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Altered Gene Expression in Liver from a Murine Model of Hyperhomocysteinemia*

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Karine Robert‡, Jean-François Chassé§, Dominique Santiard-Baron¶, Catherine Vayssettes‖, Allel Chabli‖, Joelle Aupetit‖, Nobuyo Maeda, Pierre Kamoun‖, Jacqueline London, and Nathalie Janel‡‡**

*From the EA 3508, Université Paris 7, Denis Diderot, Case 7104, 2 Place Jussieu, 75251 Paris Cedex, France, §INSERM U490, Faculté de Médecine Necker, 75743 Paris Cedex 15, France, ¶Central Nervous System Department, Aventis, 94403 Vitry sur Seine, France, ‖Laboratoire de Biochimie Médecine B, Hopital Necker-Enfants Malades, France, and **Department of Pathology, University of North Carolina, Chapel Hill, North Carolina 27599*

Cystathionine β-synthase (CBS) deficiency causes severe hyperhomocysteinemia and other signs of homocystinuria syndrome, in particular a premature atherosclerosis with multiple thrombosis. However, the molecular mechanisms by which homocysteine could interfere with normal cell function are poorly understood in a whole organ like the liver, which is central to the catabolism of homocysteine. We used a combination of differential display and cDNA arrays to analyze differential gene expression in association with elevated hepatic homocysteine levels in CBS-deficient mice, a murine model of hyperhomocysteinemia. Expression of several genes was found to be reproducibly abnormal in the livers of heterozygous and homozygous CBS-deficient mice. We report altered expression of genes encoding ribosomal protein S3a and methylthioadenosine phosphorylase, suggesting such cellular growth and proliferation perturbations may occur in homozygous CBS-deficient mice liver. Many up- or down-regulated genes encoded cytochromes P450, evidence of perturbations of the redox potential in heterozygous and homozygous CBS-deficient mice liver. The expression of various genes involved in severe oxidative processes was also abnormal in homozygous CBS-deficient mice liver. Among them, the expression of heme oxygenase 1 gene was increased, concomitant with overexpression of heme oxygenase 1 at the protein level. Commensurate with the difference in hepatic mRNA paraoxonase 1 abundance, the mean hepatic activity of paraoxonase 1, an enzyme that protects low density lipoprotein from oxidation, was 3-fold lower in homozygous CBS-deficient mice. Heterozygous CBS-deficient mice, when fed a hyperhomocysteinemic diet, have also reduced PON1 activity, which demonstrates the effect of hyperhomocysteinemia in the paraoxonase 1 activity.

Homocysteine (Hcy)[1] is a sulfur-containing amino acid that is potentially toxic. It is synthesized during the conversion of dietary methionine to cysteine. Hcy has several possible fates. It may undergo remethylation to methionine via the folic acid and B12-dependent enzyme methionine synthase or via the enzyme betaine homocysteine methyltransferase (1). Alternatively, Hcy can enter the transsulfuration pathway under conditions in which an excess of methionine is present or cysteine synthesis is required. In this pathway, Hcy first condenses with serine to form cystathionine in a rate-limiting reaction catalyzed by the B6-dependent enzyme cystathionine β-synthase (CBS), and then cystathionine is used to yield cysteine. Cysteine is a major precursor of glutathione, the principal redox metabolite in the cell.

CBS deficiency, an inborn metabolic defect that displays an autosomal recessive inheritance, is the most common identifiable cause of hyperhomocysteinemia (HH) (2). HH is associated with an increased incidence of vascular thrombosis and development of premature atherosclerosis (3). Severe monogenic HH (also referred to as homocystinuria) is commonly caused by mutations in the CBS gene that prevent the use of Hcy in the biosynthesis of cysteine and lead to abnormally high plasma total Hcy (tHcy) concentrations in patients with severe HH (100–250 μM). Normal plasma tHcy concentrations range from 5 to 15 μM. Mild HH, usually defined as plasma levels of tHcy between 15 and 30 μM, is associated with increased risk of atherosclerosis (4).

The relationship between high Hcy concentrations in the blood and thrombosis has been a subject of considerable research. Endothelial dysfunction is a key process in atherosclerosis and has been reported in patients with HH (5) and in experimental HH, which is induced by methionine loading in normal subjects (6). Administration of Hcy causes vascular injury and thrombosis in animals (7). It is thought to predispose to atherosclerosis by injuring the vascular endothelium; after exposure to Hcy, endothelial dysfunction occurs at several levels, suggesting that Hcy contributes to enhanced vascular inflammation and hemostatic changes linked to the development of atherosclerosis and a prothrombotic state.

An understanding of these processes at the molecular level is required to elucidate the mechanism of cell injury by Hcy. Expression of a range of genes has been shown to be modulated in cultured human umbilical vein endothelial cells, vascular

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‡‡ To whom correspondence should be addressed. Tel.: 33-1-44-27-77-45; Fax: 33-1-44-27-83-38; E-mail: janel@paris7.jussieu.fr.
[1] The abbreviations used are: Hcy, homocysteine; tHcy, total Hcy; apoE, apolipoprotein E; CBS, cystathionine β-synthase; CBS +/+ mice, wild type mice; CBS +/− mice, heterozygous CBS-deficient mice; CBS −/− mice, homozygous CBS-deficient mice; CYP, cytochrome P450; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HDL, high density lipoprotein; LDL, low density lipoprotein; HepG2, human hepatoma cell line; HH, hyperhomocysteinemia; HO-1, heme oxygenase 1; PON1, paraoxonase 1; RPS3a, ribosomal protein S3a; RT, reverse transcriptase; STA, sulfotransferases; SOD1, Cu/Zn superoxide dismutase 1.

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smooth muscle cells, human megakaryocytic cell line (DAMI), hepatic stellate cells, human hepatoma cell line (HepG2), and primary neuronal cells exposed to exogenous excess Hcy (8–16). *In vitro*, high levels of Hcy affect the expression of the endoplasmic reticulum stress-response genes, the acute translational responses genes, mitochondrial genes, genes encoding antioxidant enzymes and transcription factors, and genes implicated in cell growth, cellular differentiation and proliferation, and regulation of extracellular matrix homeostasis. Unfortunately, most *in vitro* studies use physiologically irrelevant concentrations or forms or both of Hcy, concentrations much higher than those found in patients with HH. Therefore, studies need to be conducted using a pathophysiologically relevant model of *in vivo* HH.

A well characterized genetic model of HH in mice was obtained by targeted deletion of the CBS gene by homologous recombination (17) and has been used to show the influence of HH on vascular function (18–22). Homozygous CBS-deficient mice (CBS −/− mice) develop very high plasma tHcy concentrations, and heterozygous CBS-deficient mice (CBS +/− mice) have mildly elevated plasma concentrations of tHcy (17–23). Both CBS +/− and CBS −/− mice exhibit some pathological features of HH such as endothelial dysfunction (18–22), which can be negated by overexpression of glutathione peroxidase (21), and hepatic steatosis (17, 24).

Although the mechanisms that lead to high plasma Hcy have been well established, the molecular basis of Hcy-mediated alteration of cellular function in whole organs is not completely understood. To elucidate the molecular mechanisms responsible for the interference in normal tissue function, we searched for genes whose expression is abnormal in CBS-deficient mice. Impaired liver function has been associated with elevated plasma levels of tHcy (25–27), evidence of the central role of the liver in the metabolism of methionine and consequently in the catabolism of Hcy (28). A recent study shows that the liver is indeed a key organ in Hcy metabolism and contributes to much of the plasma Hcy (29). We therefore focused our research on the liver of CBS-deficient mice and monitored gene expression by a combination of differential display reverse transcriptase (RT)-PCR and cDNA expression array analysis. We found that 13 genes were up- or down-regulated in liver from 3- and 4-month-old CBS +/− and/or CBS −/− mice. Several of these genes affected in CBS −/− mouse liver suggest altered cellular growth and severe oxidative processes.

