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Review

Hyperhomocysteinemia – association with renal transsulfuration and redox signaling in rats

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Abstract

Despite substantial evidence indicating the association of hyperhomocysteinemia (hHcys) and end-stage renal disease (ESRD), the pathogenic role of increased plasma homocysteine (Hcys) levels in the progression of ESRD remains unclear. This review will briefly summarize recent findings regarding the role of hHcys in the development of glomerulosclerosis, the association of hHcys with reduced renal transsulfuration and Hcys-induced changes of redox signaling in the development of glomerulosclerosis in rat kidneys. Based on these results, it is concluded that hHcys is implicated in glomerular sclerosis in hypertension, elevated plasma Hcys in Dahl salt-sensitive (SS) hypertensive rats is due to downregulation of cystathionine β-synthase (CBS) expression and consequent abnormality of transsulfuration in the kidney compared with normotensive rats. Hcys-induced superoxide ($O_2^{\bullet -}$) production by activation of NADPH oxidase as a triggering mechanism contributes to the effects of Hcys on the homeostasis of extracellular matrix and consequent sclerosis in the glomeruli, and NADPH oxidase activation by Hcys is associated with enhanced Rac GTPase activity.
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Keywords: glomerular sclerosis; homocysteine; NADPH oxidase; Rac GTPase; superoxide.

Introduction

Hyperhomocysteinemia (hHcys) has been indicated as a risk factor for many diseases, such as cardiovascular diseases, stroke, neurodegenerative diseases, osteoporotic fractures, etc. (1–3). In addition to epidemiological evidence indicating hHcys as a risk factor, studies also demonstrated increased homocysteine (Hcys) levels around tissues or cells as a pathogenic factor causing a variety of pathological damages, such as oxidative stress, endothelial dysfunction, proliferation of vascular smooth muscle cells, increased lipid peroxidation, hemostatic imbalance, DNA demethylation, protein homocysteinylation and accumulation of collagens (4–6). Recently, we have performed a series of experiments to address the involvement of hHcys in chronic renal injury, to explore the mechanisms leading to hHcys and to define the mediators of Hcys-induced pathogenic effects in the rat kidney. This review will briefly summarize recent results regarding the role of hHcys in the development of glomerulosclerosis, the association of hHcys with reduced renal transsulfuration and Hcys-induced changes of redox signaling in rat kidneys.

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Hyperhomocysteinemia and glomerular sclerosis in rat kidneys

hHcys is emerging as an independent risk factor for arteriosclerosis and cardiovascular diseases (1). Because of the pathological similarities between atherosclerosis and glomerulosclerosis, we observed glomerular injury in hHcys rats. Our result demonstrated that increase in Hcys levels resulted in proteinuria and glomerulosclerosis in Sprague-Dawley rats fed with a high methionine or folate free diet (5, 7), indicating the critical role of hHcys in the development of end-stage renal disease (ESRD). Most recently, we also observed the time course of glomerular injury and related mechanisms during hHcys in methionine-treated rats, and we also found that both urinary albumin excretion increase and mesangial expansion occurred at the 2nd week of methionine treatment (8). In these hHcys rats, podocyte effacement was also observed as processed glomerular damage. By examining several podocyte-associated proteins, including podocin, nephrin and α-actinin-4, we demonstrated that podocin and nephrin expressions were progressively reduced, while α-actinin-4 increased during hHcys. These data further confirm that increased plasma Hcys level is an important pathogenic factor resulting in glomerular injury, even at a very early stage of hHcys. These pathogenic effects of Hcys are associated with podocyte injury and changed expression and distribution of podocyte-associated proteins. In addition, we studied the role of hHcys in glomerular injury in Dahl salt-sensitive (SS) rats, a genetically hypertensive animal model with great susceptibility to glomerular injury, whose plasma Hcys levels were higher compared with normotensive rats. Normalization of plasma

Hcys levels prevented the rapid progression of glomerulosclerosis in these rats when exposed to a high salt diet (5). Further studies from our laboratory demonstrated that Hcys stimulated the formation of tissue inhibitor of metalloproteinase-1 (TIMP-1) in cultured renal mesangial cells, consequently producing the deposition of collagen in response to Hcys (9). These data strongly suggest that hHcys is an important pathogenic factor in the progression of chronic renal injury. Similarly, other investigators have also shown that hHcys is importantly implicated in glomerular injury. Even in early studies, McCully documented that renal vascular and glomerular injury occurred in a patient with homocysteinuria (10). More recently, Tyagi et al. demonstrated that Hcys-dependent glomerular injury is one of the major Hcys-induced pathological changes in hypertensive rats (11). In addition, Ozmen et al. showed that elevated plasma Hcys concentrations were associated with urinary albumin excretion and deterioration of renal function in type 2 diabetics (12). In clinical studies, renal insufficiency is strongly associated with an increased risk of elevated circulating Hcys (13). Ingram et al. reported that Hcys was indicated to link to the mesangial cell proliferation and extracellular matrix (ECM) protein production (14).

