Effects of Betaine in a Murine Model of Mild Cystathionine-β-Synthase Deficiency

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Cystathionine-β-synthase (CBS) is required for transsulfuration of homocysteine, an amino acid implicated in vascular disease. We studied homocysteine metabolism in mice with mild hyperhomocysteinemia due to a heterozygous disruption of the *Cbs* gene. Mice were fed diets supplemented with betaine or dimethylsulfonioacetate (DMSA); betaine and DMSA provide methyl groups for an alternate pathway of homocysteine metabolism, remethylation by betaine:homocysteine methyltransferase (BHMT). On control diets, heterozygous mice had 50% higher plasma homocysteine than did wild-type mice. Betaine and DMSA had similar effects in both genotype groups: liver betaine increased dramatically, while plasma homocysteine decreased by 40% to 50%. With increasing betaine supplementation, homocysteine decreased by 75%. Plasma homocysteine and BHMT activity both showed a strong negative correlation with liver betaine. Homocysteinemia in mice is sensitive to a disruption of *Cbs* and to methyl donor intake. Because betaine leads to a greater flux through BHMT and lowers homocysteine, betaine supplementation may be beneficial in mild hyperhomocysteinemia.

HOMOCYSTEINE IS the endogenous product of all transmethylation reactions that use S-adenosylmethionine (SAM) as a methyl donor. Homocysteine can be remethylated to methionine by methionine synthase (MS, EC 2.1.1.13), using 5-methyltetrahydrofolate as cosubstrate. Alternatively, betaine: homocysteine methyltransferase (BHMT, EC 2.1.1.5) can catalyze methyl transfer from betaine to homocysteine, yielding methionine and N,N-dimethylglycine (DMG). Cystathionine-β-synthase (CBS, EC 4.2.1.22) catalyzes the only catabolic pathway for homocysteine, through transsulfuration to cystathionine and subsequently cysteine. The metabolism of excessive SAM due to high dietary methionine can lead to increased production of homocysteine. CBS is activated by high SAM availability, whereas the enzyme providing methyl groups for folate-dependent homocysteine remethylation, 5,10-methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20), is inhibited by SAM; CBS activity becomes particularly important under these conditions.[1] In homocystinuria due to severe deficiency of CBS (MIM 236200), this regulatory system fails, with consequent severe hyperhomocysteinemia and life-threatening vascular complications. Betaine, or N,N,N-trimethylglycine, is an endogenous product of choline oxidation and is present in some foods.[2] In humans with homocystinuria due to pyridoxine nonresponsive severe CBS deficiency, betaine therapy has proven effective in decreasing homocysteine by approximately 75% and in improving outcome with respect to the vascular complications.[3] Usually, a dose of 100 to 200 (up to 400) mg/kg body weight (BW) per day is used. With the recognition that moderate hyperhomocysteinemia is associated with vascular disease, elucidation of homocysteine regulatory mechanisms is of increasing importance.[4]

Here, we studied the regulation of homocysteine metabolism by means of methyl donor supplementation in a mouse model of moderate hyperhomocysteinemia due to a heterozygous disruption of the *Cbs* gene.[5]

MATERIALS AND METHODS

Mice

Animal experimentation was approved by the Animal Care Committee of the Montreal Children's Hospital in compliance with guidelines of the Canadian Council for Animal Care. Mice with a heterozygous disruption of the *Cbs* gene (C57BL/6J-*Cbs*^{tm1Unc})[5] were obtained from the Jackson Laboratory and bred with C57BL/6J mice to obtain heterozygous and wild-type mice. These littermates were used for all studies. Mice were housed in our animal facility with free access to food and water. The mean starting age was 189.4 (6.7) days in study 1 and 225.1 (17.6) days in study 2, with mean BWs of 23.3 (0.6) g and 28.4 (0.6) g, respectively. There was no significant difference for these parameters between genotype or treatment groups in study 1 or study 2, respectively.

