Hepatocyte-Specific Expression of Human Carboxylesterase 1 Attenuates Diet-Induced Steatohepatitis and Hyperlipidemia in Mice

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Rodents have at least five carboxylesterase 1 (Ces1) genes, whereas there is only one CES1 gene in humans, raising the question as to whether human CES1 and mouse Ces1 genes share the same functions. In this study, we investigate the role of human CES1 in the development of steatohepatitis or dyslipidemia in C57BL/6 mice. Hepatocyte-specific expression of human CES1 prevented Western diet or alcohol-induced steatohepatitis and hyperlipidemia. Mechanistically, human CES1 induced lipolysis and fatty acid oxidation, leading to a reduction in hepatic triglyceride and free fatty acid levels. Human CES1 also reduced hepatic-free cholesterol levels and induced low-density lipoprotein receptor. In addition, human CES1 induced hepatic lipoprotein lipase and apolipoprotein C-II expression. Conclusion: Hepatocyte-specific overexpression of human CES1 attenuates diet-induced steatohepatitis and hyperlipidemia. (Hepatology Communications 2020;4:527-539).

Fat liver disease (FLD), characterized by abnormal lipid accumulation within hepatocytes, can be classified into nonalcoholic fatty liver disease (NAFLD) and alcoholic fatty liver disease (AFLD). FLD ranges from simple steatosis to steatohepatitis, which may further progress to cirrhosis and hepatocellular carcinoma. Despite extensive research, the pathophysiology of FLD is not well understood.

In addition, no Food and Drug Administration–approved therapies are available for treatment of FLD.
Carboxylesterase 1 (CES1) is a drug-metabolizing enzyme that has the ability to hydrolyze amide or ester bonds. Mouse Ces1 has eight genes that are expressed in a wide variety of cell types and tissues. Among the eight mouse Ces1 genes, Ces1d and Ces1g are relatively well-characterized, which share 78% and 74% homology in amino acids with human CES1, respectively. Mouse Ces1g has been shown to display triglyceride hydrolase (TGH) activity, and global or hepatocyte-specific inactivation of mouse Ces1g causes fatty liver and a pro-atherogenic lipid profile. On the contrary, hepatic expression of Ces1g improves liver steatosis, dyslipidemia, and insulin signaling. Global inactivation of Ces1g is also shown to aggravate alcohol-deficient or methionine/choline-deficient diet-induced liver inflammation and liver injury.

In contrast to Ces1g, global or liver-specific Ces1d−/− mice show reduced hepatic triglyceride accumulation and improved dyslipidemia and
insulin sensitivity.\(^{(10-13)}\) Interestingly, global loss of Ces1g or Ces1d attenuates the development of atherosclerosis in low-density lipoprotein receptor (Ldlr\(^{-/-}\)) mice.\(^{(12,14)}\) In mice with global Ces1g deficiency, intestinal cholesterol and fat absorption is inhibited, whereas macrophage cholesterol efflux is increased,\(^{(14)}\) which may account for the atheroprotective effect of global Ces1g deficiency. Given that Ces1d and Ces1g have differing functions in lipid metabolism and that the functions of other Ces1 isoforms remain to be characterized, it is essential to investigate the role of human CES1 in lipid metabolism directly.

Human CES1 is abundantly expressed in liver and has been shown to have cholesteryl ester hydrolase (CEH) activities.\(^{(15)}\) Liver-specific transgenic expression of human CES1 attenuates atherosclerosis in Ldlr\(^{-/-}\) mice by promoting high-density lipoprotein–mediated reverse cholesterol transport.\(^{(16)}\) In addition, macrophage-selective overexpression of human CES1 also reduces atherosclerosis in Ldlr\(^{-/-}\) mice through increased cholesterol efflux.\(^{(17)}\) So far, the role of human CES1 in regulating FLD or dyslipidemia remains to be determined.

In this report, we show that adeno-associated virus serotype 8 (AAV8)–mediated overexpression of human CES1 in hepatocytes protects against high-fat/high-cholesterol/high-fructose diet–induced or alcohol-induced steatohepatitis and hyperlipidemia. We also investigated the underlying mechanisms. Our data suggest that human CES1 in hepatocytes is protective against metabolic disorders.

