEH in metabolizing HDL-CEs and making the FC available for bile acid synthesis (14). Consistently, liver-specific deletion of Ces3, the murine homolog of human CEH (hCEH), led to a significant decrease in bile acid secretion and fecal elimination of bile acids resulting in an increase in diet-induced atherosclerosis in LDL receptor (LDLR)−/− mice (15).

In the present study we sought to further establish the anti-atherogenic role of hepatic CE hydrolysis by developing liver-specific hCEH transgenic mice. Hepatic over-expression of CEH did not affect hepatic cholesterol homeostasis but led to an increase in the flux of HDL-CEs to bile acids. The data presented here also demonstrates that by increasing fecal elimination of cholesterol as bile acids, liver-specific transgenic expression of CEH leads to attenuation of diet-induced atherosclerosis in LDLR−/− mice.

METHODS

Generation of transgenic mice

The plasmid pLIV.11, containing human apoE promoter as well as hepatic control region and all the necessary elements for chimeric transgene construction (poly linker region and heterologous intron), was used (16). Full-length hCEH cDNA (~2 kb) with an inframe 3′-myc epitope was cloned into the Mool and Mulu sites in the poly linker region (supplementary Fig. 1), and the sequence of the chimeric transgene was confirmed by sequencing. The chimeric transgene was excised by digestion with SalI and SpeI, purified by agarose gel electrophoresis, and injected into the pronuclei of fertilized mouse eggs obtained from superovulated female mice (Balb/c/C57BL/6 hybrids). The injected eggs were surgically transferred to oviducts of surrogate females. Presence of hCEH transgene was confirmed either by PCR amplification of a 682 bp product using hCEH specific primers (133–155 bp and 814–792 bp) using mouse tail genomic DNA as a template or by Southern blot analysis using full-length hCEH as a probe to identify the ~1.265 bp integrated DNA. The founder mice in the Balb-c/C57BL/6 hybrid background were backcrossed into the C57BL/6 background for 10 generations before experimentation and were labeled as liver-specific hCEH transgenic (LCEH) mice. For evaluation of atherosclerosis, LCEH mice were crossed into the LDLR−/− background. To generate macrophage- and liver-specific double transgenics in the LDLR−/− background, LDLR−/−LCEH mice were crossed with LDLR−/−CEHTg mice generated in our laboratory earlier (17). Male and female littermates were included in the study at 10 weeks of age. The total number of animals in each group was, therefore, determined by the availability of the correct genotype and gender within a litter.

For assessment of atherosclerosis, mice were fed a Western-type high-fat/high-cholesterol diet (TD88137, Harlan Teklad), which contained 21% fat, 0.15% cholesterol, and 19.5% casein by weight with no sodium cholate for 16 weeks. All procedures were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee. Littermates were used for all experiments.

Tissue distribution of hCEH in LCEH mice

Tissues were harvested from WT (C57BL/6) and LCEH mice and total RNA was extracted using an RNAeasy kit (Qiagen). hCEH mRNA expression was determined by real time PCR using optimized TaqMan gene expression assay (Hs00275607_m1), and mRNA copy number was determined using a standard curve as described earlier (12). To determine the hCEH protein expression, total protein extracts prepared from different tissues were analyzed by Western blot analysis using anti-c-myc antibody to specifically identify the c-myc tag on transgenic hCEH and species-specific fluorescently labeled secondary antibodies. Positive immune-reactivity was detected by scanning in the appropriate channels with an Odyssey infrared imaging system (LI-COR).

Sub-cellular distribution of hCEH

Primary hepatocytes were prepared, and cytoplasm as well as endoplasmic reticulum (ER) were fractionated by differential centrifugation. The presence of hCEH was detected by Western blot analysis as described above. CE hydrolytic activity was determined using a micellar substrate as described earlier (18).

