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Folic acid attenuates hyperhomocysteinemia-induced glomerular damage in rats

Lu Cao [a,1], Xiaoying Lou [a,1], Zhaoxia Zou [c], Nana Mou [a], Weikang Wu [a], Xiongqing Huang [b,**], Hongmei Tan [a,*]

[a] Department of Pathophysiology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, PR China
[b] Department of Anesthesiology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, 510080, PR China
[c] Guangdong Provincial People's Hospital, Guangzhou, 510080, PR China

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ABSTRACT

The present study investigated whether lowering plasma homocysteine (Hcy) with folic acid (FA) could attenuate hyperhomocysteinemia (HHcy)-associated glomerular damage and possible mechanisms. The HHcy animal model was established by intragastric administration with L-methionine in rats. FA was also given intragastrically. Plasma Hcy and creatinine and urinary albumin were measured. Histological and ultrastructural changes were observed by light and electron microscopes. The expression of alpha-smooth muscle actin (α-SMA), proliferating cell nuclear antigen (PCNA) and transforming growth factor-beta1 (TGF-β1) in the kidney was examined by immunohistochemical staining and western blot analysis. The administration of L-methionine induced HHcy in rats. The HHcy rats developed glomerulosclerosis and fibrosis. Plasma creatinine concentration and urinary albumin excretion were also significantly increased in HHcy rats. Effacement and extensively fusion of podocyte foot process was observed in HHcy rats, which was associated with decreased expression of nephrin protein in renal cortex of HHcy rats. Supplementation with FA lowered plasma Hcy significantly. Plasma creatinine concentration and urinary albumin excretion were also significantly attenuated by FA. Morphologically, HHcy-associated glomerulosclerosis, fibrosis, podocyte foot process effacement and loss of podocyte nephrin, were significantly improved by FA. The expressions of α-SMA, PCNA and TGF-β1 were increased in renal cortex of HHcy rats, and which were also partially reversed by FA. These data suggest that elevated plasma Hcy is an important pathogenic factor for glomerular damage. Lowering plasma Hcy by FA can inhibit TGF-β1 expression and attenuate HHcy-induced glomerular damage.

Introduction

Hyperhomocysteinemia (HHcy) is an independent cardiovascular risk factor. Li N et al. first reported that HHcy played a role in the development of glomerulosclerosis in uninephrectomized Dahl salt-sensitive rats (Li et al., 2002). Recently more studies have demonstrated that glomerulosclerosis was closely associated with the high blood concentrations of homocysteine (Hcy) (Li et al., 2002; Yi et al., 2006; Zhang et al., 2012). Despite a positive correlation between HHcy and glomerular damage, it is still uncertain whether HHcy is a causative factor or the consequence of glomerular damage. The pathogenic implications and mechanisms of HHcy-associated renal damage have not been fully elucidated yet.

Previous reports suggested the role of increased expression of alpha-smooth muscle actin (α-SMA) in myofibroblasts differentiation in case of lung fibrosis (Hamelet et al., 2007), liver fibrosis (Robert et al., 2005) and vascular fibrotic diseases (Sen et al., 2006) in HHcy animal models. Hcy was also reported to remarkably increase the expression of α-SMA in podocytes (Zhang et al., 2011), which may be associated with the development of fibrosis in the kidney (Muchaneta-Kubara and el Nahas, 1997; Van den Branden et al., 2000). In vivo and in vitro studies and human diseases have suggested that transforming growth factor beta (TGF-β) contributed to the pathogenesis of tissue fibrosis in most organs. It is widely accepted that TGF-β is a key mediator of glomerular fibrosis (Kashiwagi et al., 2000; Jiang et al., 2003; Yu et al., 2003). However, it remains unclear whether HHcy-associated glomerular fibrosis and glomerulosclerosis result, at least in part, from the enhancement of TGF-β.

Folic acid (FA) plays a critical role in the remethylation of Hcy to methionine (Met) (Homocysteine Lowering Trialists' Collaboration, 1998). Observational studies have shown an inverse relationship between FA status and plasma Hcy level. Further studies also showed that FA supplementation can considerably reduce Hcy concentrations in hemodialysis patients (Alvares Delfino et al., 2007), in chronic renal transplant recipients (Beaulieu et al., 1999) and in end-stage renal disease patients (Stam et al., 2005). Hwang et al. reported that

* Corresponding author. Fax: +86 20 87330026.
** Corresponding author. Fax: +86 20 87332200 8270.
E-mail addresses: tanhm@mail.sysu.edu.cn (H. Tan), hxiongqing@21cn.com (X. Huang).
[1] Authors made an equal contribution.

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FA could inhibit NADPH oxidase-mediated superoxide anion production in the kidney, suggesting a renal protective effect of FA (Hwang et al., 2011). However, it remains unknown whether HHcy-associated glomerular fibrosis and glomerulosclerosis could be attenuated by lowering plasma Hcy with FA. The present study was designed to answer this question and to test the hypothesis that lowering plasma Hcy levels with FA attenuates HHcy-induced glomerular damage. We investigated the effects of decreased plasma Hcy on glomerular function and structure in healthy Sprague–Dawley rats.

