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Homocysteinemia in Mice with Genetic Betaine Homocysteine *S*-Methyltransferase Deficiency Is Independent of Dietary Folate Intake[1–3]

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Abstract

Elevated homocysteine (Hcy) concentrations are associated with increased risk of several chronic diseases. Hcy can be removed by methylating it to form methionine via either the betaine homocysteine *S*-methyltransferase (BHMT) or the methionine synthase (MS) pathway. BHMT uses betaine as the methyl donor, whereas MS uses 5-methyltetrahydrofolate. We previously found that mice with the gene encoding *Bhmt* deleted ($Bhmt^{-/-}$) had altered Hcy metabolites in tissues. This study aimed to determine whether folate supplementation of $Bhmt^{-/-}$ mice reverses, and folate deficiency exacerbates, these metabolic changes. $Bhmt^{-/-}$ mice and their littermates ($Bhmt^{+/+}$ mice) were fed a folate-deficient (FD; 0 mg/kg diet), a folate control (FC; 2 mg/kg diet), or a folate-supplemented (FS; 20 mg/kg diet) diet for 4 wk. $Bhmt^{-/-}$ mice had higher plasma Hcy and hepatic *S*-adenosylhomocysteine (AdoHcy) concentrations and had lower hepatic *S*-adenosylmethionine (AdoMet) concentrations compared with $Bhmt^{+/+}$ mice for all diets. Although the FD diet increased plasma Hcy ($P < 0.05$) and hepatic AdoHcy ($P < 0.001$) concentrations in $Bhmt^{+/+}$ mice compared with FC and FS mice, the FD diet had no effect on the metabolites measured in $Bhmt^{-/-}$ mice. The FS diet did not ameliorate elevated plasma Hcy and elevated hepatic AdoHcy concentrations but did increase hepatic AdoMet concentrations in $Bhmt^{-/-}$ mice ($P < 0.001$) compared with FD and FC mice. We conclude that the BHMT pathway is a major route for the elimination of Hcy in mice and that the MS pathway has little excess capacity to methylate the Hcy that accumulates when the BHMT pathway is blocked. J. Nutr. 142: 1964–1967, 2012.

Introduction

Homocysteine (Hcy)[9] is an amino acid that is not used for protein synthesis. Plasma concentrations of Hcy are positively correlated with increased risk of cardiovascular disease, birth defects, Alzheimer disease, bone weakness, and renal dysfunction (1,2). Plasma Hcy concentrations of 5 to 15 μmol/L are considered to be normal in humans, whereas individuals with concentrations of 15 to 100 μmol/L are moderately hyperhomocysteinemic and those with concentrations >100 μmol/L are considered to be severely hyperhomocysteinemic (1,2). Normal plasma Hcy concentrations are maintained by converting Hcy to cysteine by action of the enzyme cystathionine β synthase (CBS; EC 4.2.1.22) (**Supplemental Fig. 1**) or by methylating Hcy to form methionine in reactions that can be accomplished by 1 of the 3 separate enzymatic pathways: methionine synthase (MS; EC 2.1.1.13) methylates Hcy by using 5-methyltetrahydrofolate as the methyl donor and vitamin B-12 as a cofactor; betaine homocysteine *S*-methyltransferase (BHMT; EC 2.1.1.5) methylates Hcy by using betaine as the methyl donor; and BHMT2 (EC 2.1.1.5) methylates Hcy by using *S*-methylmethionine as the methyl donor. The methionine formed from Hcy serves as the precursor of *S*-adenosylmethionine (AdoMet), the principal biological methyl donor for numerous methylations, including the methylation of DNA, histones, and phospholipids. When it donates its methyl group, AdoMet is converted to *S*-adenosylhomocysteine (AdoHcy), and AdoHcy can be recycled to Hcy. Perturbations of these pathways, either by genetic or nongenetic factors (such as diet and drugs), may result in increased Hcy concentrations.

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[9] Abbreviations used: AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; BHMT, betaine homocysteine *S*-methyltransferase; CBS, cystathionine β synthase; FC, folate control; FD, folate-deficient; FS, folate-supplemented; Hcy, homocysteine; MS, methionine synthase.
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Our laboratory previously reported that mice with the *Bhmt* gene deleted ($Bhmt^{-/-}$ mice) had absent BHMT activity and increased concentrations of Hcy in the liver (by 6-fold; $P < 0.01$) and in plasma (by 8-fold; $P < 0.001$) compared with their littermate controls (3). In addition, *Bhmt* deletion resulted in decreased concentrations of AdoMet (by 40%; $P < 0.01$) and increased concentrations of AdoHcy (by 3-fold; $P < 0.01$) in liver (3). These data suggest that the methyl-folate–mediated methylation of homocysteine cannot maintain normal Hcy concentrations in the absence of *Bhmt*.