**Materials and Methods**

**MICE AND DIETS**

Twelve-week-old male C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were fed for 16 weeks a high-fat/high-cholesterol/high-fructose diet (Western diet) containing 40% fat/0.2% cholesterol (AIN-76A; TestDiet, St. Louis, MO) and 4.2% fructose (in drinking water). The Lieber-DeCarli diet was purchased from Bio-Serv (Flemington, NJ). All of the animals received human care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health. Mice were fasted for 5 to 6 hours before euthanasia during the light cycle. All of the animal studies were approved by the Institutional Animal Care and Use Committee at Northeast Ohio Medical University.

**ADENO-ASSOCIATED VIRUSES**

The coding sequence of human CES1 was amplified by polymerase chain reaction (PCR) and cloned...
into an AAV vector under the control of a mouse albumin promoter (AAV8-ALB-hCES1). The creation of AAV8-ALB-Null has been described previously.\(^{18}\) AAV8-ALB-hCES1 or AAV8-ALB-Null (control) was produced by packaging AAV plasmids into serotype 8 and titrated by Vector Biolabs (Malvern, PA). Each mouse was intravenously injected with \(3 \times 10^{11}\) genomic copies of AAVs.

**CHRONIC PLUS BINGE ALCOHOL DRINKING**

The chronic plus binge alcohol drinking was performed as described previously.\(^{9,19}\) Briefly, C57BL/6J mice were administered a Lieber-DeCarli control liquid diet (Cat # F1259SP; BioServ) for 5 days. On the sixth day, mice were fed a Lieber-DeCarli liquid diet (Cat # F1258SP; BioServ) containing 5% (vol/vol) ethanol or pair-fed a Lieber-DeCarli control liquid diet for 10 days. On the 16th day, mice were gavaged with a single dose of ethanol (3 g/kg body weight) or isocaloric maltose dextrin.

**HEPATIC BIOCHEMICAL ANALYSES AND STAINING**

Approximately 100 mg of liver tissues were homogenized in methanol, and lipids were extracted in chloroform/methanol (2:1 vol/vol) as described.\(^{20}\) Hepatic triglycerides (TGs) and total cholesterol (TC) were measured using Infinity reagents from Thermo Fisher Scientific (Waltham, MA). Hepatic total free fatty acids (FFAs) and free cholesterol (FC) were determined using kits from Wako Chemicals USA (Richmond, VA). Hepatic fatty acid composition was analyzed by gas chromatography–mass spectrometry (GC-MS) as previously described.\(^{21}\) Hepatic hydroxyproline level was quantified using a kit from Cell Biolabs (STA675; San Diego, CA). Hepatic malondialdehyde (MDA) levels were measured using a thiobarbituric acid reactive substances assay kit (STA-330; Cell BioLabs), and hepatic reactive oxygen species (ROS) were measured using an OxiSelect in vitro ROS/RNS Assay kit (STA-347; Cell BioLabs) as described.\(^{9,18}\) Liver apoptosis was detected using a kit from Abcam (Cat # ab206386; Cambridge, United Kingdom). Hepatic neutral lipid accumulation, morphology, and fibrosis were determined by Oil Red O staining, hematoxylin and eosin staining, and picrosirius red staining, respectively, and images were acquired using an Olympus microscope.

**ANALYSIS OF PLASMA LIPIDS, LIPOPROTEINS, AND BIOCHEMISTRY**

Plasma levels of triglycerides, cholesterol, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using Infinity reagents (Thermo Fisher Scientific). Plasma lipoprotein profile was analyzed by fast protein liquid chromatography (FPLC) as previously described.\(^{22}\) Plasma high-density lipoprotein cholesterol (HDL-C) and non-HDL-C levels were determined using a high-density lipoprotein and low-density lipoprotein/very low density lipoprotein (VLDL) quantification kit from BioVision Inc. (Cat # K613; Milpitas, CA). Plasma tumor necrosis factor \(\alpha\) (TNF\(\alpha\)), interleukin (IL)-6, and IL-1\(\beta\) levels were quantified using enzyme-linked immunosorbent assay kits from PeproTech (Rocky Hill, NJ).

