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Betaine supplementation is less effective than methionine restriction in correcting phenotypes of CBS deficient mice

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Abstract

Cystathionine beta synthase (CBS) deficiency is a recessive inborn error of metabolism characterized by elevated serum total homocysteine (tHcy). Betaine supplementation, which can lower tHcy by stimulating homocysteine remethylation to methionine, is often given to CBS deficient patients in combination with other treatments such as methionine restriction and supplemental B-vitamins. However, the effectiveness of betaine supplementation by itself in the treatment of CBS deficiency has not been well explored. Here, we have examined the effect of a betaine supplemented diet on the *TgI278T Cbs*^{-/-} mouse model of CBS deficiency and compared its effectiveness to our previously published data using a methionine restricted diet. *TgI278T Cbs*^{-/-} mice on betaine, from the time of weaning until for 240 days of age, had a 40% decrease in mean tHcy level and a 137% increase in serum methionine levels. Betaine-treated *Tg-I278T Cbs*^{-/-} mice also exhibited increased levels of betaine-dependent homocysteine methyl transferase (BHMT), increased levels of the lipogenic enzyme stearoyl-coenzyme A desaturase (SCD-1), and increased lipid droplet accumulation in the liver. Betaine supplementation largely reversed the hair loss phenotype in *Tg-I278T Cbs*^{-/-} animals, but was far less effective than methionine restriction in reversing the weight-loss, fat-loss, and osteoporosis phenotypes. Surprisingly, betaine supplementation had several negative effects in control *Tg-I278T Cbs*^{+/-} mice including decreased weight gain, lean mass, and bone mineral density. Our findings indicate that while betaine supplementation does have some beneficial effects, it is not as effective as methionine restriction for reversing the phenotypes associated with severe CBS deficiency in mice.

Introduction

Cystathionine beta-synthase deficiency is an inborn error of metabolism caused by mutation in the *CBS* gene, which encodes an enzyme that catalyzes the conversion of homocysteine to cystathionine. Homocysteine is an intermediary amino acid that is derived from the

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Conflict of Interest:

Sapna Gupta, Liqun Wang, and Warren D. Kruger declare that they have no conflict of interest.

Animal Rights:

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

Author Contributions

Performed experiments: SG, LW. Analyzed data: SG, WK. Wrote Manuscript: SG, WK. Planned Experiments: SG, WK.

Conflict of interest None.

metabolism of dietary methionine via its conversion to S-adenosylmethionine in the methionine recycling pathway. Homocysteine has two metabolic fates: (1) remethylation to methionine; or (2) condensation with serine to become cystathionine followed by conversion to cysteine. In biological material homocysteine and cysteine are found both in their reduced form and cross-linked to other thiol-containing compounds. The sum total of these forms is called either total homocysteine (tHcy) to total cysteine (tCys), respectively. CBS patients have elevated plasma tHcy and methionine (Met), reduced plasma tCys, and suffer from numerous complications including thrombosis, ectopia lentis, osteoporosis, brittle and thin skin, fine fair hair, fatty liver, mental retardation and psychiatric disturbances (Mudd et al 2001). In addition, many CBS deficient patients have reduced body fat and can resemble Marfan's syndrome patients in their long and thin presentation (Brenton et al 1972; Poloni et al 2014).

The treatment goal for CBS deficient patients is to decrease tHcy levels, as lower tHcy levels are associated with improved outcomes (Yap and Naughten 1998). The first described treatment strategy was to restrict the level of methionine in the diet and supplement with cysteine (Komrower et al 1966; Perry et al 1966; Sardharwalla et al 1968). Soon after, it was discovered that a subset of patients responded to pyridoxine supplementation, which may be related to the CBS enzyme's use of pyridoxal phosphate as a co-factor (Barber and Spaeth 1969). Later, betaine supplementation was found to be effective in lowering patients' tHcy (Wilcken et al 1983). Betaine is co-substrate of the enzyme BHMT that catalyzes the formation of methionine from homocysteine in the liver. Thus, increased betaine is thought to lower homocysteine levels by decreasing the homocysteine pool and increasing the methionine pool. Because the number of CBS deficient patients is quite small, none of these different treatment strategies has ever been tested directly in a controlled clinical environment and thus, the relative effectiveness of each of these strategies in isolation is unknown. It is also important to note that dietary compliance for methionine is the most difficult clinical aspect of treating CBS deficient patients.