TG secretion from liver

Livers were harvested from WT and LCEH mice and precision cut liver slices were incubated with [3H]oleate for 3 h (19). Following three washes in PBS, total lipids were extracted and neutral lipids were separated by TLC using hexane:diethyl ether:acetic acid::90:10:1 (v/v). Spots corresponding to TG were marked, silica gel scraped, and associated radioactivity determined by liquid scintillation counting. To determine the rate of TG secretion in vivo, mice were fasted overnight and a baseline blood sample was collected via the tail vein. Mice were subsequently injected with tyloxapol (Sigma-Aldrich) at a concentration of 500 mg/kg body weight to inhibit lipoprotein lipase. Blood samples were subsequently collected at 1, 2, and 3 h postinjection and plasma TG levels were determined (L-Type TG-M kit, Wako Diagnostics). TG production rates were calculated as described (20).

Total plasma cholesterol and cholesterol distribution among plasma lipoproteins

A modified Column Lipoprotein Profile method was used. Whole plasma aliquots frozen and stored at −80°C were thawed at 4°C. Total plasma cholesterol (TPC) concentration was determined by a micro enzymatic method as described earlier (17). In brief, approximately 20 μg of cholesterol was injected onto a fast-protein liquid chromatography system (Superose 6 HR 10/30 column, Amersham Biosciences) with online mixing of the column effluent with enzymatic reagent (Cholesterol Liquid Stable,
Thermo Electron) for acquiring lipoprotein cholesterol profiles. The data were acquired on a personal computer running ChromPerfect Spirit chromatography software (Justice Software). The system was optimized so that the area under the profiles was proportional to the cholesterol mass. Area percent in each lipoprotein fraction, VLDL, LDL, and HDL, was applied to TPC to calculate cholesterol concentration in the lipoprotein fractions.

Biochemical and histological analyses of liver tissue

About 100 mg of fresh liver tissue was homogenized in PBS and total lipids were extracted and amount of total cholesterol (TC), CEs, and TGs were determined, as described before, and normalized to weight. For histological analyses, liver tissue was fixed in 10% buffered formalin and paraffin embedded. Five micron sections were stained with hematoxylin and eosin and images were acquired using a Zeiss inverted microscope fitted with a Zeiss inverted microscope fitted with a digital camera as described earlier (19).

Intraperitoneal glucose tolerance tests

Ten-week-old LDLR−/− and LDLR−/−LCEH2 littersmates were fed a Western diet for 16 weeks. After an overnight fast, a single bolus of glucose (2 mg/g body weight) was given intraperitoneally. Blood glucose levels were determined by commercially available glucometer using tail vein blood at 0, 15, 30, 60, 120, and 180 min.

Quantitative atherosclerosis analyses

The aorta was dissected from the heart to the iliac bifurcation, cleaned of any surrounding tissue, opened longitudinally, pinned down, and the area occupied by the lesions in the aortic arch and total aorta were determined using Axiovision™ image analysis software. Total lesion area (LDLR−/−LCEH2) NS

Fecal bile acid and cholesterol measurement

TC was extracted from the dried feces using chloroform-methanol (2:1, v/v). After evaporation under nitrogen, the extracts were solubilized in 2-propanol containing 10% Triton X-100 TC estimated by enzymatic assay using a Wako® Cholesterol E test kit. Bile acids extracted from dried feces were derivatized and the resulting phenacyl esters were separated using reverse phase HPLC (15). Total fecal bile acids and cholesterol were normalized to the dry weight of the feces and data presented as micrograms per gram.

Real time PCR

Total RNA was extracted using an RNeasy kit (Qiagen). cDNA was synthesized using a high capacity cDNA reverse transcription kit (Applied Biosystems). Real time PCR was performed on a Stratagene Mx3000P machine, using TaqMan Universal PCR Master Mix and optimized probe and primer sets from Applied Biosystems. The following optimized probes were used: Cos3, Mm00474816_m1; Ces1, Mm00491354_m1; Ces5, Mm00555211_m1; ES22, Mm00504141_m1; ES1, Mm00468347_m1; ES31, Mm00519905_m1; HSL, Mm00493529_m1; TG hydrolase (Tgh)2, Mm0023518_m1; Kaaa, Mm00626772_m1; BSEP, Mm00445168_m1; Abcg5, Mm00446249_m1; Abcg8, Mm00445970_m1; MDR2, Mm00435630_m1; HMGCR, Mm01282501_m1; ACAT-2, Mm00448823_m1; Ldlr, Mm00440169_m1; CYP7A1, Mm00484152_m1; and liver X receptor (LXR), Mm00437265_g1.

