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Podocyte Injury and Glomerulosclerosis in Hyperhomocysteinemic Rats

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Key Words
End-stage renal disease · Proteinuria · Cytoskeleton · Glomerulosclerosis · Hyperhomocysteinemia

Abstract
Background/Aims: We previously reported that increase in plasma homocysteine (Hcys) levels by a 6-week methionine treatment produced remarkable glomerular injury. However, the mechanism by which hyperhomocysteinemia (hHcys) produces glomerular injury remains unknown. The present study was to observe when glomerular injury happens during hHcys and to explore the possible role of podocyte injury in the progression of glomerulosclerosis associated with hHcys. ***Methods:*** Uninephrectomized Sprague-Dawley rats treated with methionine were used to examine the time course of glomerular injury induced by hHcys. ***Results:*** Creatinine clearance was not different until rats were treated with methionine for 6 weeks, although plasma Hcys levels significantly increased at the 1st week of methionine treatment. However, urinary albumin excretion increased at the 2nd week of methionine treatment. Morphological examinations showed that mesangial expansion occurred at the 2nd week and podocyte effacement was also observed as processed glomerular damage during hHcys. Immunofluorescence analyses demonstrated that podocin and nephrin expressions were reduced, while α-actinin-4 increased during hHcys. ***Conclusions:*** Increased plasma Hcys level is an important pathogenic factor resulting in glomerular injury even in the very early time of hHcys. These pathogenic effects of Hcys are associated with podocyte injury and changed expression and distribution of podocyte-associated proteins.

Introduction

Hyperhomocysteinemia (hHcys) is now known as a critical pathogenic factor in the progression of end-stage renal disease (ESRD) and in the development of cardiovascular complications related to ESRD [1–3]. Recent studies in our laboratory have demonstrated that increased plasma homocysteine (Hcys) levels result in glomerulosclerosis and proteinuria in uninephrectomized Sprague-Dawley (SD) rats fed with L-methionine in drinking water for 6 weeks [4]. Tyagi et al. [5] also demonstrated that hHcys is importantly implicated in glomerular injury in hypertensive rats. However, the time course of glomerular injury and related mechanisms during hHcys are still poorly understood.

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With respect to the mechanism of progressive glomerulosclerosis or ESRD, previous studies emphasized a central role of mesangial cells and extracellular matrix metabolism. However, the mechanism by which mesangial cells are activated remains poorly understood. In this regard, recently, our laboratory has demonstrated the role of mesangial cells in the development of glomerulosclerosis in hHcys [6, 7]. Although mesangial cells do play an important role in the deposition of extracellular matrix and consequent fibrosis, recent studies have also indicated that podocyte injury may play a more critical role in the progression of various glomerular diseases [7, 8]. Podocyte process effacement is observed in many experimental ESRD models and human glomerulopathy associated with heavy proteinuria [7, 9, 10]. A variety of pathogenic mechanisms, including immunologic processes, biochemical factors and hemodynamic alterations, are reported to induce podocyte changes [10, 11]. However, it remains unknown whether podocyte injury contributes to hHcys-induced glomerular damage. Therefore, the present study was designed to observe when glomerular injury happens during hHcys and to explore the possible role of podocyte injury in the progression of glomerulosclerosis associated with hHcys.

Materials and Methods

Animals

The experiments were performed using SD rats (200 g, 6 weeks old) purchased from Harlan Sprague Dawley Inc. (Indianapolis, Ind., USA). 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 = 54) were given drinking water containing methionine at 1 g/kg per day. The dose of methionine was chosen based on previous studies showing that it effectively produced hHcys [6]. Another group of rats were given drinking water without methionine (n = 18). All protocols were approved by the Institutional Animal Care and Use Committee of Virgina Commonwealth University. To investigate the time course of glomerular injury during hHcys, different time points were chosen in this experiment. After 1, 2, 3, 4, 5 or 6 weeks of methionine treatment, blood was taken for measurement of plasma Hcys and a 24-hour urine sample was collected for the determination of urinary albumin excretion during recording days individually. Then, methionine-treated rats were sacrificed at each time point. Rats fed with normal drinking water were sacrificed after 1 and 6 weeks, blood and 24-hour urine samples were collected at these 2 time points. All groups of kidney organs were harvested and fixed in 10% formalin solution for morphological and immunofluorescence examinations.

