Abnormal Lipid Metabolism in Cystathionine $\beta$-Synthase-deficient Mice, an Animal Model for Hyperhomocysteinemia*

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Hyperhomocysteinemia (HHCY) is a consequence of impaired methionine/cysteine metabolism and is caused by deficiency of vitamins and/or enzymes such as cystathionine $\beta$-synthase (CBS). Although HHCY is an important and independent risk factor for cardiovascular diseases that are commonly associated with hepatic steatosis, the mechanism by which homocysteine promotes the development of fatty liver is poorly understood. CBS-deficient (CBS$^{-/-}$) mice were previously generated by targeted deletion of the Cbs gene and exhibit pathological features similar to HHCY patients, including endothelial dysfunction and hepatic steatosis. Here we show abnormal lipid metabolism in CBS$^{-/-}$ mice. Triglyceride and nonesterified fatty acid levels were markedly elevated in CBS$^{-/-}$ mouse liver and serum. The activity of thiolase, a key enzyme in $\beta$-oxidation of fatty acids, was significantly impaired in CBS$^{-/-}$ mouse liver. Hepatic apolipoprotein B100 levels were decreased, whereas serum apolipoprotein B100 and very low density lipoprotein levels were elevated in CBS$^{-/-}$ mice. Serum levels of cholesterol/phospholipid in high density lipoprotein fractions but not of total cholesterol/phospholipid were decreased, and the activity of lecithin-cholesterol acyltransferase was severely impaired in CBS$^{-/-}$ mice. Abnormal high density lipoprotein particles with higher mobility in polyacrylamide gel electrophoresis were observed in serum obtained from CBS$^{-/-}$ mice. Moreover, serum cholesterol/triglyceride distribution in lipoprotein fractions was altered in CBS$^{-/-}$ mice. These results suggest that hepatic steatosis in CBS$^{-/-}$ mice is caused by or associated with abnormal lipid metabolism.

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**EXPERIMENTAL PROCEDURES**

**Detection of the CBS Transcript and Protein in Mouse Tissues**

**Northern Blot Analysis**—Total RNA was isolated from various tissues of an 8-week-old C57BL/6 male mouse using TRIzol (Invitrogen), and 10 μg of each RNA sample was separated on a 6% formaldehyde, 1% agarose gel. After the separated RNA was transferred to a Hybond-XL nylon membrane (Amersham Biosciences), the hybridization was performed as described previously (18). The entire open reading frame of rat CBS cDNA was used as a specific probe for the mouse CBS transcript. The probe was radiolabeled with [α-32P]dCTP (Amersham Biosciences), and after hybridization, the blot was scanned with a Bio-Imaging Analyzer Baa2500 (Fuji Photo Film). As a loading control, ribosomal 18 S RNA was stained with ethidium bromide.

**Western Blot Analysis**—Tissues were quickly removed from an 8-week-old C57BL/6 male, homogenized with a Teflon tissue grinder in ice-cold buffer (100 mM sodium phosphate, pH 7.8, and 1 mM phenylmethylsulfonyl fluoride), and sonicated with the Sonifier 450 (Branson Ultrasonics, Danbury, CT). The homogenate was centrifuged at 10,900 × g for 5 min at 4 °C, and the supernatant was further centrifuged at 17,400 × g for 20 min at 4 °C. The resulting supernatant was quickly frozen in liquid nitrogen and stored at −80 °C until use. Five micrograms of each tissue sample was solubilized in SDS sample buffer, boiled for 5 min, separated on a 10% SDS-polyacrylamide gel, and transferred to an Immobilon polyvinylidene difluoride transfer membrane (0.45 μm; Millipore). The mouse CBS protein was detected with an anti-rat CBS polyclonal antibody (1:1000 dilution), a horseradish peroxidase-labeled anti-rabbit IgG antibody, and the ECL Western blotting system (Amersham Biosciences).

