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Lack of global epigenetic methylation defects in CBS deficient mice

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Abstract

Cystathionine β -synthase (CBS) deficiency is a recessive inborn error of metabolism in which patients have extremely elevated plasma total homocysteine and have clinical manifestations in the vascular, visual, skeletal, and nervous systems. Homocysteine is an intermediary metabolite produced from the hydrolysis of S-adenosylhomocysteine (SAH), which is a by-product of methylation reactions involving the methyl-donor S-adenosylmethionine (SAM). Here, we have measured SAM, SAH, DNA and histone methylation status in an inducible mouse model of CBS deficiency to test the hypothesis that homocysteine-related phenotypes are caused by inhibition of methylation due to elevated SAH and reduced SAM/SAH ratio. We found that mice lacking CBS have elevated cellular SAH and reduced SAM/SAH ratios in both liver and kidney, but this was not associated with alterations in the level of 5-methylcytosine or various histone modifications. Using methylated DNA immunoprecipitation in combination with microarray, we found that of the 241 most differentially methylated promoter probes, 89% were actually hypermethylated in CBS deficient mice. In addition, we did not find that changes in DNA methylation correlated well with changes in RNA expression in the livers of induced and uninduced CBS mice. Our data indicates that reduction in the SAM/SAH ratio, due to loss of CBS activity, does not result in overall hypomethylation of either DNA or histones.

Introduction

Homocysteine (Hcy) is a non-protein incorporated amino acid that is part of the methionine-recycling pathway (Fig. 1a). Individuals lacking cystathionine β -synthase (CBS) have

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Compliance with Ethics Guidelines

Hyung-Ok Lee, Liqun Wang, Yin-Ming Kuo, Sapna Gupta, Michael J. Slifker, Yue-sheng Li, Andy J. Andrews, and Warren D. Kruger all declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

No human subjects were used in these studies.

Author contributions:

Experimental Design and planning: HOL, WDK, AJA

Experimental conduct: HOK, LW, YMK, SG, YSL

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Manuscript preparation: WDK, HOL

classical homocystinuria, which is characterized by plasma total Hcy (tHcy) levels 10–30 times greater than normal (tHcy 5~15μM). Untreated homocystinuric patients have a variety of clinical problems including a greatly increased risk of thrombosis, osteoporosis, dislocated lenses, and or intellectual disabilities (Mudd et al 2001).

To understand the biology behind tHcy toxicity, our lab has created two different mouse models of CBS deficiency. *Tg-hCBS Cbs^{-/-}* mice lack the endogenous mouse *Cbs* gene and have a wild-type human *CBS*-encoding cDNA under control of the zinc-inducible metallothionein promoter (Wang et al 2004). *Tg-I278T Cbs^{-/-}* are similar, but express a cDNA encoding for the patient-derived I278T mutation in the CBS gene (Wang et al 2005). Compared to control mice (tHcy 3~8 μM), adult *Tg-I278T Cbs^{-/-}* mice have tHcy ranging between 250–400 μM, while *Tg-hCBS Cbs^{-/-}* mice have tHcy between 150–200 μM when on regular water. *Tg-I278T Cbs^{-/-}* mice have a variety of well-characterized phenotypes including osteoporosis, decreased weight gain, low percent body fat, facial alopecia, and reduction of liver stearoyl-CoA desaturase (SCD-1) protein levels (Gupta et al 2009; Gupta and Kruger 2011). All of these phenotypes are lessened in *Tg-hCBS Cbs^{-/-}* mice (Gupta et al 2009).

