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Hyperhomocysteinemia Associated With Decreased Renal Transsulfuration Activity in Dahl S Rats

Ningjun Li, Li Chen, Rachel W. Muh, Pin-Lan Li

Abstract—Elevated plasma homocysteine (Hcys) has been reported to participate in the development of arterial and glomerular sclerosis in Dahl salt-sensitive hypertensive (SS) rats. The mechanism resulting in hyperhomocysteinemia in these animals remains unknown. Disposal of Hcys in the kidneys plays an important role in regulating the plasma Hcys level. We, therefore, examined the activities and expressions of the enzymes involved in the metabolism of Hcys in the kidneys of SS rats, compared with that of Brown Norway rats and SSBN13 rats, a consomic subcolony of SS rats that carries a substituted chromosome 13 from Brown Norway rats. High-performance liquid chromatography analysis demonstrated that plasma Hcys levels were significantly higher in SS rats. The conversion of S-adenosylhomocysteine into Hcys via S-adenosylhomocysteine hydrolase by renal tissue was not different among these 3 rat strains. However, the metabolic rate of Hcys into cysteine was markedly reduced in the SS rat kidneys. The mRNA and protein levels of cystathionine β-synthase (CBS), one of the key enzymes in the transsulfuration pathway in the kidneys, were significantly lower in SS rats. In microdissected nephron segments, CBS mRNA was shown to be mainly present in renal proximal tubules (PTs). The mRNA levels of CBS in the PTs were also significantly decreased in SS rats, accompanied by a reduced CBS activity in PTs. We conclude that hyperhomocysteinemia is associated with a decreased activity and expression of CBS in renal PTs because of the defect of chromosome 13 in SS rats. (*Hypertension*. 2006; 47:1094-1100.)

Key Words: hypertension, genetic ■ glomerulosclerosis ■ cardiovascular diseases

Hyperhomocysteinemia (hHcys) has been indicated as an independent risk factor for cardiovascular diseases[1,2] and as a pathogenic factor causing a variety of pathological changes in different cells or tissues.[3] Most recently, we have reported that increases in plasma homocysteine (Hcys) levels resulted in proteinuria and glomerulosclerosis in Sprague Dawley rats, and hHcys participated in the development of arterial and glomerular sclerosis in Dahl salt-sensitive hypertensive (SS) rats.[4] However, the mechanisms leading to increases in plasma Hcys levels in SS rats remain unknown.

Hcys is produced from S-adenosylhomocysteine (SAH) through the catalysis of SAH hydrolase. The metabolism of Hcys stands at the intersection of 2 pathways, including remethylation to methionine by methyltetra-hydrofolate-Hcys methyltransferase (MTHM) and transsulfuration to cystathionine by cystathionine β-synthase (CBS) and then cleaved to cysteine by γ-cystathionase (GCS).[5–7] It has been indicated that the activities of these metabolic pathways are the important determinants of the plasma Hcys levels.[6,8–10] The genes of these enzymes are located on rat chromosome (Chr) 3 (SAH hydrolase), Chr 17 (MTHM), Chr 20 (CBS), and Chr 2 (GCS), respectively.

It has been demonstrated that the kidneys play an important role in the regulation of plasma Hcys levels. The kidneys remove 70% of the daily Hcys load,[11,12] mainly through the transsulfuration pathway.[8,10,13,14] This important role of the kidneys in the metabolism of Hcys is also supported by studies in patients with chronic renal failure. Most (85% to 100%) patients with end-stage renal disease have hHcys.[15] It has been indicated that the hHcys in end-stage renal disease is induced by the abnormality of Hcys metabolism in the kidneys but not simply because of reduced glomerular filtration of Hcys.[10,15,16] Although these observations suggest a major role for the kidneys in Hcys elimination, the role of the kidneys in the development of hHcys and the possible mechanisms leading to impaired clearance of Hcys by the kidneys have yet to be elucidated.

The present study was designed to test the hypothesis that there were defects in the enzymes involved in the Hcys metabolism in the kidneys of SS rats, which was probably the mechanism mediating hHcys in SS rats. The SS, Brown Norway (BN), and SSBN13 rats were used for comparison. The SSBN13 rat is a normotensive consomic subcolony of SS rats that carries a substituted Chr 13 from BN rat and has been demonstrated to be resistant to high salt-induced hypertension and associated renal injury with only a 2% genetic difference from SS rats.[17] We first analyzed the plasma Hcys levels

using the fluorescence high-performance liquid chromatography (HPLC) technique to determine the presence of hHcys in SS rats. We then compared the activities and expressions of the enzymes involved in Hcys production and transsulfuration, including SAH hydrolase, CBS, and GCS, in the kidneys among these rat strains. We further determined the mRNA levels of CBS, a key enzyme for Hcys transsulfuration, along microdissected nephron segments and compared its enzymatic activity in proximal tubules (PTs) between SS and SSBN13 rats. To our knowledge, the results from the present study provide the first evidence that decreased activity and expression of CBS in renal PTs because of a defect of Chr 13 is a determinant of hHcys in SS rats.

