약학석사학위논문

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김지현의 약학석사학위논문을 인준함

2012년 7월

위 원 장 _____ (인)

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Abstract

Alterations in the Metabolomics of Sulfur-Containing Substances in Rat Kidneys After Betaine Intake

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Previous studies from this lab examined the effect of betaine treatment on liver sulfur-amino acid metabolomics. In this study, 1% of betaine dissolved in drinking water was provided to rats for two weeks in order to measure the changes in the metabolomics of sulfur-containing substances in the kidney. In rats supplemented with betaine, renal methionine level was increased by 42%. However, the activity or protein expression of betaine-homocysteine methyltransferase (BHMT) was not affected by betaine. Methionine synthase, the other enzyme which catalyzes the remethylation of homocysteine to methionine was also unchanged in the betaine treated group. S-Adenosylmethionine (SAM) concentration was increased by betaine feeding, and the protein expression of methionine adenosyltransferase II (MAT II), the type known to be expressed in extrahepatic tissues, increased in the betaine supplemented group. However, S-adenosylhomocysteine (SAH), total homocysteine, and cysteine levels as well as protein expression of cystathionine β-synthase were not affected by betaine treatment. Renal glutathione (GSH) level and the expression of ɣ-glutamylcysteine synthetase (GCS), the enzyme mediating the production of GSH, were also not changed by betaine supplementation. On the other hand, the rate limiting enzyme of taurine synthesis, cysteine dioxygenase (CDO) was decreased substantially by betaine

treatment. However, the activity and protein expression of cysteine sulfinate decarboxylase (CDC) and its product hypotaurine levels increased significantly. These results suggest that despite the negligible expression of BHMT in the kidney, betaine may affect the renal metabolomics of sulfur-containing substances significantly. The increase in hepatic methionine due to betaine treatment appears to account for the increased methionine level in plasma, consequently increasing renal methionine uptake, which serves as the major cause for the changes in the transsulfuration reactions in the rat kidney. Further studies need to be conducted to clarify the physiological and the biochemical significance of these findings.

Keywords: Betaine; kidney; sulfur-amino acid; transsulfuration reaction

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Introduction

1. Betaine

Betaine (N,N,N-trimethylglycine) is a zwitterionic quarternary ammonium compound, and a methyl derivative of the amino acid glycine. Betaine can be provided by the diet with food stuff such as wheat, shellfish, spinach, and sugar beets (Craig, 2004), or synthesized in the body from choline by the choline oxidative pathway. The process is catalyzed by choline oxidase, which produces betaine aldehyde. Betaine aldehyde dehydrogenase then catalyzes the synthesis of betaine (Barak and Tuma, 1982; Craig, 2004). Betaine is known to have physiological roles of protecting cells from increased osmolarity due to high concentrations of electrolytes or accumulations of urine, or to act as a methyl donor in transmethylation reactions in the body (Horio et al., 2001; Wettstein et al., 1998; Barak and Tuma, 1982; Finkelstein et al., 1982)

Betaine donates a methyl group to homocysteine and is thus involved in methionine synthesis in the transsulfuration reaction (Figure 1). This reaction is catalyzed by betaine homocysteine S-methyltransferase (BHMT) (Finkelstein et al., 1971, 1983) and is known to be increased with betaine intake or administration (Finkelstein et al., 1971, 1982, 1983).

2. Transsulfuration Pathway

The metabolism of sulfur-containing amino acids is mainly achieved by the transsulfuration pathway. The transsulfuration pathway is known to be most active in the liver, but there are reports that this metabolic pathway exists in a number of other tissues (Panayiotidis et al., 2004; Parsons et al., 1998; Vitvitsky et al., 2006; Finkelstein, 1998). In the liver, this process starts with the metabolism of methionine. Methionine supplied into the body produces S-adenosylmethionine (SAM) by methionine adenosyltransferase (MAT), and SAM is used in polyamine synthesis or various transmethylation reactions in the body by acting as a methyl donor (Mato et al., 2008; Chiang et al., 1996). SAM transforms into S-adenosylhomocysteine (SAH) after the donation of methyl groups in various reactions. SAH is used in homocysteine synthesis via the

action of SAH hydrolase. This reaction is reported to be reversible, and normally the equilibrium of the reaction is directed towards the synthesis of SAH. (De la Haba and Cantoni, 1959; Finkelstein, 2007).

Homocysteine is placed at a junction between two competitive metabolic pathways, remethylation and transsulfuration, and is thus an important regulator of sulfur-containing substance metabolism. Homocysteine is remethylated to methionine by the action of methionine synthase (MS) or BHMT, completing the methionine cycle, in which the sulfur atom of methionine is preserved (Finkelstein et al., 1984; Mudd and Poole, 1975).

On the other hand, homocysteine can bind with serine to synthesize cystathionine by the activity of cystathionine β-synthase (CβS). Cystathionine is then catalyzed by cystathionine ɣ-lyase (CɣL), producing cysteine (Lu, 1999), and the sulfur atom of methionine is transferred to cysteine irreversibly. Cysteine is irreversibly metabolized to taurine, GSH, and inorganic sulfate (Stipanuk et al., 2006). The liver, the main site of transsulfuration, shows high CβS and CɣL activities. However, it has been reported that the kidney also expresses these enzymes (House et al., 1997) and the metabolism of homocysteine and cysteine synthesis occurs, accordingly. During the metabolism of methionine to cysteine and its metabolites, the sulfur group of methionine is transferred to the final product taurine and GSH.

Cysteine is metabolized into glutathione (GSH) and taurine. Production of GSH from cysteine is carried out by ɣ-glutamylcysteine synthetase (GCS) and GSH synthetase. The liver produces GSH from cysteine and provides the product to other tissues via the blood stream (Hagen and Johns, 1989). Dynamic homeostasis of GSH is also known to occur in the kidney. The kidney mediates the degradation of GSH as well as uptake into the cells (Griffith and Meister, 1979; Kaplowits et al., 1985; Hagen and Jones, 1989), and the synthesis of GSH in the tissue has also been reported (Ormstad et al., 1980; Kaplowits et al., 1985).

On the other hand, cysteine is oxidized by cysteine dioxygenase (CDO) to cysteine sulfinate in the cysteine sulfinate pathway. Cysteine sulfinate decarboxylase (CDC) uses cysteine sulfinate as a substrate and produces hypotaurine. Hypotaurine is the metabolic precursor of taurine and has shown antioxidative effects in numerous reports (Aruoma et al., 1988; Mehta and Dawson, 2001; Fontana et al., 2008; Acharya and Lau-Cam, 2010). The

transformation of hypotaurine to taurine is known to be spontaneous (Pecci et al., 1999). Taurine has been reported to have significant roles in numerous diseases and its roles as an antioxidant as well as an osmolyte (Acharya and Lau-cam, 2010; Chesney et al., 2010; Eppler and Dawson Jr, 2002; Mehta and Dawson, 2001). Taurine binds to bile acid and is eliminated via the bile or is transported by the blood to be excreted in the urine.

In the transsulfuration pathway the metabolism of methionine, production of methyl donors or antioxidants such as GSH or taurine, homeostasis of sulfur-containing substances and its metabolites are strictly maintained and disruption of the transsulfuration reaction has been reported to be related to various pathological states of the body (Schnackenber et al., 2009; Tesseraud et al., 2009; Mato et al., 2008). Although the liver is known to be the major site of transsulfuration, there have been reports that the metabolism of sulfur-containing substances occurs in the kidneys as well (Finkelstein, 2007; Sullivan and Hoffman, 1983; Stipanuk et al., 1990; House et al., 1997).

3. Object of Study

It has already been reported that betaine administration affects the liver transsulfuration pathway significantly. The results from a previous study carried out from this lab reported that hepatic concentrations of methionine, SAM, SAH, and MAT activity were increased, while homocysteine and taurine levels and CDO activity were decreased in betaine fed mice.

The kidney plays an important role in GSH degradation and synthesis, which involved the metabolism of cysteine. In addition, there are reports that the metabolism of sulfur-containing substances occurs actively in the kidney. Also there are numerous reports on the disturbed homeostasis of sulfur-containing substances in subjects with renal malfunction (Hermann et al., 2005; Stam et al., 2005; Bocock and Zlotkin, 1990). These imply that the kidney will also play an important role in regulating sulfur-containing substances in the body. Therefore this study was carried out in order to investigate the effects of betaine on the renal metabolism of sulfur-containing substances in rats.

