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Inhibition of betaine-homocysteine *S*-methyltransferase in rats causes hyperhomocysteinemia and reduces liver cystathionine β -synthase activity and methylation capacity

Jana Strakova¹, Sapna Gupta², Warren D Kruger², Ryan N Dilger³, Katherine Tryon¹, Lucas Li⁴, and Timothy A Garrow^{1,*}

¹Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

²Division of Population Science, Fox Chase Cancer Center, Philadelphia, PA 19111, USA

³Department of Animal Sciences, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

⁴Metabolomics Center, University of Illinois at Urbana-Champaign, IL 61801, USA

Abstract

Methylation of homocysteine (Hcy) by betaine-homocysteine *S*-methyltransferase (BHMT) produces methionine, which is required for *S*-adenosylmethionine (SAM) synthesis. We have recently shown that short term dietary intake of *S*-(- δ -carboxybutyl)-DL-homocysteine (CBHcy), a potent and specific inhibitor of BHMT, significantly decreases liver BHMT activity and SAM concentrations but does not have an adverse affect on liver histopathology, plasma markers of liver damage or DNA methylation in rats. The present study was designed to investigate the hypothesis that BHMT is required to maintain normal liver and plasma amino acid and glutathione profiles, and liver SAM and lipid accumulation. Rats were fed an adequate (4.5 g/kg methionine and 3.7 g/kg cystine), cysteine-devoid (4.5 g/kg methionine and 0 g/kg cystine), or methionine-deficient (1.5 g/kg methionine and 3.7 g/kg cystine) diet either with or without CBHcy for 3 or 14 d. All rats fed CBHcy had increased total plasma Hcy (2- to 5-fold), and reduced liver BHMT activity (>90%) and SAM concentrations (>40%). CBHcy treatment slightly reduced liver glutathione levels in rats fed the adequate or cysteine-devoid diet for 14 d. Rats fed the methionine-deficient diet with CBHcy developed fatty liver. Liver cystathionine β -synthase activity was reduced in all CBHcy-treated animals, and the effect was exacerbated as time on the CBHcy diet increased. Our data indicate that BHMT activity is required to maintain adequate levels of liver SAM and low levels of total plasma Hcy, and might be critical for liver glutathione and triglyceride homeostasis under some dietary conditions.

Keywords

rat; betaine-homocysteine *S*-methyltransferase; betaine; homocysteine; glutathione

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*Corresponding author: Timothy A. Garrow, Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, 1201 West Gregory Drive, Urbana, IL 61801, USA, Tel.: 217-333-8455, Fax.: 217-265-0925, tagarrow@illinois.edu.

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1. Introduction

Methionine is an essential amino acid required for normal growth and development in mammals. Homocysteine (Hcy), an intermediate of methionine metabolism, lies at a branch point. It can be methylated to regenerate methionine (remethylation) or proceed through the transsulfuration pathway and donate its sulfur atom to cysteine synthesis. Reductions in remethylation or transsulfuration lead to an elevation in total plasma Hcy (tHcy), which has been classified as an independent and graded risk factor for vascular diseases and thrombosis [1,2].

The total sulfur amino acid requirement for a growing rat is estimated to be 5.5 g/kg of diet [3] and can be supplied as methionine alone or as a mixture of methionine and cysteine. It has been estimated that cysteine can supply up to 50% of the total sulfur amino acid requirement for rats [4,5], chicks [6] and pigs [7]. When diets contain adequate levels of both sulfur amino acids, the partitioning of Hcy between the remethylation and transsulfuration pathways has been estimated to be approximately equal in humans [8], perfused rat livers [9] and *in vitro* [10].

The majority of methionine metabolism occurs in the liver. There are three enzymes that catalyze Hcy remethylation: ubiquitously expressed cobalamin-dependent methionine synthase (MS), and liver- and kidney-specific betaine-homocysteine *S*-methyltransferase (BHMT) and BHMT-2, respectively. MS and BHMT have been shown to be regulated by dietary methionine excess [11] and restriction [12], but nothing is known about the regulation of BHMT-2 expression, which uses *S*-methylmethionine, a plant metabolite, rather than betaine as a methyl donor [13].

By acutely administering (i.p.) a specific and potent inhibitor of BHMT, *S*-(δ -carboxybutyl)-DL-homocysteine (CBHcy), we previously showed that this enzyme is important for maintaining the normally low tHcy concentration found in the fasted state, and repressing the elevation of tHcy following the coadministration of methionine [14]. Short term (3 d) inhibition of BHMT activity following multiple i.p. injections or dietary consumption of CBHcy altered liver and plasma amino acid profiles, reduced liver *S*-adenosylmethionine (SAM) concentrations and decreased the expression and activity of cystathionine β -synthase (CBS), the committed and rate limiting enzyme of the transsulfuration pathway, by an unknown mechanism [14,15].