Methods

Animals
The animals used in the present study were male adult inbred lines of Dahl SS rats (Dahl/SSMcwi), consomic SSBN13 rats, and BN rats (BN/MCWi). These 3 rat strains were originally generated by the Animal Resource Center and Molecular Genetic Center at the Medical College of Wisconsin (Milwaukee, WI) and are now commercially available from Charles River Laboratories (Wilmington, MA). All of the animals were maintained on a low-salt diet (0.4% NaCl, Dyets, Inc) with free access to drinking water before the experiments. It was reported that when maintained with a low-salt diet, all of the animals were normotensive.[17] All of the animal procedures were approved by the Institutional Animal Care and Use Committee.

HPLC Analysis of Hcys, Cysteine, and the Activities of SAH Hydrolase and Transsulfuration Pathway in the Kidneys
Homocysteine and cysteine concentrations were measured using fluorescence HPLC analysis as we described previously.[4] To measure the SAH hydrolase activity, increasing concentrations of SAH (10 to 500 μmol/L; Sigma-Aldrich) were incubated with renal tissue homogenates (100 μg) mixed with erythro-9-(2-Hydroxy-3-nonyl) adenine (0.03 mg/mL) at 37°C for 60 minutes. The formation of Hcys was determined by HPLC analysis, and SAH hydrolase activity was present as the conversion rate of SAH into Hcys. To determine the activities of the transsulfuration pathway, renal tissue homogenates were incubated with Hcys (1 to 500 μmol/L) or cystathionine (1 to 500 μmol/L), respectively, at 37°C for 60 minutes. The production of cysteine was determined by HPLC analysis, and the enzymatic activities were present as the conversion rate of Hcys or cystathionine into cysteine. Both the Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of these enzymes in renal homogenates were calculated using a Lineweaver–Burk plot.

Microdissection of Nephron Segments From Rat Kidney
Microdissection of rat nephron segments was performed as described previously.[18,19] Forty glomeruli and 40 mm of tubules were collected and dissolved in TRIzol solution for RNA extraction. Separated groups of freshly dissected PTs (80 mm) were sonicated in 10 μL of HEPES buffer (25 mmol/L sodium HEPES, 1 mmol/L EDTA, and 0.1 mmol/L PMSF) and incubated with Hcys to measure the enzymatic activities of the transsulfuration pathway using HPLC analysis.

RNA Extraction, Northern Blot, and Real-Time RT–PCR Analyses of the mRNA Levels of SAH Hydrolase, CBS, and GCS in Rat Kidneys
Total RNA from renal cortical tissues was extracted using TRIzol solution (Life Technologies, Inc) according to the manufacturer's protocol. The mRNA levels of SAH hydrolase, CBS, and GCS were determined by real-time RT–PCR as described below and by Northern blot analyses as we described previously.[20]

Quantitative RT–PCR Analyses of SAH Hydrolase, CBS, and GCS mRNA Levels in Rat Renal Cortical Tissue and CBS mRNA Expression in Microdissected Nephron Segments
Total RNAs from renal cortical tissue and microdissected nephron segments were reverse transcribed (cDNA Synthesis kit, Bio-Rad). The RT products were amplified by using a SYBR green real-time quantitative PCR kit (Bio-Rad). Primers were designed with a primer design computer program (Biodesigner, Bio-Rad) on the basis of rat SAH hydrolase, CBS, and GCS mRNA and 18s ribosomal RNA (rRNA) sequences from GenBank. For SAH hydrolase, the sense primer was 5′-CTTCACAAACCAGGTGAT-3′, and the antisense primer was 5′-CCTCATCCAGCTTCTTAG-3′. For CBS, the sense primer was 5′-ATTCCCCACATTACCAC-3′, and the antisense primer was 5′-TTGATTCTGACCATAGG-3′. For GCS, the sense primer was 5′-GGCTCTGGGTGCTGATAT-3′, and the antisense primer was 5′-CGTTGAGGTCATCGGAAT-3′. For 18s rRNA, the sense primer was 5′-CGCCGCTAGAGGTGA-AATTC-3′, and the antisense primer was 5′-TCTTGGCAAATG-CTTTCGC-3′. The real-time quantitative PCR was performed using an iCycler iQ Real-Time PCR Detection System (Bio-Rad) according to the manufacturer's manual. Data were gathered and analyzed by the same real-time PCR detection system. The cycle threshold (Ct) values were exported into a Microsoft Excel worksheet for calculation of gene expression in accordance with the $\Delta\Delta$Ct method. The Ct values were first normalized with respect to 18s rRNA levels to obtain ΔCt values. The ΔCt values from SSBN13 rats were used as a reference to calculate $\Delta\Delta$Ct values for all of the samples. Relative mRNA levels were expressed by the values of $2^{-\Delta\Delta Ct}$.

