

Hormone-sensitive lipase is involved in hepatic cholesteryl ester hydrolysis

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Abstract Hormone-sensitive lipase (HSL) regulates the hydrolysis of acylglycerol and cholesteryl ester (CE) in various organs, including adipose tissues. However, the hepatic expression level of HSL has been reported to be almost negligible. In the present study, we found that mice lacking both leptin and HSL (*Lep^{ob/ob}/HSL^{-/-}*) showed massive accumulation of CE in the liver compared with *Lep^{ob/ob}/HSL^{+/+}* mice, while triacylglycerol (TG) accumulation was modest. Similarly, feeding with a high-cholesterol diet induced hepatic CE accumulation in *HSL^{-/-}* mice. Supporting these observations, we detected significant expression of protein as well as mRNA of HSL in the liver. *HSL^{-/-}* mice showed reduced activity of CE hydrolase, but not of TG lipase, in the liver compared with wild-type mice. Furthermore, we confirmed the expression of HSL in viable parenchymal cells isolated from wild-type mice. The hepatocytes from *HSL^{-/-}* mice showed reduced activity of CE hydrolase and contained more CE than those from *HSL^{+/+}* mice even without the incubation with lipoproteins. Incubation with LDL further augmented the accumulation of CE in the HSL-deficient hepatocytes. From these results, we conclude that HSL is involved in the hydrolysis of CE in hepatocytes.—Sekiya, M., J-i. Osuga, N. Yahagi, H. Okazaki, Y. Tamura, M. Igarashi, S. Takase, K. Harada, S. Okazaki, Y. Iizuka, K. Ohashi, H. Yagyu, M. Okazaki, T. Gotoda, R. Nagai, T. Kadowaki, H. Shimano, N. Yamada, and S. Ishibashi. **Hormone-sensitive lipase is involved in hepatic cholesteryl ester hydrolysis.** *J. Lipid Res.* 2008. 49: 1829–1838.

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The mammalian liver plays a central role in maintaining the whole body cholesterol homeostasis. The liver delivers cholesterol to peripheral tissues via VLDL particles, and the excess peripheral cholesterol is transported to the liver through HDL acceptors (1). Cholesterol balance in the liver involves three major processes: 1) the input pathway, which consists of de novo synthesis and lipoprotein uptake from the periphery; 2) the output pathway, which consists of secretion into the circulation as VLDL and into the bile; 3) reversible conversion of cholesterol to cholesteryl esters (CEs). Esterification of cholesterol with long-chain fatty acyl-CoA is accomplished by ACAT (2). Newly formed CEs are secreted as a component of VLDL or are stored in intracellular lipid droplets in the cytoplasm, where CEs undergo a constant cycle of hydrolysis and resynthesis.

To date, at least four major CE hydrolases have been identified in the liver and shown to differ in their subcellular localizations, functions, and enzymatic properties. 1) The lysosomal acid lipase (LAL) is involved in the hydrolysis of CEs and triacylglycerols (TGs) delivered to the hepatocytes via receptor-mediated endocytosis of lipoproteins (3). 2) The cytosolic CE hydrolase from rat liver has also been purified and characterized. This lipase has been considered to be a key enzyme for releasing free cholesterol from the stores of CEs in the cytoplasm (4). 3) The microsomal CE hydrolase has also been purified from rat liver and characterized (5). 4) Carboxyl ester lipase (CEL) is another cholesterol esterase present in the he-

Abbreviations: ALT, alanine aminotransferase; ATGL, adipose triacylglyceride lipase; CE, cholesteryl ester; CEL, carboxyl ester lipase; DG, diacylglycerol; HSL, hormone-sensitive lipase; LAL, lysosomal acid lipase; LPDS, lipoprotein-deficient serum; NPC, nonparenchymal cell; PC, parenchymal cell; SREBP, sterol-regulatory element binding protein; TG, triacylglycerol.

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patic extralysosomal membrane compartment and possesses the capacity to hydrolyze HDL-CE (6).

Hormone-sensitive lipase (HSL) is an intracellular neutral lipase that catalyzes the hydrolysis of cellular CEs as well as TG, diacylglycerol (DG), monoacylglycerols, and other lipids. HSL is diffusely distributed throughout the cytosol, and upon activation, translocation of HSL from the cytosol to lipid droplets occurs (7, 8). Although HSL was initially identified as an adipose-specific lipase, it has been clarified that HSL is also expressed and functions in a wide variety of organs and cells, including heart, skeletal muscle, adrenal glands, testes, ovaries, pancreatic β -cells, and intestines (8–10). Despite the broad tissue distribution, the liver has been considered to lack HSL (11), although observations suggesting the importance of HSL in liver were reported (12).

Several laboratories, including ours, have generated HSL-deficient ($HSL^{-/-}$) mice by targeted gene disruption (12–15). Despite the decreased ability of their adipocytes to release FFAs, these mice were not obese. We have further generated leptin/HSL double mutant mice ($Lep^{ob/ob}/HSL^{-/-}$) by cross-breeding leptin-deficient $Lep^{ob/ob}$ mice with $HSL^{-/-}$ mice (16) to elucidate the role of HSL, especially in the setting of obesity. Unexpectedly, the $Lep^{ob/ob}/HSL^{-/-}$ mice gained less adiposity than $Lep^{ob/ob}$ mice, with massive accumulation of preadipocytes in adipose tissue, and HSL deficiency also suppressed the feeding activity of $Lep^{ob/ob}$ mice.

Here, we show that HSL plays a pivotal role as a lipase in the liver, especially as a CE hydrolase. Massive accumulation of CEs was observed in the liver of $Lep^{ob/ob}/HSL^{-/-}$ mice, while the hepatic TG content of $Lep^{ob/ob}/HSL^{-/-}$ mice was similar to that of $Lep^{ob/ob}/HSL^{+/+}$ mice. Moreover, feeding diets containing high cholesterol resulted in the accumulation of hepatic CEs in $HSL^{-/-}$ mice. In accordance with these results, CE hydrolase activity was decreased by HSL deficiency, while TG lipase activity was unchanged. These results suggested that HSL could serve as a CE hydrolase in hepatocytes. As expected, the expression of HSL is detected in liver homogenates, and parenchymal cells (PCs) contain more HSL protein than nonparenchymal cells (NPCs). Furthermore, to eliminate indirect effects from other tissues, we isolated primary hepatocytes. The CE hydrolase activity is reduced in the hepatocytes from $HSL^{-/-}$ mice. Intracellular CE content is increased even in the absence of lipoproteins in the hepatocytes from $HSL^{-/-}$ mice, and upon exposure to LDL, a more pronounced increase was observed. Based on these findings, we conclude that HSL is definitely expressed in hepatocytes and involved in CE hydrolysis in the liver.

