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Hyperhomocysteinemia due to cystathionine beta synthase deficiency induces dysregulation of genes involved in hepatic lipid homeostasis in mice

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***Background/Aims*: Cystathionine beta synthase (CBS) deficiency leads to severe hyperhomocysteinemia, which confers diverse clinical manifestations, notably fatty liver. Recently, abnormal lipid metabolism has been demonstrated in CBS-deficient mice, a murine model of severe hyperhomocysteinemia. To gain further insights into effects of CBS deficiency on hepatic cholesterol metabolism, the expression of hepatic genes involved in biosynthesis, uptake and efflux was determined in CBS-deficient mice.**

***Methods*: Gene expression analysis was performed on liver of CBS-deficient mice using quantitative real-time PCR.**

***Results*: We found that CBS-deficiency in liver mice significantly increases expression of genes induced by endoplasmic reticulum stress and genes that regulate the expression of enzymes required for cholesterol and fatty acid biosynthesis and uptake, notably the scavenger receptor class B type I (SR-BI), concomitant with overexpression of SR-BI at the protein level. Moreover, we also found increased mRNA levels of ABCG5, ABCG8, ABCG1 and ABCA1, which play important roles in reverse cholesterol transport, associated with an upregulation of liver X receptors and a downregulation of the peroxisome proliferators-activated receptor α.**

***Conclusions*: We found that several ATP-binding cassette transporters and nuclear hormone receptors involved in liver lipid homeostasis are differentially expressed in liver of CBS-deficient mice.**

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***Keywords*: Hyperhomocysteinemia; Cystathionine beta synthase-deficient mice; Liver; Gene expression; Cholesterol metabolism**

1. Introduction

Severe hyperhomocysteinemia is a heterogeneous autosomal recessive disease involving inborn errors of homocysteine metabolism, which lead to substantially elevated levels of total homocysteine in plasma. The most common disorder is a deficiency of cystathionine beta synthase (CBS), which catalyzes the condensation of homocysteine with serine to form cystathionine [1]. The clinical manifestations of severe hyperhomocysteinemia due to CBS deficiency are very diverse. The most common manifestations are vascular complications [2]. Patients with CBS deficiency also develop hepatic steatosis [1]. Moreover, the hepatic lesion, characterized by fatty infiltration, observed in patients with hyperhomocysteinemia, can be accompanied by perisinusoidal or central venous fibrosis and fibrosis of hepatic arterioles [3]. The pathogenesis of liver disease in hyperhomocys-

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teinemia due to CBS deficiency is not fully elucidated, even if several mechanisms have been postulated. The basic steatosis of the liver in CBS-deficient mice, a murine model of severe hyperhomocysteinemia, has been described [4]. We have recently shown that adult CBS-deficient mice not only develop steatosis, but also inflammation and fibrosis, concomitant with an enhanced expression of proinflammatory cytokines [5]. Moreover, hyperhomocysteinemia in liver of CBS-deficient mice promotes oxidative stress, which may cause mitochondrial damage in association with activation of hepatic stellate cells and Kupffer cells, leading to liver injury [5]. Previous results showed that hyperhomocysteinemia-induced endoplasmic reticulum (ER) stress in hepatocytes from heterozygous CBS-deficient mice having diet-induced hyperhomocysteinemia results in an enhanced hepatic biosynthesis and uptake of cholesterol and triglycerides, through the activation and dysregulation of the sterol regulatory element-binding proteins (SREBPs), which are important transcription factors regulating lipid homeostasis [6]. Moreover, hepatic steatosis in liver of 2–3-week-old CBS-deficient mice may be caused by deregulation of the activity and gene expression of the enzymes involved in lipid synthesis/degradation, leading to abnormal lipid metabolism in liver and serum, increased levels of hepatic and serum triglycerides and defective high density lipoprotein (HDL) maturation [7].