**Mice and Genotyping**

Heterozygous CBS-deficient mice (CBS−/−) on the C57BL/6 background were purchased from the Jackson Laboratory and subsequently bred with C57BL/6 mice (CLEA Japan, Tokyo, Japan) in our institution. CBS−/− mice were bred to produce homozygous CBS−/− mice. Tail biopsies were done at 1 week of age, and polymerase chain reaction was performed for genotyping of the targeted CBS allele (15). Wild-type (CBS+/+) heterozygous, and homozygous mice were used at 2–3 weeks of age. Age-matched mice were compared in each experiment.

**Measurement of Thiolarase, 3-Hydroxyacyl-CoA Dehydrogenase, and Lecithin-Cholesterol Acyltransferase Enzyme Activities**

Tissues were quickly removed, frozen in liquid nitrogen, and stored at −80 °C until use. The tissues were homogenized with the Polytron homogenizer (Kinematica AG, Lucerne, Switzerland) in ice-cold phosphate buffer, and the homogenate was centrifuged at 15,000 × g for 5 min. The protein content of the supernatant was determined with the Bio-Rad protein assay. Thiolarase activity was measured as described previously (19). Briefly, the reaction was initiated by adding tissue samples to a reaction mixture containing 50 μM 3-oxoacyl-CoA, 0.15 mM CoA, 25 mM MgCl2, 50 mM KCl, and 100 mM Tris-HCl (pH 8.0). The decrease in absorbance at 303 nm was measured, and the enzyme activity was expressed by the absorbance units (AU)/min/mg of protein. The 3-hydroxyacyl-CoA dehydrogenase activity was measured as described previously (20). Briefly, the reaction was initiated by adding tissue samples to a reaction mixture containing 50 mM potassium phosphate (pH 7.4), 0.15 mM NADH, and 25 μM acetoacetyl-CoA. The decrease in absorbance at 340 nm was measured, and the enzyme activity was expressed by the AU/min/mg of protein. Serum LCAT activity was determined using a commercial assay kit from Daiichi Pure Chemicals (Tokyo, Japan).

**Measurement of Lipid Components**

Lipids were extracted from tissue homogenates according to the established protocol of Bligh and Dyer (21). One volume of methanol was added to the homogenates and mixed, and then two volumes of chloroform were added and mixed. After centrifugation at 15,000 × g for 5 min, the lower phase was collected, dried, and subjected to the measurement of lipid components. TG, nonesterified fatty acid (NEFA), total cholesterol (TC), and phospholipid (PL) were independently measured with commercial enzymatic assay kits according to the manufacturers’ instructions (Wako Chemicals, Osaka, Japan). In our assay system, the detected PLs include choline-associated phospholipids such as phosphatidylcholine (lecithin), lysophosphatidylcholine, and sphingomyelin, but not phosphatidylethanolamine. The measurement of serum lipid components was similarly performed without the lipid extraction steps. Serum HDL, cholesterol, and PL were measured after the removal of other lipoproteins by the precipitation with sodium phosphotungstate-magnesium, using a commercial kit (Wako Chemicals).

**Electrophoresis of Lipoproteins**

Serum lipoproteins were analyzed by polyacrylamide disk gel electrophoresis using the Lipophor system (Jokoh, Tokyo, Japan). Serum samples were analyzed with 3% gels after blood collection. Twenty-five microliters of serum was mixed with 200 μl of the loading buffer containing Sudan Black, and the lipoproteins were separated by polyacrylamide disk gel electrophoresis. Serum HDL was analyzed by two-dimensional electrophoresis as described previously (agarose gel for the first dimension and polyacrylamide gel for the second dimension) (22). The first dimensional electrophoresis was performed using the Titan Gel Lipoprotein (Heleno Laboratories, TX). The second dimensional electrophoresis was performed using a 5–20% gradient native polyacrylamide gel. After the electrophoresis, the lipoproteins were transferred to an Immobilon polyvinylidene difluoride membrane and probed with an anti-apoA-I antibody.