Statistical analyses

Data were analyzed by two-way ANOVA using GraphPad Prizm followed by Bonferroni post hoc tests to determine genotype and gender interactions, if any, as well as the significance of genotype and gender effects. Table 1 summarizes the results of these analyses including the actual P values. The differences were considered significant with $P < 0.05$ and are indicated in all figure legends.

RESULTS

Development of LCEH2 mice

Liver-specific hCEH (gene symbol CES1) transgenic mice were developed and backcrossed into the C57BL/6 background. Total RNA from multiple tissues was used to determine the expression of hCEH. Consistent with the expression driven by the apoE promoter and the hepatocyte control region, liver-specific expression of hCEH mRNA was seen in LCEH2 mice (Fig. 1A). Furthermore, high expression of c-myc tagged hCEH protein was detected in total protein extracts from the liver, confirming liver-specific expression (Fig. 1B). The minor immune-reactive protein seen in kidney and lung extracts and the detection of a low copy number of hCEH in other tissues

| Parameter                      | Genotype Effects | Genotype Effects | Gender Effects |
|-------------------------------|------------------|------------------|----------------|
| Total 0 TPC                   | $P = 0.002$      | $P = 0.01$       | $P = 0.0002$   |
| VLDL                          | NS               | $P = 0.003$      | NS             |
| LDL                           | $P = 0.002$      | $P = 0.09$       | $P = 0.0002$   |
| HDL                           | NS               | $P = 0.02$       | $P = 0.05$     |
| Arch lesion area (LDLR−/− vs. LDLR−/−LCEH2) | NS               | $P = 0.0001$     | NS             |
| Total lesion area (LDLR−/− vs. LDLR−/−LCEH2) | NS               | $P = 0.0004$     | NS             |
| Total lesion area (LDLR−/− vs. MLCL) | NS               | $P = 0.0001$     | NS             |
| Fecal BA (LDLR−/− vs. LDLR−/−LCEH2) | NS               | $P = 0.002$      | $P = 0.001$    |
| Fecal cholesterol (LDLR−/− vs. LDLR−/−LCEH2) | NS               | $P = 0.002$      | $P = 0.001$    |

Two-way ANOVA analyses were performed for the indicated parameters and significant differences due to genotype or gender as well as genotype/gender interactions are shown. BA, bile acid.
To determine the potential effects of transgenic expression of hCEH in affecting genes involved in maintaining hepatic cholesterol homeostasis, expression of HMGCR, ACAT-2, Ldlr, and CYP7A1 was assessed. While a non-significant increase in CYP7A1 and significant increase in the expression of SR-BI ($P = 0.02$ for chow-fed mice, $P = 0.04$ for Western diet-fed mice) was observed, there was no change in the expression of other genes (supplementary Fig. III). Expression of LXR, a gene that plays an important role in hepatic lipogenesis, also remained unaltered ($1.00 \pm 0.02$ in WT mice vs. $1.05 \pm 0.11$ in LCEH2 mice).

Because FC released as a result of CEH-mediated CE hydrolysis can either be directly secreted into bile via the FC transporter AbcG5/G8 or converted into bile acids before secretion via bile acid transporter (BSEP), expression of these transporters was evaluated. Ces3 deficiency did not affect the expression of these transporters (supplementary Fig. IV). There was also no change in the expression of phospholipid transporter MDR2.

Liver-specific transgenic expression of hCEH does not affect TG secretion from the liver

In addition to catalyzing the hydrolysis of CEs, hCEH also hydrolyzes TGs, and the murine homolog of hCEH, Ces3, has been characterized as Tgh and thought to play a
role in VLDL secretion. To evaluate the effects of transgenic expression of hCEH, TG synthesis/secretion from precision cut liver slices was examined by monitoring incorporation of [3H]oleate in cellular as well as secreted TGs. There was no significant difference in [3H]oleate incorporation in cellular (tissue) as well as secreted (medium) TGs (Fig. 2A).