Western Blot
Western blot analyses were performed to determine the protein levels of CBS in the renal cortex using a mouse anti-human CBS polyclonal antibody (Abnova Co). Band intensities of CBS were normalized to that of β-actin on the same membrane after it was stripped and reprobed with anti-β-actin antibody.

Statistics
Data are presented as mean±SEM. The significance of differences within and between groups in multiple groups of experiments was evaluated using ANOVA for repeated measures, followed by Duncan's multiple range tests. The significance of differences between 2 groups was evaluated by Student t test. $P<0.05$ was considered statistically significant.

Results

Fluorescent HPLC Analysis of Plasma Hcys Levels and the Metabolites of Hcys in Renal Cortical Homogenates
Figure 1A depicts typical chromatograms showing standard Hcys and its metabolites, as well as the production of cysteine through the transsulfuration pathway in the renal cortical homogenates from BN rats. Figure 1B shows plasma total Hcys (tHcys) levels measured by fluorescent HPLC analysis. The plasma Hcys levels were significantly higher in SS rats (10.7±0.88 μmol/L) than in BN (7.5±0.32 μmol/L) and SSBN13 rats (6.7±0.50 μmol/L).

The Activities of SAH Hydrolase, Transsulfuration, and GCS in Renal Homogenates
SAH hydrolase activities were measured by the conversion rates of SAH into Hcys in renal cortical homogenates using
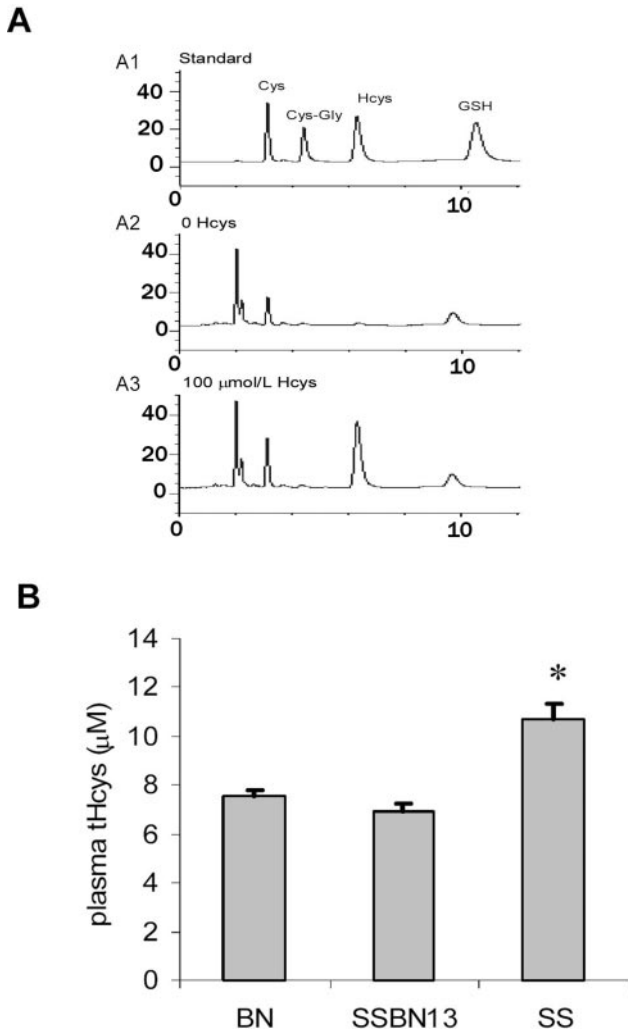
A

B

Figure 1. Fluorescent HPLC analysis of Hcys and other thiols. (A) representative chromatograms showing fluorescent derivatives of Hcys and other thiols. (A1) standard solution. (A2) production of cysteine in renal cortical homogenates from BN rats without adding Hcys as substrate. (A3) increased production of cysteine by renal cortical homogenates from BN rats after addition of Hcys. Cys indicates cysteine; CysGly, cysteinylglycine; Hcys, homocysteine; and GSH, glutathione. (B) plasma total (tHcys) concentrations in BN, SSBN13, and SS rats (n=5). *$P<0.05$ vs BN and SSBN13 rats.

