Implications of Hyperhomocysteinemia in Glomerular Sclerosis in Hypertension

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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, McCully13 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
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. 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-ft2 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. 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. Methionine can be metabolized to produce Hcys via S-adenosylmethionine and S-adenosylhomocysteine. In addition, methionine and an intermediary product, S-adenosylmethionine, can inhibit Hcys methyltransferase and thereby block the metabolism of Hcys, increasing plasma Hcys levels. 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 heys

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 1000 g 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-mercaptopetidylamine, 2.0 mmol/L) was mixed. Hcys and other thiols were reduced, derivatized, and chromatographed as described previously. 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 x 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.

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-mercaptopetidylamine). 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
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 121/110 to 165/165 mm Hg in DS rats, but it had no effect on arterial blood pressure in DR rats (118/111 mm Hg on a low salt diet versus 118/110 mm Hg on a high salt diet). When the rats were pretreated with desferrioxamine (DFX), an iron chelater, the increased tHcys concentrations and urinary protein excretion 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 (118/111 mm Hg). However, DFX pretreatment of DR rats was without effect on tHcys levels, urinary protein excretion, and glomerular structure (Figure 2).

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.

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.
phosphate, it is very difficult to quantify all these forms of Hcys. Hcys-cysteine disulfide, or Hcys-cysteinylglycine disulfide, are usually oxidized to the disulfide form, including Hcys-protein disulfide. The metabolism of these oxidized forms of Hcys in blood and tissues is complex and can be varied during measurement. The use of this method, the present study detected plasma tHcys levels in conscious rats for future studies.

Plasma tHcys concentration was higher in DS rats and in control rats than in DR rats. The DFX-induced decrease in plasma Hcys levels in the patients with ESRD or hypertension was not completely restored by a decline in GFR. In the present study, we found that high salt intake significantly increased plasma tHcys levels in the patients with ESRD or hypertension. The elevation of plasma tHcys in DS rats or ESRD patients may not depend on a decrease in GFR. In the present study, we found that high salt intake significantly increases plasma tHcys levels. Therefore, an abnormality of 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. 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. 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 Hcys produces glomerular damage in normotensive SD rats. The experimental Hcys 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 Hcys, 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 Hcys 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 Hcys 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 Hcys 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 Hcys could be a pathogenic factor responsible for arterial and renal dysfunction, consequently increasing arterial blood pressure, but a long-term persistent Hcys 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 Hcys of short duration does not contribute to sustained hypertension. Therefore, the anti-hypertensive 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 Hcys 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.29,30 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 tissue.31 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 Hcys 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 Hcys 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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**References**


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