One hypothesis to explain the phenotypes associated with elevated tHcy is the so-called “methylation hypothesis” (Fig. 1b). This hypothesis has three key elements. First, elevated serum tHcy is reflective of elevated tissue levels of SAH (Gupta et al 2008). Second, elevated SAH will cause reduced levels of DNA and protein methyltransferase activity since SAH is a competitive inhibitor with the substrate SAM (Bacolla et al 1999; Chin et al 2006). Lastly, the methylation status of DNA and histone-proteins constitute epigenetic marks that modulate gene expression (Cheng, 2010 #2522). Thus, SAH-induced hypomethylation of DNA and histones may result in altered gene expression. Although many studies have been performed examining this idea, the results to date have been confusing and inconsistent (Devlin et al 2005; Sulistyoningrum et al 2012; Glier et al 2013; Mandaviya et al 2014). Some of these inconsistencies may stem from the fact that the studies have used a variety of dietary and genetic approaches that vary not only tHcy, but also other key metabolites, such as methionine and B-vitamins. In addition, the levels of induced HHcy in most studies are quite modest. *Tg-hCBS Cbs^{-/-}* and *Tg-I278T Cbs^{-/-}* mice do not require any dietary manipulation and have much higher levels of tHcy than dietary models, and therefore may be a useful model to rigorously test the methylation hypothesis.

In previous work (Esse et al., 2014), we examined SAM/SAH ratio, arginine, and global DNA methylation status in the tissues of *Tg-I278T Cbs^{-/-}* mice. SAM/SAH ratio is thought to be an important predictor of cellular methylation capacity (Caudill et al 2001). Despite robust reductions in SAM/SAH ratios in liver, brain, heart or kidney, decreases in the concentration of protein-incorporated asymmetric and symmetric dimethylarginine (but not monomethylarginine) were observed only in liver and brain (Esse et al 2014). Moreover, the levels of non-protein incorporated asymmetric dimethylarginine and monomethylarginine in serum was actually elevated in *Tg-I278T Cbs^{-/-}* mice. Finally, no difference was observed in global DNA methylation status. These results suggest that although severe HHcy does affect SAM/SAH ratio, this does not translate into a global reduction of protein arginine or DNA methylation in *Tg-I278T Cbs^{-/-}* mice.

One significant concern in the above experiments is that *Tg-I278T Cbs*^{-/-} mice have elevated tHcy throughout their early development and this may have resulted in selection for some sort of compensatory physiologic or epigenetic changes to maintain high levels of DNA and protein methylation in spite of having low SAM/SAH ratios. In the work described here, we address this possibility by utilizing *Tg-hCBS Cbs*^{-/-} mice, in which severe HHcy can be induced in a temporally restricted window by withdrawing zinc from the drinking water. The results reported below support the conclusion that reduction in the SAM/SAH ratio, due to CBS deficiency, does not cause global hypomethylation of DNA or histones, and is probably not the major mechanism behind homocysteine toxicity.

Materials and Methods

Mouse model

All animal studies were reviewed and approved by the Fox Chase Cancer Center Institutional Animal Care and Review Board. *Tg-hCBS Cbs*^{-/-} mice were generated as previously described (Wang et al 2004). For transgene induction, the mice were given 25 mM ZnSO₄ dissolved in their drinking water. At the termination of the experiments, mice were euthanized and their tissues and serum were collected and stored at -70 °C for further analysis.

A cohort of *Tg-hCBS Cbs*^{-/-} mice, consisting of six sibling pairs, were generated and kept on zinc-water to induce transgene expression until they were 16 weeks of age. At this time, one animal from each sibling pair was taken off zinc (-Zn), while the other sibling remained on zinc (+Zn). After two additional weeks, all the animals were then euthanized and their tissues were collected.

Western blotting and serum amino acid measurements

Tissue homogenates and CBS Western blotting were performed as previously described (Gupta et al 2008). Blots were probed with rabbit anti-hCBS serum (1:10,000) and anti-actin (1:2000, Sigma). Serum tHcy and methionine levels were measured using the Biochrom 30 amino acid analyzer as performed previously (Gupta et al 2009).

Measurement of tissue SAM and SAH

For SAM and SAH quantitative measurements, ²H₃-SAM (CDN Isotopes) and ¹³C₁₀-SAH (Cambridge Isotope Laboratories) were chosen as the recovery standards, which were spiked in tissue homogenates before the initiation of solid phase extraction (SPE) as previously described (Esse et al 2014). An extracted sample was then analyzed by ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS), as described in detail previously (Struys et al 2000). Quantitative analysis was performed on a Thermo triple quadrupole mass spectrometer (TSQ Quantum Access) using instrument parameters as described previously (Kuo and Andrews 2013), and the data of individual resolved peaks of samples were processed using Xcalibur, version 2.1 (Thermo).

