Published in final edited form as:

Nutrition. 2010 ; 26(11-12): 1170–1175. doi:10.1016/j.nut.2009.10.006.

Methionine-deficient diet induces post-transcriptional down-regulation of Cystathionine β -Synthase

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Abstract

Background—Elevated plasma total homocysteine (tHcy) is a risk factor for a variety of human diseases. Homocysteine is formed from methionine and has two primary metabolic fates: remethylation to form methionine or commitment to the transsulfuration pathway by the action of cystathionine β -synthase (CBS). Here, we have examined the metabolic response in mice of a shift from a methionine-replete (M^+) to a methionine-free (M^-) diet.

Methods/Principle Findings—We found that shifting three-month old C57BL6 mice to a M^- diet caused a transient increase in tHcy, as well as an increase in the tHcy/methionine ratio. Since CBS is a key regulator of tHcy, we examined CBS protein levels and found that within 3 days on methionine-deficient diet, animals had a 50% reduction in the levels of liver CBS protein and enzyme activity. Examination of CBS mRNA and studies of transgenic animals that express CBS from a heterologous promoter indicate that this reduction is occurring post-transcriptionally. Loss of CBS protein was unrelated to intracellular levels of S-adenosylmethionine, a known regulator of CBS activity and stability.

Conclusion/Significance—Our results imply that methionine deprivation induces a metabolic state in which methionine is effectively conserved in tissue by shutdown of the transsulfuration pathway via an S-adenosylmethionine-independent mechanism that signals a rapid down-regulation of CBS protein.

Keywords

Metabolism; Genetics; Amino Acids; Cardiovascular Disease

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Introduction

In mammals, methionine is an essential sulfur-containing amino acid that can only be obtained from the diet. Besides its role in protein synthesis, it is critical because it generates S-adenosylmethionine (AdoMet), the major methyl donor for a variety of biologically important reactions. After donation of its methyl group, AdoMet is converted to S-adenosylhomocysteine (AdoHcy) which in turn is hydrolyzed to form homocysteine. Homocysteine then has two possible metabolic fates: remethylation to form methionine or entry into the transsulfuration pathway to form cysteine. Cysteine can then be used in the production of the major intracellular anti-oxidant glutathione, protein synthesis, and the formation of the osmolyte taurine. Because methionine can be converted to cysteine, addition of cysteine to the diet results in reduced dietary methionine requirements [1,2].

Entry of homocysteine into the transsulfuration pathway is controlled by the cystathionine β -synthase (CBS) enzyme [3]. Regulation of CBS activity is critical in controlling total plasma homocysteine (tHcy), a risk factor for stroke, coronary artery disease, and peripheral vascular disease [4,5]. Humans and mice with mutations in *CBS* have greatly elevated tHcy [6,7], while mice that over-express CBS have reduced tHcy [8]. CBS is known to be regulated at both the transcriptional and the post-transcriptional level. CBS mRNA is abundant in only liver and kidney, although it can be detected at much lower levels in other tissues. Exposure of cultured cells to lectins, redox stress, or glucocorticoids results in increased levels of CBS mRNA [9-11]. The enzyme is also allosterically regulated by the binding of AdoMet to the C-terminal regulatory domain [12]. *In vitro*, AdoMet addition causes a 200-300% increase in enzyme activity by stimulating enzyme turnover ($V_{\max}$) [13]. Recently, it has been reported that AdoMet binding may also affect CBS protein stability [14].

The experiments described here were motivated by a small pilot study in which we found that two of six mice placed on a methionine-free diet for eight days had large elevations in tHcy (data not shown). This finding was unexpected because methionine is the metabolic precursor of homocysteine. In the experiments described below we extend these observations, and in the process have discovered that CBS protein is down regulated post-transcriptionally.

Materials and methods

Mice and diet

All mice used in this study were from the C57BL6 strain and were approximately 12 weeks old when shifted to a methionine-free diet. Six month old female Tg-hCBS *Cbs*^{-/-} mice, which express human CBS under control of the zinc inducible metallothionein promoter, were created as described previously [8]. Tg-S466L mice were created as described [15]. Methionine-free diet was obtained from Harlan Teklad (TD 01300; Madison WI). This diet contains no methionine, 3.5g/kg cystine, 91 mg/kg B₆, 260 mg/kg B₁₂, 2.6 mg/kg folic acid, and 2.5 g/kg choline. Control animals were fed regular mouse diet (TD 2018SX) containing 4.3 g/kg methionine, 3.5 g/kg cysteine, 27 mg/kg B₆, 150 mg/kg B₁₂, 8.4 mg/kg folic acid, and 1.2 g/kg choline. Mice were fed *ad libitum*. All protocols were approved by the Fox Chase Cancer Center Laboratory Animal Facility IACUC.

