Homocysteine in Renal Injury

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Introduction

Chronic kidney disease (CKD) is a serious clinical and public health challenge globally. There is an increasing incidence and prevalence of renal failure, with poor outcomes and high cost. In China, 147 million people are affected by CKD. Although CKD is widespread, current therapeutic options for CKD are scarce and ineffective in stemming the tide of CKD progression to end-stage renal disease (ESRD). Identification of factors responsible for the progression toward ESRD is an ongoing area of interest because interventions to control risk factors (e.g. lowering of high blood pressure and high cholesterol levels) can reduce the incidence and associated cost of CKD. Several studies reported that age, blood pressure, diabetes, proteinuria, such dyslipidemia as apolipoprotein B or high-density lipoprotein cholesterol level abnormalities, and smoking were associated with the subsequent decline in glomerular filtration rate (GFR) [1–6]. However, regardless of the treatment and prevention of these traditional risk factors, patients with renal failure are increasing in number, suggesting that other factors need to be evaluated.

Key Words

- Hyperhomocysteinemia
- Chronic kidney disease
- Oxidative stress
- Endoplasmic reticulum stress
- Hypomethylation

Abstract

\textbf{Background:} Homocysteine (Hcy) is an intermediate of methionine metabolism. Hyperhomocysteinemia (HHcy) can result from a deficiency in the enzymes or vitamin cofactors required for Hcy metabolism. Patients with renal disease tend to be hyperhomocysteinemic, particularly as renal function declines, although the underlying cause of HHcy in renal disease is not entirely understood. \textbf{Summary:} HHcy is considered a risk or pathogenic factor in the progression of chronic kidney disease (CKD) as well as the cardiovascular complications. \textbf{Key Messages:} In this review, we summarize both clinical and experimental findings that reveal the contribution of Hcy as a pathogenic factor to the development of CKD. In addition, we discuss several important mechanisms mediating the pathogenic action of Hcy in the kidney, such as local oxidative stress, endoplasmic reticulum stress, inflammation and hypomethylation.
Homocysteine Metabolism and Hyperhomocysteinemia

Homocysteine (Hcy) is a thiol-containing amino acid that is derived from methionine. Methionine from dietary sources or from the breakdown of endogenous protein is converted to S-adenosyl methionine (SAM) by the enzyme SAM synthase. SAM is a major methyl group donor for various methylation reactions. When the methyl group is transferred by methyltransferases to respective acceptors, SAM is converted to S-adenosylhomocysteine (SAH) and then subsequently hydrolyzed by SAH hydrolase to form Hcy and adenosine. Once formed, Hcy can be metabolized by two alternative pathways: remethylation (RM) and transsulfuration (TS). The RM pathway regenerates methionine by methylenetetrahydrofolate reductase (MTHFR) using Hcy as a substrate and folate and vitamin B₁₂ as cofactors. TS of Hcy is catalyzed by cystathione-β-synthase (CBS) and γ-cystathionase. In this pathway, Hcy first undergoes condensation with serine to form cystathionine by CBS. Then, cystathionine is broken down into cysteine by γ-cystathionase. Finally, cysteine is metabolized into taurine and sulfate or transferred into glutathione (fig. 1). It has been shown that Hcy is primarily transsulfurated in the kidney, and deficiency of this renal TS importantly contributes to the elevation of plasma Hcy under different physiological or pathological conditions, such as hypertension, diabetes mellitus, aging, or ESRD [7, 8].

Excess Hcy is exported into circulation. In circulation, <1% of Hcy is present in the free reduced form, 10–20% of the total Hcy (tHcy) is present as a disulfide form homocysteine (Hcy-S-S-Hcy) or cysteinyl Hcy (Cys-S-SHcy), 80–90% of Hcy in circulation is oxidized Hcy that is bound to protein such as albumin and hemoglobin. In humans, the normal level of tHcy is 5–15 μM. Hyperhomocysteinemia (HHcy) is defined as an increase in the plasma level of tHcy under different physiological or pathological conditions, such as hypertension, diabetes mellitus, aging, or ESRD [7, 8].