Plasma TG levels were also measured and are shown in Fig. 2B. There was no significant difference in plasma TG levels with CEH over-expression (P = 0.76 for males, P = 0.79 for females). Consistently, the rates of in vivo secretion of VLDLs were also not significantly different between WT and LCEH2 mice (Fig. 2C). These data demonstrate that transgenic expression of hCEH does not affect hepatic TG synthesis or secretion. Cholesterol content of plasma lipoproteins also remained unchanged in LCEH2 mice (data not shown).

Liver-specific transgenic expression of hCEH increases the flux of CEs from HDLs to biliary bile acids

To examine the role of hepatic CE hydrolysis in regulating elimination of HDL-derived CEs into bile, flux of [3H]cholesterol from HDL-CEs into bile was monitored in vivo. As shown in Fig. 3, transgenic expression of hCEH significantly increased the elimination of [3H]cholesterol from HDL-CEs to biliary bile acids (2.31 ± 1.05 vs. 1.14 ± 0.45, P = 0.03). In contrast, there was no increase in the [3H]label associated with biliary FC. These data suggest that FC generated by hepatic CE hydrolysis of HDL-CEs is preferentially eliminated as bile acids and is consistent with the observed increase in bile acid secretion by adenovirus-mediated transient over-expression of hCEH in mice (13).

Effect of liver-specific hCEH expression in Western diet-fed LDLR<sup><text>-/-</text></sup> mice

LCEH2 mice were crossed into the LDLR<sup><text>-/-</text></sup> background and fed Western diet for 16 weeks and following parameters were examined.

Gene expression. Hepatic expression of members of carboxylesterase family, as well as other known hydrolases, was examined and no significant differences were noted (supplementary Fig. V). Consistent with the data obtained in the C57BL/6 background, there was no significant change in the expression of genes involved in hepatic cholesterol homeostasis (HMG-CoAR, ACAT-2, Cyp7A1, and Cyp27A1) except that no significant increase in SR-B1 expression was noted (supplementary Fig. VI). There was also no significant change in the expression of genes involved in cholesterol, phospholipid, and bile acid transport to the bile canaliculus, namely ABCC5/G8, MDR2, and BSEP, respectively (supplementary Fig. VII).

Plasma lipoprotein profiles. Fasting plasma TC and cholesterol associated with different lipoprotein fractions were measured and are shown in Fig. 4: representative fast-protein liquid chromatography profiles are shown in supplementary Fig. VIII. An overall significant effect of genotype (P = 0.008) and gender (P = 0.002) on TPC was detected by ANOVA (see Table 1). In addition, gender/genotype interaction was also considered very significant (P = 0.002). Analyses of the distribution of plasma cholesterol among the different lipoprotein fractions indicated significant effects of the genotype on VLDL (P = 0.003) and HDL (P = 0.025) cholesterol, but not LDL (P = 0.095) cholesterol. In contrast, gender/genotype interaction was considered very significant (P = 0.002) for LDL cholesterol only.

Hepatic lipids. Total hepatic lipids were analyzed for TC, FC, and CE as well as TG content. Liver-specific transgenic expression of hCEH did not affect the distribution of hepatic lipids (supplementary Fig. IXA). Histological analyses of livers from LDLR<sup><text>-/-</text></sup> and LDLR<sup><text>-/-</text></sup>LCEH2 mice showed lipid accumulation consistent with Western diet feeding but no difference was apparent between the two genotypes (supplementary Fig. IXB).

Glucose tolerance. Loss of Ces3/Tgh is reported to improve glucose tolerance (21) and therefore, intra-peritoneal glucose tolerance tests were performed in Western diet-fed LDLR<sup><text>-/-</text></sup> and LDLR<sup><text>-/-</text></sup>LCEH2 mice. Over-expression of hCEH in the liver did not significantly affect glucose
tolerance and the area under the curve (AUC) for males (LDLR<sup>+/−</sup> = 37,000 ± 2,700 and LDLR<sup>+/−</sup>/LCEH2 = 39,000 ± 1,300; P = 0.54) and females (LDLR<sup>−/−</sup> = 29,000 ± 4,300 and LDLR<sup>−/−</sup>/LCEH2 = 26,000 ± 8,000; P = 0.53) were not significantly different.