A

B

Figure 2. The activity of SAH hydrolase in renal homogenates presented as conversion rate of SAH to Hcys. (A) production rate of Hcys after incubation of renal tissue homogenates with different concentrations of SAH. (B) comparison of enzyme kinetics in SAH hydrolase among BN, SSBN13, and SS rats (n=6).

fluorescent HPLC analysis and presented in Figure 2. When incubated with SAH, tissue homogenates produced Hcys in a substrate concentration–dependent manner. There was no significant difference in SAH hydrolase activity among BN, SSBN13, and SS rats (Figure 2A). The calculated K_m by Lineweaver–Burk Plot averaged 0.008, 0.008, and 0.011 mmol/L, and V_{max} 0.71, 0.67, and 0.69 nmol/min per milligram of protein in BN, SSBN13, and SS rats, respectively (Figure 2B).

Transsulfuration activities were measured by the conversion rate of Hcys into cysteine in renal cortical homogenates and are presented in Figure 3. Incubation of renal homogenates with Hcys produced cysteine in a substrate concentration–dependent manner. However, the production rate of cysteine was significantly reduced in SS rats compared 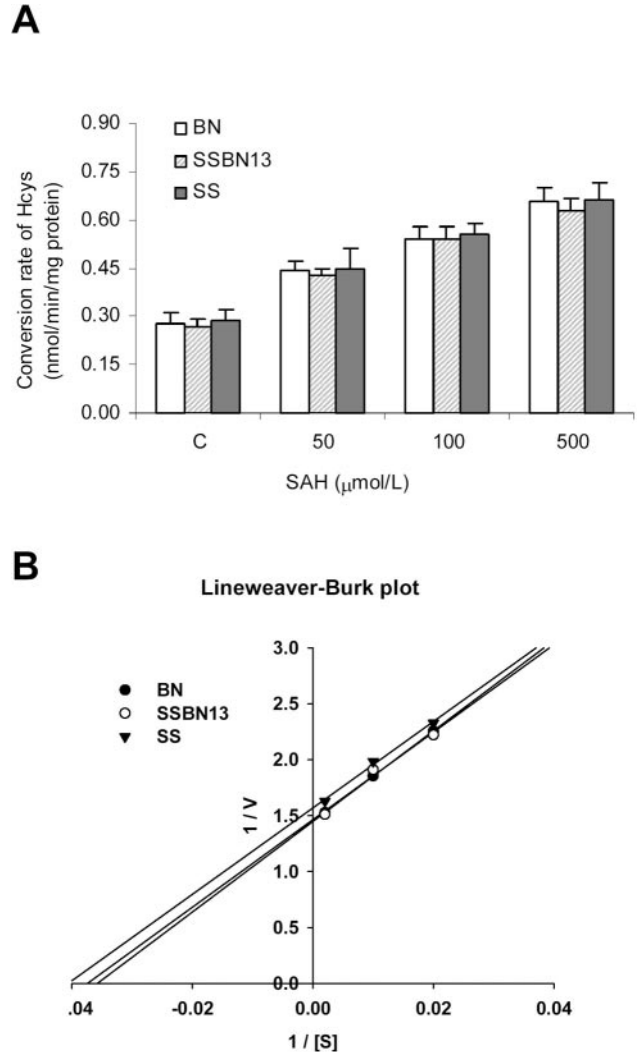with BN and SSBN13 rats (Figure 3A). This reduction of transsulfuration activity was primarily associated with a decreased V_{max} of the enzymes in transsulfuration pathway in SS rats (V_{max}, 0.85±0.06 in BN and 0.84±0.05 in SSBN13 versus 0.43±0.03 nmol/min per milligram of protein in SS rats; Figure 3B).

The conversion rate of cystathionine into cysteine was determined to represent GCS activity. When incubated with cystathionine, renal cortical tissue homogenates produced cysteine in a substrate concentration–dependent manner. However, there was no significant difference in GCS activities among these rat strains (data not shown).