The effect of CBHcy on liver CBS activity and SAM concentrations led us to hypothesize that prolonged inhibition of BHMT activity will affect glutathione synthesis and/or cause fatty liver in rats. Although CBHcy is BHMT-specific [13,14], the CBHcy-mediated reduction in CBS activity could reduce cysteine biosynthesis, the rate-limiting substrate for glutathione production. Similarly, the CBHcy-mediated reduction in liver SAM could reduce flux through phosphatidylethanolamine *N*-methyltransferase (PEMT), the liver-specific enzyme that produces phosphatidylcholine from phosphatidylethanolamine, which has been shown to be critical for triglyceride secretion from liver. Mice deficient in PEMT that are fed a choline-deficient [16] or high fat/high cholesterol diet [17] develop fatty liver. Thus, we wanted to know whether a prolonged CBHcy-dependent decrease in liver SAM would affect liver triglyceride accumulation. To test these hypotheses, we fed CBHcy (14 d) to rats in combination with diets that varied in methionine and cysteine content. The data presented here show that under certain dietary conditions, BHMT activity, besides being critical for the maintenance of normal tHcy and liver SAM levels, has a modest role in plasma and liver glutathione homeostasis and its absence promotes the development of fatty liver in rats fed a methionine deficient diet. This study also confirms that inhibition of BHMT somehow precipitates a down-regulation of CBS activity. This report adds to a

growing body of research indicating that sulfur amino acid intake and the activity levels of key enzymes of sulfur amino acid metabolism interact to modulate tHcy and therefore the risk for Hcy-related diseases in humans.

2. Methods and Materials

2.1. Animals, dietary treatments, and experimental approach

The animal study protocols were approved by the University of Illinois Laboratory and Animal Care and Use Committee. All studies were conducted using 5-week-old male Fisher 344 rats (~75 g) obtained from Harlan (Indianapolis, IN). Groups (n =6) of rats were meal-fed (3 times/day for 3 or 14 d) one of three L-amino acid defined diets that only varied in sulfur amino acid content (Dyets, Bethlehem, PA) and either contained (5 mg/meal) or were devoid of CBHcy (Orbiter, Urbana, IL). The diets varied in methionine and cysteine content as follows (cysteine supplemented as L-cystine): an adequate diet (defined as AIN-93G) [18] containing 4.5 g/kg methionine and 3.7 g/kg cystine (A); a cysteine-devoid diet containing 4.5 g/kg methionine and 0 g/kg cystine (C); and a methionine-deficient diet containing 1.5 g/kg methionine and 3.7 g/kg cystine (M). The animal trials were performed in three separate studies: (study 1) A vs. A plus CBHcy; (study 2) C vs. C plus CBHcy; and (study 3) M vs. M plus CBHcy.

Rats were individually housed (12 h/12 h light/dark cycles) in hanging wire cages and trained (3 d) to meal feed using diet A. Meals (4 g of diet) were given every 8 h and the animals were allowed to feed for 2 h (0600 to 0800, 1400 to 1600 and 2200 to 0000). Following the training period, rats were randomly assigned to a treatment group (control or CBHcy) so that initial body weights were not different. During the treatment period, the CBHcy group received 1 g of the appropriate diet (A, C or M) containing 5 mg of CBHcy at the beginning of each meal time, and after this food was consumed (~20 minutes), rats received a further 5 g of the appropriate diet that did not contain any CBHcy for the remainder of the 2 h meal period. The control group received 6 g of the appropriate diet without CBHcy for the entire 2 hour meal period. Following 3 or 14 d of treatment (i.e., 9 or 42 doses of CBHcy, respectively), rats were euthanized by CO₂ asphyxiation 4.5 h after receiving their last meal, which consisted of only 1 g of the appropriate diet (with or without CBHcy). Blood was collected via cardiac puncture into EDTA-coated tubes and liver was excised and snap frozen in liquid nitrogen and stored at -70 °C until analysis. A portion of liver was fixed in 10% buffered formalin for histological analysis. Plasma was obtained via centrifugation at 3,000×g for 20 min within 1 h of blood collection and stored at -70 °C until analysis.

2.2. Clinical chemistry

Plasma urea nitrogen, albumin, glucose, cholesterol, triglycerides, bile acids, alanine aminotransferase (ALT), creatine kinase (CK) and sorbitol dehydrogenase (SDH), were determined by the Veterinary Diagnostic Laboratory at the College of Veterinary Medicine in Illinois (Urbana, IL). All parameters were measured on Hitachi 917 chemistry analyzer (Roche Diagnostic, Indianapolis, IN) using Roche reagents for all the analytes except SDH (Catachem, Bridgeport, CT) and bile acids (Diazyme, Poway, CA).

2.3. Liver enzyme assays and BHMT abundance

Rat livers were homogenized in 4 volumes (wt/v) of homogenization buffer (50 mmol/L potassium phosphate buffer, 2 mmol/L EDTA, pH 7.4) in the presence of protease inhibitors (Calbiochem) and centrifuged at 25,000×g for 45 min. The supernatant was aliquoted and stored at -70 °C until analysis. Aliquots dedicated for the determination of BHMT and CBS activities and Western blot analysis were supplemented with β-mercaptoethanol (5 mmol/L

final), while aliquots dedicated for the determination of total glutathione concentrations and MS activity were stored without the addition of β -mercaptoethanol.