**Atherosclerosis.** Western diet-induced atherosclerosis was monitored in LDLR<sup>−/−</sup> and LDLR<sup>−/−</sup>/LCEH2 mice after 16 weeks of feeding. No mortality or morbidity was observed in mice with either genotype. Representative en face images are shown in Fig. 5A. The percent area of aorta covered with atherosclerotic lesions was determined and the quantification of the surface area occupied by atherosclerotic lesions is shown in Fig. 5B. Compared with LDLR<sup>−/−</sup> mice, liver-specific hCEH expression in LDLR<sup>−/−</sup>/LCEH2 mice led to a significant decrease in the area occupied by the lesions in the aortic arch. Total area occupied by the lesions in the entire aorta was also determined and liver-specific hCEH expression significantly decreased the total area occupied by atherosclerotic lesions.

**Fecal bile acid and cholesterol elimination.** To examine whether transgenic over-expression of hCEH-mediated increase in removal of cholesterol in feces (either as FC or as bile acids) represents a potential mechanism for the observed decrease in atherosclerosis in LDLR<sup>−/−</sup>/LCEH2 mice, total fecal bile acids as well as cholesterol levels were also monitored. As shown in Fig. 6, consistent with decreased flux of HDL-CE to bile in LCEH2 mice shown in Fig. 3, there was a significant increase in total fecal bile acids (P = 0.002) in LDLR<sup>−/−</sup>/LCEH2 mice, and the magnitude of this increase was higher in female mice; these gender effects were highly significant (P = 0.001). In contrast, there was no significant change in fecal FC content in both genders suggesting a possible preferential flux of CEH-generated FC toward bile acid synthesis. These data demonstrate that hCEH mediates the increase in HDL-CE hydrolysis and subsequent conversion of released FC to bile acids for final removal from the body in the feces. It is noteworthy that while the fecal bile acid content was significantly higher in females, fecal FC content was higher in males (P = 0.001).

**Western diet-induced atherosclerosis in macrophage and liver hCEH double transgenics in LDLR<sup>−/−</sup> background**

We earlier reported a significant reduction in diet-induced atherosclerosis in macrophage-specific CEH transgenic mice and the data presented above demonstrate the antiatherogenic role of hepatic CEH (17). To examine whether transgenic over-expression of CEH in macrophage and liver would have additive effects, if any, double CEH transgenics in the LDLR<sup>−/−</sup> background (MLCL) were generated and Western diet-induced atherosclerosis was evaluated. While there was a significant reduction in aortic arch (P = 0.0004, Fig. 7A) and total (P < 0.0001, Fig. 7B) lesion area in double transgenic MLCL mice of both genders compared with LDLR<sup>−/−</sup> mice, this reduction was not significantly different from that seen in macrophage (P = 0.86 for arch, P = 0.53 for total lesion area) or liver single transgenic mice (P = 0.52 for arch, P = 0.29 for total lesion area). These data point to a potential maximum reduction that can possibly be achieved by enhancing CEH-mediated flux of cholesterol from macrophage foam cells to feces as bile acids by enhancing CEH-mediated elimination of cholesterol from the body either by increasing efflux from macrophage foam cells or by hepatic removal.

**DISCUSSION**

The liver plays a central role in the final elimination of cholesterol from the body either as FC or as bile acids, and lipoprotein-derived CEs represent the major source for biliary FC and bile acids. The data presented here clearly demonstrate that increasing hepatic CEH-mediated hydrolysis...
Liver CEH increases cholesterol removal and is anti-atherogenic.

