Short Communication

Quantification of Biological Thiols in the Plasma of a Homocystinuria Model with Cystathionine β-Synthase Deficiency Utilizing Hydrophilic Interaction Liquid Chromatography and Fluorescence Detection

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Abstract
Biological thiols – homocysteine (Hcy), cysteine (Cys), γ-glutamylcysteine (γGluCys), glutathione (GSH), and cysteinylglycine (CysGly) – in plasma samples of cystathionine β-synthase (CBS, EC 4.2.1.22)-deficient mice were quantified using hydrophilic interaction liquid chromatography (HILIC) and fluorescence detection. Mice deficient in CBS provide a model mouse of homocystinuria, an inherited metabolic disease characterized by the abnormal accumulation of Hcy in urine and blood. For quantification, the thiols were derivatized with ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F), followed by separation with an amide column containing a mobile phase of acetonitrile–40 mM ammonium formate buffer (pH 3.0) (75/25, v/v). The total concentrations of Hcy in the plasma samples of CBS-wild type (-WT), -heterozygous (-Hetero), and -knockout (-KO) mice were 24.7, 29.3, and 237.7 μmol/L, respectively; 236.4, 178.7, and 78.5 μmol/L for Cys; 5.80, 4.87, and 0.75 μmol/L for γGluCys; 64.6, 74.0, and 51.2 μmol/L for GSH; and 2.48, 3.98, and 0.90 μmol/L for CysGly. The concentration of Hcy was significantly increased, while Cys, γGluCys, and CysGly concentrations were significantly decreased in CBS-KO mice compared to those in CBS-WT mice. The results indicated that biological thiols other than Hcy could also be utilized for the evaluation and investigation of homocystinuria pathologies.

Keywords: Hyperhomocystinuria; HPLC; Ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate; Sulfur amino acids

1. Introduction
Homocystinuria is an inherited metabolic disease characterized by the abnormal accumulation of homocysteine (Hcy) in urine and blood (homocysteinemia). This disease is tested for during newborn mass screenings in Japan, the United States, and other countries [1,2]. In human, Hcy is converted to methionine in the remethylation pathway or to cystathionine in the trans-sulfuration pathway as shown in Fig. 1. Conversion to cystathionine is suppressed by a deficiency of cystathionine β-synthase (CBS), which occurs in the majority of patients with homocystinuria [1,2]. Severe symptoms of untreated patients include intellectual disabilities, osteoporosis, dislocated lenses, and thrombosis [1,2]. These pathologies are thought to be caused by chemical activities of the thiol group in Hcy; Hcy can generate reactive oxygen species via disulfide bond formation, and can suppress enzyme activities by binding to proteins [1-3]. On the other hand, Hcy metabolites including cysteine (Cys), γ-glutamylcysteine (γGluCys), glutathione (GSH), and cysteinylglycine (CysGly) (Fig. 1) also possess a thiol group. GSH, in particular, functions as a reductant and detoxicant in biological fluids and tissues [3,4]. Hence, the balance between levels of Hcy and these metabolites is essential in the pathology of homocystinuria. As the accumulation of Hcy caused by CBS deficiency should alter...
the amount of downstream thiol metabolites, a comprehensive analysis of biological thiols should offer useful information to understand the pathology of disease. However, only Hcy, Cys, and GSH have been measured in humans and animal models of homocystinuria [5-11]. Considering that γGluCys and CysGly are precursors and degradation products of GSH, respectively, simultaneous analysis of all the thiols mentioned above should offer helpful information to reveal how Hcy accumulation leads to diverse symptoms and to investigate suitable indicators of disease progression.

In most previous reports, analytical methods for biological thiols employed thiol-selective fluorescence derivatization and reversed-phase liquid chromatography (RPLC) [4]. Major problems with these techniques included low detection sensitivity and low retention of derivatized thiols, of which polarity is high. Recently, we developed an analytical method utilizing ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F) as a fluorogenic derivatization reagent and hydrophilic interaction liquid chromatography (HILIC) as a separation mode to tackle these problems [12-14]. Since HILIC is more suitable for retention and separation of highly polar compounds than RPLC, and the fluorescence of SBD-thiols is enhanced in the acetonitrile-rich mobile phase of HILIC [12,15,16], this newly developed method enables simultaneous quantification of all biological thiols described above in human and mouse plasma samples.

