Low Dose of Folic Acid Can Ameliorate Hyperhomocysteinemia-Induced Cardiac Fibrosis and Diastolic Dysfunction in Spontaneously Hypertensive Rats

Ping Cao,1,2 MD, Wangmeng Zhang,2 MD, Guicheng Wang,2 MD, Xuan Zhao,3 MD, Ning Gao,1 MD, Zhen Liu,1 MD and Rui Xu,1,4 MD

Summary
To evaluate whether lowering plasma homocysteine (Hcy) levels at different doses of folic acid (FA) could reduce cardiac fibrosis and diastolic dysfunction in spontaneously hypertensive rats (SHRs) with hyperhomocysteinemia (Hhcy) and investigate the possible mechanism of action.

We randomly divided 32 male SHRs into control, Hhcy, Hhcy + low-dose FA (LFA), and Hhcy + high-dose FA (HFA) groups. Echocardiography and Masson staining of cardiac tissue were used to assess diastolic function and cardiac fibrosis. Blood pressure (BP) and Hcy levels were measured during the experiment. We also measured the indicators of oxidative stress (OS) and examined the expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) genes and proteins using real-time polymerase chain reaction (PCR), immunohistochemistry, and western blotting to explore the possible mechanism of action.

FA treatment reversed SHR cardiomyocyte interstitial and perivascular collagen deposition and diastolic dysfunction exacerbated by Hhcy. These effects were associated with promoting the translocation of Nrf2 from the cytoplasm to the nucleus, activating HO-1 expression and inhibiting OS. However, HFA did not show any additional benefit from LFA in reducing cardiac injury.

Even at a low dose, FA can ameliorate Hhcy-induced cardiac fibrosis and diastolic dysfunction in SHRs by activating Nrf2/HO-1 pathway and inhibiting OS, independent of BP, providing evidence for the efficacy of LFA in the treatment of hypertension associated with Hhcy.

Key words: Hhcy, Hypertension, Nrf2/HO-1 pathway

Hypertension and other conditions of pressure overload, such as aortic stenosis, can lead to adverse cardiac changes characterized by the hypertrophy of cardiomyocytes, myocardial and perivascular collagen deposition, diastolic dysfunction, and possibly subsequent systolic dysfunction. A plasma homocysteine (Hcy) level exceeding 15 μM is referred to as hyperhomocysteinemia (Hhcy), an independent risk factor for cardiovascular disease, type 2 diabetes, diabetic nephropathy, and thoracoabdominal aortic aneurysms.1-3 Data from a spontaneously hypertensive rat (SHR) model indicated that the interaction between hypertension and Hhcy led to coronary arteriolar remodeling, cardiac fibrosis, and diastolic dysfunction.4 Oxidative stress (OS) paralleled these structural and functional changes, indicating that myocardial redox state might be an important determinant of Hhcy-induced cardiac injury.5 More common in Chinese patients with hypertension because of their particular genetic characteristics,6 about 75% of Chinese patients with hypertension have Hhcy.7 Therefore, further studying whether intervention to reduce plasma Hcy level can relieve cardiac injury is of great clinical significance.

The Hcy-lowering effect of folic acid (FA) has been demonstrated in clinical trials.8,9 However, the use of FA to reduce plasma Hcy levels in the treatment of cardiovascular disease is controversial. Studies10 have provided evidence that FA ameliorates Hcy-mediated endocardial endothelial dysfunction in homocysteinemic hypertensive rats by decreasing matrix metalloproteinase activity and one of the D- or L-isomer of Hcy. FA mitigated cardiac dysfunction by normalizing the levels of tissue inhibitor of metalloproteinase and homocysteine-metabolizing enzymes after myocardial infarction in mice.11 Another...
study supported the hypothesis that FA supplementation had no effect on arterial stiffness and cardiovascular outcomes in elderly patients with hyperhomocysteinemia. So far, whether reducing Hcy level with FA can improve Hcy-related hypertensive cardiac fibrosis and the possible mechanism of action are unclear, and there is no unified understanding on the efficacy of different doses of FA.