We hypothesized that $Bhmt^{-/-}$ mice would be dependent on the folate pathway for remethylation of Hcy, and that they would have higher Hcy concentrations when made folate deficient. Conversely, if MS activity is normally limited by the availability of folate, supplementation with folate would lower Hcy concentrations in $Bhmt^{-/-}$ mice.

Materials and Methods

Animals and diets. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. $Bhmt^{-/-}$ mice were generated as described previously (3). Heterozygous breeding pairs were used to generate $Bhmt^{-/-}$ mice and their wild-type littermates ($Bhmt^{+/+}$ mice) for all experiments. *Bhmt* genotypes were determined by a PCR-based method as described previously (3). Mice were housed in a temperature-controlled environment at 24°C and exposed to a 12-h light/dark cycle. Six-week-old mice were given free access to water and to the assigned pelleted diets (Dyets) for 4 wk. Mice were assigned to 1 of 3 dietary groups: folate-deficient (FD; AIN-76A diet with 0.0 mg folic acid/kg diet, 1.1 g choline chloride/kg diet, and 1% succinyl sulfathiazole), folate control (FC; AIN-76A diet with 2 mg folic acid/kg diet, 1.1 g choline chloride/kg diet, and 1% succinyl sulfathiazole), or folate-supplemented (FS; AIN-76A diet with 20 mg folic acid/kg diet, 1.1 g choline chloride/kg diet, and 1% succinyl sulfathiazole) as previously described (4). Succinyl sulfathiazole was added to all diets to kill intestinal bacteria that are able to synthesize folate.

Tissue collection. On the last day of the study, mice were moved to clean cages and feed-deprived for 4 h before tissue collection. Mice were anesthetized by inhalation of isoflurane (Hospira). Body weight was assessed by using a scale. Blood was collected via cardiac puncture. Plasma was separated from blood by centrifugation at 3000 × *g* for 5 min at room temperature, frozen immediately in liquid nitrogen, and stored at −80°C until analysis. Livers were harvested, weighed, snap-frozen in liquid nitrogen, and stored at −80°C until used.

Plasma and hepatic metabolites. Plasma total folate concentrations were measured by using a microbiological assay (5). Plasma total Hcy concentrations were measured by using an HPLC method (6). Cysteamine (10 μmol/L) was used as an internal standard to correct for recovery. The concentrations of hepatic AdoMet and AdoHcy were measured by using an HPLC method as previously described (7,8).

Statistical analysis. Values are presented as means ± SEM. Diet × genotype effects were analyzed by 2-way ANOVA. Differences between the 3 dietary groups within the same genotype were determined by using 1-way ANOVA and Tukey-Kramer's honestly significant difference test. Differences between the 2 genotype groups fed the same diet were determined by using Student's *t* test. JMP version 9.0 (SAS Institute) was used to perform all statistical analysis.

Results

Body and liver weights. $Bhmt^{+/+}$ and $Bhmt^{-/-}$ mice appeared grossly normal on all 3 experimental diets. After 4 wk of consuming the various folate diets, no significant difference was observed in the body weights of mice either by genotype or by diet. The liver weights of both $Bhmt^{+/+}$ and $Bhmt^{-/-}$ mice were not affected by diets but were affected by genotype. $Bhmt^{-/-}$ mice had livers 34% and 30% heavier than those of $Bhmt^{+/+}$ mice fed the FD diet ($P < 0.001$) and the FS diet, respectively ($P < 0.05$) (**Table 1**). The heavier livers observed in the $Bhmt^{-/-}$ mice were consistent with our previous findings (3).