Genotyping

Cbs genotypes were determined by a polymerase chain reaction (PCR)-based method. Genomic DNA was extracted from mouse tail biopsies. A 0.4-kb fragment of *Cbs* intron 2 was amplified on a Perkin-Elmer (Boston, MA) TC1 using 0.25 μg sense Primer 1 (5′-TAC TAC CAC TGC CCA GCT TT-3′) and 0.05 μg antisense Primer 2 (5′-CCG AGC CAA CTT AGC CCT TA-3′) to identify the wild-type allele. The *Cbs* disruption was identified by amplifying a 0.2-kb fragment of intron 2 and the inserted *neo* gene using 0.25 μg sense Primer 1 and 0.25 μg antisense Primer 3 (5′-GAG GTC GAC GGT ATC GAT A-3′). Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA) was used with the buffer recommended by the manufacturer with PCRx

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Enhancer Solution (Invitrogen) at 1 × concentration in a final volume of 50 μL. DNA was amplified during 35 cycles with the following PCR conditions: 94°C for 1 minute, 62°C for 1 minute, and 72°C for 2 minutes. Genotypes were deduced by electrophoretic analysis of the amplification products.

Dietary Experiments

Study 1. Four female wild-type mice and 4 female mice, heterozygous for the *Cbs* disruption, were fed for 2 weeks with an amino acid–defined control diet (TD 00310; Harlan Teklad, Madison, WI). This control diet had essentially the same composition, including folate content, as the reference rodent diet, AIN-93M,[6] but contained choline at 5 instead of 10 mmol/kg diet, 0.30% L-methionine, 0.35% L-cystine, and 0.35% L-serine. Total labile methyl group content (sum of methionine, choline, betaine) was 25.1 mmol/kg as compared with 32.1 (AIN-93M). Our control diet thus contained only 20% less labile methyl groups than the AIN-93M reference diet, and similar diets have been considered to be methyl-sufficient in other dietary studies in rodents. The lower choline concentration was chosen to avoid saturating conditions for the choline-dependent remethylation pathway.

Another 2 groups, each with 4 wild-type and 4 heterozygous female mice, were fed the same diet for 2 weeks, containing either a betaine (Sigma, St Louis, MO) or a dimethylsulfonioacetate (DMSA or DMAT (NutriQuest, Chesterfield, MO)) supplement of 25 mmol/kg diet. Anhydrous betaine was purchased from Sigma. DMSA was obtained by custom synthesis for T. Garrow. BW and food intake were recorded weekly.

After 2 weeks, mice were killed in a CO_2 chamber. Blood was collected by heart puncture, anticoagulated with EDTA (MicrovetteR 500, Sarstedt, Germany) and immediately put on ice. Plasma was quickly separated by centrifugation at 10,000 × *g* for 5 minutes and immediately frozen at −70°C until analysis. Livers were dissected and liver aliquots were frozen on dry ice and stored at −70°C until analysis.

Study 2. Four groups, each comprising 4 adult heterozygous male mice, were placed on control diet TD 00310 and supplemented with increasing amounts of anhydrous betaine in drinking water, ranging from 0 to 100 mmol/L, for 2 weeks. Water was changed twice weekly. BW and food and water consumption were monitored. Betaine intake by drinking water was calculated. After 2 weeks, mice were processed as in study 1.

Metabolites

Total homocysteine and total cysteine concentrations were measured after chemical reduction of a plasma sample by high-performance liquid chromatography (HPLC) as previously described.[7] Plasma amino acid concentrations were measured by HPLC using a previously described method[8] in 3 of the 4 groups of study 2. Choline compounds in tissues were extracted by the method of Bligh and Dyer.[9] Choline, glycerophosphocholine, phosphocholine (PCho), betaine, and phosphatidylcholine (PtdCho) were then measured using liquid chromatography-electrospray ionization-isotope dilution mass spectrometry (LC-ESI-IDMS).[10]

BHMT activity. BHMT activity in crude liver extracts was analyzed as previously described.[11]

Statistical Analyses

Results are provided as mean ± SEM. Metabolite levels between groups were compared using the Kruskall-Wallis nonparametric test. If a significant test result was found, single parameters were compared with the 2-sided Wilcoxon test. Linear correlation between 2 parameters for mice on control diet was calculated and Spearman's linear regression coefficient provided. The nonparametric Spearman's rank test was used to correlate betaine and homocysteine for the mice on varying betaine supplements, and Spearman's rank correlation coefficient was calculated. For all analyses, a *P* level of .05 was considered significant. For the correlation studies of mice on the control diet, the 8 female mice from study 1 and 4 male heterozygous mice from study 2 were analyzed together.

