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Sulfur amino acid metabolism in Zucker diabetic fatty rats

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ABSTRACT

The present study was aimed to investigate the metabolomics of sulfur amino acids in Zucker diabetic fatty (ZDF) rats, an obese type 2 diabetic animal model. Plasma levels of total cysteine, homocysteine and methionine, but not glutathione (GSH) were markedly decreased in ZDF rats. Hepatic methionine, homocysteine, cysteine, betaine, taurine, spermidine and spermine were also decreased. There are no significant difference in hepatic *S*-adenosylmethionine, *S*-adenosylhomocysteine, GSH, GSH disulfide, hypotaurine and putrescine between control and ZDF rats. Hepatic SAH hydrolase, betaine-homocysteine methyltransferase and methylene tetrahydrofolate reductase were up-regulated while activities of gamma-glutamylcysteine ligase and methionine synthase were decreased. The area under the curve (AUC) of methionine and methionine-d_4 was not significantly different in control and ZDF rats treated with a mixture of methionine (60 mg/kg) and methionine-d_4 (20 mg/kg). Moreover, the AUC of the increase in plasma total homocysteine was comparable between two groups, although the homocysteine concentration curve was shifted leftward in ZDF rats, suggesting that the plasma total homocysteine after the methionine loading was rapidly increased and normalized in ZDF rats. These results show that the AUC of plasma homocysteine is not responsive to the up-regulation of hepatic BHMT in ZDF rats. The present study suggests that the decrease in hepatic methionine may be responsible for the decreases in its metabolites, such as homocysteine, cysteine, and taurine in liver and consequently decreased plasma homocysteine levels.

1. Introduction

Although a recent study suggested that elevated plasma homocysteine is a marker, rather than a cause, of atherosclerotic disease [1], elevated plasma homocysteine levels may be a risk factor for cardiovascular disease [2]. A recent meta-analysis and Mendelian randomization analysis performed among 4011 cases and 4303 controls provided a strong evidence for a causal association of homocysteine levels with the development of type 2 diabetes [3]. Homocysteine is an intermediate of the sulfur amino acid metabolic pathway. Altered homocysteine metabolism, particularly in the liver, is a major cause of hyperhomocysteinemia (HHcy). It has been demonstrated that total homocysteine in blood is either lower or normal in type 2 diabetes patients, whereas an increased homocysteine level was observed only in the presence of nephropathy [4].

Zucker diabetic fatty (ZDF) rats, an experimental type 2 diabetic model, have a defective leptin receptor and a decreased plasma total homocysteine level, which appears to be due to increased homocysteine clearing enzymes, such as cystathionine beta-synthase (CβS) and betaine-homocysteine methyltransferase (BHMT) [5]. Our previous studies showed that changes in plasma homocysteine level in obese or diabetic conditions were dependent on the type of experimental animal model used [6–8]. Plasma total homocysteine concentration increased by 1.6-fold in obese mice fed a high-fat diet for 12 weeks [7], but was 40% lower in db/db mice, obese type 2 diabetic animals with a G-to-T point mutation of the leptin receptor [6]. In contrast, plasma homocysteine levels were similar between control rats and non-obese type

Abbreviations: HHcy, hyperhomocysteinemia; ZDF, Zucker diabetic fatty; CβS, cystathionine β-synthase; BHMT, betaine-homocysteine methyltransferase; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; MS, methionine synthase; GSH, glutathione; CDO, cysteine dioxygenase; GCL, γ-glutamylcysteine ligase; GSSG, glutathione disulfide; MPG, *N*-(2-mercaptopropionyl)-glycine; EDTA, ethylenediaminetetraacetic acid; TCEP, tris-(2-carboxyethyl)-phosphine hydrochloride; SBD-F, ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid; DTNB, 5,5-dithiobis-2-nitrobenzoic acid; MAT, adenosyltransferase; MTHFR, methylenetetrahydrofolate reductase; SAHH, *S*-adenosylhomocysteine hydrolase; GCLC, γ-glutamylcysteine ligase catalytic subunit; GCLM, γ-glutamylcysteine ligase modifier subunit; LC, liquid chromatography; ESI, electrospray ionization; MS, mass spectrometry; HPLC, high performance liquid chromatography; SD, standard deviation.