TABLE 1 Body and liver weights of $Bhmt^{+/+}$ and $Bhmt^{-/-}$ mice fed the FD, FC, or FS diet for 4 wk[1]

Genotype	Diet	Body weight	Liver weight
		g	*g*
$Bhmt^{+/+}$	FD	22.3 ± 1.38	0.92 ± 0.05
$Bhmt^{+/+}$	FC	22.7 ± 1.38	1.10 ± 0.09
$Bhmt^{+/+}$	FS	22.3 ± 1.26	0.96 ± 0.05
$Bhmt^{-/-}$	FD	21.0 ± 1.15	1.23 ± 0.06**
$Bhmt^{-/-}$	FC	21.7 ± 1.89	1.30 ± 0.09
$Bhmt^{-/-}$	FS	19.5 ± 1.52	1.23 ± 0.08*
P value			
D		0.65	0.21
G		0.14	<0.0001
D × G		0.78	0.73

[1] Values are means ± SEM, $n = 8$ or 9. Asterisks indicate different from $Bhmt^{+/+}$ fed the same diet: $*P < 0.05$, $**P < 0.01$. D, diet; FC, folate control; FD, folate-deficient; FS, folate-supplemented; G, genotype.

Plasma folate. To confirm that the mice were fed the experimental diets, we measured plasma total folate concentrations. $Bhmt^{+/+}$ and $Bhmt^{-/-}$ mice fed the FS diet had increased plasma folate concentrations that were 40% ($P < 0.001$) and 60% ($P < 0.001$) greater, respectively, compared with those fed the FC diet (**Fig. 1**). $Bhmt^{+/+}$ and $Bhmt^{-/-}$ mice fed the FD diet had plasma folate concentrations that were 84% ($P < 0.001$) and 95% ($P < 0.001$) less, respectively, compared with those fed the FC diet. $Bhmt^{-/-}$ mice fed the FD diet had lower plasma folate concentrations than did $Bhmt^{+/+}$ mice fed the same diet ($P < 0.05$). There was no interaction between diet and genotype for plasma folate concentration.

Plasma homocysteine and cysteine. Mean plasma Hcy concentration in the $Bhmt^{+/+}$ mice fed the FC diet was 4.61 ± 0.36 μmol/L, which is similar to published values (9) (**Fig. 2**). $Bhmt^{-/-}$ mice fed the FC diet had 8.4-fold greater plasma Hcy

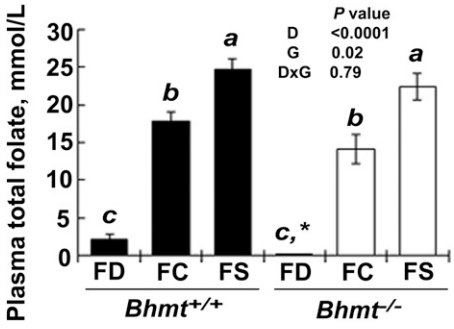

FIGURE 1 Plasma total folate concentrations of $Bhmt^{+/+}$ and $Bhmt^{-/-}$ mice fed the FD, FC, or FS diet for 4 wk. Values are means ± SEM, $n = 8$–10. Means with a genotype without a common letter differ, $P < 0.001$. *Different from $Bhmt^{+/+}$ mice fed the same diet, $P < 0.05$. D, diet; FC, folate control; FD, folate-deficient; FS, folate-supplemented; G, genotype.

concentrations than did *Bhmt*$^{+/+}$ mice fed the same diet ($P <$ 0.001), which is consistent with our previous findings (3). Four weeks of consuming the FS diet did not result in altered plasma Hcy concentrations in either *Bhmt*$^{+/+}$ or *Bhmt*$^{-/-}$ mice compared with those fed the FC diet. With this supplemented diet, *Bhmt*$^{-/-}$ mice had 10.6-fold greater plasma Hcy concentrations than did *Bhmt*$^{+/+}$ mice ($P < 0.0001$). Four weeks of consuming the FD diet resulted in a significant increase in plasma Hcy concentrations in *Bhmt*$^{+/+}$ mice ($P < 0.05$) but not in *Bhmt*$^{-/-}$ mice compared with mice fed the FC diet. With this deficient diet, *Bhmt*$^{-/-}$ mice had 7.6-fold greater plasma Hcy concentrations than did *Bhmt*$^{+/+}$ mice ($P < 0.0001$). Because homocysteine could also be removed by forming cysteine via the CBS pathway, we measured plasma cysteine. No significant difference was observed in plasma cysteine concentration by diet or by genotype (**Supplemental Fig. 2**). There was no interaction between diet and genotype for plasma Hcy or cysteine concentration.