In the present study, we quantified Hey-related biological thiols – Hey, Cys, γGluCys, GSH, and CysGly – in the plasma of CBS-wild type (-WT), -heterozygous (-Hetero), and -knockout (-KO) mice as total thiol (=disulfide+thiol) concentration. Changes in thiol concentrations in models of homocystinuria were comprehensively evaluated. As CBS-KO and Hetero mice have been used as models of severe and mild-to-moderate hyperhomocystinuria [17,18], where Hcy is accumulated in urine and blood because of environmental and genetic factors, changes in thiol metabolism associated with disease progression were also investigated.

2. Experimental

2.1. Chemicals and reagents

L-Cys, DL-Hcy, L-glutathione, CysGly, and γGluCys were obtained from Merck (Darmstadt, Germany). Tiopronin (N-(2-mercaptopropionyl)glycine, MPG) and trichloroacetic acid (TCA) were purchased from Wako (Osaka, Japan). SBD-F was from Dojindo (Kumamoto, Japan). Tris(2-carboxyethyl)phosphine (TCEP) was obtained from Tokyo Chemical Industry (Tokyo, Japan). Water was purified using a Milli-Q system (Merck Millipore, Darmstadt, Germany). HPLC-grade acetonitrile from Merck was used. All other chemicals were of analytical-reagent grade.

2.2. Animals

Wild-type (CBS+/-, WT), heterozygous (CBS +/-, Hetero), and knockout (CBS-/-, KO) mice were produced by breeding CBS+/- mice (B6.129P2-Cbstm1Unc/J, originally purchased from The Jackson Laboratory, Bar Harbor, ME, USA). Genotyping of mice was performed as described on The Jackson Laboratory homepage [19]. All mice were bred and housed at the Center of Biomedical Research, Graduate School of Medical Sciences, Kyushu University (Fukuoka, Japan), under a 12:12-h light-dark cycle and with free access to food (CLEA Rodent Diet CA-1, CLEA Japan, Tokyo, Japan) and water. The experiments were performed with permission from Animal Care and Use Committee of Kyushu University (A27-061-0).

2.3. Sample preparation

Mice aged from 15 to 18 days (WT, n=5; Hetero, n=4; and KO, n=3) were anesthetized and euthanized by exsanguination from the inferior vena cava. The obtained blood was collected in heparinized tubes (Nippon Becton Dickinson, Tokyo, Japan) and cooled immediately on ice. Plasma was separated by centrifugation at 8,000 rpm for 15 min. Plasma samples were stored at –80°C until use. Pretreatment of plasma samples was carried out according to a previous report [14]. Briefly, MPG was added to plasma samples, and disulfides in the samples were reduced to their thiol forms by TCEP for total biothiol (=disulfide+thiol) quantification. Proteins were removed...
by adding TCA and centrifugation. Thiols in the supernatant were derivatized with SBD-F, followed by 5× dilution with acetonitrile for injection to avoid deterioration of peak shape [13,20]. For the analysis of Hcy in CBS-KO samples, an additional 4× dilution was conducted and final samples were composed of 80% acetonitrile. Five microliters of sample were injected into a HPLC system for analysis.

2.4. Instruments for HPLC–fluorescence detection and chromatographic conditions

HPLC system consisted of a pump (PU-2080 Plus, JASCO, Tokyo, Japan), a column oven (CO-1560, JASCO), and a fluorescence detector (RF-20A, Shimadzu, Kyoto, Japan). Inertsil Amide column (150 mm × 3.0 mm i.d., 5 µm, GL Sciences, Tokyo, Japan) was used. A mobile phase consisting of acetonitrile–40 mM ammonium formate buffer (pH 3.0) (75/25, v/v) was used at the flow rate of 0.4 mL/min. The column temperature was set at 35°C, and the SBD-thiols were detected by fluorescence with excitation and emission wavelengths of 375 and 510 nm, respectively. The chromatograms were analyzed using Chromato-Pro ver. 5.00 software (Run Time Corporation, Hachioji, Japan). Quantification was conducted using relative peak heights compared to the internal standard, SBD-MPG.

2.5. Statistical analysis

Statistical analysis was carried out using Prism 6 (GraphPad Software, La Jolla, CA, USA). Comparisons of each biological thiol concentration among different genotype groups were conducted using one-way ANOVA followed by Dunnett’s multiple comparison test.