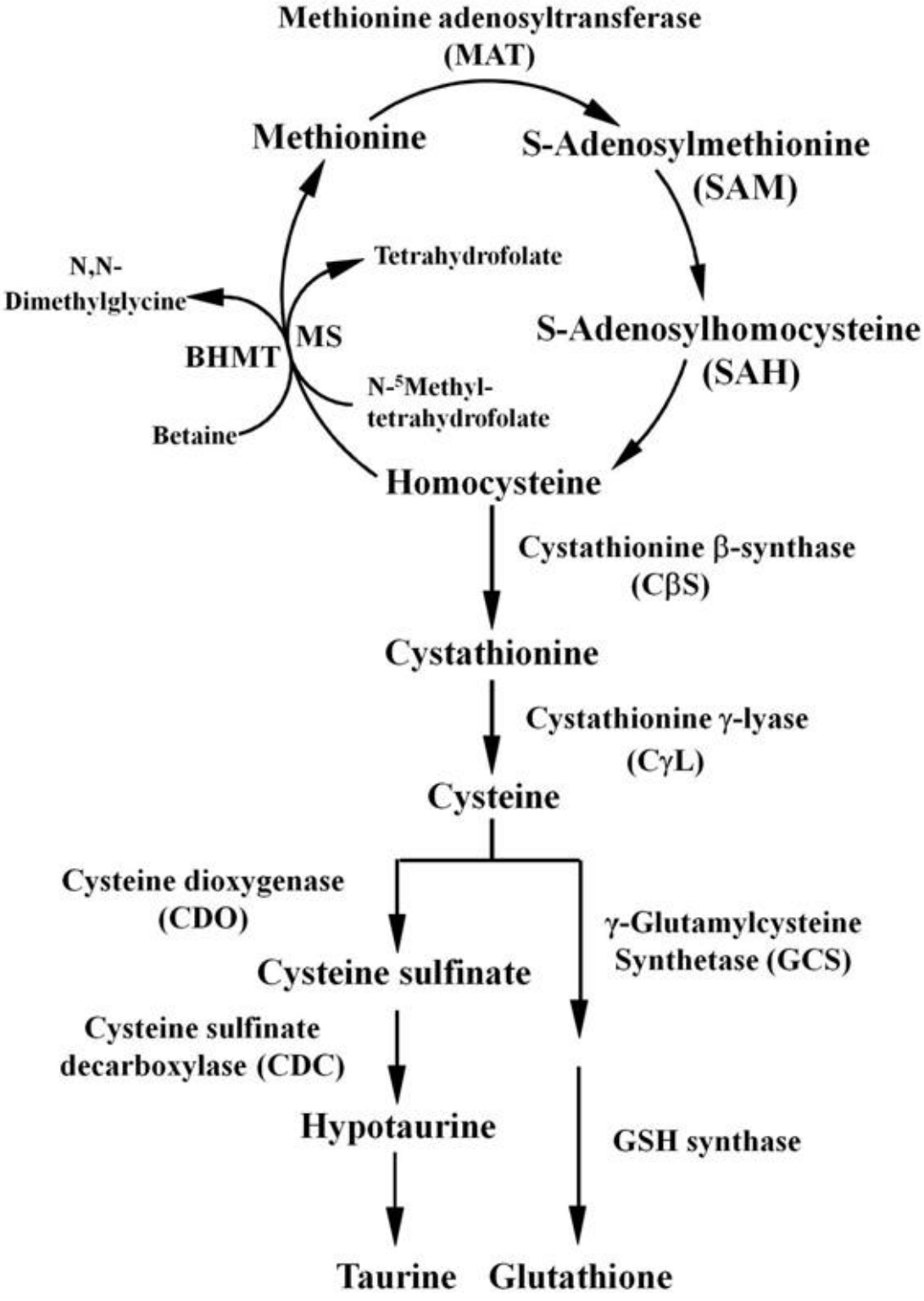

FIGURE 1. Metabolic reactions in the transsulfuration pathway

Methods and Materials

1. Materials

1.1. Animals and treatments

Male Sprague-Dawley rats purchased from Orient Bio (Sung-Nam, Kyunggido) were used for the experiments. Animals were acclimated to temperature (22 ± 2 ℃) and humidity (55 ± 5 %) controlled rooms with a 12-hr light/dark cycle (light 0700-1900; dark 1900-0700) at the institute of laboratory animal resources building (Seoul National University, College of Pharmacy) for at least 1 week prior to use. Laboratory chow and tap water were allowed ad libitum.

Betaine (1 %) dissolved in tap water replaced regular tap water at the initiation of betaine supplementation, while regulat tap water was provided for the control group animals. Same lab chows were provided for control and betaine supplemented groups. Animals were sacrificed 2 weeks after betaine supplementation. Rats were under anesthesia with diethyl ether during extraction of blood from the abdominal aorta and during the excision of kidneys.

1.2. Chemicals

Drugs and chemicals used in this study are as follows. 1-Hepatne sulfonic acid, 2-mercaptoethanol, amino acid standards, ammonium phosphate, ATP, betaine, boric acid, BPB (Bromophenol blue), Bradford reagent, cysteine HCl, cysteine sulfinic acid (cysteine sulfinate), $_{DL}$-homocysteine, DTNB (diethylene triamine penta acetic acid) , EDTA disodium salt, ferrous ammonium sulfate, GR (glutathione reductase), hydroxylamine HCl, L-cysteine, L-glutathione, $MgCl_2$, NAD^+, NADPH (β-nicotinamide adenine dinucleotide phosphate reduced form), naphthol blue black, ninhydrin, OPA (*O*-phthalaldehyde), PLP (pyridoxal 5-phosphate), SAH (S-adenosylhomocysteine), SAM (S-adenosylmethionine iodide salt), SBD-F (ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid), sodium acetate, TCA (trichloroacetic acid), TCEP (tris-(2-carboxyethyl)-phosphine hydrochloride), TEMED

(tetramethylethylenediamine), THF (tetrahydrofuran) and tween 20 were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).
Ethanol (99.9 %) (EtOH), acetic acid, perchloric acid, potassium chloride (KCl), potassium phosphate dibasic (K_2HPO_4), potassium phosphate monobasic (KH_2PO_4) and sodium hydroxide (NaOH) were purchased from Duksan Pure Chemical Co. (Ahn-San, Kyunggi-Do), while c-HCl, diethyl ether, sodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$) and sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$) were products from Dae Jung chemicals (Si-Heung, Kyunggi-Do). Glycerol, glycine and tris acid were purchased from Amresco Inc. (Cochran Road Solon, OH, USA), and ammonium persulfate, bis-acrylamide solution and sodium dodecyl sulfate (SDS) were products from Bio-Rad Laboratories Inc. (Hercules, CA, USA). Acetonitrile and methanol were products purchased from Burdick and Jackson Co. (Muskegon, MI, USA), tris base from USB Corporation (Cleveland, OH, USA), bovine serum albumin from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and skim milk from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). ECL$^+$solution (ECL^{TM} reagent) was purchased from GE Healthcare Life Sciences (Little Chalfont, Buckinghamshire (HP79NA), UK) / (Piscataway Township, NJ, USA). All the chemicals and solvents used were of reagent grades or better.

1.3. Antibodies

The primary antibodies used as probes in assessing protein expression are as follows. Goat polyclonal antibody for MAT II, (CAT No. sc-28031), rabbit polyclonal antibody for CβS (CAT No. sc-67154) and mouse monoclonal antibody for GAPDH (CAT No. sc-32233) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The goat anti-BHMT primary antibody (CAT No. EB07943) and goat antibody for MS (CAT No. EB05055) was the bought from Everest Biotech (Upper Heyford, Oxfordshire, UK). Rabbit polyclonal antibody for GCS was from Neomarkers (Lab Vision Corporation) (Kalamazoo, MI, USA). The mouse monoclonal antibody for CDC (CAT No. ab82613) was a product of Abcam (Cambridge, MA, USA) and the rabbit anti serum for CDO was a gift from Dr. Hosokawa (National Institute of Health and Nutrition, Tokyo, Japan). For secondary antibodies, HRP-conjugated goat-anti-rabbit (GAR), dog-anti-mouse (DAM), products of Jackson

ImmunoResearch Laboratories Inc. (West Grove, PA, USA) and the donkey-anti-goat (DAG, CAT No. sc-2020) from Santa Cruz were used.
All primary antibodies were diluted with 5 % BSA solutions by appropriate ratios, and were stored at 4 ℃ after use. Secondary antibodies were diluted with 5 % skim milk solutions by appropriated ratios. The dilution ratios of each antibody were selected according to the datasheet provided each.

2. Method

2.1. Measurement of sulfur-containing substances

2.1.1. Measurement of GSH

Renal and plasma GSH were measured using the enzymatic recycling method (Griffith, 1980). Rats were anesthetized using diethyl ether and then blood was drawn from the abdominal aorta. Blood was collected in an Ep tube pretreated with heparin, then centrifuged immediately at 15000 rpm (approximate 8000g) for 30 seconds to gain plasma. 10 % sulfosalicylic acid was added and protein was precipitated to prevent GSH degradation. Hemolysis affects the plasma GSH concentration and therefore such blood was not used.
Three-fold volume of ice-cold 1.15 % KCl solution was added to kidney and homogenized using a polytron (ULTRA-TURRAX T-25, IKA-Labortechnik, Germany). Equal volume of 10 % PCA solution containining 2 mM EDTA was added to the homogenate and centrifuged at 15,000 rpm (approximate 8000g) for 10 minutes for protein precipitation. The supernatant was collected and stored in a deep freezer (Model ULT-1490, REVCO, Asheville, NC, USA) at below - 70 ℃ until use for analysis.
Phosphate buffer (0.125 M phosphate, 6.3 mM EDTA, pH 7.5) was added to the supernatant and used for dilution for the sample GSH concentration to be within the standard range, and used for analysis of total GSH concentration. Solutions of 0.3 mM NADPH 0.7 ml, 6 mM DTNB 0.1 ml, sample or GSH standard 0.2 ml were added into a EP tube, mixed well and left to react in room temperature for 4 minutes. 60 ㎕ of 12.5 units / ml of GSH reductase was added for renal GSH measurement, and 50 units / ml of GSH reductase for the measurement of

plasma GSH measurement. The reaction mixture was mixed well, and the change in absorbance was measured for 2 minutes at 412 nm using a spectrometer. The slope of change within a minute range of linear change was used to calculate the concentration of GSH from the standard slope.

2.1.2. Measurement of cysteine

Renal cysteine was measured using the samples made for GSH analysis, using the method of Gaitonde (1967). Acid ninhydrin 100 ㎕, acetic acid 100 ㎕ and sample 100 ㎕ were mixed and incubated in 100 ℃ for 10 minutes then immediately cooled in ice. 0.67 ml of 95 % ethanol was added for stabilization and 20 - 30 minutes later the absorbance was measured at 560 nm using a spectrometer. The concentration of cysteine in sample was calculated using the standard curve.