Global DNA methylation status in tissues

Genomic DNA was purified using either the Gentra Puregene Tissue kit (Qiagen) for liver and kidney, or the QiAmp Genomic DNA Kit (Qiagen) for bone marrow, following the manufacturer's instruction. The percentage of 5-methyldeoxycytidine (% MdC) present in tissue genomic DNA was determined by stable-isotope dilution LC-MS/MS, as described previously (Quinlivan and Gregory 2008).

Histone modification

Histones were extracted from 100 mg of liver tissue using the EpiQuik Histone Extraction Kit (EpiGentek) following the manufacturer's protocol. Western blotting was performed for the analysis of asymmetric dimethylation of arginine 3 on histone H4 and total H4 probed with anti-H4R3me2a (1:800, Active Motif) and anti-H4 (1:1000, Abcam), respectively. For measuring histone H3 modification in the liver tissue, we used the EpiQuik H3 modification Multiplex Assay (EpiGentek) following the manufacturer's instructions.

Methylated DNA immunoprecipitation array

Methylated regions of the genome from liver genomic DNA were isolated and labeled as described in the Agilent Microarray Analysis of Methylated DNA Immunoprecipitation protocol (http://www.agilent.com/cs/library/usermanuals/public/G4170-90012_Methylation_2.3.pdf). The labeled DNAs were then hybridized onto the Agilent mouse CpG Island 2×105k microarray (Agilent G4811A). Data was obtained using an Agilent scanner at 5 µm resolution, followed by processing using the Agilent Feature Extraction (v9.5) software.

Examination of the distribution of the raw probe signals and analysis of background revealed that one of the samples was a clear outlier, so this sample and its sibling control were dropped from the analysis (mice pair #2). In addition, probes were removed from the analysis if called absent in less than four of the five remaining pairs or flagged as poor quality in any of the arrays. For the remaining samples, data was log₂ transformed, and the difference between the Cy3 and Cy5 samples was determined. Differential methylation was then assessed using the limma package in Bioconductor. A list of differentially methylated promoter probes between -Zn and +Zn mice was selected on the basis of the probe being located in the promoter and a P<0.01. This list was then used for Gene Ontology Analysis using Web-Gestalt (Wang et al 2013).

RNA expression microarray

RNA was extracted using TRIzol Reagent (Invitrogen) followed by cleaning of contaminating DNA using the RNeasy Mini Kit (Qiagen). Quality and quantity of RNA were determined using an Agilent 2100 bioanalyzer (Agilent Technologies). Agilent 4×44k expression arrays (Agilent G4122F) were used and RNA was labeled with Cy3 fluorescent dye. The microarray images were quantified and preprocessed using Agilent's feature extraction software. Median signal intensities were quantile-normalized and log₂-transformed, and only probes with no absent values (based on Agilent Feature Extraction flags) in at least one condition (+Zn or -Zn) were retained. Differential expression was assessed using limma package in Bioconductor. A list of differentially regulated genes

between $-Zn$ and $+Zn$ mice was selected on the basis of $P < 0.01$. This list was then used for functional pathway analysis using WebGestalt (Wang et al 2013).

Statistic analysis

Values in text are mean + SEM. Differences between two groups were analyzed by the Mann-Whitney U test (unpaired and non-parametric test). Significance between more than two groups was determined using One-way ANOVA followed by Tukey's multiple comparison tests employing GraphPad Prism 6.0 software. Statistical significance was accepted at the value of $P < 0.05$.

Results

Loss of CBS elevates serum tHcy, tissue SAM and SAH and reduces SAM/SAH ratio

A cohort of *Tg-hCBS Cbs*^{-/-} mice was kept on zinc-water to induce transgene expression until they were 16 weeks of age. Then each sibling pair was off ($-Zn$) or on zinc ($+Zn$) for two additional weeks. Examination of hCBS expression in the liver revealed that all $+Zn$ animals had robust expression, whereas, $-Zn$ animals had greatly reduced levels (Fig. 2a). This resulted in the mean serum tHcy rising from 12.8 μM to 158 μM ($P < 0.002$), and an elevation of methionine from 54 to 76 μM , although this did not quite achieve statistical significance ($P < 0.093$) (Fig. 2b).