**REAL-TIME PCR AND WESTERN BLOT ASSAYS**

Total RNA was isolated with TRIzol reagent (Thermo Fisher Scientific), and mRNA levels were quantified by quantitative real-time PCR using Powerup SYBR Master mix (Thermo Fisher Scientific) on a 7500 Real Time PCR machine (Applied Biosystems, Foster City, CA). mRNA expression levels were normalized to 36B4. Western blot assays were performed using total cell lysates, microsomes, or nuclear extracts of the liver samples, as described previously.\(^{21}\) Antibodies against CES1 (cat # ab45957) and Tubulin (cat # ab4074) were purchased from Abcam. Antibodies against histone (Cat # 9671) or caspase 3 (CASP3; total [cat # 9661] or cleaved [cat # 9662]) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against LDLR (cat # NB100-191), scavenger receptor group B type 1 (cat # NB400-101), sterol regulatory element-binding protein 2 (SREBP-2; cat # NBP2-20481), or calnexin (cat # NB1001965) were purchased from Novus Biologicals.
Cluster differentiation factor 36 (CD36) antibody (cat # PA116813) was purchased from Thermo Fisher Scientific. Cholesterol 7α-hydroxylase (CYP7A1) antibody was a gift from Dr. David Russell at the University of Texas Southwestern Medical School.

Bile Acid Extraction and Measurement

Bile acids in the liver, intestine, and gallbladder were extracted using ethanol. Bile acid levels in the liver, intestine, or gallbladder were determined using a bile acid kit (Diazyme, San Diego, CA). Total bile acids are the sum of bile acids in the liver, intestine, and gallbladder.

TGH Activity Assays

Hepatic microsome proteins were isolated, and TGH activity was measured using 3H-triolein as substrates, as described previously.(21)

Fatty Acid Oxidation

C57BL/6 mice were injected intravenously with AAV8-ALB-NULL or AAV8-ALB-hCES1 at a dose of 3 × 10¹¹ genomic copies per mouse. After 72 hours, mouse primary hepatocytes were isolated and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum in 12-well dishes. Hepatocytes were then treated for 36 hours with media in the presence or absence of 100 mM ethanol. Fatty acid oxidation (FAO) was performed using 3H-palmitate as substrates, as described previously.(5)

Body Fat Content Measurement

Body fat content was detected using Echo-MRI (Echo-MRI, LLC, Houston, TX) as described.(23)

Statistical Analysis

Statistical significance was analyzed using unpaired Student t test and analysis of variance (GraphPad Prism, GraphPad Software, San Diego, CA). All data were expressed as mean ± SEM. Differences were considered statistically significant at P < 0.05.

Results

Hepatocyte-Specific Expression of Human CES1 Reduces Liver Steatosis by Promoting FAO in Western Diet-Fed Mice

Given that multiple Ces1 genes exist in mice and they do not appear to function similarly, we reasoned that it was critical to investigate human CES1 functions directly. We therefore generated an AAV that expressed human CES1 under the control of a mouse albumin promoter (AAV8-ALB-hCES1). AAV8-ALB-hCES1 or AAV8-ALB-Null was injected intravenously into C57BL/6 mice, which were then fed a Western diet for 16 weeks. Overexpression of hCES1 in hepatocytes had no effect on body weight (Supporting Fig. S1A), but reduced body fat content (Supporting Fig. S1B) and hepatic levels of TC, FC (Fig. 1A), TGs (Fig. 1B), and FFAs (Fig. 1C). Analysis of hepatic fatty acid composition using GC-MS showed that human CES1 overexpression reduced C16:1, C18:0, and C18:1 fatty acids by 48%, 38% and 44%, respectively, in Western diet-fed mice (Fig. 1D). Histological staining studies showed that human CES1 overexpression reduced neutral lipid accumulation and improved liver histology (Fig. 1E).

Consistent with a reduced hepatic TG levels, overexpression of human CES1 increased hepatic TGH activity by 166% (Fig. 1F). Newly released FFAs from lipolysis are known to serve as ligands for peroxisome proliferator-activated receptor α (PPARα) to activate FAO.(5,21,24) Consistent with these findings, overexpression of human CES1 increased hepatic mRNA levels of Ppara and its target genes Cpt1 (carnitine palmitoyltransferase 1), Cpt2 and Pdk4 (pyruvate dehydrogenase kinase isozyme 4) (Fig. 1G), and increased FAO (Fig. 1H). The increased FAO may account for the reduced hepatic FFA levels (Fig. 1C). There was no change in hepatic levels of Ces1d, Ces1g or Hnf4α (Supporting Fig. S1C), or genes involved in de novo lipogenesis (Srebplc, Cebpa, Acc, and Fasn) or VLDL secretion (ApoB and Mttp) (Supporting Fig. S1D,E).