RESULTS

Food and Drug Intake

Study 1. Supplementing the diet with 25 mmol/kg betaine led to a slightly, but significantly, increased food intake (118.3 [2.7]) compared with the control group (108.6 [2.9] g/kg BW per day) in the second week. Betaine intake was calculated as 346 [8] mg/kg BW per day. The treatment group receiving DMSA voluntarily decreased food intake during the first days of the study by 25% as previously described[12]; this resulted in a drug intake of 246 [2] mg/kg BW per day. During the first week, mice on the DMSA diet showed a weight loss of 13.3% [0.7], which was significantly higher than that of mice on the control (5.5% [0.5]) or betaine diet (5.9% [0.7]). Weights did not change significantly in week 2 of the study.

Study 2. Food intake amounted to 120.0 [2.6] g/kg BW per day in week 1 and 107.6 [1.4] g/kg BW per day in week 2 and was not significantly different between treatment groups in week 2. Betaine intake by water was not different between weeks 1 or 2 and amounted to 14 [1], 220 [13], and 1,549 [60] mg/kg BW per day for the 3 treatment groups.

Metabolite Concentrations and BHMT Activities

Study 1. On the control diet, heterozygous mice had significantly (49.8% [18.4]) higher plasma homocysteine than did wild-type mice, whereas cysteine concentrations in plasma were not significantly different (Table 1). Liver concentrations of betaine, choline, and PCho were each moderately, but not significantly, decreased in heterozygous mice compared with wild-type mice. Liver BHMT activity was not genotype-dependent. Plasma homocysteine (Fig 1) and BHMT activity (Fig 2) both showed a strong negative correlation with liver betaine.

The effects of betaine treatment were similar in both genotype groups: in wild-type mice plasma homocysteine was 37.5% [18.2] lower and in heterozygous mice, 48.8% [7.0] lower compared with mice on the control diet (Table 1). Liver betaine was much higher in betaine-treated animals compared with mice on the control diet. Other choline metabolites did not show consistent changes. BHMT activity in liver was similar in the control and treated groups.

DMSA supplementation had similar effects to those of betaine. Plasma homocysteine was significantly lower in both genotype groups compared with mice on control diet: by 55.2% [4.5] in wild-type mice and by 50.4% [4.2] in heterozygous mice; homocysteine thus remained 1.6-fold elevated in DMSA-treated heterozygous mice compared with DMSA-treated wild-type mice. Liver betaine was much higher in both genotypes. Other choline metabolites and BHMT activity in liver were similar to those of the control group.

Study 2. At the maximum level of betaine supplementation in this study, homocysteine was decreased to 25.2% [2.3] of the control level, while methionine and serine were 49.2% [5.3] and 41.0% [6.0] elevated, respectively (Fig 3). BHMT activity

Table 1. Plasma Total Homocysteine and Total Cysteine (μmol/L), Liver Choline Metabolite Concentrations (μmol/kg wet weight), and Specific Activity of BHMT in Liver (U/mg protein) of Female *Cbs*-Mice Stratified for Genotype and Diet

	Control		Betaine		DMSA	
	Wild-Type	Heterozygous	Wild-Type	Heterozygous	Wild-Type	Heterozygous
t.Homocysteine	15.4	23.0*	9.6†	11.8†	6.9†	11.4†
	0.4	2.8	2.8	1.6	0.7	1.0
t.Cysteine	149.2	154.9	168.1	167.8	158.8	157.2
	7.7	10.5	7.1	11.3	4.8	6.74
Betaine	183.5	142.8	1,031.9†	672.8†	1287.9†	1,555.9†
	15.8	22.1	373.4	154.9	250.3	697.7
Choline	119.4	86.3	80.7†	128.5†	96.5	116.9†
	13.7	5.7	7.7	7.4	13.1	4.9
PCho	239.9	172.6	224.7	697.3	235.8	276.6
	27.5	26.8	44.1	409.3	62.3	49.6
GPC	538.5	517.8	499.7	364.7	457.9	411.2
	39.6	19.2	27.9	92.8	26.6	44.4
PtdCho	17,087	17,033	16,568	17,123	17,449	17,619
	157	302	297	504	694	359
BHMT	135.3	126.2	149.5	118.7	120.9	100.0
	8.4	12.9	14.5	12.2	17.0	10.1

NOTE. Results are presented as mean and SEM of 4 mice per group.

Abbreviations: PCho, phosphocholine; GPC, glycerophosphocholine; PtdCho, phosphatidylcholine.