We determined SAM and SAH concentrations in liver and kidney tissue (Fig. 3). In the liver, SAM increased 72% from 55.8 to 96.1 nmoles/g ($P < 0.002$), SAH increased 276% from 45.2 to 170 nmoles/g ($P < 0.002$), and the SAM/SAH ratio changed from 1.7 to 0.6 ($P < 0.009$) in $-Zn$ vs. $+Zn$ mice. In the kidney, SAM increased 65% from 40.3 to 66.7 nmoles/g ($P < 0.002$), SAH increased 376% from 8.1 to 24.2 nmoles/g ($P < 0.002$), and the SAM/SAH ratio changed from 5.0 to 2.8 ($P < 0.065$).

We also examined the correlation between tHcy and tissue SAM, SAH, and SAM/SAH ratios in individual mice (Supplementary Fig. 1). In both liver and kidney, we observed strong positive correlations between tHcy and both SAM and SAH, and a negative correlation between tHcy and SAM/SAH ratio.

Loss of CBS does not affect global levels of DNA or histone methylation

We determined the percentage of 5-MdC in DNA derived from liver, kidney, and bone marrow. Bone marrow was included because it is a fast dividing tissue, potentially requiring higher levels of DNA methyltransferase activity. The mean percentage 5-MdC was significantly different in each of the three tissues (Fig. 4a), but was not different between $+Zn$ and $-Zn$ animals for any of the tissues (Fig. 4b).

We also examined the levels of 21 specific histone modifications including 15 different methylation modifications, 4 acetylations, and 2 phosphorylations in the livers of $+Zn$ and $-Zn$ treated animals using ELISA technology. We observed no statistically significant differences in any of the modifications (Fig. 5a). An additional assessed modification was the presence of asymmetric dimethyl-arginine on histone H4 arginine residue 3 (H4R3me2a) (Fig. 5b). This modification was previously reported to have been significantly down-

regulated in *Tg-I278T Cbs*^{-/-} mice versus controls (Esse et al 2014). Here, we found no significant difference in the overall level of this modification, but we did observe that the levels of this modification seem to vary greatly between livers of different animals. We suspect that the earlier report of differences was simply a statistical artifact due to the small number of samples that were examined. Overall, these experiments show that there are no major global changes in the levels of various histone modifications associated with altered SAM/SAH ratios.

Gene Specific DNA methylation shows an excess of hypermethylated genes in hyperhomocysteinemic mice

Despite our inability to detect any overall DNA methylation levels, it still might be possible that there is a subset of DNA sites whose methylation status is altered by changes in tHcy. To examine this possibility, we measured gene specific DNA methylation in the liver using methylated DNA immunoprecipitation followed by hybridization to a DNA microarray (MeDIP-Chip) containing 88,678 CpG probes. For each sample, the relative amount of methylated DNA for each locus was quantified by comparing the signal of the Cy3 labeled immunoprecipitated DNA to the Cy5 labeled total DNA in a dual hybridization format. The log transformed data were analyzed by comparing the log₂ fold-change (LogFC) between +Zn to -Zn animals. A positive LogFC indicates that the fragment is hypomethylated in -Zn treated mice, while negative LogFC indicates hypermethylation in -Zn treated, hyperhomocysteinemic animals.

An examination of the distribution of the LogFC for each of the probes reveals that the mean LogFC is -0.038 and the median is -0.012. The deviance from 0 (the expected mean if there was no difference in methylation) is driven by the excess of probes in the negative (hypermethylated) tail (Supplementary Figs. 2a and 2b). To examine this more closely, we selected differentially methylated promoter probes using the criteria of a P<0.01. We found 215 out of 241 probes (89 %) had negative LogFC values (Supplemental Table 1), indicating that most were actually more methylated in -Zn treated animals, precisely the opposite of our initial hypothesis. This list was also examined for pathway enrichment using the WebGestalt tool including the KEGG and Gene Ontology databases. This tool gives both raw and adjusted P-values and takes into account all of the genes on the chip. The most significant enrichment probes were involved in process of transcription or DNA binding (Supplemental Table 2). There were no significant enrichments identified in the KEGG database.