Our lab has been studying the relative effectiveness of different dietary treatments of CBS deficiency using the *Tg-I278T Cbs*^{-/-} mouse model of CBS deficiency. This mouse contains a homozygous deletion of the endogenous mouse *Cbs* gene and a transgene that encodes for a mutant human missense CBS protein (*Tg-I278T*) downstream of the mouse Mt-I promoter (Wang et al 2005). By giving the mice zinc water until weaning, they can survive the neonatal lethality that occurs between three and six weeks of age in *Cbs*^{-/-} mice lacking the transgene. *Tg-I278T Cbs*^{-/-} mice have extremely elevated serum tHcy, elevated serum methionine, and reduced serum tCys. In addition, they have a variety of well-characterized phenotypes including osteoporosis, decreased mean survival, decreased weight gain, low percent body fat, and facial alopecia (Gupta et al 2009; Gupta and Kruger 2011). *Tg-I278T Cbs*^{-/-} mice expressing human I278T CBS did not respond to pyridoxine supplementation in their drinking water, which was somewhat surprising given the observation that human I278T homozygotes are generally pyridoxine responsive (Chen et al 2006). In other dietary studies, we showed that *Tg-I278T Cbs*^{-/-} benefited greatly from a low methionine diet (8.3% of the normal level), but did not benefit noticeably from a diet in which cysteine was raised by adding the cysteine analog N-acetyl L-cysteine (NAC) to drinking water (Gupta

and Kruger 2011; Gupta et al 2014). These findings indicate that the main drivers of the *Cbs*^{-/-} phenotypes are related to the elevated tHcy and methionine and not a shortage of cysteine.

In this study, we have examined a cohort of *Tg-I278T Cbs*^{-/-} and *Tg-I278T Cbs*^{+/-} mice that have been given betaine supplementation in their drinking water. The mice were characterized after seven months of supplementation. Our results show that betaine supplementation does have beneficial effects on *Tg-I278T Cbs*^{-/-} mice, but the benefits are not as strong as that observed for methionine restriction.

Materials and methods

Mouse model

The mouse model of CBS deficiency, *Tg-I278T Cbs*^{-/-}, was described previously and is on the C57BL6 strain background (Wang et al 2005). Transgene positive *Cbs*^{-/-} and *Cbs*^{+/-} animals were created by mating transgene positive *Cbs*^{-/-} males with transgene positive *Cbs*^{+/-} females on zinc sulfate water to induce the human transgene. Transgene induction during neonatal period prevents lethality due to lack of endogenous mouse CBS protein in pups. At 10 days of age, animals were genotyped as previously described (Wang et al 2004). All mice were fed a standard mouse chow (Teklad 2018SX, Harlan Teklad, Madison, WI, USA) containing 6 g Met / kg, 3 g Cystine, and 0.017 g pyridoxine / kg. Weaning was done at age day 30, when mice were put into cages containing either regular water or water supplemented with 2 % (w/v) betaine (Sigma; B2629). Mice given regular water are referred to as the RD (regular-diet) group, while mice given betaine water are referred to as the BSD (betaine supplemented diet) group. Weight and photographs of the mice were taken every 30th day. Mice were sacrificed at 240 days of age at which time liver, kidney, and serum were extracted. Corpses and tissues were then stored at -80° for bio-analysis and body scans. A portion of the liver was fixed and stained with hematoxylin and eosin as previously described (Wang et al 2005).

Dual energy X-ray absorptiometry (DEXA) analysis

DEXA analysis was performed using a Lunar PIXimus II densitometer as previously described (Gupta and Kruger 2011). DEXA data used for RD mice is the consolidation of values obtained from the previous work (Gupta and Kruger 2011) and additional values obtained along with the current study.

Measurement of serum tHcy and methionine

Serum total homocysteine (tHcy) and methionine levels were measured by using the Biochrom 30 amino acid analyzer as performed previously (Gupta et al 2009; Gupta and Kruger 2011). Serum was reduced in the presence of dithiothreitol, and reaction was then stopped by sulfosalicylic acid. The supernatant obtained after centrifugation was used to analyze tHcy (a sum total of free and disulfide bonded homocysteine) and methionine.