2.1.3. Meausrement of SAM and SAH

Three-fold volume of ice-cold 1.15 % KCl solution was added to kidney and homogenized using a polytron (ULTRA-TURRAX T-25, IKA-Labortechnik, Germany). Equal volume of 10 % PCA solution was added to the homogenate and centrifuged at 15,000 rpm (approximate 8000g) for 10 minutes for protein precipitation. The supernatant was collected and stored in a deep freezer (Model ULT-1490, REVCO, Asheville, NC, USA) at below - 70 ℃ until use for analysis.

For the analysis of SAM and SAH concentration, 1-heptanesulfonic acid sodium salt was used for ion pairing and was analyzed using a HPLC system, installed with a reverse-phase chromatography column (3.5 ㎛ Kromasil C18, 4.6 x 250 mm, Eka Chemicals, Bohus, Sweden) and a UV detector (UV-975 UV/VIS detector, Jasco Co., Tokyo, Japan) (She et al., 1994). The mobile phase consisted of 18 % methanol, 40 mM $NH_4H_2PO_4$ and 8 mM 1-heptanesulfonic acid sodium salt. The flow was maintained at 1.0 ml / min at 35 ℃ using a column oven.

2.1.4. Meausurement of methionine, hypotaurine and taurine

Three-fold volume of ice-cold 1.15 % KCl solution was added to kidney and homogenized using a polytron (ULTRA-TURRAX T-25, IKA-Labortechnik, Germany). Three-fold volume of ice cold methanol was added to the homogenate and centrifuged at 15,000 rpm (approximate 8000g) for 10 minutes for protein precipitation. The collected supernatant was mixed with twice-fold volume of distilled water and stored in a deep freezer (Model ULT-1490, REVCO, Asheville, NC, USA) at below - 70 ℃ until use for analysis.
Blood was centrifuged immediately after collection for extraction of plasma, and three-fold volume of ice-cold methanol was added to precipitate the proteins. The same procedure was carried out as for the kidney samples.
For the quantitative analysis of amino acids and taurine, the *O*-phthaldialdehyde induced derivatives were measured using a HPLC system installed with a reverse-phase chromatography column (3.5 ㎛ Kromasil C18, 4.6 x 100 mm, Eka Chemicals, Bohus, Sweden) and a fluorescent detector (FP-920 fluorescence detector, Applied Biosystem, Foster, CA, USA). 0.1 M sodium acetic acid (Solvent A; pH 7.2) and methanol + tetrahydrofuran = 97 + 3 (v/v) (Solvent B) were used as mobile phases, and for the concentration gradient two pumps (Jasco Model PU-980, Jasco Co., Tokyo, Japan) were used. The concentration gradient were made modifying the method of Rajendra (1987), and was changed as follows at a flow rate of 1.2 ml / min: Solvent A at 0 min 90%, 0 ~ 4 min 82 %, 4 ~ 16 min 82 %, 16 ~ 24 min 72 %, 24 ~ 27.5 min 72 %, 27.5 ~ 35 min 55 %, 35 ~ 42.5 min 55 %, 42.5 ~ 48 min 46 %, 48 ~ 50 min 30 %, 50 ~ 51 min 90 %

2.1.5. Meausrement of homocysteine

The method of Nolin et al. (2007) was used for the measurement of homocysteine levels. Samples for analysis were equal to the samples for the measurement of SAM and SAH, SBD-F induced derivatives were measured using HPLC system installed with a reverse-phase chromatography column (3.5 ㎛, Waters Symmetry C18, 4.6 x 150 mm, Waters Corporation, Milford, MA, USA) and a fluorescent detector (FP-920 fluorescence detector, Applied Biosystem, Foster, CA, USA). 0.1 M sodium acetic acid + methanol = 97 + 3 (v/v) (Solvent A) and methanol (Solvent B) were used as mobile phases, and

for a concentration gradient two pumps (Jasco Model PU-980, Jasco Co., Tokyo, Japan) were used. The concentration gradient were as follows at a flow rate 1.0 ml / min and oven temperature 40 ℃: Solvent A at 0 min 100 %, 0 ~ 7 min 100 %, 7 ~ 8 min 80 %, 8 ~ 13 min 80 %, 13 ~ 14 min 80 %, 14 ~ 20 min 100 %.

2.2. Enzyme Activity Assay

2.2.1. Extraction of cytosol

In rats under anesthesia using diethyl ether, the abdominal cavity was cut open and after blood collection from the abdominal aorta. Kidneys were excised and homogenized with a four-fold volume of ice-cold 1.15 % KCl solution containing 1 mM EDTA. The homogenate was centrifuged at 10,000 g at 4 ℃ for 23 minutes, and the supernatant was collected. The supernatant was centrifuged again at 100,000 g at 4 ℃ for 65 minutes. The supernatant, cytosol, was collected and used for enzyme activity measurements and were stored until and after use in a deep freezer below - 70 ℃.

2.2.2. Protein Assay

Protein assay was carried out using the method of Lowry et al. (1952). For the assay 1 ml of diluted protein sample was mixed with 5 ml of Lowry complex [2 % (w / v) Na_2CO_3 : 1% (w/v) copper sulfate : 2 % (w/v) potassium sodium tartarate = 100 : 1 : 1]. After 10 minutes 0.5 ml of 0.1 N folin-ciocalteau's phenol was added and 30 minutes later the absorbance was measured at 750 nm using a UV / VIS spectrophotometer (Model V-530, JASCO, Tokyo, Japan).The standard curve was formed using bovine serum albumin, and the protein concentration of samples was calculated from the standard curve.

2.2.3. MAT (Methionine Adenosyltranferase) Activity

MAT activity was measured using the method of Mudd et al. (1975). The

reaction mixture consisted of 80 mM Tris-HCl / 50 mM KCl (pH 7.4), 5 mM ATP, 40 mM $MgCl_2$, 0.1mM methionine and 1mg of cytosolic protein, and the total volume was 1ml. The substrate methionine was added to start the reaction, and the mixture was incubated at 37℃ for 30 minutes. 10% perchloric acid 0.5 ml was added to end the reaction. The product SAM concentration was quantified using the method of She et al (1994) above.

2.2.4. BHMT (Betaine-Homocysteine Methyltransferase) Activity

BHMT activity was assayed according to the method of Ericson et al. (1967). The reaction mixture was composed of 1.15 % KCl / 1 mM EDTA / 50 mM Tris base (pH 7.4), 0.5 % homocysteine in 10 mM DTT, 1 % betaine, methanol and 1mg of cytosolic protein, and the total volume of the reaction mixture was 0.6 ml. the mixture was incubated at 37 ℃ for 1 hr and 0.9 ml methanol was added to end the reaction. The methionine produced was quantified using the method of Rahendra (1987) mentioned above.

2.2.5. CDO (Cysteine Dioxygenase) Activity

CDO activity was measured using the method of Bagley et al. (1995). The reaction mixture consisted of 0.5 mM ferrous ammonium sulfate, 5 mM hydroxylamine HCl, 2 mM NAD^+, 5mM cysteine and 1mg cytosolic protein, and the total volume was 1ml. The mixture was incubated at 37℃ for 17 minutes and 2.5 ml of cold methanol was added to end the reaction. The cysteine sulfinate was quantified using by measuring the *O*-phtaldialdehyde / 2-mercaptoethanol induced derivatives using a fluorescence detector and a 3.5 ㎛ Kromasil C18 column (4.6 x 100 mm, Eka Chemicals, Bohus, Sweden) installed HPLC system.

2.2.6. CDC (Cysteine sulfinate Decarboxylase) Activity

CDC activity was measured by quantifying the amount of hypotaurine produced. 100 ug of protein, and 0.1 M potassium phosphate buffer (pH 7.4) with 5 mM

dithiothreitol, 0.2 mM pyridoxal 5-phosphate was mixed and preincubated at 37 ℃ for 3 minutes. 20 mM cysteine sulfinate 50 ㎕ was added to start the reaction and after 30 minutes of reaction time 0.6 ml of cold methanol was added to end the reaction. The hypotaurine produced was induced into a derivative using *O*-phtaldialdehyde / 2-mercaptoethanol and measured using the method of Ide (1997).

2.3. Western Blot Assay

According to the method of Laemmli (1970) the protein mixture was separated by SDS-PAGE (sodium dodecyl-sulfate-polyacrylamide gel electrophoresis). To make a 7.5 % separating gel 3.95 ml DDW, 2.5 ml 1.5 M Tris (pH 8.8), 3 ml 30 % Bis / Acrylamide solution and 10 % SDS 100 ㎕ were mixed. To the mixture a 10 % ammonium persulfate 60 ㎕ and TEMED 5 ㎕ were added, poured into a gel cassette. Small amounts of DDW were added on the top layer to flatten the top surface of the gel and the gel was left to harden. After the separating gel had hardened the DDW on the surface was carefully removed the stacking gel was poured and the well-comb was inserted for sample loading. The stacking gel was made by mixing 3.36 ml DDW, 1.5 ml 0.5 M tris (pH 6.8), 780 ㎕30 % Bis/Acrylamide solution, 60 ㎕ 10 % SDS, 45 ㎕10 % ammonium persulfate and 6 ㎕ TEMED. Samples for electrophoresis were made by mixing sample dilution buffer (2.5 ml 1 M Tris (pH 6.8), 5 ml 80 % glycerol, 5 ml 20 % SDS, 0.2 ml 1 % bromophenol blue, 2 ml 2-meraptoethanol and 5.3 ml DDW) to dilute the cytosolic protein and then heated at 95 ℃ for 5 minutes. Samples were loaded on to the gel and electrophoresed in running buffer (3.04 g trizma base, 14.42 g glycine, and 5 ml 20 % SDS in 1 L of solution) using a Bio-Rad Mini Protein II machine. The voltage was maintained at 100 V during running within the stacking gel and 160 V in the separating gel. After electrophoresis proteins were transferred to a NC (nitrocellulose) membrane at 80 V in transfer buffer (3.04 g trizma base, 14.42 g glycine and 200 ml methanol in 1 L solution). After transfer an amido black solution was used to dye the protein on the NC membrane. the membrane was then submerged in 5 % non-fat milk solution for an hour to prevent unspecific binding, washed at least three times with tris buffer (TBS-T) containing 0.05 % tween 20, then was probed with the

appropriate primary antibody at 4 ℃ for an overnight. The next day the primary antibody solution was removed, washed, and the membrane was submerged in a secondary antibody solution, diluted with 5 % non-fat milk for 1 hour. ECL^{+} chemiluminescence system was used to develop the probes and Kodak X-OMAT film (Sigma-Aldrich, St.Louis, MO, USA) was used to detect the enhanced chemiluminescence. GAPDH was used as the housekeeping protein probe.

