Human liver cholesteryl ester hydrolase: cloning, molecular characterization, and role in cellular cholesterol homeostasis

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Zhao, Bin, Ramesh Natarajan, and Shobha Ghosh. Human liver cholesteryl ester hydrolase: cloning, molecular characterization, and role in cellular cholesterol homeostasis. Physiol Genomics 23: 304–310, 2005. First published August 30, 2005; doi:10.1152/physiolgenomics.00187.2005.—The liver regulates cholesterol homeostasis and eliminates excess cholesterol as bile acids or biliary cholesterol. Free cholesterol for bile acid synthesis or biliary secretion is obtained by the hydrolysis of stored cholesteryl esters or from cholesteryl esters taken up by the liver from high-density lipoproteins via a selective uptake pathway. The present study was undertaken to characterize the enzyme catalyzing this reaction, namely, cholesteryl ester hydrolase (CEH) from the human liver, and demonstrate its role in regulating bile acid synthesis. Two cDNAs were isolated from the human liver that differed only in the presence of an additional alanine at position 18 in one of the clones. Transient transfection of COS-7 cells with a eukaryotic expression vector containing either of these two cDNAs resulted in significant increase in the hydrolysis of cholesteryl esters, authenticating these clones as human liver CEH. CEH mRNA and protein expression in human hepatocytes were demonstrated by real-time PCR and Western blot analyses, respectively, confirming the location of this enzyme in the cell type involved in hepatic cholesterol homeostasis. Overexpression of these CEH clones in human hepatocytes resulted in significant increase in bile acid synthesis, demonstrating a role for liver CEH in modulating bile acid synthesis. This CEH gene mapped on human chromosome 16, and the two clones represent two different transcript variants resulting from splice shifts at exon 1. In conclusion, these data identify that human liver CEH was expressed in hepatocytes, where it potentially regulates the synthesis of bile acids and thus the removal of cholesterol from the body.

IN MAMMALIAN SYSTEMS, the liver plays a central role in the synthesis, redistribution, and regulation of whole body cholesterol as well as the regulation of plasma lipoprotein concentrations. Cholesterol balance in the liver is maintained by three major processes: 1) input pathways (cholesterol synthesis and lipoprotein uptake); 2) output pathways (formation of bile acids and biliary secretion of cholesterol as well as secretion of very-low-density lipoproteins (VLDL)); and 3) reversible conversion of cholesterol to cholesteryl esters (CEs). The hepatic free cholesterol (FC) concentration is maintained at low physiological levels to prevent cytotoxicity and is therefore tightly regulated. Excess FC is esterified with long-chain fatty acids by the action of acyl-CoA:cholesterol acyltransferase-2 (ACAT2), which is an integral membrane protein present mainly in the rough endoplasmic reticulum and is the major enzyme for cholesterol esterification in the human liver (20). The CEs thus formed either constitute part of the neutral lipid core of VLDL or are accumulated as cytoplasmic lipid droplets. Cytoplasmic CE droplets are in a dynamic state, undergoing a constant cycle of hydrolysis and reesterification (16) where esterification of FC is catalyzed by ACAT2 and the hydrolysis of CE is thought to occur in the cytoplasm by a neutral cholesteryl ester hydrolase (CEH) (2). For biliary cholesterol secretion as well as bile acid synthesis, FC is released from the intracellular stores of CE by CEH-mediated hydrolysis.

Conversion of cholesterol to bile acid or direct secretion into the bile are the only two recognized mechanisms for cholesterol elimination from the body. In humans, hepatic conversion of cholesterol to bile acids accounts for ~50% of cholesterol elimination, whereas the remaining 50% takes place almost entirely via biliary cholesterol secretion (23). Because only a small portion (5–20%) of biliary cholesterol is derived from de novo synthesis (6, 25), the bulk must be supplied by the hepatic uptake of lipoproteins. High-density lipoprotein (HDL) is the main lipoprotein that transports cholesterol from peripheral tissue, including artery wall-associated macrophages, to the liver by the process named reverse cholesterol transport. The clinical importance of this process is underscored by the observed strong inverse correlation between HDL-cholesterol and the risk for cardiovascular disease. HDL CE must be hydrolyzed by hepatocytes to release cholesterol and triglycerides into the cell, where they can be converted to very-low-density lipoproteins (VLDL) and subsequently secreted into the circulation. This hydrolysis is thought to occur in the nonlysosomal compartment presumably, by a neutral CEH (21, 27). Hepatic CEH is, therefore, required not only to release FC from the intracellular stores of CE (generated by ACAT2-mediated esterification) but also to hydrolyze CE delivered via selective uptake and represents the key enzyme required for releasing the pool of metabolically active FC from intracellular stores of CE, providing a substrate for bile acid synthesis and for biliary secretion of cholesterol. Despite the importance of CEH in regulating the availability of FC in the liver, the identity of this enzyme in the human liver remains obscure.