Expressions of SAH Hydrolase, CBS, and GCS mRNAs in the Renal Cortical Tissues

Real-time RT–PCR analyses showed that CBS mRNA levels in renal cortical tissues were significantly lower in SS rats than in BN and SSBN13 rats (Figure 4A), whereas there was no significant difference in the mRNA expressions of SAH
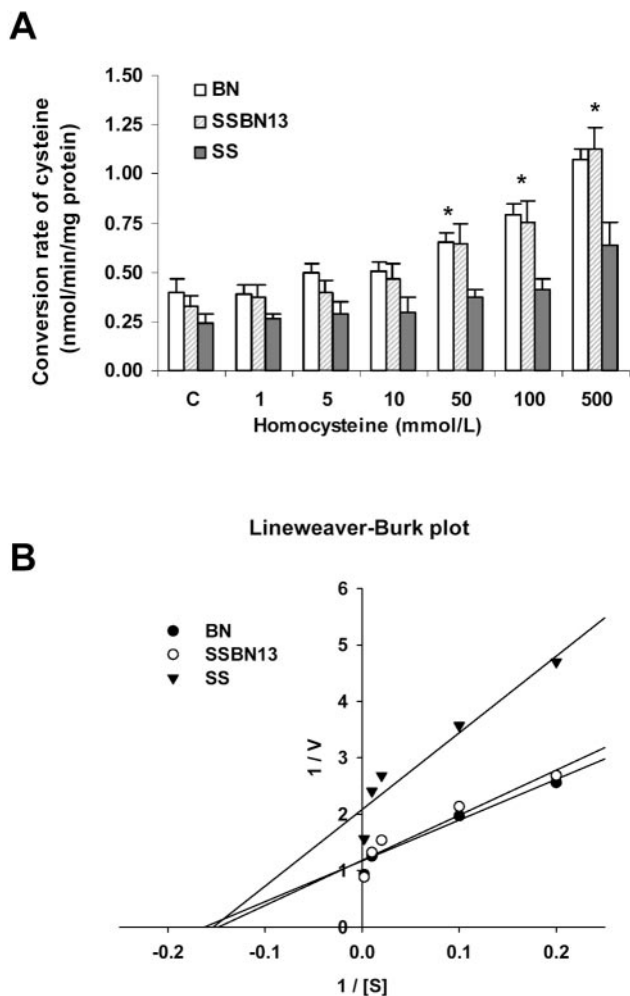
A

B

Lineweaver-Burk plot

Figure 3. The activities of Hcys transsulfuration in renal homogenates as measured by conversion rate of Hcys to cysteine. (A) production rate of cysteine after incubation of renal tissue homogenates with different concentrations of Hcys. (B) comparison of enzyme kinetics in transsulfuration activities among BN, SSBN13, and SS rats. *$P<0.05$ vs SS rats (n=6).

A

B

Figure 4. The mRNA expressions of SAH hydrolase, CBS, and GCS in renal cortical tissues. (A) relative mRNA levels in CBS by real-time RT–PCR analysis. *$P<0.05$ vs BN and SSBN13 rats (n=5). (B) abundances of mRNA in CBS, SAH hydrolase, and GCS by Northern blot analysis.

hydrolase and GCS in the kidneys among these rat strains (data not shown). The comparison of the mRNA levels in these enzymes was confirmed by Northern blot analysis, which also showed that the abundance of CBS mRNA was lower in SS rats than in SSBN13 rats, whereas there was no difference in the mRNA levels of SAH hydrolase and GCS between these 2 strains (Figure 4B).

CBS Protein Levels in the Renal Cortical Tissues in BN, SSBN13, and SS Rats

The data from the Western blot analyses of CBS protein levels in the kidneys from BN, SSBN13, and SS rats are shown in Figure 5. The immunoblots with the immunoreactive 63-kDa CBS protein bands are depicted in Figure 5A. The quantitative data from these experiments were summarized in Figure 5B. The blot intensities of CBS proteins were significantly decreased in the kidneys from SS rats compared with BN and SSBN13 rats.
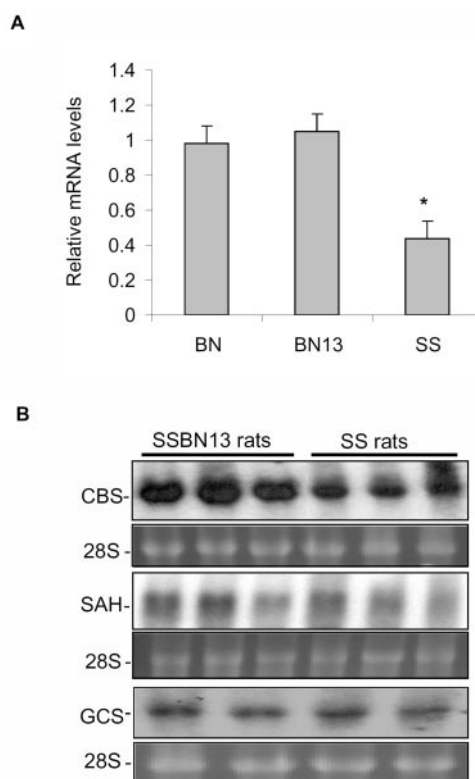
Relative Distribution of CBS mRNA in Nephron Segments