3. Statistics

All data are expressed as mean ± SE and were analyszed using a two-tailed Student's *t*-test. The acceptable level of significance was established at $P < 0.05$.

Results

1. Effects of betaine on the sulfur-containining substances and related enzymes in the methionine cycle in the kidney

The concentrations of metabolites involved in the methionine cycle, as well as related enzyme activities and protein expressions were measured in control and betaine-fed rats. Renal methionine level was increased in the betaine treated group compared to the control group (Figure 2). SAM, produced from methionine by MAT also showed an increase in the betaine supplemented group, but the following metabolite SAH did not show any differences between control and betaine-fed groups (Figure 3). Homocysteine levels were also unchanged with betaine treatment (Figure 4).

MAT, which uses methionine as a substrate, showed increased activity in the betaine supplemented group. The protein expression of MAT II, known to be distributed in extrahepatic tissues, was increased in the betaine-treated group. The protein expression of BHMT, an enzyme known to remethylate homocysteine to methionine was negligible (data not shown). The activity of BHMT did not show any changes with betaine treatment, and MS, the other remethylating enzyme also showed no difference in the protein expression between the control and the betaine supplemented group (Figure 5).

2. Effects of betaine on the plasma sulfur-containing substances

Methionine and homocysteine levels were measured in the plasma of animals. Plasma methionine concentration increased as in the kidneys with betaine treatment, but homocysteine level was not different in betaine supplemented group compared to control (Figure 6).

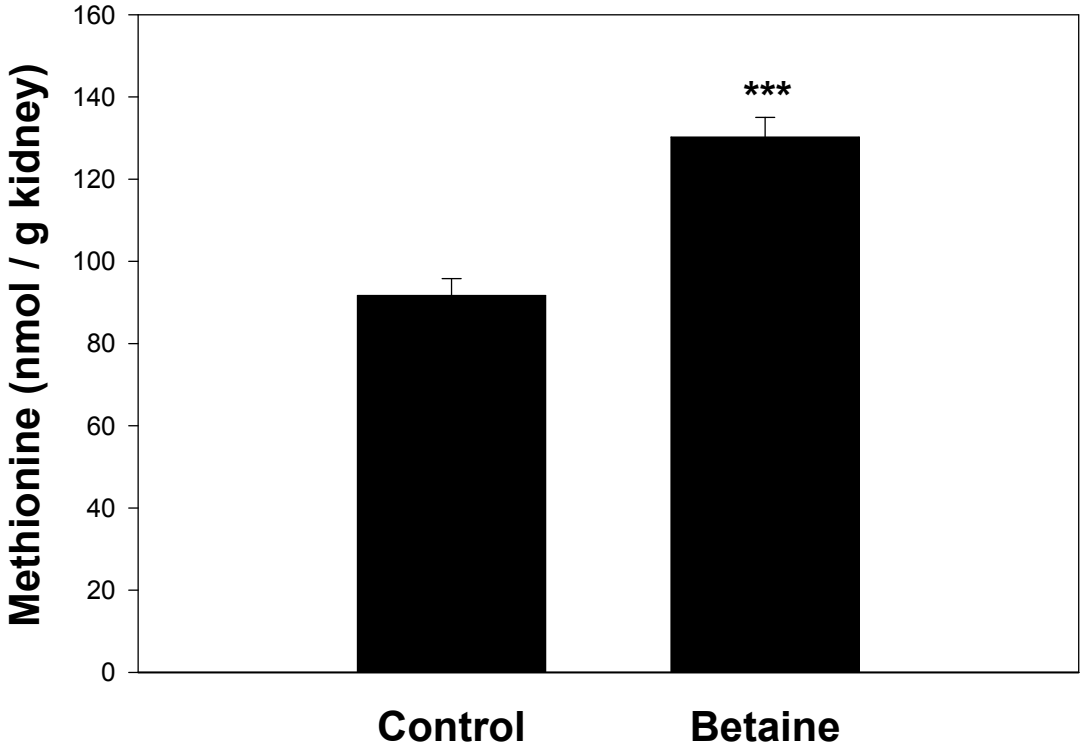

Figure 2. Effect of betaine on renal methionine level. Each value represents the mean ± SE for six rats. *** Significantly difference from control at $P < 0.001$ (Student's t-test)

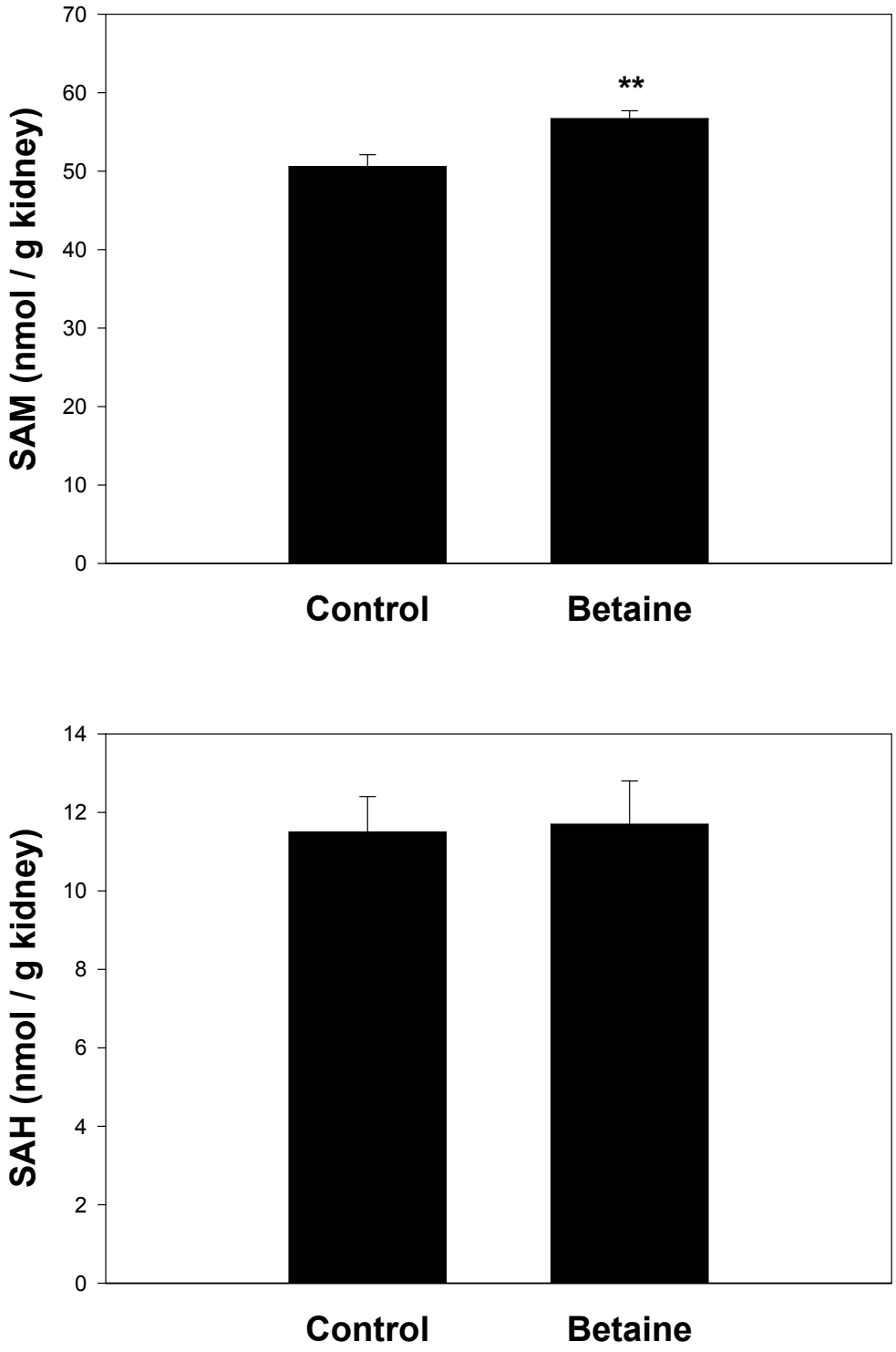

Figure 3. Effect of betaine on renal SAM and SAH levels. Each value represents the mean ± SE for six rats. ** Significantly difference from control at $P < 0.01$ (Student's t-test)

