Modulation of Cystathionine β-Synthase Level Regulates Total Serum Homocysteine in Mice

Liqun Wang, Kwang-Hwan Jhee, Xiang Hua, Patricia M. DiBello, Donald W. Jacobsen, Warren D. Kruger

Abstract—Elevated total plasma homocysteine is an independent risk factor in the development of vascular disease in humans. Cystathionine β-synthase (CBS) is an enzyme that condenses homocysteine with serine to form cystathionine. In this article, we describe the effects of modulating CBS activity using a transgenic mouse that contains the human CBS cDNA under control of the zinc-inducible metallothionein promoter (Tg–CBS). In the presence of zinc, Tg–CBS mice have a 2- to 4-fold increase in liver and kidney CBS activity compared with nontransgenic littermates. Transgenic mice on standard mouse chow had a 45% decrease in their serum homocysteine (12.1 to 7.2 μmol/L; P<0.0001) when zinc was added to drinking water, although zinc had minimal effect on their nontransgenic siblings (13.2 μmol/L versus 13.0 μmol/L; P=NS). Tg–CBS mice maintained on a high-methionine, low-folate diet also had significantly lower serum homocysteine compared with control animals (179 μmol/L versus 242 μmol/L; P<0.02). CBS overexpression also significantly lowered serum cysteinylglycine (3.6 versus 2.8 μmol/L; P<0.003) levels and reduced the levels of many amino acids in the liver. We also found that expression of Tg-CBS rescued the severe hyperhomocysteinemia and neonatal lethality of Cbs deletion animals. Our results show that elevating CBS activity is an effective method to lower plasma homocysteine levels. In addition, the creation of an inducible mouse system to modulate plasma homocysteine will also be useful in the study of homocysteine-related vascular disease. (Circ Res. 2004;94:1318-1324.)

Key Words: metabolism ■ genetics ■ amino acids ■ cardiovascular diseases

In the past decade, elevated plasma total homocysteine (tHcy) has emerged as an important risk factor for the development of vascular disease. More than 80 clinical and epidemiological studies, including more than 10 000 patients, have established tHcy as a risk factor for disease of the coronary, cerebral, and peripheral arteries, and for arterial and venous thromboembolism. Elevated tHcy is a graded risk factor with no threshold and is independent of other known risk factors. Analysis of prospective studies suggests that elevated tHcy accounts for a significant fraction of coronary artery disease and stroke in the general population.

Emerging evidence indicates that elevated tHcy is not simply a marker for vascular disease, but actually has pathological effects on vascular endothelium. Monkeys and mice with elevated tHcy levels caused by diet or mutations have impaired vascular endothelium characterized by abnormal vasodilator properties. Elevated tHcy has also been shown to cause oxidative stress by inhibition of NO, resulting in lower intracellular glutathione (GSH) pools in endothelial cells. Finally, elevated homocysteine in both cell culture and in livers has been shown to stimulate the unfolded protein response, leading to activation and dysregulation of the sterol response pathway and apoptosis in vascular endothelium.

Because of the link between tHcy and vascular disease, there has been much interest in strategies to lower tHcy. Several studies have shown that folic acid and multivitamin supplementation can lower plasma homocysteine. In fact, as a result of the fortification of grain with folic acid in the United States, there has been a 50% decrease in the number of individuals with elevated tHcy concentrations (>13 μmol/L) in the general population. However, not all individuals respond to vitamin therapy. In a study of 304 individuals, it was found that 20% actually had elevations in plasma homocysteine levels after 3 weeks of folic acid supplementation, which correlated with the status of single-nucleotide polymorphisms in the cystathionine β-synthase (CBS) gene. It has also been observed that end-stage renal disease patients as a group have extremely elevated plasma homocysteine despite vitamin therapy. Thus, there is a need for additional strategies to lower plasma homocysteine.