Given the high prevalence of HHcy in China is 27.5%, which is much higher than that of some developed countries with folic acid fortification policies. Geographically, the prevalence of HHcy in China was high in northern areas, and was higher in inland versus coastal areas. One of the main factors that contribute to these geographical disparities may be different dietary habits in these regions. Folate and vitamin B₁₂ intake is lower in the diet of Chinese northerners than in that of the southerners [10]. Seafood (rich in betaine and vitamin B₁₂) consumption in coastal regions is higher than that in inland regions [11, 12]. Genetic background may be another essential factor. The MTHFR C677T polymorphism is the most common genetic determinant of HHcy especially under the conditions of low dietary folate. It has been reported that the frequencies of 677T allele and 677 TT genotype showed a decreasing trend from northern to southern China [13].

In recent years, researchers have conducted a number of epidemiological studies to investigate the prevalence of HHcy in China. A meta-analysis based on 60,754 subjects derived from 36 studies revealed that the overall pooled prevalence of HHcy in China is 27.5%, which is much higher than that of some developed countries with folic acid fortification policies. Geographically, the prevalence of HHcy in China was high in northern areas, and was higher in inland versus coastal areas. One of the main factors that contribute to these geographical disparities may be different dietary habits in these regions. Folate and vitamin B₁₂ intake is lower in the diet of Chinese northerners than in that of the southerners [10]. Seafood (rich in betaine and vitamin B₁₂) consumption in coastal regions is higher than that in inland regions [11, 12]. Genetic background may be another essential factor. The MTHFR C677T polymorphism is the most common genetic determinant of HHcy especially under the conditions of low dietary folate. It has been reported that the frequencies of 677T allele and 677 TT genotype showed a decreasing trend from northern to southern China [13].

Given the high prevalence of HHcy in the Chinese population, it is possible that HHcy is a key risk factor for the development of CKD in the Chinese population.

Fig. 1. Hcy metabolism. Hcy has two main alternative pathways: to be remethylated to methionine and to enter the cysteine biosynthetic pathway. MET = Methionine; MTs = methyltransferases; R = methyl acceptor; THF = tetrahydrofolate; BHMT = betaine-Hcy methyltransferase; DMG = dimethylglycine.
The Pathogenesis of HHcy in Renal Failure

The tHcy level in patients with ESRD is 3–5 times higher than normal, and the prevalence of HHcy in this patient group is 85–100%. Almost every study has shown a highly significant positive correlation between the concentrations of creatinine and Hcy. GFR values estimated from serum creatinine or calculated creatinine clearance are consistently and inversely correlated with plasma Hcy levels. This relationship is a powerful indirect evidence that elevated Hcy levels in renal disease are intimately linked to kidney function.

The close relationship between plasma Hcy and GFR suggests that Hcy is cleared from the body by urinary excretion after glomerular filtration, just like creatinine. It has been shown that kidney plays a major role in the maintenance of Hcy plasma homeostasis in rats [14, 15]. However, studies performed in humans have not shown the occurrence of any significant arteriovenous gradient of Hcy across this organ [16, 17]. In hypertensive patients, the fractional extraction of Hcy across the kidney is positively related to renal plasma flow but not to GFR, indicating that in humans the removal of Hcy is less than it occurs in rodents, and that it is limited to conditions characterized by elevated renal blood flow. In addition, many studies showed that clinically stable renal transplant recipients have an excess prevalence of HHcy, suggesting that improvement of the GFR in these patients does not completely restore plasma Hcy to normal.