We have previously shown that fasting represses CES1 protein expression in the liver, likely due to reduced glucose availability.(8) Consistent with this
finding, fasting repressed the mRNA levels of Ces1d and Ces1g (Supporting Fig. S2). Thus, although CES1 has TGH activity, hepatic CES1 likely promotes lipolysis during the fed state, as glucose is shown to induce CES1 expression. In addition, we could not fully rule out the possibility that CES1 also regulates de novo lipogenesis. Taken together, the data of Fig. 1 demonstrate that human CES1 prevents Western diet–induced hepatosteatosis, likely by inducing lipolysis and FAO.

**HEPATOMYCIE-SPECIFIC EXPRESSION OF HUMAN CES1 AMELIORATES WESTERN DIET-INDUCED LIVER INFLAMMATION, APOPTOSIS, AND FIBROSIS**

Excessive FFAs or FC can cause endoplasmic reticulum stress, mitochondrial dysfunction, apoptosis and inflammation, which play a key role in the progression of FLD from simple steatosis to steatohepatitis. The data of Fig. 1 and Table 1 indicate that human CES1 overexpression reduces hepatic FFA and FC levels, leading us to investigate whether human CES1 regulated apoptosis, inflammation, and liver injury. Hepatocyte-specific overexpression of human CES1 reduced plasma ALT (Fig. 2A) and AST levels (Fig. 2B), and inhibited hepatic mRNA levels of inflammatory genes (Tnfα, Il-6, Il-1β, and Mcp1 [monocyte chemoattractant protein 1]) (Fig. 2C) and fibrogenic genes (Tgfβ [transforming growth factor β], α-Sma [α smooth muscle actin], Timp1 [tissue inhibitor of metalloproteinase 1], Col1a1 [alpha-1 type I collagen], and Col1a2 [alpha-2 type I collagen]) (Fig. 2D).

Smad2 and Smad3 (mothers against decapentaplegic homolog 2/3 [Smad2/3]) are closely related downstream effectors in TGFβ–induced apoptosis. Consistent with the inhibition of Tgfβ expression (Fig. 2D), phosphorylated Smad2/3 levels as well as CASP3 levels were significantly reduced by human CES1 overexpression (Fig. 2E,F). TUNEL (terminal

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**FIG. 1.** Hepatocyte-specific expression of human CES1 prevents Western diet–induced hepatosteatosis by promoting lipolysis and FAO. C57BL/6 mice were injected intravenously with AAV8-ALB-Null or AAV8-ALB-hCES1 at a dose of 3 × 10^{11} genomic copies/mouse and fed a Western diet for 16 weeks (n = 8). (A) Hepatic cholesterol levels. (B) Hepatic TG levels. (C) Hepatic FFA levels. (D) Hepatic fatty acid composition analyzed by GC-MS (n = 6-8). (E) Representative images of liver sections stained with Oil Red O (top panel) or hematoxylin and eosin (bottom panel). (F) Liver microsome proteins were isolated and TGH activity was measured using 3H-triolein as substrates. (G) Hepatic mRNA levels were quantified by quantitative real-time PCR. (H) Mouse primary hepatocytes were isolated from C57BL/6 mice injected with AAV8-ALB-Null or AAV8-ALB-hCES1. FAO was determined using 3H-palmitic acid as substrates (n = 5). *P < 0.05, **P < 0.01. Abbreviations: CE, cholesteryl ester; H&E, hematoxylin and eosin; ORO, Oil Red O.
deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling) studies showed that human CES1 overexpression significantly suppressed apoptosis (Fig. 2G,H). Overexpression of human CES1 also reduced fibrosis (Fig. 2G), hydroxyproline levels (Fig. 2I), reactive oxygen species (ROS) (Fig. 2J), and MDA levels (Fig. 2K) in the liver. These data demonstrate that hepatocyte-specific expression of human CES1 attenuates Western diet–induced steatohepatitis by inhibiting ROS production, lipid peroxidation, apoptosis, and inflammation.