**Detection of apoB100 and apoA-I**

ApoB100 and apoA-I levels were determined by the Western blot analysis. Serum samples were diluted (1:30) with SDS sample buffer. LPL was homogenized in 50 mM potassium phosphate buffer containing the protease inhibitor mixture (Roche Applied Science). The protein contents of the samples were determined by the Bio-Rad protein assay, and the samples were subjected to SDS-polyacrylamide electrophoresis. Western blot analyses were performed with antibodies against mouse apoB100 and apoA-I (Santa Cruz). Apo-A1 in HDL fractions was detected from samples prepared with sodium phosphotungstate-magnesium (Wako Chemicals). The electrophoresis was performed using 2–15% and 5–20% gradient gels for apoB100 and apoA-I, respectively. The apoB100 band intensity was quantified using NIH Image software.

**Gene Expression of Thiolarase and LCAT**

Thiolarase (both mitochondrial and peroxisomal) and lcat expression levels were determined by the Northern blot analysis. Total RNA was isolated from liver with TRIzol, and 5 μg of each RNA sample was separated by 6% formaldehyde, 1% agarose gel electrophoresis followed by blotting to the Hybond-XL nylon membrane. Partial mouse cDNA sequences for peroxisomal thiolarase, mitochondrial thiolarase, and LCAT (nucleotides 1–627, 1–700, and 1–687, respectively), were used as specific cDNA probes. The cDNA probes were isolated from adult mouse liver cDNA by PCR amplification and labeled with digoxigenin using the DIG DNA labeling Mix (Roche Applied Science). The hybridization was performed at 50 °C for 16 h. Hybridized DNA probes were detected with a horseradish peroxidase-labeled anti-digoxigenin antibody (Roche Applied Science) and the ECL system. The expression levels of thiolarase and lcat were normalized by the band intensities of ethidium bromide-stained ribosomal 18 S RNA. The average expression levels in the wild-type mice were expressed as 100%.

**Lipolysis by LPL**

LPL hydrolysis of VLDL was measured as described previously (23, 24) with some modifications. Briefly, serum samples were incubated with purified bovine LPL (0.2 mg/ml) in 100 mM Tris (pH 8.5) at 37 °C for 10 min. The reaction was stopped by the addition of 100 mM Tris (pH 8.5) and 0.1% Triton X-100. The samples were immediately placed on ice, and the NEFA contents were measured using the commercial enzymatic assay kit as described. The NEFA levels in samples without LPL addition represented the basal levels.

**Lipoprotein Analysis by HPLC System**

HPLC analysis of serum lipoproteins was performed as described (25, 26). In brief, 100-μl samples of diluted serum were applied onto two columns (WQK Lipoprotein HPLC) connected in tandem. Two conventional FPLC system cannot separate CM from VLDL, but, in contrast, this HPLC system provides higher resolution especially in the fraction range of large lipoproteins in which CM and VLDL fractions can be separated. Both TG and cholesterol levels in post-column effluents were measured with the enzymatic reactions as described above.

**Statistical Analysis**

Statistical analyses were performed by the Student’s t test. The data presented are the means ± S.E. The p values < 0.05 were considered to be statistically significant.
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RESULTS

Predominant Expression of the Cbs Transcript and CBS Protein in Mouse Liver—The tissue distribution of the Cbs mRNA and CBS protein was determined in the adult mouse. Total RNA was isolated from eight different tissues and analyzed by Northern blotting. A single 3-kb Cbs transcript was detected in brain, liver, and kidney, but not in heart, lung, thymus, spleen, or skeletal muscle (Fig. 1A). Expression of the CBS protein was examined in the same eight tissues by the Western blotting using an anti-CBS antibody. The mouse 61.5-kDa CBS protein was most highly expressed in liver followed by kidney (at one-third of the liver level). Faint CBS expression was detected in brain but not in any other tissues tested (Fig. 1B). CBS expression was not detected in CBS−/− tissues (liver, kidney, and brain) (data not shown).