Metabolite measurements

Blood was collected by retroorbital bleed. Fifty microliters of serum was assessed for amino acids using a Biochrom 30 amino acid analyzer as previously described [8]. For liver measurements, livers were collected fresh, immediately homogenized and soluble extracts were prepared as previously described [6]. Protein concentration in the extract was determined by the Coomassie blue protein assay reagent (Pierce, Rockford, IL) using BSA as a standard.

Three hundred micrograms of protein were then analyzed for amino acids using a Biochrom 30 amino acid analyzer. Amino acids were quantitated by comparing peak area to a known standard using E-Z Chrom Elite 2.0 software. AdoMet and AdoHcy were measured as previously described [16].

CBS protein analysis and enzyme activities

CBS protein was assessed using Western Blot analysis as previously described [6]. Blots were quantified by collecting the chemiluminescent signal directly on an Alpha Imager FluorChem gel documentation system (San Leandro, CA). CBS enzyme activity was measured as previously described [8]. Units were measured as nmoles of cystathionine formed per hour per milligram of protein. Activity was always measured in the presence of 400 μ M AdoMet. For betaine-dependant homocysteine methyltransferase (BHMT), 30 μ g of dialyzed mouse liver extract was added to a reaction mixture containing 2 mM DL-homocysteine, 2 mM betaine, and 10 mM Tris (pH 8.0) for one hour at 37°C. The reaction was stopped by heating at 100°C and the methionine produced was measured using a Biochrom 30 amino acid analyzer.

Methionine synthase activity was determined using spectrophotometric assay as previously described [17]. For each reaction 200 μ g of dialyzed mouse liver extract was used.

Real Time PCR

Mouse livers were harvested and total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instruction. Mouse CBS gene-specific probe and primer sets for quantitative assay were obtained from Applied Biosystems (Foster, CA). Quantitative real time PCR was carried out according to the TaqMan Assay-on-Demand one-step protocol of Applied Biosystems with universal thermal condition. Mouse β -actin primer and probe sets from the same supplier were used as endogenous normalization standard. Each assay was performed in triplicate.

Statistical analysis

When comparing multiple time points to control animals one-way ANOVA was used followed by Dunnett's multiple comparison test using GraphPad Prism 4.0. For comparison between two groups we used a two-sided Student's T-test. Unequal variances were assessed using Bartlett's test for equal variances. P-values less than 0.05 are considered significant. In the text all values are given mean $\pm$ the standard error. Model fitting for either linear or exponential decay was performed using GraphPad Prism 4.0.

Results

Methionine depletion results in transient elevation of tHcy and altered tHcy/methionine ratio

Mice were placed on a methionine-free (M^-) diet and were examined for plasma total homocysteine (tHcy) and methionine after day 3, day 7, day 11, and day 33. Serum methionine levels decreased significantly after only three days on the M^- diet, and then stabilized between day 3 and day 11 before declining further by day 33 (Fig. 1A).

Surprisingly, we observed that animals at day 7 that had unusually high elevations in tHcy (Fig. 1B), with a mean tHcy more than double that of the animals on the control diet (6.9 μ M vs. 16.6 μ M, $P < 0.05$). However, by day 33 tHcy levels had dropped below starting value (6.9 μ M vs. 2.0 μ M, $P < 0.006$). Examination of the tHcy/methionine ratio shows that it is significantly elevated at both the seven and 33 day time point relative to the M^+ control animals (Fig. 1C). From these findings we conclude that a M^- diet induces a transient elevation in serum tHcy and a sustained increase in tHcy/methionine ratio.

Methionine depletion associated with decrease in CBS protein and activity

One possible cause of elevated tHcy and an increased tHcy/methionine ratio is loss of CBS activity [6]. Therefore, we measured the levels of CBS protein in liver that were on M⁻ diet for 3, 7, 11, and 33 days. CBS protein levels decreased within three days after shifting to M⁻ diet and stayed decreased through 33 days (Fig. 2). We did not observe a decrease in actin or two other enzymes examined (Table 1) indicating that loss of CBS was not due to non-specific effects of methionine deficiency. Measurement of CBS enzyme activity in liver extracts from mice on M⁻ diet for 33 days (Table 1) shows that the average CBS activity from the mice supplemented with methionine was 330 units compared to only 158 units from the methionine deficient animals (n=5, P<0.0004). These results confirm that methionine depletion results in loss of endogenous CBS activity and protein.

