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Abstract—Hyperhomocysteinemia (hHcys) has been recognized as a new risk factor for cardiovascular diseases independent of plasma lipid levels or other factors. However, it remains unknown whether hHcys is implicated in the target organ damages associated with hypertension. The present study first examined the possible role of hHcys in the development of glomerulosclerosis in Dahl salt-sensitive (DS) hypertensive rats. High-performance liquid chromatography showed that plasma total homocysteine (tHcys) concentration was 7.64 ± 0.29 μmol/L in conscious DS rats on a low salt (0.4% NaCl) diet, which was higher than 5.23 ± 0.25 μmol/L in Dahl salt-resistant normotensive rats. When these rats were exposed to a high salt (4% NaCl) diet, plasma tHcys markedly increased in DS rats (14.7 ± 1.31 μmol/L) but not in Dahl salt-resistant rats (5.34 ± 0.54 μmol/L). An iron chelater, desferrioxamine (0.3 mg/kg IV per day), completely normalized high salt–induced elevations of plasma tHcys and significantly attenuated the sclerotic changes in the glomeruli in DS rats. To further determine whether hHcys has an independent effect in the development of glomerulosclerosis, Sprague-Dawley rats were fed drinking water containing methionine (1 g/kg per day) for 6 weeks to produce hHcys. In these rats, plasma tHcys increased to 12.5 ± 1.9 μmol/L (versus 6.1 ± 2.6 μmol/L in control rats), and the aorta exhibited typical sclerotic changes, but arterial pressure was not altered. Urinary protein excretion increased to 52 ± 2 mg/24 hours (versus 17 ± 2 mg/24 hours in control rats), and the glomerular mesangium was expanded with glomerular hypercellularity, capillary collapse, and fibrous deposition in the rats with hHcys. These results suggest that elevated plasma homocysteine may be an important pathogenic factor for glomerular damage in hypertension independent of arterial pressure. (*Hypertension*. 2002;39[part 2]:443-448.)

Key Words: renal disease ■ glomerulosclerosis ■ blood pressure ■ homocysteine

Recent studies have indicated that hyperhomocysteinemia (hHcys) is an independent risk factor of arteriosclerosis, involving coronary, cerebral, and peripheral arteries.[1–6] There is accumulating clinical evidence that plasma total homocysteine (tHcys) levels increase in the patients with essential hypertension or end-stage renal disease (ESRD).[1,2,5,7–9] In the patients with ESRD, plasma tHcys levels may become 3 to 5 times higher than normal, and even dialysis therapy or kidney transplantation cannot restore plasma tHcys to a normal level.[9–12] This epidemic and clinical evidence has indicated that persistent hHcys may be involved in the progression of ESRD associated with hypertension and in the development or exaggeration of cardiovascular complications associated with ESRD.

Despite substantial evidence indicating the association of hHcys and ESRD, little is known regarding the pathogenic role of increased plasma homocysteine (Hcys) in the progression of glomerular damage associated with hypertension. Given the similarity of pathological changes between glomerular injury associated with hypertension and Hcys-induced arterial damages, such as endothelial injury, cell proliferation or growth, increased matrix formation, and aggregated proteoglycans,[12,13] the increase in plasma Hcys may also play a crucial role in initiating and facilitating glomerular injury in hypertensive individuals. Moreover, impaired renal function will lead to a further increase in plasma tHcys, which, in turn, exaggerates the progression of glomerular injury, resulting in a vicious cycle and, consequently, in glomerulosclerosis.[9,12] Indeed, even in the first report about the arteriosclerotic role of hHcys, McCully[13] described that there was a moderate increase in mesangial matrix, associated with some enlargement, and a moderate increase in the numbers of endothelial and mesangial cells in a patient with homocystinuria. Recent studies have shown that elevated plasma tHcys can occur before a decrease in renal function in patients with essential hypertension.[5,6,14,15] This raised a question of whether Hcys directly induces glomerular damage independent of hypertension. The present study was designed to answer this question and to test the hypothesis that elevations of plasma tHcys levels produce glomerular dysfunction and consequent sclerosis. We examined whether the effects of Hcys are independent of increased arterial blood pressure and contribute to the

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great susceptibility to glomerular sclerosis in Dahl salt-sensitive (DS) hypertensive rats. Using fluorescent high-performance liquid chromatography (HPLC), we first measured plasma tHcys levels in DS rats and their normotensive controls, Dahl salt-resistant (DR) normotensive rats, on a low or high salt diet to determine the association of plasma tHcys levels with glomerular dysfunction or sclerosis. Then, we determined the effects of a decrease in plasma tHcys concentrations on glomerular function or structure. To further confirm the role of Hcys in glomerular damage, we produced an experimental hHcys model without hypertension and determined whether only elevations of plasma tHcys levels induce glomerular damage or sclerosis.