EXPERIMENTAL PROCEDURES

Mice and Genotype Determination—The CBS gene in mouse embryonic stem cells was inactivated by homologous recombination (17). CBS +/− mice were bred to produce CBS −/− mice. Tail biopsies were obtained from mice at 4 weeks of age, and the PCR was used for genotyping for the targeted CBS allele (17). CBS −/− mice, CBS +/− mice, and wild type control (CBS +/+) mice from the same litter were used at three and four months of age. CBS −/− mice fed a standard laboratory diet died young. Therefore, CBS +/+, CBS +/−, and CBS −/− mice were fed standard A04 rodent chow (Usine d'Alimentation Rationnelle, Epinay sur Orge, France) supplemented with 1.592 g/kg choline chloride salt.

Plasma and Hepatic tHcy Assays—At the time of sacrifice, blood samples were collected in tubes containing a ⅒ volume of 3.8% sodium citrate and placed on ice immediately, and plasma was isolated by centrifugation at 2500 × *g* for 15 min at 4 °C. Liver was harvested, snap-frozen, and stored at −80 °C until use. tHcy was assayed in plasma and crude liver extracts with a fluorescence polarization immunoassay using an Abbott IMX analyzer (Abbott Laboratories, Mississauga, Ontario, Canada) (30) and normalized to total protein concentration for liver extracts.

Western Blot Analysis—Total protein liver extracts (20 μg) were subjected to SDS electrophoresis on 12% gels under reducing conditions by the method of Laemmli (31). Proteins were transferred electrophoretically to nitrocellulose membranes (Bio-Rad). The membranes were blocked with Tris buffer (1.5 mM Tris base, pH 8, 5 mM NaCl, 0.1% Tween 20) containing 5% skimmed milk, and the immobilized proteins were immunostained with a polyclonal antibody directed against rat CBS (1:10000 dilution) (32) or with anti-Cu,Zn superoxide dismutase 1 (SOD1) (1:1000 dilution) (Calbiochem) or with anti-HO-1 (1:200 dilution) (Sigma-Aldrich), or with anti-α-tubulin (1:2000 dilution) (Sigma-Aldrich). After incubation with the primary and peroxidase-conjugated secondary antibodies, blots were developed with the enhanced chemiluminescence reagent (Amersham Biosciences).

Total RNA Isolation—Total RNA was prepared from two mixtures of three different livers from 3- or 4-month-old mice by the guanidinium thiocyanate procedure (33). The quantity and purity of the RNA was assessed by measuring absorbance at 260 and 280 nm.

Analysis of Differential Gene Expression Using Differential Display—Total RNA was analyzed by RT-PCR-based differential display as described by Liang and Pardee (34). DNase-treated RNA (400 ng) was reverse-transcribed using anchored primers D1 (5′-T_{11}GC-3′) or D2 (5′-T_{11}GG-3′) or D4 (5′-T_{11}CG-3′) and superscript II reverse transcriptase (Invitrogen). One-fortieth of the reverse transcriptase mixture was used for the subsequent amplification by PCR with 2.5 μM concentrations of the same D1 and D2 and D4 as 3′ primers and 0.5 μM concentrations of an arbitrary 5′ primer: U2 (5′-TTGATCCGAG-3′), 2.5 mM $MgCl_2$, 10 μM dNTPs, 1 μCi of [α-^{33}P]dATP (PerkinElmer Life Sciences), and 1 unit of *Taq* polymerase (Invitrogen) in 20 mM Tris-HCl, pH 8.0, 50 mM KCl. The mixture was subjected to the following cycles: 1× (5 min at 94 °C, 5 min at 40 °C, 5 min at 72 °C), 39× (15 s at 95 °C, 2 min at 40 °C, 1 min at 72 °C), 1× (5 min at 72 °C). The amplified cDNAs were separated on a 6% DNA sequencing gel. Total RNA from two extractions of mixtures of three different tissues was used, and duplicate RT-PCRs were performed and repeated at least twice to ensure reproducibility.

Cloning and Sequencing of cDNA Fragments—DNA bands of interest were cut from the sequencing gels and eluted, then reamplified by PCR with the same primer pairs. The amplified fragments were inserted directly into the pCR2.1 TA vector by using the TA cloning kit (Invitrogen) and sequenced using the T7 sequencing kit (Amersham Biosciences). The sequences were compared with the GenBank™ data base using the BLAST program.

Analysis of Differential Gene Expression Using a Mouse cDNA Array—Radiolabeled cDNA probes were generated by reverse transcription of total RNA (2.5 μg) with Moloney murine leukemia virus reverse transcriptase and 35 μCi of [α-^{32}P]dATP (PerkinElmer Life Sciences). The radiolabeled cDNA probes were purified from unincorporated nucleotides by column chromatography using the NucleoSpin extraction kit (Clontech) and hybridized overnight at 68 °C to an atlas mouse stress cDNA expression array consisting of 140 known mouse genes as described by the manufacturer (Clontech catalog number 7749-10). The membranes were washed four times for 30 min in 2× saline-sodium citrate (SSC; 1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), 1% SDS followed by one time for 30 min in 0.1× SSC, 0.5% SDS at 68 °C and exposed to x-ray film (Kodak Biomax MS). The x-ray films were scanned, and the images were analyzed using the AtlasImage™ Software (Clontech).

Northern Blot Analysis—Samples of denatured RNA (15 μg) from each group were electrophoresed for 3 h on a 2.2 M formaldehyde, 1% agarose gel. After a capillary transfer onto a nylon membrane (Appligene Oncor), the membrane was cross-linked using a UV transilluminator (Stratalinker, Appligene Oncor). The isolated cDNA fragments of RT-PCR analysis were used as probes for verification of differential expression by Northern blotting. cDNA-specific probes of genes included on the cDNA array were obtained by reverse transcription of DNA-free RNA (500 ng) from liver tissues with specific primers complementary to the studied cDNA and superscript II reverse transcriptase (Invitrogen) followed by PCR amplification using nested primers before labeling. Specific probes were generated by labeling the cDNA fragments with [α-^{32}P]dCTP (PerkinElmer Life Sciences) using the Multiprime DNA labeling system (Amersham Biosciences). After an overnight incubation in the probe solution (ExpressHyb solution, Clontech) at 68 °C, the membrane was washed 3 times for 30 min in 0.1× SSC, 0.1% SDS at 68 °C. An autoradiogram was generated by exposing the washed membrane to film (Fuji) for overnight at −80 °C. We ensured that loading was equal and the RNA from the liver samples had not been degraded by hybridization with a probe specific for mouse SOD1 gene. Changes in steady-state mRNA levels were quantified by densitometric scanning using Image Analysis Software and normalized to SOD1 to correct for differences in gel loading.

Paraoxonase Enzyme Assays—PON activity toward phenyl acetate (Sigma-Aldrich) was quantified spectrophotometrically using 50 mM

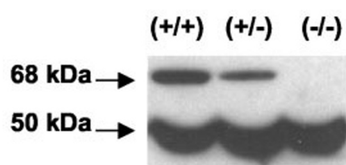

FIG. 1. **CBS expression in liver of CBS +/− and CBS −/− mice.** Liver protein extracts from CBS +/+ (+/+), CBS +/− (+/−), and CBS −/− (−/−) mice were analyzed by Western blotting, with a polyclonal antibody against rat CBS. The band at 68 kDa was significantly less intense than wild type in liver of CBS +/− mice and was undetectable in liver of CBS −/− mice. The band at 50 kDa did not differ significantly between the three extracts and was, thus, nonspecific; it could serve as an internal control.