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diabetic Goto–Kakizaki rats [8]. Interestingly, db/db mice exhibited decreases in hepatic and plasma sulfur amino acids and their metabolites, including methionine, homocysteine, cysteine, *S*-adenosylmethionine (SAM), *S*-adenosylhomocysteine (SAH) and hypotaurine. These results raise the possibility that depletion of methionine, a precursor for all sulfur-containing intermediates in the sulfur amino acid metabolic pathway, may be responsible in part for decreased homocysteine, as well as decreased cysteine, SAM, SAH and hypotaurine.

The liver plays a significant role in the regulation of plasma homocysteine levels because of its full complement of enzymes involved in sulfur amino acid metabolism [9]. Dynamic processes regulate sulfur amino acid homeostasis [10,11]. Homocysteine intersects two competitive metabolic pathways: (i) remethylation to methionine by two independent enzymes, methionine synthase (MS) or BHMT, and (ii) transsulfuration to cysteine via cystathionine by the consecutive actions of CβS and cystathionine γ-lyase (Fig. 1). Cysteine is metabolized to taurine, glutathione (GSH), and inorganic sulfate. Cysteine dioxygenase (CDO) catalyzes the oxidation of cysteine to cysteine sulfinate, which is converted to hypotaurine by cysteine sulfinate decarboxylase. Hypotaurine is non-enzymatically transformed into taurine. Meanwhile, γ-glutamylcysteine ligase (GCL) and GSH synthetase consecutively mediate GSH synthesis.

To expand on previous studies, the present study aimed to investigate the metabolomics of sulfur amino acids in ZDF rats, an obese type 2 diabetic animal model. Hepatic levels and activities of enzymes involved in sulfur amino acid metabolism were investigated to determine whether differences in sulfur amino acid metabolic profiles reflected changes in the activities of their metabolizing enzymes. Moreover, we performed plasma kinetic analysis of methionine and homocysteine in ZDF rats intravenously treated with a mixture of methionine and methionine-d_4. The present study suggests that the decrease in hepatic methionine may be responsible for the decreases in its metabolites, such as homocysteine, cysteine, and taurine in liver and consequently decreased plasma homocysteine levels in ZDF rats.

2. Materials and methods

2.1. Chemicals and antibodies

Chemicals including amino acid standard, DL-homocysteine, L-cysteine, L-methionine, GSH, GSH disulfide (GSSG), GSH reductase, SAH, SAM, taurine, hypotaurine, L-serine, putrescine, spermidine, spermine, cystathionine, betaine hydrochloride, hexamethylenediamine, adenosine, homocysteine thiolactone, *N*-(2-mercaptopropionyl)-glycine (MPG), *O*-phthaldehyde, dansyl chloride, ATP, $MgCl_2$, ethylenediaminetetraacetic acid (EDTA), pyridoxal-5-phosphate, 5-methyl-tetrahydrofolate, hydroxocobalamin, 1-heptanesulfonic acid, trichloroacetic acid, tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP), ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid (SBD-F), 2-mercaptoethanol, dithiothreitol, chloroform, KCl, HCl, sodium acetate, methanol, acetonitrile, ferrous ammonium sulfate, 5,5-dithiobis-2-nitrobenzoic acid (DTNB), hydroxylamine, NAD, 2-vinyl pyridine, sodium hydroxide, sodium carbonate and boric acid were purchased from Sigma–Aldrich (St. Louis, MO). Perchloric acid, sodium dihydrogenphosphate and disodium hydrogenphosphate were purchased from Junsei Chemical (Tokyo, Japan). Deuterium labeled betaine-d_9 hydrochloride was purchased from CDN isotopes Inc. (Quebec, Canada). Deuterium labeled methionine-d_3 and methionine-d_4 were purchased from Cambridge Isotope Laboratories (Andover, MA). Antibodies against methionine adenosyltransferase (MAT)I/III, methylene tetrahydrofolate reductase (MTHFR) and α-tubulin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and the anti-SAH hydrolase (SAHH), anti-MS and anti-CDO antibodies were purchased from Abcam (Cambridge, UK). The anti-BHMT antibody was purchased from Everest Biotech (Oxfordshire, UK). The anti-GCL catalytic subunit (GCLC) and anti-GCL modifier subunit (GCLM) antibodies were purchased from NeoMarker Inc. (Fremont, CA). The anti-CβS antibody was kindly provided by Dr. Matherly (Wayne State University School of Medicine, Detroit, MI). Horseradish peroxidase-conjugated goat anti-rabbit and rabbit anti-goat antibodies were obtained from BioRad Laboratories (Hercules, CA). Enhanced chemiluminescence detection reagent solutions were purchased from Thermo scientific (Rockford, IL). All chemicals and solvents used in this study were reagent grade or higher.