EXPERIMENTAL PROCEDURES

Animals

Cross-breeding experiments used leptin-deficient $Lep^{ob/ob}$ mice. $HSL^{-/-}$ mice (13) and $Lep^{ob/ob}/HSL^{-/-}$ mice (16) were generated, and genotyping was performed as described previously

(16). Mice were housed in a temperature-controlled environment with a 12 h light/dark cycle and allowed free access to water and a standard chow diet (Oriental MF, composed of 60% carbohydrate, 13% fat, and 27% protein on a caloric basis; Oriental Yeast, Tokyo, Japan). Mice were euthanized at 16 weeks after a 6 h fast, except in the fasting/refeeding study, in which mice were starved for 24 h or refed for 12 h after a 24 h starvation.

Dietary cholesterol load experiments

Eight-week-old female $HSL^{-/-}$ mice and their littermate wild-type mice were fed a standard chow with or without 2% cholesterol for 7 weeks. Mice were euthanized after a 12 h starvation. All experiments were performed in accordance with our institutional guidelines.

Liver lipid content

Liver lipid was extracted by the chloroform-methanol method, and total cholesterol and free cholesterol were determined by fluorometric microassay according to a modified method of Heider and Boyett (17), with some modifications as described previously (18).

Immunoblotting of HSL

Livers (two mice for each genotype) were excised from 16 week old male mice after 6 h of fasting, homogenized in buffer A (20 mM Tris-HCl, pH 7.0, 250 mM sucrose, 5 μ g/ml leupeptin, and 2.8 μ g/ml aprotinin), and microcentrifuged at $14,000\times g$ for 15 min. Fifty micrograms of the supernatant fraction was subjected to SDS-PAGE analysis. Homogenate (10 μ g) from epididymal white adipose tissues of a wild-type male mouse (16 weeks old) was used as a control. Immunoblot analysis was performed using an ECL kit (Amersham Biosciences, Piscataway, NJ) (13).

Hepatocyte isolation

Isolation of viable PCs and NPCs from mouse liver was performed as described previously (19, 20). In brief, the portal vein was cannulated, and the liver was perfused with 0.05% collagenase (Wako Pure Chemicals, Tokyo, Japan). After removing undigested tissues, the cell suspension was centrifuged at $100\times g$ for 2 min. The pellet was used for PC preparation, while for NPC preparation the supernatant was filtered through a nylon mesh to eliminate PCs. The cell suspension was layered on top of 70% (v/v) Percoll (Amersham Bioscience, Tokyo, Japan) and then centrifuged at $800\times g$ for 10 min. The pellet was saved as intact PCs. The supernatant located on the top of Percoll, which contained most of the NPCs but also some PCs and debris, was further centrifuged through a two-step Percoll gradient (25% + 50%) at $800\times g$ for 30 min. A pure NPC band at the interface between the two density cushions was collected. The cells were washed twice with PBS and pelleted by centrifugation. The purity of the cells was morphologically assessed by light microscopic examination. More than 90% of the cells in the PC fraction were hepatocytes, whereas less than 10% of the cells in the NPC fraction were hepatocytes. The cell pellet was redissolved in buffer A and sonicated with a Branson sonifier at power 2 for two 10 s pulses. After microcentrifugation at $14,000\times g$ for 10 min, an aliquot of the supernatant was analyzed by immunoblot analysis.

Northern blot analysis

Total RNA was isolated using Trizol Reagent (Life Technologies, Helgerman, CT). Ten microgram RNA samples equally pooled from four to six mice of each genotype were electrophoresed on a 1% agarose gel containing formaldehyde and

transferred to a nylon membrane. The membranes were hybridized with probes that were labeled with [α - 32 P]dCTP using the Megaprime DNA Labeling System kit (Amersham Biosciences) in Rapid-hyb Buffer (Amersham Biosciences) and analyzed using a BAS2000 Bioimaging Analyzer (Fuji Photo Film, Tokyo, Japan). Loading was normalized by the expression of 36B4 (acidic ribosomal phosphoprotein P0) mRNA.

TG lipase and neutral CE hydrolase activity assay

Enzyme activity was assayed basically as described previously (13) with some modifications in the sample preparation. In brief, liver S-100 protein in buffer A was obtained by ultracentrifugation at 100,000 $\times g$ for 45 min. Buffer exchange to PBS containing 1 mM EDTA was carried out by dialysis for 4 h, because the liver homogenate turned out to include some unknown factor(s) that interfered with assay linearity (data not shown). The samples were incubated at 37°C for 30–60 min in a final volume of 200 μ l of a reaction mixture containing 105 μ mol/l tri[3 H]oleoylglycerol (99.4 μ Ci/ μ mol), 23.7 μ mol/l lecithin, 12.5 μ mol/l sodium taurocholate, 1 mol/l NaCl, and 85 mmol/l potassium phosphate (pH 7.0). The high concentration of NaCl was included to inactivate LPL. CE hydrolase activity was measured using a reaction mixture containing 6.14 μ mol/l cholesterol [1- 14 C]oleate (48.8 μ Ci/ μ mol).

Microsomal ACAT activity

Liver microsomal protein was obtained by ultracentrifugation at 100,000 g for 45 min. The precipitates were resuspended and ultracentrifuged again at 100,000 $\times g$ for 45 min. The precipitates were resuspended and used for the assay. ACAT activity in microsomes was determined by the rate of incorporation of [1- 14 C]oleoyl-CoA into the CE fraction according to Yagyu et al. (18).

Biochemical analyses

Blood was collected from the retro-orbital venous plexus after a 6 h or a 12 h fast. Cholesterol (Determiner TC; Kyowa Medex, Tokyo, Japan), TG (TG LH; Wako Pure Chemicals), FFA (NEFA C; Wako Pure Chemicals), and alanine aminotransferase (ALT) (transaminase C-II; Pyruvate oxidase *Pedococcus* sp. (POP) N-(2-hydroxy-3-sulfopropyl)-m-toluidine (TOOS) method; Wako Pure Chemicals) were measured enzymatically. HPLC was performed as described previously (21).

TLC

Liver lipid was extracted by the chloroform-methanol method, and aliquots were pooled for each genotype. Evaporated lipid was dissolved in chloroform, and lipid from 0.6 mg of liver tissue was loaded in each lane. Lipids were separated by TLC with hexane-diethylether-acetic acid (80:20:1) or chloroform-acetone-acetic acid (96:4:1) as solvent. Visualization was done with 10% sulfuric acid.

Histology

Mice (16 weeks) were euthanized after a 6 h fast, and their livers were excised and fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were stained with hematoxylin-eosin.

Primary culture of hepatocytes and CE formation

Hepatocytes were isolated from nonfasted $HSL^{-/-}$ mice and their littermate $HSL^{+/+}$ mice (10 weeks) by the collagenase perfusion method as described previously (22). Cells were resuspended in Williams' E medium supplemented with 5% fetal calf serum, and seeded on collagen-coated dishes at a final density of 2×10^4 cells/cm 2 , and attached for 6 h.

