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Original Contribution

A critical life-supporting role for cystathionine γ-lyase in the absence of dietary cysteine supply

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ABSTRACT

This study examined the important relationship between cystathionine γ-lyase (CSE) functionality and cysteine supply for normal growth and life span. Mice with a targeted deletion of the CSE gene (CSE-KO) were fed a cysteine-limited diet and their growth and survival patterns as well as levels of cysteine, homocysteine, glutathione, and hydrogen sulfide (H_2S) were measured. CSE-KO mice fed a cysteine-limited diet exhibited growth retardation; decreased levels of cysteine, glutathione, and H_2S; and increased plasma homocysteine level. However, histological examinations of liver did not reveal any abnormality and plasma levels of aspartate aminotransferase, alanine aminotransferase, and albumin were normal in these animals. No CSE-KO mice survived after 12 weeks of feeding with the cysteine-limited diet. Supplementation of H_2S to the CSE-KO mice failed to reverse the aforementioned abnormalities. On the other hand, supplementation of cysteine in the drinking water of the CSE-KO mice significantly increased plasma cysteine and glutathione levels. This eventually led to an increase in body weight and rescued the animals from death. In conclusion, CSE is critical for cysteine biosynthesis through the transsulfuration pathway and the combination of CSE deficiency and lack of dietary cysteine supply would threaten life sustainability.

Cysteine is an intermediate for the synthesis of glutathione, taurine, and sulfate. Several in vivo and in vitro studies have demonstrated that the availability of cysteine is the rate-limiting factor for glutathione (GSH) biosynthesis, in which cysteine serves as a substrate through reactions of glutamate synthase and glutathione ligase [1]. Taurine is synthesized by the reduction and decarboxylation of cysteine, which has numerous biological functions, including its antioxidant role. Furthermore, the free sulfhydryl group present in cysteine is considered crucial for the biological functions of proteins.

As a semiessential amino acid, cysteine is not exclusively provided by the diet. In mammals, cysteine is synthesized in a limited and insufficient amount by the transsulfuration pathway, which involves the transfer of sulfur from methionine to serine to form cysteine. Cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), two pyridoxal 5′-phosphate-dependent enzymes, are involved in the metabolism of cysteine. Deficiency of CSE expression decreases the levels of cysteine, glutathione, taurine, and hydrogen sulfide (H_2S) [2,3]. Mutation or deficiency of CBS [4] and CSE [5] results in increased homocysteine and cystathionine, leading to mental retardation, thromboembolism, fatty liver, and cardiovascular complications with early and aggressive atherosclerosis [6–8]. Rao et al. [2] reported that freshly isolated hepatocytes incubated with L-[^{35}S]methionine showed accumulation of ^{35}S-labeled cysteine, GSH, and sulfate. Treatment of hepatocytes with 2 mM propargylglycine (PPG) to inhibit CSE activity decreased 93% of GSH, 88% of sulfate, and 89% of cysteine levels. In a rat in vivo experiment, the inhibition of CSE by PPG impaired cysteine biosynthesis. Subsequently, liver protein degradation is increased to keep cysteine concentration within normal range [9]. Intermediates of the urea cycle such as ornithine, citrulline, and arginine were also significantly increased and the administration of *N*-acetylcysteine to PPG-treated rats reversed the changes in blood urea level [9]. However, using PPG to assess the role of CSE in cysteine metabolism has suffered from nonspecific effects of PPG and incomplete inhibition of CSE activities. Some other enzymes, such as alanine aminotransferase [10] and aspartate aminotransferase [11], are also affected by PPG albeit to a lesser extent. Furthermore, all aforementioned studies focused on the elimination or inhibition of CSE without considering the exogenous uptake of cysteine. Therefore, the relationship between cysteine supplementation and CSE functionality has been unclear.

The CSE gene is not expressed until after birth, and as such human infants may require cysteine supplementation because of the absence of an alternative pathway for cysteine synthesis. The required cysteine is supplemented by human milk, which is relatively high in cysteine and low in methionine content [12]. Sufficient cysteine supply through the diet not only is essential for infants, it may also become a serious concern in cases in which the CSE enzyme of the

Abbreviations: CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; CSE-KO, CSE gene knockout; GSH, glutathione; PPG, propargylglycine; WT, wild type.

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transsulfuration pathway is lacking or malfunctioning. Clinical studies have shown that people in Western countries consume suboptimal levels of cysteine in their diet although they are superoptimal in calories [13]. Thus, the importance of dietary supplementation of cysteine in the face of deficient CSE activity should be better clarified.

In this study, the critical role of CSE in cysteine metabolism was examined using a mouse strain with a targeted deletion of the CSE gene, CSE-KO mice. To manipulate the amount of dietary cysteine supply, a casein-based diet was used, which contains only trace amounts of cysteine. The growth and survival patterns as well as changes in products of the transsulfuration pathway were examined in CSE-KO mice fed the cysteine-limited diet. Finally, cysteine rescue experiments were conducted to confirm the role of dietary cysteine and the related mechanisms were investigated.