Methods

Animals

Experiments using DS, DR, and Sprague-Dawley (SD) rats (250 g, 8 weeks old) purchased from Harlan Sprague Dawley Inc (Indianapolis, Ind) were performed. All animals were housed individually in the Animal Resource Center at the Medical College of Wisconsin. DS and DR rats were maintained on a low salt (0.4% NaCl) or high salt (8.0% NaCl) diet with free access to drinking water. SD rats were maintained on a low salt diet (0.4% NaCl) with free access to drinking water. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin.

Implantation of Chronic Catheters for Measurement of Arterial Pressure

The rats were prepared for measurement of arterial blood pressure while they were conscious; then, they were anesthetized with ketamine (15 mg/kg IM) and xylazine (2 mg/kg IM). A polyvinyl catheter was implanted in the left femoral artery and advanced to 2 cm below the branching of the renal arteries by use of aseptic techniques. The catheter was tunneled to the back of the neck and passed through a flexible spring, which was secured to a leather jacket that fits around the chest. After surgery, the rats were given 40 000 U IM penicillin G to prevent infections, and a 7-day recovery period was allowed before any data were collected.[16] In some rats, a polyvinyl catheter was inserted into the left femoral vein for intravenous infusion.

Measurement of Arterial Blood Pressure and Urinary Protein

The arterial blood pressure of rats in the conscious state was measured in a 900-ft^2 shared department space designed for chronic small animal monitoring of hemodynamic variables and metabolic balance studies. The amplified analog signal of arterial blood pressure from a pressure transducer was converted to a digitized signal at 100 Hz, and the signal was processed with the use of Significat data acquisition software and a Unix computer as described previously.[16] A 24-hour urine sample was collected, and urinary protein excretion was measured.

Production of hHcys in SD Rats

To speed up the damaging effect of Hcys on the glomeruli, uninephrectomized SD rats were used. After a 1-week recovery from uninephrectomy, a group of rats (n=7) was given bottle water containing methionine (Sigma Chemical Co) at 1g/kg per day for 6 weeks. The dose of methionine was chosen on the basis of previous studies showing that it can effectively produce hHcys.[17] Methionine can be metabolized to produce Hcys via *S*-adenosylmethionine and *S*-adenosylhomocysteine. In addition, methionine and an intermediate product, *S*-adenosylmethionine, can inhibit Hcys methyltransferase and thereby block the metabolism of Hcys, increasing plasma Hcys levels.[3,17] Another group of rats was given bottle water without

methionine. After a 6-week methionine treatment, arterial blood pressure was measured as described above. Blood samples (0.3 mL) from these rats in the conscious state were collected for tHcys assay. On last day of the experiment, a 24-hour urine sample was collected for protein assay. Then, the aorta and kidney were harvested and fixed in 10% neutral formalin solution for morphological examination.

HPLC Analysis of Hcys

To measure plasma tHcys, a whole-blood sample of 0.3 mL was collected into prechilled Vacutainer tubes containing sodium heparin (Becton Dickinson). The blood samples were immediately centrifuged at 1000g for 5 minutes at 4°C, and resulting plasma was stored at −20°C until analysis. To quantify plasma tHcys, a 50-μL aliquot of plasma or standard solution with 10 μL of the internal standard solution (2-mercaptoethylamine, 2.0 mmol/L) was mixed. Hcys and other thiols were reduced, derivatized, and chromatographed as described previously.[18]

HPLC was performed with a Hewlett-Packard Model 1090 Series II system with 2 pumps and an autosampler. Separation was carried out at ambient temperature with an analytical column, Supelco LC-18-DB (150-mm×4.6-mm internal diameter, 5-μm particle size) with a Supelco guard column. Fluorescence intensities were measured with an excitation wavelength of 385 nm and an emission wavelength of 515 nm. The analytical column was eluted with 0.1 mol/L potassium dihydrogenphosphate buffer (pH 2.1) containing 6% acetonitrile (vol/vol) as a mobile phase with a flow rate of 2.0 mL/min.