Cooperation between SR-BI and CEH in mediating the hydrolysis of HDL-CEs and the flux of FC generated toward bile acid synthesis (14). The data presented here also demonstrate that transgenic over-expression of hCEH increases the flux of HDL-CEs to biliary bile acids. It is noteworthy that hCEH over-expression led to a significant increase in the expression of SR-BI in LCEH2 mice and future studies will examine the coordinated regulation, if of lipoprotein-derived CEs leads to increased elimination of cholesterol as biliary and fecal bile acids. This enhanced elimination of cholesterol from the body is anti-atherogenic, resulting in significant attenuation of Western diet-induced atherosclerosis in LDLR⁻/⁻/LCEH2 mice. These data confirm our previous results demonstrating a significant increase in atherogenesis by liver-specific deficiency of the murine homolog of hCEH, namely Ces3 (15). However, in contrast to liver-specific Ces deficiency, which did not alter plasma lipoprotein profiles, transgenic expression of hCEH significantly reduced total plasma and VLDL cholesterol levels and these effects were more pronounced in males. Although these changes in the plasma cholesterol levels can be viewed as anti-atherogenic, it needs to be emphasized that increasing evidence, however, points to an apparent lack of correlation between plasma lipid profiles and atherogenesis (22–25), and flux of cholesterol from the peripheral tissue, including the artery wall-associated macrophage foam cells, to feces is a more relevant parameter for predicting the risk for the development of atherosclerosis (26). In addition, Rowlan et al. (27) have recently demonstrated that most atherosclerosis susceptibility loci are distinct from those for plasma lipids.

In earlier studies, we have demonstrated a significant increase in bile acid secretion as well as flux of cholesterol from HDL to bile by adenovirus-mediated transient over-expression of hCEH, a process dependent on hepatic expression of SR-BI (13). Consistently, transgenic expression of hCEH significantly increased the flux of cholesterol from HDL to bile and this increase was associated with biliary bile acids and not biliary FC. FC associated with HDL is thought to be directly/rapidly secreted into bile (28) and intracellular sterol carrier protein-2 (SCP-2) plays an important role in the transport of FC from the basolateral to the cannilicular side of the hepatocyte (29). However, the fate of HDL-CEs taken up by selective uptake via SR-BI is not well-established. We have recently demonstrated cooperation between SR-BI and CEH in mediating the hydrolysis of HDL-CEs and the flux of FC generated toward bile acid synthesis (14). The data presented here also demonstrates that transgenic over-expression of hCEH increases the flux of HDL-CEs to biliary bile acids. It is noteworthy that hCEH over-expression led to a significant increase in the expression of SR-BI in LCEH2 mice and future studies will examine the coordinated regulation, if...
Transgenic expression of human CES1 in liver and macrophages reduces diet-induced atherosclerosis. LDLR−/− and MLCL mice were fed a high-fat/high-cholesterol Western diet for 16 weeks and development of atherosclerosis was assessed by en face analyses. Data are presented as percent lesion area in aortic arch (A) or total aorta (B). The effects of genotype (transgenic expression of CEH) on lesion area and aortic cholesterol content were determined to be significant by two-way ANOVA analyses and individual *P* values are included in Table 1.

Fig. 7. Transgenic expression of human CES1 in liver and macrophages reduces diet-induced atherosclerosis. LDLR−/− and MLCL mice were fed a high-fat/high-cholesterol Western diet for 16 weeks and development of atherosclerosis was assessed by en face analyses. Data are presented as percent lesion area in aortic arch (A) or total aorta (B). The effects of genotype (transgenic expression of CEH) on lesion area and aortic cholesterol content were determined to be significant by two-way ANOVA analyses and individual *P* values are included in Table 1.

Fig. 8 summarizes the role of CEH in cholesterol elimination from the body. In artery wall-associated macrophage cells, CEH catalyzes the rate-limiting and obligatory first step in CE mobilization. The FC generated is effluxed to lipid-poor apoA1 or HDL and is carried to the liver via HDL predominantly as CEs. While HDL-FC is thought to be rapidly secreted into bile (29), HDL-CEs need to be hydrolyzed by hepatic CEH to generate FC; and currently it is not known whether CEH-generated FC equilibrates with other FC pools, including HDL-FC. Nonetheless, CEH-generated FC can theoretically have four fates: 1) resecretion via ABCA1 to apoA1 to generate nascent HDL; 2) translocation to the ER for esterification by ACAT-2 and secretion of the resulting CEs associated with VLDL; 3) transport to mitochondria or the ER to serve as a substrate for CYP27A1 or CYP7A1, respectively, for bile acid synthesis and secretion of bile acids; and 4) direct secretion into bile via ABCG5/G8, a process facilitated by intracellular sterol carrier proteins SCP-2 or FABP1 (30). While fates 1 and 2 recirculate the cholesterol returning from the periphery, fates 3 and 4 contribute to the final elimination of cholesterol from the body, and are hence anti-atherogenic. The data presented herein establishes the role of CEH in hydrolysis of HDL-CEs and elimination of the resulting FC primarily as bile acids, indicating a possible preferential translocation of CEH-generated FC to either mitochondria or ER for bile acid synthesis. Intracellular mechanisms