Because CBS was the major contributor to the decreased Hcys metabolism in SS rat kidneys, we further analyzed whether this alteration occurred in any renal tubular segments. In microdissected nephron segments, real-time quantitative RT–PCR analysis demonstrated that CBS was present in all of the nephron segments and mainly expressed in PTs. Similar to the data from renal cortical tissue, the mRNA levels of CBS in PTs were significantly lower in SS rats than that in SSBN13 rats (Figure 6A).

CBS Activities in Microdissected Renal PTs

Microdissected renal PTs were incubated with Hcys, and the generation of cysteine was analyzed by HPLC analysis. CBS activity was presented as the production rate of cysteine from Hcys by PTs at nmol/min per millimeter. The conversion rate of Hcys into cysteine by PTs was significantly lower in SS rats than in SSBN13 rats, which is consistent with the results from renal cortical tissues (Figure 6B).

Discussion

The present study showed that SS rats exhibited higher plasma Hcys levels compared with BN and SSBN13 rats even on a low-salt diet, suggesting that introgression of BN Chr 13 into an SS rat genome normalized plasma Hcys levels. Given the fact that the susceptibility to renal injury in SSBN13 rats was decreased compared with SS rats,[17] and normalization of
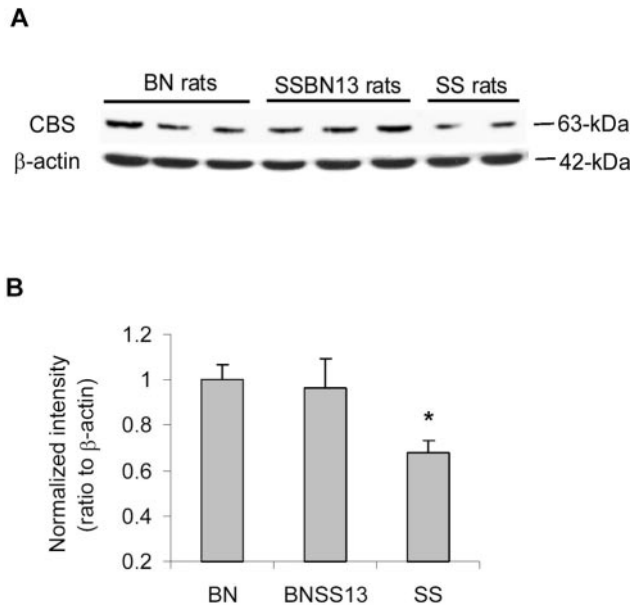
A

B

Figure 5. Western blot analyses of CBS protein levels in renal cortical tissues. (A) representative blot images. (B) densitometric summary of the blot analysis after normalized to β-actin (n=6).

plasma Hcys attenuated glomerular injury in SS rats,[4] the findings in the present study indicate that the decrease in plasma Hcys levels may be one of the important mechanisms attenuating glomerular injury in SSBN13 rats. It should be point out that, although hHcys is associated with hypertension in human studies,[21,22] there is no hHcys in some other hypertensive animal models, including spontaneously hypertensive rats and deoxycortisone acetate rats.[23,24] Therefore, there may be difference about the mechanism and contribution of hHcys in different forms of hypertension.

The kidneys are the major sites for the metabolism or removal of plasma Hcys.[11,12] Therefore, to explore the mechanisms leading to increased plasma Hcys levels in SS rats, we compared the activities of enzymes involved in the metabolism of Hcys in the kidneys among SS rats, their consomic SSBN13 counterparters, as well as BN rats, the other parent strain of SSBN13 rats. A recent study in our laboratory has demonstrated that renal cortical tissue is an important resource of Hcys and adenosine via SAH hydrolase and that this SAH-derived Hcys or adenosine importantly participates in the regulation of renal function.[25] Because SAH hydrolase is a primary enzyme to produce Hcys by converting SAH into Hcys,[26] we first analyzed the SAH hydrolase activities in renal cortical tissues from SS, SSBN13, and BN rats. It was found that tissue homogenates produced Hcys in a SAH concentration–dependent manner. However, there was no difference in SAH hydrolase activities among these rat strains, indicating that overproduction of Hcys is not involved in the mechanisms of hHcys in SS rats.