For the neutral CE hydrolase assay, the attached cells were maintained in Medium 199 containing Earle's salts (Invitrogen) and 5% fetal calf serum. After incubation for 20 h, the medium was replaced with the same medium and the cells were further incubated for 12 h. The cells were washed twice with PBS, scraped into buffer A, and sonicated with a Branson sonifier at power 2 for two 10 s pulses. After microcentrifugation at 14,000 g for 10 min, an aliquot of the supernatant was analyzed for neutral CE hydrolase assay.

For CE formation, attached cells were incubated in Williams' E medium containing 5% human lipoprotein-deficient serum (LPDS) for 48 h and subsequently incubated in the same medium containing human LDL (100 μ g/ml) and [1- 14 C]oleate-albumin complex with vehicle or 10 μ M CS-505, an ACAT inhibitor, which were provided by Sankyo and Kyoto Pharmaceuticals (Tokyo, Japan), for 24 h as described previously (23) with minor modifications. Intracellular lipids were separated by TLC, and spots corresponding to the CE fraction were scraped and radioactivities were measured. LPDS (d > 1.21 g/ml) and LDL (d = 1.019–1.063 g/ml) were isolated with sequential ultracentrifugation from plasma of healthy human volunteer subjects (24). Informed consent was obtained from the volunteers according to our institutional guideline for the use of human materials.

Statistics

Statistical differences between groups were analyzed by one-way ANOVA and a posthoc Tukey-Kramer test, unless stated otherwise.

RESULTS

Both leptin deficiency and dietary cholesterol load induce massive accumulation of CEs in the liver of $HSL^{-/-}$ mice

Leptin-deficient $Lep^{ob/ob}$ mice exhibit many characteristics of obesity, including hepatic steatosis (25). To determine the effect of HSL deficiency on hepatic lipid metabolism, especially in a leptin-deficient obesity model, $HSL^{-/-}$ mice were cross-bred with leptin-deficient $Lep^{ob/ob}$ mice and the liver lipid content of each genotype was measured (6 h fasted mice; Fig. 1A). To our surprise, in the $Lep^{ob/ob}$ background, the hepatic CE content was remarkably increased (173% for males and 214% for females) in $Lep^{ob/ob}/HSL^{-/-}$ mice compared with $Lep^{ob/ob}/HSL^{+/+}$ mice. There was no difference in acylglycerol content between $Lep^{ob/ob}/HSL^{+/+}$ and $Lep^{ob/ob}/HSL^{-/-}$ mice. In the $Lep^{+/+}$ background, acylglycerol content was reduced in $HSL^{-/-}$ mice compared with $HSL^{-/-}$ mice, consistent with previous reports (26, 27). There was a trend toward an increase in hepatic CE content in $HSL^{-/-}$ compared with $HSL^{+/+}$ mice, especially in female mice (17% for males and 137% for females). Free cholesterol content was not affected by either the absence or the presence of HSL or leptin.

$HSL^{-/-}$ mice have reduced plasma FFA levels caused by the impediment of the mobilization of FFA from adipose tissues (12–16). In the fasted state, HSL normally stimulates adipose lipolysis, thus increasing the influx of FFA to the liver and contributing to hepatic TG accumulation. Therefore, we evaluated both fasted and refed states (fasting, 24 h of fasting; refeeding, 12 h of feeding after 24 h of fasting; Fig. 1B). In the fasted state, hepatic TG and CE tended to accumulate to a greater extent, and the differences between