BHMT activity was determined by measuring the formation of radiolabeled methionine and dimethylglycine from [^{14}C -methyl]-betaine (Moravek Biochemicals) as described by Garrow [19]. To determine the abundance of liver BHMT, liver homogenates (0.5 $\mu\text{g}/\text{well}$) were separated on 10% SDS-PAGE, blotted onto a nitrocellulose membrane and blocked with 5% non-fat milk. The membrane was then probed with primary antibody, anti-BHMT (1 to 3,500 dilution), overnight at 4 °C followed by horseradish peroxidase conjugated secondary antibody (1 to 15,000 dilution) for 1 h at room temperature.

CBS activity was determined by measuring the formation of radiolabeled cystathionine from [^{14}C]-serine (Moravek Biochemicals). The reaction mixture was prepared according to Lambert et al. [20] as detailed in Collinsova et al. [14]. Serine was separated from cystathionine as described by Taoka et al. [21].

MS activity was determined by measuring the formation of radiolabeled methionine from 5- [^{14}C]-methyltetrahydrofolate (Amersham Biosciences UK limited) as described by Banerjee et al. [22] and detailed in Collinsova et al. [14].

2.4. Sulfur amino acid metabolites—Total cysteine, cysteinyl-glycine, Hcy and glutathione were determined in plasma and liver samples. Samples were reduced with TCEP and then derivatized with 7-fluorobenzo-2-oxa-1, 3-diazole-4-sulfonic acid ammonium (SBD-F, Sigma-Aldrich) as described by Garcia et al. [23]. To determine liver SAM and S-adenosylhomocysteine (SAH), rat livers were homogenized in 4 volumes (wt/v) of 400 mmol/L HClO_4 , centrifuged at 10,000 $\times g$ for 15 min and analyzed by HPLC method according to Wang et al. [24].

2.5. Plasma betaine, choline, and total amino acid analyses

An extract for choline and betaine determination was prepared from plasma samples as described by Holm et al. [25]. Briefly, samples (30 μl) were deproteinized by three volumes of acetonitrile that contained 10 $\mu\text{mol/L}$ of d_9 -betaine (*N*-(carboxymethyl)-*N,N,N*-trimethyl- d_9 -ammonium chloride; CDN Isotopes, Pointe-Claire, Quebec, Canada) and 10 $\mu\text{mol/L}$ of d_9 -choline (*N,N,N*-trimethyl- d_9 - choline chloride; CDN Isotopes, Pointe-Claire, Quebec, Canada). Samples were then centrifuged at 5,800 $\times g$ for 2 minutes and supernatant was analyzed by LC/MS as described previously in detail [15]. The free amino acid pools of liver and plasma samples were measured on BioChrom 30 amino acid analyzer (Cambridge, UK) using procedures previously described [26,27].

2.6. Histochemistry

Portions of rat livers and kidneys were fixed in 10% buffered formalin for 22 h and stored in 70% ethanol until embedded in paraffin. Paraffin slices (3 μm) were stained with hematoxylin and eosin and evaluated for presence of fat droplets.

2.7. Statistical analyses

All data were subjected to a 2-way ANOVA using the GLM procedure of SAS software (SAS Inst. Inc.) unless otherwise noted. The statistical model included 2 factors, including duration (3 d vs. 14 d) and dietary provision (absence vs. presence) of CBHcy treatment. It is important to note that no statistical comparisons were made between diet types (A, C, M) because of experimental limitations (i.e., not all diet types were represented in the 3 separate feeding studies required to generate the data shown herein). For growth performance, as well as plasma and liver concentrations of amino acids and other metabolites, a 1-way ANOVA

was conducted with only dietary CBHcy provision in the model; these response variables were only measured after rats had received dietary treatments for 14 d. Data were analyzed with procedures appropriate for a completely randomized design with significance set at an α -level of 0.05. All data are expressed as means $\pm$ SEM.

3. Results

3.1. Growth performance

Generally speaking, there were only small differences in food intake and weight gain between CBHcy treated animals and their respective controls (Table 1). Animals that consumed the A diet with CBHcy for 14 d and those that consumed the C diet with CBHcy for 3 d gained more weight than those that were fed diets without CBHcy ($P < 0.05$). As expected, animals that consumed the M diet regardless of CBHcy treatment gained less weight and consumed less diet than animals that consumed the A and C diets alone or in combination with CBHcy.