HEPATOMC-SPECIFIC EXPRESSION OF HUMAN CES1 IMPROVES HYPERLIPIDEMIA IN WESTERN DIET–FED MICE

In addition to attenuating steatohepatitis, hepatocyte-specific overexpression of human CES1 also reduced plasma levels of non-HDL-C (Fig. 3A), TG (Fig. 3B), and FFAs (Fig. 3C). Analysis of plasma lipoprotein profiles by FPLC showed that human CES1 reduced plasma low-density lipoprotein cholesterol (LDL-C) levels (Fig. 3D). In the liver, overexpression of human CES1 significantly induced the mRNA levels of Srebp2, Ldlr, Cyp7a1, ATP-binding cassette group G type 5 (Abcg5), Abcg8, apolipoprotein C-II (ApoC2), and lipoprotein lipase (Lpl), but repressed Cds36 mRNA levels (Fig. 3E). In addition, overexpression of human CES1 increased hepatic protein levels of LDLR, CYP7A1, and mature SREBP2 by 2.7, 2.2 and 2.9 fold, respectively, but reduced hepatic CD36 protein levels by 58% (Fig. 3F,G). Consistent with the induction of hepatic CYP7A1 expression, overexpression of human CES1 significantly increased bile acid levels in the intestine, liver, and gallbladder (Fig. 3H). The increased Abcg5 and Abcg8 expression may account for the reduced hepatic FC levels, which may subsequently promote the induction of SREBP2 and LDLR expression and a decrease in plasma LDL-C levels. The induction in hepatic Apoc2 and Lpl may account for the reduced plasma TG levels.

| Fatty Acids | Control Diet | Control Diet | EtOH Diet | EtOH Diet |
|------------|--------------|--------------|-----------|-----------|
|            | AAV-Null     | AAV-hCES1    | AAV-Null  | AAV-hCES1 |
| C14:0      | 0.128 ± 0.02 | 0.106 ± 0.017| 0.308 ± 0.09*| 0.121 ± 0.02*|
| C16:0      | 21.93 ± 3.36 | 37.49 ± 10.17| 48.38 ± 4.98*| 34.26 ± 2.73*|
| C16:1      | 0.338 ± 0.05 | 0.366 ± 0.06 | 0.768 ± 0.205| 0.178 ± 0.037*|
| C18:0      | 7.68 ± 0.31  | 6.2 ± 0.35*  | 12.85 ± 1.13'| 8.41 ± 0.38*|
| C18:1      | 25.27 ± 3.63 | 27.36 ± 3.22 | 61.71 ± 10.41'| 42.77 ± 0.65|
| C18:2      | 11.64 ± 1.85 | 10.7 ± 1.26  | 43.36 ± 5.85*| 27.31 ± 1.78*|
| C20:4      | 0.53 ± 0.15  | 0.146 ± 0.04*| 0.225 ± 0.05'| 0.023 ± 0.007*|
| C22:6      | 7.99 ± 2.26  | 2.57 ± 0.51* | 3.34 ± 0.776*| 0.912 ± 0.05*|

Note: C57BL/6 mice were injected with AAV8-ALB-Null or AAV8-ALB-hCES1 and fed a control diet or EtOH diet. Hepatic fatty acid composition was analyzed by GC-MS.
*P < 0.05 versus control diet–fed AAV-Null group.
†P < 0.05.
‡P < 0.01 versus control diet–fed AAV-Null group.
§P < 0.05.
||P < 0.01 versus EtOH diet–fed AAV-Null group.

HEPATOMC-SPECIFIC EXPRESSION OF HUMAN CES1 INCREASES LIPOLYSIS AND FAO AND ATTENUATES ALCOHOL-INDUCED HEPATOSTEATOSIS

Although NAFLD and AFLD share the similar pathogenic spectrum (simple steatosis and steatohepatitis), their pathogenic causes are completely different. To investigate whether human CES1 also had a similar effect on AFLD, we fed C57BL/6 mice with a Lieber-Decarli diet containing 5% ethanol or pair-fed a control diet following a National Institute on Alcohol Abuse and Alcoholism protocol.(19) The chronic plus binge ethanol feeding did not affect mouse body weight (Supporting Fig. S3A) or body fat content (Supporting Fig. S3B). Ethanol feeding...
significantly increased plasma levels of ALT (Fig. 4A) and AST (Fig. 4B) as well as hepatic levels of TG (Fig. 4C) and FFA (Fig. 4D). These increases were significantly attenuated by hepatocyte-specific overexpression of human CES1 (Fig. 4A-D). Oil red O staining showed that human CES1 reduced ethanol-induced neutral lipid accumulation (Fig. 4E). In contrast, overexpression of human CES1 had no effect on ethanol-induced changes in genes involved in de novo lipogenesis (Srebp1c, Acc, and Fasn) or VLDL secretion (Apob and Mttp) (Supporting Fig. S3C-G).