any, of hepatic CEH and SR-BI to further delineate the role of these two proteins in regulating the flux of HDL-CEs to bile acids. The lack of increase in flux of HDL-CEs to biliary FC in LCEH2 mice points to a preferential movement of CEH-generated FC toward bile acid synthesis and it remains to be seen whether intracellular cholesterol transport proteins such as SCP-2 or StAR proteins are involved in this process (30).

The murine homolog of hCEH, Ces3, is also known as Tgh, and as members of the carboxylesterase family of enzymes, both catalyze the hydrolysis of ester bonds in multiple substrates including CEs and TGs. Based on its role in TG hydrolysis, murine hepatic Ces3 is thought to play a role in VLDL secretion. Transgenic expression of hCEH did not affect hepatic TG synthesis or TG secretion in vivo; the observed secretion rates were comparable to those reported for C57BL/6 mice (21). Using a Zn-inducible metallothionein promoter-driven human TGH minigene in mice, Wei et al. (31) also reported unchanged hepatic TG secretion rates compared with WT mice.

Consistent with its role in regulating the flux of lipoprotein CEs to bile acids and biliary excretion, transgenic expression of hCEH significantly increased fecal sterol elimination. This increased fecal sterol elimination was predominantly associated with acidic sterol or bile acids. Furthermore, significant gender-related differences were observed; while total fecal FC was higher in males of both genotypes, total fecal bile acids were higher in females of both genotypes. Consistently, Turley et al. (32) have earlier reported a significantly higher bile acid synthesis and bile acid pool, as well as fecal bile acid excretion in female mice. Gender specificity of bile acid metabolic pathways has also been shown in Cyp7a1-deficient mice, where attenuated BSEP and changes in gall bladder bile composition is only observed in female mice (33). Charach et al. (34) described a strong correlation between bile acid secretion and coronary artery disease (CAD) in humans, where patients with established CAD excreted significantly lower amounts of bile acids, and concluded that impaired ability to excrete cholesterol may be an additional risk factor for CAD development. Additionally, while normal bile acid secretion in heterozygous familial hypercholesterolemic patients is not associated with CAD, suboptimal bile acid synthesis, especially in males, is strongly related to development of CAD (35). It remains to be seen whether these gender-related differences in bile acid secretion and elimination in feces is related to the reduced risk for CAD in premenopausal women. However, it is noteworthy that increased risk for CAD in postmenopausal women is associated with altered cholesterol metabolism and inefficient fecal elimination of cholesterol (36).
involved in the translocation of FC to appropriate subcellular destinations (marked by “?” in Fig 8) are currently undefined. Interactions between intracellular sterol binding proteins, such as SCP-2, FABP1, or StAR proteins and CEH, could potentially regulate this process and facilitate the preferential flux of CEH-generated FC from HDL-CEs toward bile acid synthesis and final elimination from the body.

An increase in hepatic CEH in addition to an increase in macrophage CEH in MLCL mice did not result in additive reduction in atherosclerosis. It is likely that the maximum possible effect is obtained by either increasing macrophage or hepatic CEH. Nonetheless, these data confirm that a simultaneous increase in CEH-mediated CE hydrolysis in both macrophages and liver does not have any deleterious effects, opening the way for the development of pharmacological agents for increasing CEH activity. This is specifically relevant because LXR ligands that enhance FC efflux from macrophages and reduce atherosclerosis in mice (38) have undesirable lipogenic effects in the liver (39).

In conclusion, by mediating the hydrolysis of HDL-CEs in the liver and increasing elimination of cholesterol as bile acids in the feces, hepatic CEH is anti-atherogenic. The authors thank Dr. Liqing Yu of Wake Forest University School of Medicine for providing the pLIV.11 vector.

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