Western blotting

Liver homogenates (20 % w/v) were made in RIPA buffer (Thermo Scientific, USA) in the presence of protease inhibitor cocktail tablet (Complete mini, Roche, Germany) as described previously (Gupta and Kruger 2011). Protein concentration was measured by BCA protein assay kit (Thermo Scientific), and 30 µg of total protein extract was used to immunoblot SCD-1 by using goat polyclonal antibody (1:200; sc-14719) and secondary anti-goat antibody (1:5,000; sc-2020) from Santa Cruz Biotechnology. Beta actin was probed as a control by using mouse monoclonal primary antibody (Sigma; A-5441). Western blotting procedure was performed as previously described using 10% Bis tris SDS gel and MOPS buffer from Invitrogen (Life Technologies). (Gupta and Kruger 2011).

BHMT activity assay

Liver homogenates were dialyzed overnight in 10 mM Tris-HCl (pH 8.0 with 15% glycerol) and 150 µg of dialyzed protein extract was used for BHMT activity assay. The reaction mixture (50 µl) contained Tris-HCl buffer (10 mM; pH 8.0), Betaine (0.33 mM), and DL-homocysteine (0.33 mM). Reaction proceeded for 1 hr at 37 °C and was terminated by addition of 10 % sulfosalicylic acid at a 1:1 ratio. Activity was determined by measuring the amount of methionine formed using a Biochrom 30 amino acid analyzer.

Statistical analysis

For growth curve analysis, we fitted the data from each group using a cubic polynomial model with relative weighting using the non-linear curve fitting functions in GraphPad Prism 4.0. Due to large difference between genders, males and females were analyzed separately. The mean goodness of fit (R^2) for all eight (8) curves was 0.82 (range 0.61–0.95). Confidence intervals were determined from the standard error by GraphPad Prism.

For all other analysis, significance was determined using ANOVA followed by Tukey's multiple comparison tests employing GraphPad Prism 4.0 software (La Jolla CA). To compare only two sets of samples, a two-sided students t-test was used. For all tests, a $P < 0.05$ was considered significant.

Results

Betaine supplementation lowers tHcy and raises Met in *Cbs*^{-/-} mice

To see if a betaine supplemented diet (BSD) could lower tHcy in *Tg-I278T Cbs*^{-/-} (referred to as *Cbs*^{-/-}, going forward) mice, we first performed a short-term study in which *Cbs*^{-/-} and *Cbs*^{+/-} mice on standard mouse chow were given either normal water or water supplemented with 2 % (w/v) betaine for two weeks. This dosage was chosen based on a previous study in which betaine was shown to lower tHcy in another mouse model of CBS deficiency (Maclean et al 2010). Since the average mouse drinks about 5 ml of water per day, this amount of betaine corresponds to dosage of approximately 4000 mg/kg. We observed that short-term treatment resulted in a decrease in serum tHcy from 349 µM for mice on a regular diet (RD) to 159 µM ($P < 0.0001$) for mice on BSD, and an increase in serum Met from 157 µM to 611 µM ($P < 0.0001$) in *Cbs*^{-/-} animals (Figure 1). In *Cbs*^{+/-} animals, betaine supplementation did not have a significant effect on either metabolite.

Based on these findings, we decided to examine the effect of long-term BSD (Fig. 1). In these studies, *Cbs*^{-/-} and *Cbs*^{+/-} animals were given BSD starting at the time of weaning (30 days of age) and were followed until 240 days of age. Mean tHcy in the BSD *Cbs*^{-/-} mice was 209 μ M, but this was still significantly lower than that observed in RD mice (349 μ M, $P < 0.0001$). Similarly, serum Met levels in *Cbs*^{-/-} mice were elevated compared to RD animals (373 μ M vs. 157 μ M, $P < 0.0001$), although not as much as in the short-term study. In control *Cbs*^{+/-} mice, we observed a small but statistically significant lowering of tHcy levels (8.7 μ M vs. 5.3 μ M, $P < 0.001$), but no difference in Met (83 μ M vs. 86 μ M, $P = \text{ns}$).