It should be noted that results from clinical observation on the correlation of plasma Hcys concentration and kidney disease progression are controversial (13, 15–18). In this regard, we had an interesting discussion with Rensma et al. (17) in *Circulation*. Rensma et al. argued that Hcys is noxious to the human vascular endothelium and that the renal microvasculature appears to be protected from this effect. However, by analyzing their results we found that failure to correlate changes in renal function and plasma Hcys level may be related to the sensitivity of their measurements to represent renal function, the small sample sizes and criteria for selecting patients. For example, in their studies, they used creatinine clearance (CRc) to measure glomerular filtration rate (GFR). However, CRc overestimates GFR and cannot precisely represent renal function under certain circumstances. The Cockcroft-Gault formula used in these studies usually overestimates GFR and produces an error two times larger than other methods (19). In addition, hyperhomocystinuric patients usually suffer from severe metabolic disorders; therefore, the creatinine metabolism may be disturbed in these patients, which would affect CRc. In fact, some studies reported that hHcys markedly altered the activity of creatinine phosphokinases (20). Therefore, one should be cautious in using CRc for evaluation of renal function during hHcys.

Another cautious factor for clinical studies is patient sample size and status. For example, in the studies of Rensma et al., they concluded that renal function or glomerular function is somehow protected, because they found that there was no significant difference in Hcys levels in patients with rapid vs. slow decline of renal function. However, they reported that plasma Hcys level was 19.3 μM in a group of patients with rapid decline of renal function, which in fact was higher, but not significantly higher than the 13.7 μM in patients with slow decline of renal function. It seems that the sample size of patients was too small to reach a significant difference in Hcys levels. If a sample size is large enough, an average increase of 5.6 μM should easily reach statistical significance. In addition, because their patients were chosen without diet control and were suffering from different diseases and receiving different therapeutic regimens, one cannot exclude the effects of these factors on the relationship between Hcys levels and renal function. In fact, many other studies have shown that GFR decline is associated with elevation of plasma Hcys (21, 22). This discussion gives an example of how some clinical results regarding the role of Hcys in renal injury should be cautiously interpreted.

Hyperhomocysteinemia and renal transsulfuration in Dahl SS hypertensive rats

Hcys is a non-protein forming sulfur amino acid that is produced from S-adenosylhomocysteine (SAH) through the catalysis of SAH hydrolase. Its metabolism stands at the intersection of two pathways: remethylation to methionine by methyltetrahydrofolate-Hcys methyltransferase, which requires folate and vitamin B_{12} (or betaine in an alternative reaction) and transsulfuration to cystathionine by cystathionine β-synthase (CBS) and then cleaved to cysteine by γ-cystathionase (GCS), which requires pyridoxal-5′-phosphate (23–25). It has been indicated that the activities of these metabolic pathways are the important determinants of plasma Hcys levels (1, 24, 26, 27).

Despite that numerous studies have demonstrated that hHcys is indicated as an independent risk factor for cardiovascular diseases and a pathogenic factor in the development of hypertension-associated end-stage organ damages (1, 5, 6, 28–36), the mechanisms leading to hHcys are not quite clear. Severe hHcys is due to rare genetic defects resulting in deficiencies in the enzymes involved in Hcys metabolism and/or methyl-B_{12} synthesis, while the pathogenesis of mild hHcys, a more common form of hHcys, is complex and considered to be multifactorial, including genetic, nutritional and life style factors (35, 37, 38). It has been reported that the contribution of genetic factors to hHcys is relatively small ($<9\%$) in the general population (39–41), while folate, cobalamin and pyridoxine deprivation are responsible for approximately 28%–40% of the variability in plasma Hcys (39, 42, 43), and also some other factors, such as age, renal function, smoking, coffee consumption, alcoholism and drugs (e.g., folate antagonists, NO and L-dopa) may affect plasma Hcys (35). Nevertheless, a large portion of hHcys (47%) are not associated with micronutrient deficiencies, impaired renal function and/or currently known genetic mutations (44). Further work is needed to explore the causes of currently unexplained cases

of mild hHcys, such as interaction of genes, unknown mutations, other environmental factors or nutrients.

