Endogenous hydrogen sulfide and ERK1/2-STAT3 signaling pathway may participate in the association between homocysteine and hypertension

Lin SHI¹, Xiao-Yun LIU¹, Zhi-Gang HUANG¹,², Zhi-Yi MA¹, Yang XI¹, Lu-Yan WANG¹, Ning-Ling SUN¹,#
¹Hypertension Research Laboratory, Department of Cardiology, Peking University People’s Hospital, Beijing, China
²Emergency Department, Peking University Shenzhen Hospital, Shenzhen, China

Abstract

Background Homocysteine (Hcy) is a risk factor for hypertension, although the mechanisms are poorly understood. Methods We first explored the relationship between Hcy levels and blood pressure (BP) by analyzing the clinical data of primary hypertensive patients admitted to our hospital. Secondly, we explored a rat model to study the effect of Hcy on blood pressure and the role of H2S. An hyperhomocysteinemia (HHcy) rat model was induced to explore the effect of Hcy on blood pressure and the possible mechanism. We carried out tissue histology, extraction and examination of RNA and protein. Finally, we conducted cell experiments to determine a likely mechanism through renin-angiotensin-aldosterone system (RAAS) and extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway. Results In primary hypertensive inpatients with HHcy, blood pressure was significantly higher as compared with inpatient counterparts lacking HHcy. In the rat model, blood pressure of the Wistar rats was significantly increased with increases in serum Hcy levels and decreased after folate treatment. Angiotensin converting enzyme 1 (ACE1) expression in the Wistar Hcy group was enhanced comparing to controls, but was decreased in the Wistar folate group. Angiotensin II receptor type 1 (AGTR1) levels in the kidney tissue increased in the Wistar folate group. Both serum H2S and kidney cystathionine γ-lyase decreased with elevated levels of serum Hcy. In vitro, increased concentrations and treatment times for Hcy were associated with increased expression of collagen type 1 and AGTR1. Both serum H2S and kidney cystathionine γ-lyase decreased with elevated levels of serum Hcy. This dose and time dependent response was also observed for p-STAT3 and p-ERK1/2 expression. Conclusion Endogenous H2S might mediate the process of altered blood pressure in response to changes in serum Hcy levels, in a process that is partly dependent on activated RAAS and ERK1/2-STAT3 signaling pathway. Keywords: Angiotensin converting enzyme 1; Blood pressure; ERK1/2-STAT3 signaling pathway; Homocysteine; Hydrogen sulfide

1 Introduction

Homocysteine (Hcy) is a thiol-containing, non-protein, amino acid that is generated during nucleic acid methylation and demethylation of methionine. Hcy has emerged as a new independent risk factor for peripheral arterial disease, hypertension and coronary heart disease. Hcy levels increase significantly with age, and hyperhomocysteinemia (HHcy) prevalence has been reported to be higher in the elderly than in other age groups. Animal experiments have shown that Hcy accelerates endothelial damage and atherosclerosis by inducing inflammation, endoplasmic reticulum stress, vascular remodelling and by other mechanisms. Hcy is a precursor for endogenous hydrogen sulfide (H2S) generation. H2S, a crucial signaling molecule that has been shown to relax blood vessels and reduce inflammation; moreover, H2S also regulates a number of diverse physiological processes that include atherosclerosis, hypertension, lipid metabolism, diabetes, and neuromodulation through physiological processes like inflammation. Cystathionine γ-lyase (CSE) is the key enzyme that catalyzes H2S production in the kidney, liver, vascular smooth muscle and cardiovascular system. A number of studies have shown that H2S has a key role in regulating blood pressure (BP), and by doing so, effects the development of hypertension. However, whether high levels of Hcy increase blood pressure remains controversial and the extent that H2S participates in this process is unclear. It has been shown that activation of outer membrane fibroblasts and increased collagen synthesis participated in vascular remodelling in the process of atherosclerosis, hypertension, vascular injury, and in other diseases.
tiating the membrane lipid peroxidation chain reaction, Hcy is able to damage vascular endothelial cells, can promote the proliferation of smooth muscle cells, and can promote increased collagen synthesis and decrease vascular smooth muscle cell metabolism by a variety of ways.\(^{[12–14]}\) The renin-angiotensin-aldosterone system (RAAS) is important in the pathogenesis and development of vascular remodelling. HHcy has been shown to aggravate aneurysm formation that is induced by angiotensin II.\(^{[15]}\) The MAPK/ERK signaling pathway plays an important role in a variety of growth and differentiation-inducing pathways, wherein ERK signaling is a core component of the pathway.\(^{[16]}\) Activation of the ERK1/2 signal pathway could promote vascular smooth muscle cells to proliferate, to damage endothelial cells, and promote atherosclerosis.\(^{[17]}\)