We also determined the effect of methionine starvation on two other homocysteine metabolizing enzymes, methionine synthase (MS) and betaine-dependent homocysteine methyltransferase (BHMT) in the livers of mice on M⁻ diet for 33 days (Table 1). We did not observe any significant effect of methionine starvation on liver MS activity, but we did observe a 2.5-fold increase in BHMT activity. This increase in BHMT activity appears to occur early in the methionine starvation process, as we saw a similar increase in activity from animals that had only been on M⁻ diet for three days (Table 1). These results show that when confronted with methionine starvation, there is stimulation of the remethylation pathway via upregulation of BHMT and concurrent down-regulation of the transsulfuration pathway via CBS.

Methionine-free diet does not decrease CBS mRNA

We next determined if the down-regulation of CBS protein occurred via reduction in CBS mRNA. Liver CBS mRNA from animals either on a M⁺ diet or on a M⁻ diet for 3, 7, 11, or 33 days was isolated and quantitated using quantitative RT-PCR (Fig. 3). We found that over time the animals on the M⁻ diet had a small increase in the amount of CBS mRNA compared to the M⁺ diet animals, although this did not reach statistical significance. These results imply that the loss of CBS protein observed in the methionine-deficient animals was not due to decreased transcription, but rather due to translational or post-translational mechanisms.

Other effects of long term methionine starvation—After 33 d M⁻ mice weighed 38% less than M⁺ mice (P<0.001) and their livers were 37% smaller (P<0.001). When soluble extracts were made from these livers, the ratio of soluble protein to wet-weight remained the same (data not shown) suggesting that although the livers are smaller, their composition is not grossly altered. M⁻ mice also exhibited hair loss on their backs starting at about 30 d. Examination indicated that the hair was much more brittle than the hair on M⁺ mice (data not shown). The fact that the hair loss was only observed on their backs was likely due to their rubbing against the top of the cage at the point where the water bottle enters the cage. No significant changes in activity or behavior were observed.

Methionine depletion affects human CBS driven by a heterologous promoter

We also examined the effect of methionine-depletion on transgenic mice that are null for mouse CBS and express human CBS cDNA using a zinc inducible metallothionein promoter (*Tg-hCBS Cbs^{-/-}*) [8]. We first measured serum tHcy and methionine when the animals were on M⁺ diet and normal water, i.e., the transgene was uninduced and the animals would be expected to behave as CBS null animals. Consistent with previous studies, we found that the average tHcy was 194 ± 20 μ M, while serum methionine measured 65 ± 6 μ M. We next induced transgene expression while the animals were still on M⁺ diet. Human CBS expression caused the average tHcy to lower to 11.1 ± 6.3 μ M, but there was minimal change in serum methionine (58.4 ± 5.4 μ M). These results confirm that CBS is being expressed in these animals.

We next examined the effect of introducing a M⁻ diet. After one week on M⁻ diet we found that mice had a significant decrease in mean methionine ($52.3 \pm 3.5 \mu\text{M}$ vs. $23.1 \pm 10.3 \mu\text{M}$, $p < 0.002$) and a significant increase in tHcy ($8.6 \pm 2.6 \mu\text{M}$ vs. $17.6 \pm 10.8 \mu\text{M}$, $p < 0.05$) compared to animals on the M⁺ diet (Fig. 4a). The tHcy/methionine ratio rose from 0.16 in the controls up to 0.76 ($P < 0.0007$) in the methionine-deficient animals. These results show that the transgenic animals have similar elevation in serum tHcy and elevated tHcy/methionine ratios as mice expressing endogenous CBS.

We also examined measured CBS protein levels by immunoblot analysis. As shown in Fig. 4b, three of the four mice on M⁻ diet had substantially lower levels of CBS protein than the M⁺ mice. Since the human CBS expressing transgene is driven off a different promoter than endogenous CBS, and has different 5' and 3' untranslated sequences, our results imply that loss of CBS protein due to methionine starvation is likely to be due to post-translational mechanisms, i.e., decreased protein half-life as opposed to decreased translational efficiency.

