Long-term betaine therapy in a murine model of cystathionine beta-synthase deficient homocystinuria: Decreased efficacy over time reveals a significant threshold effect between elevated homocysteine and thrombotic risk

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ABSTRACT

Classical homocystinuria (HCU) is caused by deficiency of cystathionine β-synthase and is characterized by connective tissue disturbances, mental retardation and cardiovascular disease. Treatment for pyridoxine non-responsive HCU typically involves lowering homocysteine levels with a methionine-restricted diet and dietary supplementation with betaine. Compliance with the methionine-restricted diet is difficult and often poor. Investigating optimization of the efficacy of long-term betaine treatment in isolation from a methionine-restricted diet is precluded by ethical considerations regarding patient risk. The HO mouse model of HCU developed in our laboratory, exhibits constitutive expression of multiple pro-inflammatory cytokines and a hypercoagulative phenotype both of which respond to short-term betaine treatment. Investigation of the effects of long-term betaine treatment in the absence of methionine-restriction in HO HCU mice revealed that the ability of betaine treatment to lower homocysteine diminished significantly over time. Plasma metabolite analysis indicated that this effect was due at least in part, to reduced betaine-homocysteine S-methyltransferase (BHMT) mediated remethylation of homocysteine. Western blotting analysis revealed that BHMT protein levels are significantly repressed in untreated HCU mice but are significantly induced in the presence of betaine treatment. The observed increase in plasma homocysteine during prolonged betaine treatment was accompanied by a significant increase in the plasma levels of TNF-alpha and IL-1beta and reversion to a hypercoagulative phenotype. Our findings are consistent with a relatively sharp threshold effect between severely elevated plasma homocysteine and thrombotic risk in HCU and indicate that the HO mouse model can serve as a useful tool for both testing novel treatment strategies and examining the optimal timing and dosing of betaine treatment with a view toward optimizing clinical outcome.

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1. Introduction

Classical homocystinuria (HCU) is caused by deficiency of cystathionine β-synthase (CBS) (EC 4.2.1.22). This enzyme sits at the branch point between the methionine cycle and transsulfuration and catalyzes the condensation of serine and homocysteine (Hcy) into cystathionine which is subsequently converted to cysteine by cystathionine γ-lyase (CGL) (EC 4.4.1.1) [1]. In humans, HCU is characterized by a range of connective tissue disturbances, mental retardation and a dramatically increased incidence of vascular disorders particularly thromboembolic disease [1]. Cardiovascular complications are the major cause of morbidity in HCU and it has been calculated that an untreated patient with the severest form of this disease has a 27% chance of having a thrombotic event by the age of 15 [2]. Treatment strategies for pyridoxine non-responsive HCU typically attempt to lower plasma and tissue levels of Hcy by a combination of restricting dietary intake of the Hcy precursor methionine and dietary supplementation with trimethylglycine, more commonly referred to as betaine. This latter compound serves as a methyl donor in the remethylation of Hcy to methionine in a reaction occurring almost exclusively in the liver and catalyzed by betaine-homocysteine S-methyltransferase (BHMT) (EC 2.1.1.5) [1]. Early intervention with this treatment can prevent or ameliorate the clinical sequelae of HCU resulting in significantly improved survival and clinical outcome [1,2]. However, compliance with the methionine-restricted diet is difficult and often poor. If the efficacy of betaine treatment could be increased, it is conceivable that strict adherence to the methionine-restricted diet could be relaxed thus constituting a significant improvement in quality of life for individuals with HCU. A major

impediment to achieving this goal is the fact that prolonged experimentation on the pharmacokinetics, dosing and frequency of long-term betaine treatment, in isolation from a methionine-restricted diet, is precluded by ethical considerations regarding patient risk.

Prior work in our laboratory has shown that a previously described *cbs* (−/−) null mouse model of HCU does not recapitulate the human HCU patient response to betaine due to severe liver damage [3]. We have subsequently generated and characterized a transgenic mouse model of HCU, designated HO, that expresses very low levels of the human CBS gene and incurs severely elevated plasma and tissue total homocysteine (tHcy) but does not exhibit hepatic steatosis or fibrosis [4]. The HO mouse model of HCU exhibits a hypercoagulative phenotype and constitutive induction of multiple pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-a) and interleukin-1beta (Il-1b) [4,5]. Crucially, the HO mouse model responds biochemically to the Hcy lowering effects of betaine and one week of this treatment results in significant amelioration of the hypercoagulative phenotype [4] and virtual ablation of most of the pro-inflammatory cytokine expression [5].