The possible association of Hcy with hypertension has been studied comparatively less intensively. Herein, we tried to examine the hypothetical mechanisms of a putative association between Hcy and hypertension in the clinical and laboratory settings. Firstly, we explored the relationship between Hcy levels and blood pressure (BP) by analyzing the clinical data of primary hypertensive patients admitted to our hospital. Secondly, we explored a rat model to study the effect of Hcy on blood pressure and the role of H\(_2\)S. Finally, we conducted cell experiments to determine a likely mechanism through renin-angiotensin-aldosterone system (RAAS) and extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway in RNA and protein level.

2 Methods

2.1 Correlation of HCY and BP for inpatients presenting with primary hypertension

In this part we analyzed the clinical data of primary hypertensive inpatients to explore the relationship between Hcy levels and blood pressure. Patients with primary hypertension that who were continuously hospitalized in the hypertension ward at our hospital from January 2016 to November 2017 were included. Patients were hospitalized for an unsatisfied control of BP under current treatment or newly discovered high blood pressure. Inclusion criteria included two points: (1) inpatients whose measurements of blood pressure find systolic blood pressure (SBP) \(\geq 140\) mmHg and (or) diastolic blood pressure (DBP) \(\geq 90\) mmHg for three times on different days without using of antihypertensive drugs; and (2) patient has a history of hypertension and is currently taking drugs to lower blood pressure, even if the patient’s current blood pressure is lower than 140/90 mmHg he/she still should be diagnosed with hypertension and be enrolled. Exclusion criteria included secondary hypertension, serious valvular heart disease, acute coronary syndrome, severe liver or kidney dysfunction, NYHA heart function defined as class III or more, malignant tumors, severe infections or non-infectious inflammatory disease states, thyroid hyperfunction, autoimmune disease, severe electrolyte disorders, pregnancy or lactation, and trauma, surgery or stroke within the last three months. Enrolled patients were classified into a non-HHcy group and an HHcy group according to their plasma Hcy levels. In this study, the diagnostic criterion for HHcy was an Hcy \(\geq 15\) \(\mu\)mol/L, which is the same as the recommended standard in the new guidelines.\(^{[18]}\) Information with regard clinical history, medical use and physical examination was collected on admission. In addition, the Helsinki Declaration guidelines were strictly implemented. The biochemical indices and other test results were provided by the corresponding department. Hcy was detected by cyclic enzyme assay with automatic biochemistry analyzer with the intent of inducing HHcy.\(^{[19–21]}\) In addition, both groups were fed an eight-week period. Rats in the control group were fed a regular diet, and those in the Hcy group were fed a methionine-rich diet with 1.7% L-methionine with the intent of inducing HHcy.\(^{[19–21]}\) In addition, both groups were fed for an eight-week period. Rats in the folate group were fed a methionine-rich diet for eight weeks to induce HHcy, and were then placed on a methionine-and-folate diet containing 1.7% L-methionine and 0.008% folate for an additional four weeks as therapy.\(^{[22]}\) The animal experiment protocols were approved by the local Animal Experimental Ethical Review Committee of the People’s Hospital of Peking University. Blood pressure measurements were obtained and recorded for all rats in each group at baseline and during the final week of the study using a non-invasive tail blood pressure measuring instrument. Rats were conscious and undisturbed during the measurement process.