HPLC Analysis of Plasma Hcys Levels

Plasma total Hcys was measured by fluorescence HPLC analysis as we described [6, 12].

Measurement of Urinary Albumin Excretion and Creatinine Clearance

Twenty-four-hour urine was collected with a metabolic cage. Urinary albumin concentration was measured using the albumin blue 580 method (Molecular Probes, Eugene, Oreg., USA). Plasma and urinary creatinine (Pcr and Ucr) were measured as described before [4]. Creatinine clearance (Ccr) was calculated using the following formula: Ccr (milliliters/minute) = (Ucr/Pcr) × urine volume (milliliters)/1,440.

Immunofluorescence Staining

Immunoflurescence staining was performed as described previously [13]. The slides were stained with rabbit anti-α-actinin 1:100 (Sigma), rabbit anti-podocin 1:200 (Sigma) and rabbit anti-nephrin 1:100 (Abcam). The sections were examined by fluorescent microscopy with a Nikon 40 Plan Apo oil immersion lens. The images were captured with a Spot CCD camera (Diagnostic Instruments Inc., Sterling Heights, Mich., USA) and exported into Adobe Photoshop 7.0. All exposure settings were kept constant for each group of kidneys.

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 we described before [4, 6]. To examine the ultrastructural changes in glomeruli in the hHcys kidney, the kidney was fixed for electron microscopic examinations. Endothelial cells, podocytes, mesangial cells and diaphragm slit were examined. Electron microscopic sample handling and detection were performed by the electron microscopic core lab of Medical College of Virginia.

Statistical Analysis

Data are presented as means ± SEM. The significance of differences in mean values within and between multiple groups was examined with an analysis of variance for repeated measures followed by Duncan's post hoc test. The Student's t test was used to evaluate the significance of differences between 2 groups of experiments (Sigma Stat; SPSS Inc., Chicago, Ill., USA). A value of $p < 0.05$ was considered statistically significant.

Results

Plasma Total Hcys Levels Significantly Increased in Rats with Methionine Treatment

As shown in figure 1, plasma total Hcys concentration was significantly increased in rats fed with methionine, starting from the 1st week of methionine treatment. After the 2nd week of methionine treatment, the plasma Hcys levels were above 10 μM, which was considered as hHcys.

Progressive Increase in Urinary Albumin Excretion in Rats with Methionine Treatment

As shown in figure 2a, albuminuria was progressively increased in rats with hHcys starting from the 2nd week of methionine treatment (43.3 ± 6.1 vs. 20.6 ± 3.1 mg/24 h of control). After 6 weeks of treatment with methionine, the urinary albumin excretion of rats increased to 86.2 ± 4.7 mg/24 h.

No Significant Time-Dependent Change in Ccr in Rats with Methionine Treatment

The summarized data in figure 2b shows that Ccr was not significantly different until the rats were treated with methionine for 6 weeks, although there was a tendency of decrease in Ccr in the first 5 weeks of methionine treatment.

Glomerulosclerotic Damage in Rats with Methionine Treatment

Morphological analysis showed that the glomerular extracellular matrix was increased and that the glomerular mesangium was expanded with hypercellularity, capillary collapse and fibrous deposition in the glomerulus in hHcys. There were significant progressive increases in glomerular injuries, starting from the 2nd week of methionine treatment in the hHcys rats compared with controls (fig. 3a). The summarized semiquantitative injury score of glomeruli is shown in figure 3b. Electron microscopic examination showed that fibrotic pathological change was observed in glomerular filtration membranes of hHcys rats starting from the 2nd week of methionine treatment. At the 2nd week, the foot processes broadened and the podocyte appeared to swell. At the 4th week, widespread fusion and effacement of the podocyte appeared. At the 6th week, the podocytes were dramatically damaged; most of the podocytes were effaced, as shown in figure 3c.