Homocysteine has 2 possible metabolic fates: remethylation to form methionine or transsulfuration to form cystathionine (Figure 1). The transsulfuration reaction is performed by the enzyme cystathionine β-synthase (CBS). This enzyme condenses homocysteine with serine to form cystathionine and requires pyridoxine (vitamin B₆) as a cofactor. Previous work has shown that it is possible to increase the activity of human CBS (hCBS) by deletion or mutation of the C-terminal regulatory domain. These findings suggest...
Overexpression of CBS Lowers Homocysteine

Materials and Methods

Hemagglutinin-Tagged hCBS

A hemagglutinin (HA) epitope tagged version of hCBS was created as follows. Primers HA CBS (5'-ATGTACCCATACGATGTTCC-AGATTACGCTCCTTCTGAGACCCCC-3') and WK90 (5'-TTACCAATCACGCGTGTGTTTAGGGC-3') were used to polymerase chain reaction (PCR) amplify hCBS using pHCBS as template. The resulting PCR product contains the HA epitope (YPYDVPDYA) fused in frame to the second amino acid of hCBS. The PCR product was then cloned into PCR-cloning vector PCR 2.1 (Invitrogen) to create PCR 2.1::hCBS. To test the epitope-tagged version of the protein for function, PCR 2.1::hCBS was digested with EcoRI and cloned into the EcoRI site of pUC18. To make cloning easier, we modified the plasmid by converting the NrdI site to MfiI by inserting a linker (GCAATTGTC). The resultant plasmid was dubbed pUC-MT-EMfi.

PCR 2.1::hCBS was digested with EcoRI and the hCBS fragment was then cloned into the MfiI site of pUC-MT-EMfi, creating pLW1. To create a better translation initiation environment, pLW1 was further modified by site-directed mutagenesis to convert the −3 position nucleotide from C to A, creating pLW2. pLW2 was then digested with EcoRI, and this fragment was then cloned into 2999 digested with EcoRI to create pLW3.

Production and Screening of Transgenic Mice

PLW3 was digested with SulI to drop out bacterial vector sequences, and the mouse–human sequences were isolated using Qiagen II Gel Extraction Kit (Qiagen). The recovered DNA was then purified by extraction with phenol/chloroform and ethanol precipitation. DNA was resuspended in 10 mmol/L Tris, 0.1 mmol/L EDTA, pH 7.3, at 20 ng/mL.

The purified transgene DNA fragment was microinjected into pronuclei of day 0.5 C57B6/CH F2 embryos from C57B6/CH F1 × C57B6/CH F1 matings. Injected embryos were implanted into the oviducts of day 0.5 pseudopregnant female Swiss Webster mice. Tails from the resulting pups were clipped 2 weeks after birth for genotype analysis. Presence of the transgene was confirmed by PCR amplification with primers 5'AAGAGTTCGGCCTCAAGTGTGAG3' and 5'ATGTAGTTCCGCA-CTGAGTCGGGCAGAATG3', which amplify an 858-bp fragment of human CBS. DNA-positive founders were then crossed to C57B6 animals, and these pups were analyzed for germline transmission of the transgene and were tested for transgene expression as described in Figure 2.

B6.129P2-Cbs<sup>tm1Unc</sup> mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Genotyping for the Cbs<sup>tm1Unc</sup> allele was performed using a 3 primer PCR system developed by the Loscalzo Laboratory. The primers used were 5'GGTCTGGAATTCACTAT-GTCAG3' (forward primer, intron 2), 5'CCTGAGTACCTGACG-ATCGCT3' (reverse primer, intron 2), and 5'AGGTGCAGCG-GTACGTAA3' (reverse primer, neo linker). The wild-type allele gives a 300-bp product, whereas the deletion allele gives a 176-bp band product.

Mouse Diets

Mice were fed either the Fox Chase Cancer Center animal facility standard rodent chow (LabDiet 5013) or a special high-methionine, low-folate diet from Harlan Teklad (TD98272) for hyperhomocysteinemia-inducing experiments. This diet contains 5× more methionine (20 g/kg versus 4.1 g/kg) and three-quarters the amount of folate (1.5 mg/kg versus 2.0 mg/kg) per kilogram compared with the standard rodent chow. For transgene induction, ZnSO₄ was added to the water at a concentration of 25 mmol/L.