Tris-HCl, pH 8, containing 1 mM $CaCl_2$ and 5 mM phenyl acetate. The reaction was monitored for 10 min at 35 °C by measuring the appearance of phenol at 270 nm with the use of continuously and automated recording spectrophotometer (PowerWaveX microplate spectrophotometer of Biotek instruments with KC4 software). All rates were determined in duplicate and corrected for non-enzymatic hydrolysis (35). The enzyme activity is expressed as ΔA_{270} per min and per mg of cellular proteins of liver.

RESULTS

Hepatic CBS Protein Production and tHcy Levels—CBS −/− mice fed a standard laboratory diet died before 1 month of age. Betaine-homocysteine methyltransferase in liver and kidney cells can transfer a methyl group to methionine from betaine, the oxidized form of choline, providing a secondary pathway for homocysteine remethylation (36). Therefore, to allow us to study adult mice (3- and 4-month-old), CBS +/+, CBS +/−, and CBS −/− mice were fed standard A04 rodent chow (Usine d'Alimentation Rationnelle) enriched in choline chloride, necessary to the survival of CBS −/− mice. CBS +/+ and CBS +/− mice were also fed supplemented chow to avoid gene expression modulations due to the diet.

Hepatic CBS protein production and hepatic tHcy concentrations were measured. We carried out Western blotting experiments using a polyclonal antibody against rat CBS (32) to probe protein extracts from the livers of CBS +/+, CBS +/−, and CBS −/− mice. Two bands, 68 and 50 kDa, were detected (Fig. 1). The band at 50 kDa was found to be nonspecific; its intensity did not differ significantly between the three extracts and, thus, could serve as an internal control. The intensity of the specific band of 68 kDa in crude liver extracts from CBS +/− mice was half that in CBS +/+ mice; the band was absent from extracts from CBS −/− mice (Fig. 1). The same results were obtained with protein samples isolated from the brains of CBS +/+, CBS +/−, and CBS −/− mice (37).

Commensurate with this difference in hepatic CBS protein abundance, the mean hepatic concentration of tHcy in CBS −/− mice was approximately 20-fold higher than that in CBS +/+ mice (tHcy concentrations were 6.44 ± 3.86 *versus* 0.34 ± 0.14 nmol/mg of cellular protein; $p < 0.001$ by Student's t test $n = 5$ for each). In contrast, hepatic tHcy concentrations in CBS +/− mice fed a standard rodent chow enriched in choline chloride that is sufficient in folate and vitamin B6 were not significantly different from those in CBS +/+ mice (tHcy levels were 0.43 ± 0.15 *versus* 0.34 ± 0.14 nmol/mg of cellular protein; $p < 0.35$ by Student's t test $n = 5$ for each).

Plasma tHcy concentration was also higher in CBS −/− mice than in CBS +/+ mice (205 ± 86 *versus* 3.9 ± 0.9 μM; $p < 0.0001$ by Student's t test $n = 4$ for each). CBS +/− mice had intermediate plasma tHcy levels ~2-fold that in CBS +/+ mice (tHcy levels were 9.1 ± 2.4 *versus* 3.9 ± 0.9 μM; $p < 0.0001$ by Student's t test $n = 8$ for each). Plasma levels of tHcy in mice are generally lower than those in humans; nevertheless, the plasma tHcy concentrations resulting from partial CBS deficiency in mice have been shown to be sufficient to cause endothelial dysfunction (18, 21, 22). Therefore, CBS +/− mice and CBS −/− mice may be suitable for pathophysiological study of mild and severe HH and can be used to identify the genes involved.

Identification by Differential Display of Transcripts Differentially Expressed in Liver from CBS +/− and CBS −/− Mice—We sought to identify genes that are differentially expressed in the liver of CBS-deficient mice. We compared liver RNA fingerprint patterns from CBS +/+ mice with those from CBS +/− mice and CBS −/− mice of 3- or 4-month-old mice. After screening using 20 primer combinations, a total of 15 cDNA fragments differentially expressed in the livers of CBS −/−, CBS +/−, and CBS +/+ were identified on sequencing gels. They were isolated, cloned, and sequenced. Fig. 2*A* shows the typical banding patterns for the PCR products obtained with three different primer combinations. Band D contained two different cDNAs (Table I). To confirm the results of differential display RT-PCR of mRNA, Northern blot analysis was performed using probes obtained from the isolated differential display fragments (Fig. 2*B*). The genes that were confirmed by Northern blot analysis as having differential expression are listed in Table I. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) is widely used as an internal control in mRNA expression studies. Unexpectedly, G3PDH expression was lower in livers from CBS −/− mice than in controls (Fig. 2*B*). The gene encoding SOD1, an antioxidant enzyme, was expressed at equal levels in all three groups of mice (Fig. 2*B*). We have also shown no difference in hepatic SOD1 protein production (Fig. 5) and in SOD1 activity (data not shown) between CBS +/+, CBS +/−, and CBS −/− mice. The mouse SOD1 probe was therefore used as the loading control. The relative expression levels of these genes were also quantified by densitometric scanning of Northern blots (Table I). Three of the cDNAs corresponding to genes down-regulated in livers of CBS −/− mice had sequences similar to those of known mouse genes (Table I): mitochondrial cytochrome *c* oxidase subunit I gene (GenBank™ accession number NP 008110); the gene for cytochrome P450 (CYP) 3A25 (GenBank™ accession number AF 204959), a testosterone 6β- hydroxylase; and the gene for ribosomal protein S3a (RPS3a) (GenBank™ accession number NM_016959), a ribosomal protein that is essential for cell growth and cellular proliferation (38, 39). One of the cDNAs revealing up-regulation in CBS −/− mice was similar to the methylthioadenosine phosphorylase gene (GenBank™ accession number AB 056100), encoding an enzyme implicated in the salvage pathways of purines and methionine (40), and another had sequences EST (expressed sequence tag) identical to those in the cDNA clone 620429009 (GenBank™ accession number AV 324910), confirming the expression of this novel transcript of unknown function. These results demonstrate notably altered mitochondrial gene expression in livers of 3- and 4-month-old CBS −/− mice. Mitochondrial alterations in the liver of CBS −/− mice are known to generate reactive oxygen species. Therefore, we searched for specific changes in the expression of stress-response genes.

Identification of Differentially Expressed Stress Gene Products—Atlas mouse stress cDNA expression membranes (Clontech) were used to identify genes modulated in response to hepatic stress in CBS-deficient mice. The membrane used contains 140 cDNA species involved in stress. The complete list of genes is available at www.Clontech.com. Total RNA obtained from two mixtures of three different livers of 3- or 4-month-old CBS +/+ and CBS −/− mice were used to generate ^{32}P-labeled cDNA probes. The probes were hybridized to the membranes. Identical arrays were differentially screened in triplicate to