Changed Podocin, Nephrin and α-Actinin-4 Expression in Podocyte Correlates with the Abnormalities of the Podocytes Induced by hHcys

As shown in figure 4, in control rats, podocin and nephrin staining was detected as a fine, linear-like pattern along the glomerular capillary loop. In hHcys rats, both podocin and nephrin expression showed a trend toward gradual decrease. In control rats, α-actinin-4 staining was detected as a dotted, linear-like pattern. In hHcys rats, α-actinin-4 expression was significantly increased starting at the 2nd week. After the 2nd week, the α- actinin-4 expression level had no more significant increases.

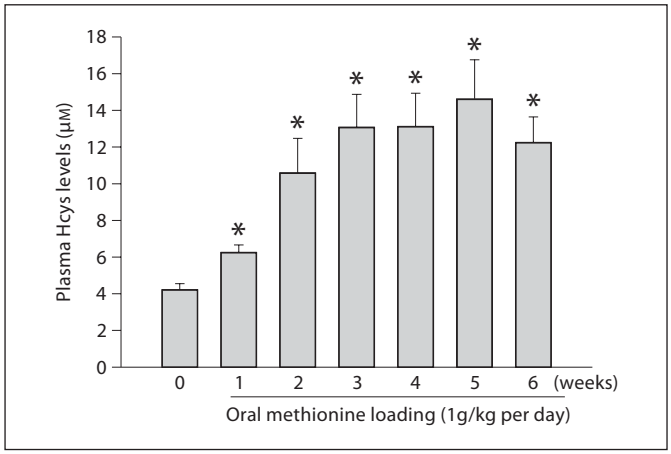

Fig. 1. Plasma total Hcys concentrations in different groups of rats with or without methionine treatment at the different time points (n = 9). * $p < 0.05$ compared with control.

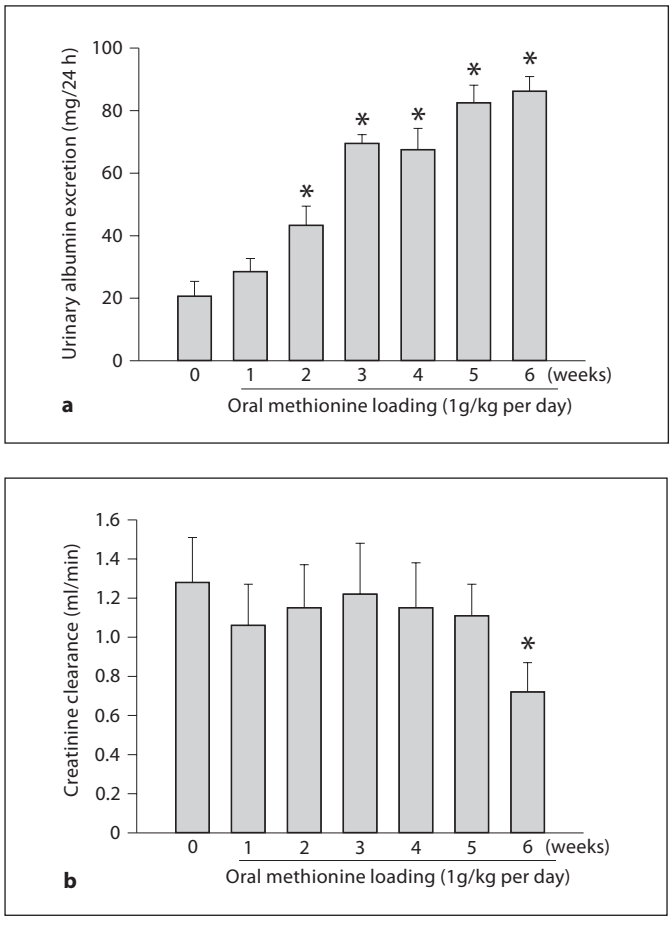

Fig. 2. Urinary albumin excretion (**a**) and Ccr (**b**) in different groups of rats with or without methionine treatment at the different time points (n = 9). * $p < 0.05$ compared with control.