We also performed global RNA expression analysis on liver. For this analysis, we selected, as differentially expressed, a group of 406 probes (P<0.01) that mapped to 386 unique genes (Supplemental Table 3). Gene Ontology analysis indicated that this set was enriched for genes involved in small molecule metabolic processes, particularly processes involving organic acids (Supplemental Table 4). Interestingly, we did not observe any overlap between the groups of genes identified in the methylation and RNA expression analysis.

We next examined the relationship between methylation differences and mRNA levels in the most differentially methylated gene set. First, we compared the LogFC RNA expression versus the LogFC DNA methylation for each of the 241 promoter probes described earlier

(Supplementary Fig. 2c). While we did observe a weak negative correlation between differential expression and promoter methylation differences, it was not very strong and was almost entirely driven by three outlier points. We also performed the inverse analysis, examining DNA methylation in genes that were selected because they were differentially expressed at the RNA level. For these studies, we identified the 20 most differentially regulated transcripts that had probes present on both the RNA and DNA microarrays. These 20 genes had a total of 154 promoter methylation probes associated with them. We did not observe any significant relationship between the LogFC of the DNA with that of the RNA for this gene set (Supplementary Fig. 2d). Taken together, these results do not show that there is much of a correlation between changes in DNA promoter methylation and RNA expression in the liver of mice with inducible hyperhomocysteinemia.

Discussion

In this study, we used a zinc-regulated CBS transgenic mouse model to explore the hypothesis that severe hyperhomocysteinemia may affect epigenetic methylation by altering the SAM/SAH ratio. We found that loss of CBS expression in adult animals resulted in severe hyperhomocysteinemia in serum, large increases in liver and kidney SAH, and a significant reduction in SAM/SAH ratio. However, we did not observe any significant decrease in the amount of epigenetic associated methylations. This was shown by determining DNA methylation (global and specific) and histone specific methylation. No statistically significant differences were found either in the percentage of 5-MdC in DNA in either liver, kidney and bone marrow. In liver, there were no significant differences in the levels of several histone methylations. The examining of specific DNA methylation using MeDIP-Chip indicated that there was actually an increase in the number of hypermethylated DNA probes in the CBS deficient mice. In addition, we found no correlation between CBS-induced differences in promoter methylation and RNA expression. Our data does not support the view that CBS deficiency and hyperhomocysteinemia-induced hypomethylation promote pathology via epigenetic methylation.

The strongest part of this study involves the analysis of DNA methylation. Global DNA methylation was determined by hydrolyzing DNA down to individual bases and then calculating the ratio of 5-MdC to unmethylated dC. This assay is extremely robust and has a very low coefficient of variation, making it highly likely that we could detect a 5–10% difference in 5-MdC content. In addition, we performed genome-wide methylation analysis using MeDIP-Chip. This procedure did reveal a slight difference in the methylation pattern between –Zn and +Zn treated animals, with the –Zn treated animals having an increase in the number of probes that were hypermethylated. This finding is actually the opposite of what was predicted as –Zn animals have elevated serum tHcy and decreased SAM/SAH ratios. Thus, there is no indication that altered SAM/SAH ratios are inhibiting DNA methylation.

Our inability to find evidence of hypomethylation, despite altered SAH and SAM/SAH ratios, was unexpected. Because the chemical structure of S-adenosylmethionine and S-adenosylhomocysteine are nearly identical, SAH can bind to the active site of methyltransferase enzymes that utilize SAM as a substrate and act as a potent competitive