3.2. Liver histology and clinical chemistry

Two rats from each treatment (control, CBHcy) and time point (3 d, 14 d) were screened for fatty liver. Animals that consumed the A and C diets with or without CBHcy had normal liver histology. One of two rats that received the M diet for 3 d developed mild lipidosis, and the level of this condition was severe in one of two rats receiving the M diet for 14 d. All screened animals that were fed the M diet with CBHcy developed lipidosis, which progressed from mild to moderate as time on the treatment progressed (not shown). Future studies may benefit from a more thorough investigation of the CBHcy-induced lipidosis anecdotally observed here.

Apart from a 20% elevation in urea nitrogen in rats receiving the C plus CBHcy treatment (relative to the C treatment), there were no significant effects of dietary treatment or time on any measure of clinical chemistry, including markers of liver damage (data not shown).

3.3. Plasma choline, betaine, and total thiols

Independent of dietary treatment, rats receiving CBHcy had elevated levels of plasma betaine ranging from 5 to 8- fold ($P < 0.05$; Table 2). Plasma choline concentrations were approximately 20% lower ($P < 0.05$) in the animals that consumed the A and C diets with CBHcy. After 3 days of treatment, rats consumed the M diet with CBHcy also had lower plasma choline (30%, $P < 0.05$), but plasma choline did not differ from the control group at day 14.

The reduction of liver BHMT activity caused by the consumption of CBHcy in combination with the three dietary treatments led to a 2 to 5- fold increase in tHcy ($P < 0.05$), which was directly proportional to the length of the inhibition (Table 2). The concentration of plasma cysteinylglycine was lower in animals that consumed the C diet with CBHcy for 3 d and in animals that consumed the M diet with CBHcy for 3 and 14 d. CBHcy treatment did not affect plasma cysteine and glutathione levels in any treatment group.

3.4. Plasma and liver amino acids and other metabolites

Plasma and liver amino acids were determined only in rats that were fed the experimental diets for 14 d (Table 3). Independent of diet, CBHcy consumption caused a significant decrease in plasma methionine ($P < 0.05$) and glutamine ($P < 0.05$) and a significant increase in plasma glycine ($P < 0.05$). Animals fed the C and M diets had slightly decreased plasma serine, but this was still significant compared to the non-treated animals ($P < 0.05$). Animals

consuming the A and C diet with CBHcy had elevated plasma cystathionine ($P < 0.05$) and histidine ($P < 0.05$). The treatment had no effect on plasma ammonia and ethanolamine.

Liver serine ($P < 0.05$) and ethanolamine ($P < 0.05$) significantly decreased and ammonia ($P < 0.05$) increased in all CBHcy fed rats (Table 3). Liver methionine significantly decreased only in the rats that consumed the C and M diets with CBHcy ($P < 0.05$). Liver cystathionine was elevated only in rats fed the C diet with CBHcy. The treatment had no effect on liver glycine levels.

The differences in tissue glycine, serine and histidine, noted above, were also observed and commented upon in our prior study [15]; readers are referred to that work for a discussion of these CBHcy-induced metabolite changes.

3.5. Liver enzyme activities and abundance

Animals that consumed diets containing CBHcy had >90% reduction in liver BHMT activity ($P < 0.05$; Table 4) and significantly increased liver immunodetectable BHMT protein (approximately 2-fold higher) when compared to their respective controls (Figure 1).

CBHcy treatment significantly reduced liver CBS activity ($P < 0.05$; Table 4), except in the CBHcy treated animals that consumed the A diet for only 3 d. The reduction in CBS activity was time dependent, with the lowest values observed following 14 d of treatment. The rats consuming the M diet had the lowest CBS activities.

CBHcy treatment in combination with the A and C diet did not affect liver MS activity (Table 4). Animals that were maintained on the M diet with CBHcy had slightly lowered MS activity at day 3 (~20% reduction; $P < 0.05$) and day 14 (~15% reduction; $P < 0.05$) compared to animals fed the methionine-deficient diet alone.

3.5. Concentrations of thiol-containing compounds in the liver

Liver cysteine and cysteinyl-glycine were not affected by CBHcy (Table 5). Liver glutathione decreased in animals that were fed the A diet with CBHcy for 14 d ($P < 0.05$). Total liver Hcy concentrations were below the detection limit of our HPLC method [24].

Independent of dietary treatment, liver SAM concentrations decreased by 30 to 55% in all CBHcy treated animals ($P < 0.05$; Table 5). The liver SAH concentrations of the animals that consumed the A and M diets containing CBHcy did not differ from their respective controls. Liver SAH levels of animals fed the C diet plus CBHcy were approximately 2-fold higher than that of animals consuming the C diet alone. The indicator of liver methylation capacity, the SAM-to-SA ratio, was lower in the animals that consumed CBHcy, although the decrease was not always statistically significant.

4. Discussion

We investigated how prolonged inhibition of BHMT affects amino acid and sulfur amino acid metabolite profiles, glutathione levels and/or cause fatty liver. Results indicated that while animal growth was not generally affected by this prolonged inhibition, substantial differences were observed in circulating and tissue metabolite profiles, activities of pertinent enzymes, and accumulation of hepatic lipid. It should be noted that our experimental design does not permit comparison among the dietary treatments because of several confounding factors: (i) the three studies were conducted at different times, (ii) data were analyzed separately, and (iii) animals were not pair-fed. Thus comparisons between the dietary treatment groups need to be interpreted with caution.