OS extensively participates in the pathophysiological process of cardiac injury. The antioxidant effect of FA has also been demonstrated. Nuclear factor erythroid 2-related factor 2 (Nrf2) is an important regulator of OS. It is widely found in the muscle tissue, heart, brain, liver, kidney, blood vessels, and other oxygen-consuming organs. Nrf2 activation can induce the synthesis of antioxidant enzymes, binomial detoxification enzymes, and anti-inflammatory factors. Under physiological conditions, Nrf2 binds to the adapter protein keap1 present in the cytoplasm and is rapidly degraded by the ubiquitin-proteasome system, thus maintaining the activity of Nrf2 at a relatively low level. However, under excessive OS, Nrf2 is released from the complex, activated, and then transferred to the nucleus to bind to the antioxidant reaction element. The formation of this complex leads to the activation of downstream heme oxygenase-1 (HO-1). However, no studies have determined whether Nrf2/HO-1 pathway regulation is involved in the cardiac injury of Hhcy-associated hypertension. Therefore, in this study, we took the animal model of Hhcy-associated hypertension as the research object to investigate whether FA can alleviate cardiac fibrosis and diastolic function by regulating the Nrf2/HO-1 pathway and inhibiting OS and evaluate the efficacy of high-dose FA (HFA) and low-dose FA (LFA).

Methods

Experimental animals: Thirty-two adult male SHRs (260-280 g, 12 weeks) were purchased from Vital River Laboratories Co., Ltd. (Beijing, China). The rats were maintained at the optimal temperature of 22°C ± 2°C with humidity of 50% ± 5%. The rats were maintained under a 12-hour light/dark cycle and had free access to standard food and tap water.

Experimental design: After 2 weeks of adaptation, the rats were randomly divided into four groups (n = 8/group).

Control group: physiological saline (PS) 5 mL/kg, intraperitoneal injection twice daily + PS (0.5 mL) by gavage once daily.

Hhcy group: 2% DL-Hcy (H4628, Sigma-Aldrich, St. Louis, USA) 5 mL/kg, intraperitoneal injection twice daily + PS (0.5 mL) by gavage once daily.

Hhcy + LFA group: 2% DL-Hcy 5 mL/kg, intraperitoneal injection twice daily + 0.4 mg/kg FA (F7876, Sigma) by gavage once daily.

Hhcy + HFA group: 2% DL-Hcy 5 mL/kg, intraperitoneal injection twice daily + 4 mg/kg FA by gavage once daily.

All animals were given intraperitoneal injection for 12 weeks. The PS and FA gavage intervention commenced 4 weeks after the start of the experiment and 1 hour after DL-Hcy treatment. Blood pressure (BP) and plasma Hcy level were measured at week 4, 8, and 12. At week 12, we performed cardiac echocardiography in SHRs.

Approximately 12 hours after the last injection, all rats were euthanized with 3% sodium pentobarbital. Whole blood was collected via the vena cava, and the left ventricle (LV) was aseptically excised for the follow-up analysis.

Plasma Hcy analysis: At week 4 and 8 of the experiment, blood was collected from the tail vein of rats, and at week 12 of the experiment, blood was collected from the vena cava of rats after anesthesia. After whole blood centrifugation, plasma Hcy concentration was determined using the Cobas8000 automatic biochemical analyzer (Roche, Switzerland).

Measurement of BP: The systolic blood pressure (SBP) and diastolic blood pressure (DBP) of the caudal artery of rats were monitored by a noninvasive BP measurement system under the awake state. To ensure the measurement accuracy, temperature was controlled at 37°C-39°C, and the BP was measured after the rats were quiet and the pulse was stable. Each rat was measured three times and averaged.

Echocardiographic examination: Echocardiography was conducted on rats anesthetized with 10% chloral hydrate (0.3 mL/100 g) using a Vevo 770 cardiac system (Visual Sonics Inc., Toronto, Canada). The LV end-systolic and end-diastolic diameters, LV posterior wall systolic and diastolic thicknesses, interventricular septum thickness during systole and diastole, left ventricular ejection fraction (LVEF), and fractional shortening (FS) were measured according to the American Society of Echocardiography guidelines. Diastolic function was determined by the E/A ratio, according to the Doppler inflow velocity (E, mitral peak early filling velocity; A, mitral peak flow velocity at atrial contraction). The values of three consecutive heart cycles were averaged for all echocardiographic measurements.