Altered Lipid Components and Thiolase Activity in CBS−/− Mouse Liver—Liver is the major locus for the Cbs/CBS expression (15) in which the Cbs/CBS is also expressed (Fig. 1). We measured several lipid contents (TG, NEFA, TC, and PL) by enzyme assays in CBS-deficient liver (Table I). The hepatic TG and NEFA levels were 11.4- and 3.1-fold greater, respectively, in CBS−/− mice than those in wild-type mice. The hepatic TC levels were not significantly different among the CBS+/+, CBS+/−, and CBS−/− mice, although the PL levels in CBS−/− liver were significantly lower than those in CBS+/+ or CBS+/− liver. Accumulation of TG and NEFA in CBS−/− liver could result from the impairment of lipid export, and 3) inhibition of β-oxidation. We measured the enzymatic activity of 3-oxoacyl-CoA thiolase, which is involved in the final step of β-oxidation of fatty acids in both mitochondria and peroxisomes and is a rate-limiting enzyme of β-oxidation. The hepatic thiolase activity in CBS−/− mice was significantly lower than that in the wild-type mice (42.8 ± 1.9 versus 75.3 ± 5.9 AU/min/g protein; p < 0.0002) (Fig. 2A). Next, we examined the expression of both peroxisomal and mitochondrial thiolase genes in liver. The expression of peroxisomal thiolase was significantly elevated in CBS−/− liver (139 ± 8% of the wild-type levels, p < 0.014) (Fig. 2B), whereas that of mitochondrial thiolase was not significantly different among the three genotypes (Fig. 2C). We have also measured hepatic enzymatic activity of 3-hydroxyacyl-CoA dehydrogenase, another enzyme in β-oxidation. No significant differences were observed among the three genotypes (Fig. 2D).

Altered Lipid and Lipoprotein Components in CBS−/− Mouse Serum—Lipid components in serum were determined (Table II). Serum TG and NEFA levels were significantly higher in CBS−/− mice than those in wild-type mice, whereas TC levels were not significantly different (Table II), as observed in liver (Table I). Serum NEFA levels were also significantly higher in CBS−/− mice than those in the wild-type mice. In contrast, serum PL levels were not significantly different among the three genotypes.

We further examined serum apolipoprotein B100 (apoB100) levels. ApoB100 is an unusually large (~550 kDa) secretory protein and is the major component of VLDL and low density lipoprotein (LDL) particles. Liver is a major locus for apoB100 expression and a site where apoB100 assembles endogenous lipids to form TG-rich lipoproteins such as VLDL (27). Serum apoB100 levels were measured by Western blotting using an anti-apoB100 antibody. Serum apoB100 protein levels were much higher in CBS−/− mice than those in the wild-type or CBS+/− mice (Fig. 3A). This observation suggests that serum VLDL and/or LDL levels are much higher in CBS−/− mice than those in the wild-type or CBS+/− mice.

To confirm the above observation, we analyzed the distribution of serum lipoproteins by polyacrylamide disk gel electrophoresis. In this system, lipoproteins were stained with Sudan Black and then separated by gel electrophoresis with mobility that depends on their particle sizes. CBS−/− mouse serum contained more VLDL particles and less HDL particles than the wild-type or CBS+/− mouse serum, whereas serum LDL levels were comparable among the three genotypes (Fig. 3B). This finding suggests that the increased serum apoB100 may be a reflection of the increased serum VLDL (not LDL) in CBS−/− mice. To examine whether VLDL secretion from liver is enhanced, hepatic apoB100 levels were analyzed by Western blotting. CBS−/− mouse liver contained less apoB protein than the wild-type or CBS+/− mouse liver (Fig. 3C), suggesting that the VLDL secretion from the liver into serum is down-regulated in CBS−/− mice.

Next, we examined whether serum lipoproteins (VLDL and CM) in CBS−/− mice are metabolized by LPL as those in wild-

| TABLE I  |
|---------------------------------|
| **CBS genotypes**               |
|---------------------------------|
| **TG (mg/g protein)**           |
| +/+ (n = 8)     | 734 ± 52        | 637 ± 50        | 3832 ± 846     |
| −/− (n = 7)     | 947 ± 1.7b      | 9.47 ± 1.7b     |
| **NEFA (mEq/g protein)**        |
| +/+ (n = 8)     | 3.04 ± 0.28     | 2.60 ± 0.41     |
| −/− (n = 7)     | 147 ± 12        | 153 ± 11        |
| **TC (mg/g protein)**           |
| +/+ (n = 8)     | 147 ± 12        | 153 ± 11        |
| −/− (n = 7)     | 124 ± 25        | 142 ± 25        |
| **PL (mg/g protein)**           |
| +/+ (n = 8)     | 1007 ± 37       | 965 ± 36        |
| −/− (n = 7)     | 684 ± 105a      | 684 ± 105b      |

*p < 0.0001.