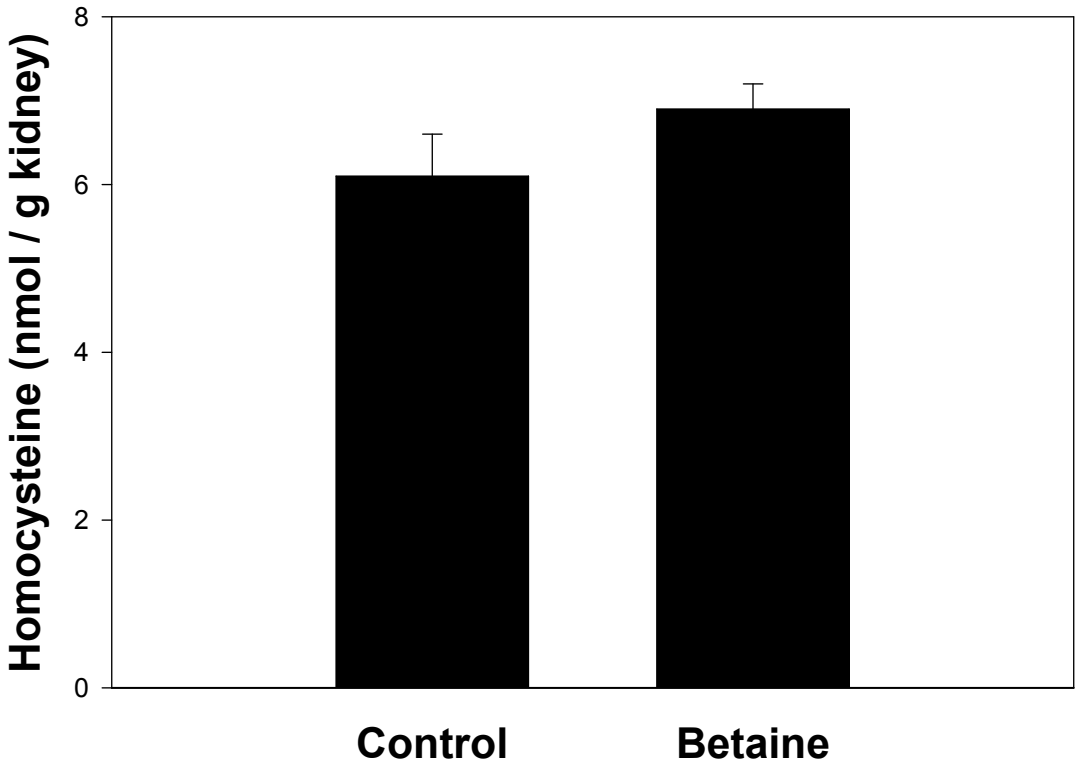

Figure 4. Effect of betaine on renal homocysteine level. Each value represents the mean ± SE for six rats.

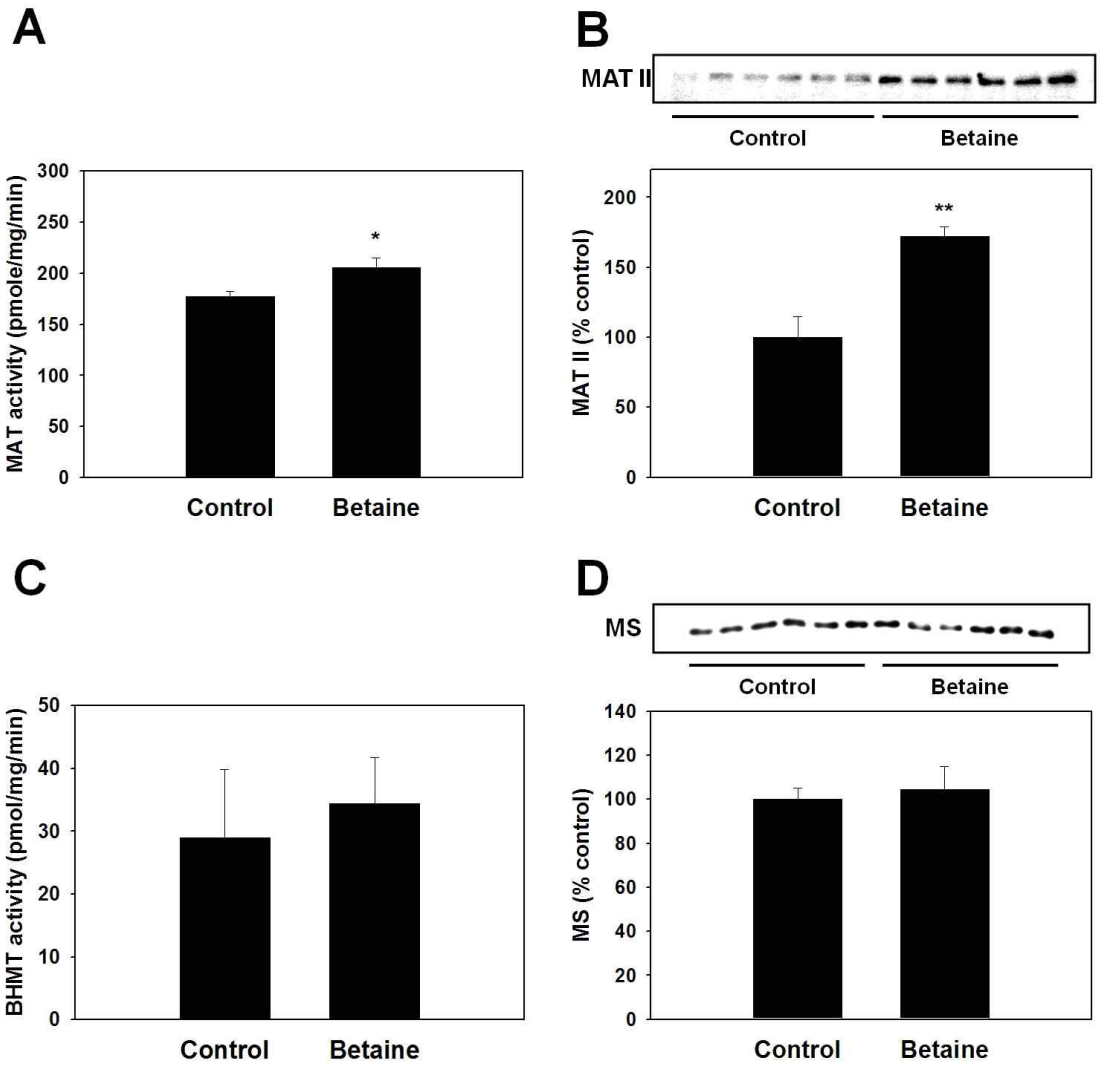

Figure 5. Effect of betaine administration on renal MAT activity (A), MAT protein (B), BHMT activity (C), and MS protein (D). Each value represents the mean ± SE for six rats. *,** Significantly different from control at $P < 0.05$, 0.01, respectively (Student's t-test).

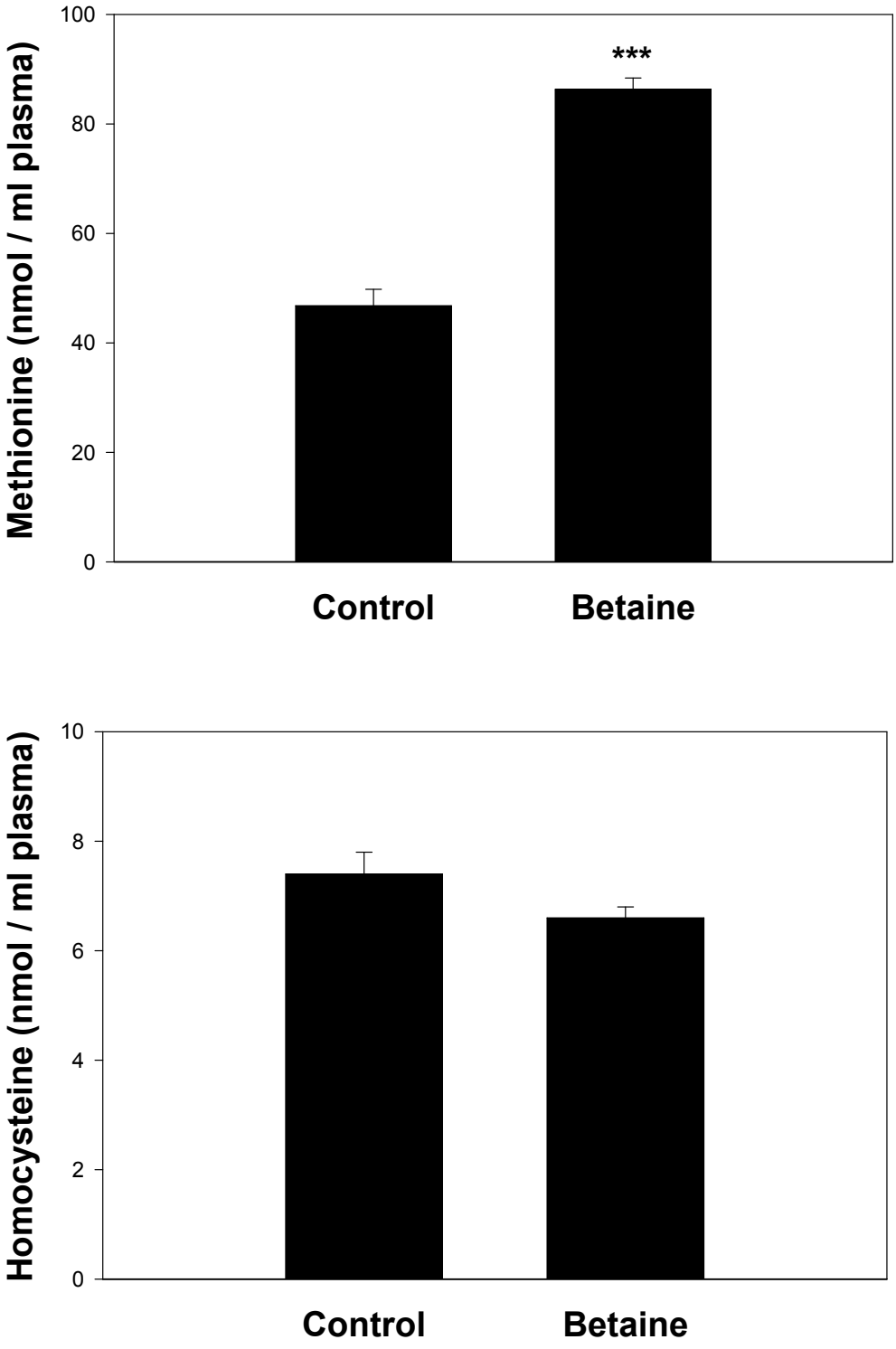

Figure 6. Effect of betaine on plasma methionine and homocysteine level. Each value represents the mean ± SE for six rats. *** Significantly difference from control at $P < 0.001$ (Student's t-test)

3. Effects of betaine on the sulfur-containing substances and related enzymes in the cysteine metabolism in kidney

Changes in metabolite levels, activity and protein expression of related enzymes related to cysteine metabolism by betaine treatment were measured in the kidney.