Many studies have shown an association between premature atherosclerosis and impaired HDL maturation [8]. Hepatic cholesterol uptake from serum coupled to intracellular processing and biliary excretion are important features in the removal of excess cholesterol from the body [9]. Accumulation of cholesterol in the arterial wall is a critical step in atherogenesis, and the transport of excessive cholesterol from the periphery to the liver by the HDL alleviates atherosclerosis. This process is called reverse cholesterol transport and consists of three different stages: the efflux of peripheral cellular cholesterol to HDL, the transport of cholesterol esters through the blood to the liver, and the uptake of cholesterol esters by the liver [10]. Cholesterol esters from HDL are taken up into the liver through selective uptake by the HDL receptor, scavenger receptor class B type I (SR-BI), where they are primarily catabolized to bile acids, through conversion by cholesterol 7α-hydroxylase (CYP7A1) and sterol 27-hydroxylase (CYP27) for biliary excretion via the bile salt export protein (BSEP1) [9,11–14]. A second major catabolic route is the direct efflux of cholesterol from the liver into the bile via the ABC half-transporters ABCG5 and ABCG8, which together function as a biliary sterol efflux regulator [15]. Another member, ABCG1, has been proposed to play a role in the intracellular trafficking and efflux of cholesterol [16]. Native HDL might also be formed by the ABC transporter A1 (ABCA1)-mediated cholesterol efflux [17].

To gain further insights into effects of CBS deficiency on hepatic cholesterol metabolism, the expression of hepatic genes involved in biosynthesis, uptake and efflux was determined in CBS-deficient mice.

2. Materials and methods

2.1. Animals

Animal care was conducted in accordance with internal guidelines of the French Agriculture and Forestry Ministry for animal handling. Mice were housed in a controlled environment with unlimited access to food and water on 12-h light/dark cycle. We made every effort to minimize suffering and the number of animals used. Mice heterozygous for targeted disruption of the *Cbs* gene (*Cbs* +/−) [4] were generously donated by Dr. N. Maeda (Department of Pathology, University of North Carolina, Chapel Hill, NC, USA). *Cbs* +/− mice were bred to obtain homozygous Cbs-deficient (*Cbs* −/−) mice. This produced *Cbs* −/− and wild-type (*Cbs* +/+) mice from the same breed of mice. Tail biopsies were performed on mice at 4 weeks of age and polymerase chain reaction was used for genotyping of the targeted CBS allele [4]. At weaning and during the life, *Cbs* −/− and *Cbs* +/+ mice from the same litter were fed on a diet supplemented with 1.592 g/kg choline chloride salt with free access (Usine d'Alimentation Rationnelle, Epinay sur Orge, France) because *Cbs* −/− mice died young when fed a standard laboratory diet. The wild-type littermates were also fed the same supplemented chow to avoid differences due to diet.

2.2. Preparation of serum samples and plasma homocysteine assays

At the time of sacrifice, blood samples were collected into tubes containing a 1/10 volume of 3.8% sodium citrate, placed on ice immediately, and plasma was isolated by centrifugation at 2500*g* for 15 min at 4 °C. Plasma total homocysteine, defined as the total concentration of homocysteine after quantitative reductive cleavage of all disulfide bonds, was assayed by using the fluorimetric high-performance liquid chromatography method described by Fortin and Genest [18].

2.3. Analysis of gene expression by real-time quantitative reverse transcription-polymerase chain reaction

The mRNA levels were assessed by real-time quantitative reverse transcription-polymerase chain reaction (Q-PCR). At the time of sacrifice, liver was harvested, snap-frozen, and stored at −80 °C until use. Total RNA was prepared from livers from mice by the guanidinium thiocyanate procedure. The quantity and the purity of the RNA were assessed by measuring absorbance at 260 and 280 nm. Reverse transcription was carried out on 2 μg total RNA as described by the manufacturer (Ambion, UK). cDNA (0.2 μl) was diluted with PCR mix (Light Cycler FastStart DNA Master SYBR Green I Kit, Roche Diagnostics) containing a final concentration of 3 mM $MgCl_2$ and 0.5 μM of primers in a final volume of 20 μl. The primers were designed with Primer 3 software. The primer pairs were selected to yield a single amplicon based on dissociation curves. Primer sequences are given in Table 1. Q-PCR was performed in a Lightcycler system (Roche Diagnostics). The thermal cycler parameters were as follows: hold for 8 min at 95 °C for one cycle followed by amplification of cDNA for 40 cycles with melting for 5 s at 95 °C, annealing for 5 s at 65 °C and extension for 10 s at 72 °C. Each reaction was performed in triplicate. The mouse superoxide dismutase-1 (SOD1) mRNA was used as an endogenous control [19]. ΔΔCp analysis of the results allows us to assess the ratio of the target mRNA versus SOD1 mRNA [20].