Immunoblot, Enzyme Analysis, and Amino Acid Analysis of Liver

Mice were euthanized with CO₂, and livers and kidney were harvested and immediately put on ice. The liver was homogenized using a dounce homogenizer in extract buffer [50 mmol/L Tris-HCl, pH 7.8, 25 mmol/L NaCl], to which a protease inhibitor pill (Complete-Mini, Roche) was added. Extracts were then centrifuged at 12,000g at 4°C and the supernatant was retained. Protein concentration was determined by the coomassie blue protein assay reagent (Pierce) using BSA as a standard. For Western blot analysis, 25 µg of extract were run on precast 7% NuPage Tris–acetate sodium dodecyl sulfate gel (Invitrogen) and transferred to polyvinylidene difluoride membrane as described. Samples were probed with a mouse sequences and can be used to remove the Bluescript-based vector sequences.

Because we had difficulties cloning directly into the NrdI site on this large plasmid, we used the following strategy. We digested 2999 with EcoRI and isolated a 1.4-kb fragment containing 814 bp of the MT-I promoter, the NrdI cloning site, and 650 bp of the hGH 3' untranslated region. This fragment was then inserted into the EcoRI site of pUC18. To make cloning easier, we modified the plasmid by converting the NrdI site to MfiI by inserting a linker (GCAATTGTC). The resultant plasmid was dubbed pUC-MT-EMfi.

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The transgene and the endogenous mouse gene. We confirmed zinc results in a large increase in hCBS levels but does not the liver and kidney in all 3 lines. In the liver, the presence of 2A, the human transgene is tightly regulated by zinc in both

Blot using anti-human CBS antiserum. As shown in Figure 2A, the human transgene is tightly regulated by zinc in both the liver and kidney in all 3 lines. In the liver, the presence of zinc results in a large increase in hCBS levels but does not affect the levels of mCBS. In the kidney, zinc induces both the transgene and the endogenous mouse gene. We confirmed that the upper band was HA-tagged hCBS by Western blot with an anti-HA antibody (data not shown). We also performed Western analysis on extracts derived from a variety of other mouse tissues, including brain, skeletal muscle, spleen, lungs, colon, stomach, and adenoids. The only tissues that expressed detectable levels of hCBS were the stomach and colon. This is consistent with previously published expression patterns of transgenes being expressed from the MT-I promoter.21

Initially, we examined expression of the transgene in liver and kidney. Mice from each line were either given regular water or water containing 25 mmol/L ZnSO4 for 10 days, after which their livers and kidneys were harvested and total protein was isolated. HA-tagged hCBS (HAhCBS) and endogenous mCBS were detected by immunoblot analysis using anti-human CBS antibody. B, Homogenous expression of HAhCBS. Mice derived from the indicated founders were backcrossed to C57B6 for 3 generations, put on ZnSO4 water, and then had their livers, and in some cases kidneys, harvested and analyzed for CBS expression by immunoblot.

**Figure 2.** A, Zinc (Zn) regulation of Tg-CBS mice. Offspring 4- to 6-weeks old of the indicated founder mice were fed either normal water or water containing 25 mmol/L ZnSO4 for 10 days, and their livers and kidneys were harvested and analyzed. HA-tagged hCBS by Western blot.

<table>
<thead>
<tr>
<th>Zinc/Tissue</th>
<th>Tg–CBS</th>
<th>No Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>−Zn/liver</td>
<td>992 ± 206</td>
<td>1025 ± 222</td>
</tr>
<tr>
<td>+Zn/liver</td>
<td>2417 ± 1108</td>
<td>1115 ± 284</td>
</tr>
<tr>
<td>−Zn/kidney</td>
<td>129 ± 13</td>
<td>107 ± 20</td>
</tr>
<tr>
<td>+Zn/kidney</td>
<td>935 ± 230</td>
<td>260 ± 32</td>
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Units are nanomoles cystathionine/milligrams per hour.