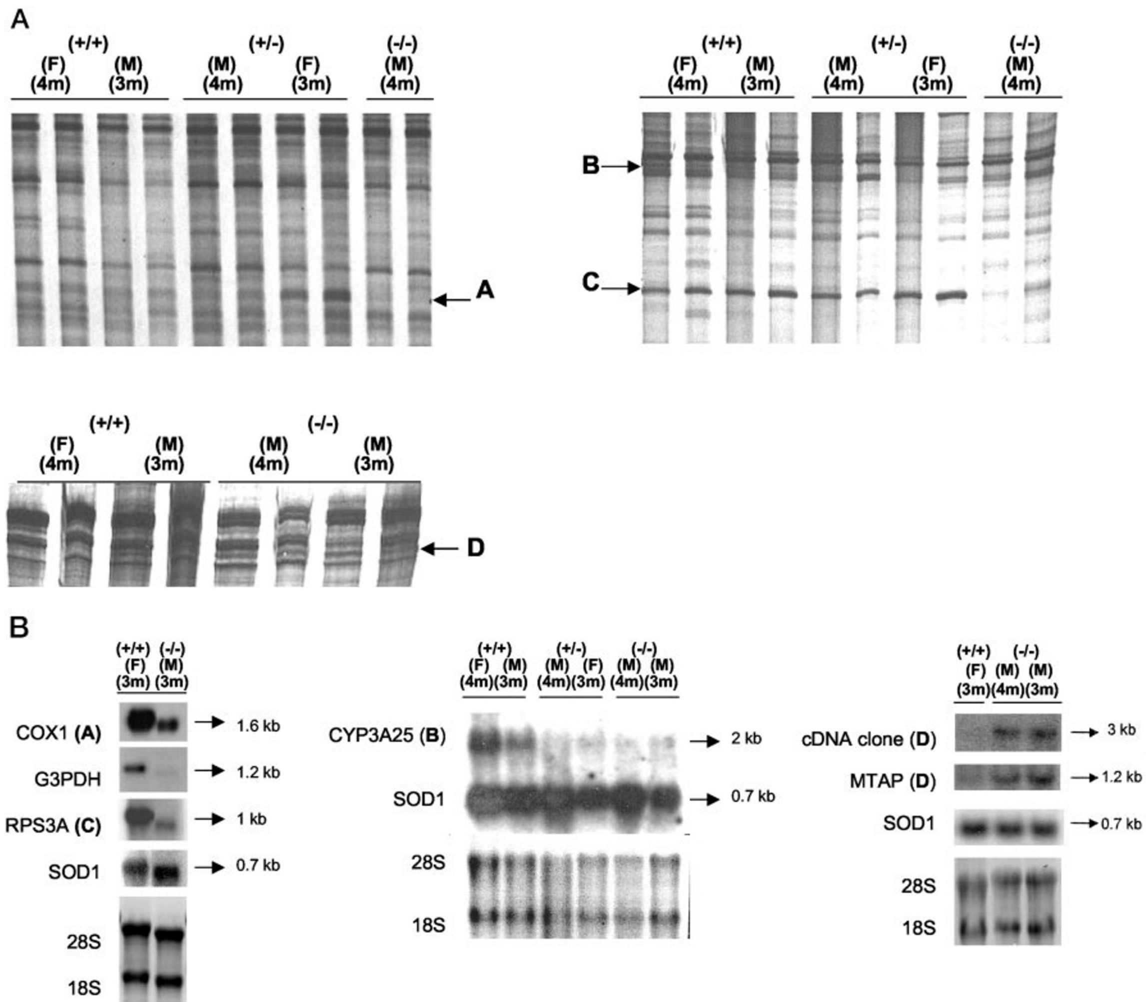

FIG. 2. **Differential display analysis of mRNA from liver tissues of CBS +/−, CBS −/− mice, and CBS +/+ mice from the same litter.** *A*, sequencing gel electrophoresis of cDNAs amplified by PCR (in duplicate) from liver RNAs of male (*M*) and female (*F*) CBS +/+ (+/+), CBS +/− (+/−), and CBS −/− (−/−) mice of 3 (*3m*)- and 4- (*4m*)-month-old mice. Differentially displayed fragments (*arrows A*, *B*, *C*, and *D*) were detected, isolated, and sequenced. *B*, Northern blot hybridization of cDNAs A, B, C, and D and G3PDH probes with 15 μg of total RNA isolated from liver tissues of male (*M*) or female (*F*) CBS +/− (+/−), CBS −/− (−/−), and control CBS +/+ (+/+) mice of 3 (*3m*)- and 4 (*4m*)-month-old mice. A control hybridization with mouse SOD1 was used to evaluate the amount of RNA loaded. The blot is representative of two independent analyses. A photo of the ethidium bromide staining of the same gel is shown, demonstrating that equal amounts of RNA were loaded. Ribosomal RNA bands (28 S and 18 S) are indicated on the *left*. This experiment is representative of two separate experiments yielding similar results. *COX1*, mitochondrial cytochrome *c* oxidase subunit I; *CYP3A25*, cytochrome P4503A25; *cDNA clone*, cDNA clone 620429009 3′; *MTAP*, methylthioadenosine phosphorylase; kb, kilobase(s).

eliminate false positives, and only hybridization signals of at least 3-fold background levels were considered. Fig. 3 is an example of one hybridization. To allow comparisons, hybridization signals were normalized to the signals obtained from housekeeping gene controls on the same array and to the relative decrease in expression of the G3PDH gene in CBS −/− liver (Fig. 3, *arrow 1*, and Fig. 2*B*).

Computer analysis of hybridization intensities of the three hybridizations indicated that seven known mouse genes were differentially expressed. All of the cDNA spots indicated with an *arrow* in Fig. 3 were selected for further examination. The differential expression was evaluated by Northern blot analysis; this confirmed four down-regulated and three up-regulated CBS −/− mice mRNA in two independent experiments. In each case, the relative expression levels of these genes observed on Northern blots were consistent with the differential gene expression identified by array hybridization (Table II). We also analyzed by Northern blotting the modulations in liver RNA of CBS +/− mice. Seven comparative Northern blots were obtained with the CBS −/−, CBS +/−, and CBS +/+ livers probed with the differentially expressed cDNAs listed in Table II (Fig. 4). The genes with higher expression in CBS −/− liver included HO-1, encoding stress-response protein that is induced by a variety of agents causing oxidative stress (41), CYP7B1 (which was also overexpressed in liver from male +/− mice), encoding an oxysterol 7α-hydroxylase, which plays a crucial role in lipid and bile acid metabolism (42), and CYP3A11, encoding a testosterone 6β-hydroxylase (Fig. 4). Genes found to be down-regulated in CBS −/− liver included CYP2B9/10 (which were also underexpressed in liver from female CBS +/− mice), encoding testosterone 16α-hydroxylases, CYP2C29, encoding an aldehyde oxygenase, the genes for sulfotransferases 1,2 (STA1/2) (which were also underexpressed in liver from female CBS +/− mice), two members of alcohol/hydroxysteroid sulfotransferase gene family that catalyze the sulfation of hydroxysteroids, and PON1, encoding a high density lipoprotein (HDL)-associated enzyme that can protect low density lipoprotein (LDL) against oxidation by aortic wall cells (43). CYP2B9 and CYP2B10 mRNAs are very similar in size and nucleotide sequence. Similarly, STA1 and STA2 are very similar. Consequently, in both cases, probes recognize both members of the pair, which cannot be easily discriminated.

TABLE I
cDNA fragments corresponding to mRNAs differentially expressed in the liver of CBS −/− and CBS +/− mice detected by differential display analysis

↑ indicates up-regulation, and ↓ indicates down-regulation in the liver of CBS +/− and (or) CBS −/− mice.