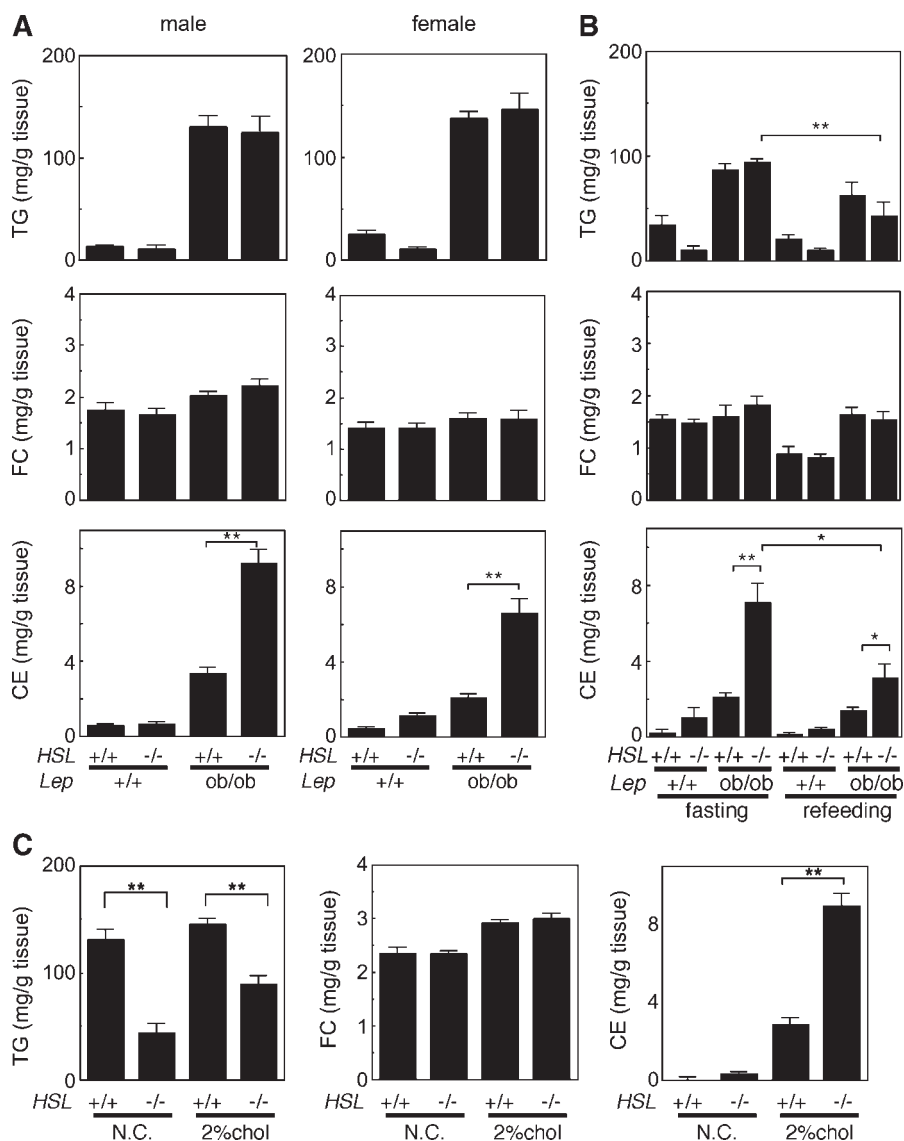


Fig. 1. Liver lipid contents of each genotype in the cross-breeding experiment with *Lep^{ob/ob}* mice. A: After a 6 h fast (n = 10, 16 weeks of age). B: Fasting/refeeding study (n = 4, 16 weeks of age; female mice were starved for 24 h or refed for 12 h after a 24 h starvation). C: Liver lipid content of hormone-sensitive lipase-deficient (*HSL^{-/-}*) mice fed a high-cholesterol diet (n = 5–6; female mice were fed a standard chow diet with or without 2% cholesterol for 7 weeks.). Livers were excised, and lipid was extracted by the chloroform-methanol method. Total cholesterol and free cholesterol (FC) were determined by fluorometric microassay. CE, cholesteryl ester; TG, triacylglycerol. Data are expressed as means ± SEM. * *P* < 0.05, ** *P* < 0.01 as determined by ANOVA followed by the Tukey-Kramer posthoc test.

HSL^{+/+} and *HSL^{-/-}* mice were more pronounced than in the refed state. In both states, the accumulation of CEs in the *HSL^{-/-}* mice described above was conserved.

Moreover, hepatic accumulation of CEs in *HSL^{-/-}* mice was confirmed with the diet-induced model. *HSL^{-/-}* mice were challenged with dietary cholesterol (2%) for 7 weeks (euthanized after a 12 h fast; Fig. 1C). Dietary cholesterol induced hepatic CE accumulation in *HSL^{-/-}* mice (217% increase).

HSL is expressed in PCs

Liver has been reported to express HSL at a negligible level (11), whereas our data suggested that there was he-

patic HSL expression. Therefore, we performed immunoblot analysis of liver homogenates (Fig. 2A). The hepatic expression of HSL was detected in both *Lep^{+/+}* and *Lep^{ob/ob}* mice using our polyclonal anti-HSL antibody, and the expression level was not affected by the presence or absence of leptin. Quantification of the immunoblotting signals indicated that the expression levels of HSL in liver were approximately 5% of those in adipose tissue.

HSL has been considered to be present in macrophages (13, 28–30), although there are some conflicting reports (31, 32). Since Kupffer cells are the resident macrophages in liver, we examined whether or not HSL was expressed in purified hepatocytes. After digesting liver by collage-

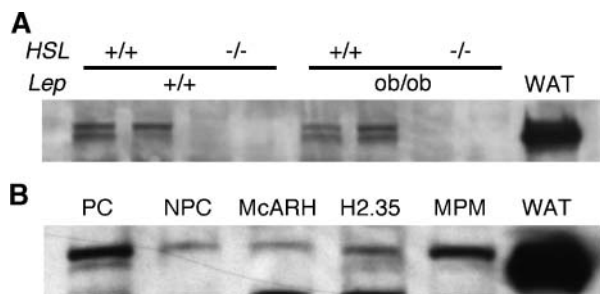


Fig. 2. Immunoblot analysis of HSL protein in the liver. A: Homogenate of liver (50 μ g) was subjected to SDS-PAGE in duplicate for each genotype. In the right lane, homogenate (10 μ g) of epididymal white adipose tissue (WAT) from wild-type male mice was loaded as a control. B: Immunoblot analysis of different types of liver cells and murine hepatocyte cell lines using polyclonal anti-HSL antibody. Homogenate (20 μ g) of each cell population was loaded. In the right lane, homogenate (4 μ g) of epididymal white adipose tissue from wild-type male mice was loaded as a control. PC, parenchymal cell; NPC, nonparenchymal cell; H2.35, H2.35 hepatocyte cell line; McARH, McARH-7777 hepatoma cell line; MPM, mouse peritoneal macrophage.

nase perfusion, viable PCs and NPCs were purified on a Percoll gradient. The expression of HSL was detected in PCs as well as in NPCs, which contain Kupffer cells, and HSL was also expressed in H2.35 and McARH7777 murine hepatocyte cell lines (Fig. 2B). HSL was more highly expressed in PCs than in NPCs (Fig. 2B). Taken together, these findings indicate that HSL is indeed expressed in hepatocytes and contributes especially to hepatic CE hydrolase activity. For some samples from whole liver homogenates and liver PCs, the bands immunoreactive with our anti-HSL antibody were duplets. For samples from NPCs, McARH7777, H2.35, or mouse peritoneal macrophages, however, the bands were single, with the same molecular weight as the upper bands of the duplets. We speculate that the lower band shown in some samples from whole liver homogenates or PCs is a product of proteolysis or nonspecific cross-reactivity of the antibody.

Furthermore, Northern blot analysis provided additional evidence of hepatic HSL expression. As shown in Fig. 3A, the expression of HSL was almost negligible in a refed state, and fasting induced the expression, which indicates that the hepatic expression of HSL is regulated by nutritional status. The expression pattern of HSL might explain the fact that the hepatic accumulation of CEs in $HSL^{-/-}$ mice was more pronounced in the fasted state.

HSL deficiency decreases CE hydrolase activity but not TG lipase activity in the liver

To further explore the underlying mechanisms, neutral lipase activities of liver homogenates were measured. In a dietary cholesterol load experiment (12 h of fasting), CE hydrolase activity was reduced in $HSL^{-/-}$ mice (49% reduction in the standard chow group and 53% reduction in the 2% cholesterol chow group; Fig. 4A). In a leptin-deficient model, CE hydrolase activity was reduced in fasted $HSL^{-/-}$ mice (31% reduction), and the difference