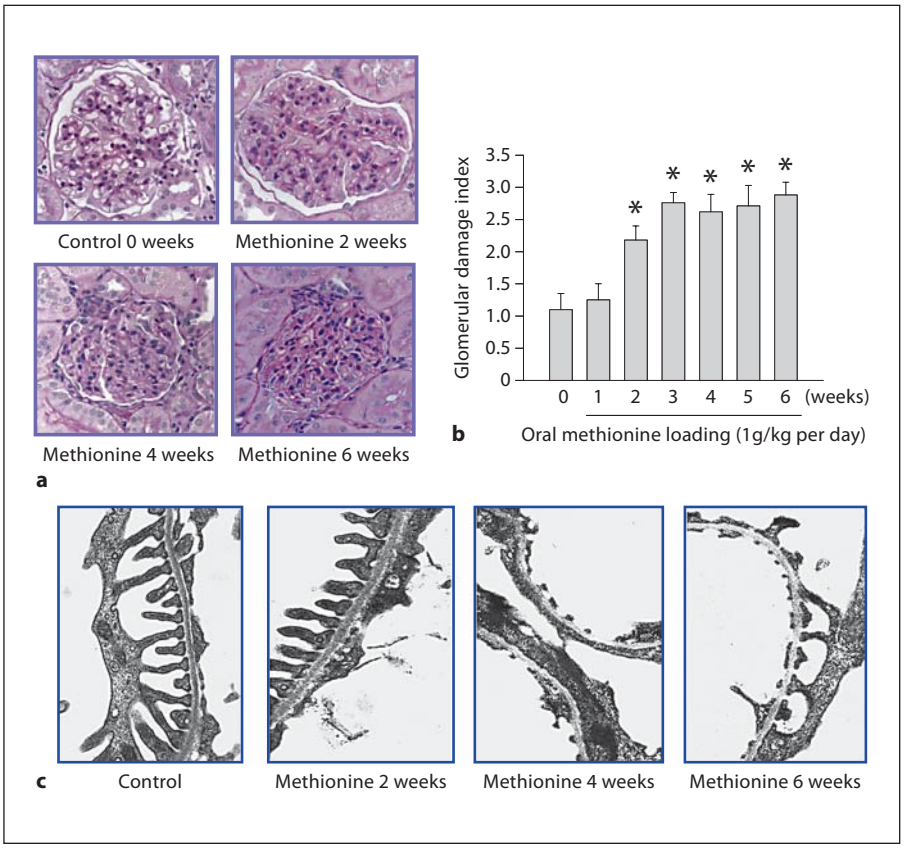

Fig. 3. Morphological features of glomeruli from different groups of rats with or without methionine treatment at the different time points (n = 9). **a** Photomicrographs showing typical glomerular structure in different groups of rats. Original magnification ×250. **b** Semiquantitative score of glomerular damage index. * $p < 0.05$ compared with control. **c** Morphological change in the podocyte foot process. The foot processes in control rats were tall and narrow. After 2 weeks of methionine treatment, the foot processes broadened. After 4 weeks and 6 weeks, the fusion and effacement of foot processes was observed. Electron microscopy ×46,000.