Fragment name	Primer used in cDNA synthesis	Primers used in PCR	Reason for excision	Northern blot[a]	Similar known mouse genes GenBank™ accession number[b]
A	D1 (T_{11}GC)	U2 (TTGATCCGAG), D1 (T_{11}GC)	↓	Down-regulated 3-fold only in liver of CBS −/−	Cytochrome c oxidase subunit I (COX1), NP 008110
B	D4 (T_{11}CG)	U2 (TTGATCCGAG), D4 (T_{11}CG)	↓	Down-regulated 3-fold in liver of CBS −/− and CBS +/−	Cytochrome P4503A25 (CYP3A25), AF 204959
C	D4 (T_{11}CG)	U2 (TTGATCCGAG), D4 (T_{11}CG)	↓	Down-regulated 22-fold only in liver of CBS −/−	RPS3a, NM_016959
D	D2 (T_{11}GG)	U2 (TTGATCCGAG), D2 (T_{11}GG)	↑	Up-regulated 6-fold only in liver of CBS −/−	Methylthioadenosine phosphorylase, AB 056100
D	D2 (T_{11}GG)	U2 (TTGATCCGAG), D2 (T_{11}GG)	↑	Up-regulated 25-fold only in liver of CBS −/−	cDNA clone 620429009 3′, AV 324910

[a] After autoradiography, signal density of each RNA band, normalized to SOD1, was expressed as relative fold-change in gene expression *vs.* control levels. Results represent the average of two independent hybridization experiments.
[b] As found by searching the GenBank™ and the EMBL databases using BLAST and FASTA.

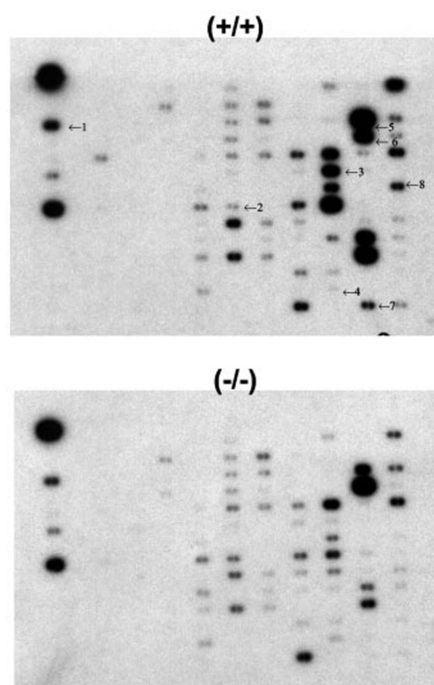

FIG. 3. **Analysis of differential stress gene expression in liver of CBS −/− mice.** mRNAs from CBS +/+ and CBS −/− mouse liver were studied with Atlas mouse stress cDNA expression arrays. The images show an example of the stress membranes hybridized with ^{32}P-labeled cDNA probes generated from total RNA isolated from livers of CBS +/+ (+/+) or CBS −/− (−/−) mice. The image intensities have been adjusted according to the average of the relative intensities of the housekeeping genes (*first line*) and the relative decrease in expression of G3PDH in liver of CBS −/− (*arrow 1*) as shown by Northern blot analysis in Fig. 2*B*. *Arrows* with *numbers* indicate increased or decreased intensities representative of three separate hybridization experiments.

Therefore, the mRNA detected on the Northern blot is referred to as CYP2B9/10 and STA1/2, respectively (Fig. 4).

Analysis of HO-1 Protein Expression and PON1 Activity— Expression of HO-1 was examined in CBS +/+, CBS +/−, and CBS −/− liver mice by Western blotting to determine whether HO-1 is also overexpressed at the protein level. In agreement with increased levels of HO-1 mRNA, HO-1 protein expression was induced in liver of CBS −/− mice (Fig. 5).

Activity of PON1 was also examined in liver extracts of CBS +/+, CBS +/−, and CBS −/− mice. The mean hepatic activity of PON1 in CBS +/− mice was not different from those in CBS +/+ mice (data not shown). Commensurate with the difference in hepatic mRNA PON1 abundance, the mean hepatic activity of PON1 in CBS −/− mice was ~3-fold lower than that in CBS +/+ mice (PON1 activities were 8.8 ± 2.6 *versus* 24.4 ± 4.2 ΔA_{270}/min/mg of cellular protein; $p < 0.03$ by Student's t test $n = 4$ for each).

To determine whether the difference in PON1 activity is due to the loss of CBS expression or due to the resultant HH, activity of PON1 was also examined in liver extracts of CBS +/+ and CBS +/− fed a hyperhomocysteinemic diet. At the time of weaning, CBS +/+ mice and CBS +/− mice were provided with drinking water that was supplemented with 0.5% L-methionine (defined as high methionine diet) (20), and mice were studied at 3 months of age. The mean hepatic activity of PON1 in CBS +/+ mice fed a hyperhomocysteinemic diet was not different from those in CBS +/− mice fed a standard rodent diet (data not shown), and the mean plasma concentration of tHcy was 2-fold that in CBS +/+ mice fed a standard rodent diet (tHcy levels were 9.3 ± 1 *versus* 3.9 ± 0.9 μM; $p <$ 0.0001 by Student's t test $n = 4$ for each). However, CBS +/− mice fed a hyperhomocysteinemic diet have a significant decrease in mean hepatic activity of PON1 (PON1 activities were 13.2 ± 1.7 *versus* 24.4 ± 4.2 ΔA_{270}/min/mg of cellular protein; $p < 0.005$ by Student's t test $n = 4$ for each), and the mean plasma concentration of tHcy was 2-fold that in CBS +/+ mice fed a hyperhomocysteinemic diet and in CBS +/− mice fed a standard rodent diet (tHcy levels were 15 ± 0.6 *versus* 9.2 ± 1.8 μM; $p < 0.0001$ by Student's t test $n = 4$ for each). These results show that the decrease in PON1 activity is inversely correlated with moderate to severe HH in mice.

DISCUSSION

Severe HH, defined as plasma tHcy concentrations greater that 30 μM, is most often caused by mutations in CBS. Several groups have investigated the cellular processes affected by HH. They looked for genes induced in cells cultured *in vitro* in media with high concentrations of Hcy. Hcy is an intermediate amino acid formed during intracellular demethylation of methionine, and extracellular Hcy results from cellular export of Hcy. The plasma tHcy concentration is higher in CBS −/− mice than in CBS +/+ mice, reflecting the higher hepatic level. However, CBS +/− mice have mildly elevated plasma tHcy concentrations, and this does not reflect the hepatic tHcy level.

We used differential screening of mRNAs prepared from the liver of CBS −/−, CBS +/−, and CBS +/+ mice to provide insight into perturbations associated with CBS deficiency and, thus, with high hepatic tHcy concentrations. We also analyzed by Northern blotting the modulations in the liver RNA of CBS

TABLE II
Identification of genes corresponding to mRNAs differentially expressed in liver of CBS −/− and CBS +/− mice as assessed by stress cDNA expression arrays and northern blot analysis

Relative change in gene expression[a]	Mouse gene name, GenBank™ accession number	Northern blot
↓ 1	Glyceraldehyde-3-phosphate dehydrogenase (G3PDH, GAPDH), M32599	Down-regulated 5-fold only in liver of CBS −/− (GAPDH)
↑ 2	HO-1; p32 protein, M33203	Up-regulated 3-fold only in liver of CBS −/− (HO-1)
↓ 3	Cytochrome P450 IIB9 (clone PF26; CYP2B9); P450-16α, RIP; testosterone 16α-hydroxylase + cytochrome P450 IIB10 (clone PF3/46; CYP2B10) + testosterone 16α-hydroxylase type A (16AOH-A) + testosterone 16α-hydroxylase type B (16AOH-B), M21855 + M21856 + M60273 + M60358	Down-regulated 2-fold in liver of female CBS −/− and female CBS +/− (CYP2B9/10)
↑ 4	Cytochrome P450 VIIB1 (CYP7B1); oxysterol 7α-hydroxylase; HCT-1, U 36993	Up-regulated 68-fold in liver of male CBS −/− and male CBS +/− (CYP7B1)
↓ 5	Cytochrome P450 IIC29 (CYP2C29); P450-MUT-2; aldehyde oxygenase, D 17674	Down-regulated 33-fold only in liver of CBS −/− (CYP2C29)
↑ 6	Cytochrome P450 IIIA11 (CYP3A11); P450-UT, X60452	Up-regulated 2-fold only in liver of CBS −/− (CYP3A11)
↓ 7	STA1; hydroxysteroid sulfotransferase + STA2 (STH2), L02335 / L27121	Down-regulated 8-fold in liver of female CBS −/− and female CBS +/− (STA1/2)
↓ 8	PON1; serum aryldiakylphosphatase 1; aromatic esterase 1 (A-esterase 1), L40488	Down-regulated 10-fold only in liver of CBS −/− (PON1)

[a] Relative gene expression levels were determined by normalizing the hybridization signals to the signals obtained for the housekeeping genes and the relative decrease in expression of G3PDH in the liver of CBS −/− mice. ↑ indicates up-regulation and ↓ indicates down-regulation in the liver of CBS +/− and (or) CBS −/− mice.