Ethanol feeding repressed hepatic TGH activity (Fig. 4F) and hepatic genes involved in FAO, including Ppara (Fig. 4G), Cpt1 (Fig. 4H), Cpt2 (Fig. 4I) and Pdk4 (Fig. 4J), which were all normalized by overexpression of human CES1 in hepatocytes (Fig. 4F-J). In line with these findings, ethanol inhibited FAO in hepatocytes, which was normalized by overexpression of human CES1 (Fig. 4K). Thus, human CES1 attenuates alcohol-induced hepatosteatosis likely by enhancing lipolysis and FAO.

In addition, we also analyzed hepatic fatty acid composition by GC-MS. Alcohol treatment markedly increased C14:0, C16:0, C18:0, C18:1, and C18:2 fatty acid levels by 167%-372%, and interestingly decreased C20:4 and C22:6 fatty acid levels, but did not change C16:1 fatty acid levels (Table 1). Overexpression of human CES1 in hepatocytes significantly attenuated alcohol-induced increases in C14:0, C16:0, C16:1, C18:0, and C18:2 fatty acid levels, and reduced C20:4 and C22:6 fatty acid levels (Table 1). These data are consistent with a role of human CES1 in promoting...
FAO. Together, our data indicate that human CES1 attenuates alcohol-induced hepatosteatosis likely by enhancing lipolysis and FAO.

HEPATOCYTE-SPECIFIC EXPRESSION OF HUMAN CES1 AMELIORATES ALCOHOL-INDUCED HYPERLIPIDEMIA

In addition to improving alcohol-induced hepatosteatosis, human CES1 expression also reduced ethanol-induced increases in plasma non-HDL-C levels (Fig. 5A). There was no change in plasma HDL-C levels (Fig. 5B). In addition, human CES1 normalized ethanol-induced increases in plasma TG (Fig. 5C) and FFA (Fig. 5D) levels. Consistent with these findings, overexpression of human CES1 normalized or increased hepatic expression of Ldlr (Fig. 5E), Cyp7a1 (Fig. 5F), Srebp2 (Fig. 5G), Abg5 (Fig. 5H), or Abg8 (Fig. 5I) in alcohol-fed mice. Alcohol increased Cd36 mRNA levels, which were completely normalized by human CES1 (Fig. 5J). In addition, human CES1 increased Apoc2 (Fig. 5K) and Lpl (Fig. 5L) expression in alcohol-fed mice. Our western blot assays further confirmed the mRNA expression data shown for Ldlr, Cyp7a1, and Srebp2 (Fig. 5M).

HEPATOCYTE-SPECIFIC EXPRESSION OF HUMAN CES1 INHIBITS INFLAMMATION IN ALCOHOL-FED MICE

Inflammation plays an important role in the pathogenesis of alcoholic liver disease. Alcohol
feeding significantly increased hepatic mRNA levels of inflammatory genes including Tnfα (Fig. 6A), Il-6 (Fig. 6B), Il-1β (Fig. 6C) and Mcp1 (Fig. 6D), as well as fibrogenic genes including Tgfβ (Fig. 6E), α-Sma (Fig. 6F), Col1a1 (Fig. 6G), and Col1a2 (Fig. 6H). Overexpression of human CES1 decreased or normalized alcohol-induced increases in these inflammatory or fibrogenic gene expression (Fig. 6A-H). In addition, overexpression of human CES1 also normalized alcohol-induced increases in plasma levels of TNFα (Fig. 6I), IL-6 (Fig. 6J), and IL-1β (Fig. 6K). Thus, human CES1 can prevent alcohol-induced inflammatory response.

**HUMAN CES1 PREVENTS ALCOHOL-INDUCED APOPTOSIS**

Alcohol-induced apoptosis plays an important role in the development of AFLD. Alcohol significantly induced hepatic apoptosis, which was blunted by human CES1 overexpression (Fig. 7A,B). In addition, human CES1 also attenuated alcohol-induced increases in hepatic MDA (Fig. 7C) and ROS (Fig. 7D) levels. Consistent with these observations, alcohol induced hepatic phosphorylated Smad2/3 and cleaved CASP3 levels, which were normalized or attenuated by overexpression of human CES1 (Fig. 7E-G). Thus,
hepatocyte-specific expression of human CES1 prevents alcohol-induced apoptosis likely by inhibiting ROS production, lipid peroxidation, and the TGFβ-Smad2/3 pathway.