This study was undertaken to clone and characterize human liver CEH. Using the strategy of homology cloning, we obtained two cDNA clones from the human liver that code for an active CEH. This CEH is expressed in human hepatocytes, and its overexpression results in an increase in bile acid synthesis, suggesting a role for this enzyme in hepatic cholesterol homeostasis.

EXPERIMENTAL PROCEDURES

Materials. COS-7 cells were purchased from the American Type Culture Collection (Rockville, MD). The ThermoScript RT-PCR system, cell culture media, fetal bovine serum, and cell culture reagents were obtained from Invitrogen (Carlsbad, CA). phorbol 12-myristate 13-acetate and Kodak LS film were obtained from Sigma (http://physiolgenomics.physiology.org). Address for reprint requests and other correspondence: S. Ghosh, Pulmonary and Critical Care Division, Dept. of Internal Medicine, Virginia Commonwealth Univ., Richmond, VA 23298-0050 (e-mail: shobha@hsc.vcu.edu).
Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Bedford, MA). GENESCREEN membranes and \(^{32}P\)dCTP were obtained from NEN Research Products (Boston, MA). TaqMan Universal PCR Master Mix was from Applied Biosystems (Foster City, CA). All other reagents and chemicals were purchased from Fisher Chemical (Cincinnati, OH).

**Cell culture.** COS-7 cells were maintained in DMEM containing 10% fetal bovine serum according to the instructions supplied.

**Cloning of CEH cDNA using RT-PCR.** Total RNA from the human liver was prepared by CsCl gradient centrifugation according to the procedure of Chirgwin et al. (5). Total RNA (10 \(\mu\)g) was reverse transcribed using the ThermoScript RT-PCR system (Invitrogen) according to the manufacturer's instructions. No reverse transcriptase was added to negative controls. Oligo dT\(_{30}\) was used as a primer for first-strand cDNA synthesis. PCR primers used for CEH amplification were based on the sequence of human macrophage CEH (Accession No. AY268104). The forward primer sequence was 5’-CGAGAAGCTTGTGGCTTCGCTGCCTTTATCCTGGCC-3’, and the reverse primer sequence was 5’-CAGAGGCCCATGAGGACCGAAG-3’. Amplification reactions were carried out for 30 cycles with denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 2 min. The extension was increased to 10 min for the final cycle. PCR products were analyzed on a 1% agarose gel. The 1,870-bp PCR product was cloned into eukaryotic TA cloning vector pCDNA3.1/V5/His-TOPO (Invitrogen). Plasmid DNA was prepared from several colonies and analyzed for the presence of insert by BstXI digestion. Six recombinant plasmids containing the 1,870-bp

**Fig. 1.** cDNA nucleotide sequence and deduced amino acid sequence of human liver cholesterol ester hydrolase (CEH). The sequence for clone 1 is shown; clone 2 contains an additional Ala at position 18 between Trp and Gly, as marked by the solid diamond. The conserved active site motif GXSXG is shown in bold and underlined. Putative residues of the catalytic triad (Glu\(^{354}\) and His\(^{468}\)) are also shown in bold.
PCR product were sequenced in both directions by an ABI automated DNA sequencing system.

*Transcript transfection of COS-7 cells.* COS-7 cells were plated in six-well tissue culture dishes (6 × 10⁵ cells/well) and transfected with recombinant plasmid DNA (clones 1 and 2) containing CEH cDNA in the correct orientation using Effectene (Qiagen) as described previously (10). Controls were simultaneously transfected with the empty plasmid pcDNA3.1/V5/His-TOPO vector (pCMV). After 48 h of incubation, cells were harvested in homogenizing buffer containing protease inhibitors (8). The cell suspension was sonicated using a Heat Systems Ultrasonic Processor, and cell lysates were assayed for CEH activity as described previously (7). The substrate cholesteryl [1-14C]oleate was dissolved in acetone and presented as droplets representing the physiological state of cytosolic stores of CEs.

*Northern blot analysis.* Total RNA from transfected COS-7 cells was extracted using the RNeasy Kit (Qiagen), and 20 μg RNA was electrophoresed on a 1% agarose gel in the presence of formaldehyde. RNA was stained with ethidium bromide, and the integrity of RNA was verified by the presence of 28S and 18S rRNA bands. RNA was transferred to a Genescreen membrane and hybridized with 32P-labeled full-length CEH cDNA probe. After a high-stringency wash, the blot was autoradiographed as described in *Northern blot analysis*. B: no hybridization was observed when cells were transfected with the empty vector (lane 1), but a strongly hybridizing band at 2.2 kb was evident in cells transfected with either clone 1 (lane 2) or clone 2 (lane 3).