Independent of diet, CBHcy treatment caused a significant reduction in liver BHMT activity, an induction of BHMT protein expression, and an elevation of tHcy that was exacerbated as time on the treatment progressed. The induction of BHMT protein expression was likely the result of decreased liver SAM concentrations. Previous studies have shown that SAM downregulates BHMT transcription *in vitro* [28,29], and that BHMT expression is increased (4-fold) in methionine *S*-adenosyltransferase 1A-deficient mice, which have reduced (by 74%) SAM concentrations [30].

CBHcy does not inhibit MS or CBS activities in mouse liver homogenates [14], indicating that these enzymes do not bind CBHcy. However, in the current study, MS activity was slightly reduced in rats consuming the M diet with CBHcy, and CBS activity was dramatically reduced in all rats receiving CBHcy independent of the dietary treatment. Although MS and CBS protein expression were not determined, it is likely that the observed decrease in these enzyme activities reflect changes in protein abundance by an unknown mechanism. CBS catalyzes the rate limiting reaction of the transsulfuration pathway, which produces cysteine for protein, glutathione and taurine synthesis. SAM is a required allosteric activator of CBS [31] and stabilizes the enzyme *in vitro* [32]. CBS activity was determined in the presence of saturating levels of SAM and so lower CBS protein levels, rather than decreased liver SAM concentration, likely explain the observed reduction in CBS activity. Indeed, we have previously shown that rats fed CBHcy for 3 d have decreased CBS protein abundance (26% reduction) [15]. Among the three dietary treatments, rats fed the M diet displayed the lowest CBS activity, which further decreased with CBHcy treatment, and liver and plasma methionine levels decreased in a similar manner to CBS activity. In other studies using rats, methionine restriction decreased CBS activity [33] and low dietary protein decreased both CBS activity and mRNA expression [34]. A more recent study showed that switching mice to a methionine-free diet resulted in the down-regulation of CBS expression via a post-translational mechanism involving reduced stability of CBS protein, and this effect was unrelated to intracellular SAM concentrations [35]. In contrast, the methionine *S*-adenosyltransferase 1A-deficient mice show significantly elevated methionine concentrations (776%) and their CBS activity increased (3 to 4-fold) [30]. Combined these studies suggest that inhibition of BHMT causes reductions in liver methionine and SAM, and using a combination of mechanisms yet to be fully detailed, the liver cell responds by down-regulating CBS to bolster the recycling of Hcy back to methionine.

Despite the marked decrease in liver CBS activity, plasma cystathionine was elevated (3-fold) in CBHcy treated rats fed the A and C diets (14 d), a phenomena that is currently not understood. CBS has a high K_m for Hcy (1–25 mmol/L) [21], and so it could be argued that total flux through CBS might be increased in the CBHcy-induced hyperhomocysteinemic rats. However, both total CBS activity and SAM, a required activator of the enzyme, decreased during CBHcy treatment, suggesting total flux through CBS must decrease. In fact, the progressive increase in tHcy caused by CBHcy treatment could be attributed, at least in part, to the progressive decrease in CBS activity during CBHcy treatment. A recent study [36] showed that CBS knockout mice have 10-fold higher plasma cystathionine compared to wild type controls, which currently is unexplainable. Metabolic tracer studies are needed to elucidate why cystathionine increases in CBHcy-treated rats and CBS knockout mice. It is possible that the cystathionase catalyzed reaction becomes the rate limiting step of Hcy transsulfuration in CBHcy-treated animals as it does in B₆ deficient rats [37].

Short term CBHcy-mediated inhibition of BHMT (3 d) did not affect liver glutathione concentrations [15]. We wanted to investigate whether long term CBHcy treatment, combined with sulfur amino acid restriction, affected glutathione concentrations via a putative CBHcy-induced down-regulation of the transsulfuration pathway. Interestingly,

plasma and liver cysteine concentrations were not affected by CBHcy treatment, but liver glutathione was significantly lower in rats fed the A diet plus CBHcy (14 d) compared to their respective controls, suggesting that BHMT activity is important for maintaining glutathione levels under normal dietary conditions. Of the three control diets, liver glutathione was the lowest in animals fed the C diet, suggesting that cysteine concentrations are maintained at the expense of glutathione production. This was not unexpected because liver glutathione synthesis was previously shown to depend on dietary cysteine content [38].