Note: Data are the means ± SE from seven or eight mouse samples for each group. Student’s t test was performed for the pairwise comparison with the wild type (+/+). Lipid concentrations were calculated from lipid contents per protein contents (mg/g protein). TG, triglyceride; NEFA, nonesterified fatty acid; TC, total cholesterol; PL, (choline-associated) phospholipid.
Because LPL catalyzes the conversion of TG in VLDL (or CM) to NEFA, purified bovine LPL was added to serum samples, and NEFA production during 10-min incubation was measured. Although the basal NEFA levels in CBS−/− serum lipoproteins were comparable with (or rather higher than) those in wild-type or CBS+/− samples, the NEFA levels produced by exogenously added LPL in CBS−/− samples was much less than those in samples from either wild-type or CBS+/− mice (Fig. 3D). These results suggest that VLDL and CM in CBS−/− mouse serum could be inefficient substrates for endogenous LPL, which may cause abnormal accumulation of TG in both VLDL and CM.

Altered HDL Components and LCAT Activity in CBS−/− Serum—Because serum HDL levels were decreased in CBS−/− mice (Fig. 3B), the lipid components of HDL (cholesterol and PL) were investigated in serum. Total RNA isolated from liver was analyzed by the Northern blotting using digoxigenin-labeled peroxisomal and mitochondrial thiolase cDNA probes. The band intensity was quantified using NIH Image. The data are the means ± S.E. of five mouse samples for each group. *, p < 0.014 versus the wild-type. The insets show the Northern blot images of peroxisomal (B) and mitochondrial (C) thiolase mRNA, and UV-detected 18 and 28 S rRNA as loading controls. D, 3-hydroxyacyl-CoA dehydrogenase enzyme activity in CBS−/− mouse liver. The enzyme activity was calculated by following the decrease in absorbance at 340 nm and is expressed as the AU/min/mg of protein. The data are the means ± S.E. of five mouse samples for each genotype group.

**TABLE II**

The levels of TG, NEFA, TC, and PL in serum

Data are the means ± S.E. from seven or eight mouse samples for each group. Student’s t test was performed for the pairwise comparison with the wild type (+/+). Lipid concentrations were calculated from lipid contents per protein contents (mg/g protein). TG, triglyceride; NEFA, nonesterified fatty acid; TC, total cholesterol; PL, (choline-associated) phospholipid.

| CBS genotypes | +/+ (n = 7) | +/− (n = 10) | −/− (n = 7) |
|---------------|-------------|-------------|------------|
| TG (mg/ml)    | 5.52 ± 0.90 | 8.63 ± 2.50 | 17.95 ± 5.00a |
| NEFA (μEq/l)  | 459 ± 40    | 622 ± 30b   | 830 ± 60   |
| TC (mg/ml)    | 10.8 ± 0.5  | 9.9 ± 0.5   | 11.9 ± 1.1 |
| PL (mg/ml)    | 21.2 ± 1.8  | 18.5 ± 2.0  | 24.8 ± 3.2 |

a p < 0.04.  
b p < 0.004.  
c p < 0.0005.