**RESULTS**

**Cloning of human liver CEH.** With the use of primers based on human macrophage CEH, a 1,870-bp product was amplified from total RNA from the human liver. Direct cloning and sequencing of this PCR product resulted in the identification of two different clones: clone 1 was shorter by one amino acid and lacked an alanine at position 18 compared with clone 2. The complete sequence and predicted amino acid sequence of clone 1 are shown in Fig. 1. The first ATG codon, 20 bp downstream from the 5′-end, was identified by the sequence CCACGATG, differing only in the fifth nucleotide from the

tained in William's E medium supplemented with insulin (0.25 U/ml) and penicillin (100 U/ml) in a 5% CO₂ atmosphere at 37°C. Culture medium also contained 0.1 μM dexamethasone and 1.0 μM L-thyroxine, as described previously (19). The next day, cells were transfected with either the empty vector pCMV or clone 1 or clone 2 using Effectene. During transfection, [3H]cholesterol (1 μCi/ml) was also included in the medium. After 48 h, the culture medium was subjected to Bligh and Dyer extraction. Radioactivity associated with the methanol-water phase (incorporation into bile acids) and the chloroform phase (unincorporated cholesterol) was determined. Bile acid biosynthesis was measured as the conversion of [3H]cholesterol to 3H-labeled bile acids in culture media (14). Cells were lysed, and cell-associated radioactivity was determined to obtain the total incorporation of [3H]cholesterol that remained constant.

**Real-time PCR for CEH.** Total RNA was extracted with the RNeasy Mini Kit (Qiagen). Total RNA (5 μg) was reverse transcribed with the Thermoscript RT-PCR System (Invitrogen), and first-strand cDNA was used to perform real-time PCR using the Stratagene Mx3000p real-time PCR system with TaqMan Human CEH Expression Assays (HS00275607_m1, Applied Biosystems). A standard curve, plotted as the observed cycle threshold value versus log DNA copy number, was generated by serial dilution of clone 2 plasmid DNA (copy number range: 2.35–4.7 × 10⁷). Tissue expression was calculated as the absolute copy number from this standard curve. Copy number per microgram RNA in hepatocytes, adipose tissue, human monocyte cell line THP1, and THP1 macrophages is shown.

**measurement of bile acid synthesis.** Primary human hepatocytes were purchased from a National Institutes of Health-approved facility (Liver Tissue Procurement Distribution System, University of Minnesota). Cells were plated in six-well tissue culture plates and main-

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**Fig. 2.** Expression of CEH mRNA in transfected COS-7 cells. COS-7 cells were transiently transfected with either the empty vector (lane 1) or with clone 1 (lane 2) or clone 2 (lane 3). Total RNA was extracted and electrophoresed on an agarose gel. The 28S and 18S rRNA bands were visualized by ethidium bromide staining (A). RNA was transferred to a membrane and hybridized to a radiolabeled full-length CEH cDNA probe. After a high-stringency wash, the blot was autoradiographed as described in *Northern blot analysis*. B: no hybridization was observed when cells were transfected with the empty vector (lane 1), but a strongly hybridizing band at 2.2 kb was evident in cells transfected with either clone 1 (lane 2) or clone 2 (lane 3).

**Fig. 3.** CEH activity in transfected COS-7 cells. COS-7 cells were transiently transfected with either the empty vector (pCMV) or with clone 1 or clone 2. Cells were harvested, and cell lysates were assayed for CEH activity as described in *Transcript transfection of COS-7 cells*. CEH specific activity is expressed as nanomoles of oleate released per hour per milligram of protein. Three protein concentrations were used to ensure the linear range of protein concentration, and mean specific activity was calculated. Data are means ± SD for 3 independent experiments. *P < 0.05.
consensus sequence CCACCATG, described by Minchiotti et al. (17). The nucleotide sequence surrounding this ATG codon also corresponds to the optimal consensus sequence AXXATG-GXG for the initiation of translation by eukaryotic ribosomes (12). Beginning with this start codon, a long open reading frame coding for 567 amino acids was observed. A termination codon, TGA, is present at position 1701, leaving a 146-bp 3'-untranslated region. The polyadenylation signal as well as the poly(A) tail is excluded from these clones because the 3'-primer used for the amplification of these clones was upstream of the polyadenylation sequence of macrophage CEH.