Homocysteine can be remethylated or metabolized to produce cysteine at its location in the transsulfuration pathway. No differences in the level of this metabolite, cysteine, was shown by betaine-feeding compared to control (Figure 7). Glutathione, a well-known antioxidant and a metabolite of cysteine, also showed no changes between the two groups (Figure 8). On the other hand, hypotaurine, produced from cysteine via an alternative pathway, was increased in the betaine treated group, while taurine, the end product of this pathway, was shown to be decreased significantly in the group supplemented with betaine (Figure 9).

The production of cysteine from homocysteine is catalyzed by CβS. The protein expression of CβS did not show differences between the control and the betaine-treated group of rats (Figure 10). Also no changes were seen between the groups in the protein expression of GCS, the rate limiting enzyme in the production of GSH from cysteine. However, the enzyme system responsible for the production of taurine from cysteine showed changes between the control and the betaine-supplemented groups. CDO was decreased significantly in activity and protein expression, whilst surprisingly CDC was increased in both parameters in contrast to the changes of CDO (Figure 11).

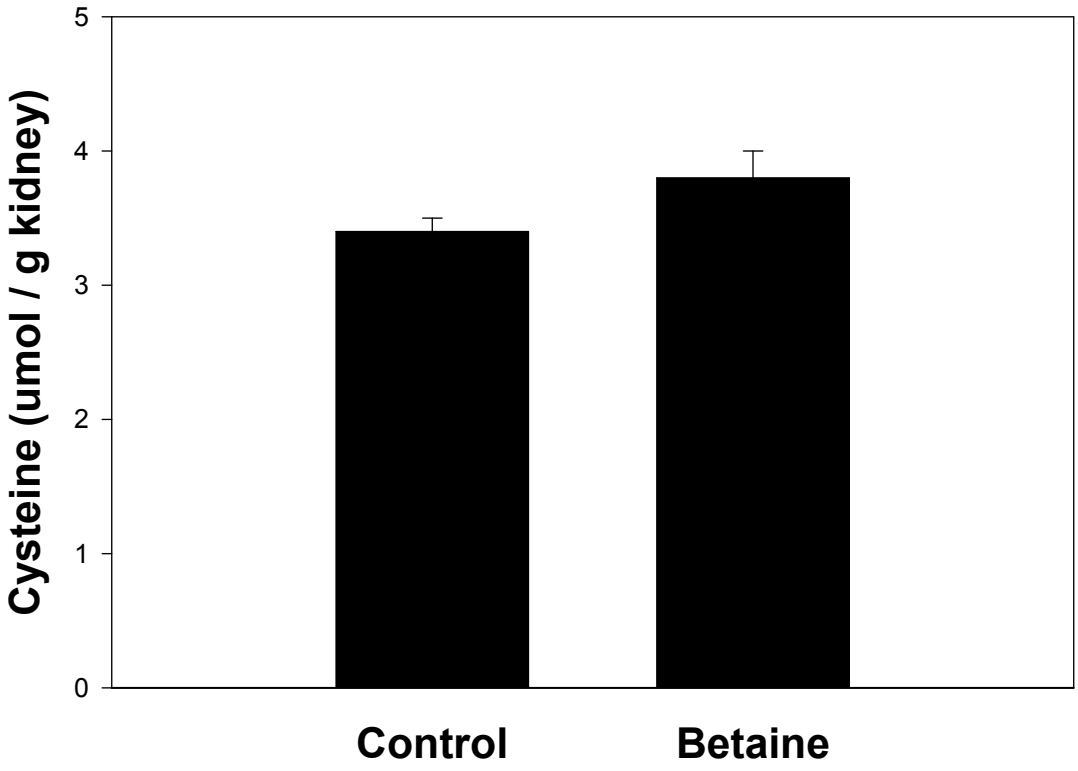

Figure 7. Effect of betaine on renal cysteine level. Each value represents the mean ± SE for six rats.

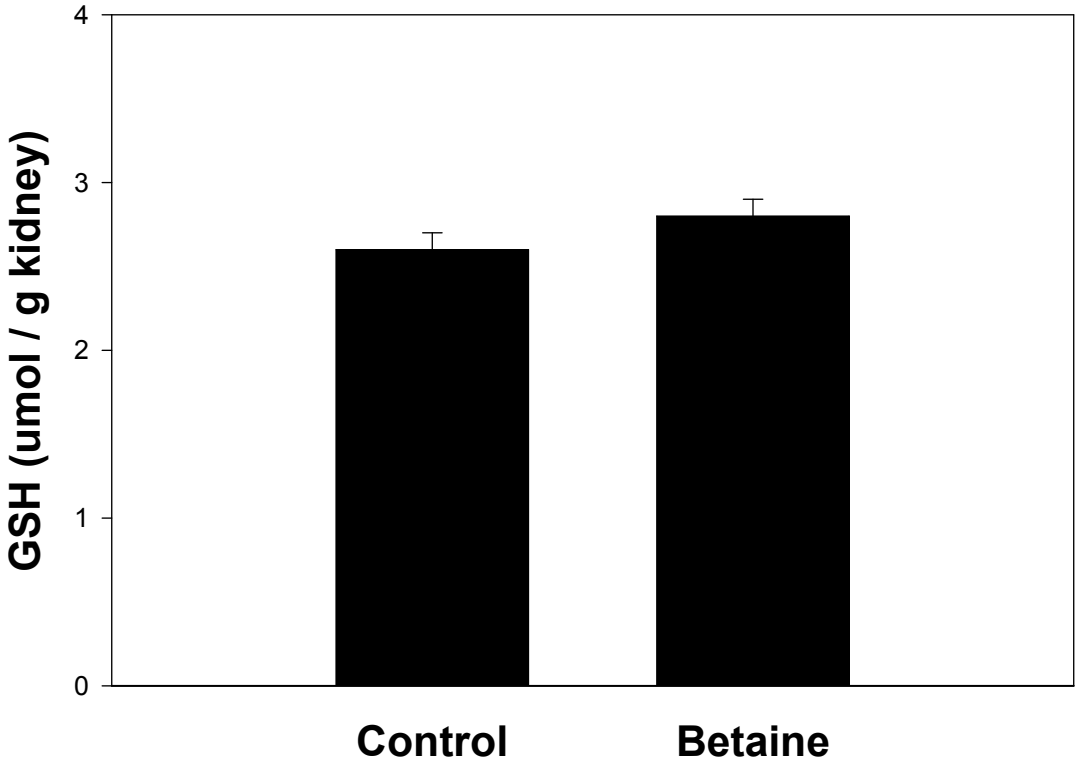

Figure 8. Effect of betaine on renal GSH level. Each value represents the mean ± SE for six rats.