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**Fig. 2.** Enzyme activity/gene expression of thiolase and enzyme activity of 3-hydroxyacyl-CoA dehydrogenase in CBS-deficient liver. A, thiolase enzyme activity. The activity was measured by following the decrease in absorbance at 303 nm and is expressed as the AU/min/mg of protein. The data are the means ± S.E. of six or seven mouse samples for each genotype group. *, p < 0.0002 versus the wild-type. B and C, peroxisomal (B) and mitochondrial (C) thiolase mRNA levels. Total RNA isolated from liver was analyzed by the Northern blotting using digoxigenin-labeled peroxisomal and mitochondrial thiolase cDNA probes. The band intensity was quantified using NIH Image. The data are the means ± S.E. of five mouse samples for each group. *, p < 0.014 versus the wild-type. The insets show the Northern blot images of peroxisomal (B) and mitochondrial (C) thiolase mRNA, and UV-detected 18 and 28 S rRNA as loading controls. D, 3-hydroxyacyl-CoA dehydrogenase enzyme activity in CBS−/− mouse liver. The enzyme activity was calculated by following the decrease in absorbance at 340 nm and is expressed as the AU/min/mg of protein. The data are the means ± S.E. of five mouse samples for each genotype group.
of fatty acids from phosphatidylcholine to cholesterol and thus produces esterified cholesterol-rich HDL (28, 29). We examined the gene expression and the enzymatic activity of LCAT in CBS-deficient mice. The hepatic lcat gene expression levels and LCAT activity in serum were significantly decreased in CBS/H11002/H11002 mice (64 and 11% of the wild-type levels, respectively) (Fig. 5). Although the lcat gene expression in CBS/H11001/H11002 liver was similar to that in the wild-type liver (Fig. 5A), the serum LCAT activity reduced in CBS/H11002/H11002 mice (~75% of the wild-type level) (Fig. 5B). Therefore, the impaired LCAT enzyme activity (rather than the decreased lcat expression) may underlie the decreased serum HDL-cholesterol in CBS/H11002/H11002 mice.

We measured free cholesterol (nonesterified cholesterol) levels in serum, because LCAT activity is correlated with serum esterified cholesterol level (30). The free cholesterol level was elevated in CBS−/− mouse serum (Fig. 6). We further examined both the serum and hepatic levels of apoA-I, a major structural protein of HDL particles that is required for the LCAT activation (31). The apoA-I levels in HDL fractions were comparable between the wild-type and CBS-deficient mice (Fig. 7A). Moreover, there were no significant differences in either serum or hepatic apoA-I levels between those mice (Fig. 7A). Therefore, the decreased LCAT activity in CBS−/− liver was not caused by apoA-I deficiency.
Small HDL Appeared in CBS−/− Mouse Serum—Serum HDL fractions were separated by two-dimensional gel electrophoresis, and apoA-I in HDL fractions was detected by Western blotting using an anti-mouse apoA-I antibody. A previous study showed that mature HDL particles were detected at the α site, whereas nascent HDL particles (whose particle sizes are smaller than those of mature ones) were at the pre-β site on the agarose gel electrophoresis (30). In the wild-type mice, apoA-I-containing lipoprotein migrated at the α site in the first-dimensional gel and at a single spot in the second-dimensional gel (Fig. 7B), indicating the predominant expression of mature HDL. In contrast, in CBS−/− mice, apoA-I-containing lipoprotein that migrated at the α site was found as two spots in the second-dimensional gel, suggesting the coexpression of mature and immature HDL.

Lipoprotein Analysis by HPLC—Finally, to confirm that HDL cholesterol is decreased and VLDL TG is increased in CBS−/− mouse serum, we performed HPLC analyses to directly measure their levels in each lipoprotein fraction (see “Experimental Procedures”). Using this high resolution system, serum levels of cholesterol and TG in lipoprotein fractions (corresponding to CM, VLDL, LDL, and HDL) can be separated (25, 26). Serum TG was mainly retained in LDL, CM, and VLDL fractions in the wild-type mice (Fig. 8A). The serum TG levels in CM and VLDL fractions were remarkably increased,
A

B

FIG. 7. ApoA-I-containing lipoprotein fractions in CBS-deficient mice. A, apoA-I levels in serum HDL fractions, whole serum, and liver extract. Each sample was analyzed by the Western blotting using an anti-apoA-I antibody. B, analysis of apoA-I-containing serum HDL fractions by two-dimensional gel electrophoresis. Serum HDL fractions were separated on a 0.7% agarose gel (for the first dimension) and subsequently on a non-denaturing 5–20% gradient polyacrylamide gel (for the second dimension). After the electrophoresis, the lipoproteins were transferred to a nylon membrane and analyzed by the Western blotting using an anti-apoA-I antibody.

whereas those in LDL fractions were unchanged in CBS<sup>−/−</sup> mice (Fig. 8A).