*$P < .05$ compared with wild-type genotype, †$P < .05$ compared with control diet of same genotype.

gradually decreased to 76.5% [3.1], whereas liver betaine concentration increased up to 150-fold (45 [3] μmol/kg wet weight (ww) with no betaine, compared with 6,645 [1,770] μmol/kg ww at the maximum level, $P < .05$). Other choline metabolites did not show consistent changes. Plasma homocysteine and liver betaine showed a highly significant negative correlation in this study, as well (Fig 4). Plasma methionine and liver betaine showed a significant positive correlation (Fig 4).

DISCUSSION

We used mice with a heterozygous knockout of the *Cbs* gene as a model of disrupted homocysteine metabolism. These mice showed frank, although moderate, hyperhomocysteinemia compared with wild-type littermates, as reported in the original description of this animal model.[5] Mean levels of tissue metabolite concentrations were not particularly informative due to high variability. However, the observations in individual mice were highly informative and demonstrated a significant negative correlation between plasma homocysteine and liver betaine, as well as between BHMT activity and liver betaine. These results indicate an increased consumption of betaine in hyperhomocysteinemia due to enhanced flux through the betaine-dependent remethylation pathway. In another mouse model of hyperhomocysteinemia, due to a disruption of the *Mthfr* gene, we also found a clear correlation between the extent of hyperhomocysteinemia and the degree of betaine depletion. However, in the *Mthfr* mice, betaine depletion was also associated with choline deficiency as indicated by low concentrations of liver PCho, the intracellular storage form of choline.[13] Addition of betaine to the diet in *Cbs* mice led to increased liver betaine and lower plasma homocysteine concentrations without affecting the concentrations of other choline compounds or specific BHMT activity. Betaine supple-

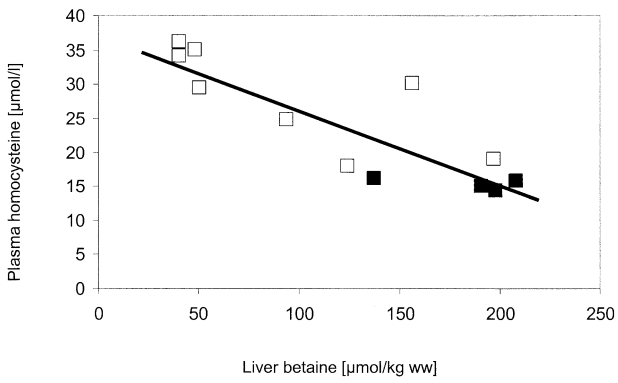

Fig 1. Linear correlation between plasma homocysteine and liver betaine concentrations in *Cbs* mice on the control diet. Wild-type (■), heterozygous (□); $r = -.8558$, $P = .0004$, n = 12.

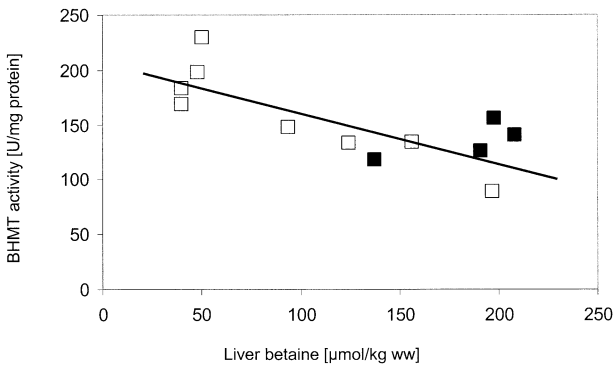

Fig 2. Linear correlation between specific BHMT activity and liver betaine concentrations in wild-type (■) and heterozygous (□) *Cbs* mice on the control diet; $r = -.7494$, $P = .0050$, n = 12.

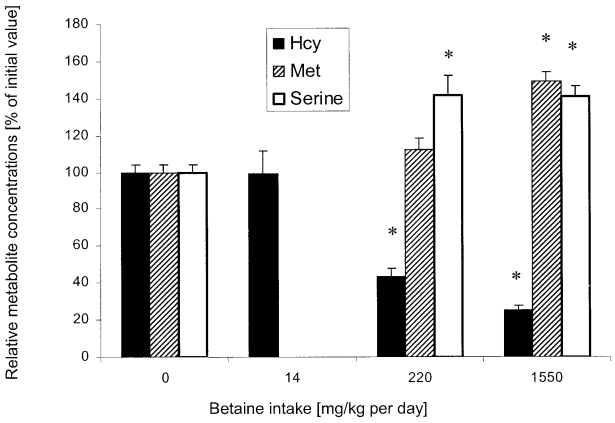

Fig 3. Mean [SEM] relative concentrations of homocysteine, methionine, and serine in plasma in heterozygous male *Cbs* mice on increasing betaine supplements. Values for methionine and serine were not obtained at the lowest dose of betaine (14 mg/kg), but it is unlikely that they would differ from the 0 betaine value at this low dose, particularly because plasma homocysteine was unchanged. *$P < .05$ *v* 0, n = 4 per group.