**Table 1. CBS Enzyme Activity**
kidney. In the liver, the mean activity of the zinc-treated transgenic animals was 2.2-fold higher than the zinc-treated nontransgenic control animals (2742 U versus 1115; \( P = 0.016 \)). In the kidney, there was 3.5-fold elevation in CBS activity between transgenic and nontransgenic animals (935 U versus 260; \( P = 0.007 \)). We also confirmed that zinc elevates endogenous mouse CBS in the kidney (107 U versus 260 U; \( P = 0.002 \)).

**Effects of CBS Overexpression**

We next examined how CBS overexpression affects the levels of tHcy and other thiols in transgenic and control animals. We collected serum from 16 transgenic animals and 17 nontransgenic littermates on standard mouse chow and zinc-containing water, which induces MT-controlled hCBS activity. We then shifted these animals to normal water for 2 weeks and re-bleed them. Serum was analyzed for tHcy, GSH, cysteine, and cysteinyl–glycine (Figure 3). The mean tHcy (±SEM) for the nontransgenic control animals was 13.0±1.1 \( \mu \text{mol/L} \) for animals on normal water and 13.2±1.0 \( \mu \text{mol/L} \) on zinc-containing water (paired \( t \) test; \( P = 0.99 \)). For the transgenic animals, tHcy was 12±0.7 \( \mu \text{mol/L} \) in the absence of zinc but decreased 45% when the animals were put on zinc water (7.2±0.3 \( \mu \text{mol/L} \); paired \( t \) test; \( P = 0.000003 \)). Comparing the tHcy levels between zinc-treated control and zinc-treated transgenic animals, there was also a highly significant difference (13.2 versus 7.2 \( \mu \text{mol/L} \); unpaired \( t \) test; \( P < 0.0001 \)). We did not observe any difference in the levels of serum GSH between transgenic and nontransgenic animals, although the addition of zinc to the water did lower GSH levels significantly in both control and transgenic animals. Both serum cysteine and cysteinyl–glycine levels were lower in the zinc-treated transgenic animals compared with identically treated control animals, although only in cysteinyl–glycine levels were changes statistically significant (3.6 versus 2.8 \( \mu \text{mol/L} \); \( P = 0.003 \)). Addition of zinc had no effect on either serum cysteine or cysteinyl–glycine levels in control animals. These results show that CBS overexpression can significantly lower serum homocysteine.

We also examined how CBS overexpression affected tHcy in mice that had abnormally elevated tHcy caused by dietary intervention. Sixteen control and 12 transgenic mice were put on mouse chow containing 5\( \times \) the level of methionine and 25\% less folate than the standard mouse chow in the presence of zinc water. After 3 weeks on this chow, blood was collected and analyzed for tHcy, GSH, cysteine, and cysteinyl–glycine (Figure 4). Total serum homocysteine was 23\% lower in the CBS-overexpressing animals compared with control animals (179 versus 242 \( \mu \text{mol/L} \); \( P < 0.02 \)), indicating that elevated CBS activity could help alleviate homocysteine elevations caused by abnormal diet. We did not see any effect of CBS overexpression on GSH or cysteine levels, although cysteinyl–glycine was reduced in CBS-overexpressing animals compared with controls.

At the completion of this experiment, we euthanized 6 transgenic animals and 6 nontransgenic littermates and examined free amino acid pools in total liver homogenates (Table 2) using an amino acid analyzer. The amino acids present at detectable levels were threonine, serine, asparagine, glutamine, glutamic acid, glycine, alanine, valine, homocysteine, methionine, cystathionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, cysteine, ornithine, glutathionine, urea, taurine, phosphoserine, and \( \alpha \)-butyric acid. Statistically significant differences were observed in the levels of phenylalanine, valine, leucine, asparagine, isoleucine, \( \alpha \)-aminobutyrate, and ammonia. In all cases, the pools of these amino acids were low in the transgenic compared...
with the control animals. Interestingly, none of the changed amino acids were part of the methionine or cysteine metabolic pathway. This suggests that the control of amino acid homeostasis in the liver is extremely complex.