We hypothesized that loss of BHMT activity might cause fatty liver due to a reduction in liver phosphatidylcholine synthesis as a direct result of the CBHcy-induced decrease in liver methylation capacity. Phosphatidylcholine is required for VLDL formation and triglyceride export from liver. There are two pathways to synthesize phosphatidylcholine: 1) SAM-dependent methylation of phosphatidylethanolamine catalyzed by PEMT, and 2) a SAM-independent pathway (Kennedy pathway), which uses choline as the precursor. The PEMT pathway accounts for approximately 30% of total hepatic PC production and is liver specific. Mice deficient in PEMT do not develop fatty liver unless they are fed a choline deficient [16] or high-fat/high cholesterol [17] diet. This is because under optimal dietary intakes of choline, the Kennedy pathway compensates for the PEMT deficiency. We observed a decrease in plasma choline concentrations in CBHcy treated rats fed the A and C diets, suggesting that more choline was used for phosphatidylcholine production via the Kennedy pathway, which was presumably sufficient for normal VLDL export since those animals had normal liver histology. In contrast, animals fed the M diet developed fatty liver and it appeared to be exacerbated by CBHcy treatment. Interestingly, plasma choline levels tended to be the lowest in the rats fed the M diet, suggesting that more choline was used for phosphatidylcholine synthesis via the Kennedy pathway. However, it is possible that the reduction in plasma choline was because more was oxidized to betaine, and overall, more rats need to be screened for fatty liver to confirm this effect of CBHcy.

Sulfur amino acid metabolism has a large capacity to adapt to changes in dietary methionine and cysteine by regulating the metabolic fate of Hcy. Here we report that prolonged inhibition of BHMT in rats consuming an adequate, cysteine-devoid or a methionine deficient diet causes a substantial elevation in tHcy, despite an elevation in the liver BHMT protein and a wild type folate-mediated remethylation pathway. Inhibition of BHMT also reduces liver SAM. The data indicate that BHMT activity is required to maintain normal liver glutathione levels and prevent the accumulation of liver fat under certain dietary conditions.

Abbreviations

ALT	alanine aminotransferase
BHMT	betaine-homocysteine <i>S</i> -methyltransferase
BHMT2	betaine-homocysteine <i>S</i> -methyltransferase 2
CBHcy	<i>S</i> -(α -carboxybutyl)- <i>DL</i> -homocysteine
CBS	cystathionine β -synthase
CK	creatine kinase
Hcy	homocysteine
methyl-THF	methyltetrahydrofolate
MS	methionine synthase

PEMT	phosphatidylethanolamine <i>N</i> -methyltransferase
SAH	<i>S</i> -adenosylhomocysteine
SAM	<i>S</i> -adenosylmethionine
SDH	sorbitol dehydrogenase
tHcy	total plasma homocysteine

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Figure 1. Liver BHMT expression is increased in rats fed CBHcy

Liver immunodetectable BHMT levels of rats fed adequate (A; n=6), cystine-devoid (C; n=6) and methionine-deficient (M; n=6) diets for 3 and 14 days either without (Con) or with CBHcy (CB). A representative westernblot is shown.

Table 1

Growth performance of rats fed adequate (n=6), Cys-devoid (n=6) and Met-deficient (n=6) diets for 14 days either without (control) or with (CBHcy) an inhibitor of BHMT activity¹.

Diet	Measure	Control	CBHcy
Adequate	Weight gain, g	44 ± 1	54 ± 2 *
	Food intake, g	147 ± 3	156 ± 4
	Gain:food, g/kg	297 ± 7	344 ± 12 *
Cys-devoid	Weight gain, g	45 ± 2	47 ± 2
	Food intake, g	150 ± 3	161 ± 4
	Gain:food, g/kg	302 ± 6	292 ± 9
Met-deficient	Weight gain, g	22 ± 4	30 ± 4
	Food intake, g	117 ± 6	128 ± 5
	Gain:food, g/kg	183 ± 19	228 ± 24

¹ Values are means ± SEM. Control and CBHcy treatments were compared using a 1-way ANOVA.

* Significant effect of CBHcy treatment over 14 days ($P < 0.05$).

Table 2

Plasma concentrations of betaine, choline, and total thiolin rats fed adequate (n=6), Cys-devoid (n=6) and Met-deficient (n=6) diets for 3 or 14 days either without (control) or with (CBHcy) an inhibitor of BHMT activity¹.

