Free Radical Biology & Medicine 52 (2012) 1716–1726

Contents lists available at SciVerse ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Methionine excess in diet induces acute lethal hepatitis in mice lacking cystathionine γ-lyase, an animal model of cystathioninuria

Hidenori Yamada [a,b], Noriyuki Akahoshi [a,b,c], Shotaro Kamata [d], Yoshifumi Hagiya [d], Takako Hishiki [a], Yoshiko Nagahata [c], Tomomi Matsuura [c], Naoharu Takano [c], Masatomo Mori [b], Yasuki Ishizaki [b], Takashi Izumi [b], Yoshito Kumagai [e], Tadashi Kasahara [d], Makoto Suematsu [a,c], Isao Ishii [a,b,d,*]

[a] Department of Biochemistry, School of Medicine, Keio University, Tokyo 160-8582, Japan
[b] Department of Molecular & Cellular Neurobiology, Department of Medicine & Molecular Science, and Department of Biochemistry, Graduate School of Medicine, Gunma University, Gunma 371-8511, Japan
[c] Suematsu Gas Biology Project, Exploratory Research for Advanced Technology, Japan Science and Technology Agency, Tokyo 160-8582, Japan
[d] Department of Biochemistry, Graduate School of Pharmaceutical Sciences, Keio University, Tokyo 105-8512, Japan
[e] Environmental Medicine Section, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki 305-8575, Japan

ARTICLE INFO

Article history:
Received 2 August 2011
Revised 15 February 2012
Accepted 22 February 2012
Available online 3 March 2012

Keywords:
Antioxidants
Homocysteine
Metabolome
Methionine sulfoxide
Transamination
Transsulfuration

ABSTRACT

Physiological roles of the transsulfuration pathway have been recognized by its contribution to the synthesis of cytoprotective cysteine metabolites, such as glutathione, taurine/hypotaurine, and hydrogen sulfide (H_2S), whereas its roles in protecting against methionine toxicity remained to be clarified. This study aimed at revealing these roles by analyzing high-methionine diet-fed transsulfuration-defective cystathionine γ-lyase-deficient (*Cth*$^{-/-}$) mice. Wild-type and *Cth*$^{-/-}$ mice were fed a standard diet (1×Met: 0.44%) or a high-methionine diet (3×Met or 6×Met), and hepatic conditions were monitored by serum biochemistry and histology. Metabolome analysis was performed for methionine derivatives using capillary electrophoresis– or liquid chromatography–mass spectrometry and sulfur-detecting gas chromatography. The 6×Met-fed *Cth*$^{-/-}$ (not 1×Met-fed *Cth*$^{-/-}$ or 6×Met-fed wild type) mice displayed acute hepatitis, which was characterized by markedly elevated levels of serum alanine/aspartate aminotransferases and serum/hepatic lipid peroxidation, inflammatory cell infiltration, and hepatocyte ballooning; thereafter, they died of gastrointestinal bleeding due to coagulation factor deficiency. After 1 week on 6×Met, blood levels of ammonia/homocysteine and hepatic levels of methanethiol/3-methylthiopropionate (a methionine transamination product/methanethiol precursor) became significantly higher in *Cth*$^{-/-}$ mice than in wild-type mice. Although hepatic levels of methionine sulfoxide became higher in 6×Met-fed wild-type mice and *Cth*$^{-/-}$ mice, those of glutathione, taurine/hypotaurine, and H_2S became lower and serum levels of homocysteine became much higher in 6×Met-fed *Cth*$^{-/-}$ mice than in wild-type mice. Thus, transsulfuration plays a critical role in the detoxification of excessive methionine by circumventing aberrant accumulation of its toxic transamination metabolites, including ammonia, methanethiol, and 3-methylthiopropionate, in addition to synthesizing cysteine-derived antioxidants to counteract accumulated pro-oxidants such as methionine sulfoxide and homocysteine.