**Tg–CBS Rescues Cbs<sup>tm1Unc</sup> Animals**

Watanabe et al created a knockout allele for CBS (Cbs<sup>tm1Unc</sup>) and found that animals homozygous for this allele showed severe homocysteinemia and growth retardation and rarely survived beyond 6 weeks.25 We confirmed these findings in our own colony of Cbs<sup>tm1Unc</sup> mice. We found that at the time of genotyping (aged 4 to 6 weeks), only 2 of 258 offspring of Cbs/ Cbstm1Unc mating were homozygous for Cbs<sup>tm1Unc</sup>. To determine whether our transgene could rescue these effects, we mated our Tg–CBS animals to animals Cbs/ Cbstm1Unc and obtained Tg–CBS Cbs/ Cbs<sup>tm1Unc</sup> offspring. These animals were then put on zinc water and backcrossed to Cbs/ Cbs<sup>tm1Unc</sup> mice. Our belief was that if the transgene rescued lethality, we would expect approximately one-eighth (12.5%) of the offspring to be both homozygous for Cbs<sup>tm1Unc</sup> and hemizygous for the transgene. Furthermore, we would also expect to see significantly more viable homozygous animals with the transgene than without the transgene. Of 182 offspring born, 21 were homozygous for the knockout allele, and of these, 18 also contained the transgene (9.9% of the total). These results show that the transgene is able to rescue the lethality associated with homozygosity for Cbs<sup>tm1Unc</sup>.

We also examined thcy levels in all 18 Tg–CBS Cbs<sup>tm1Unc</sup>/ Cbs<sup>tm1Unc</sup> animals in both the presence and absence of induction by zinc. As shown in Figure 5, thcy was significantly elevated when mice were switched from zinc-containing to nonzinc-containing water. The mean thcy went from 67.3 μmol/L to 190.37 μmol/L (P=0.00015; n=18). Interestingly, we saw a greater effect in female animals than in male animals. In females, mean thcy increased from 25 μmol/L to 231 μmol/L (P=0.000078; n=7), whereas in males, thcy went from 93 μmol/L to 179 μmol/L (P=0.0005; n=11). We hypothesize that the relatively high levels of thcy observed in the presence of zinc in some animals (especially males) may be attributable to attenuation of zinc response because of the prolonged exposure to zinc. However, despite this possibility, approximately one-third of all the animals had thcy within the normal range when on zinc and extreme elevations in thcy when zinc was removed. In summary, our results show that using the Tg–CBS, it is possible to obtain healthy adult animals with extremely elevated thcy.

**Discussion**

Despite the identification of plasma homocysteine as an independent risk factor for vascular disease and of CBS as a key enzyme in homocysteine catabolism, the in vivo feasibility of reducing thcy by elevating CBS activity was unknown. On the basis of the enzymatic kinetics of CBS and the other enzymes in the pathway, it was unclear that CBS activity would be rate limiting for the clearance of homocysteine. The K<sub>m</sub> of CBS for homocysteine is estimated to be ~5 mmol/L, whereas the K<sub>m</sub> for both methionine synthase