The kidneys have been shown to be major sites for the removal of plasma Hcys. Most tissues, except liver and kidney, lack a functional transsulfuration pathway and in these tissues Hcys metabolism seems fully dependent on remethylation pathway or on Hcys export from the cells (25, 45, 46). To maintain low intracellular Hcys concentration and balance between Hcys production and catabolism, the intracellular Hcys is exported when its production exceeds the metabolic capacity (46–49). After entering blood stream, Hcys is mainly removed in the kidneys. It has been calculated that kidneys remove 70% of the daily Hcys load (50, 51). Hcys transsulfuration and remethylation enzymes are both present in the kidneys, but Hcys is metabolized mainly through transsulfuration pathway in the kidneys (26). The important role of kidney in the metabolism of Hcys is supported by many studies in patients with chronic renal failure (45, 52). Most of the patients (85%–100%) with ESRD have hHcys (21, 53–55), as Hcys clearance in these patients was only 22% of normal (56). Previous studies have shown that increased plasma Hcys levels are attributed to a reduction of GFR (21, 57–59). However, the clearance of Hcys is not affected to the same extent as the clearance of creatinine (60) and hemodialysis can only transiently decrease plasma Hcys levels in ESRD (61, 62), indicating that hHcys is not simply due to reduced glomerular filtration of Hcys in ESRD. Actually, urinary Hcys excretion is trivial (27, 63). During acute hHcys produced by the infusion of L-Hcys (active form of Hcys) in rats, its uptake was 85% of the infused dose, while urinary excretion remained negligible ($<2\%$) (27). Therefore, hHcys in ESRD appears to be induced by the abnormality of Hcys metabolism in the kidneys.

In Dahl SS hypertensive rats, plasma Hcys levels are higher compared with normotensive rats (5, 64). Therefore, these SS rats could be an ideal model for studies of the mechanisms responsible for hHcys associated with hypertension (64). To explore the mechanisms leading to hHcys in SS rats, we analyzed the activities of various possible enzymes involved in Hcys metabolism in SS rat kidneys compared to SSBN13 rats, which is a consomic subcolony of SS rats with substitution of chromosome (Chr) 13 from Brown Norway (BN) rats and resistant to high salt-induced hypertension and hHcys (65). In addition, BN rats, the other parent stain of SSBN13 rats, were also used for comparison. We found that the conversion rate of SAH into Hcys by SAH hydrolase in renal cortical tissue homogenate was not different among these three rat strains. In contrast, the conversion rate of Hcys to cysteine by a transsulfuration pathway via CBS and GCS was markedly reduced in SS rat kidneys compared to SSBN13 and BN rats. Because Hcys is first converted to cystathionine by CBS, and cystathionine is then cleaved to cysteine by GCS, we further dissected the contribution of these two enzymes to the decreased transsulfuration activity in SS rats. It was demonstrated that reduced transsulfuration activity in SS rat kidneys is associated with decreased activity of CBS. Real time RT-PCR and Northern blot analysis showed that the abundance of CBS mRNA in the renal cortex was significantly lower in SS rats than in SSBN13 and BN rats, but SAH and GCS expression in the kidney were not different among these rat stains. Western blot analysis also confirmed that CBS levels were significantly lower in SS rats. These results are in accordance with the data from analyses in the enzymatic activities and suggest that decreased activity of renal CBS in SS rats is attributed to the reduced expression of this enzyme, and thereby diminishing renal transsulfuration activity. In microdissected nephron segments, CBS defect in mRNA expression and enzyme activities was shown mainly in renal proximal tubes in SS rats (64), which may be responsible for the decreased transsulfuration in SS rat kidneys, as this nephron segment has been reported to be a major site for transsulfuration (26).

The important role of CBS in the regulation of plasma Hcys levels has also been supported by the results obtained from gene knockout mice and transgenic mice in recent reports. In the mice with heterozygous disruption of the *CBS* gene, plasma Hcys levels increased by 50% (66). In contrast, elevating CBS activity by overexpression of CBS decreased plasma Hcys by 45% in transgenic mice (67). In addition, plasma Hcys levels significantly decreased in patients with Down syndrome, because that *CBS* gene is located on human Chr 21 and overexpressed due to trisomy 21 in these patients (68). Our findings that decreased expression and activity of CBS occurred in the kidneys of SS rats further support the role of CBS in hHcys as a causative factor. However, it is not known why CBS expression was altered in these SS rats, although introgression of Chr 13 from BN rats into SS rats restored CBS expression and activity in the kidneys. Bioinformatics analysis of rat genome showed that the restoration of the CBS expression and activity might be associated with recovery of some regulatory mechanisms related to rat Chr 13, because the *CBS* gene is not located in 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 SS hypertension.