Introduction

Methionine is an essential amino acid in mammals; however, it is the most toxic among the constituent amino acids of proteins, whether expressed in terms of percentage of the diet or an increase over the requirement (0.5–0.6% of the diet). When the methionine level in voluntary food intake is maintained at four to six times the estimated requirement, food intake and growth are suppressed [1]. Excessive methionine intake causes oxidative/nitrosative injuries in the liver, hepatic encephalopathy, altered erythrocyte morphology, and the resultant splenic hemosiderosis in rats [1–4]. Some metabolic change or adaptation may occur because supplementation of serine or glycine could restore growth after a few days of feeding on a high-

Abbreviations: Ac-Met sulfoxide, *N*-α-acetyl-L-methionine sulfoxide; ALP, alkaline phosphatase; ALT, alanine aminotransferase; APTT, activated partial thromboplastin time; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CE–MS, capillary electrophoresis–mass spectrometry; CBS, cystathionine β-synthase; CK, creatine kinase; CTH, cystathionine γ-lyase; DIC, disseminated intravascular coagulation; GSH, reduced glutathione; GSSG, oxidized glutathione; KMTB, 2-keto-4-methylthiobutyrate; LC, liquid chromatography; LDH, lactate dehydrogenase; MAT, methionine adenosyltransferase; MTP, 3-methylthiopropionate; PT, prothrombin time; ROS, reactive oxygen species; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; TBARS, thiobarbituric acid-reactive substrates; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

* Corresponding author at: Department of Biochemistry, Graduate School of Pharmaceutical Sciences, Keio University, Tokyo 105-8512, Japan. Fax: +81 3 5400 2671.
E-mail address: isao-ishii@umin.ac.jp (I. Ishii).

0891-5849/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2012.02.033

methionine diet in rats [1] and patients with innate hypermethioninemia by methionine adenosyltransferase (MAT)[1] I/III deficiency are asymptomatic [5].

Methionine is normally metabolized in the liver to cysteine via the methionine cycle and transsulfuration [6]. Serine is a substrate of the transsulfuration enzyme cystathionine β-synthase (CBS), and glycine is a precursor of serine; therefore, supplementation of serine or glycine may lead to effective clearance of methionine by activating CBS. On the other hand, excessive methionine can be converted via transamination to 2-keto-4-methylthiobutyrate (KMTB), 3-methylthiopropionate (MTP), and methanethiol (CH_3SH; methyl mercaptane), all of which are extremely toxic [1,7,8]. Increased amounts of methionine transamination products are present in plasma and urine of patients with severe hypermethioninemia due to either MAT I/III deficiency or CBS deficiency [5]. Poisonous ammonia (NH_3) is also generated from glutamate, a transamination product from a reaction between methionine and α-ketoglutarate. There is a significant association between the severity grades of hepatic encephalopathy and serum concentrations of methionine, KMTB, NH_3, or CH_3SH-mixed disulfides [7]. Excess CH_3SH/NH_3 causes hepatic encephalopathy/coma in experimental animals [9]. Meanwhile, *S*-adenosylmethionine (SAM; a MAT product) is sold as a nutritional supplement to improve liver function and depression under the marketing name SAM-e [10]. The pathophysiology of methionine-induced liver toxicity remains to be elucidated.

To date, five genetic mouse models with targeted deletion of methionine cycle/transsulfuration enzymes (except multiple SAM-dependent methyltransferases) have been reported: mice lacking *S*-adenosylhomocysteine (SAH) hydrolase [11], methionine synthase [12], MAT I/III [13], CBS [14], and cystathionine γ-lyase (CTH) [15,16]; the first two of these are embryonically lethal. MAT I/III-deficient mice are apparently normal but predisposed to liver injury [13] and spontaneously develop liver tumors [17], whereas CBS-deficient ($Cbs^{-/-}$) mice (a model of homocystinuria; OMIM ID: 236200) suffer from severe hepatic dysfunction/steatosis and most die before 4 weeks of age [14,18]; importantly, both mice display hypermethioninemia [13,18]. Here we investigated the pathogenesis upon excessive methionine intake utilizing transsulfuration-defective CTH-deficient ($Cth^{-/-}$) mice, a model of cystathioninuria (OMIM ID: 219500), which develop apparently normally and are free of hypermethioninemia and hepatic dysfunction [16].