[b] After autoradiography, signal density of each RNA band, normalized to SOD1, was expressed as relative fold-change in gene expression *vs.* control levels. Results represent the average of two independent hybridization experiments.

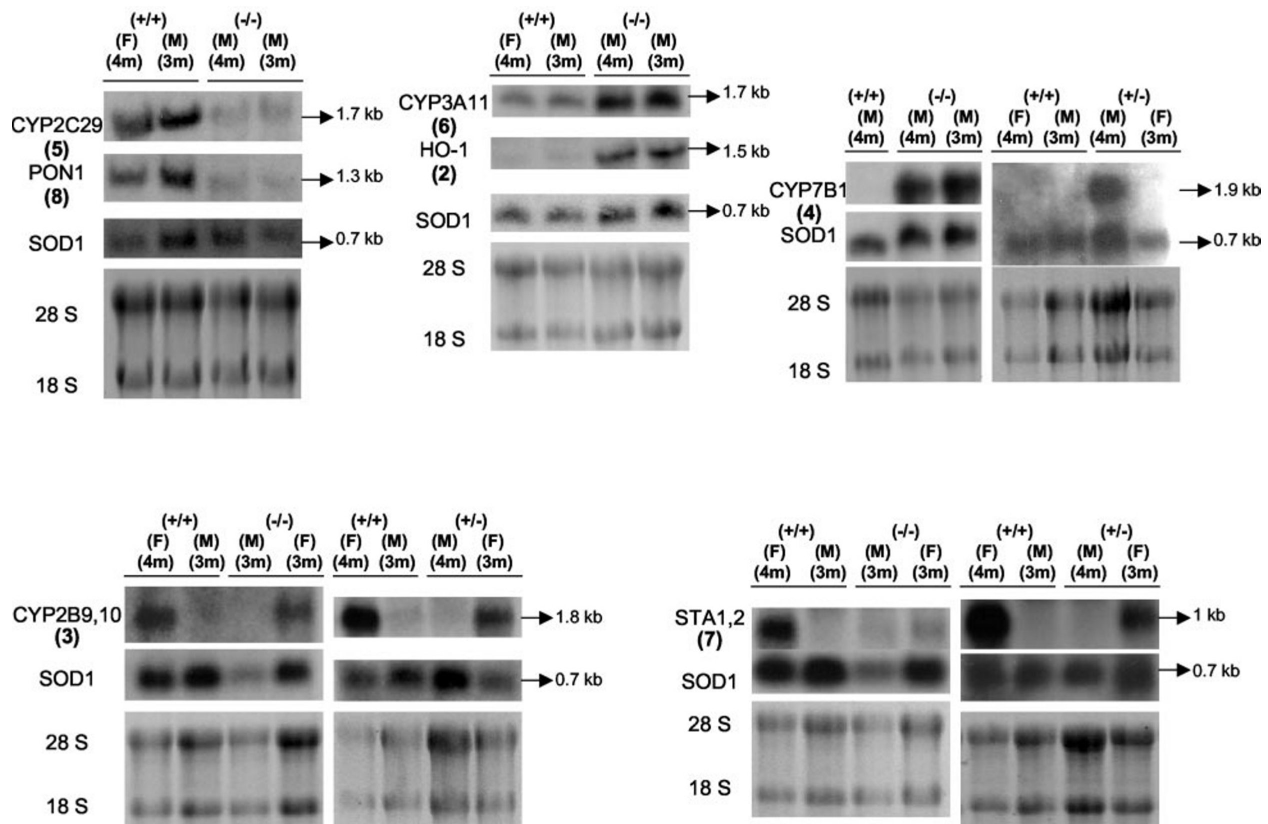

FIG. 4. **Northern blot analysis confirming the cDNA microarray results.** Total RNA was isolated from liver of male (*M*) or female (*F*) CBS +/+ (+/+), CBS +/− (+/−), CBS −/− (−/−) mice of 3 (*3m*)- and 4 (*4m*)-month-old mice. Gels were stained with ethidium bromide (ribosomal RNA bands are indicated on the *left* as 28 S and 18 S). Northern blots were hybridized with ^{32}P-labeled probes for the given gene and SOD1 for the loading control (shown below for each). Numbers corresponding to the increased or decreased intensities shown on the arrays are indicated. The results are representative of two experiments. *CYP2C29*, cytochrome P450 IIC29; *CYP3A11*, cytochrome P450 IIIA11; *CYP7B1*, cytochrome P450 VIIB1; *CYP2B9/10*, cytochrome P450 IIB9/10; *kb*, kilobase(s).

+/+ and CBS +/− mice with or without the supplemented diet with choline, and we have obtained the same results (data not shown). Some of the modifications of expression were only observed in the livers of CBS −/− mice, and others were only observed in the livers of CBS −/− mice and CBS +/− mice. Note that hepatic tHcy levels in CBS +/− mice do not differ significantly from those in CBS +/+ mice. We did not observe modifications of genes in the liver of CBS +/− mice that were not modified in CBS −/− mice.

We report altered expression of genes encoding RPS3a and

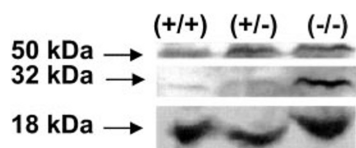

FIG. 5. **SOD1 and HO-1 expression in liver of CBS +/− and CBS −/− mice.** Liver protein extracts from CBS +/+ (+/+), CBS +/− (+/−), and CBS −/− (−/−) mice were analyzed by Western blotting with anti-SOD1 (18 kDa) and with anti-HO-1 (32 kDa). A control hybridization with anti-α-tubulin (50 kDa) was used to evaluate the amount of protein loaded.

methylthioadenosine phosphorylase, suggesting such cellular growth and proliferation perturbations may occur in homozygous CBS-deficient mice liver. The gene encoding RPS3a was down-regulated, and it has been shown that apoptosis is induced in certain cell lines by lowering constitutively high levels of RPS3a expression using antisense RPS3a gene sequences (44). The gene encoding methylthioadenosine phosphorylase was up-regulated. Methylthioadenosine, the substrate of methylthioadenosine phosphorylase, is a potent inhibitor of *S*-adenosylmethionine-dependent transmethylation reactions (40). CBS −/− mice have elevated plasma levels of tHcy and a concomitant increase in *S*-adenosyl-L-Hcy, a potent inhibitor of methyltransferases, notably in liver (45). Thus, other metabolic perturbations caused by CBS deficiency might have possible effects upon gene expression patterns in liver of CBS −/− mice.