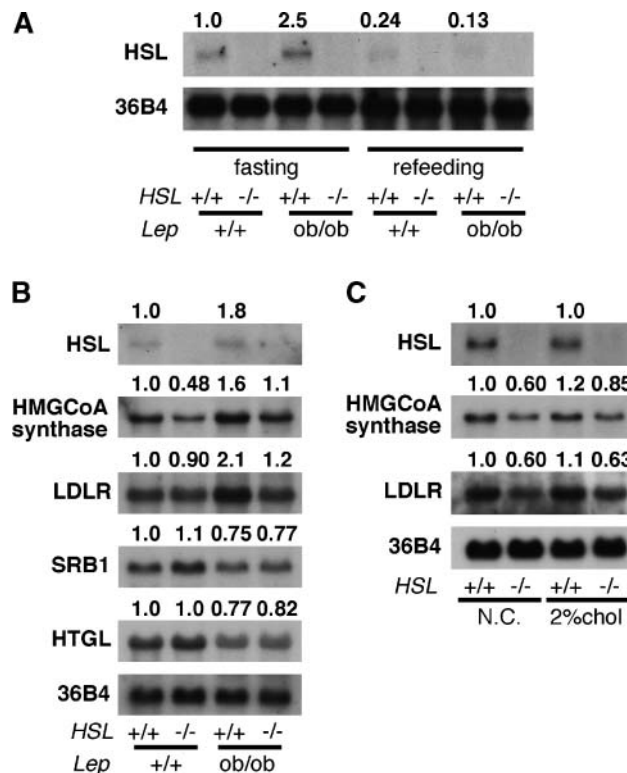


Fig. 3. Gene expression profile of $HSL^{-/-}$ mice cross-bred with $Lep^{ob/ob}$ mice. A: Mice were starved for 24 h or refed for 12 h after a 24 h starvation (female, 16 weeks of age; $n = 4$). B: Mice were starved for 6 h (male, 16 weeks of age; $n = 5$). C: $HSL^{-/-}$ mice fed a high-cholesterol diet ($n = 5-6$; female mice were fed a standard chow diet with or without 2% cholesterol for 7 weeks, and mice were starved for 12 h). Their livers were excised and total RNA was isolated. Ten micrograms of total RNA from each liver was pooled and subjected to Northern blotting, followed by hybridization with the indicated cDNA probes. HTGL, hepatic triglyceride lipase; HSL, hormone-sensitive lipase; LDLR, low density lipoprotein receptor; SRB1, scavenger receptor class B type I. Loading was normalized by the expression of 36B4 (acidic ribosomal phosphoprotein P0) mRNA, and the fold changes compared with the control are displayed above each blot.

between $HSL^{+/+}$ and $HSL^{-/-}$ mice was decreased in the refed state, which was in accordance with the expression pattern. On the other hand, neither leptin nor HSL affected TG lipase activity, and TG lipase activity was increased in the fasted state (Fig. 4C).

Excess cholesterol in the endoplasmic reticulum is esterified by ACAT and stored in cytoplasmic lipid droplets (2). The ACAT activity was not different between $HSL^{+/+}$ and $HSL^{-/-}$ mice, and the ACAT activity did not explain the excess accumulation of CEs in $HSL^{-/-}$ mice (Fig. 4B).

HSL deficiency alters the expression profiles of genes controlling liver lipid metabolism

Next, we evaluated the hepatic expression profiles of genes regulating lipid homeostasis (Fig. 3). Cholesterol input through de novo synthesis, represented here by HMG-CoA synthase, and lipoprotein uptake, represented by the LDL receptor (1), were decreased in both $HSL^{-/-}$

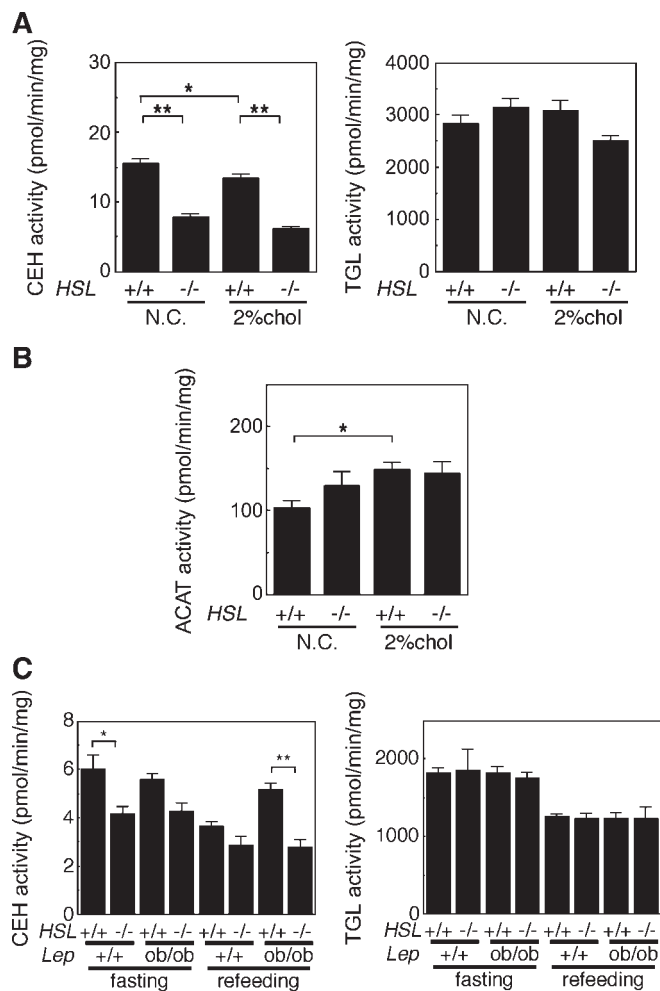


Fig. 4. Enzymatic activity of liver homogenates. A, B: $HSL^{-/-}$ mice fed a high-cholesterol diet ($n = 5-6$; female mice were fed a standard chow diet with or without 2% cholesterol for 7 weeks, and mice were starved for 12 h). C: $HSL^{-/-}$ mice cross-bred with $Lep^{ob/ob}$ mice. Mice were starved for 24 h or refed for 12 h after a 24 h starvation (female, 16 weeks of age; $n = 4$). A and C show enzymatic activity of liver homogenates from each genotype. Livers were excised and homogenized in buffer A (250 mM sucrose, 20 mM Tris-HCl, pH 7.4, and protease inhibitors). S-100 protein was obtained by ultracentrifugation at 100,000 g for 45 min. Buffer exchange to phosphate-buffered saline containing 1 mM EDTA was carried out by dialysis for 4 h. The samples were incubated at 37°C for 30–60 min in a reaction mixture containing tri^3H oleoylglycerol or $[1-^{14}C]$ oleate. TGL and CEH denote TG lipase and CE hydrolase, respectively. For B, microsomal ACAT activity was measured. Liver microsomal protein was obtained by ultracentrifugation at 100,000 g for 45 min. The precipitates were resuspended and used for assay. ACAT activity in microsomes was determined by the rate of incorporation of $[1-^{14}C]$ oleoyl-CoA into the CE fraction. Data are expressed as means \pm SEM. * $P < 0.05$, ** $P < 0.01$ as determined by ANOVA followed by the Tukey-Kramer posthoc test.

and $Lep^{ob/ob}/HSL^{-/-}$ mice. The reverse cholesterol transport pathway mediated by HDL also transports CE to the liver. The expression levels of two key players in this pathway, hepatic triglyceride lipase and scavenger receptor class B type I, were not changed.

HSL deficiency affects the plasma lipoprotein profile

Hepatic lipoprotein secretion is one of the pathways regulating cholesterol homeostasis in the liver. $HSL^{-/-}$ mice were reported to have reduced levels of VLDL caused by decreased FFA mobilization from adipose tissues to the liver (26). The reduction of FFA and VLDL by HSL disruption was also confirmed in the $Lep^{ob/ob}$ background (Fig. 5A). Increased plasma HDL-cholesterol in $HSL^{-/-}$ mice was also reported (26) and confirmed in our study, but the increase of HDL-cholesterol was blunted in the $Lep^{ob/ob}$ background.