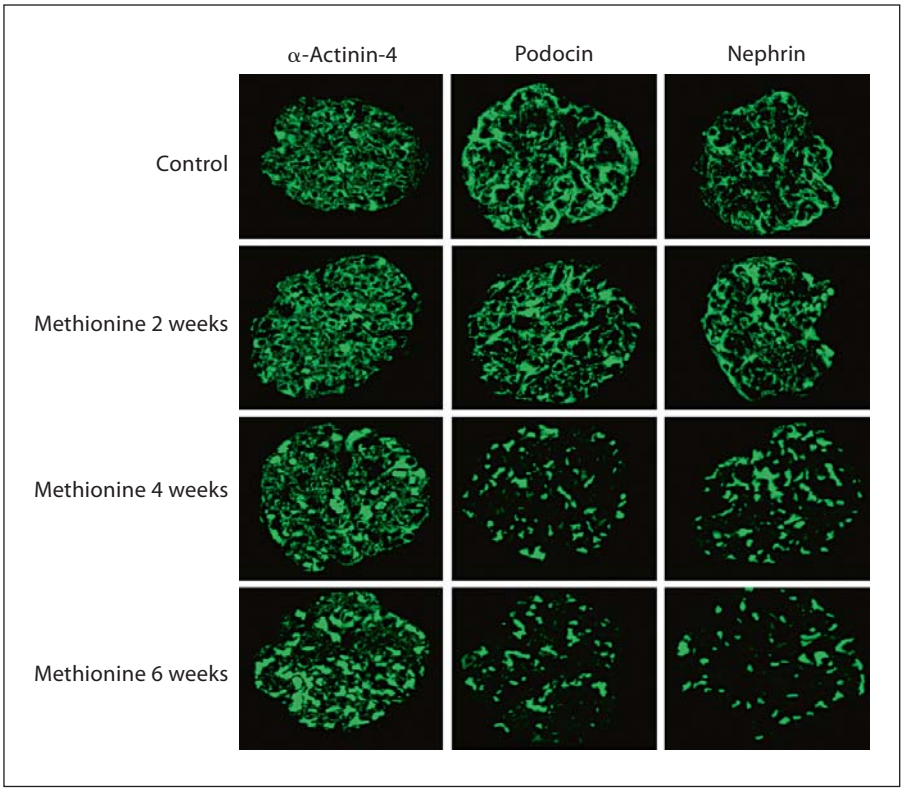

Fig. 4. Representative immunofluorescent micrographs of glomeruli from control and methionine-treated rats at the different time points with staining of podocin, nephrin and α-actinin-4 (n = 6).

All the rats without methionine treatment had no change in these results including Hcys level, albumin extraction, morphological structure of glomeruli and podocyte-associated protein expression at the 1st and the 6th weeks.

Discussion

The present study indicates that increased plasma Hcys level is an important pathogenic factor resulting in glomerular injury, which leads to microalbuminuria and glomerulosclerosis even in the early stage of hHcys. This pathogenic effect of Hcys is associated with podocyte injury and changed expression or distribution of podocyte-associated proteins.

Although it is well accepted that Hcys contributes to progressive glomerular injury in experimental animal models and human patients, little is known about the time course of the development of glomerular injury and related mechanisms during hHcys. In the present study, we first determined the starting time point at which hHcys induced renal injury in methionine-treated rats by measurement of albumin excretion, Ccr and morphological examination of glomerular structure at the different time points. It was found that albuminuria is progressively increased in rats with hHcys starting from the 2nd week of methionine treatment. It is well known that alteration in mesangial cell function is important to the progression of glomerular disease. Numerous models of chronic renal failure including hHcys-induced glomerular injury are characterized by mesangial cell proliferation and elaboration of extracellular matrix protein resulting in glomerulosclerosis [6, 14]. In agreement with a deterioration in albuminia, histological examinations in this study showed that the glomerular mesangium was expanded with glomerular hypercellularity, capillary collapse and fibrous deposition in the rats with hHcys. There were significant progressive increases in glomerular injuries in the hHcys rats starting from the 2nd week of methionine treatment in the hHcys rats. These results support the view that the effects of Hcys on mesangial cells could contribute to the progression of chronic renal failure. Recent studies have also correlated podocyte loss with the onset and magnitude of glomerulosclerosis. Podocytes are highly differentiated cells with a complex cellular morphology. They function as glomerular basement membrane (GBM) turnover, maintenance of the glomerular filtration barrier and regulation of glomerular filtration [15–17]. Podocyte loss leads to the area of denuded GBM where podocytes were reduced. The lack of tensile support normally provided by the podocytes is lost in these areas and leads to outward bulging of the capillary loop because of hydrostatic capillary pressures. Since many forms of glomerular disease are associated with increased intraglomerular hydrostatic capillary pressure, this process is further augmented. Then, the expanding capillary loop causes the denuded basement membrane to abut on Bowman's capsule, leading to synechia formation, which is the 1st step to the development of focal segmental glomerular sclerosis. Finally, inspissated proteins and hyalinosis develop in the capillary loops and progressive scarring ensues [18, 19]. However, to our knowledge, to date no reports indicate that podocyte injury contributes to hHcys-induced glomerular damage. We wondered whether podocyte number is a critical determinant factor for the development of glomerulosclerosis and that a decrease in podocyte number leads to progressive renal failure in hHcys. Using electron microscopy analysis, we found that the development of glomerular dysfunction was associated with the progressive podocyte injury. These discoveries could give new insight into the pathophysiology and mechanism of Hcys-induced glomerular injury.