Because there was no difference in the production of Hcys by SAH activity in the kidneys among BN, SSBN13, and SS rats, we next analyzed the activities of the enzymes responsible for Hcys metabolism in the kidneys. Although there are 2 pathways for the metabolism of Hcys, remethylation of Hcys into methionine by MTHM, and transsulfuration of

Hcys into cysteine via CBS and GCS, it has been reported that Hcys is metabolized mainly through the transsulfuration pathway in the kidneys.[8,13,14] Our results demonstrated that conversion rates of Hcys into cysteine in renal homogenates were significantly lower in SS rats than in SSBN13 and BN rats, suggesting that decreased activity in the Hcys transsulfuration pathway may contribute to the hHcys in SS rats. However, transsulfuration activity as measured by the conversion of Hcys into cysteine includes the consecutive effects of 2 enzymes, namely CBS and GCS. To dissect the contributions of CBS and GCS to the decreased transsulfuration activity in SS rats, we examined the conversion rate of cystathionine, an intermediate product in the transsulfuration pathway, into cysteine to represent GCS activity. The major reason for this experimental design is that, to date, there is no reliable method to quantitate cystathionine during the converting process of Hcys into cysteine. Therefore, it is difficult to directly measure CBS activity by detecting the conversion of Hcys into cystathionine. Our studies showed that there was no significant difference in GCS activity among SS, SSBN13,

A

B
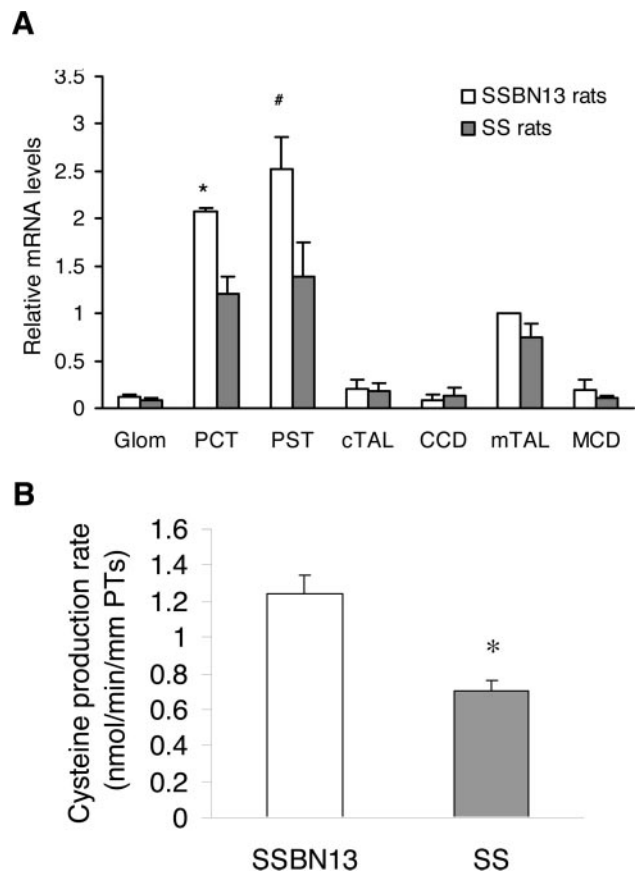
Figure 6. CBS mRNA expression and activities in microdissected rat nephron segments. (A) real-time quantitative RT–PCR analysis of CBS mRNA expression. Glom indicates glomeruli; PCT, proximal convoluted tubule; PST, proximal straight tubule; mTAL, medullary thick ascending limb of Henle's loop; cTAL, cortical thick ascending limb of Henle's loop; CCD, cortical collecting duct; MCD, medullary collecting duct. The ΔCt values from mTAL of SSBN13 rats were used as a reference to calculate $\Delta\Delta$Ct values for all other samples *$P<0.05$ vs SS rats; #$P<0.01$ vs SS rats (n=5). (B) CBS activities in microdissected renal PTs. *$P<0.05$ vs SSBN13 rats (n=5).

and BN rats. It seems that reduced transsulfuration activity in the kidneys of SS rat is because of decreased activity of CBS. To our knowledge, these results provide the first evidence indicating that hHcys is associated with an impaired renal CBS activity. Given the important role of the kidneys in the regulation of plasma Hcys,[12,16,27] this alteration of Hcys metabolism in the kidneys may present an important mechanism for the development of hHcys.