We demonstrated the presence of oxidative stress in liver of CBS-deficient mice. The expression of many CYP was affected in CBS −/− and CBS +/− liver cells, indicating perturbation of the redox potential. One mitochondrial mRNA transcript (mitochondrial cytochrome *c* oxidase subunit I) was down-regulated in CBS −/− liver. This suggests that all mitochondrial transcripts are down-regulated because all mitochondrial transcription depends on a single promoter (46). This effect could also be attributed to oxidative damage, which is particularly important in the mitochondrial compartment. High concentrations of Hcy on human megakaryocytic cell line cells decrease mitochondrial gene expression in the presence of physiological concentrations of Cu^{2+} (14). The down-regulation of mitochondrial RNAs may be a general mechanism by which cells of liver of CBS −/− mice protect themselves against oxidative stress.

Changes in the intracellular redox state associated with elevated tHcy levels in liver of CBS −/− mice can alter the expression of intracellular defense genes induced in response to stressful conditions. The activity of several antioxidant enzymes including glutathione peroxidase was decreased in liver of CBS −/− mice (47). Glutathione, which plays a key role in the cellular defense against oxidative stress, is less abundant in liver of CBS −/− than control mice (47). Approximately half of the intracellular glutathione pool in human liver cells is derived from Hcy via the transsulfuration pathway (48). The Hcy-dependent transsulfuration pathway is important for maintaining the intracellular glutathione pool, and abnormality in this pathway compromises the redox buffering capacity of cells. Therefore, the susceptibility of hepatic tissues to oxidative stress may be enhanced by CBS deficiency. We have shown that expression of HO-1 is powerfully induced in liver of CBS −/− mice at the transcriptional and protein expression levels. Agents that are oxidants or can generate active intermediates lead to induction of HO-1 in cultured human skin fibroblasts (49). Induction of HO-1 mRNA may be a result of oxidative stress in the liver of CBS −/− mice, and overexpression of HO-1 protein could contribute to a cytoprotective mechanism to prevent further oxidative injury in the liver.

Werstuck *et al.* (24) demonstrate that HH-induced endoplasmic reticulum stress in hepatocytes results in an enhanced lipid biosynthesis by activation of the sterol regulatory element-binding protein, thus leading to hepatic steatosis. CBS +/− mice fed a hyperhomocysteinemic diet have increased hepatic cholesterol and triglyceride levels through increased hepatic expression of genes involved in cholesterol and triglyceride synthesis, uptake, and storage (24). We have also seen by histological analysis of liver sections from CBS −/− mice that hepatocytes are engorged with lipid vesicles (data not shown), but we have not found expression modulations of genes involved in hepatic metabolism of cholesterol. Even if we have not screened all the hepatic transcripts of CBS −/− mice, we have used a mouse stress cDNA expression membrane that contains the gene encoding peroxisome proliferator-activated receptor γ, and overexpression of peroxisome proliferator-activated receptor γ has been associated to the development of hepatic steatosis. Nevertheless, we have not found gene expression modulation of peroxisome proliferator-activated receptor γ in the liver of CBS −/− mice.

There is growing evidence that elevated plasma levels of tHcy are associated with an increased risk of atherosclerosis. Several studies show that Hcy induces endothelial dysfunction and injury in CBS +/− (18, 19, 21, 22) and CBS −/− (50) mice. Moreover, endothelial dysfunction is believed to be an important mechanism initiating the pathogenesis of atherosclerosis. We have found altered expression of gene encoding PON1 concomitant with reduced PON1 activity in the liver of CBS −/− mice but also in the liver of CBS +/− mice fed a hyperhomocysteinemic diet, which demonstrates the importance of HH in the PON1 activity. Most serum PON1 is synthesized in the liver, and PON1 is a protein component of HDL. It destroys biologically active lipids in mildly oxidized LDL, thereby preventing oxidized LDL build-up when antioxidant protection is not sufficient (43, 51). This protection is very important because oxidized LDL is highly cytotoxic to the endothelium, promotes conversion of macrophages to foam cells, and stimulates monocyte recruitment and immunological responses (52). PON1-deficient mice, when crossed to apolipoprotein E (apoE, a protein that mediates removal of plasma lipoproteins via the LDL receptors and other receptors)-deficient mice and fed on a high fat high cholesterol diet, also exhibited increased expression of genes related to oxidative stress like HO-1 and were more susceptible to atherosclerosis than wild type from the same litter (53). Moreover, HO-1 expression in cultured artery wall cells is up-regulated by mildly oxidized LDL (54). Therefore, increased and decreased levels of HO-1 and PON1, respectively, in the liver of CBS −/− mice provide indirect evidence that severe HH may lead to higher LDL oxidation in mice. A recent study shows that PON1 contributes to the protective role of HDL against the oxidative damage and against toxicity exerted by Hcy involved in the development of atherosclerosis (55). Moreover, mice deficient in CBS and apoE develop accelerated aortic atherosclerosis (56). Increased lesion formation was observed in 1-year-old CBS −/−/apoE −/− mice on regular diet and in a diet-induced milder HH in 5-month-old CBS +/−/apoE −/− mice (56) and suggests that isolated HH is atherogenic. We have not observed atherosclerosis lesions in CBS −/− mice at 3 or 4 months of age (data not shown). Nevertheless, mice used in our study are younger than mice used by Wang *et al.* (56).

In summary, the reduced PON1 activity indicates that HH in mice could lead to higher LDL oxidation, which may influence the development and progression of atherosclerosis lesions. Our work also increases our understanding of the gene pathways involved in the pathophysiological consequences of HH.