Table 1
Primer sequences for Q-PCR (all primers are listed 5′ to 3′)

mRNA	Forward	Reverse
ABCA1	CTTCCCACATTTTTGCCTGG	AAGGTTCCGTCCTACCAAGTCC
ABCG-1	AGGCCTACTACCTGGCAAAGA	GCAGTAGGCCACAGGGAAACA
ABCG-5	TGTCCTACAGCGTCAGCAACC	GGCCACTCTCGATGTACAAGG
ABCG-8	AGAGTTGCATCCCCCTAGCC	TCCTTGACACAGGCATGAAGC
BiP/GRP78	CATCGACTTGGGGACCACCTAT	TCTGGGGCAAATGTCTTGGTTT
BSEP1	GGGAGCAGTGGGTGTGGTAAAAG	TCCTGGGAGACAATCCCAATGTT
CHOP-10/gadd153	GGAAGTGCATCTTCATACACCACC	TGACTGGAATCTGGAGAGCGAGGGC
CYP7A1	TGTGCATGTGTGTAGAGGCTGGA	GATGTGGCAACCTCCTGCAATTC
CYP27	TGCCTGGGTCGGAGGAT	GAGCCAGGGCAATCTCATACTT
HMGCoA reductase	TCTGGCAGTCAGTGGGAACTATT	CCTCGTCCTTCGATCCAATTT
LCAT	CTTCAACTACACAGGCCAAG	CATCTTCATAGAGTGCAGCC
LDL-r	CTGTGGGCTCCATAGGCTATCT	GCGGTCCAGGGTCATCTTC
LXRα	CCTTCCTCAAGGACTTCAGTTACAA	CATGGCTCTGGAGAACTCAAAGAT
LXRβ	AAGGACTTCACCTACAGCAAGGA	GAACTCGAAGATGGGATTGATGA
PPARα	GGACCTTCGGCAGCTGGT	TCGGACTCGGTCTTCTTGATG
PPARγ	CATTCTGGCCCACCAACTTC	TCAAAGGAATGCGAGTGGTCTT
PPARδ	GACCAGAACACACGCTTCCTTC	CCATCACAGCCCATCTGCA
SHP1	ACGATCCTCTTCAACCCAGATGT	CAGTGCCCAGTGAGCCTCCTGTT
SOD1	TGGGGACAATACACAAGGCTGT	TTTCCACCTTTGCCCAAGTCA
SR-BI	GGCTGCTGTTTGCTGCG	GCTGCTTGATGAGGGAGGG
SREBP1a	GGCCGAGATGTGCGAAC	GTTGATGAGCTGGAGCATGT
SREBP1c	AGCTGTCGGGGTAGCGTCTG	GAGAGTTGGCACCTGGGCTG
SREBP2	CAAGTCTGGCGTTCTGAGGAA	ATGTTCTCCTGGCGCAGCT

2.4. Western blot analysis

Western blot analyses were performed to determine the protein levels of SR-BI as described [5], using a goat anti-human SR-BI polyclonal antibody (Santa Cruz Biotechnology, Tebu, France). Band intensities of SR-BI were normalized to that of β-actin on the same membrane after it was stripped and reprobed with anti-β-actin antibody (Sigma–Aldrich, France). Digitized images of the immunoblots obtained using a LAS-3000 imaging system (Fuji Photo Film Co., Ltd.) were used for densitometric measurements with an image analyzer (Multi Gauge software, Fuji Photo Film Co., Ltd.).

2.5. Data analysis

Statistical analysis was done with one-way ANOVA followed by Student's unpaired *t*-test using Statview software. The results are expressed as means ± SEM. Data were considered significant when $P < 0.05$.

3. Results

3.1. Total plasma homocysteine levels in CBS-deficient mice

We and other have found that *Cbs* −/− mice fed a standard laboratory diet died before 1 month of age [4]. We also have shown that adult CBS-deficient mice not only develop steatosis, but also inflammation and fibrosis [5]. In order to study the effect of CBS deficiency on lipid metabolism of adult mice, 3- and 4-month-old male *Cbs* −/− mice were fed standard A03 rodent chow (Usine d'Alimentation Rationnelle) enriched in choline chloride, necessary to the survival of *Cbs* −/− mice. Male *Cbs* +/+ mice were also fed supplemented chow to avoid gene expression modulations due to the diet. Plasma total homocysteine concentration was fortyfold higher in *Cbs* −/− mice, characterized as a murine model of severe hyperhomocysteinemia [4], than that in *Cbs* +/+ mice (166.3 ± 27.1 vs. 4.1 ± 0.3 μM; $P < 0.004$ by Student's *t*-test ($n = 6$ for each)), which is similar to the levels observed in severe hyperhomocysteinemic patients [1].