When animal experiments were concluded, rats were narcotized and maintained under isoflurane in a specific pathogen-free environment. Rat kidneys and aortas were
isolated and separated into parts. After anesthetization, blood samples were collected from the heart of each rat with a disposable sterilized syringe. Hydrogen sulfide in the serum was determined using a sensitive sulfur electrode device that was provided by Peking University First Hospital. Serum Hcy was detected using specific ELISA kits. Analysis of serum was carried out following the corresponding manufacturer’s instructions. Aortas were collected and then fixed in 4% paraformaldehyde and embedded in paraffin. Each paraffin block was cut into sections of 4 μm. Gamma cystathionase rabbit polyclonal antibody was obtained from the Proteintech Group. A rabbit enhanced polymer detection system, and diaminobenzidine were applied in the immunohistochemical staining of CSE sections. Staining was performed following the manufacturer’s instructions. Areas of interest were documented and examined using Image J.

Total RNA in kidney tissue was isolated using the HiPure Total RNA Mini Kit and reverse transcribed using the Revert Aid First Strand cDNA Synthesis Kit. Real-time PCR was performed and a melting curve was used to detect non-specific products. The fold increases in target gene expression were calculated with the 2^(-ΔΔCT) approach. RIPA buffer was used to extract total protein in kidney tissue after liquid nitrogen grinding. Western immunoblotting was used to estimate protein expression of ACE1 and AGTR1. Anti-angiotensin converting enzyme 1 antibody was obtained from Abcam PLC. Rabbit anti-GAPDH polyclonal antibody, AGTR1 rabbit polyclonal antibody, and HRP goat-anti-rabbit IgG antibody were obtained from Proteintech Group, Inc. Enhanced chemiluminescence Western Blotting substrate was used to visualize the immunoreactive bands in a rapid chemical optical imaging system by the Image Quant 350. Band densities were measured using Image J software.

2.3 The mechanisms of vascular remodeling induced by Hcy in adventitial fibroblasts of rat

Male Sprague-Dawley rats were raised for one week to adapt to the environment, following which, rats were narcotized and thoracic aorta were isolated. The paste-tissue pieces method was used to culture the original generation of adventitia fibroblasts that were isolated from the thoracic aorta. Generation 3-5 AF cells were used for the following experiment: AFs were divided into four groups: (1) the control group cells were cultured without Hcy; (2) in the Hcy group, AFs were dose-dependently stimulated with Hcy (i.e., at 50, 100, and 200 μmol/L) and time-dependently (i.e., at 12, 24, and 48 h). For the Hcy + U0126 group, AFs were pretreated with U0126 (at 10 μmol/L) for 30 min and then stimulated by Hcy (at 100 μmol/L) for 24 h. U0126 is a specific inhibitor of ERK1/2. For the DMSO group, AFs were treated with DMSO (at 10 μmol/L) for 24 h. Subsequent expression of collagen type 1, AGTR1, p-ERK1/2, p-STAT3 and t-ERK1/2 were measured by RT-PCR and Western immunoblotting.

2.4 Data analysis

SPSS version 22.0, GraphPad Prism 7 and ELISAcalc were utilized for statistical analysis. The one sample Kolmogorov-Smirnov (KS statistic) measured whether the data was distributed normally. Next, the Student’s t test, one-way analysis of variance (ANOVA), LSD-t tests, Mann-Whitney U test, Kruskal-Wallis test, and Chi-square test, Pearson Chi-square test and Fisher’s exact test were employed for comparative analyses of the data. Correlation analysis of two sets of non-normally distributed continuous numerical variable data was carried out by measuring Spearman’s rank correlation. Covariance analysis was used to test for difference between two or more adjusted means to limit the influence of covariates that might have affected the dependent variables and which could not be artificially controlled in ANOVA. In all tests, an alpha value of P < 0.05 was considered statistically significant.

3 Results

3.1 Correlation of homocystein and blood pressure for inpatients presenting with primary hypertension

We report that 620 inpatients with primary hypertension that met the criteria were included in this study. The base-lines of the non-HHcy group and the HHcy group are shown in Table 1. In this part of the study, we found 185 patients in the HHcy group and stratified to approximately 29.84% of our patients in total. The mean age was 61.23 ± 11.15 years for the non-HHcy group and 63.22 ± 11.86 years for the HHcy group. There were also significant statistical differences in gender composition when comparing the non-HHcy and the HHcy groups (P < 0.001). We also found differential smoking habits between both groups, which might be due to the variable gender composition.