Materials and methods

Animals

Sprague–Dawley rats (150–180 g, 6 weeks) were provided by Laboratory Animal Center of Sun Yat-sen University. Rats were randomized into control group, HHcy group and HHcy + FA group with intragastrically administration of purified water, L-Met (1 g/kg/d, Sigma, USA), L-Met plus FA (100 mg/kg/d, Sigma, USA) for 3 months, respectively. Each group has 8 rats. The animal experiment was approved by the Animal Care and Use Committee of Sun Yat-sen University. All animal care and procedures conform to the Council for International Organizations of Medical Sciences (CIOMS) guidelines.

Plasma Hcy and creatinine analysis

At the end of the experiment, rats were fasted overnight and anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Blood was collected from vena cava. The plasma Hcy levels were measured by fluorescence immunoassay (Abbott IMx analyzer, Abbott Laboratories, Berkshire, UK) as previously reported (Yan et al., 2010; Lan et al., 2011). The plasma creatinine concentrations were assayed by the picric acid assay.

Urinary albumin measurement

To collect urine samples, rats were placed on a clean platform until spontaneous voiding was observed. The urine sample was collected immediately and urinary albumin excretion was examined by using a commercially available rat albumin ELISA kit (CUSABIO, Wuhan, China).

Histological examination

For renal histology studies, renal tissues were fixed with 10% formalin solution. Paraffin-embedded 4 μm-thick sections were stained with the periodic acid-Schiff (PAS) and Masson's trichrome staining (MTS). Photomicrographs were obtained from six different fields (40× magnification) in each sample of fringe cortex. The total number of points for each sample falling on the renal vascular ball was used for calculating the glomerular damage index. The severity of glomerular damage was graded from 0 to 4 using a semiquantitative score: 0, no matrix expansion; 1, minor; 2, weak; 3, moderate; and 4, strong. Collagen-positive area in glomeruli was determined by computer-assisted color gated measurement of MTS sections using the updated Image-Pro Plus program and expressed the data as a percentage.

For transmission electron microscopic (TEM) observation of ultrastructural changes in podocytes, the kidneys were fixed with 3% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer. The kidneys were sliced longitudinally and cortical strips were cut into 1 mm^3 tissue blocks. After dehydration with ethanol, the samples were embedded in Durcupan resin for ultra-thin sectioning and TEM examinations.

Immunohistochemical staining

For immunohistochemistry, slides were deparaffinized and incubated with proteinase K for antigen retrieval. The slides were immunostained with rabbit anti-α-SMA (1:250; Leica Biosystems, UK) and mouse anti-proliferating cell nuclear antigen (PCNA) (1:500; Leica Biosystems, UK) antibodies. The specificity of immunohistochemical staining was confirmed by omitting primary antibodies. The slides were lightly counterstained with hematoxylin to reveal nuclei and photographed.

Western blot analysis

The kidney cortical tissues were minced into pieces and incubated with RIPA protein extraction solution with fast agitation. Homogenates were centrifuged at 12000 ×*g* for 20 min at 4 °C. Supernatants from each sample were collected and protein concentrations were measured. Western blot procedures were carried out as described previously (Yan et al., 2010) with specific antibodies against α-SMA (1:1000; Abcam, UK), PCNA (1:1000; Santa Cruz, USA), TGF-β1 (1:50; RD, USA) and nephrin (1:1000; Abcam, UK). Incubation with GAPDH antibody (1:5000; Santa Cruz, USA) was carried out as the loading control. The blots were detected on Kodak X-Omat film by enhanced chemiluminescence (Applygen Technologies, Beijing, China). Quantification of band intensity was carried out using Image J software (NIH, Bethesda, MD, USA).

Statistical analysis

Data was presented as mean ± SD. Significant differences among groups were carried out by one-way ANOVA test followed by LSD test using SPSS 17.0 software. $p < 0.05$ was considered statistically significant.

Results

L-Met treatment induced HHcy and FA reduced plasma Hcy

In the L-Met treatment group, the rats showed a significant higher level of plasma Hcy compared with control rats (25.24 ± 7.23 vs 4.04 ± 1.07, $p < 0.001$). Daily supplementation with FA significantly reduced, but didn't normalize plasma Hcy concentrations compared to HHcy rats (Fig. 1A).

FA reduced HHcy-induced increased creatinine level and albuminuria

To assess the effect of HHcy and the protection of FA on the kidneys, plasma creatinine and urinary albumin were measured. The plasma creatinine level in HHcy rats was significant higher than that in control rats, and supplementation with FA significantly decreased plasma creatinine levels compared with HHcy group (Fig. 1B). In parallel with the increased plasma Hcy, urinary albumin excretion was significantly increased in HHcy group, whereas FA treatment significantly reduced HHcy-induced urinary albumin excretion (Fig. 1C), indicating a protection of FA on HHcy-induced kidney injury.

FA attenuated HHcy-induced glomerular damage and collagen deposition

Kidney weights were significantly higher as expressed as kidney mass relative to body weight in L-Met treated HHcy rats than in control rats (Fig. 2B). The renal histological injury was examined by evaluating the glomerular damage index and relative area of glomerular fibrosis at the end of experiment. Representative images of PAS and MTS staining are shown in Fig. 2A. In rats with experimental HHcy, the semiquantitative analysis data showed that the glomerular damage index was significantly greater than that in control rats.