Expression of human liver CEH in transiently transfected COS-7 cells. The authenticity of cloned human liver CEH was verified by expression of clone 1 and clone 2 in COS-7 cells. Expression was driven by the cytomegalovirus promoter located upstream of the 5'-end of the cDNA. Expression of mRNA was monitored by Northern blot analysis, and a single band with positive hybridization was observed in cells transfected with either clone 1 (Fig. 2B, lane 2) or clone 2 (Fig. 2B, lane 3), confirming the expression of CEH mRNA in these transfected cells. No hybridization was seen in cells transfected with vector alone (Fig. 2B, lane 1). The integrity of RNA samples is demonstrated by intact 28S and 18S bands stained with ethidium bromide (Fig. 2A).

To establish that the mRNA produced in transfected cells indeed coded for active CEH, cell lysates were assayed for CEH activity. Lysates from cells transfected with either clone 1 or clone 2 had greater than threefold higher CEH activity compared with cells transfected with empty vector, with both clones (Fig. 3) authenticating the cloned cDNA as a functional CEH.

Tissue distribution of CEH. The multiple tissue blot was probed with human liver CEH cDNA, and the results are shown in Fig. 4. A single band corresponding to a size of 2.2 kb was seen in most of the tissues. Although the liver had the highest expression of CEH, significant expression was also observed in the heart, lung, small intestine, colon, spleen, and...
leukocytes, suggesting a wide tissue distribution of same or highly similar CEH in the majority of the tissues.

Detection of CEH expression in human hepatocytes. Although total liver RNA contains RNA from all hepatic cells, hepatic cholesterol homeostasis is maintained by coordinated regulation of enzymes in hepatocytes and not Kupffer cells. To determine whether CEH was indeed expressed in hepatocytes to play a role in cholesterol homeostasis, CEH mRNA levels were determined in human hepatocytes by quantitative RT-PCR. A standard curve was generated by using serial dilutions were determined in human hepatocytes by quantitative RT-PCR. A standard curve was generated by using serial dilutions of recombinant plasmid DNA, and the range of linearity for this assay system extended from 23 to $10^7$ CEH copy numbers (Fig. 5). The copy number of CEH in human hepatocytes was determined to be $1.6 \times 10^6/g$ total RNA compared with $3.14 \times 10^6/g$ total RNA in THP1 monocytes and THP1 macrophage and $8.19 \times 10^6/g$ total RNA in adipose tissue, respectively.

Expression of CEH protein in human hepatocytes was further confirmed by Western blot analysis. Increased immunoreactivity was observed when an increasing concentration of total cellular protein was loaded onto a SDS-PAGE gel (Fig. 6). Taken together with mRNA expression, these data establish the expression of CEH in human hepatocytes, placing this enzyme in the correct cell type to regulate hepatic cholesterol homeostasis.

Effect of CEH overexpression on bile acid synthesis. CEH hydrolyses hepatic CEs that are either produced by esterification of FC by ACAT2 or taken up by hepatocytes via selective uptake from HDL to release FC for bile acid synthesis. We examined the effects of CEH overexpression in human hepatocytes on bile acid synthesis. Differences in bile acid synthesis were determined by monitoring the conversion of $[^{3}H]$cholesterol to methanol-water-soluble materials and $[^{3}H]$labeled bile acids as described in Measurement of bile acid synthesis. A significant increase ($P < 0.05$) in bile acid synthesis was observed when either clone 1 or clone 2 was expressed compared with the empty vector (Fig. 7). Therefore, overexpression of cloned hepatic CEH indeed plays a role in the synthesis of bile acids by human hepatocytes.

**Table 1. Comparison of clone 1 with liver carboxylesterase (L07764), macrophage CEH (AY268104), and the human gene (CES1)**

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<th>Nucleotide In</th>
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Sequences were aligned using BLAST, and single nucleotide changes and the corresponding amino acid changes in the coding region are shown. CEH, cholesteryl ester hydrolase.

**DISCUSSION**

We report here the cloning and identification of human liver CEH. Two clones were obtained, which differed in only one amino acid. The clones were authenticated by demonstrating the ability of the cDNAs to increase CE hydrolysis in transiently transfected COS-7 cells. The expression of this CEH mRNA was demonstrated in several human tissues including the liver. In the liver, CEH mRNA and protein were detected in hepatocytes, confirming CEH expression in the cell regulating hepatic cholesterol homeostasis. The cloned cDNAs exhibited a high degree of homology with rat hepatic CEH (9), a member of carboxylesterase family shown to be involved in hepatic cholesterol homeostasis and regulated by sterols via sterol response elements at the transcriptional level (18). Direct evidence for the role of human liver CEH in intracellular cholesterol metabolism is provided by the observed increase in bile acid synthesis by transiently transfected human hepatocytes. These results demonstrate the ability of liver CEH to increase FC availability for bile acid synthesis.