Morphological Examination

The fixed kidneys were paraffin-embedded, and sections were prepared and stained with periodic acid–Schiff stain. Glomeruli were evaluated (scored from 0 to 4) on the basis of the degree of glomerulosclerosis and mesangial matrix expansion, as previously described.[19]

Statistical Analysis

Data are presented as mean±1 SE. The Student t test was used to evaluate statistical significance of differences between 2 groups. A value of $P < 0.05$ was considered statistically significant.

Results

HPLC Analysis of Plasma tHcys

Figure 1A presents a typical HPLC chromatogram illustrating fluorescent derivatives of Hcys and some other thiols, including cysteine, cysteinylglycine, and glutathione, in rat plasma with an internal standard (2-mercaptoethylamine). Using this HPLC analysis, we determined plasma tHcys concentrations in DS and DR rats. These rats were catheterized and allowed to recover for 1 week. Blood samples were taken through arterial catheters with the animals in the conscious state. It was found that plasma tHcys concentrations were 5.23±0.55 μmol/L in DR rats and 7.64±0.29 μmol/L in DS rats fed a low salt (0.4% NaCl) diet. When these rats were exposed to a high salt (8% NaCl) diet, plasma tHcys in DS rats was markedly increased to 14.7±1.31 μmol/L, whereas it was not significantly altered in DR rats (Figure 1B).
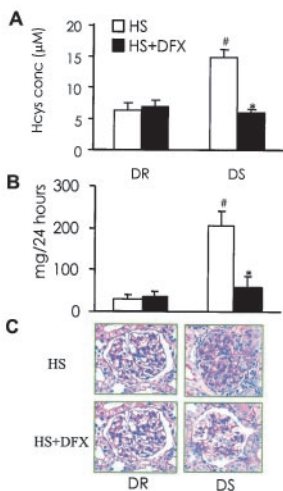
Plasma tHcys Levels and Glomerular Damage in DS or DR Rats on a High Salt Diet

In parallel to the increase in tHcys concentrations, urinary protein excretion was significantly increased in DS rats on a high salt diet, and glomerulosclerotic damage, such as mesangial expansion, hypercellularity, and capillary collapse, were observed. In DR rats, however, there was no change in
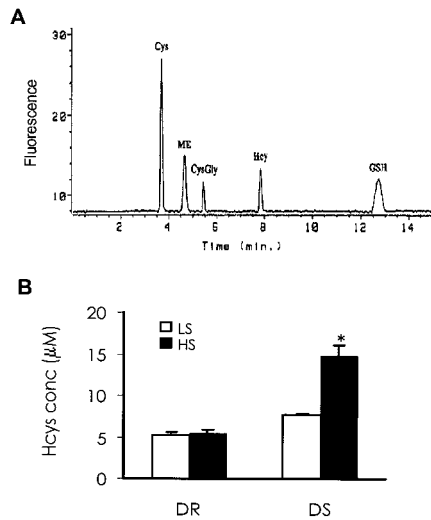
Figure 1. Fluorescent HPLC analysis of plasma Hcys and other thiols. A, A typical representative chromatogram showing fluorescent derivatives of Hcys and other thiols. Cys indicates cysteine; ME, internal standard, 2-mercaptoethylamine; CysGly, cysteinylglycine; Hcy, homocysteine; and GSH, glutathione. B, Plasma total homocysteine concentration (Hcys conc) in DS and DR rats (n=7) on a low salt (LS, 0.4% NaCl) or high salt (HS, 8% NaCl) diet. *$P<0.05$ compared with the values obtained from rats on an LS diet.

tHcys levels, urinary protein excretion, and glomerulosclerotic damage when they were exposed to a high salt diet (Figure 2). A high salt diet for 2 weeks increased arterial blood pressures from 121 ± 5 to 165 ± 6 mm Hg in DS rats, but it had no effect on arterial blood pressure in DR rats (115 ± 4 mm Hg on a low salt diet versus 118 ± 5 mm Hg on a high salt diet). When the rats were pretreated with desferrioxamine (DFX), an iron chelator, the increased tHcys concentrations and urinary protein excretion