Histological analysis: Heart samples were fixed in 4% formaldehyde and embedded in paraffin, before being cut into 4-5-μm-thick cross sections. The tissue sections were stained with Masson’s trichrome stain to enable the evaluation of myocardial interstitial fibrosis and perivascular fibrosis. After Masson staining, cardiomyocytes were stained red, whereas collagen fibers were stained blue. The Image-Pro Plus 6.0 software (Media Cybernetics, MD, USA) was used to calculate the cardiac collagen volume fraction (CVF; CVF = collagen area/total area) and the ratio of the perivascular collagen area to the luminal area (PVCA/LA).

Serum OS biomarkers: MDA was used as the index of peroxidation damage, and superoxide dismutase (SOD) was used as antioxidant markers in cardiac injury. Serum MDA and SOD levels were estimated using commercial kits according to the manufacturer’s instructions (Jiancheng Institute of Biological Technology, Nanjing, Jiangsu, China).

Real-time quantitative polymerase chain reaction (PCR): Total RNA was extracted from the heart tissue us-
ing TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was conducted with Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN, USA). Primer sequences (Biosune Biotechnology, Shanghai, China) are listed in Table I. Real-time PCR was performed using SYBR Green PCR Reagents (Roche, Indianapolis, IN, USA). Primer sequences (Biosune Biotechnology, Shanghai, China) were used to perform the statistical analyses. One-way ANOVA was used to detect differences among the groups, and P-values < 0.05 were considered statistically significant.

**Results**

*Establishment of an animal model of SHRs with HhcY and FA-decreased Hcy level:* Four weeks after intraperitoneal injection of DL-HhcY, HhcY was observed in SHRs. In the HhcY, HhcY + LFA, and HhcY + HFA groups, compared with the control group (6.87 ± 0.62 μmol/L), HhcY had higher levels, which were 25.82 ± 0.78, 26.61 ± 0.95, and 26.08 ± 0.84 μmol/L, respectively. With the intervention of different doses of FA (HhcY + LFA and HhcY + HFA groups) at the beginning of the fourth week, both LFA and HFA significantly decreased HhcY levels (27.57 ± 0.43, 14.07 ± 0.81, and 13.74 ± 0.72 μmol/L in the HhcY, HhcY + LFA, and HhcY + HFA groups at week 8 and 28.46 ± 0.57, 12.95 ± 1.02, and 11.63 ± 0.96 μmol/L at week 12, respectively); however, there was no statistically significant difference between the HhcY + LFA and HhcY + HFA groups (Figure 1A).

SBP ≥ 150 mmHg and/or DBP ≥ 100 mmHg were used as diagnostic criteria for hypertension in rats.

*Echocardiography and Masson staining suggest that HhcY-induced diastolic dysfunction and cardiac fibrosis can be reversed even at low doses of FA:* The results of Masson staining in the four groups are shown in Figure 2. Cardiac fibrosis, especially an increase in myocardial and perivascular collagen deposition, was observed in the hearts of the SHRs administered DL-HhcY only compared with the controls and other groups. The increased CVF and PVCA/LA demonstrated the changes and disorders in the structure of the interstitial and perivascular collagen networks. In comparison with the HhcY group, CVF and PVCA/LA were significantly reduced in the groups treated with LFA (7.3%, 0.99-fold, P < 0.05) or HFA (8.6%, 1.27-fold, P < 0.05). Among the FA treatment groups, there was a lower interstitial and perivascular collagen deposition in the HhcY + LFA group compared with the HhcY + HFA group. However, there was no statistically significant difference between the two groups (P > 0.05).

The echocardiographic results are summarized in Ta-
The levels of plasma homocysteine (Hcy), systolic blood pressure (SBP), and diastolic blood pressure (DBP).