Because the values of V_{max} in CBS activities were different among SS, SSBN13, and BN rats, it was possible that there was an abnormality in the availability or expression of this enzyme in SS rats. To address this issue, we examined the mRNA levels of these enzymes and found that the mRNA levels in CBS were significantly lower in SS rats, whereas the expressions of mRNA in SAH hydrolase and GCS were not different in these 3 rat strains. Western blot analyses further demonstrated that CBS protein levels were also decreased in SS rats compared with SSBN13 and BN rats. These results agree with the data from analyses in the enzymatic activities and suggest that decreased activity of CBS in SS rats is attributed to the reduced expression of this enzyme, thereby diminishing renal transsulfuration activity.

The important role of CBS in the regulation of plasma Hcys levels has also been supported by the result obtained from gene knockout mice and transgenic mice in recent reports. In the mice with heterozygous disruption of the CBS gene, the plasma Hcys levels were increased by 50%.[28] In contrast, elevating CBS activity by overexpression of CBS decreased plasma Hcys by 45% in transgenic mice.[29] In addition, plasma Hcys levels were significantly decreased in patients with Down syndrome, because that CBS gene is located on human Chr 21 and overexpressed because of trisomy 21 in these patients.[30] Therefore, decreased expression and activity of CBS in the kidneys of SS rats from our data are suggested to be responsible for the development of hHcys in these rats.

In addition to the above studies in CBS gene deficiency, several mutations of the genes for the enzymes involved in Hcys metabolism, including methylenetetrahydrofolate reductase, methionine synthase reductase, and methionine synthase, have also been associated with hHcys in human studies.[31–38] However, the defect of the CBS gene is unlikely the reason for the decreased mRNA expression of CBS in SS rats, because the rat CBS gene is not located on Chr 13, whereas introgression of Chr 13 from BN rats into SS rats corrects the impaired mRNA expression of CBS. The mechanisms mediating reduced mRNA expression of CBS in the kidneys of SS rats may be related to the regulation of CBS gene expression affected by Chr 13.

To determine which part of the nephron is responsible for the Hcys metabolism in the kidneys, relative distribution of CBS mRNA along the nephron was examined in microdissected nephron segments using quantitative RT–PCR analysis. CBS was shown to be mainly present in PTs, indicating that PTs are major sites for Hcys metabolism in the kidneys, which is consistent with previous reports that PTs were capable of catalyzing the metabolism of Hcys.[8] In those studies, >90% of the total activity of the transsulfuration pathway was found in the cortex, primarily in proximal convoluted tubules prepared by Percoll gradient centrifugation. Interestingly, our study demonstrated a defect in mRNA expression and enzyme activities of CBS in renal PTs in SS rats, which may be responsible for the decreased transsulfuration in SS rat kidneys.

In summary, the present study showed that plasma tHcys levels were higher in SS rats than that in SSBN13 and BN rats, and there was a significant decrease in the activities of transsulfuration in the kidneys of SS rats because of a defect of CBS activity. In contrast, there was no difference in the enzyme activity of SAH hydrolase in these 3 rat strains. These results indicate that the hHcys in SS rats is associated with a decreased metabolism of Hcys through the transsulfuration pathway and not because of an excessive production of Hcys in the kidneys. We also demonstrated that renal PTs were the primary sites for determining decreased CBS activity in the kidneys of SS rats. This decreased CBS activity was found to be related to decreased V_{max} and expression of this enzyme, which may be a result of the deficiency in the regulation of CBS gene expression controlled by factors linked to Chr 13. In conclusion, downregulation in the expression and activity of CBS in renal PTs because of a defect in Chr 13 represents one of the mechanisms for decreased metabolism of Hcys through the transsulfuration pathway in the kidneys, consequently resulting in hHcys in SS rats.

Perspectives

The present study demonstrated that introgression of Chr 13 from BN rats into SS rats restored CBS expression and activity in the kidneys. The differences in the activities and expressions of CBS between the SS rat and SSBN13 rat are probably attributed to the genes carried on rat Chr 13. Because the CBS gene is not located in rat Chr 13, the restoration of the CBS expression and activity may be associated with recovery of some regulatory mechanisms related to rat Chr 13. These regulatory mechanisms for CBS expression determined by rat Chr 13 remain to be defined, which would be of importance in elucidating the pathogenesis of hHcys associated with salt-sensitive hypertension.

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