3.2. Effect of CBS-deficiency on ER stress in mice

In order to investigate the capacity of CBS deficiency to cause an ER stress response, we analyzed the mRNA encoding the diagnostic unfolded protein response (UPR) marker BiP/GRP78, and the transcription factor C/EBP homologous protein/growth arrest/DNA damage 153 (CHOP-10/gadd153) which has been shown to be induced at the transcriptional level in response to ER stress [21]. We found that BiP/GRP78 and CHOP-10/gadd153 gene expression were significantly increased in liver of *Cbs* −/− mice compared to *Cbs* +/+ mice (Table 2). As the ER stress also initiates cellular lipid synthesis by activation of SREBPs, we have analyzed the gene expression of SREBP1a, SREBP1c and SREBP2, and found a significant increase in liver of *Cbs* −/− mice compared to *Cbs* +/+ mice (Table 2). These results also confirm previous results showed by another technical approach (see Table 2).

Table 2
Effect of CBS deficiency on immunoglobulin heavy chain-binding protein/glucose-regulated protein 78 (BiP/GRP78), protein/growth arrest and DNA damage 153 (CHOP-10/GADD153), sterol regulatory element-binding protein (SREBP)1a, SREBP1c, SREBP2 mRNA expression in liver of mice

mRNA	CBS +/+ ($n = 4$)	CBS −/− ($n = 4$)	t	df	P	Data from the literature
BiP/GRP78	1.01 ± 0.06	1.46 ± 0.21	−2.51	6	0.046	Up-regulated in liver of *Cbs* −/− mice (Northern blot data, [31])
CHOP-10/gadd153	0.99 ± 0.05	6.36 ± 0.47	−7.4	5	0.0007	Up-regulated in liver of mice having diet-induced hyperhomocysteinemia (Northern blot data, [6])
SREBP1a	1.03 ± 0.05	3.4 ± 0.19	−8.1	5	0.0005	Up-regulated in liver of mice having diet-induced hyperhomocysteinemia (Northern blot data, [6])
SREBP1c	1.04 ± 0.05	4.9 ± 0.1	−46.85	5	<0.0001	
SREBP2	1.00 ± 0.96	2.56 ± 0.06	−13.02	5	<0.0001	Up-regulated in liver of rats having diet-induced hyperhomocysteinemia (Western blot data, [32])

Gene expression analysis was performed by means of Q-PCR. Values are expressed as fold induction of gene expression in liver of four male *Cbs* −/− mice compared to the gene expression in liver of four male *Cbs* +/+ mice, and the statistical analysis was done with one-way ANOVA followed by Student's unpaired *t*-tests. The degrees of freedom (df), the *t* values and the *P* values are given according to the Student's *t*-tests.

3.3. Effect of CBS deficiency on genes involved in the biosynthesis and uptake of cholesterol

SREBPs regulate the expression of enzymes required for cholesterol and fatty acid biosynthesis and uptake, like hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase and low-density lipoprotein receptor (LDL-r) [22–24]. As we have found an increased gene expression of SREBPs (Table 2), we have also analyzed the expression of genes encoding HMG-CoA reductase and LDL-r, and also found an increase in liver of *Cbs* −/− mice compared to *Cbs* +/+ mice, which confirms previous results showed by another technical approach (see Table 3). Moreover, the mRNA expression of the receptor involved in the selective uptake of HDL cholesterol esters (SR-BI) [11] was also significantly increased (Table 3). Expression of SRB-I was examined by Western blotting to determine whether SR-BI is also overexpressed at the protein level. In agreement with increased levels of SR-BI mRNA, SR-BI protein expression was markedly increased in liver of *Cbs* −/− mice (Fig. 1A and Table 4), compared with protein extracts from *Cbs* +/+ mice. To determine whether the difference in SR-BI expression is due to the loss of CBS expression by genetic deletion or due to the resultant hyperhomocysteinemia, we used the combination of genetic and dietary approaches. For this, protein expression of SR-BI was determined in liver extracts of *Cbs* +/− mice fed a control diet and *Cbs* +/− mice fed a control diet with drinking water supplemented with 0.5% L-methionine [19] for three months. Such a diet, defined as high methionine diet, led to an intermediate hyperhomocysteinemia in mice (~50 μM; Table 4). Cbs +/− mice fed a hyperhomocysteinemic diet have also a significant increase in protein expression of SR-BI (Fig. 1B and Table 4).