mentation decreased homocysteine levels in wild-type and heterozygous mice, demonstrating the sensitivity of rodents towards betaine (or choline) intake, independent of the *Cbs* genotype. Betaine intake at approximately 350 mg/kg BW per day, in study 1, lowered homocysteine levels in *Cbs* heterozygotes to values that were below those of wild-type mice on the control diet. However, despite the greatly elevated betaine concentrations in liver, *Cbs* heterozygotes on the betaine supplement maintained homocysteine concentrations that were still clearly above those of their wild-type littermates on the same diet. This indicates that betaine status is a strong determinant of plasma homocysteine, but that repletion of hepatic betaine stores cannot completely compensate for the effects of heterozygous CBS deficiency on plasma homocysteine. The same was true in our study of mice with MTHFR deficiency.[13]

Using an alternate methyl donor, DMSA, we were able to mimic the effects of betaine. DMSA is also exclusively metabolized by BHMT, but has some theoretical advantages, because its demethylated product has a higher K_i towards BHMT than the demethylated product of betaine, dimethylglycine, and may therefore be less likely to cause feedback inhibition.[12] Interestingly, the homocysteine-lowering effect of DMSA was very similar to that of betaine in study 1, and the DMSA supplement led to greatly increased liver betaine concentrations, without significantly affecting other choline metabolites or BHMT activity. Augmentation of betaine stores could be due to decreased catabolism or increased synthesis of betaine. Betaine can be generated via 3-fold methylation of phosphatidylethanolamine (PtdEth) to PtdCho, which is converted to choline, the precursor of betaine. DMSA catabolism yields 1 SAM molecule that could be used to methylate PtdEth. Liver PtdCho concentration remained unchanged in our study, but its large cellular pool could have masked a small increase. Although we cannot exclude an augmentation of methylation-dependent synthesis of PtdCho and hence betaine due to the DMSA supplement, the increase in liver betaine is more likely due to a betaine-sparing effect of DMSA, because DMSA is preferentially bound to the catalytic domain of BHMT compared with betaine.[12]

To determine whether an increase in betaine intake would lead to further homocysteine-lowering, we exposed heterozygous *Cbs* mice to betaine doses up to 1,550 mg/kg BW per day. We observed an additional decrease of plasma homocysteine to 25% of the initial level to an extent that corresponds very well to the described effects in humans.[3] In heterozygous *Mthfr* mice, we had observed a maximum homocysteine-lowering to 40% of the initial level. In parallel with the dose-dependent decrease in homocysteine levels at betaine intakes from 0 to 220 and 1,550 mg/kg BW per day, we observed a dose-dependent increase in plasma methionine. An examination of the correlation between plasma homocysteine or plasma methionine with liver betaine (Fig 4) reveals that there is only a small, if any, benefit in terms of homocysteine-lowering beyond a liver betaine concentration of 240 μmol/kg ww, which was largely achieved with a supplement of 220 mg/kg BW per day. In contrast, in *Mthfr*-deficient mice, we found no additional effect above a dose of 53 mg/kg BW.[13] It appears, therefore, that betaine may be more effectively used in CBS deficiency than in remethylation defects, such as MTHFR deficiency. Another possible explanation is that betaine stores were more depleted in *Cbs* mice than in *Mthfr* mice before the study period. Because both homocystinuria mouse models had been backcrossed to different genetic backgrounds, direct comparison of absolute metabolite concentrations has to be performed with caution. However, plasma concentrations of homocysteine and cysteine and hepatic BHMT activity were not significantly different between wild-type mice of the *Mthfr* strain (on a BALB/c background) and wild-type mice of the *Cbs* strain (on a C57BL/6 background) on the control diet, whereas liver concentrations of Cho, PCho, and PtdCho were significantly lower, and betaine concentrations showed a trend toward lower values in *Cbs* mice compared with *Mthfr* mice (not shown).