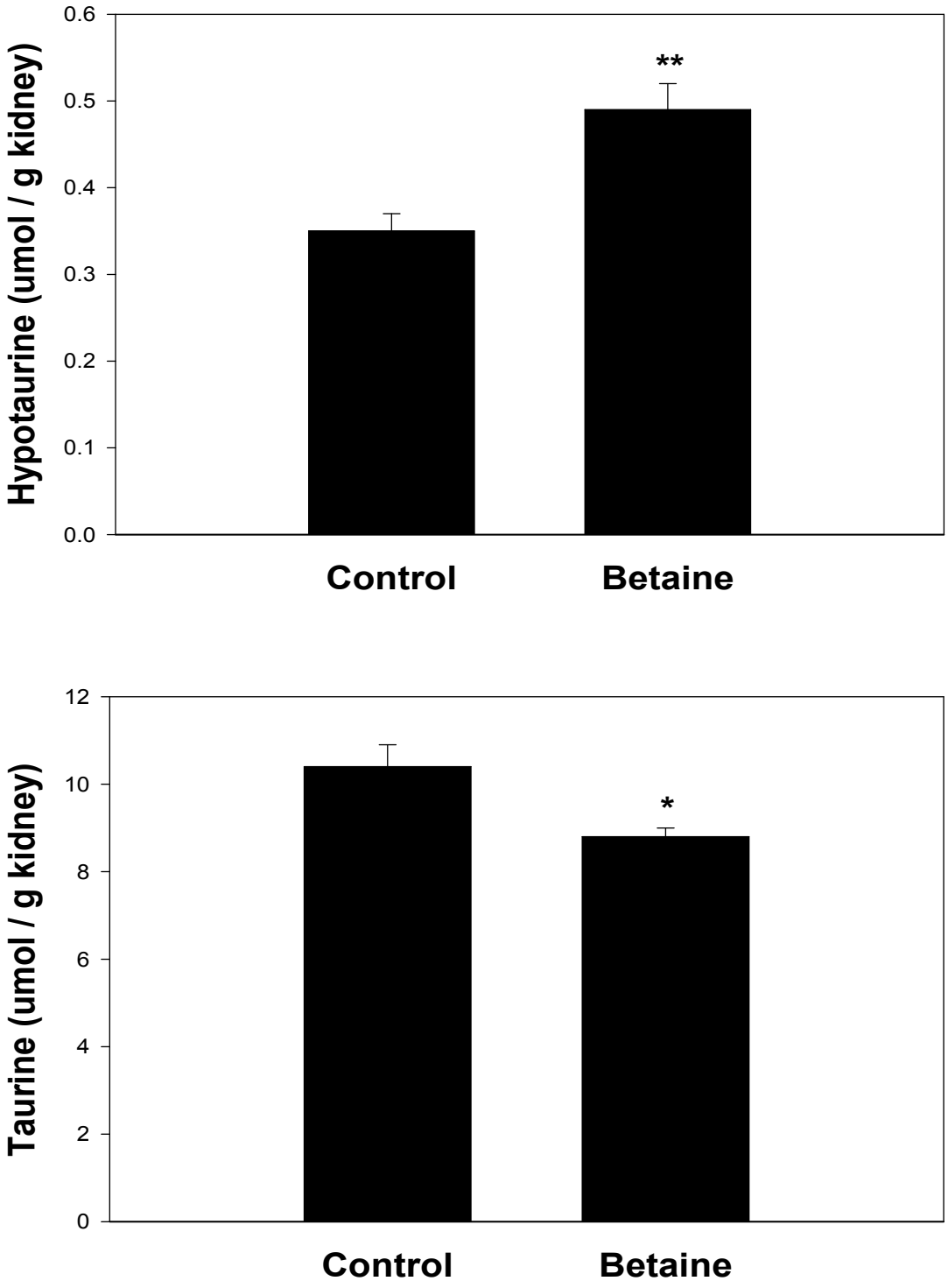

Figure 9. Effect of betaine on renal hypotaurine and taurine level. Each value represents the mean ± SE for six rats. *, ** Significantly difference from control at $P < 0.05$, 0.01 respectively (Student's t-test)

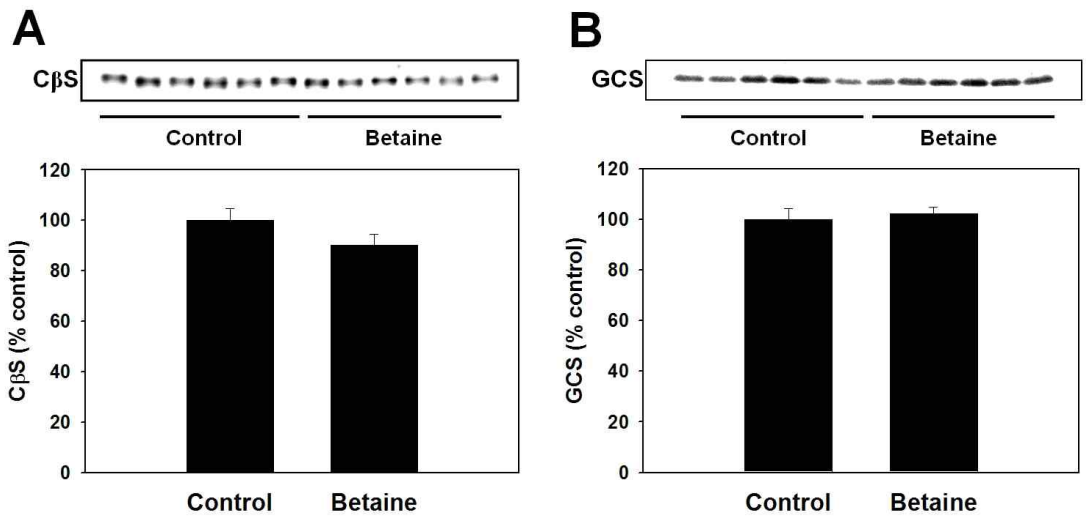

Figure 10. Effect of betaine administration on renal CβS (A) and GCS (B) proteins. Each value represents the mean ± SE for six rats.

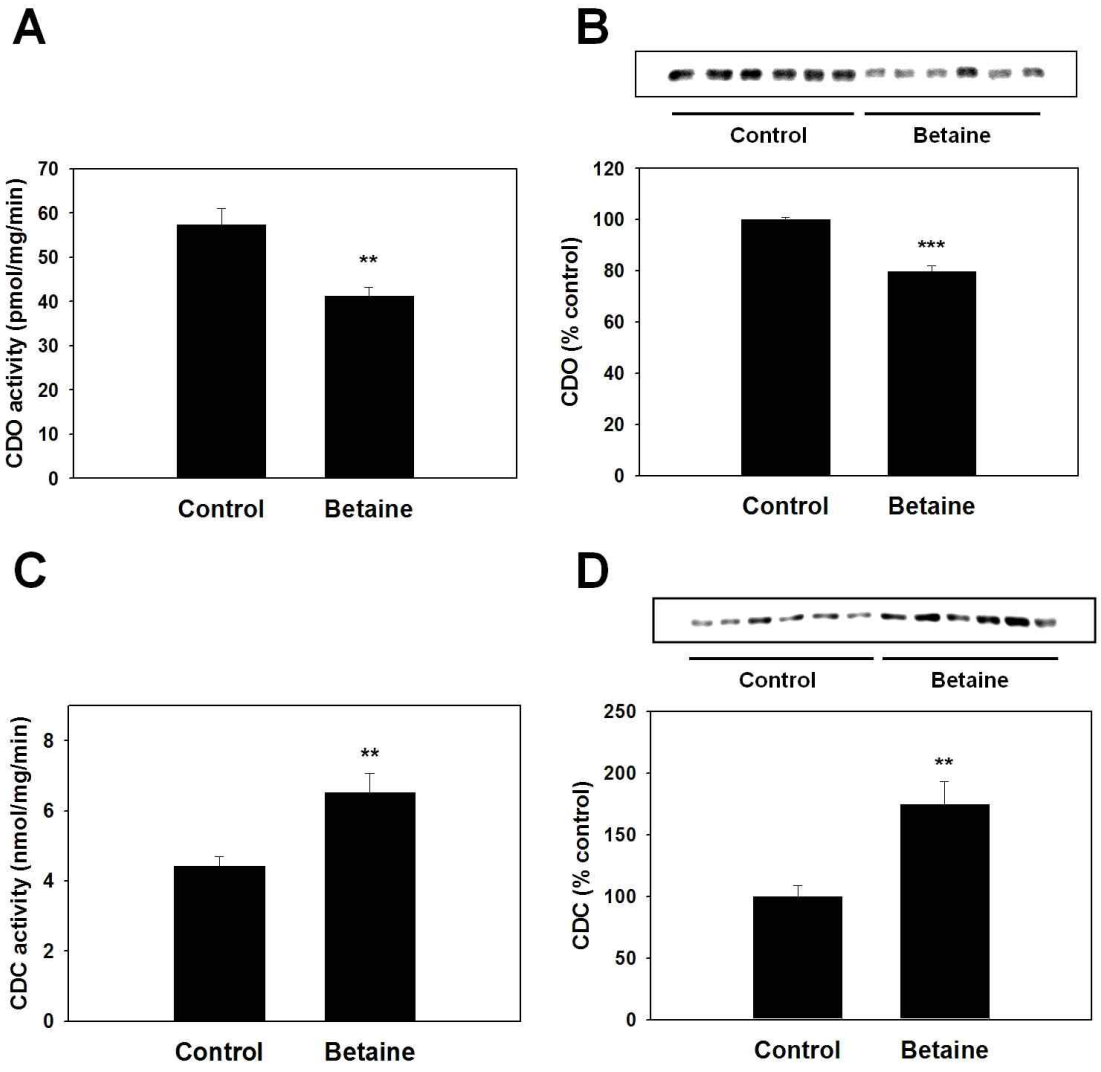

Figure 11. Effect of betaine on renal CDO activity (A), CDO protein (B), CDC activity (C), and CDC protein (D). Each value represents the mean ± SE for six rats. **, *** Significantly different from control at $P < 0.01$, 0.001, respectively (Student's t-test).

Discussion

Betaine is a well-known osmolyte to control renal osmolarity (Lohr et al., 1991; Horio et al., 2001). Betaine also reacts with homocysteine by the catalysis of BHMT to produce methionine. Numerous studies have reported on the effect of betaine on the transsulfuration pathway of the liver such as increases in methionine level, SAM : SAH ratio, and decreased taurine production to facilitate GSH production (Kim and Kim 2005; Purohit et al., 2007).

The transsulfuration system is present in the kidney, and the metabolism of sulfur-containing substances occurs actively via this pathway. This implies an important role of kidney in the regulation of sulfur-containing substances in the body. In this study the effects of betaine on the metabolomics of renal sulfur-containing substance were investigated.

The results of this study show that betaine affects the renal transsulfuration pathway significantly, including changes in the sulfur-containing substances as well as the related enzyme system. Rats fed betaine in drinking water showed approximately 42 % increase in renal methionine concentration. Changes in renal BHMT were measured to determine if the change in methionine level was due to either of this enzyme. However, the protein expression of BHMT was almost non-detectable (data not shown), and the activity, which is known to be significantly lower than that in the liver, did not show any changes associated with betaine intake.