In contrast to serum TG, serum cholesterol was mainly retained in HDL and LDL fractions in the wild-type mice (Fig. 8B). However, when compared with the wild-type mice, the serum cholesterol levels in HDL fractions were remarkably decreased, whereas those in VLDL were slightly increased in CBS<sup>−/−</sup> mice (Fig. 8B). Interestingly, the cholesterol-containing lipoproteins in CBS<sup>−/−</sup> mouse serum showed a broad distribution over the lipoprotein fraction ranges (Fig. 8B), suggesting the occurrence of abnormal (cholesterol-containing) lipoproteins with intermediate particle sizes.

DISCUSSION

CBS is the key enzyme of the transsulfuration pathway for the biosynthesis of cysteine from methionine and catalyzes the condensation of homocysteine and serine into cystathionine. CBS deficiency leads to abnormal accumulation of homocysteine in serum (homocystinemia) and in urine (homocystinuria). Although liver is the major organ for the Cbs/CBS expression (Fig. 1) and the transsulfuration pathway (18), and hepatic steatosis is the common pathology observed in severe HHcy patients (2) and CBS<sup>−/−</sup> mice (15), the underlying mechanism has not been elucidated. CBS<sup>−/−</sup> mice suffer from severe growth retardation and some of them start to die after 2 weeks of age (15). Within 5 weeks after birth, the majority of them die, probably from severely impaired hepatic functions (15). In this study, we examined hepatic (and serum) lipid metabolism in CBS-deficient mice at 2–3 weeks of age.

In the present study, we showed that TG and NEFA levels were up-regulated in CBS<sup>−/−</sup> mouse liver and serum (Tables I and II). This finding is consistent with a previous report that showed the increased TG levels in diet-induced HHcy mouse liver, as well as increased TG (or cholesterol) levels in homocysteine-treated hepatoma cells (4). In addition, in CBS<sup>−/−</sup> mouse liver, the thiolase activity was markedly decreased (Fig. 2A), although the levels of gene expression of peroxisomal and mitochondrial thiolases were elevated and unchanged, respectively (Fig. 2, B and C). The reason for this is currently unknown; however, it was previously shown that two free cysteine residues that exist near the active center of thiolase are necessary for its enzymatic activity (32). The accumulated homocysteine may covalently cross-link with these cysteine residues of thiolase via disulfide bonds and inhibit its enzymatic activity. The apoB100 level was significantly elevated in CBS<sup>−/−</sup>
mouse serum (Fig. 3A), whereas it was decreased in CBS/−/− mouse liver (Fig. 3C). In addition, VLDL levels were much higher in CBS/−/− mouse serum than those in wild-type or CBS/+/− mouse serum (Fig. 3B). These lines of evidence suggest that the decreases in thiolase activity and TG release from the liver (as forms of VLDL particles) might cause or promote hepatic steatosis in CBS/−/− mice.

Choline-associated PL levels were significantly decreased in CBS/−/− liver (68% of the wild-type level) (Table I). A major choline-associated PL in liver is phosphatidylcholine (33), and phosphatidylcholine has been shown to play an important role in VLDL secretion from liver (34, 35). Also, phosphatidylcholine is a major PL involved in the membrane secretory pathway as well as a major PL component of VLDL particles. Therefore, the decreased phosphatidylcholine levels might lead to inhibition of VLDL secretion from the CBS/−/− liver.

Serum lipids are carried by particles containing several lipoproteins such as VLDL, LDL, and HDL. ApoB100-containing TG-rich VLDL particles are synthesized in and released from the liver and function to transport fatty acids to peripheral tissues such as adipose tissues and muscle. In peripheral tissues, VLDL particles are metabolized to LDL particles by the removal of core TG and dissociation of apolipoprotein (other than apoB100). We found that VLDL but not LDL levels were up-regulated in CBS/−/− mouse serum (Figs. 3B and 8A), suggesting that VLDL lipolysis might be impaired in CBS/−/− mice. Indeed, we found the decreased lipolysis by exogenously added LPL in CBS/−/− mouse serum (Fig. 3D). A previous study showed that oral administration of homocysteine thiolactone induces the elevation of plasma TG levels in rats (36). Therefore, the elevated serum homocysteine may inhibit intravascular VLDL lipolysis, resulting in hypertriglyceridemia. Hypertriglyceridemia is a potential risk marker for coronary heart diseases, especially when it is associated with low levels of HDL-cholesterol (37). Serum levels of HDL-cholesterol were markedly decreased in CBS/−/− mice (Fig. 4A), suggesting that CBS/−/− mice may be susceptible to atherosclerosis.