Hepatic AdoMet and AdoHcy. Hepatic AdoHcy concentrations in mice were not affected by the different folate diets but were affected by genotype (**Fig. 3***A*). *Bhmt*$^{-/-}$ mice fed the FC diet had 2.7-fold greater hepatic AdoHcy concentrations than did *Bhmt*$^{+/+}$ mice fed the same diet ($P < 0.001$), which is consistent with our previous findings (3). Four weeks of consuming the FS diet or the FD diet did not change hepatic AdoHcy concentrations in either *Bhmt*$^{+/+}$ or Bhmt$^{-/-}$ mice compared with those fed the FC diet. *Bhmt*$^{-/-}$ mice had 2-fold ($P < 0.0001$) and 1.6-fold ($P < 0.0001$) greater hepatic AdoHcy concentrations than did *Bhmt*$^{+/+}$ mice when fed the FS diet or the FD diet, respectively.

Bhmt$^{-/-}$ mice fed the FC diet had hepatic AdoMet concentrations that were 54% less than those of *Bhmt*$^{+/+}$ mice fed the same diet ($P < 0.01$), which is consistent with our previous findings (3) (Fig. 3*B*). Four weeks of consuming the FS diet had no effect on hepatic AdoMet concentrations in *Bhmt*$^{+/+}$ mice compared with those fed the FC diet but increased hepatic AdoMet concentrations in *Bhmt*$^{-/-}$ mice by 30% ($P < 0.001$). With this supplemented diet, *Bhmt*$^{-/-}$ mice had hepatic AdoMet concentrations that were 36% less than those of *Bhmt*$^{+/+}$ mice ($P < 0.0001$). Four weeks of consuming the FD diet reduced hepatic AdoMet concentrations in *Bhmt*$^{+/+}$ mice by 37% ($P <$ 0.05) compared with those fed the FC diet but had no effect on hepatic AdoMet concentrations in *Bhmt*$^{-/-}$ mice. With this deficient diet, *Bhmt*$^{-/-}$ mice had hepatic AdoMet concentrations that were 43% less than those of *Bhmt*$^{+/+}$ mice ($P < 0.01$).

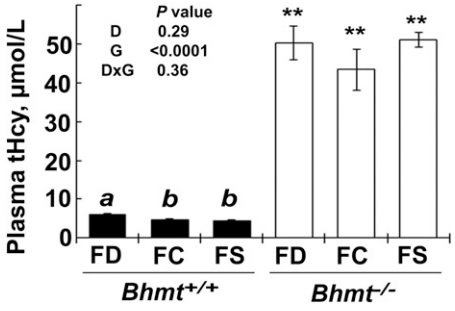

FIGURE 2 Plasma tHcy concentrations of *Bhmt*$^{+/+}$ and *Bhmt*$^{-/-}$ mice fed the FD, FC, or FS diet for 4 wk. Values are means ± SEM, n = 8–9. Means within a genotype without a common letter differ, $P <$ 0.05. **Different from *Bhmt*$^{+/+}$ mice fed the same diet, $P < 0.001$. D, diet; FC, folate control; FD, folate-deficient; FS, folate-supplemented; G, genotype; tHcy, total homocysteine.

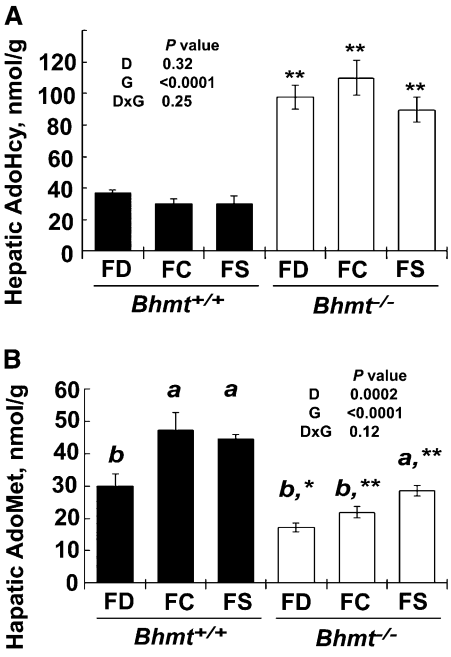

FIGURE 3 Hepatic AdoHcy (*A*) and AdoMet (*B*) concentrations of *Bhmt*$^{+/+}$ and *Bhmt*$^{-/-}$ mice fed the FD, FC, or FS diet for 4 wk. Values are means ± SEM, n = 8–9. Means within a genotype without a common letter differ, $P < 0.05$. Asterisks indicate different from *Bhmt*$^{+/+}$ mice fed the same diet:*$P < 0.05$, **$P < 0.001$. AdoHcy, adenosylhomocysteine; AdoMet, adenosylmethionine; D, diet; FC, folate control; FD, folate-deficient; FS, folate-supplemented; G, genotype.