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**Figure 4.** Thiol-containing compounds in mouse serum from mice on a high-methionine, low-folate diet. Transgenic animals (n=12) and littermate control animals (n=16) were fed a high-methionine, low-folate diet for 4 weeks with ZnSO<sub>4</sub> water, and then serum was collected and analyzed. Eight of the transgenic mice are derived from founder 25, 1 from founder 21, and 3 from founder 2 after at least 3 generations of back-crossing. The error bars indicate the 95% confidence interval for each measurement. Brackets indicate significant differences between the transgenic and nontransgenic animals. C-G, cysteinyl-glycine.
and betaine-dependent homocysteine methyltransferase are in the low micromolar range. This has led to the view that CBS activity was used only in situations in which there was “homocysteine” overload, such as immediately after a methionine-loaded meal. However, we observed that overexpression of CBS lowered plasma homocysteine effectively when animals were maintained on a standard diet. This suggests that even under normal dietary conditions, methionine synthase and betaine-dependent homocysteine methyltransferase are not able to convert homocysteine to methionine fast enough to prevent a substantial buildup of homocysteine to the point at which it could be used by CBS as a substrate. The fact that the percentage lowering was worse in the high methionine diet suggests that under extreme conditions, it may be possible to saturate the transsulfuration pathway such that overexpression of CBS is less effective.

Our results also bear on the question of the relative importance of the liver and kidney in the control of plasma homocysteine levels. Unexpectedly, we found that the endogenous mouse CBS gene is induced in the kidney (but not in the liver) with exposure to zinc. However, from our serum homocysteine data, it is clear that nontransgenic animals did not have any difference in their plasma homocysteine levels when exposed to zinc (Figure 3A). This implies that the kidney is not a key player in control of plasma homocysteine levels in the mouse. If this were true as well in humans, then the hyperhomocysteinemia observed in humans with end-stage renal disease may not be caused by the failure of the kidney to metabolize plasma homocysteine but may be attributable to an indirect effect on homocysteine metabolism in the liver.

Other than the above-described metabolic consequences, we did not observe any other differences between control and Tg–CBS mice. The mice exhibited normal growth, normal behavior, and had normal-looking pups. These findings indicate that elevated CBS activity in the liver is essentially benign and suggests that treatments that activate CBS activity could potentially be useful in lowering plasma homocysteine in human patients. One possibility is that gene therapy could be used to deliver either extra copies of CBS gene or CBS protein to the liver. Although in theory a powerful approach, the technical hurdles are still great. A potentially more feasible strategy would be to use small-molecule drugs that could elevate activity of existing CBS protein in the liver. We have shown previously that the C-terminal domain of CBS acts as a negative regulatory domain (ie, deletion of the C terminus results in a protein with significantly elevated CBS activity). It should be possible to design or screen for drugs that bind to this domain in such a way as to cause activation of endogenous CBS. Because we were able to achieve reduction of serum homocysteine levels with a relatively modest 2.2-fold increase in CBS activity, it seems possible that such effects might be possible to achieve by targeting compounds to the regulatory domain. Such compounds could be useful in the treatment of hyperhomocysteinemia in

Figure 5. tHcy of Tg-CBS Cbs<sup>tm1Unc</sup>/ Cbs<sup>tm1Unc</sup> animals with (Zn) and without (−Zn) zinc. Tg-CBS Cbs<sup>tm1Unc</sup>/ Cbs<sup>tm1Unc</sup> animals between 2 and 8 months of age on ZnSO<sub>4</sub> water had serum collected and were then shifted to regular water for 2 weeks, at which time serum was collected again. All of the animals were derived from founder 25. The squares connected by the filled in lines represent the male animals, whereas the circles connected by the dotted lines represent the female animals.
populations, such as end-stage renal disease patients, that are resistant to homocysteine lowering by B-complex vitamin therapy.16

We also demonstrated that expression of human CBS can functionally complement deletion of mouse Cbs. By crossing in the transgene, we were able to obtain normal-sized, healthy-looking Cbs deletion animals that lived well into adulthood. Plasma homocysteine on these animals was somewhat elevated on average, but approximately one-third of the animals had homocysteine levels that were within the normal range. Thus, we were able to rescue the neonatal lethality defect and at least partially rescue the severe homocysteinemia with the transgene. When zinc was removed, we were able to obtain extremely elevated plasma homocysteine levels as high as that observed in the surviving Cbs homozygotes described by Watanabe et al17 The ability to produce healthy adult animals whose tHcy levels can be modulated by zinc should be extremely useful to investigators interested in the pathophysiology of tHcy.

Acknowledgments

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References


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