Diet	Measure	Control		CBHcy	
		3 days	14 days	3 days	14 days
Adequate	Betaine ³	172 ± 6	141 ± 5	1184 ± 66	1047 ± 56
	Choline ³	16 ± 1	15 ± 1	13 ± 0	12 ± 0
	Cys ²	76.8 ± 4.4	88.0 ± 2.4	79.3 ± 4.5	89.0 ± 1.9
	Cys-Gly ²	3.9 ± 0.1	3.5 ± 0.1	3.7 ± 0.1	3.4 ± 0.0
	tHcy ^{2,3,4}	7.1 ± 0.3	8.0 ± 0.3	15.7 ± 1.4	37.2 ± 3.5
	Glutathione ^{2,3}	68.5 ± 9.0	43.3 ± 6.3	51.6 ± 6.2	36.0 ± 3.5
Cys-devoid	Betaine ^{2,3,4}	178 ± 9	178 ± 8	1591 ± 96	1401 ± 56
	Choline ^{2,3}	21 ± 1	18 ± 1	15 ± 1	14 ± 1
	Cys ²	58.8 ± 5.0	74.4 ± 2.5	58.5 ± 6.2	77.2 ± 3.8
	Cys-Gly ^{2,3}	3.4 ± 0.2	2.8 ± 0.1	2.9 ± 0.1	2.5 ± 0.1
	tHcy ^{2,3,4}	8.8 ± 0.4	12.1 ± 1.2	33.9 ± 1.9	61.8 ± 1.9
	Glutathione ²	51.8 ± 6.1	38.6 ± 1.5	49.6 ± 2.7	44.8 ± 1.7
Met-deficient	Betaine ^{2,3}	201 ± 19	136 ± 7	1108 ± 25	998 ± 47
	Choline ^{3,4}	16 ± 2	13 ± 1	11 ± 0	12 ± 1
	Cys ^{2,3}	92.5 ± 2.6	101.9 ± 2.9	101.1 ± 2.8	105.6 ± 6.6
	Cys-Gly ³	3.2 ± 0.1	3.0 ± 0.1	2.3 ± 0.1	2.1 ± 0.2
	tHcy ^{2,3}	8.3 ± 0.7	13.2 ± 1.5	15.5 ± 1.6	22.7 ± 2.9
	Glutathione	67.3 ± 6.8	60.4 ± 8.5	70.8 ± 6.1	52.1 ± 2.8

¹ Values are means ± SEM, and all measures are expressed as μmol/L of plasma.

² Main effect of time (P < 0.05).

³ Main effect of CBHcy treatment (P < 0.05).

⁴ Time × CBHcy treatment interaction (P < 0.05).

Table 3

Plasma and liver concentrations of amino acids and other metabolites in rats fed adequate (n=6), Cys-devoid (n=6) and Met-deficient (n=6) diets for 14 days either without (control) or with (CBHcy) an inhibitor of BHMT activity¹.

Diet	Measure	Plasma (μmol/L)		Liver (nmol / mg protein)	
		Control	CBHcy	Control	CBHcy
Adequate	Glutamine	874 ± 29	787 ± 22*	79 ± 6	58 ± 4*
	Glycine	182 ± 9	309 ± 5*	15 ± 1	17 ± 1
	Histidine	33 ± 1	49 ± 2*	2.8 ± 0.1	3.3 ± 0.1*
	Methionine	43 ± 2	35 ± 1*	0.8 ± 0.1	0.7 ± 0.0
	Serine	269 ± 12	240 ± 7	9.3 ± 1.0	4.4 ± 0.2*
	Ammonia	67 ± 7	67 ± 5	19 ± 1	24 ± 1*
	Cystathionine	0.7 ± 0.1	2.3 ± 0.2*	0.2 ± 0.0	0.2 ± 0.0
	Ethanolamine	10 ± 1	8 ± 1	0.9 ± 0.1	0.5 ± 0.0*
Cysteine-devoid	Glutamine	1087 ± 39	890 ± 42*	93 ± 6	58 ± 5*
	Glycine	203 ± 11	344 ± 10*	21 ± 1	20 ± 1
	Histidine	41 ± 1	55 ± 2*	3.5 ± 0.2	3.5 ± 0.1
	Methionine	43 ± 1	35 ± 1*	0.8 ± 0.0	0.6 ± 0.0*
	Serine	363 ± 14	293 ± 14*	15 ± 1	6 ± 1*
	Ammonia	67 ± 4	74 ± 3	20 ± 1	23 ± 1*
	Cystathionine	0.9 ± 0.1	2.6 ± 0.2*	0.21 ± 0.02	0.15 ± 0.01*
	Ethanolamine	8 ± 0	7 ± 1	1.0 ± 0.1	0.5 ± 0.1*
Methionine-deficient	Glutamine	1134 ± 44	886 ± 48*	95 ± 6	80 ± 4
	Glycine	196 ± 7	278 ± 19*	14 ± 1	15 ± 1
	Histidine	42 ± 2	44 ± 2	2.9 ± 0.2	3.5 ± 0.1*
	Methionine	33 ± 2	20 ± 1*	0.6 ± 0.0	0.4 ± 0.1*
	Serine	438 ± 17	309 ± 16*	13 ± 1	7 ± 0*
	Ammonia	80 ± 8	69 ± 4	21 ± 0	24 ± 1*
	Cystathionine	0.5 ± 0.0	0.7 ± 0.1	0.2 ± 0.0	0.2 ± 0.0

Diet	Measure	Plasma (μmol/L)		Liver (nmol / mg protein)	
		Control	CBHcy	Control	CBHcy
Ethanolamine		8 ± 0	7 ± 0	0.9 ± 0.1	0.4 ± 0.0 *

Values are means ± SEM. Control and CBHcy treatments were compared within sample type using a 1-way ANOVA.