Comparison of blood pressure levels between the non-HHcy group and the HHcy group showed that blood pressure measurements in the HHcy group were significantly higher than that found in the non-HHcy group with comparative SBP outcomes giving blood pressure measurements of 152 (140, 166) vs. 145 (130, 160); P = 0.004, and the DBP outcomes giving measurements of 80 (80, 98), 88 (80, 98), P = 0.010 (Table 2). When accounting for covariates that included gender, age, blood glucose levels, blood glucose levels, blood glucose levels, blood glucose levels.
Table 1. The baselines of the non-HHcy group and the HHcy group.

| Demographics                  | Non-HHcy group (n = 435) | HHcy group (n = 185) | P value |
|-------------------------------|--------------------------|----------------------|---------|
| Age, yrs                      | 61.23 ± 11.86            | 63.22 ± 11.15        | 0.666   |
| Male                          | 163 (37.5%)              | 135 (73.0%)          | < 0.001*|
| Smoking                       | 95 (25.3%)               | 71 (47.0%)           | < 0.001*|
| Body mass index, kg/m²        | 26.62 ± 4.08             | 27.08 ± 4.49         | 0.219   |
| Heart rate, beats/min         | 72.84 ± 9.42             | 73.27 ± 9.71         | 0.614   |
| Coronary heart disease        | 34 (7.8%)                | 16 (8.6%)            | 0.728   |
| Diabetes                      | 113 (26.0%)              | 42 (22.7%)           | 0.389   |
| Hyperlipidemia                | 171 (40.0%)              | 89 (48.4%)           | 0.056   |
| Medication                    |                          |                      |         |
| ACE inhibitor or ARB          | 239 (54.9%)              | 85 (45.9%)           | 0.040*  |
| Calcium antagonists           | 264 (60.7%)              | 119 (64.3%)          | 0.394   |
| β-blocker                     | 162 (37.2%)              | 60 (32.4%)           | 0.253   |
| o-blocker                     | 15 (3.4%)                | 11 (5.9%)            | 0.156   |
| Statin                        | 190 (43.7%)              | 82 (44.3%)           | 0.882   |
| LVEF                          | 72.37% ± 5.87%           | 72.17% ± 5.35%       | 0.712   |

Blood glucose status

| FBG, mmol/L                       | 5.43 (4.91, 6.32) | 5.36 (4.74, 5.97) | 0.535   |
| HbA1c (%)                         | 5.90 (5.60, 6.50) | 5.80 (5.50, 6.10) | 0.787   |

*P < 0.05, **P < 0.01. Data presented were n (%) or mean ± SD for symmetric variables, or median (25th, 75th centiles) for skewed variables. The tests performed were Student’s t test for symmetric variables, Mann-Whitney U test for skewed variables, and Chi-square test or Fisher exact test for categorical variables. ACE: angiotensin-converting enzyme; ARB: angiotensin II receptor blocker; FBG: fasting blood glucose; HbA1c: glycosylated hemoglobin; HHcy: hyperhomocysteinemia; LVEF: left ventricular ejection fraction.

Table 2. Comparison of blood pressure levels between the non-HHcy group and the HHcy group.

| Blood pressure, mmHg | Non-HHcy group (n = 435) | HHcy group (n = 185) | Z value | P value |
|----------------------|--------------------------|----------------------|---------|---------|
| SBP                  | 145 (130, 160)           | 152 (140, 166)       | −2.917  | 0.004***|
| DBP                  | 88 (80,98)               | 90 (80,100)          | −2.593  | 0.010** |

*P < 0.05, **P < 0.01. Data presented were levels of blood pressure, median (25th, 75th centiles) for skewed variables. The test performed was Mann-Whitney U test for skewed variables. DBP: diastolic blood pressure; HHcy: hyperhomocysteinemia; SBP: systolic blood pressure.

Hcy in patients with grade 3 hypertension was higher compared with patients in normal, high normal and grade 1 hypertension category, *P < 0.05, **P < 0.01. Data presented was median (25th/75th centiles) for skewed variables. The test performed was Mann-Whitney U test for skewed variables. Hcy: homocysteine.

Figure 1. Linear regression analysis on relationship between logarithm of blood pressure and Hcy level. Hcy: homocysteine; SBP: systolic blood pressure.