A: The levels of Hcy at week 4, 8, and 12. B: The levels of SBP at week 4, 8, and 12. C: The levels of DBP at week 4, 8, and 12. *P < 0.05 versus control group, *P < 0.05 versus Hhc group (n = 8). Vertical bars represent SE.

Figure 1. The levels of plasma homocysteine (Hcy), systolic blood pressure (SBP), and diastolic blood pressure (DBP). A: The levels of Hcy at week 4, 8, and 12. B: The levels of SBP at week 4, 8, and 12. C: The levels of DBP at week 4, 8, and 12. *P < 0.05 versus control group, *P < 0.05 versus Hhc group (n = 8). Vertical bars represent SE.

LFA may play a role in ameliorating cardiac fibrosis and diastolic dysfunction by promoting nuclear transport of Nrf2 and activating Nrf2/HO-1 pathway: To verify that the Nrf2 and HO-1 are involved in the cardioprotective effect of FA against Hhc toxicity, the expression levels of these genes were evaluated using the real-time PCR technique. As shown in Figure 4A, the Hhc group significantly reduced the expression level of Nrf2 and HO-1 genes in comparison with the control group (P < 0.05), but this effect was reversed by FA treatment (P < 0.05). However, there was no significant statistical difference in the mRNA levels of Nrf2 and HO-1 in the Hhc + LFA and Hhc + HFA groups (P > 0.05). These results underline that the Hhc inhibited the expression of these genes, and the FA caused cardioprotective against Hhc via upregulating Nrf2/HO-1 pathways under OS conditions.

The immunohistochemical staining for Nrf2 and HO-1 in the heart tissues is shown in Figure 4B and C. In the FA intervention groups, we observed increased Nrf2 staining in the nuclei of cardiomyocytes. Thus, FA intervention promoted Nrf2 nuclear transfer in SHRs with Hhc. The staining of HO-1 in cardiomyocytes was elevated in the FA intervention groups compared with the Hhc group.

To elucidate the protective mechanism of FA in Hhc-induced OS in the SHR model, we also measured the protein expression level of Nrf2 and HO-1. Western blot analysis showed that FA treatment significantly enhanced the Hhc-induced decrease in HO-1 protein ex-
Figure 2. FA prevented Hhcy-induced cardiomyocyte interstitial and perivascular collagen deposition. A, B: Representative pictures of the myocardial tissue sections stained with Masson trichrome showing interstitial and perivascular collagen deposition. C, D: Quantitative analysis of the cardiac collagen volume fraction (CVF) and the ratio of the perivascular collagen area to the luminal area (PVCA/LA). *P < 0.05 versus control group; #P < 0.05 versus Hhcy group. Vertical bars represent SE.

Table II. Parameters Measured by Echocardiogram

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Hhcy</th>
<th>Hhcy + LFA</th>
<th>Hhcy + HFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEDD (mm)</td>
<td>6.09 ± 0.96</td>
<td>6.12 ± 0.38</td>
<td>6.07 ± 0.67</td>
<td>6.10 ± 0.43</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.97 ± 1.22</td>
<td>3.28 ± 1.06</td>
<td>3.13 ± 0.27</td>
<td>3.12 ± 0.49</td>
</tr>
<tr>
<td>IVSD (mm)</td>
<td>2.23 ± 0.35</td>
<td>2.36 ± 0.76</td>
<td>2.28 ± 0.41</td>
<td>2.32 ± 0.44</td>
</tr>
<tr>
<td>IVSS (mm)</td>
<td>3.21 ± 0.61</td>
<td>3.41 ± 0.27</td>
<td>3.23 ± 0.42</td>
<td>3.28 ± 0.39</td>
</tr>
<tr>
<td>LVPWD (mm)</td>
<td>2.24 ± 0.41</td>
<td>2.60 ± 0.15</td>
<td>2.39 ± 0.37</td>
<td>2.40 ± 0.55</td>
</tr>
<tr>
<td>LVPWS (mm)</td>
<td>3.36 ± 0.67</td>
<td>3.43 ± 0.26</td>
<td>3.41 ± 0.29</td>
<td>3.44 ± 0.32</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>80.23 ± 8.54</td>
<td>76.93 ± 9.30</td>
<td>79.88 ± 7.81</td>
<td>78.17 ± 5.82</td>
</tr>
<tr>
<td>FS (%)</td>
<td>0.53 ± 0.11</td>
<td>0.47 ± 0.05</td>
<td>0.51 ± 0.06</td>
<td>0.48 ± 0.08</td>
</tr>
<tr>
<td>E/A ratio</td>
<td>1.79 ± 0.35</td>
<td>1.41 ± 0.22*</td>
<td>1.79 ± 0.19*</td>
<td>1.63 ± 0.31*</td>
</tr>
</tbody>
</table>