2.2. Animal experiments

This study used male ZDF rats (ZDF/Gmi *fa/fa*) aged 5 and 11 weeks and male lean rats (ZDF/Gmi *fa/?*) of the same ages. All animals were maintained at Korea Research Institute of Bioscience and Biotechnology (Ochang, Korea). The rats were housed at 22 ± 2 °C and 50 ± 5% humidity controlled rooms with a 12-h light/dark cycle for at least 1 week prior to experimentation. Laboratory chow and tap water were allowed ad libitum. The rats were euthanized using CO_2. All animal experiments were approved by the Institutional Animal Care and Use Committee, and were performed in accordance with institutional guidelines.

2.3. Preparation of plasma and hepatic samples

Following euthanasia, blood samples were obtained from the abdominal aorta. Plasma was obtained by centrifugation of blood at 10,000 g for 15 min at 4 °C. The supernatant fraction and remaining plasma were stored at −70 °C until analysis. The liver was rapidly removed and homogenized in a 3-fold volume of ice-cold buffer consisting of 0.154 M KCl, 50 mM Tris–HCl, and 1 mM EDTA (pH 7.4). All subsequent steps were performed at 0–4 °C. The liver homogenates were deproteinized in a 3-fold volume of ice-cold methanol to measure methionine, hypotaurine and taurine, or in an equal volume of 10% PCA to measure SAM, SAH, homocysteine, cysteine, GSH, GSSG, putrescine, spermidine and spermine. After centrifugation at 10,000 g for 20 min, the supernatant fraction was collected and refrigerated in −70 °C. The liver homogenates were centrifuged at 10,000 g for 20 min. The supernatant fraction was further centrifuged at 104,000 × *g* for 65 min. The supernatant fraction was collected and refrigerated in −70 °C (the cytosol samples). The total protein concentration was measured using a bicinchoninic acid protein assay kit (Thermo Scientific, IL).

2.4. Determination of sulfur amino acids and their metabolites in liver and plasma

The methionine concentrations were measured using liquid chromatography (LC) triple quadruple electrospray ionization (ESI)/mass spectrometry (MS)/MS and methionine-d_3 as an internal standard. The LC-ESI/MS/MS system consisted of a Shimadzu LC-20AD XR high performance liquid chromatography (HPLC) system (Shimadzu, Tokyo, Japan) and an API 3200 QTRAP® LC–MS/MS system (AB Sciex, Framingham, MA) equipped with a Turbo V™ Ion Source (Applied Biosystems, Foster City, CA) operated in the positive multiple reaction monitoring ion mode with the following transition: methionine *m/z* 150 → 104; methionine-d_3 153 → 107. Plasma samples were deproteinized with 10-fold methanol and diluted in 50% acetonitrile containing methionine-d_3 (200 nM). The sample