A search of the National Center for Biotechnology Information (NCBI) nucleotide database revealed that the isolated cDNAs were highly homologous to two liver carboxylesterases (Accession Nos. L07764 and L07665) reported by Kroetz et al. (13), which differed with respect to two 3-bp in-frame deletions leading to the loss of alanine at position 18 and glutamine at position 362 in L07765. Clones 1 and 2 reported here, however, differed only with the absence of alanine at position 18 in clone 1. When clone 1 was compared with liver carboxylesterase (L07764), in addition to the absence of Ala at position 18, only nine other nucleotide differences were noted, three of which lie in the 5’-untranslated region. Nucleotide changes and the corresponding amino acid changes are shown in Table 1. Single amino acids have profound effects on the catalytic properties and substrate specificities of enzymes, although the mechanism of these effects remains to be elucidated. It remains to be seen whether the above-mentioned substitutions are essential for conferring the ability to hydrolyze CEs because CEs were not substrates tested by Kroetz et al., who only described hydrolysis of aromatic and aliphatic esters by human liver carboxylesterase. A high degree of sequence homology also exists between human macrophage
CEH (11) and human liver CEH; the two proteins differed only in five amino acid residues (Table 1). This is in contrast with ACAT, where the macrophage and liver isoforms [ACAT1 (4) and ACAT2 (3), respectively] are only 44% homologous.

Sequence alignment of human liver CEH with the human genome (BLAST search) identifies the gene on chromosome 16 with the official symbol CES1 (carboxylesterase 1) and one of the gene aliases as CEH. This gene is 30 kb long, coding with 14 exons (Fig. 8). Three transcriptional variants are described based on NCBI RefSeq analyses, and the two clones described here are indeed two of the three variants. Detailed analyses of exon/intron boundaries showed that lack of alanine on position 18 in clone 1 is due to the splice shift at the end of exon 1. In addition to the lack of alanine in position 18, one of the two liver carboxylesterases reported by Kroetz et al. (L07765) also lacked glutamate in position 362, and this is the result of another splice shift at the end of exon 10. Although Kroetz et al. (13) demonstrated that the isoform lacking two amino acids was inefficiently glycosylated, the differences in the isoforms with or without alanine in position 18 remain to be explored. Table 1 also shows the nucleotide present at the positions where clone 1 differs either from liver carboxylesterase (L07764) or macrophage CEH (AY268104). It is noteworthy that at all the positions where differences exist, at least two of these three transcripts have the same nucleotide or amino acid residue. Future studies will define whether these differences represent true single nucleotide polymorphisms. Single amino acid differences in rat carboxylesterases significantly alter the substrate specificities, and lack of critical residues results in the loss of the ability to hydrolyze CEs (29). Identification of human liver CEH will now permit the analyses of hepatic CES1 isoform(s) expression and how it may regulate the metabolism of hepatic cholesterol.

As a member of the carboxylesterase family, human liver CEH also contains the catalytic triad of serine, histidine, and an acidic residue as indicated in Fig. 1. Thus human liver CEH becomes the third member of the carboxylesterase family (rat hepatic CEH and human macrophage CEH being the other two) to be involved in intracellular CE metabolism. The ability of CEH expressed in transiently transfected COS-7 cells to hydrolyze CEs presented as lipid droplets in the assay system establishes its role in the hydrolysis of intracellular CEs stored as cytoplasmic lipid droplets. However, future studies will determine whether this hepatic CEH can also hydrolyze CEs delivered to hepatocytes via selective uptake from HDL particles. Raeven et al. (22) have shown that CEs delivered via selective uptake pathway accumulate prominently in cytoplasmic lipid storage droplets of the cells and would therefore be in the ideal physical state to be hydrolyzed by the hepatic CEH identified here. The observed stimulation of bile acid synthesis from human hepatocytes transiently transfected with clone 1 and clone 2 provides the first evidence that this enzyme can stimulate cholesterol removal from the liver as bile acids by increasing the availability of FC.

In conclusion, the identification of human liver CEH and demonstration of its expression in human hepatocytes represents the first step toward understanding the role of CEH in bile acid synthesis and hepatic cholesterol homeostasis. Future studies will determine the additional role of this enzyme in hydrolysis of CEs delivered via selective uptake from HDL and thereby defining its role in reverse cholesterol transport.

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GRANTS

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