Hcy in patients with grade 3 hypertension was higher compared with patients in normal, high normal and grade 1 hypertension category, *P < 0.05, **P < 0.01. Data presented was median (25th/75th centiles) for skewed variables. The test performed was Mann-Whitney U test for skewed variables. Hcy: homocysteine.
presenting with primary hypertension gradually increased with increases in the plasma Hcy level. Since gender, smoking, HDL-C levels, creatinine and eGFR might affect Hcy levels, so we placed these data into the covariate factor for partial correlation analysis between arterial systolic blood pressure and plasma Hcy levels. After controlling for these covariates, we found that there remained a significant correlation between SBP and Hcy levels ($P = 0.015$).

Blood lipid levels for both the non-HHcy group and the HHcy group were measured by determining the levels of low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides, and total cholesterol (Table 4). Statin use was compared between both groups since it can lower total cholesterol and LDL-C levels in the blood. The results showed no statistical difference in statin use for both groups. LDL-C values aligned to a normal distribution, and analyses from the Student’s $t$ test for skewed variables. HDL-C, triglycerides, and total cholesterol levels were non-normally distributed, and the results of the Mann-Whitney $U$ test showed that HHcy patients presenting with hypertension had lower levels of HDL-C than non-HHcy patients; i.e., 1.11 (0.95, 1.26) vs. 1.13 (1.00, 1.31); $Z = -2.636$ ($P = 0.008$).

Renal function status was measured by measuring serum creatinine and the estimated glomerular filtration rate (eGFR) and urinary microalbumin levels. The results of the statistical analysis are shown in Table 5. Serum creatinine levels in the HHcy group were significantly higher than that shown for the non-HHcy group. Further, the HHcy group had a significantly reduced eGFR and increased microalbumin as compared the non-HHcy group.

### 3.2 Association of Hcy with BP in rat models and its possible mechanisms

At baseline, the three groups of Wistar rats had similar SBP ($F = 0.455, P = 0.641$). At the end of the study, Wistar rats in the Hcy group had a higher SBP than did the control group; additionally, SBP was lowest in the folate group (Table 6, $F = 3.956, P = 0.038$). A higher SBP was found in the Wistar Hcy group while the SBP in the Wistar folate group decreased to those levels found in the control group—an observation that indicated elevated serum Hcy levels being associated with an increased blood pressure.

Similarly, for the SHR rats, at the end of the study, a high SBP was observed in the control and Hcy groups, and a lower SBP was observed in the folate group ($F = 4.237, P = 0.028$, Figure 2). Mean SBP of the SHR folate group was found to have decreased by 17.12 mmHg as compared the SHR Hcy group ($t = 3.386, P = 0.010$). SBP for the SHR rats in the folate group was significantly decreased as compared with the Hcy group, which is in keeping with observations found in Wistar rats. Folate treatment lowered the SBP in Hcy hypertensive rats.

On concluding the animal studies, the mean serological values of Hcy for Wistar Hcy rats and SHR Hcy rats were 23.83 μmol/L and 24.05 μmol/L, respectively. A significant increase in Hcy was also shown in both Wistar Hcy rats ($t = 3.063, P = 0.028$) and SHR Hcy rats ($t = 2.525, P = 0.035$) and both animal groups developed HHcy after eight weeks of being fed the high methionine diet (Figure 3). Compared with the Wistar control group, serum hydrogen sulfide levels in the Wistar Hcy group decreased after being fed the methionine rich diet, and were increased on being treated with folate ($F = 4.928, P = 0.019$; Table 6). The Wistar Hcy group had decreased levels of serum-borne hydrogen sulfide than did the Wistar folate group ($t = -3.947, P = 0.002$; Figure 4). Minimal differences in serum hydrogen sulfide levels were found for the SHR groups, and none were statist-

### Table 4. Comparison of blood lipid levels between the non-HHcy group and the HHcy group.

| Lipid status | Non-HHcy group ($n = 435$) | HHcy group ($n = 185$) | $T$ value or $Z$ value | $P$ value |
|--------------|----------------------------|------------------------|-----------------------|-----------|
| LDL-C        | 3.16 ± 0.84                | 3.16 ± 0.90            | -0.026                | 0.979     |
| HDL-C        | 1.13 (1.00, 1.31)          | 1.11 (0.95, 1.26)      | -2.636                | 0.008**   |
| Triglycerides| 1.66 (1.13, 2.33)          | 1.63 (1.01, 2.58)      | -0.684                | 0.494     |
| Total cholesterol | 4.92 (4.18, 5.75) | 4.71 (4.05, 5.57) | -0.539                | 0.590     |