Hcy TS and RM enzymes are present in animal and human kidneys. Rat kidney extracts contain TS (cystathionine β-synthase and cystathionase) and RM (MS) enzymes in significant amounts [18]. In human subjects, appreciable levels of these enzymes are found primarily in liver and kidney tissue. Although both enzymatic pathways can theoretically be used, whether renal disease affects the specific metabolic pathways is not clear. Stable-isotope studies in nondiabetic [19, 20] and diabetic patients [21] with CKD have shown impaired metabolic clearance of Hcy by both the TS and the RM pathways. Consistently, Stam et al. [20] reported that, in ESRD patients, metabolic Hcy clearance by TS and the RM is severely reduced, absolute RM rate is reduced to a much lesser extent, whereas the TS rate is unchanged. Of note, Garibotto et al. [22] observed that the human kidney plays a unique role in the removal of SAH from the circulation, which suggests that the kidney may have an important role in the control of body transmethylation reactions. The SAH arteriovenous difference across the kidney represents ~40% of the SAH arterial plasma concentration, positioning the human kidney as a major tissue in the metabolic disposal of plasma SAH, suggesting a new role for the kidney in tissue methyl transfer reactions in humans.

An alternative theory involves currently unidentified uremic substances that inhibit normal extrarenal Hcy metabolism. The liver is considered the most likely target organ, given its major regulatory role in protein metabolism, its high levels of Hcy metabolizing enzymes, and its capacity to export massive amounts of Hcy in vitro [23]. In addition, extrarenal metabolic disturbances have also been reported to contribute to the occurrence of HHcy in ESRD. These metabolic disturbances are associated with a generalized downregulation of the methionine cycle and its catabolism, which may be the inhibitory action of various metabolites accumulated or retained during ESRD, such as SAH, sulfate, and dimethylglycine.

Role of HHcy in CKD: Clinical Evidence and Hcy-Lowering Therapy

It is well known that elevated Hcy level is associated with kidney disease. The next question is whether elevated Hcy is an independent risk factor of CKD. In 2004, Ninomiya et al. [24] reported the first population-based cohort study of 1,477 Japanese community-dwelling individuals without CKD. After following up for 5 years, age-adjusted 5-year incidences of CKD were 2.2% in the low tertile (≤8.3 μM), 5.4% in the middle tertile (8.3–10.5 μM), and 8.6% in the high tertile (≥10.6 μM) of Hcy levels for men and 3.3, 6.0, and 6.9% for women, respectively. In a multivariate analysis, these relationships remained substantially unchanged, even after adjustment for other confounding factors, such as systolic blood pressure, antihypertensive medication, hemoglobin A1c level, total cholesterol level, high-density lipoprotein cholesterol level, habitual smoker status, regular alcohol intake, proteinuria, and baseline kidney function. This study suggests that moderately elevated serum tHcy level is a significant risk factor for the development of CKD in the general population. Consistently, in 2014, Levi et al. [25] reported a historical prospective study on 3,602 subjects with normal estimated GFR (eGFR) and without proteinuria in Israel between the years 2000 and 2012. Annual eGFR decline was 25% higher in subjects with elevated (>15 μM) versus normal mean Hcy level (≤15 μM).

MTHFR catalyzes the conversion of 5, 10-methylene-tetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for Hcy RM to methionine. C677T, a single nucleo-
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Homocysteine (C→T) at nucleotide position 677, leads to Ala→Val codon substitution at amino acid position 222. MTHFR C677T is a major genetic determinant for HHcy and has been shown to be associated with risks for stroke, coronary heart disease, neural tube defect, depression, schizophrenia, and cancer. We performed a cross-sectional analysis of the association of MTHFR C677T polymorphism with eGFR in 18,814 Chinese adults, and demonstrated that MTHFR C677T is a risk allele for decreased kidney function in hypertensive Chinese males.

Microalbuminuria is a strong indicator of the risk of future cardiovascular disease and renal dysfunction. A couple of cross-sectional studies have shown that slightly increased level of Hcy is associated with the presence of microalbuminuria. Stehouwer’s group [26] performed a prospective study in an age, sex and glucose tolerance-stratified sample (n = 316) of a population-based cohort study. The mean follow-up duration is 6.1 years. Logistic regression analyses showed development of (micro)albuminuria is significantly associated with baseline Hcy levels >19.0 μM compared with Hcy levels <9.1 μM, indicating that HHcy is an independent determinant of the development of (micro)albuminuria among nondiabetic subjects, even after adjustment for estimates of GFR. A 5 μM increase in the Hcy level was associated with an increased risk of developing (micro)albuminuria. This study indicates that increased Hcy levels precede the occurrence of (micro)albuminuria. The pathophysiological pathway linking Hcy level and risk of (micro)albuminuria is unknown. Some evidence suggests that HHcy enhances oxidative stress, which could induce endothelial and mesangial cell (MC) dysfunction, result in increased intraglomerular pressure and/or decreased glomerular charge and size selectivity, and thus cause microalbuminuria.