**Discussion**

FLD is one of the most common chronic liver diseases worldwide. So far, the pathogenic mechanisms remain to be determined. In patients with alcoholic liver disease, hepatic CES1 expression is reduced by 75%. However, the role of human CES1 in FLD remains unknown. Because mice have eight Ces1 genes (Ces1a-Ces1h) that have shown inconsistent functions, it is unlikely to infer what functions human CES1 may have based on mouse studies in which specific mouse Ces1 gene is overexpressed or depleted. Therefore, it is imperative to investigate human CES1 using an appropriate approach. In this study, we overexpressed human CES1 specifically in mouse hepatocytes in vivo. Our data show that hepatocyte-specific expression of human CES1 protects against Western diet or alcohol-induced steatohepatitis, thus suggesting a role of human CES1 in the pathogenesis of FLD.

Previous studies have shown that human CES1 has CEH activity. When human CES1 hydrolyzes, cholesterol esters release FC, which may be oxidized to serve as a ligand for liver X receptors (LXRs). Activation of LXRs is known to induce the expression of CYP7A1, ABCG5, and ABCG8. When CYP7A1 is induced, more FC is converted to bile acids, leading to a reduction in hepatic FC levels. The reduced hepatic FC levels can induce SREBP2

**FIG. 5.** Human CES1 prevents alcohol-induced hyperlipidemia. C57BL/6 mice were fed a control diet or alcohol as described in Fig. 4 (n = 8 per group). Plasma levels of non-HDL-C (A), HDL-C (B), TG (C), and FFA (D) were determined. Hepatic mRNA levels of Ldlr (E), Cyp7a1 (F), Srebp2 (G), Abcg5 (H), Abcg8 (I), Cd36 (J), Apoc2 (K), and Lpl (L) were quantified by quantitative real-time PCR. (M) Hepatic proteins were analyzed by western blotting. *P < 0.05, **P < 0.01.
expression and processing, leading to an increase in LDLR expression and uptake of LDL by hepatocytes. As a result, overexpression of human CES1 in the liver reduces plasma LDL-C levels. In addition, human CES1 also induces hepatic ApoC-II and LPL expression, which may account for the reduced plasma TG levels. The overexpression of human CES1 in the liver also prevents alcohol-induced increases in pro-inflammatory and fibrogenic genes. C57BL/6 mice were fed a control diet or alcohol as described in Fig. 4 (n = 8 per group). Hepatic mRNA levels (A-H) and plasma levels of TNFα (I), IL-6 (J), and IL-1β (K) were determined. *P < 0.05, **P < 0.01.

Abbreviation: DCF, 2',7'-dichlorodihydrofluorescein.
levels. Bile acids are endogenous ligands for farnesoid X receptor (FXR). Activation of the FXR is known to induce ApoC-II, a co-activator of LPL. Because human CES1 induces CYP7A1 expression and bile acid synthesis, CES1 induces ApoC-II expression likely by activation of FXR.

In addition to the CEH activity shown by the Ghosh et al., (19) we demonstrate that human CES1 has TGH activity. By hydrolyzing TG to release FFAs, human CES1 promotes FAO to lower hepatic TG and FFA levels. Interestingly, human CES1 does not regulate genes involved in de novo lipogenesis or VLDL secretion. Although we cannot fully rule out the possibility that human CES1 may regulate de novo lipogenesis or VLDL secretion, our data suggest that human CES1 lowers hepatic TG levels likely by inducing lipolysis and subsequent FAO.

The progression of FLD from simple steatosis to steatohepatitis needs additional “hits,” such as ROS, apoptosis, and/or inflammation. FFA or FC-induced lipotoxicity contributes to lipid peroxidation, ROS production, apoptosis, and inflammation. In our current study, we demonstrate that human CES1 overexpression reduces hepatic FFA and FC levels, lipid peroxidation, ROS production, apoptosis and inflammation, which together help address how human CES1 overexpression attenuates steatohepatitis.

In summary, our present data have demonstrated an important role of human CES1 in the pathogenesis of FLD and hyperlipidemia. Importantly, we have also uncovered the underlying mechanisms, which likely involve both the CEH and TGH activities of CES1. Our data suggest that human CES1 may be a useful drug target for treatment of FLD and related hyperlipidemia.

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Supporting Information

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