LVEDD indicates left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; IVSD, intraventricular septum thickness in diastole; IVSS, intraventricular septum thickness in systole; LVPWD, left ventricular posterior wall thickness in diastole; LVPWS, left ventricular posterior wall thickness in systole; LVEF, left ventricular ejection fraction; and FS, fractional shortening. Values are means ± SE (n = 8), *P < 0.05, compared with the control group. **P < 0.05, compared with the Hhcy group.

expression (Figure 4D and E, P < 0.05). The lower nuclear Nrf2 and higher cytoplasm Nrf2 expression were observed in response to Hhcy intervention. FA significantly upregulated the level of nuclear Nrf2 protein and reduced the level of cytoplasmic Nrf2 protein in a concentration-dependent manner compared with the Hhcy group (Figure 4D and E, P < 0.05), suggesting that FA promoted nuclear translocation of Nrf2 expression. At the same time, we found that the nuclear and cytoplasmic Nrf2 protein and the total HO-1 protein expression were statistically different in the Hhcy + HFA and Hhcy + LFA groups (P < 0.05). The overall results suggest that FA reduces Hhcy-
induced OS in SHR cardiac tissue by promoting nuclear transport of Nrf2 and activating the Nrf2/HO-1 pathway.

Discussion

In this study, considering the high incidence of Hhcy combined with hypertension in China, an animal model was established by intraperitoneal injection of DL-Hcy for 12 weeks to investigate the therapeutic effect and mechanism of FA lowering Hhcy level on cardiac fibrosis and diastolic function in Hhcy-related hypertension.

In the early stage of cardiac remodeling, it can be manifested as increased myocardial fibers, and fibrous collagen is the most common type of collagen. The inhibition of collagen can significantly reduce ventricular remodeling.23) In our study, Masson staining of SHR heart tissue demonstrated that Hhcy promoted changes and disorders in the structure of the interstitial and perivascular collagen networks, which is consistent with the results of animal studies24) and clinical findings.25) However, the FA intervention reversed the interstitial and perivascular fibrous collagen deposition. Cardiac fibrosis can lead to impaired diastolic function. Tissue Doppler echocardiography showed that the E/A ratio was the best echocardiographic parameter for evaluating diastolic function. We found that Hhcy aggravated diastolic dysfunction in SHRs, which tended to improve after the FA intervention. It is well known that diastolic dysfunction occurs earlier in patients with hypertension as opposed to systolic dysfunction. Studying the efficacy and mechanism of FA in the treatment of Hhcy-related hypertensive cardiac damage is of clinical relevance and could lead to novel pharmacological interventions.

In previous studies, there were significant differences in FA doses used for Hhcy intervention, with different FA doses producing different results.13,14,26) To clarify the effects of different doses of FA on Hhcy-related cardiac injury in SHRs, the LFA and HFA groups were established. Remarkably, our findings indicated that HFA did not show any additional benefit from LFA, either in reducing cardiac fibrosis or improving diastolic dysfunction. There have also been studies showing that HFA did not have more benefit in measuring vascular reactivity in atherosclerotic subjects.27) We examined the BP of four SHR groups at three time periods and found that FA did not affect the BP of the rats. We speculated that FA affected cardiac fibrosis and diastolic function through other factors independent of BP.