Figure 2. Plasma tHcys levels, urinary protein excretion, and glomerular structure in DS and DR rats on an HS diet. A, Effects of DFX on plasma tHcys levels in DS (n=7) and DR (n=6) rats on an HS diet. B, Effects of DFX on urinary protein excretion in DS and DR rats on an HS diet. C, Photomicrographs (original magnification ×250) showing glomerular structure in DS rats with and without pretreatment with DFX. *$P<0.05$ compared with the values obtained from rats on an HS diet. #$P<0.05$ compared with the values obtained from DR rats.
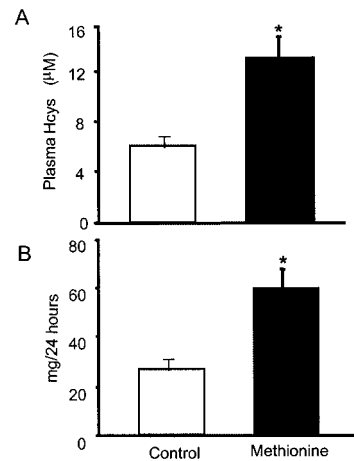

Figure 3. Effects of methionine on plasma tHcys levels and urinary protein excretion in SD rats. A, Plasma tHcys levels in control and methionine-treated rats. B, Urinary protein excretion in control and methionine-treated rats (n=7). *$P<0.05$ compared with control.

induced by high salt intake in DS rats were substantially blocked. Glomerulosclerotic damage was also significantly attenuated. In these DFX-pretreated DS rats, the increase in arterial blood pressure induced by a high salt intake was also normalized (118 ± 11.1 mm Hg). However, DFX pretreatment of DR rats was without effect on tHcys levels, urinary protein excretion, and glomerular structure (Figure 2).

Plasma tHcys and Urinary Protein in SD Rats With Methionine Pretreatment

To further determine the role of hHcys in the development of glomerular damage or sclerosis, we examined plasma tHcys levels, urinary protein excretion, and glomerular structure morphology in an experimental rat model of hHcys. In uninephrectomized SD rats, methionine (1 g/kg per day) in the drinking water for 6 weeks significantly increased plasma tHcys levels in animals in the conscious state (Figure 3A). In parallel, urinary protein was significantly increased (Figure 3B). However, arterial blood pressure was normal in these methionine-treated rats (116 ± 5 mm Hg). In these rats with experimental hHcys, the aorta exhibited pronounced endothelial damage and hyperplasia or thickening of medial smooth muscle. A thickened subendothelial space with some fibrous deposits and fragmented or disrupted elastic laminae was observed. These pathological changes in the aorta represent a typical feature of arteriosclerosis induced by hHcys (Figure 4).
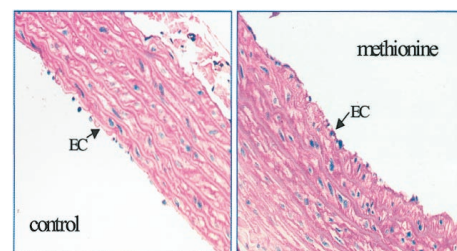
Figure 4. Photomicrographs (original magnification ×250) showing sclerotic changes in the aorta from control and methionine-treated rats. EC indicates endothelial cells.
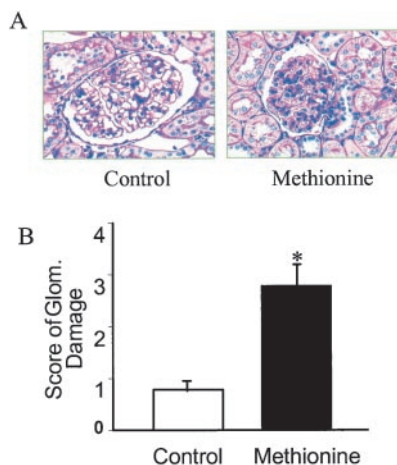
Figure 5. Morphological features of the glomeruli from control and methionine-treated SD rats. A, Photomicrographs (original magnification ×250) showing typical glomerular structure in control and methionine-treated rats. B, Semiquantitative score of glomerular (Glom.) damage (n=6). *$P<0.05$ compared with control.