Intravascular VLDL lipolysis is regulated by LPL, which is activated by hepatic LCAT secreted into blood vessels (23, 38). In the present study, we found that the lcat expression and LCAT activity were significantly decreased in CBS/−/− mouse liver and serum, respectively (Fig. 5). Hepatic lcat expression levels in CBS/−/− mice were similar to those in the wild-type mice, whereas the serum LCAT activity in CBS/−/− mice was significantly lower than that in the wild-type mice (Fig. 5). The decreased LCAT activity resulted in increased nonesterified cholesterol levels in CBS/−/− mouse serum (Fig. 6). Therefore, the decrease in hepatic lcat expression in CBS/−/− mice may not be solely responsible for the reduced serum LCAT activity. A site-directed mutagenesis study revealed that thiol-specific reagents (such as 5,5′-dithiobis-2-nitrobenzoic acid) inhibit LCAT activity by covalent cross-linking with two free cysteine residues near the active site, suggesting that homocysteine may covalently cross-link with these cysteine residues via disulfide bonds, leading to the enzyme inhibition (39, 40). It may be possible to detect protein modification by homocysteine using physicochemical approaches (e.g. matrix-assisted laser desorption ionization mass spectrometry), which will be pursued in the future studies. It has been shown that LCAT-deficient mice have the decreased levels of esterified and HDL-cholesterol as well as increased levels of TG and free cholesterol (23, 30, 41), features that were also observed in our CBS-deficient mice. Therefore, CBS/−/− mice may have increased TG production from the liver caused by LCAT deficiency, resulting in the development of hepatic steatosis (23).

Serum HDL was analyzed in CBS/−/− mouse serum (Fig. 7). Two-dimensional electrophoresis revealed that small HDL migrating at the α site (but not the pre-β site) was observed in CBS/−/− serum. It has been shown that nascent HDL particles migrate at the pre-β site in first dimensional gel electrophoresis and exhibit faster migration than mature HDL particles in second dimensional gel electrophoresis. In LCAT-deficient mice, the increased levels of pre-β-migrating and α-migrating small HDL were observed (30). Because LCAT activity is impaired and HDL-cholesterol levels are decreased in CBS/−/− mice, α-migrating small immature HDL may appear in CBS/−/− serum.

Although total serum cholesterol levels were not significantly different between the wild-type and CBS/−/− mouse (Table II), the cholesterol distribution in lipoprotein fractions was remarkably altered; HDL cholesterol levels were down-regulated, whereas VLDL cholesterol levels were up-regulated in CBS/−/− mouse serum (Figs. 5A and 8B). It is well known that the decreased HDL cholesterol levels lead to the promotion of atherosclerosis. It will be intriguing to examine whether serum cholesterol contents in each lipoprotein fraction are normal in HHCHY patients.

In conclusion, we have analyzed lipid metabolism in CBS-deficient mice. In CBS/−/− mouse serum, TG, NEFA, and non-esterified cholesterol levels were up-regulated, whereas HDL-cholesterol and HDL-PL levels were down-regulated. The thiolase activity and apoB100 levels were reduced in CBS/−/− mouse liver, suggesting that hepatic steatosis may be caused by the impaired β-oxidation of fatty acids and the decreased VLDL secretion from liver. Furthermore, a small immature form of HDL was observed, and TG/cholesterol distributions in lipoprotein fractions (CM, VLDL, LDL, and HDL) were altered in CBS/−/− mouse serum. These observations suggest that homocysteine may regulate both the activity and gene expression of the enzymes involved in lipid synthesis/degradation (thiolase and LCAT) and that the excessive homocysteine accumulation caused by CBS deficiency leads to the dysregulation of lipid metabolism.

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