Taken together, the upregulation of SREBPs, associated with a dysregulation of HDL receptors, supported

Table 3
Effect of CBS deficiency on HMGCoA reductase, LDL-receptor (LDL-r), scavenger receptor class B1 (SR-BI) mRNA expression in liver of mice

mRNA	CBS +/+ ($n = 4$)	CBS −/− ($n = 4$)	t	df	P	Data from the literature
HMGCoA reductase	0.99 ± 0.07	5.36 ± 0.63	−4.5	5	0.006	Up-regulated in liver of rats having diet-induced hyperhomocysteinemia (RNase protection assay and Western blot data, [32,33])
LDL-r	1.1 ± 0.08	2.66 ± 0.46	−2.2	5	0.05	Up-regulated in liver of mice having diet-induced hyperhomocysteinemia (Northern blot data, [6])
SR-BI	1.01 ± 0.08	5.5 ± 0.51	−7.5	5	0.0007	

Gene expression analysis was performed by means of Q-PCR. Values are expressed as fold induction of gene expression in liver of four male *Cbs* −/− mice compared to the gene expression in liver of four male *Cbs* +/+ mice, and the statistical analysis was done as stated in Table 2.

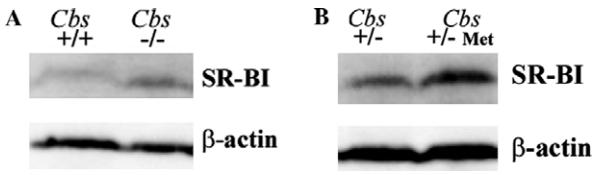

Fig. 1. Western immunoblots showing SR-BI expression in liver of (A) wild-type (*Cbs* +/+) mice and homozygous (*Cbs* −/−) mice, (B) heterozygous (*Cbs* +/−) mice and heterozygous mice fed a hyperhomocysteinemic diet (*Cbs* +/− Met). Proteins were subjected to immunoblot analysis using antibodies specific to SR-BI. After stripping, the membranes were reprobed with anti-β-actin antibody for the control. The blots are representative of three independent experiments.

a role for both pathways, increased biosynthesis and increased uptake of cholesterol in liver of *Cbs* −/− mice.

3.4. Effect of CBS deficiency on genes involved in cholesterol metabolism and efflux

The fact that the reduction of plasma HDL cholesterol induced by CBS deficiency was not accompanied by increased total intrahepatic cholesterol [7] led us to hypothesize that increased hepatic uptake of HDL-cholesterol via SR-BI is paralleled by elimination of cholesterol. We next examined CBS deficiency effect on expression of genes encoding CYP7A1, CYP27 and BSEP1, and found no difference compared to *Cbs* +/+ mice (Table 5). Even if the expression of an important repressor of CYP7A1 mRNA expression, small heterodimer partner (SHP1) [25], was increased, the small decrease of CYP7A1 expression, detected in liver of *Cbs* −/− mice, fails to reach statistical differences compared to *Cbs* +/+ mice (Table 5).

We also investigated the effect of CBS deficiency of genes encoding ABC transporters involved in biliary efflux, and found that the gene expression of ABCG1, ABCG5 and ABCG8 was higher in liver of *Cbs* −/− mice compared to *Cbs* +/+ mice (Table 5). We also found that not only ABCA1 gene expression was increased, but still the gene expression encoding lecithin:cholesterol acetyltransferase (LCAT), which is essential for maturation of HDL, was also decreased in liver of *Cbs* −/− mice compared to *Cbs* +/+ mice, and confirms previous results showed by another technical approach (see Table 5).