Methionine can also be produced by MS in the transsulfuration pathway. However, no changes in MS were observed either, and this led to the assumption that the increase in renal methionine level was not due to changes in the two enzymes. Garcia and Stipanuk (1992) have reported that methionine is taken up by the kidney from the blood for its excretion and metabolism. Therefore the plasma methioinine level was measured. The result showed that plasma methionine concentration increased in the betaine-administered group of rats. The liver exports methionine into the blood stream (Wilson et al 2009, Shinohara et al 2006, Korendyaseva et al 2010). Therefore it seems that the plasma methionine level was increased subsequently from export of the enhanced hepatic methionine concentration from betaine intake into the bloodstream. The uptake of the larger amount of methionine from blood into the kidney seems to have resulted in the increase of the renal methionine level.

MAT mediates the synthesis of SAM from methionine. Renal MAT activity was higher in the betaine fed group compared to control. MAT is known to have three types, and MAT II is expressed in extrahepatic tissues (Sullivan and Hoffman 1983, Mato et al 1997, Finkelstein 1990). Therefore the protein expression of MAT II was measured. The results showed that MAT II protein expression was enhanced in the betaine treated group of rats. SAM concentration was also higher in the betaine-fed group, and therefore it seems that the increase in renal methionine level is responsible for the induced MAT II activity and its product. Unlike its metabolic precursor, SAH concentration did not differ between the two groups of rats. SAM is used in the body as a biological methyl donor resulting in the methylation of lipids, proteins, and nucleic acids, the production of polyamines, and an antioxidant (Chiang et al., 1996; Finkelstein, 2007; Gonzalez-Correa et al., 1997; Evans et al., 1997). In addition, SAM itself can act as an inhibitor of MAT II, and an activator of CβS (Finkelstein, 2007). While the ratio of SAM : SAH shows the methylation potential of cells, it has also been reported that changes in this ratio is correlated to alterations of certain enzymes in the transsulfuration pathway (Williams and Schalinkske, 2007). Thus the increase in renal SAM level, and consequently the SAM : SAH ratio in the results imply significance of betaine treatment in modifying the renal metabolism of sulfur-containing substances.

Homocysteine, placed at the junction between the remethylation and the transsulfuration process, was not affected by betaine intake in the kidney. A previous study reported that betaine intake in mice decreased hepatic homocysteine concentration (Kim and Kim, 2005). Unlike the liver, however, plasma homocysteine level was not changed. The kidney is known to import homocysteine from the blood (Bostom et al., 1995; House et al., 1997, 1998). Therefore, if any changes in plasma homocysteine occured, it seems likely that the renal homocysteine level will reflect such modification. The absence of changes in renal homocysteine level was in accordance with the plasma homocysteine level, implying that betaine treatment affects neither the plasma nor the renal homocysteine concentrations.

Homocysteine is metabolized to cystathionine by CβS in the transsulfuration pathway, and this enzyme has been shown to be expressed in the kidney (Finkelstein, 1990). Cysteine can then be metabolized to produce GSH by GCS that is well expressed in the (Meister and Tate, 1976; Stipanuk et al., 2002). In

the previous study, hepatic concentrations of cysteine or its metabolite GSH were unchanged by betaine treatment and similar results were shown in this study for renal concentrations. The enzymes responsible for mediating the synthesis of these substances, CβS and GCS, were also unaffected, which is in accordance with the previous data for hepatic CβS and GCS.

Cysteine has two metabolic processes in the transsulfuration pathway. One is GSH synthesis, and the other the synthesis of taurine. Cysteine can be metabolized by CDO to cysteine sulfinate. In the following step, CDC uses cysteine sulfinate as substrate to produce hypotaurine. The transformation of hypotaurine to taurine is known to be spontaneous (Pecci et al., 1999). CDO is expressed in the kidney (Stipanuk et al., 1990; Stipanuk et al., 2002; Stipanuk and Ueki, 2011). The results showed that renal CDO activity decreased in the betaine-treated rats. To investigate at what stage this change was made, CDO protein expression was measured. The protein expression of CDO was decreased in the betaine-fed rats, and from this it was suggested that the protein level and activity of CDO was down-regulated by betaine in the kidney. The final product of CDO, taurine, was also lowered in the betaine administered group. Taurine is known to act as an osmolyte (Graham and Wilkinson, 1992; Yancey, 2005; Chesney et al., 2010) as is betaine (Graham and Wilkinson, 1992; Horio et al., 2001; Wettstein et al., 1998; Lohr et al., 1991). Since betaine was provided in this study, it is likely that the total concentration of osmolytes in renal cells would have increased. Graham and Wilkinson (1992) reported on the protective effects of betaine and taurine under high levels of osmotic stress. The results showed that betaine was more effective as an osmoprotectant than taurine. It is possible that the cellular osmolarity was adjusted by decreasing taurine level and synthesis as an adaptive modification, maintaining the total concentration of osmolytes when betaine, an exogenous osmolyte and a better osmoprotectant, was provided. This is also supported by the report of Burg (1994) that experimentally altering the concentration of one osmolyte changes the others to maintain a constant total sum level.

CDC is involved in the process of taurine synthesis. The activity and protein expression of this enzyme were shown to be enhanced with betaine treatment, resulting in the increase of the direct product, hypotaurine. Rentschler et al (1986) showed that when taurine was deficient CDC activity is induced, and Eppler and Dawson Jr (2001) showed similar results in rats. Therefore in

contrast to the decreases in CDO and taurine, increases in CDC activity and protein expression seem to be induced to compensate for the decrease in taurine level.

Conclusion

The results of this study show that betaine feeding results in changes in the metabolomics of sulfur-containing substances in kidney, even with very low expression of BHMT. The changes induced in the renal transsulfuration pathway are comparable to those reported in the liver. When rats were fed betaine for two weeks renal methionine, SAM, and the related enzyme MAT II activity were enhanced. The increase in methionine, however, does not seem to be due to increased production in the kidney, but by an increased uptake from the blood which contained higher concentrations of methionine from other tissues, especially the liver. No changes were induced by betaine in levels of SAH, homocysteine, cysteine, GSH or CβS and GCS, enzymes responsible for the synthesis of cysteine and glutathione, respectively. However, in the pathway for taurine synthesis CDO activity and protein expression decreased significantly leading to diminished taurine concentration but CDC activity and protein expression were enhanced, inducing an increase in hypotaurine level.

In our previous study (Kim and Kim, 2005) betaine intake led to a significant increase in methionine and SAM levels. SAM is not only a principle methyl donor, but acts also as an antioxidant and a regulator of enzymes involved in the transsulfuration pathway (Chiang et al., 1996; Evans et al., 1997; Finkelstein, 2007). The decrease in SAM and SAM : SAH ratio has been reported to be related to various diseases, including those of the kidney (Herrmann et al., 2005; Loehrer et al., 1998), and numerous studies have reported on the beneficial effect of SAM treatment in restoring the hepatic transsulfuration pathway to normal range (Mato et al., 2008, Kim et al., 2008). The protein expression of BHMT was negligible in the kidney, and the activity detected was substantially lower than that known in the liver. Therefore BHMT did not seem to have a significant role in the betaine induced changes in renal metabolomics of the sulfur-containing substances. However, not only were the changes induced by betaine feeding in the kidney were similar to those in the liver, but as expected, SAM level and SAM : SAH ratio was significantly increased in the kidney of betaine treated rats. The results imply the significance of betaine treatment in modifying the renal metabolism of sulfur-containing substances, and thus, future studies on the physiological significance of betaine administration on kidney malfunction or diseases seem necessary.

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국문초록

선행연구에서 betaine 투여가 랫트의 간 황 함유 아미노산 대사에 영향을 미친다는 것이 보고된 바 있다. 본 연구에서는 betaine 이 신장 황 함유 아미노산의 대사에 일으키는 변화를 측정하기 위해 랫트에 2 주간 1% 의 betaine 을 식수로 공급하였다. Betaine 은 신장 내 methionine 수치를 42 % 증가시켰다. 하지만 신장 내의 betaine-homocysteine methyltranferase (BHMT) 효소 함량이나 활성에는 변화가 없었다. Homocysteine 에서 methionine 으로의 remethylation 과정에 관여하는 또 다른 효소인 methionine synthase 역시 betaine 에 의해 변화하지 않았다. Betaine 투여에 의해 S-adenosylmethionine (SAM) 이 증가하였는데 SAM 을 생성하는 효소로 알려진 methionine adenosyltransferase I/III (MAT I/III) 는 측정되지 않았으나 extrahepatic tissue 에 존재한다고 알려진 methionine adenosyltransferase II (MAT II) 는 그 발현이 증가하였다. 하지만 S-adenylhomocysteine (SAH), total homocysteine, cysteine level 그리고 cystathionine β-synthase 단백질 발현에는 betaine 투여에 의한 변화가 관찰되지 않았다. 또한 glutathione (GSH) level 이나 GSH 의 생성을 매개하는 효소인 γ-glutamylcysteine synthetase (GCS) 의 발현 역시 betaine 섭취에 의한 차이가 보이지 않았다. 한편, taurine 생성 과정의 율속인자인 cysteine dioxygenase (CDO) 는 betaine 에 의해 현저히 감소하였다. 그러나 이와는 반대로, cysteine sulfinate decarboxylase (CDC) 의 발현과 hypotaurine level 은 유의적으로 증가하였다. 이상의 결과들은 betaine 이 신장 BHMT 의 결핍에도 불구하고 신장 내 황 함유 아미노산의 대사에 유의적인 영향을 미친다는 것을 시사하며, betaine 투여에 의해 증가한 간 내 methionine 이 혈액을 통해 신장으로 공급되어 신장 내 methionine 의 양을 증가시킨 것으로 추정된다. 본 연구결과의 생리학적 및 생화학적 중요성을 밝히기 위한 추가적인 연구가 필요할 것으로 생각된다.

주요어: Betaine; kidney; sulfur-amino acid; transsulfuration reaction

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