* Significant effect of CBHcy treatment over 14 days within sample type ($P < 0.05$).

Table 4

Activities of liver Hcy-metabolizing enzymes in rats fed adequate (n=6), Cys-devoid (n=6) and Met-deficient (n=6) diets for 3 or 14 days either without(control) or with (CBHcy) an inhibitor of BHMT activity¹.

Diet	Measure	Control		CBHcy	
		3 days	14 days	3 days	14 days
Adequate	BHMT ³	64.8 ± 4.2	72.8 ± 1.8	2.4 ± 0.3	4.1 ± 0.1
	MS ²	6.4 ± 0.2	5.7 ± 0.2	6.6 ± 0.2	5.1 ± 0.2
	CBS ^{2,3,4}	204.6 ± 19.4	220.9 ± 14.6	174.7 ± 25.0	84.8 ± 4.6
Cys-devoid	BHMT ^{3,4}	46.2 ± 1.6	40.2 ± 2.3	4.3 ± 0.2	6.4 ± 0.3
	MS ²	4.3 ± 0.2	3.4 ± 0.0	4.7 ± 0.3	3.4 ± 0.2
	CBS ^{2,3}	197.6 ± 7.1	184.9 ± 13.7	119.3 ± 6.1	70.0 ± 4.5
Met-deficient	BHMT ³	49.9 ± 3.2	51.0 ± 2.5	2.1 ± 0.2	3.1 ± 0.2
	MS ^{2,3}	7.9 ± 0.4	6.1 ± 0.2	6.3 ± 0.3	5.2 ± 0.2
	CBS ^{2,3}	122.3 ± 4.6	67.6 ± 2.2	75.5 ± 5.2	17.8 ± 0.9

¹ Values are means ± SEM, and all measures are expressed as units of enzyme activity/mg of tissue.

² Main effect of time (P < 0.05).

³ Main effect of CBHcytreatment (P < 0.05).

⁴ Time × CBHcytreatment interaction (P < 0.05).

Table 5

Total liver thiols, SAM and SAH in rats fed adequate (n=6), Cys-devoid (n=6) and Met-deficient (n=6) diets for 3 or 14 days either without (control) or with (CBHcy) an inhibitor of BHMT activity¹.

Diet	Measure	Control		CBHcy	
		3 days	14 days	3 days	14 days
Adequate	Cys	62.9 ± 7	57.9 ± 4.0	49.0 ± 8.2	57.6 ± 1.7
	Cys-Gly ²	14.7 ± 1.0	20.8 ± 1.5	15.8 ± 1.2	17.3 ± 0.8
	Glutathione ³	3981 ± 356	4997 ± 145	3813 ± 271	3581 ± 197
	SAM ³	51.9 ± 4.5	55.2 ± 3.2	35.7 ± 1.8	29.3 ± 2.9
	SAH ³	12.0 ± 1.6	11.5 ± 1.8	10.2 ± 0.5	12.1 ± 1.5
	SAM/SAH	4.5 ± 0.9	5.1 ± 1.1	3.6 ± 0.2	2.5 ± 0.2
Cys-devoid	Cys ²	60.2 ± 2.9	81.3 ± 3.8	64.2 ± 3.0	78.5 ± 1.8
	Cys-Gly ³	14.5 ± 1.2	17.3 ± 1	12.3 ± 0.9	14.6 ± 1.7
	Glutathione ^{2,3}	1999 ± 270	2681 ± 310	1740 ± 227	2273 ± 287
	SAM ³	56 ± 2.4	59.8 ± 1.8	35.1 ± 2.1	37.0 ± 1.6
	SAH ³	9.0 ± 0.9	6.3 ± 0.3	16.3 ± 2.7	18.2 ± 1.9
	SAM/SAH ³	6.5 ± 0.5	9.7 ± 0.7	2.3 ± 0.1	2.0 ± 0.2
Met-deficient	Cys	66.8 ± 12.8	72.6 ± 4.4	64.3 ± 9.9	81.0 ± 4.5
	Cys-Gly	18.4 ± 3.6	18.6 ± 1.6	18.0 ± 2.6	21.5 ± 2.5
	Glutathione	3860 ± 437	3797 ± 228	4024 ± 271	4097 ± 353
	SAM ³	44.7 ± 1.7	49.3 ± 4.3	23.2 ± 2.0	21.0 ± 2.8
	SAH ³	6.2 ± 0.5	7.5 ± 0.4	7.3 ± 0.5	8.2 ± 0.9
	SAM/SAH ^{2,3,4}	7.5 ± 0.8	6.6 ± 0.4	3.2 ± 0.3	2.7 ± 0.3

¹ Values are means ± SEM. Total liver thiols, SAM and SAH measures are expressed as nmol/g of liver tissue and the ratio of SAM/SAH is in arbitrary units.

² Main effect of time (P < 0.05).

³ Main effect of CBHcy treatment (P < 0.05).

⁴ Time × CBHcy treatment interaction (P < 0.05).