Subsequently, we measured the activity of serum MDA and SOD related to OS. In our study, the activity of the defense molecule SOD in the serum of the Hhcy group was lower than that in the control group. The FA treatment increased SOD activity, whereas MDA showed an opposite trend. The effect of FA on the reduction of Hhcy level and inhibition of OS was consistent with previous studies.16) Based on the important role of Nrf2 in mammalian cellular defense system, especially in OS regulation,28,29) our research focused on the Nrf2/HO-1 defense pathway. HO-1, a member of the heat-shock protein family, is an important antioxidant, anti-inflammatory, and cellular protective enzyme regulated by the activation of the major transcription factor Nrf2.30,31) Previous studies have shown that HO-1 plays a key role in regulating ROS.32) During excessive OS, Nrf2 underwent nuclear translocation and induced the transcription of several antioxidant genes, including SOD subtype, NQO1, and HO-1. Nrf2 pathway plays an important role in heart protection. It has been demonstrated that astaxanthin protects ochratoxin a-induced OS and apoptosis in the heart via the Nrf2 pathway.33) Resveratrol protects against chronic intermittent hypoxia-induced myocardial injury by also targeting Nrf2.34) Therefore, to explore the possible mechanism of FA to alleviate Hhcy-induced cardiac fibrosis and diastolic dysfunction, we detected the changes of Nrf2 and HO-1 genes using PCR, immunohistochemistry, and western blot; they were used to detect the changes of Nrf2 and HO-1 protein expression in the nucleus and cytoplasm of the four groups. In this study, we found that FA evidently promoted Nrf2 translocation from the cytoplasm to the
nucleus and activated the HO-1 expression to upregulate the antioxidant enzyme activity. At the same time, we found that, compared with the Hhcy + LFA group, the HFA treatment promoted more Nrf2 protein migration from the cytoplasm to the nucleus and promoted HO-1 activation; the difference was statistically significant. Considering the fact that HFA had no redundant benefits in inhibiting cardiac fibrosis and diastolic function in SHRs with Hhcy, we hypothesized that FA had certain limits in improving cardiac fibrosis and diastolic dysfunction in hypertensive rats with Hhcy through the Nrf2/HO-1 pathway.

Previous epidemiological studies have shown that elevated plasma Hcy levels are popular in the general population and are associated with increased risk of hypertension, cardiovascular disease, and stroke.\(^35,36\) FA supplementation improves arterial endothelial function in adults with relative Hhcy, with potentially beneficial effects on the atherosclerotic process.\(^37\) Enalapril maleate and FA tablets reduced Hcy levels in hypertensive rats with Hhcy and alleviated endoplasmic reticulum stress and vascular remodeling.\(^38\) In our study, FA (0.4 and 4 mg/kg/day) improved Hhcy-induced anomalies in SHR hearts, including the decline in cardiac diastolic function and cardiac interstitial and perivascular collagen accumulation. However, a high dose of FA did not improve Hhcy-aggravated cardiac fibrosis and diastolic dysfunction in SHRs compared with a low dose.

In summary, this study established a model of Hhcy and hypertensive rats and confirmed that Hhcy combined with hypertension can enhance cardiac fibrosis and decrease diastolic function. FA alleviates Hhcy-induced hypertensive cardiac fibrosis and diastolic dysfunction independent of BP by promoting the translocation of Nrf2 from the cytoplasm to the nucleus and by activating Nrf2/HO-1 pathway. However, HFA treatment provides no additional benefit, providing evidence for LFA treatment of hypertension associated with Hhcy.

**Disclosure**

**Conflicts of interest:** The authors declare that they have no conflict of interest.

**Author contributions:** Ping Cao and Wangmeng Zhang conducted research and wrote and revised the manuscript; Xuan Zhao, Guicheng Wang, and Ning Gao performed the experiments and collected data; Zhen Liu collected and
analyzed data; and Rui Xu designed the study, contributed to the interpretation of results, provided supervision, and revised the manuscript.

**Ethical approval:** All animal studies were performed in accordance with the guide for the Care and Use of Laboratory Animals of the National Institutes of Health (eighth edition, 2011). The animal use protocol was approved by the Committee on the Ethics of Animal Experiments of Shandong University.

**References**

34. Sun ZM, Guan P, Luo LF, et al. Resveratrol protects against CHF-induced myocardial injury by targeting Nrf2 and blocking...