Glomerulosclerotic Damage in SD Rats With Methionine Pretreatment

With a microscope, we observed typical sclerotic changes in the glomeruli from these rats with experimental hHcys. It was found that glomerular extracellular matrix was increased and that the glomerular mesangium was expanded with hypercellularity, capillary collapse, and fibrous deposition in the glomerulus in hHcys (Figure 5A). The semiquantitative injury score of glomeruli was substantially higher in methionine-treated rats than in control rats (Figure 5B).

Discussion

In the present study, we measured plasma tHcys levels in conscious rats by use of fluorescent HPLC analysis. This HPLC analysis has been considered to be a standard method for the measurement of plasma or serum tHcys levels, which is more precise and sensitive compared with other methods used to determine tHcys as a risk factor in general human populations.[3–5,14,18] It is well known that there are different forms of Hcys in blood and that >98% of blood Hcys is oxidized to the disulfide form, including Hcys-protein disulfides, Hcys-cysteine disulfide, or Hcys-cysteinylglycine disulfide.[3,18] It is very difficult to quantify all these forms of Hcys because they are varied and convertible during measurement. In clinical practice, therefore, plasma tHcys was measured as a risk factor for cardiovascular diseases by an in vitro reduction of all oxidized Hcys forms to release free Hcys. It has been demonstrated that plasma tHcys concentrations measured by this method can more precisely predict cardiovascular risk than can individual forms of Hcys.[3,14,18] With the use of this method, the present study detected plasma tHcys levels in conscious normotensive DR and SD rats on a low salt diet that are comparable to the levels in human populations. These results provide a reference of plasma tHcys levels in conscious rats for future studies.

Plasma tHcys concentration was slightly higher in DS rats than in DR rats. If these rats were exposed to a high salt diet,

plasma tHcys was markedly elevated in DS rats but not in DR rats. In parallel with elevations of plasma tHcys in DS rats, increased urinary protein excretion and glomerulosclerotic damage were observed. Pretreatment of DS rats with DFX, an iron chelator, completely blocked both homocysteinemia and proteinuria. Glomerular damage in these rats was also substantially attenuated by DFX. These results provide the first evidence that glomerular damage or sclerosis in DS rats is associated with elevations of plasma tHcys levels. This association of glomerular dysfunction or damage with plasma Hcys levels is similar to that in humans. As reviewed by Brattstroem and Wilcken,[2] almost all epidemic or clinical case-control studies in which plasma tHcys was measured in patients with renal diseases have shown a highly significant positive correlation between serum creatinine and plasma tHcys levels.

Despite a positive correlation between hHcys and glomerular damage found in DS rats, it remains to be determined whether the elevation of plasma tHcys is a pathogenic factor or a functional consequence of glomerular damage in these rats. Previous studies have shown that increased plasma Hcys levels in patients with ESRD are attributed to a reduction of glomerular filtration rate (GFR).[9,12] However, recent data have challenged this view and suggest that hHcys associated with ESRD is not simply due to decreased glomerular filtration of Hcys.[2,10] It has been demonstrated that clinically stable renal transplant recipients have an excess prevalence of hHcys, suggesting that improvement of GFR in these patients does not completely restore plasma Hcys to normal.[10] Similarly, many studies have reported that hHcys still occurs in ESRD patients receiving hemodialysis therapy.[3,9,11] In some patients with hypertension, in addition, a long-term diuretic therapy resulted in hHcys, but their GFR or renal functions were normal.[5,15] All these data indicate that elevated plasma tHcys levels in the patients with ESRD or hypertension may not depend on a decrease in GFR. In the present study, we found that high salt intake significantly increases plasma Hcys levels, suggesting that plasma Hcys levels may be associated with tubular ion transport activity. It is well known that filtered amino acids are primarily reabsorbed in proximal tubules; this occurrence is linked to sodium reabsorption in this nephron segment. Previous studies have reported that Hcys filtered from the glomeruli can be transported into tubular cells, where it is metabolized to cysteine. This metabolic pathway is the most important way to dispose of excess Hcys from the body.[20,21] Therefore, an abnormality of tubular sodium reabsorption associated with amino acid transport may be one of the important mechanisms producing hHcys in DS rats exposed to a high salt diet. The DFX-induced decrease in plasma Hcys levels may be associated with the normalization of tubular function. It has been reported that DFX can prevent oxidative stress, increase the expression of some protective enzymes (such as heme oxygenase-1), and increase the tolerance to hypoxia in tubular cells.[22–24] On the basis of these findings, we proposed that elevations of plasma tHcys levels are not the consequence of glomerular damage in DS hypertensive rats. Given the sclerotic effects of Hcys on arteries or arterioles, it is possible

that increased Hcys is a pathogenic factor resulting in glomerular damage.