CKD patients have a markedly elevated risk for cardiovascular disease (CVD) [27, 28]. Approximately 50% of patients with ESRD die from a CVD cause, which is 15–30 times higher than the age-adjusted CVD mortality in the general population [29]. Thus, prevention and treatment of CVD are major considerations in the management of individuals with CKD [27]. However, the extent of cardiovascular risk is not accounted for by traditional risk factors. Since a high level of total plasma Hcy was first identified as a risk factor for atherosclerosis, it has been proposed that HHcy is a crucial element in the pathogenesis of uremic cardiovascular complications [30–32]. In 1996, Robinson et al. [33] conducted a cross-section study of 176 dialysis patients and found that in these patients a tHcy concentration >27.8 μM is an independent risk factor for vascular complications of ESRD. Most recently, Elias et al. [34] conducted a prospective study with 498 participants with and without early-stage CKD (eGFR <60 ml/min/1.73 m²) in a sample of community-living individuals free from ESRD, dialysis, stroke, and dementia. Levels of tHcy were positively related to carotid-femoral pulse wave velocity measured 4–5 years later for participants with early-stage CKD, suggesting that plasma tHcy is an important predictor of arterial stiffness in individuals with modest CKD.

From many Hcy-lowering intervention studies in renal failure patients, it becomes clear that folate acid has the most powerful Hcy-lowering effect. The beneficial results of vitamin supplementation from clinical trials are not consistent. We conducted a meta-analysis of relevant randomized trials to examine the efficacy of folate therapy to lower Hcy levels in an effort to reduce CVD risk in patients with ESRD or advanced CKD. This meta-analysis included 3,886 patients with ESRD/CKD from 7 qualified randomized trials using folate acid therapy and with CVD reported as one of the end points. Folic acid therapy reduced the risk of CVD by 15%. A decrease in Hcy level >20% was observed when treatment duration was longer than 24 months.

**Role of Hcy in Renal Damage: Potential Cellular and Molecular Mechanisms**

With respect to the direct action of Hcy to damage cells or induce sclerotic changes, recent studies focus on several important cellular and molecular mechanisms including oxidative stress, inflammation, endoplasmic reticulum (ER) stress and hypomethylation. It is our belief that understanding these mechanisms will help further clarify the pathogenesis of ESRD.

**Hcy-Induced Oxidative Stress**

Oxidative stress is possibly the most detrimental stressor in the pathogenesis of most diseases. Hcy contains a highly reactive thiol group and can undergo rapid autooxidation in the presence of oxygen and metal ions (iron and copper) generating potent ROS including superoxide anion, and hydrogen peroxide and hydroxyl radical, suggesting that auto-oxidation of Hcy is one of the mechanisms of ROS formation. NADPH oxidase is a major source of superoxide production in mammalian cells. It has been shown that Hcy directly mobilized the cytosolic subunits p47phox and p67phox to the cell membrane to ac-
tivate the NADPH oxidase. In phagocytic monocytes, Hcy has been shown to increase the phosphorylation of cytosolic subunits p47\textsuperscript{phox} and p67\textsuperscript{phox} via protein kinase C-β activation that promoted the assembly of the active oxidase enzyme. Tyagi et al. [35] demonstrated that Hcy increases the mRNA level of NADPH oxidase in a dose- and time-dependent manner accompanied by an increase in ROS production in endothelial cells. In addition, they found that Hcy increases NOX-4, and causes its translocation to the mitochondria [36]. Collectively, these data support that HHcy-mediated superoxide production from NADPH oxidase is a critical biochemical mechanism in the pathogenesis of various diseases.