inhibitor of those enzymes. Kinetic studies have shown that many DNA and protein methyltransferases bind SAH as well, or even slightly tighter than SAM (Lee et al 1977; Flynn and Reich 1998; Yokochi and Robertson 2002; Patnaik et al 2004; Chin et al 2006). Recently, Barroso et al. demonstrated that addition of a SAH hydrolase inhibitor to human coronary artery endothelial cells resulted in a significant increase of SAH, a decrease of E2H2 methyltransferase, and a reduction of H3K27me3 methylation (Barroso et al 2016). These findings suggest that SAH accumulation can affect epigenetic regulation. Even stronger evidence from comes genetic studies in *Arabidopsis*. In a genetic screen designed to find mutations that reduce cytosine methylation and silencing of the *Pai2* gene, Bender and colleagues identified a mutation that was shown to be a hypomorphic allele of the *Sah1* gene, which encodes SAH hydrolase (Mull et al 2006). In this same screen, they identified multiple alleles of *Cmt3* (cytosine methyltransferase) and *Suvh4* (histone methyltransferase), suggesting that elevated SAH can mimic loss of methyltransferase function.

Why were we unable to detect alterations in histone and DNA methylation levels? One possible explanation is that the differences in the SAM/SAH ratio were simply not great enough. Although SAH levels rose between 2.7 and 3.8-fold in liver and kidney, respectively, SAM levels also increased, resulting in only a 62% and 46% reduction in SAM/SAH ratio. Interestingly, mice homozygous for mutations in the methylene tetrahydrofolate reductase (*Mthfr*) gene, encoding a key enzyme in providing the methylfolate needed for the conversion of Hcy to methionine, have reduced SAM and elevated SAH levels and a 78–82% reduction in SAM/SAH in the liver, brain, testes and ovary (Chen et al 2001). These mice also show reduced global DNA methylation (Chen et al 2001) and some differences in sperm methylation (Chan et al 2010). In *Tg-I278T Cbs*^{-/-} mice, we observed greater reduction of SAM/SAH in brain and heart and a similar reduction in kidney, and yet, observed no difference in global DNA methylation (Esse et al 2014). Taken as a whole, these findings suggest that, *in vivo*, SAM/SAH ratio alone may not be as critical a factor in regulating DNA methylation levels as previously thought. A second possible explanation is that not only the ratio, but also the absolute levels of the substrate SAM or SAH might be important. This would make kinetic sense, especially if the SAM levels were below the K_m of the enzyme. This idea may help explain the phenotypic differences observed between homocysteine remethylation defects, like MTHFR deficiency, and CBS deficiency. It is tempting to speculate that the neurologic phenotypes seen in MTHFR deficient patients, but not CBS deficient patients, may be related specifically to DNA methylation effects.

In summary, the data in this paper does not support the idea that the phenotypes associated with CBS deficiency are caused by alteration in the intracellular SAM/SAH ratio and inhibition of epigenetic methylation. Rather, the data suggests that the unique phenotypes observed in CBS deficiency are much more likely to be directly related to the effects of high tHcy itself. The precise mechanism by which tHcy is toxic remains to be elucidated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Methylation inhibition hypothesis. (a) Methionine recycling pathway in wild-type tissue. Met=methionine, SAM=S-adenosylmethionine, MT=methyl transferase enzyme, CH₃-DNA=methylated DNA, CH₃-Histone=histone methylated at either lysine or arginine residues, SAH=S-adenosylhomocysteine, Hcy=homocysteine, Cysta=cystathionine. (b) Predicted changes in key metabolites in CBS deficient mouse.

Fig. 2.
Zinc-regulated induction of hyperhomocysteinemia. (a) Western of CBS protein in zinc-treated and non-zinc treated animals. (b) Serum tHcy and serum Met in same animals.

Fig. 3.
Liver and kidney SAM, SAH, and SAM/SAH ratios. Bars show mean and SEM.

Fig. 4. 5-methyldeoxycytidine (5-MdC) content of tissues. (a) 5-MdC content of liver, kidney, and bone marrow of all twelve animals. (b) Stratification of each tissue by zinc treatment. Bars show mean and SEM.

Fig. 5.
Histone methylation and modifications in liver. (a) Indicated histone H3 modifications were assessed by Elisa assay using the Epiquick Histone Modification Multiplex Assay kit. Error bars show SEM. (b) Western blot of liver nuclear extracts using an antibody specific for the H4R3me2a. Total H4 levels on same blot are shown below. Bar graph shows quantitation of western.