Dietary cholesterol load also altered these lipoprotein profiles. Plasma total cholesterol was increased by the increased HDL-cholesterol in $HSL^{-/-}$ mice fed normal chow. Measurement of plasma cholesterol levels revealed a significant increase in $HSL^{-/-}$ mice fed a 2% cholesterol diet (Fig. 5D), and the increase was caused by increased VLDL/LDL-cholesterol (VLDL-cholesterol and LDL-cholesterol were increased by 92% and 50%, respectively) (Fig. 5C).

Excessive TG accumulation plays a role in the progression of liver injury. On the other hand, it is not well known whether CE accumulation leads to liver injury. Therefore, we examined plasma ALT levels as an indicator of liver injury. Excessive accumulation of TG induced by leptin deficiency increased the plasma ALT level, while accumulation of CEs in $HSL^{-/-}$ mice did not affect plasma ALT (Fig. 5B). In addition, we examined whether histological alteration occurred following the CE accumulation in $Lep^{ob/ob}/HSL^{-/-}$. Despite the striking increase of CE content in $Lep^{ob/ob}/HSL^{-/-}$ mice, we found no histological difference between $Lep^{ob/ob}/HSL^{+/+}$ and $Lep^{ob/ob}/HSL^{-/-}$ mice (data not shown).

DG accumulation was not noticeable in $HSL^{-/-}$ mice

It has been speculated that HSL is a rate-limiting enzyme in the DG catabolism in adipose tissues, since DG accumulation and TG lipase activity were observed in adipose tissues of $HSL^{-/-}$ mice (15, 33). In our study, the acylglycerol content in $Lep^{ob/ob}/HSL^{-/-}$ mice was not significantly different from that in $Lep^{ob/ob}/HSL^{+/+}$ mice. We performed TLC to examine whether DG was accumulated in the liver of $Lep^{ob/ob}/HSL^{-/-}$ mice. Almost all of the acylglycerol detected in the livers of $HSL^{-/-}$ mice consisted of TG, while DG accumulation in adipose tissue was confirmed in both $Lep^{+/+}/HSL^{-/-}$ and $Lep^{ob/ob}/HSL^{-/-}$ mice (Fig. 6). It is necessary to further elucidate whether or not there might be a difference that was undetectable by our methods in the DG content between $HSL^{+/+}$ and $HSL^{-/-}$ mice.

CE hydrolysis was impaired in primary cultured hepatocyte from $HSL^{-/-}$ mice

HSL is widely expressed in multiple tissues, and HSL deficiency alters systemic lipid metabolism, including the impediment of the mobilization of FFA from adipose tissues and increased remnant lipoprotein clearance via LPL activity in muscle and white adipose tissue (26). To eliminate these indirect effects through systemic factors

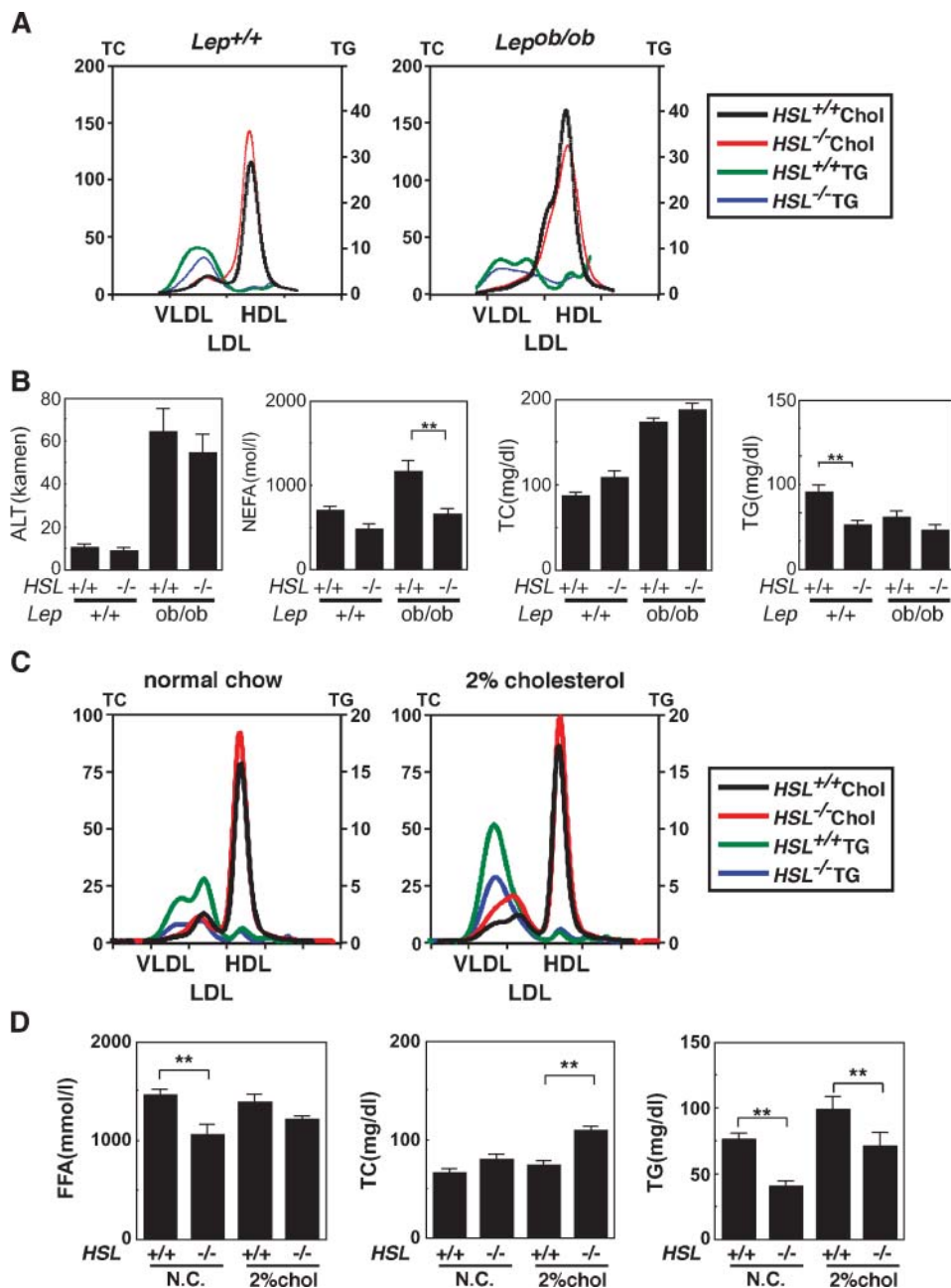


Fig. 5. Plasma analysis. A, B: *HSL^{-/-}* mice cross-bred with *Lep^{ob/ob}* mice (12 weeks of age, male; n = 10 for each genotype) were starved for 6 h, and blood was collected from the retro-orbital venous plexus. C, D: *HSL^{-/-}* mice fed a high-cholesterol diet [n = 5–6; female mice (8 weeks) were fed a standard chow diet with or without 2% cholesterol for 7 weeks, and mice were starved for 12 h]. A and C show analysis of plasma lipid by HPLC. Chol and TG denote cholesterol and triacylglycerol, respectively. For B and D, plasma alanine aminotransferase (ALT) levels and plasma lipid profiles are shown. TC, total cholesterol. Data are expressed as means \pm SEM. ** $P < 0.01$ as determined by ANOVA followed by the Tukey-Kramer posthoc test.