3. Results and discussion

3.1. Separation of biological thiols in CBS deficient mice plasma

Total thiols (=disulfide+thiol) such as Hcy (tHcy), Cys (tCys), and GSH (tGSH) were frequently quantified to compare biological thiol metabolism among normal and pathological cases. Hence, in the present study, the total concentration of each biological thiol – tHcy, tCys, total CysGly (tCysGly), tGSH, and total γGluCys (tγGluCys) – was quantified. Fig. 2 shows representative chromatograms of a standard sample, and CBS-WT, -Hetero, and -KO mice plasma samples. As shown in Fig. 2A, five SBD-thiols were separated in 16 min. In mice plasma samples, the peaks of five SBD-thiols were also shown (Figs. 2B-D). As mentioned in the Introduction, we have already developed an analytical method for biological thiols, and this is the first application of the method to the CBS-KO mice plasma. Therefore, to confirm that each SBD-thiol peak in the CBS-KO mice
calculated concentrations, except for tCysGly were unchanged, which confirmed that the previous method enabled thiol quantification in the plasma of CBS-KO mice. tCysGly could not be determined under the changed conditions because an interfering peak was co-eluted.

In our previous study, we found two unknown thiols in the plasma of 129SV mice, which were confirmed to possess an SBD-S moiety and molecular weights of 1184.519 (SBD-T1) and 800.281 (SBD-T2) by HILIC-electrospray negative ionization-mass spectrometry [14]. In this study, two peaks (peaks 7 (SBD-T1) and 8 (SBD-T2) in Fig. 2) were found in all mice samples analyzed. Peak identification was performed by comparing retention times of the peaks between 129SV and CBS mice samples. The presence of the peaks of unknown compounds, not only in the 129SV mice but also in the CBS mice, whose background was C57BL/6, indicated that biological thiols T1 and T2 exist in the blood of mice regardless of their species.

### 3.2. Comparison of biological thiol concentrations in plasma of mice with different CBS genotypes

The results of biological thiol concentrations in the plasma of mice with different CBS genotypes are summarized in Fig. 3. The values for SBD-T1 and SBD-T2 were shown as relative peak heights compared to an internal standard, SBD-MPG, not as the concentrations. tHcy concentrations in the plasma of CBS-WT, -Hetero, and -KO mice (24.7±7.4, 29.3±2.8, and 237.7±52.3 μmol/L, respectively) were similar to those previously reported (6.1±0.8, 13.5±3.2, and 203.6±65.3 μmol/L, respectively) [18]. Slight concentration differences in -Hetero and -WT mice between the present study and those in previous reports may be due to differences in age (15–18 days vs 21 days, respectively) and rearing conditions. Results of tCys and tGSH were also consistent with those of previous

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As expected, tHcy in KO mice plasma was significantly increased compared with that in WT mice. In contrast, tCys, tCysGly and tγGluCys were decreased in KO over those in WT mice plasma, and there was no significant difference in tGSH. CBS deficiency should suppress the trans-sulfuration pathway (Fig. 1), which results in a decrease of Hcy metabolites. Hence, the observation of tCys, tγGluCys, and tCysGly reduction in the plasma of KO mice compared with that in WT mice reconfirmed that CBS deficiency influences downstream thiol metabolism.

As for the unidentified thiols, significant reduction in KO compared with that in WT mice plasma was observed for T1, while there was no significant change in T2. Trends of T1 concentrations among the three genotypes were similar to those observed with tCysGly and tGSH as shown in Fig. 3. Therefore, it is possible that T1 has some relevance to GSH and its metabolites, including CysGly in mice. Further studies are necessary to clarify the relationship in greater detail.

### 4. Conclusions

In the present study, total biological thiols (tHcy, tCys, tCysGly, tγGluCys, and tGSH) in mice plasma samples were quantified, and the concentrations were compared among CBS-WT, -Hetero, and -KO mice. tHcy concentration in KO mice was increased drastically compared with that in WT mice as expected, while tCys, tCysGly, and tγGluCys concentrations were decreased. The results showed that biological thiols other than Hcy could be utilized for the evaluation and investigation of mild-to-severe hyperhomocystinuria pathologies, since CBS-Hetero and -KO mice have been used as animal models for these pathologies. As trends in the concentration of an unidentified thiol compound (T1) among the three genotypes were similar to those of tCysGly
and tGSH, it was assumed that T1, CysGly, and GSH may have a close relationship. However, further studies, such as preclinical research using a larger number of animal models and clinical research, are necessary to confirm whether the simultaneous evaluation of these biological thiols could work as a biomarker or indicator for disease.

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