3.5. Effect of CBS deficiency on the expression of nuclear hormone receptors

Several members of the nuclear hormone receptor family are involved in the transcriptional control of genes involved in cholesterol and fatty acids metabolism, as well as genes involved in the efflux, transport, and excretion of cholesterol [26–29]. We found that liver receptor (LXR)α/β gene expression was threefold higher in liver of *Cbs* −/− mice compared to *Cbs* +/+ mice (Table 6). Moreover the peroxisome proliferators-activated receptor (PPAR)δ (also known as PPARβ), like PPARγ, was significantly increased in liver of *Cbs* −/− mice compared to *Cbs* +/+ mice (Table 6). However, the PPARα expression was significantly reduced in liver of *Cbs* −/− mice compared to *Cbs* +/+ mice, which confirms previous results showed by another technical approach (see Table 6).

4. Discussion

Previous work reports abnormal lipid metabolism in CBS-deficient mouse liver and serum, which may account for the development of hepatic steatosis [7]. The basic steatosis of the liver in CBS-deficient mice has been characterized by staining methods which revealed lipid droplets containing triglycerides but not cholesterol [5]. Moreover, Namekata et al. [7] found that the reduction of plasma HDL cholesterol induced by CBS deficiency was not accompanied by increased total intrahepatic cholesterol. Then the expression of hepatic genes involved in cholesterol metabolism was determined in CBS-deficient mice.

We have recently found that hyperhomocysteinemia in liver of CBS-deficient mice promotes oxidative stress [5], which can activate the ER stress pathway [30]. Here we found an increased expression of BiP/GRP78 and CHOP-10/gadd153 in liver of CBS-deficient mice, which has also been found in hepatocytes from homozygous

Table 4
Plasma total homocysteine (tHcy) concentrations, and relative expression of SR-BI in liver of CBS-deficient mice and CBS wild-type mice

CBS genotype	tHcy (μM)	Relative expression of SR-BI (% of wild-type[a] or heterozygous on control diet[b])
Wild-type (*Cbs* +/+)	3.5 ± 0.4	1
Homozygous (*Cbs* −/−)	233 ± 45.7 (0.007; −5.017; 4)[c]	2.16 ± 0.15[a] (0.002; −7.578; 4)[c]
Heterozygous (*Cbs* +/−) (control diet)	9.97 ± 0.7	1
Heterozygous (*Cbs* +/−) (high Met diet)	48.3 ± 7.6 (0.007; −5.023; 4)[d]	1.53 ± 0.08[b] (0.003; −6.339; 4)[d]

Relative protein expression was determined by normalization of the density of images from SR-BI with that of β-actin on the same blot. The values of SR-BI of 3 *Cbs* −/− mice or 3 *Cbs* +/− fed a hyperhomocysteinemic diet (high Met diet) were normalized to the mean of 3 *Cbs* +/+ mice[a] or 3 *Cbs* +/− mice[b]. Data correspond to means ± SEM and the statistical analysis was done with ANOVA followed by Student's unpaired *t*-test using Statview software. The *P* values, the *t* values and the degrees of freedom (df) are given according to the Student's *t*-tests vs wild-type mice[c] or heterozygous on control diet[d].

Table 5
Effect of CBS deficiency on lecithin:cholesterol acetyltransferase (LCAT), cholesterol 7α-hydroxylase (CYP7A1), sterol 27-hydroxylase (CYP27), bile salt export pump (BSEP1), small heterodimer partner (SHP1), ABC half-transporters ABCG1, ABCG5, ABCG8, ABC transport 1 (ABCA1) mRNA expression in liver of mice

mRNA	CBS +/+ ($n=4$)	CBS −/− ($n=4$)	t	df	P	Data from the literature
ABCA1	0.99 ± 0.05	1.2 ± 0.06	−2.4	5	0.02	
ABCG1	0.98 ± 0.01	11.85 ± 1.1	−6.4	5	0.001	
ABCG5	1.13 ± 0.3	2.4 ± 0.3	−2.2	5	0.05	
ABCG8	0.94 ± 0.15	1.9 ± 0.18	−3.1	5	0.02	
BSEP1	1.05 ± 0.08	0.91 ± 0.13	0.855	5	0.44	
CYP7A1	1.08 ± 0.04	0.76 ± 0.04	2.21	6	0.07	
CYP27	1.06 ± 0.03	1.07 ± 0.09	0.2	6	0.85	
LCAT	1.1 ± 0.05	0.4 ± 0.08	5.2	5	0.003	Down-regulated in liver of *Cbs* −/− mice (Northern blot data, [7])
SHP1	1.1 ± 0.5	4.15 ± 0.51	−3.9	5	0.01	