and reinforce the in vivo results, we also examined cholesterol metabolism in primary cultured hepatocytes. The CE hydrolase activity in *HSL^{-/-}* hepatocytes was reduced compared with that in *HSL^{+/+}* hepatocytes (65% reduction; Fig. 7A). Furthermore, we exposed these cells to human LDL (100 μ g/ml) and examined [14 C]oleate incorporation into the CE fraction. Even when the cells

were cultured in medium supplemented with LPDS, the *HSL^{-/-}* hepatocytes contained more CE compared with *HSL^{+/+}* hepatocytes (47% increase), although the extent of CE accumulation was small. In the presence of LDL, more pronounced CE accumulation was observed (54% increase). These responses were blunted by ACAT inhibitor (10 μ M CS-505) (Fig. 7B).

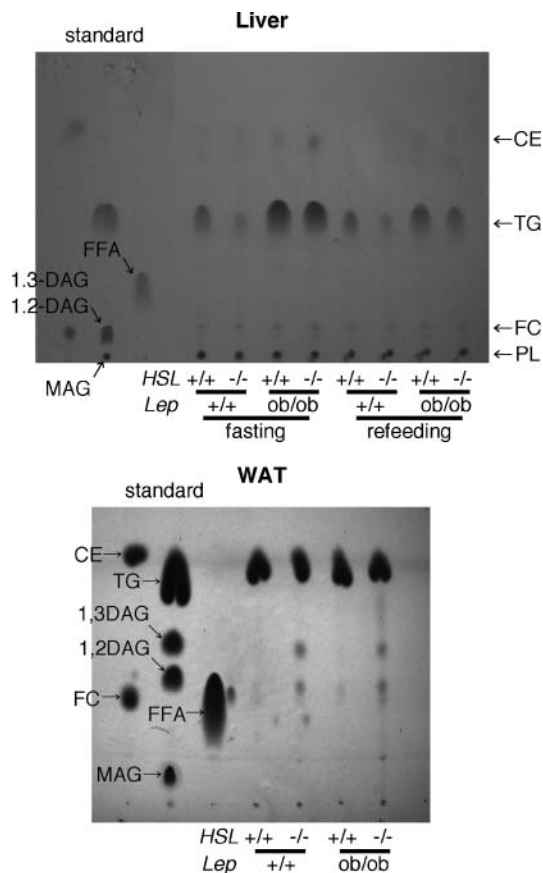


Fig. 6. Lipid separation by TLC. Liver lipid was extracted by the chloroform-methanol method, and aliquots were pooled for each genotype ($n = 4$). Pooled lipids from 0.6 mg of liver tissue were loaded in each lane. Lipids were separated with hexane-diethylether-acetic acid (80:20:1) or chloroform-acetone-acetic acid (96:4:1) as solvent. Standards are shown at left. As a control, lipids from epididymal white adipose tissue (WAT) were also separated and visualized. CE, cholesterol ester; DAG, diacylglycerol; FC, free cholesterol; FFA, free fatty acids; MAG, monoacylglycerol; PL, phospholipid; TG, triacylglycerol.

DISCUSSION

In the present report, we attempted to investigate whether HSL is involved in the hydrolysis of neutral lipids in the liver. We show here that HSL was indeed expressed in hepatocytes and that a deficiency of HSL induced hepatic CE accumulation, especially under two different nutritional conditions: leptin deficiency and dietary cholesterol load.

To date, several lipases that have CE hydrolase activity have been identified in hepatocytes, including hepatic cytosolic CE hydrolase (4), CEL (34, 35), LAL (3), and microsomal CE hydrolase (5). In our study, HSL deficiency decreased hepatic CE hydrolase activity and induced hepatic CE accumulation. Among a wide variety of lipases expressed in hepatocytes, HSL definitely plays an important role in CE metabolism.

On the other hand, lipases with TG lipase activity include adipose triglyceride lipase (ATGL) (36), TG hydrolase (37), TG hydrolase-2 (38), hepatic triglyceride lipase (39), LPL, and LAL. Among these lipases, ATGL

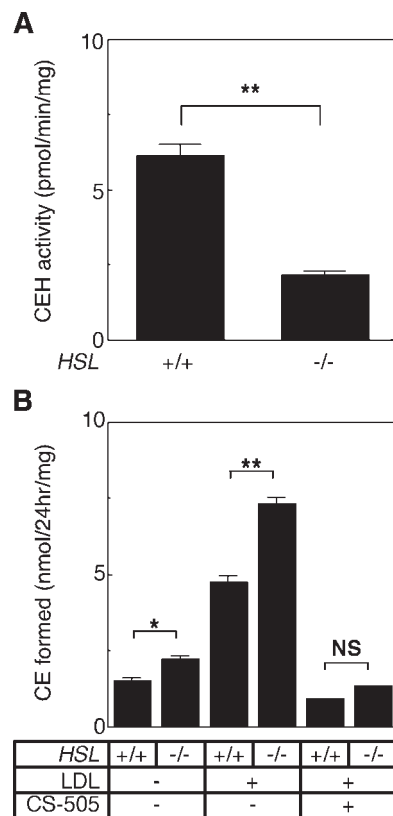


Fig. 7. Primary cultured hepatocytes. Hepatocytes were isolated from nonfasted $HSL^{-/-}$ mice and their littermate $HSL^{+/+}$ mice by the collagenase perfusion method. A: After isolation (male, 10 weeks; $n = 5$ for each genotype), the attached cells were maintained in Medium 199 containing Earle's salts and 5% fetal calf serum for 20 h. The medium was replaced with the same medium, and the cells were further incubated for 12 h. The whole cell lysates were assayed for CE hydrolase (CEH) activities. Data are expressed as means \pm SEM. ** $P < 0.01$ as determined by Student's t -test. B: CE formation. Attached cells were incubated in Williams' E medium containing 5% human LPDS for 48 h and subsequently incubated in the same medium containing 100 μ g/ml LDL and [14 C]oleate-albumin complex with vehicle or the ACAT inhibitor CS-505 (10 μ M) for 24 h. Intracellular lipids were separated by TLC, spots corresponding to the CE fraction were scraped, and radioactivities were measured. Data are expressed as means \pm SEM. * $P < 0.05$, ** $P < 0.01$ as determined by ANOVA followed by the Tukey-Kramer posthoc test.

would play an important role in hepatic TG metabolism. ATGL-null mice have been reported to exhibit hepatic TG accumulation and reduced TG lipase activity (36). In our study, HSL deficiency affected neither TG lipase activity nor hepatic TG content. Other TG lipases could compensate for HSL deficiency, and DG lipase activity might not be regulated by HSL in hepatocytes, although it is regulated by HSL in adipocytes.