In heterozygous CBS deficiency in mice, plasma serine in-

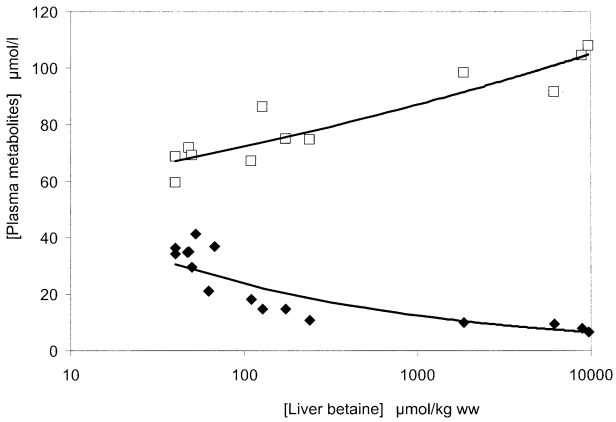

Fig 4. Nonlinear correlation between plasma homocysteine and liver betaine (■, $r = -.5441$, $P = .0351$) and between plasma methionine and liver betaine concentrations (□, $r = .7273$, $P = .0159$) in 16 male heterozygous *Cbs* mice on various betaine supplements.

creased with betaine supplementation (Fig 3). Serine plasma concentrations were shown to be depressed in 16 humans with CBS deficiency and were normalized with betaine supplementation, whereas serine concentrations in patients with remethylation defects were normal.[14] There may be an increased requirement for serine in CBS deficiency due to its role as a 1-carbon donor for folate-dependent remethylation. These results are consistent with earlier work in humans, in which it was observed that dietary choline supplementation decreased the de novo production of 1-carbon units used to support SAM-dependent transmethylation reactions.[15,16]

The dramatic increase of liver betaine, following betaine supplementation, demonstrated that betaine was well absorbed and stored. However, other choline compounds remained unchanged, and specific BHMT activity decreased slightly. Liver betaine concentrations with the highest dose (mean = 6.6 mmol/kg ww with 1,550 mg betaine per kg BW) were probably beyond substrate saturation of BHMT, whose K_m was determined to be 2.2 mmol/L[17] or even less.[18] In this study, we did not observe an increase of BHMT activity in wild-type or heterozygous *Cbs* mice with increasing betaine doses. In our previous study, however, BHMT activity was induced in heterozygous *Mthfr* mice receiving betaine supplementation. These data are consistent with previous work on methionine and methyl donor intake on hepatic BHMT expression in rats.[12] In these studies, BHMT induction was observed to require some degree of methionine deficiency, along with betaine or DMSA supplementation. Induction was found to be directly correlated to the level of dietary methyl donor supplementation and the degree of concomitant methionine deficiency. The exact molecular signals that mediate this response are not known, although we have shown that BHMT induction may be due to low intracellular SAM levels, because we observed that SAM inhibits BHMT transcription in HepG2 cells.[19] Because dietary methionine was not restricted in our dose-response study in heterozygous *Cbs* mice, the hepatic concentrations of methionine and SAM would be expected to be either normal or even slightly elevated. Therefore, the combination of the dietary conditions and genetically impaired CBS activity used in this study would not be expected to result in the induction of BHMT activity in wild-type mice, but rather in a slight repression, as observed. In contrast, *Mthfr* mice have reduced liver homocysteine remethylation rates and low methionine and SAM levels.[21] These conditions are favorable for the induction of BHMT expression following betaine supplementation, as observed.[13] As an alternative explanation for any effects intracellular SAM may have had on BHMT expression in this study, the small decrease in BHMT activity observed at the highest betaine dose in *Cbs* mice could have been caused by increasing DMG levels.[21]

In conclusion, increased plasma homocysteine can be viewed as an indicator of limited betaine supply for remethylation, a condition that readily occurs in mice, especially when homocysteine metabolism is impaired. It is intriguing to assume that the same could be true in humans, particularly because we recently demonstrated a negative correlation between plasma homocysteine and plasma betaine in human subjects.[13] Although the *Cbs* mouse is a suitable model to study the pharmacologic action of betaine in moderate hyperhomocysteinemia, human studies are warranted to clarify the role of betaine in hyperhomocysteinemic conditions.

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