To test this hypothesis, we examined whether chronic experimental hHcys produces glomerular damage in normotensive SD rats. The experimental hHcys rat model was produced by the addition of methionine in the drinking water for 6 weeks. It was found that plasma tHcys levels significantly increased, and the rat aorta exhibited sclerotic changes after a 6-week methionine treatment. In these rats with experimental hHcys, remarkable glomerular damage or sclerosis was observed, with an average injury score of 2.8 ± 0.6. However, mean arterial blood pressure in these rats was not different from that in control rats. These results indicate that increased plasma Hcys may directly act on the glomeruli to produce sclerotic changes. On the basis of these results, we have proposed that elevations of plasma Hcys levels contribute at least partially to glomerular damage or sclerosis in DS rats.

We were concerned about why methionine-induced experimental hHcys produced arteriosclerosis and glomerulosclerosis but not increased arterial blood pressure. A recent study also demonstrated that the exposure of SD rats to a 4-week methionine-rich diet produced a significant endothelial dysfunction or injury, but it had no effect on the arterial blood pressure measured in rats in the anesthetized state.[25] It seems that hHcys is not implicated in the development of hypertension, despite arteriosclerosis and glomerulosclerosis. However, many epidemiological and clinical case-control studies have observed a significant positive association of plasma tHcys levels with arterial blood pressure, especially in the young populations, suggesting that hHcys may be an independent risk factor for hypertension.[1,5,8] In animal experiments, when minipigs were fed a methionine-rich diet for 4 months, plasma tHcys concentrations were doubled, and arteriosclerosis and high blood pressure were observed.[26] These results have indicated that hHcys could be a pathogenic factor responsible for arterial and renal dysfunction, consequently increasing arterial blood pressure, but a long-term persistent hHcys may be needed to produce a sustained hypertension. In DS rats exposed to high salt diet, DFX was found to decrease arterial blood pressure. This DFX effect on arterial blood pressure may not be associated with a decrease in plasma Hcys levels, because hHcys of short duration does not contribute to sustained hypertension. Therefore, the antihypertensive effect of DFX may be due to its direct antioxidant effects in the kidney. There is accumulating evidence indicating that oxidative stress in the kidney plays an important role in the development of hypertension.[27,28]

The present study did not attempt to explore the mechanisms mediating the effects of hHcys to produce glomerular damage or sclerosis. Previous studies have demonstrated that an increase in plasma Hcys can lead to overproduction of one of its highly reactive forms, Hcys-thiolactone, which activates oxidative stress in the arterial wall.[3,29] As a result, highly reactive oxygen radicals would accumulate within cells, damaging the cells lining the arteries, promoting blood clot formation, stimulating the growth of arterial smooth muscle cells, and ultimately resulting in the formation of fibrous tissue, mucoid matrix, and degenerative elastic tissues.[3] Moreover, Hcys has been found to reduce NO production and directly trap NO, which may cause endothelial dysfunction and damage, activating oxidative stress in the arterial wall.[29–31] Recent studies have further emphasized the critical role of oxidative stress in initiating and promoting endothelial dysfunction and arteriosclerosis.[3,4] Enhanced oxidative stress may be associated with Hcys-induced NO decrease and the direct stimulation of superoxide production through the redox receptor of Hcys.[32,33] In addition, a decrease in adenosine production and a reduction of the methylation of many cellular elements during hHcys may also activate or promote the sclerotic process.[3,4,34] These mechanisms, especially Hcys-induced oxidative stress, may play an important role in mediating the sclerotic process in the glomeruli.

In summary, the present study demonstrated that plasma tHcys levels increased in DS hypertensive rats and that a reduction of tHcys levels prevented or attenuated urinary protein excretion and glomerulosclerosis. Experimentally increased plasma tHcys levels produced glomerular damage without an increase in arterial blood pressure. These results indicate that hHcys may be an injury factor resulting in glomerular damage, which may be importantly implicated in the development of glomerulosclerosis in DS rats.

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