Liver has two populations of cells that exhibit neutral CE hydrolase activity: hepatocytes and macrophages. The expression of HSL in macrophages is widely accepted but remains controversial to some extent (13, 28–32). The relative contribution of the resident macrophages to the reduced neutral CE hydrolase activity of $HSL^{-/-}$ mice would be almost nil because 1) the neutral CE hydrolase activity

of $HSL^{-/-}$ macrophages is not blunted (13) and 2) the relative number of Kupffer cells in the liver is small (PCs, 65%; liver endothelial cells, 21%; Kupffer cells, 8.5%; stellate cells, 5.5%) (40). We demonstrated that HSL is definitely expressed in PC and mediates hepatic lipid homeostasis.

It should be necessary to eliminate indirect effects from systemic factor(s) because HSL is widely expressed among tissues and HSL deficiency alters systemic lipid metabolism. HSL is also involved in cholesterol metabolism in adrenal cells (13, 41, 42). $HSL^{-/-}$ mice have normal basal levels of corticosteroids but are unable to increase secretion in response to ACTH stimulation. The endocrinological alteration in $HSL^{-/-}$ mice might also contribute to hepatic CE accumulation. We examined primary cultured hepatocytes and demonstrated that CE hydrolase activity was reduced in $HSL^{-/-}$ hepatocytes and that intracellular CE content was increased in $HSL^{-/-}$ hepatocytes. A normal membrane polarity is vital for hepatocytes to perform their functions, such as bile secretion and serum lipoprotein secretion. There were some inconsistencies between in vivo and in vitro, such as the extent of accumulation of CE. These inconsistencies could be partially explained by the loss of polarity induced by cell isolation or by the systemic alteration of lipid metabolism induced by HSL deficiency. To further validate the function of HSL in the liver, liver-specific HSL knockout mice would be helpful.

The neutral CE hydrolase activity in the liver homogenate of $HSL^{-/-}$ mice was reported elsewhere to be approximately 20% of that in $HSL^{+/+}$ mice (12), while our results showed a milder decrease in HSL-deficient mice. Several factors could explain the discrepancy between our results and those of Mulder et al. (12). 1) We found that liver crude homogenates include some unknown factors that interfere with assay linearity, and to exclude those factors, buffer exchange to PBS was carried out. 2) The genetic background of the mice used by Mulder et al. (12) was SV129/C57BL/6J hybrid, whereas our mice were back-crossed to C57BL/6J. 3) The difference in diet composition may also affect the results: our study showed that feeding conditions affect lipase activity and that the neutral CE hydrolase activity of $HSL^{-/-}$ mice was reduced especially in the fasted state. The differences of feeding conditions may partially explain the discrepancy. 4) The reaction conditions used are different. Our enzymatic activities were measured in the presence of taurocholate, which is known to be an activator of CEL (6).

Wolman disease and cholesteryl ester storage disease are caused by defective LAL activity, resulting in massive accumulation of CEs and TGs in hepatocytes (43). $HSL^{-/-}$ and $Lep^{ob/ob}/HSL^{-/-}$ mice are similar to mice with LAL deficiency regarding the fact that CEs are accumulated in the liver, but the resultant phenotypes caused by defective activities of HSL and LAL deficiency are not identical. HSL deficiency did not lead to the hepatosplenomegaly observed in LAL-deficient mice (44). Patients with defective LAL activity show clinical sequelae of liver dysfunction. Although $Lep^{ob/ob}$ mice had elevated serum ALT levels accompanied by increased hepatic TG content, HSL deficiency did not affect serum ALT levels. These phenotypic differences

might be explained by several factors, including differences in the subcellular localization of lipases and lipid and the amount of accumulated CEs.

It was reported that hepatic VLDL synthesis is reduced in $HSL^{-/-}$ mice as a result of decreased FFA transport from the adipose tissue to the liver (26). It was also reported that the decreased VLDL-TG in $HSL^{-/-}$ mice is associated with increased LPL activity in muscle and white adipose tissue (26). The reduced plasma VLDL in $HSL^{-/-}$ mice was also confirmed in our experiments, and these findings were applicable to $Lep^{ob/ob}/HSL^{-/-}$ mice. Despite the reduced VLDL secretion in $HSL^{-/-}$ mice, dietary cholesterol load increased plasma VLDL/LDL-cholesterol. The direct involvement of hepatic HSL in lipoprotein metabolism should be evaluated using primary cultured hepatocytes or liver-specific HSL-deficient mice. As shown in Results, the LDL receptor and HMG-CoA synthase, which are representative genes regulated by sterol-regulatory element binding protein (SREBP)-2, were downregulated in $HSL^{-/-}$ mice; therefore, reduced lipoprotein uptake via the LDL receptor would partially explain these lipoprotein profiles. Upon activation, SREBP-2 is released from the membrane into the nucleus as a mature protein by a sequential two-step cleavage process, and the activation is regulated by cellular cholesterol (45). The precise mechanism underlying the transcriptional regulation in $HSL^{-/-}$ mice should be further elucidated.

Our experiments revealed that the expression of hepatic HSL is regulated transcriptionally by nutritional status. It was reported that the expression of HSL is regulated by glucose using an adipocyte cell line (46) and that upstream stimulatory factors 1 and 2 are involved in the transcriptional regulation of HSL (47). We observed similar transcriptional regulation in vivo, although the precise mechanisms have not been elucidated. HSL in adipose tissue is known to play a pivotal role in providing fatty acids to supply energy for demand in the fasted state. HSL in liver might play a similar role by hydrolyzing stored CEs and providing fatty acids for ATP synthesis in the fasted state.

In conclusion, HSL is definitely expressed in hepatocytes, and the deficiency of HSL reduced hepatic CE hydrolase activity but not TG lipase activity. This reduction of CE hydrolase activity induced the accumulation of CE in liver. This study provides important new insights into hepatic cholesterol metabolism, especially regarding hepatic CE hydrolysis. **LR**

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