Materials and methods

Animal use and treatment

In-house-bred male CSE-KO mice [3] were used in this study. All animal experiments were conducted in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996) and approved by the Animal Use Committee of Lakehead University (Canada). All animals were housed in a controlled environment with unlimited access to food and water on a 12-h light–dark cycle. The mice were initially fed a standard rodent chow diet (22% protein, 5% fat, 63% carbohydrate, 0.43% cystine, and 0.42% methionine; Rodent RQ 22–5; Zelgler Bros., Gardners, PA, USA) until 6 weeks of age and then switched to cysteine amino acid-limited casein-based rodent diet (17.3% protein, 5.2% fat, 63.5% carbohydrate, 0.05% cystine, and 0.75% methionine; Cat. No. TD.05230; Harlan Teklad, Madison, WI, USA). The two diets are isocaloric but differ mainly in their cysteine content. Mice were allowed free access to this diet and water for 10 weeks. In a parallel study, the mice fed the casein diet were supplemented with DL-cysteine in the drinking water (1 mg/ml) for 10 weeks. After the 10-week treatment period, all the mice were sacrificed and their body weight, organ weights (liver, white adipose fat, kidney, heart, and stomach), and blood glucose levels were measured.

H_2S production rate

H_2S production rate was measured as described previously [14]. Briefly, liver tissues were isolated from mice and homogenized in 50 mM ice-cold potassium phosphate buffer (pH 6.8). The tissue homogenates were first incubated with L-cysteine (10 mM) for 90 min at 37 °C, and then trichloroacetic acid was added to stop the reaction. The level of methylene blue generated by the interaction of H_2S with *N,N*-dimethyl-*p*-phenylenediamine sulfate was determined at 670 nm with a FLUOstar OPTIMA microplate spectrophotometer (BMG Labtech, Germany).

Liver histology and function analyses

Mouse livers were dissected out and fixed in 4% paraformaldehyde for 18 h and then cryoprotected in 30% sucrose/phosphate-buffered saline at 4 °C for 3 days. The samples were embedded in optimal cutting temperature compound (OCT; Calbiochem), frozen, and sectioned with a cryostat in 10-μm-thick sections and then picked up on poly-L-lysine-coated slides. The sections were stained with hematoxylin and eosin in the standard fashion. Sections were viewed under light microscopy. At least two arbitrarily chosen fields per tissue cross section and four sections per tissue sample were examined. Plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and albumin were measured using a Roche Modular P800 (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's procedures, with reagents provided by the same company.

Plasma cysteine and homocysteine measurements

Mouse plasma was collected at the end of the experiment and its cysteine concentration was measured according to the spectrophotometric method of Gaitonde [15] with minor modifications. Briefly, 450 μl of the plasma was transferred to a centrifuge tube containing 140 μl of perchloric acid (6%, v/v) and centrifuged at 12,000 *g* for 10 min. A 500-μl sample of this supernatant was transferred to another centrifuge tube containing 500 μl of 1 M glacial acetic acid, 200 μl of 1 M HCl, and 300 μl of ninhydrin reagent (0.25 g ninhydrin dissolved in 6 ml of 1 M glacial acetic acid). The pH was adjusted to between 0.3 and 0.9 with 1 M HCl. This reaction mixture was mixed thoroughly and heated in boiling water (100 °C) for 10 min to develop the pink product. The mixture was then rapidly cooled in cold water (4 °C). This pink product was stabilized by diluting the reaction mixture to 1 ml with 95% ethanol and the absorbance was read at 560 nm. A reagent blank without cysteine was prepared under the same conditions. The plasma cysteine concentration was calculated from the standard curve (100 μM to 1 mM cysteine). Plasma homocysteine was measured as described before [16].

Glutathione assay

Total GSH concentrations were measured in plasma, liver, and heart using a commercial GSH assay kit (Cayman Chemical). The sulfhydryl group of GSH reacts with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB; Ellman's reagent) and produces yellow-colored 5-thio-2-nitrobenzoic acid (TNB). Measurement of the absorbance of TNB at 412 nm provides an estimation of total glutathione in the samples [17]. Briefly, 100 μl of plasma/tissue homogenate was added to an equal volume of the metaphosphoric acid and then centrifuged at 2000 *g* for 2 min to remove protein. Then, 50 μl of 4 M triethanolamine was added for each milliliter of homogenate to increase the pH. For total GSH assay, 50 μl of sample was added to 150 μl of a reaction mixture containing 0.4 M 2-(*N*-morpholino)ethanesulfonic acid, 0.1 M phosphate (pH 6.0), 2 mM EDTA, 0.24 mM NADPH, 0.1 mM DTNB, and 0.1 unit of glutathione reductase. The reaction was carried out for 25 min, and then total glutathione was determined by absorbance at 412 nm using GSSG (oxidized glutathione) as the standard.

Statistical analysis

Data are presented as means ± SEM. The statistical analyses for the data were carried out by using Excel 2007 (Microsoft, Redmond, WA, USA) with Student's *t* test, and the significance level was set at $p<0.05$.

Results

Growth pattern and mortality of CSE-KO mice on cysteine-limited diet

The body weight of CSE-KO mice was significantly decreased during the 10-week casein diet feeding, whereas wild-type (WT) mice showed increased body weight with the same feeding treatment (Fig. 1A). The decrease in body weight of CSE-KO mice started from the first week after switching to the casein diet and continued until the end of the experiment or death of the mouse. The number of living CSE-KO mice significantly decreased and reached 92% mortality by the end of 10 weeks of the casein diet feeding, whereas WT mice appeared healthy without any mortality (Fig. 1B). Water intake by CSE-KO mice was significantly higher than that of WT mice and there was no significant difference in the food intake between the two