Loss of CBS does not involve AdoMet-dependent mechanism

Recently, Produva et al. have presented evidence that binding of AdoMet to CBS may be important in regulating protein stability [14]. Therefore, we measured AdoMet as well as other methionine cycle metabolites including AdoHcy, methionine, and cysteine in liver extracts from mice on M⁺ and mice on M⁻ diet for 3-11 days (Fig. 5). We did not observe statistically significant depletion in the levels of any of these metabolites over this period.

We also studied the effect of M⁻ diet on mice expressing human CBS with a S466L mutation. This alteration is known to make the human CBS protein “constitutively” active and not responsive to allosteric stimulation by AdoMet, although it is not known if it no longer binds AdoMet [15,18]. We found that S466L CBS was down-regulated in response to methionine starvation just as the endogenous mouse CBS protein (Fig. 6). This finding, along with the fact that tissue concentrations of AdoMet were not depleted in the methionine-starved animals, suggests that AdoMet is not the key regulator of CBS down-regulation in the methionine-starvation response.

Discussion

In this paper we report the finding that dietary deprivation of methionine in mice causes a post-transcriptional down regulation of CBS. We found that switching to a M⁻ diet, resulted in a 50% decrease in CBS protein and activity. Loss of CBS occurred within 3 days and persisted for at least 33 days. This down-regulation of CBS occurs at the post-transcriptional level. We did not observe any decrease in CBS mRNA in the methionine-depleted animals, but rather a slight increase in mRNA. We also saw CBS down-regulation in transgenic mice expressing a human CBS cDNA as a transgene. Since this transgene construct has a heterologous promoter, and the resulting transcript has heterologous 5' and 3' untranslated regions, it implies that the loss of CBS protein is likely occurring at the post-translational level as opposed to alternations in translation efficiency. A possible explanation is that the rate to translation of the CBS protein is down regulated due to an overall shortage of methionine. However, we do not favor that hypothesis because the control protein, actin, was not affected by methionine depletion. Thus, the simplest explanation for our findings is that CBS protein is down-regulated by alteration in its rate of degradation. Consistent with this idea are studies from *S. cerevisiae* in which the proteosome inhibitor MG132 has been shown to increase steady-state levels of human CBS [19].

The reduction in CBS activity offers a possible explanation for the finding that serum tHcy increases transiently in animals that have been shifted from a M⁺ to a M⁻ diet. Homocysteine is catabolized either by remethylation via the enzymes methionine synthase and betaine-

dependent homocysteine methyltransferase or by entry to the transsulfuration pathway via the action of CBS. We hypothesize that the increase in tHcy occurs because CBS activity is reduced early in response to the switch from a methionine-replete to a methionine-free diet, resulting in the reduction of homocysteine catabolism. The transient nature of the tHcy elevation may be due to decreased liver homocysteine synthesis from methionine as methionine levels decrease. Thus there may be a brief window in which homocysteine catabolism is lower but homocysteine production is not. Alternatively, our finding that BHMT levels increase after shifting to a M⁻ diet may also explain this transient increase. An argument against BHMT being involved is BHMT is already up regulated at 3 days which is prior to the observed elevation in tHcy. Our findings are also consistent with studies in both rats and humans that show that diets that are low in protein paradoxically cause elevated plasma homocysteine [20,21].

One surprising finding was that the levels of AdoMet and SAH found in the liver did not change significantly following methionine starvation. On the surface, this is somewhat at odds to earlier work by Finkelstein et al. performed in rats [22]. In the Finkelstein experiments, rats were given a 1% methionine diet had AdoMet levels three times higher than those given 0.25% methionine diet. However, in the experiments described here we found no difference in liver AdoMet levels in animals with diets having either 0% methionine or 0.43% methionine. A key difference in these two studies is that our “high” methionine diet is actually more similar to their “low” methionine diet. Thus it may be that AdoMet levels go up under conditions of excess methionine, but do not go down below a certain threshold when methionine levels are low. The ability to maintain a threshold AdoMet level may be related to the fact that there is a dramatic decrease in the weight of the liver after 33 days on the M⁻ diet. We speculate that catabolism of the liver is providing the methionine and AdoMet that is necessary to maintain this basal level of AdoMet and thus maintain cellular function.

Prudova *et al.* [14] found that mammalian cells grown in tissue culture media in which homocysteine had been substituted for methionine had reduced levels of CBS protein due to reduced half-life. In that paper they present evidence that AdoMet is a key regulator of CBS protein stability. Our *in vivo* mouse data are not consistent with their conclusion that the binding of AdoMet to CBS is the mechanism for this protein stabilization. Direct measurement of AdoMet levels in the livers of methionine-starved mice did not reveal any differences in the AdoMet concentrations despite clear differences in the amount of CBS protein. In addition, mice expressing a mutant form of human CBS that does not respond to AdoMet stimulation (Tg-S466L) showed an identical down-regulation. Our findings indicate that another mechanism independent of AdoMet is involved in the regulation of CBS stability.