Discussion

Normal plasma Hcy concentrations are maintained by the CBS, MS, or BHMT pathways (Supplemental Fig. 1). Perturbations of the CBS and MS pathways, either by single nucleotide polymorphisms in the genes or by deficiencies in nutrients involved, result in hyperhomocysteinemia in both humans and rodents (10,11). On the other hand, limited information is available on the BHMT pathway. It has been assumed that the CBS and the MS pathways have adequate capacity to handle any Hcy load and that the BHMT pathway provides excess capacity. We, and others, found that the role of BHMT in Hcy metabolism is important because *Bhmt*$^{-/-}$ mice had a substantial increase in plasma and hepatic Hcy concentrations (3,12). In addition, *Bhmt*$^{-/-}$ mice had a significant increase in hepatic AdoHcy and a decrease in hepatic AdoMet concentrations. Because the enzyme MS also methylates Hcy to methionine by using 5-methyltetrahydrofolate as the methyl donor, we asked whether dietary folate supplementation would ameliorate, and folate deficiency would exacerbate, these phenotypes presented in *Bhmt*$^{-/-}$ mice. There is a second pathway capable of remethylating Hcy to methionine by using *S*-methylmethionine as the methyl donor, catalyzed by the enzyme encoded for by *BHMT2*, but this enzyme uses *S*-methylmethionine as a substrate and this compound is not found in the purified diet used in this study. We did not see evidence of BHMT2 activity in this study.

Consistent with our previous findings, *Bhmt*$^{-/-}$ mice had heavier livers and higher plasma Hcy, higher hepatic AdoHcy, and lower hepatic AdoMet concentrations compared with those of *Bhmt*$^{+/+}$ mice fed a diet adequate in folate (3). Although folate deficiency resulted in elevated plasma Hcy and hepatic AdoHcy concentrations in *Bhmt*$^{+/+}$ mice, we found that the diminished

availability of dietary folate did not further exacerbate the high plasma Hcy, high hepatic AdoHcy, or low hepatic AdoMet concentrations observed in *Bhmt*$^{-/-}$ mice. The lack of effects of folate deficiency on these variables in *Bhmt*$^{-/-}$ mice suggests that MS does not make a substantial contribution toward methylating Hcy in these mice. Increased availability of folate did not ameliorate the high plasma Hcy and the high hepatic AdoHcy concentrations observed in *Bhmt*$^{-/-}$ mice. Once again, these data suggest that the MS pathway is not folate-limited in these mice and that there was not excess capacity in this pathway that could be used to make up for loss of BHMT-mediated methylation of Hcy. However, increased folate intake did increase the hepatic AdoMet concentrations in *Bhmt*$^{-/-}$ mice, but only to concentrations similar to those of *Bhmt*$^{+/+}$ mice on an FD diet. This may reflect some increased flux through MS pathway but could also be due to activation of methionine adenosyltransferase by AdoHcy.

Our findings show that BHMT-mediated methylation of Hcy is more important than previously appreciated. In mice, the BHMT pathway appears to be the predominant pathway for removal of Hcy, whereas the MS pathway does not appear to have excess capacity to make up for the loss of this pathway (even when mice are provided with excess folate). Mice and humans differ in the relative importance of several steps in Hcy metabolism: for example, mice use much more cysteine as a precursor for hair formation. Despite these differences, the knockout mouse provides a useful guide as to phenotypes that should be examined in humans. A number of single nucleotide polymorphisms in *BHMT* exist in humans, and we suggest that those that result in loss of BHMT activity could have a phenotype that includes hyperhomocysteinemia. Our current study in mice suggests that these individuals will not be responsive to folate supplementation.

Acknowledgments

Y.-W.T. and I.C. conducted all experiments equally; Y.-W.T. designed the experiments, analyzed the data, and wrote the manuscript; and S.H.Z. oversaw the overall design for the experiments, assisted with the preparation of the manuscript, and had primary responsibility for the final content. All authors read and approved the final manuscript.

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