Materials and methods

Animals

CTH heterozygous ($Cth^{+/-}$) mice were generated and backcrossed for 10 generations to a C57BL/6J inbred strain (CLEA Japan, Tokyo, Japan) [16]. CBS heterozygous ($Cbs^{+/-}$) mice (B6.129P2-Cbs^{tm1Unc}/J) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and backcrossed for 10 generations to C57BL/6J [16,18]. The $Cth^{+/-}$ (or $Cbs^{+/-}$) males and females (backcrossed 10 generations; N10) were bred to obtain $Cth^{-/-}$ (or $Cbs^{-/-}$) mice. Mice were allowed free access to CE-2 standard dry rodent diet (1×Met; CLEA Japan), which contains 0.44% methionine, or to high-methionine diets (3×Met (CE-2 + 0.88% Met) and 6×Met (CE-2 + 2.20% Met)). All procedures involving animals were approved by the Animal Care Committee of Keio or Gunma University.

Measurement of amino acid contents and biochemical parameters in serum

Levels of total homocysteine and other free amino acids were measured as described previously [16,18]. Levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, lactate dehydrogenase (LDH), creatine kinase (CK), albumin, blood urea nitrogen (BUN), creatinine, and NH_3 were measured using Dri-Chem 4000 (Fuji Film). Levels of glucose and triglyceride were measured with enzymatic assay kits (Wako, Osaka, Japan). Total antioxidant capacity (to reduce Cu^{2+} to Cu^{+} [16]) was measured using an antioxidant assay kit (Cayman Chemical, Ann Arbor, MI, USA). Levels of lipid peroxidation and protein carbonyls were examined using a TBARS (thiobarbituric acid-reactive substrates) assay kit and a protein carbonyl fluorometric assay kit, respectively (Cayman Chemical). Corticosterone levels were determined using an AssayMax corticosterone ELISA kit (Assay Pro, St. Charles, MO, USA).

Histochemistry

Tissues were quickly dissected out from ether-anesthetized mice, fixed in 10% formalin, and embedded in paraffin. Five-micrometer sections were cut with a cryostat, deparaffinized, and stained with Mayer's hematoxylin/eosin Y or Prussian blue/nuclear fast red for detection of splenic hemosiderosis (focal iron accumulation) [19]. A terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed using an in situ apoptosis detection kit (Takara Bio, Tokyo, Japan) [16]. For oil red O staining, livers were fixed overnight in 4% paraformaldehyde and cryoprotected in 30% sucrose. After sinking, they were embedded, frozen, sectioned (5 μm), and stained with Mayer's hematoxylin/oil red O solutions [18]. The slides were examined with a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan) fitted with Plan Apo 20× or 40× lens (Nikon).

Measurement of bleeding times, blood counts, and coagulation tests

Tail bleeding time was determined by removing 2 mm of the distal tail and immersing the tail in 37 °C isotonic saline. Complete cessation of bleeding was defined as the bleeding time with a cut-off of 6 min. Blood samples were quickly collected from the heart and two types of plasma pools were prepared: one treated with sodium heparin (final 1 U/ml) and the other with sodium citrate (final 3.8%). Blood counts were performed using the former pool with a MEK-6038 automatic hematology analyzer (Nihon Koden, Tokyo, Japan). Prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen levels were measured using the second type of pool with a COAG2V blood coagulation analyzer (A&T Corp., Kanagawa, Japan). A cut-off of 50 mg/dl was set for low fibrinogen levels.

Hepatocyte culture and cell viability assay

Hepatocytes were prepared using collagenase perfusion [16] and dispersed onto collagen I-coated dishes/slides. After overnight incubation with serum (10%)-containing Dulbecco's modified Eagle's medium (DMEM), the cells were washed twice and incubated with methionine/cystine-free DMEM with or without various concentrations of L-methionine, DL-homocysteine, L-cysteine, or DL-methionine sulfoxide. The cells in 96-well dishes were subjected to a CellTiter-Glo luminescence cell viability assay (Promega) to measure cellular ATP levels. The cells on eight-well slides were stained with a GFP-Certified apoptosis/necrosis detection kit (Enzo Life Sciences, Plymouth Meeting, PA, USA), followed by mounting with ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). Numbers of annexin V–EnzoGold-stained apoptotic cells, 7-amino actinomycin D-stained necrotic cells, and DAPI-positive live adherent cells were counted (>1000 total cells in each condition) using BZ-H1C cell counting software (Keyence).

Metabolome analysis

Livers were quickly dissected out, snap-frozen in liquid nitrogen, and stored at −80 °C until use. Hepatic levels of most methionine