Gene expression analysis was performed by means of Q-PCR. Values are expressed as fold induction of gene expression in liver of four male *Cbs* −/− mice compared to the gene expression in liver of four male *Cbs* +/+ mice, and the statistical analysis was done as stated in Table 2.

and heterozygous CBS-deficient mice having diet-induced hyperhomocysteinemia [6,31]. The cellular reaction to ER stress initiates cell-specific responses that can notably include the dysregulation of lipid metabolism and inflammation [6], which we have found in liver of CBS-deficient mice [5]. ER stress has been shown to affect lipid metabolism through the activation and dysregulation of the SREBPs [6], which regulate the expression of enzymes required for cholesterol and fatty acid biosynthesis and uptake [22–24]. Gene expression of HMG-CoA reductase and LDL-r was also significantly increased in liver of CBS-deficient mice, concomitant with an increased expression of SREBP1a, SREBP1c, and SREBP2. Previous works have also demonstrated an increased expression of LDL-r, HMG-CoA reductase, SREBP-1 and SREBP-2 in liver of heterozygous CBS-deficient mice and rats having diet-induced hyperhomocysteinemia [6,32,33]. Taken together, these results emphasize the role of homocysteine in biosynthesis and uptake of cholesterol induced by CBS-deficiency.

We have then analyzed the expression of genes involved in hepatic cholesterol transport and metabolism. Cellular and whole-body cholesterol homeostasis is maintained through a network of transcriptional programs. Hepatic cholesterol uptake from serum coupled with cellular processing and bile excretion are important features in the last step of reverse cholesterol transport, which is an HDL-mediated pathway [10]. Hepatic overexpression of SR-BI in mice is accompanied by reduced plasma HDL and a substantial increase in biliary cholesterol [34]. SR-BI may mediate directly these effects by increasing hepatic HDL cholesterol uptake or by increasing cholesterol secretion into bile, or both [35]. We have found an increased hepatic SR-BI expression in CBS-deficient mice at the transcriptional and protein expression levels, which may account for decreased plasma HDL cholesterol [7]. Moreover, the protein expression was also markedly increased in liver of *Cbs* +/− mice fed a hyperhomocysteinemic diet, which demonstrates the importance of hyperhomocysteinemia in the expression of SR-BI. Our study also shows that the decrease in plasma HDL levels in CBS-deficient mice does not result from altered mRNA expression of ABCA1, a major contributor to plasma HDL levels. LCAT, by transesterification of free cholesterol accepted by nascent HDL, maintains a free cholesterol gradient between peripheral cells and the HDL particle surface and thus promotes efflux of free cholesterol from the peripheral tissues [10]. We and other [7] have found a decrease in LCAT gene expression in liver of CBS-deficient mice. Given the central role of SRBI and LCAT in reverse cholesterol transport by HDL, their gene expres-

Table 6
Effect of CBS deficiency on liver X receptor (LXR)α, LXRβ, peroxisome proliferators-activated receptor (PPAR)α, PPARγ, PPARδ mRNA expression in liver of mice

mRNA	CBS +/+ ($n=4$)	CBS −/− ($n=4$)	t	df	P	Data from the literature
LXRα	1.06 ± 0.1	3 ± 0.5	−2.79	6	0.03	
LXRβ	1.1 ± 0.1	3.2 ± 0.3	−4.45	5	0.007	
PPARα	1 ± 0.09	0.64 ± 0.06	2.89	5	0.03	Down-regulated in liver of *Cbs* +/− mice (Western blot data, [42])
PPARγ	1.2 ± 0.14	2.78 ± 0.3	−3.35	5	0.02	
PPARδ	0.99 ± 0.06	11.2 ± 2.9	−2.4	7	0.05	

Gene expression analysis was performed by means of Q-PCR. Values are expressed as fold induction of gene expression in liver of four male *Cbs* −/− mice compared to the gene expression in liver of four male *Cbs* +/+ mice, and the statistical analysis was done as stated in Table 2.

sion modulation can account for abnormalities of plasma lipid profile in CBS-deficient mice [7]. The upregulation of SRB-I and downregulation of LCAT, associated with the upregulation of ABCA1, indicate that the decrease in plasma HDL levels may be due to an increased of HDL catabolism rather than an altered production of HDL in CBS-deficient mice.