We also compared the effects of BSD to our previously published work on using a methionine-restricted diet (MRD) that contained one-twelfth the Met found in normal chow (Gupta et al 2014). MRD *Cbs*^{-/-} mice had significantly lower serum tHcy (81 μ M vs. 209 μ M, $P < 0.0004$) and Met (27 μ M vs. 373 μ M, $P < 0.0001$) compared to BSD *Cbs*^{-/-} mice (Fig. 1). Thus, BSD was less effective than MRD in controlling tHcy in *Cbs*^{-/-} mice.

BSD rescues hair loss in CBS deficient mice

Facial alopecia is the most striking phenotype of *Cbs*^{-/-} mice on RD, occurring between 105–120 days of age in both males and females (Robert et al 2004; Gupta and Kruger 2011). Previously, we have shown that MRD completely eliminated the facial alopecia phenotype in *Cbs*^{-/-} mice. Here, we found that BSD also prevented facial alopecia in both male and female *Cbs*^{-/-} mice (Fig. 2). However, as the animals aged, we did start to notice some loss of hair around the eye, suggesting that it might not be quite as effective as MRD.

Effects of BSD on weight gain, fat mass, lean mass and bone mineral density

Cbs^{-/-} mice on RD weighed significantly less than the control mice at all ages from weaning (day 30) to 240 days of age (Gupta and Kruger 2011). To examine the potentially beneficial effects of a BSD, we compared weight gain of *Cbs*^{-/-} and *Cbs*^{+/-} mice on BSD with our previously published growth data for mice on a RD and MRD (Gupta et al 2014) (Fig. 3). We observed a significant increase in weight gain in the male *Cbs*^{-/-} BSD cohort compared to the *Cbs*^{-/-} RD cohort, but the increase was less than that observed in male *Cbs*^{-/-} mice on MRD. We observed no beneficial effect of BSD on female *Cbs*^{-/-} mice. Unexpectedly, in both male and female *Cbs*^{+/-} mice, we observed inhibition of weight gain in the BSD group although not as significant in males as observed in MRD. From these experiments, we conclude that BSD has modest beneficial effect on weight gain in male *Cbs*^{-/-} mice, and a negative effect on both male and female *Cbs*^{+/-} mice.

DEXA was used to examine both fat mass and lean mass at the end of the experiment. Male *Cbs*^{-/-} mice showed statistically significant gain in fat mass, but the increase was not as robust as observed in male *Cbs*^{-/-} mice on MRD. In *Cbs*^{-/-} female mice, no significant difference in either fat mass or lean mass between RD and BSD mice was observed (Fig. 4A, B). However, in *Cbs*^{+/-} control animals, we did observe a statistically significant decrease in lean mass in both male and female mice (22.5 g vs. 19.9 g, $p < 0.003$, male; 17.3 g vs. 14.7 g, $p < 0.003$, female). This suggests that the decreased weight gain observed in the growth curves of *Cbs*^{+/-} is primarily driven by the loss of lean mass.

DEXA was also used to determine if BSD, like MRD, could increase bone mineral density in *Cbs*^{-/-} mice (Fig. 4C). No significant increase in bone mineral density was observed in either male or female *Cbs*^{-/-} mice on BSD compared to RD. However, in both male and female *Cbs*^{+/-} mice, bone mineral density was reduced in the BSD group compared to the RD group ($P<0.005$ and $P<0.006$, respectively).

Betaine supplementation causes increased liver lipid accumulation, SCD-1, and BHMT expression in CBS deficient mice

Liver pathology was examined in all animals at the end of the experiment. Both *Cbs*^{-/-} and *Cbs*^{+/-} mice on BSD had a high frequency of fatty livers as evidenced by the accumulation of lipid vacuoles (Supplementary Fig. 1). *Cbs*^{-/-} mice on BSD exhibited a higher percentage of fatty livers compared to *Cbs*^{+/-} animals (20% vs 65%), but this difference was not statistically significant ($p=0.15$).