**$P < 0.01$. Data presented were levels of lipid, mean ± SD for symmetric variables, or median (25th, 75th centiles) for skewed variables. The tests performed were Student’s $t$ test for symmetric variables and Mann-Whitney $U$ test for skewed variables. HDL-C: high-density lipoprotein cholesterol; HHcy: hyperhomocysteinemia; LDL-C: low-density lipoprotein cholesterol.

### Table 5. Comparison of renal function in the two groups of patients.

| Renal function status | Non-HHcy group ($n = 435$) | HHcy group ($n = 185$) | $T$ value or $Z$ value | $P$ value |
|-----------------------|----------------------------|------------------------|-----------------------|-----------|
| Serum creatinine, mg/dL | 66.45 ± 18.16             | 90.49 ± 36.48          | -10.894               | < 0.001*  |
| eGFR, ml/min per 1.73 m² | 94.76 ± 17.12             | 80.57 ± 23.51          | 8.369                 | < 0.001*  |
| Microalbumin, mg/L    | 7.45 (2.9, 17.95)          | 13.65 (5.48, 36.8)     | -4.491                | < 0.0011  |

*$P < 0.001$. Data presented was mean ± SD for symmetric variables, or median (25th/75th centiles) for skewed variables. The tests performed were Student’s $t$ test for symmetric variables and Mann-Whitney $U$ test for skewed variables. eGFR: estimated glomerular filtration rate; HHcy: hyperhomocysteinemia.
Figure 2. Final SBP levels in all three groupings of Wistar and SHR rats. At the end of the study, Wistar rats in the Hcy group had a higher SBP than did the control group; additionally, SBP was lowest in the folate group ($F = 3.956, P = 0.038$). For SHR rats the folate group had the lowest SBP ($t = 3.386, P = 0.010$). For both types of rats, compared with the control group, SBP increased in the Hcy group and decreased after the addition of folic acid in the diet. $*P = 0.010$. Hcy: homocysteine; SBP: systolic blood pressure; SHR: spontaneously hypertensive rats.

Figure 3. Homocysteine levels at baseline and final for Wistar and SHR rats. On concluding the animal studies, a significant increase in homocysteine was shown in both the Wistar Hcy rats ($t = 3.063, P = 0.028$) and the SHR Hcy rats ($t = 2.525, P = 0.035$) and both groups developed hyperhomocysteinemia after eight weeks of being fed the high methionine diet, $P < 0.05$. Hcy: homocysteine; SHR: spontaneously hypertensive rats.

Table 6. Blood pressure and serum hydrogen sulfide in each grouping of rats.

| Group          | N  | Baseline SBP, mmHg | Final SBP, mmHg | Final H$_2$S, μmol/L |
|----------------|----|--------------------|-----------------|----------------------|
| Wistar-Control | 8  | 109.00 ± 9.37      | 124.00 ± 6.17   | 24.75 ± 6.73         |
| Wistar-Hcy     | 6  | 107.75 ± 9.05      | 144.67 ± 28.80  | 19.21 ± 3.01         |
| Wistar-Folate  | 7  | 104.31 ± 11.87     | 119.67 ± 11.31  | 27.85 ± 4.94         |
| SHR-Control    | 8  | 143.88 ± 17.25     | 171.67 ± 14.58  | 20.99 ± 2.02         |
| SHR-Hcy        | 8  | 149.71 ± 11.35     | 178.75 ± 14.00  | 18.96 ± 3.41         |
| SHR-Folate     | 8  | 134.13 ± 7.13      | 161.63 ± 3.31   | 16.50 ± 1.97         |

* Among the three Wistar groups, rats in the Hcy group had the highest final SBP and those in the folate group had the lowest final SBP, $P < 0.05$; ** Among the SHRs, rats in the folate group had the lowest final SBP, $P < 0.01$. Hcy: homocysteine; SBP: systolic blood pressure; SHR: spontaneously hypertensive rats.