In the current study, we sought to determine the effects of long-term betaine treatment in the absence of methionine-restriction upon methionine cycle metabolites, hemostasis and pro-inflammatory cytokine expression in the HO mouse model of HCU. We report here that the Hcy lowering effects of betaine treatment diminish significantly over time in a manner that is consistent at least in part, with decreased BHMT mediated remethylation of Hcy. The increase in plasma tHcy over time was accompanied by a significant increase in the plasma levels of the pro-inflammatory cytokines TNF-a and IL-1b and reversion to a hypercoagulative phenotype. Collectively, our findings are consistent with a relatively sharp threshold effect between severely elevated plasma homocysteine and thrombotic risk in HCU and that the mouse model can serve as a useful tool for examining the optimal dosing and frequency of this treatment with a view towards optimizing clinical outcome.

2. Materials and methods

2.1. Animal studies

The generation of the human transgenic HO mouse model of HCU has been described previously [4]. For the experiments reported here, we used four experimental groups each consisting of either 8 HO or 8 C57BL/6J control mice bred in house. Group one consisted of wild type (WT) mice on normal water. Group two consisted of untreated HO mice. Group three consisted of HO mice treated with betaine for one week and group four consisted of HO mice treated with betaine for 6 weeks. Mice in all groups were male, aged between 3 and 4 months and were maintained on standard chow (LabDietNIH5K67, PMI nutrition international, Brentwood, MO). Male mice were used exclusively in order to conserve females for breeding and to avoid the possible influence of changes in the menstrual cycle upon inflammatory cytokine expression levels. To prevent fighting between non-sibling males, mice were kept in individual cages on a 12 hour light/-dark cycle at a mean temperature of 22 °C. Trimethylglycine (betaine) (Sigma-Aldrich) was administered by dissolving this compound in drinking water (20 g/l) and was supplied ad libitum for one or 6 weeks. Betaine water was replenished twice per week. A paired-feeding design was used to ensure isocaloric intake between all experimental groups and body weights were measured once per week. There was no significant difference in average body weights for any of the experimental groups either before or after completion of the experimental trial. Betaine treatment had no discernible effect on either food consumption or water uptake. Mice used in this experiment typically weighed around 30 g and consumed approximately 3.5 ml of water a day. Thus the typical daily dose of betaine was approximately 2333 mg/kg.

Mice from groups one, two and three were sacrificed after one week and mice from group four were sacrificed after 6 weeks. Upon completion of the study, animals were anesthetized via intraperitoneal injection with sodium pentobarbital and euthanized by exsanguination. Blood was collected from the inferior vena cava and plasma was separated through centrifugation. Liver and kidney tissues were taken from all mice for biochemical and histological analysis at sacrifice. All experiments were approved by the University of Colorado Health Sciences Center institutional animal care and use committee and were performed according to the NIH standards for animal care and use.

2.2. Genotype determinations

Mouse genotypes were determined initially by PCR analysis of genomic DNA obtained from tail snips as described previously [4]. The genotypes of all animals used in this study were confirmed by determination of tHcy levels in plasma samples obtained by non-lethal tail bleeding.

2.3. Biochemical analysis

Plasma tHcy, methionine, total cysteine (tCys), dimethylglycine (DMG), methylglycine (MG), glycine and serine levels were determined from 20 μl plasma samples as described previously [6]. Plasma S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy) levels were determined as described previously [7].

2.4. Histological examination of mouse tissues and assessment of liver injury

Tissues were immersion-fixed overnight in 4% paraformaldehyde in PBS (pH 7.3). Paraffin embedded sections were stained with hematoxylin and eosin to evaluate gross histopathological changes including steatosis and inflammation.

Liver injury was assessed by determining plasma levels of alanine aminotransferase (ALT) activity using an enzyme-coupled assay with lactic dehydrogenase (LDH) as described previously [8].

2.5. SDS-PAGE and Western blotting

Liver samples were homogenized in buffer containing 100 mM KPi, pH 7.4, 1 mM EDTA, and 1:50 (v/v) protease inhibitor cocktail from Sigma. The ratio of liver tissue to lysis buffer was 1 g of liver tissue to 5 ml of lysis buffer. The homogenate was subsequently centrifuged at 4 °C at 20,000 g for 20 min. The supernatant thus formed was used as a crude extract. The protein concentration of crude extracts was determined by the Bradford method using bovine serum albumin as a standard [9].

Denatured proteins were separated by SDS-PAGE using a 9% separating gel with a 4% stacking gel under reducing conditions [10]. Proteins were then transferred onto PVDF membrane using a semi-dry transfer cell (Bio-Rad). Resulting blots were probed with primary antibodies to BHMT (ARP41474_T100; Aviva Systems Biology used at 1:1000 v/v dilution) and β-actin as a loading control (a2228; Sigma used at a 1:10,000 v/v dilution). Signals were detected using a Typhoon 9400 system (Amersham Pharmacia) after incubation with the appropriate Fluorescein- or Texas red-conjugated secondary antibodies (Vector Laboratories) or Alexa Fluor 647-conjugated secondary antibody (Invitrogen). The relative intensities of protein bands were quantified using Quantity One version 4.6.5 software (Bio Rad). Signal intensity from BHMT bands was calculated relative to signal intensity from β-actin in liver.