Taken together, our data indicate that the mice are able to down-regulate CBS protein levels prior to large decreases in tissue levels of methionine or AdoMet. This implies that there is a homeostatic maintenance mechanism that responds early to alterations in dietary methionine. Thus we hypothesize that mice may have a specific sensing system (perhaps involving the portal vein) that can determine if methionine uptake is reduced and can provide a signal to the liver to activate a mechanism to reduce CBS protein levels. Since the mechanism for down-regulation of CBS involves protein stability, this process may involve the ubiquitin/proteasome pathway.

Acknowledgments

This work was supported by grants from the National Institutes of Health, HL57299 and CA06927, a grant from the American Heart Association, 0555423U, and a grant from the Pennsylvania Department of Health. We thank Doug Markham and Al Knudson for critical reading of the manuscript. We thank the transgenic facility, the laboratory animal facility, and the DNA sequencing facility of the Fox Chase Cancer Center for their assistance.

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Figure 1. Effect of M⁻ diet on serum methionine (A), serum tHcy (B), and serum tHcy/methionine ratio (C)

C57BL6 mice were placed on M⁻ diet and serum was collected after 3, 7, 11, and 33 days and methionine and tHcy were measured as described in Methods. Control animals (+Met) were maintained on standard methionine containing diet. Bars show SEM for each group with each group having a minimum of seven animals. Single asterisk indicates distribution is significantly different from control at $p < 0.05$.

Figure 2. Depletion of CBS in response to methionine deprivation

(A) C57BL6 animals were fed M⁻ diet for the indicated number of days. Four mice from each time point were analyzed for liver CBS protein by Western blot. (B) Data from (A) were analyzed by densitometry and normalized to actin. Bars show relative CBS levels and error bars show standard deviation. Asterisk indicates P<0.01 compared to M⁺ (day 0) control mice. (C) Eight C57BL6 mice fed either M⁻ or M⁺ diet for 33 days were analyzed for liver CBS by Western blot. (D) Densitometry of results shown in C. Asterisk indicates P<0.0008.

Figure 3. CBS mRNA quantitation by qRT-PCR

C57BL6 Mouse liver mRNA was isolated from animals fed either a M^+ diet or an M^- diet for the indicated number of days and was then analyzed using TaqMan based qRT-PCR as described in Materials and Methods. Results are shown as relative RNA levels normalized to M^+ animals. Error bars show the SEM ($n=5$).

Figure 4. Effect of methionine-free diet on Tg-hCBS Cbs^{-/-} animals

Eight Tg-hCBS Cbs^{-/-} animals were placed either on M⁺ or M⁻ diet with zinc water. (A) After seven days serum was analyzed for tHcy and Methionine. Error bars show SEM (n=4). (B) Western blot analysis of livers of same animals after 10 days. The arrow shows the location of human CBS.

A.

B.

Figure 5. Analysis of methionine-related metabolites in the liver of M^- diet

C57BL6 animals were placed on M^- diet for either 3, 7, or 11 days and free pools of liver amino acids were analyzed. (A) S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) levels were determined as described in Materials and Methods. Error bars show SEM (n=5). (B) Cysteine and methionine in liver lysates. All values are nmoles/mg protein. Error bars show SEM (n=5).

Figure 6. Immunoblot analysis of Tg-S466L expressing mice on M⁺ and M⁻ diet
Mice on either M⁺ or M⁻ diet for two weeks were sacrificed and examined for mouse and human CBS by immunoblot. The transgene status of the mice is indicated above. The S466L human CBS protein runs slightly above the endogenous mouse CBS (mCBS).

Liver enzyme activity**Table 1**

Enzyme activity	+Methionine Diet	−Methionine Diet	P-value
CBS (33d)(n=5)	330 ± 48.0	158 ± 43.8	<0.0004
MS (33d)(n=4)	63.3 ± 5.0	64.8 ± 3.2	0.64
BHMT (33d)(n=4)	92.4 ± 11.4	248.8 ± 41.5	<0.0004
BHMT 3d (n=2)	152 ± 20.5	256 ± 49.3	0.11

* Units are nmoles of product formed per milligram of protein extract per hour