The relationship between hHcys and renal dysfunction needs to be clarified. As discussed above, hHcys is a pathogenic factor in the progression of renal injury, while kidney damage can also induce hHcys, because the kidneys are major sites for Hcys to be metabolized. However, in the general population the contribution of renal dysfunction to hHcys is negligible. In our study on SS rats, hHcys occurs before renal injury is developed. Therefore, hHcys is normally produced by factors other than renal dysfunction, such as genetic factors, diet and lifestyle factors, as well as unknown reasons (35, 39–43). Our results demonstrated that diminished renal transsulfuration activity in the kidneys may be responsible for hHcys in SS

rats, which may represent a novel mechanism inducing hHcys and shed light onto the pathogenesis of hHcys, whereby causes are currently unexplained.

Hyperhomocysteinemia and redox signaling in the rat kidney

As discussed above, studies from our laboratory and others have indicated that chronic elevations of plasma Hcys importantly contribute to the development of glomerular disease independent of hypertension (5). Despite substantial evidence indicating the association between hHcys and ESRD, the mechanisms by which Hcys promotes the development of glomerulosclerosis have not been fully illustrated. In recent years, we have focused mainly on the early mechanism by which Hcys produces glomerular damage. It was found that one such early mechanism is related to the changes in redox signaling during hHcys. In this regard, NADPH oxidase is considered to contribute to more than 90% of superoxide ($O_2^{\bullet -}$) production under physiological conditions in kidney cells (69–71). By studying the role of this enzyme in mediating the pathogenic action of Hcys, we found that in mesangial cells from rat glomeruli, Hcys dose-dependently increased $O_2^{\bullet -}$ production, which was blocked by inhibition of NADPH oxidase activity using pharmacological blockers or antisense oligonucleotide of $p22^{phox}$, a crucial subunit of NADPH oxidase (9, 72), indicating that Hcys stimulates $O_2^{\bullet -}$ production via activation of NADPH oxidase. This Hcys-induced NADPH oxidase activation is responsible for the ECM accumulation in mesangial cells by upregulation of TIMP-1 (9). In in vivo rat experiments, we further demonstrated the contribution of this NADPH oxidase to hHcys-induced glomerular injury (7). In rats fed with folate-free diet, plasma Hcys levels and renal NADPH oxidase activities were significantly increased, which was accompanied by marked glomerular injury. Treatment of these rats with apocynin, one of the NADPH oxidase inhibitors, significantly improved hHcys-induced glomerular injury, as shown by decreased urinary albumin excretion and reduced glomerular damage index. Inhibition of NADPH oxidase also normalized ECM components changes with decreased TIMP-1 and increased matrix metalloproteinase-1 activity in these hHcys rats. These data demonstrated that Hcys-induced oxidative stress by stimulating NADPH oxidase is an important early mechanism responsible for its pathological actions on glomeruli. Hcys-induced NADPH oxidase activation has also been confirmed by many recent reports of other investigators in different tissue or cells (73–76).

A further question that remains is how Hcys activates NADPH oxidase in glomerular cells. By determining the effects of L-Hcys on the expression level of NADPH oxidase subunits in cultured rat mesangial cells and glomeruli in hHcys rats, we found that L-Hcys had no effect on the expression of NADPH oxidase subunits (72). It is indicated that increased NADPH oxidase activity may be associated with its activation rather than expression level. In many studies, NADPH oxidase activation was reported to rely on two major initiating steps including $p47^{phox}$ translocation from cystosol to cell membrane and Rac GTPase activation. In more experiments, comparison of $p47^{phox}$ levels in cell membrane and cystosol fractions of rat mesangial cells demonstrated that $p47^{phox}$ was already present in membrane fraction under resting condition and that Hcys stimulation had no effect on the translocation of this NADPH oxidase subunit. It seems that in these kidney cells, $p47^{phox}$ is already in cell membrane, and therefore its regulation must be different compared to those in other cells, such as macrophages or endothelial cells. We then tested another important mechanism related to Rac GTPase activation. Incubation of rat mesangial cells with Hcys increased Rac GTPase activity, as shown by GTP-bound Rac through a pull-down assay. However, Hcys had no effect on total Rac protein levels. It is suggested that Rac GTPase activation mediates the effect of Hcys on NADPH oxidase activity. We recently also investigated the mechanism by which Hcys induced Rac GTPase activation and found that phosphorylation of Vav-2, a subfamily of guanine nucleotide exchange factors, importantly contributed to Hcys-induced increase in Rac1 activity and consequent activation of NADPH oxidase in rat mesangial cells (77).

In conclusion, hHcys is implicated in glomerular sclerosis in hypertension, elevated plasma Hcys in SS hypertensive rats is due to downregulation of CBS expression and consequent abnormality of transsulfuration in the kidney. $O_2^{\bullet -}$ production by NADPH oxidase as a triggering mechanism contributes to the effects of Hcys on the homeostasis of ECM and consequent sclerosis in the glomeruli, and NADPH oxidase activation by Hcys is associated with enhanced Rac GTPase activity.

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