Numerous studies have demonstrated that NADPH oxidase is importantly involved in the progressive glomerular injury associated with HHcy [37–39]. In cultured rat MC, 40–160 μM of Hcy markedly increased the mRNA levels of tissue inhibitor of metalloproteinase-1 and led to the accumulation of collagen type I which was accompanied by enhanced NADPH oxidase activity. The NADPH oxidase inhibitor DPI can substantially attenuate Hcy-induced biochemical and functional changes. In the rat model of HHcy induced by a folate-free diet, glomerular sclerosis occurred which is characterized by enhanced local oxidative stress, mesangial expansion, podocyte dysfunction, and fibrosis. When these rats were treated with the NADPH oxidase inhibitor apocynin, the glomerular injuries were significantly attenuated [38, 40]. It is concluded that Hcy-induced local oxidative stress and consequent cell dysfunction and extracellular matrix metabolism in glomerular cells are associated with enhanced NADPH oxidase activity. In addition, Hcy has been reported to cause mesangial apoptosis via ROS generation and p38-mitogen-activated protein kinase activation [37].

**Inflammation**

Studies in humans have identified an association between Hcy and inflammation. For instance, in a subject aged ≥65 years, IL-6 and IL-1 cytokines were independent predictors of plasmatic Hcy concentrations [41]. Similarly, in another study, serum Hcy levels and C-reactive protein levels were significantly higher in patients with stage 3 CKD compared to those with stage 1 disorder [42]. In this regard, the potential consequences of HHcy on inflammation in the kidney have been studied by assessing the impact of Hcy on monocyte chemoattractant protein-1 (MCP-1) expression by glomerular MC [43]. Hcy (50–200 μM) induced MCP-1 protein and mRNA levels in glomerular MC via NF-κB activation, a process found to be mediated by generation of oxidative stress [43]. Hcy has also been shown to increase the expression of MCP-1 and IL-8 in cultured endothelial cells. In methionine-induced HHcy rats, MCP-1 protein and mRNA levels were increased in the kidneys, and this increase was dependent on NF-κB, supporting the concept that HHcy accelerates the progression of kidney disease by causing inflammation [44].

Nucleotide-binding oligomerization domain containing 2 (NOD2), a member of the NOD-like receptor family, plays an important role in innate immune and adaptive response. In addition to being present in inflammatory cells, NOD2 is also highly expressed in the kidney, including renal proximal tubule epithelial cells, glomerular MC, endothelial cells, and podocytes. Yi’s group [45] showed that NOD2 expression was significantly increased in the kidney from HHcy mice accompanied with upregulation of several proinflammatory cytokines, including IL-1β, IL-6, TNF-α, MCP-1, and intracellular adhesion molecule-1. In NOD2\textsuperscript{−/−} mice, HHcy-induced expression of proinflammatory mediators was inhibited accompanied with ameliorated podocyte injury and glomerulosclerosis, suggesting an involvement of the intracellular NOD-like receptors in HHcy-induced inflammation [45].

**ER Stress**

The ER is an intracellular compartment that plays a critical role in protein synthesis and folding, calcium storage, and calcium release. It also serves as a site of biosynthesis for steroids, cholesterol, and other lipids. Proteins that are translocated into the ER lumen undergo post-translational modifications and folding required for optimal function. Properly folded proteins are allowed to reach their destiny via the secretory pathway, whereas unfolded and misfolded proteins are exported or dissociated from the ER and then degraded by cytoplasmic proteasomes [46, 47]. ER stress is referred to as a condition under which unfolded and misfolded proteins are accumulated. Accumulation of these unfolded or misfolded proteins activates an unfolded protein response (UPR) which includes increased expressions of UPR-responsive genes, reduced global protein translation, and unfolded protein degradation.