Previously, we showed that Stearoyl-coenzyme A desaturase (SCD-1), a key lipogenic enzyme involved in fat regulation (Paton and Ntambi 2009), was down-regulated in *Cbs*^{-/-} mice on RD, and speculated that this might be related to the loss of fat mass in these animals (Gupta and Kruger 2011). Therefore, we examined the effect of BSD on SCD-1 protein levels (Fig. 5). In *Cbs*^{+/-} mice, levels of SCD-1 were not altered by BSD. However, in *Cbs*^{-/-} mice BSD caused a significant increase in SCD-1 protein levels (Fig. 5). We also examined the levels of liver BHMT protein in *Cbs*^{-/-} and *Cbs*^{+/-} mice on RD and BSD. On RD, *Cbs*^{-/-} animals had reduced steady state BHMT levels compared to *Cbs*^{+/-} animals, while BSD caused very large induction of BHMT in both *Cbs*^{-/-} and *Cbs*^{+/-} animals (Fig. 5). These observations were also confirmed by measuring BHMT enzyme activity (Supplementary Fig. 2). These findings show that BSD induces both BHMT protein and activity.

DISCUSSION

The current study was designed to determine the effect of long-term betaine supplementation on the phenotypes of the *Tg-I278T Cbs*^{-/-} mouse model of CBS deficiency. We performed these studies in an identical fashion to our earlier dietary studies examining the effect of N-acetyl L-cysteine supplementation and dietary methionine restriction, thus allowing us to compare results. Our major finding is that while we observed some beneficial effects with betaine, it was not as effective as MRD in reversing the phenotypes of *Cbs*^{-/-} animals. The amount of betaine supplementation used in these studies was more than forty times the dose given to human patients when adjusted for body mass, which seems like a massive amount. However, it should be noted that dose translation between human and mouse are better approximated using normalization to body surface area (Reagan-Shaw et al 2008). When done this way, our dose would be the equivalent to 324 mg/kg/day, within the 160 to 423 mg/kg/day range of that used in human patients (Wilcken et al 1983; Sakamoto and Sakura 2003). Thus we feel that the dose of betaine used here is a reasonable approximation of the human situation.

As expected, we found that a BSD lowered serum tHcy and raised serum methionine in both short and long-term treatments. However, we saw somewhat greater effectiveness of tHcy

lowering in the mice that were only treated for two (2) weeks compared to thirty weeks. This finding is similar to that reported by Maclean et al. for the HO model of CBS deficiency (Maclean et al 2012). In the HO model, the neonatal lethality of the CBS deletion is rescued by a transgene carrying the entire human CBS gene. This transgene is functional, but expresses very little functional CBS protein. The reported levels of tHcy and methionine in this model are similar to the *Tg-I278T Cbs^{-/-}* mice. Unlike our *Tg-I278T Cbs^{-/-}* animals, the HO model has not been reported to exhibit alopecia, fat-loss, or osteoporosis phenotypes. However, it has been reported to have decreased clotting times in a tail bleeding assay and that this effect is reversible by short-term betaine treatment. No differences in clotting behavior have been observed in *Tg-I278T Cbs^{-/-}* mice (Gupta et al 2009; Dayal et al 2012). It is unclear why the two models behave so differently, although strain background differences are a distinct possibility. An important strength of our dietary studies is that we always include a control group of sibling *Cbs^{+/-}* animals, which is critical for proving that certain effects are only due to CBS deficiency and not some other strain difference.