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REFERENCES

1. Ueland, P. M., Refsum, H., Stabler, S. P., Malinow, M. R., Andersson, A., and Allen, R. H. (1993) *Clin. Chem.* **39,** 1764–1779
2. Kraus, J. P. (1998) *Eur. J. Pediatr.* **157,** 50–53
3. Cattaneo, M. (1999) *Thromb. Haemostasis* **81,** 165–176
4. Nygard, O., Nordrehaug, J. E., Refsum, H., Ueland, P. M., Farstad, M., and Vollset, S. E. (1997) *N. Engl. J. Med.* **337,** 230–236
5. Tawakol, A., Omland, T., Gerhard, M., Wu, J. T., and Creager, M. A. (1997) *Circulation* **95,** 1119–1121
6. Bellamy, M. F., McDowell, I. F., Ramsey, M. W., Brownlee, M., Bones, C., Newcombe, R. G., and Lewis, M. J. (1998) *Circulation* **98,** 1848–1852
7. Lentz, S. R., Sobey, C. G., Piegors, D. J., Bhopatkar, M. Y., Faraci, F. M., Malinow, M. R., and Heistad, D. D. (1996) *J. Clin. Invest.* **98,** 24–29
8. Chacko, G., Ling, Q., and Hajjar, K. A. (1998) *J. Biol. Chem.* **273,** 19840–19846
9. Kokame, K., Kato, H., and Miyata, T. (1996) *J. Biol. Chem.* **271,** 29659–29665
10. Outinen, P. A., Sood, S. K., Liaw, P. C. Y., Sarge, K. D., Maeda, N., Hirsh, J., Ribau, J., Podor, T. J., Weitz, J. I., and Austin, R. C. (1998) *Biochem. J.* **332,** 213–221
11. Outinen, P. A., Sood, S. K., Pfeifer, S. I., Pamidi, S., Podor, T. J., Li, J., Weitz, J. I., and Austin, R. C. (1999) *Blood* **94,** 959–967
12. Tsai, J. C., Perrella, M. A., Yoshizumi, M., Hsieh, C. M., Haber, E., Schlegel, R., and Lee, M. E. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 6369–6373
13. Tsai, J. C., Wang, H., Perrella, M. A., Yoshizumi, M., Sibinga, N. E. S., Tan, L. C., Haber, E., Chang, T. H. T., Schlegel, R., and Lee, M. E. (1996) *J. Clin. Invest.* **97,** 146–153
14. Austin, R. C., Sood, S. K., Dorward, A. M., Singh, G., Shaughnessy, S. G., Pamidi, S., Outinen, P. A., and Weitz, J. I. (1998) *J. Biol. Chem.* **273,** 30808–30817
15. Torres, L., Garcia-Trevijano, E. R., Rodriguez, J. A., Carretero, M. V., Bustos, M., Fernandez, E., Eguinoa, E., Mato, J. M., and Avila, M. A. (1999) *Biochim. Biophys. Acta* **1455,** 12–22
16. Althausen, S., and Paschen, W. (2000) *Brain Res. Mol. Brain Res.* **84,** 32–40
17. Watanabe, M., Osada, J., Aratani, Y., Kluckman, K., Reddick, R., Malinow, M. R., and Maeda, N. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 1585–1589
18. Eberhardt, R. T., Forgione, M. A., Cap, A., Leopold, J. A., Rudd, M. A., Trolliet, M., Heydrick, S., Stark, R., Klings, E. S., Moldovan, N. I., Yaghoubi, M., Goldschmidt-Clermont, P. J., Farber, H. W., Cohen, R., and Loscalzo, J. (2000) *J. Clin. Invest.* **106,** 483–491
19. Lentz, S. R., Erger, R. A., Dayal, S., Maeda, N., Malinow, M. R., Heistad, D. D., and Faraci, F. M. (2000) *Am. J. Physiol. Heart Circ. Physiol.* **279,** 970–975
20. Dayal, S., Bottiglieri, T., Arning, E., Maeda, N., Malinow, M. R., Sigmund, C. D., Heistad, D. D., Faraci, F. M., and Lentz, S. R. (2001) *Circ. Res.* **88,** 1203–1209
21. Weiss, N., Zhang, Y. Y., Heydrick, S., Bierl, C., and Loscalzo, J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 12503–13508
22. Weiss, N., Heydrick, S., Zhang, Y. Y., Bierl, C., Cap, A., and Loscalzo, J. (2002) *Arterioscler. Thromb. Vasc. Biol.* **22,** 4–41
23. Caudill, M. A., Wang, J. C., Melnyk, S., Pogribny, I. P., Jernigan, S., Collins, M. D., Santos-Guzman, J., Swendseid, M. E., Cogger, E. A., and James, S. J. (2001) *J. Nutr.* **131,** 2811–2818
24. Werstuck, G. H., Lentz, S. R., Dayal, S., Hossain, G. S., Sood, S. K., Shi, Y. Y., Zhou, J., Maeda, N., Krisans, S. K., Malinow, M. R., and Austin, R. C. (2001) J. *Clin. Invest.* **107,** 1263–1273
25. Hultberg, B., Berglund, M., Andersson, A., and Frank, A. (1993) *Alcohol Clin. Exp. Res.* **17,** 687–689
26. Cravo, M. L., Gloria, L. M., Selhub, J., Nadeau, M. R., Camilo, M. E., Resende, M. P., Cardoso, J. N., Leitao, C. N., and Mira, F. C. (1996) *Am. J. Clin. Nutr.* **63,** 220–224
27. Halsted, C. H., Villanueva, J., Chandler, C. J., Stabler, S. P., Allen, R. H., Muskhelishvili, L., James, S. J., and Poirier, L. (1996) *Hepatology* **23,** 497–505
28. Finkelstein, J. D. (1998) *Eur. J. Pediatr.* **157,** Suppl. 2, S40–S44
29. Stead, L. M., Brosnan, M. E., and Brosnan, J. T. (2000) *Biochem. J.* **350,** 685–692
30. Shipchandler, M. T., and Moore, E. G. (1995) *Clin. Chem.* **41,** 991–994
31. Laemmli, U. K. (1971) *Nature* **227,** 680–685
32. Omura, T., Sadano, H., Hasegawa, T., Yoshida, Y., and Kominami, S. (1984) *J. Biochem.* **96,** 1491–1500
33. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
34. Liang, P., and Pardee, A. B. (1992) *Science* **257,** 967–971
35. Deakin, S., Leviev, I., Gomarashi, M., Calabresi, L., Franceschini, G., and James, R. W. (2002) *J. Biol. Chem.* **277,** 4301–4308
36. Finkelstein, J. D. (2000) *Semin. Thromb. Hemostasis* **26,** 219–225
37. Robert, K., Vialard, F., Thiery, E., Toyama, K., Sinet, P. M., Janel, N., and London, J. (2003) *J. Histochem. Cytochem.* **51,** 363–371
38. Seshadri, T., Uzman, J. A., Oshima, J., and Campisi, J. (1993) *J. Biol. Chem.* **268,** 18474–18480
39. Kho, C. J., Wang, Y., and Zarbl, H. (1996) *Cell Growth Differ.* **7,** 1157–1166
40. Williams-Ashman, H. G., Seidenfeld, J., and Galletti, P. (1982) *Biochem. Pharmacol.* **31,** 277–288
41. Choi, A. M., and Alam, J. (1996) *Am. J. Respir. Cell Mol. Biol.* **15,** 9–19
42. Li-Hawkins, J., Lund, E. G., Turley, S. D., and Russell, D. W. (2000) *J. Biol. Chem.* **275,** 16536–16542
43. Watson, A. D., Berliner, J. A., Hama, S. Y., La Du, B. N., Faull, K. F., Fogelman, A. M., and Navab, M. (1995) *J. Clin. Invest.* **96,** 2882–2891
44. Naora, H., Nishida, T., Shindo, Y., Adachi, M., and Naora, H. (1998) *Leukemia* (*Baltimore*) **12,** 532–541
45. Choumenkovitch, S. F., Selhub, J., Bagley, P. J., Maeda, N., Nadeau, M. R., Smith, D. E., and Choi, S. W. (2002) *J. Nutr.* **132,** 2157–2160
46. Shan, B., Vazquez, E., and Lewis, J. A. (1990) *EMBO J.* **9,** 4307–4314
47. Chabli, A., Chadefaux-Vekemans, B., Jacob, N., Janel, N., Nicole, A., and Kamoun, P. (2002) *Ann. Biol. Clin.* **60,** 220 (abstr.)
48. Mosharov, E., Cranford, M. R., and Banerjee, R. (2000) *Biochemistry* **39,** 13005–13011
49. Applegate, L. A., Luscher, P., and Tyrrell, R. M. (1991) *Cancer Res.* **51,** 974–978
50. Denis, C. V., Terraube, V., Robert, K., and Janel, N. (2003) *Thromb. Haemostasis* **90,** 262–264
51. Aviram, M., Rosenblat, M., Bisgaier, C. L., Newton, R. S., Primo-Parmo, S. L., and La Du, B. N. (1998) *J. Clin. Invest.* **101,** 1581–1590
52. Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C., and Witztum, J. L. (1989) *N. Engl. J. Med.* **320,** 915–924
53. Shih, D. M., Xia, Y. R., Wang, X. P., Miller, E., Castellani, L. W., Subbanagounder, G., Cheroutre, H., Faull, K. F., Berliner, J. A., Witztum, J. L., and Lusis, A. J. (2000) J. *Biol. Chem.* **275,** 17527–17535
54. Ishikawa, K., Navab, M., Leitinger, N., Fogelman, A. M., and Lusis, A. J. (1997) *J. Clin. Invest.* **100,** 1209–1216
55. Ferretti, G., Bacchetti, T., Marotti, E., and Curatola, G. (2003) *Metabolism* **52,** 146–151
56. Wang, H., Jiang, X., Yang, F., Gaubatz, J. W., Ma, L., Magera, M. J., Yang, X., Berger, P. B., Durante, W., Pownall, H. J., and Schafer, A. I. (2003) *Blood* **101,** 3901–3907