Hcy-induced ER stress response has received much attention. The basis for the impairment in ER function by Hcy has been speculated that Hcy disrupts disulphide
bond formation perturbing protein folding. In addition, Hcy was found to activate the expression of genes known to be under the control of signaling pathways that respond to load on the ER. These include the BiP/GRP78 gene, encoding an ER chaperone [48]; CHOP/GADD153, encoding a transcription factor implicated in cellular responses to ER stress [49, 50], and HERP, encoding a protein that may be involved in degradation of malfolded ER proteins [51]. Other mechanisms by which Hcy induces cell injury through ER stress may be associated with activation of IRE-1, a signaling molecule that leads to a rapid and sustained activation of JNK protein kinases and cell apoptosis [52, 53] or dysregulation of the sterol response to Hcy. Recently, we found that Hcy causes ER stress in proximal tubular cells. HHcy mice fed high-methionine diet are more sensitive to cisplatin-induced acute kidney injury by enhancing ER stress [unpubl. data], suggesting HHcy could aggravate renal damage by causing ER stress.

Hypomethylation

SAH is a powerful competitive inhibitor of SAM-dependent methyltransferases. It has been proposed that increased Hcy elevates the level of SAH, which inhibits the transferring methyl group from SAM to acceptors, leading to hypomethylation [54, 55]. Indeed, SAH concentrations are elevated both intracellularly and extracellularly in various models of HHcy. DNA methylation, in peripheral mononuclear cells, has been shown to be associated with plasma total Hcy concentration even in healthy individuals [39]. Hcy-induced hypomethylation has been recognized as a key biochemical mechanism by which Hcy induces vascular injury. Wang’s group [56] demonstrated that Hcy inhibits cyclin A transcript by demethylating a CpG island at the promoter and thus suppresses endothelial cell growth. In patients with ESRD, a hypomethylation status of cellular proteins and DNA can be detected, as shown by high SAH intracellular concentrations, low SAM/SAH concentration ratios [57, 58], and inhibited protein methylation and repair [59]. Ingrosso et al. [60] reported that the level of global DNA methylation was reduced in male patients with uremia and HHcy. This DNA hypomethylation was linked to defects in the expression of genes regulated by methylation. Moreover, folate therapy, a common method to reduce HHcy, restored DNA methylation to normal levels and corrected the patterns of gene expression.

Although Hcy-induced DNA hypomethylation has been shown to be involved in endothelial cell injury, results have been inconsistent in many studies. In CBS null mice, higher SAH concentrations were detected in all tissues studied, but lower DNA methylation status was only detected in the liver [61], suggesting other mechanisms might be implicated in Hcy-modulated gene expression. We recently reported that Hcy downregulated the expression of histone methyltransferase G9a, which in turn decreased the level of H3K9me2 at the promoter of COL1A1. H3K9 hypermethylation serves as a docking site for the chromatin modifier protein heterochromatin protein 1, which in turn recruits DNMT1 and stimulates its activity, leading to DNA hypermethylation in the surrounding area [62, 63]. It is possible that Hcy-mediated G9a repression leads to DNA hypomethylation to reinforce the epigenetic activation of gene transcription.

Conclusion

HHcy is an integral component of several disorders including cardiovascular and cerebrovascular diseases, neurodegeneration, liver steatosis, and CKD. HHcy can result from deficiencies of vitamin cofactors (B₆, B₁₂, folic acid) required for Hcy metabolism and/or from genetic disorders of its metabolism. This review provides evidence that supports the causal role for HHcy in the development of CKD and outlines several cellular and molecular mechanisms by which Hcy induces renal injury. These mechanisms include oxidative stress, inflammation, ER stress, and DNA hypomethylation. Due to space limitation, some other mechanisms such as increased Hcy thiolactone levels, homocysteinylation, and mitochondrial dysfunction are not included. In perspective, further studies are much needed to provide more convincing evidence demonstrating the pathogenic role of HHcy in the progression of CKD in animal studies and clinical studies. The value of folate acid therapy in CKD remains to be confirmed by large clinical trials.

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Conflict of Interest Statement

The authors have no competing financial interests to declare.
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