Because of the well characterized age-related phenotypes in *Tg-I278T Cbs^{-/-}* mice, we are in a position to assess the effects of long term (210 day) betaine supplementation. We found that BSD reversed the facial alopecia and low *Scd-1* expression phenotypes, but did not fully correct the weight gain and loss of fat mass, with no effect on bone density phenotype. This result was in contrast to MRD, which corrected all of the assessed phenotypes (Gupta et al 2014). A possible reason for this difference may be related to the levels of tHcy in each of the treatments. MRD lowered tHcy to a mean of 81 μ M vs. 209 μ M for betaine supplemented animals. In previous studies, there was a large difference in the phenotypes of *Tg-hCBS Cbs^{-/-}* compared to *Tg-I278T Cbs^{-/-}* mice, suggesting that there is a tHcy threshold effect, which occurs between 296 μ M and 169 μ M. It is possible that the tHcy levels achieved by betaine may lie quite close to this critical level. Another possibility is that differences in methionine levels may play a role in the effectiveness of the two treatments. *Tg-I278T Cbs^{-/-}* mice on MRD have lower levels of both serum tHcy and methionine, while those on BSD have lower tHcy but elevated methionine. Thus, it is possible that the elevated methionine may be masking some of the beneficial effects of lower tHcy. There are two reports of cerebral edema occurring in human CBS deficient patients treated with betaine and in one MATI/III deficient patient mistakenly treated with betaine, suggesting that extremely elevated methionine maybe detrimental (Yaghmai et al 2002; Devlin et al 2004). However, in another study following CBS deficient patients for a total of 825 patient years, it was found that betaine was a safe treatment (Yap et al 2001). To our knowledge, the exact relationship between both tHcy and Met with clinical endpoints in CBS deficient patients has not been determined.

At the molecular level, we examined the two proteins, SCD-1 and BHMT, in the liver. Since, genetic deficiency of *Scd-1* is linked to both reduced body adiposity and resistance to diet and genetically induced obesity (Cohen et al 2002), it has been suggested that low SCD-1 levels might be the cause the low fat mass phenotype in *Cbs^{-/-}* animals (Gupta and Kruger 2011). Here, we observed that *Cbs^{-/-}* mice on BSD had elevated SCD-1 levels compared to *Cbs^{-/-}* on RD, but had only a very marginal effect on fat mass. This observation suggests that although SCD-1 levels are influenced by tHcy, elevating SCD-1

levels cannot correct the fat loss phenotype in isolation. With regards to BHMT expression, we found that BSD induces both BHMT protein and enzyme activity in the liver, similar to the findings reported previously (Maclean et al 2012). The mechanism by which betaine increases BHMT protein is not known.

In summary, the studies described here suggest that while dietary betaine supplementation does have some beneficial effects on *Cbs*-deficient mice, it is not as effective as severe methionine restriction. Since compliance with severe methionine restriction is difficult in CBS deficient patients, future studies focusing on combining betaine supplementation with less severe methionine restriction may be useful in finding an optimum balance between these two nutritional strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

The effect of *Cbs* genotype and diet on serum tHcy and methionine (met). The graphs show the mean concentrations of tHcy (**A**) and methionine (**B**). Error bars show the 95% confidence interval (CI). Table on bottom shows the genotype, diet, duration of diet, and the number of animals (N) for each bar.

Fig. 2. Correction of alopecia of *Cbs*^{-/-} animals by BSD. Pictures show a sibling pair of *Cbs*^{-/-} mice, one on RD and the other on BSD, from 60 to 240 days of age. The mouse on the BSD diet is indicated by the asterisk.

Fig. 3.

Comparison of the weight of $Cbs^{-/-}$ and $Cbs^{+/-}$ mice on RD, BSD, and MRD. Male and female $Cbs^{-/-}$ mice (A) and $Cbs^{+/-}$ mice (B) were placed on the indicated diets at 30 days of age and weighed once a month till 240 days of age. The color scheme is: RD blue, BSD green, and MRD red. The symbol at each time point shows the mean weight, with the error bar showing the 95% confidence interval of each curve. At least 10 mice were measured for each data point. An asterisk indicates a $p < 0.001$ for the curve being identical to RD.

Fig. 4. DEXA body composition studies on 240 day-old *Cbs*^{-/-} and *Cbs*^{+/-} mice on RD, BSD, and MRD. Effects of genotype and diet on fat mass (A), lean mass (B), and bone mineral density (C) are shown in male and female mice separately. The dietary treatment and number of animals examined are shown in table on the bottom. All graphs show mean ± 95% CI.

Fig. 5.

The effect of RD and BSD on SCD-1 and BHMT protein levels in liver of *Cbs*^{-/-} and *Cbs*^{+/-} male mice. Top panel shows *Cbs*^{-/-}, while bottom panel shows *Cbs*^{+/-} mice. SCD-1 runs as a two bands, the bottom one missing N-terminal sequences (Heinemann and Ozols 1998). In the BHMT blot, the lower band is a cross-reacting protein of unknown identification.