To further explore the possible mechanisms of podocyte injury in the progression of glomerulosclerosis associated with hHcys at the molecular levels, the expression and distribution of podocyte-associated proteins were investigated. Previous studies showed that nephrin, podocin and CD2AP are the 3 important slit-diaphragm-associated proteins and form the slit diaphragm complex with other podocyte proteins such as P-cadherin, FAT and NEPH1–3 [20–24]. Podocin, encoded by the gene called *NPHS2*, localizes to the silt diaphragm, accumulates there in an oligomeric form in lipid rafts and associates via its C-terminus with CD2AP and nephrin. Further studies revealed direct interaction of podocin and CD2AP. Hence, podocin may act as a scaffolding protein, serving in the structural organization of the slit diaphragm and the regulation of its filtration function. In this study, we found that the podocin protein levels were significantly and progressively decreased in hHcys rats. The expression patterns of podocin were also changed. Moreover, nephrin, which plays a critical role in maintaining the glomerular filtration barrier, was also significantly and progressively decreased and the expression patterns were changed in hHcys rats, which is consistent with previous studies [25, 26].

α-Actinin-4 is an actin-bundling protein thought to have important roles both in loose cross-linking of actin

filaments into contractile bundles and in helping to form the anchoring complex for the ends of actin stress fibers where they terminate on the plasma membrane at focal contacts [27]. This protein is present in podocyte foot processes. Increased immunofluorescence staining of α-actinin-4 has been observed in podocytes during foot process effacement in nephrotoxic serum nephritis [28]. In puromycin aminonucleoside nephrosis, significant induction of α-actinin-4 has been demonstrated before the development of foot process effacement [29]. In addition, α-actinin-4 has already been shown to bind the cytoplasmic domain of the β_1-subunit of integrin molecules which serves to attach the podocyte cell membrane to extracelluar matrix proteins in GBM, suggesting its important role in anchoring podocyte actin microfilaments to integrin at the base of foot process. These findings suggest that altered expression or distribution of α-actinin-4 may play an important role in the development of foot effacement in renal diseases. In the present study, the significant increase of α-actinin-4 expression was found at the 2nd week of methionine treatment, when the podocyte started to broaden and swell, but not effaced at that time. These results are consistent with previous studies showing that significant induction of glomerular α-actinin-4 had occurred before the development of foot process effacement [29]. These results further confirm the importance of cytoskeleton-associated proteins in the regulation of podocyte effacement in the development of glomerulosclerosis induced by hHcys and suggest that induction of α-actinin-4 by hHcys may result in dysregulation of actin microfilament bundling in foot processes, causing disruption of normal foot process structure, thereby leading to glomerular injury.

It should be noted that the present study did not attempt to explore the mechanism by which Hcys induce podocyte injury and glomerulosclerosis. However, the oxidative-stress-mediated pathogenic mechanism has been demonstrated to contribute to Hcys-induced glomerular injury. Recent studies in our laboratory have demonstrated that ceramide-activated NAD(P)H oxidase is responsible for glomerular injury associated with hHcys [6, 7]. The release of superoxide leads to proteinuria by affecting glomerular mesangial, endothelial and epithelial cells and disturbing normal glomerular permselectivity. Furthermore, NAD(P)H oxidase is a major superoxide source in podocytes [30]. Therefore, it is possible that increased production of superoxide by Hcys-induced NAD(P)H oxidase could be attributed to podocyte injury in hHcys rats. Further studies are needed to address this hypothesis.

In summary, our results provide direct evidence that increased plasma Hcys level is an important pathogenic factor resulting in glomerular injury and first confirm the involvement of podocin, nephrin and cytoskeleton-associated protein α-actinin-4 in podocyte effacement in the development of glomerulosclerosis induced by hHcys. These results suggest a new pathogenic pathway contributing to glomerular injury associated with hHcys, which may direct toward the development of new therapy of end stage renal diseases related to hHcys.

Acknowledgment

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