The liver plays an important role in the catabolism of cholesterol, via the bile acid pathways or by the direct efflux into the bile. The secretion of bile acids into the bile is notably mediated by BSEP1, and the secretion of cholesterol is mediated by ABCG5/ABCG8. We did not found difference in gene expression of BSEP1, CYP7A1 and CYP27 in liver of CBS-deficient mice, compared to wild-type mice. Bile acid synthesis is also regulated via a negative feedback mechanism mediated notably by SHP1, which suppress expression of CYP7A1. Even if the expression of SHP1 was increased, the small decrease of CYP7A1 expression fails to reach statistical differences in liver of CBS-deficient mice compared to wild-type mice. Another mechanism of CYP7A1 gene expression regulation has been reported that involved inflammatory cytokines TNF-α, which we have found to be enhanced in liver of CBS-deficient mice [5]. TNF-α, induced by bile acid activation of Kupffer cells, blocks hepatic expression of CYP7A1 in adjacent hepatocytes [35]. Moreover, we have found an increased expression of genes encoding ABCG5, ABCG8 and ABCG1 in liver of CBS-deficient mice compared to wild-type mice. It has been shown that overexpression of ABCG5/G8 in mice enhances biliary cholesterol secretion [36]. Therefore, upregulation of hepatic ABCG5/ABCG8, associated with upregulation of ABCG1, can promote the rate of excretion of cholesterol into the bile, thus promoting reverse cholesterol transport in CBS-deficient mice.

In vivo and *in vitro* data have indicated the importance of nuclear receptors in the regulation of several hepatic genes involved in hepatic fatty acids and cholesterol metabolism and reverse cholesterol transport. Among these genes, hepatic SR-BI gene expression has been reported to be stimulated by LXRα and PPARγ [37,38], which we have found to be up-regulated in liver of CBS-deficient mice. LXRα and LXRβ were also shown to be positive regulators of the hepatic expression of ABCG5 and ABCG8 [39]. Coupling the regulation of cholesterol catabolic and efflux pathways to de novo lipogenesis enables LXR to more efficiently dispose of excess sterol and prevent cholesterol-induced cytotoxicity.

Recent results also indicate a role for PPARα and PPARδ in regulating levels of serum triglycerides in mice by modulating both very low density lipoprotein (VLDL) production and catabolism of VLDL-triglycerides [40,41]. Here we found an increased expression of PPARδ, which has been shown to be a key regulator of intracellular fatty acid metabolism gene expression [28], and a decreased expression of PPARα. Previous works have also demonstrated a decreased expression of PPARα in liver of heterozygous CBS-deficient mice at the protein level [42]. Recent data have also showed a direct effect of homocysteine on PPARα expression [43]. Apolipoprotein B (apoB) functions as an obligatory platform for the assembly of lipid by way of microsomal triglyceride transfer protein (MTP), which has been shown to be up-regulated by PPARα [40], and as a vehicle for secretion of hepatic triglycerides in the form of VLDL. Even if hepatic apoB100 protein levels and VLDL secretion from liver were reduced in CBS-deficient mice compared to wild-type mice [7], we did not found expression differences for genes encoding apoB and MTP (data not shown). Recent results have shown that lipid peroxidation and oxidant stress regulate the apoB100 degradation and VLDL secretion from liver cells [44]. An enhanced lipid peroxidation, which we have found in liver of CBS-deficient mice [5], may account for apoB100 degradation, thus leading to triglyceride accumulation [5,7].

We have not defined how hyperhomocysteinemia could interact with gene expression modulation. One possible mechanism may involve changes in methylation of DNA, an important epigenetic factor in regulation of gene expression. The relationship between changes in DNA methylation and expression of many genes with homocysteine metabolism has already been demonstrated [45–47]. Therefore, it will be interesting to determine whether the methylation status of the promoter region of genes found to be modulated is altered in hyperhomocysteinemia.

In conclusion, we have provided data showing that several ABC transporters and nuclear hormone receptors involved in liver lipid homeostasis are differentially expressed in liver of CBS-deficient mice. These results can explain the fact that CBS deficiency in mice was accompanied by increased hepatic triglycerides content but not intrahepatic cholesterol.

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