Figure 4. Final hydrogen sulfide levels in the Wistar rat groups. Compared with the Wistar-control group, serum hydrogen sulfide levels in the Wistar Hcy group decreased after the 8-week methionine diet, and increased after the 4-week folate treatment. Hcy: homocysteine; $**P = 0.002$.

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Real-time PCR of CSE mRNA showed that for SHR rats, mRNA expression of CSE in kidney tissues isolated from the folate group was significantly enhanced ($F = 6.302, P = 0.008$; Figure 5). In Wistar rats, mRNA expression of CSE in the folate group was also enhanced, although the differences were not statistically significant ($F = 1.201, P = 0.322$; Figure 5). In Figure 5, lg2$\Delta$CT was calculated for each group to show CSE mRNA expression because the value determined by the 2$^{\Delta\Delta\text{CT}}$ approached gave values that exhibited too large a difference that could not be appropriately displayed in the figure. Immunohistochemical CSE staining of in aortic tissue from Wistar rats and SHRs displayed minimal differences (Figure 6). CSE was identified in the smooth muscle layer of the arterial wall; however, this was not found in the endothelial layer—an observation that was consistent with a previous study.[26] These results indicate enhanced CSE expression in the kidney tissue of rats in the folate group, which indicates an increase in H$_2$S production, whereas dampened CSE expression in kidney tissues might tentatively indicate decreased serum H$_2$S in HHcy rats.
Figure 5. Real-time PCR of CSE mRNA in rat kidney tissue in each group of Wistar and SHR rats. For SHR rats, expression of CSE mRNA in kidney tissue isolated from the folate group was significantly enhanced \((F = 6.302, P = 0.008)\), while in Wistar rats, mRNA expression of CSE in the folate group was also enhanced, although the difference was not statistically significant. CSE: cystathionine γ-lyase; SHR: spontaneously hypertensive rats.

Results of the Western immunoblotting assay for Wistar rats indicates that ACE expression in the Hcy group was enhanced as compared to the control group \((t = -4.206, P = 0.002;\) Figure 7) while in the folate group it was suppressed \((t = 2.453, P = 0.043;\) Figure 7). Western blot assays also indicated that the expression of AGTR1 was increased in the Wistar folate group as compared to the control group \((F = 3.810, P = 0.009;\) Figure 8). Additionally, the SHR Hcy group displayed dampened expression of AGTR1 as compared the Wistar Hcy group \((t = 2.910, P = 0.022;\) Figure 8). Expression of ACE was elevated in Wistar HHcy rats, and this might be associated with reduced levels of H\(_2\)S.

3.3 The mechanisms of vascular remodeling induced by Hcy in adventitial fibroblasts of rat

The expression of ACE mRNA was higher than that of the control group. Compared with the control group, the difference was statistically significant following 48 h of Hcy stimulation \((P < 0.05)\). AGTR1 mRNA expression of was higher than the control group; however, when compared with the control group, no significant statistical differences were found (Figure 9). The expression of ACE in fibroblasts increased gradually with increasing Hcy concentrations, although this was not statistically significant (Figure 10). With increases in the concentration and treated time of Hcy, we found that the expression of collagen type 1 and AGTR1 were both increased, and the maximal effect under these condition, appeared at a Hcy concentration of 100 \(\mu\)mol/L at 48 h of stimulation (Figure 11).

Figure 6. Immunohistochemical staining of rat aorta in all three groupings of Wistar (A–C) and SHR (D–E) rats. Immunohistochemical staining of rat aorta showed that expression of CSE was identified in the smooth muscle layer of the artery wall but not in the endothelial layer. The vascular smooth muscle layers in both Hcy groups were significantly thickened. The scale of the figure is 1: 100 \(\mu\)m. CSE: cystathionine γ-lyase; Hcy: homocysteine; SHR: spontaneously hypertensive rats.

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Figure 7. Expression of ACE in rat kidney tissue for Wistar and SHR groups. Expression of ACE in rat kidney tissue showed that for Wistar rats, compared to the control group, ACE expression in the Hcy group was enhanced ($t = -4.206$, $^*P = 0.002$) and in the folate group it was suppressed ($t = 2.453$, $^*P = 0.043$). For SHRs this trend was similar but the difference was not statistically significant. ACE: angiotensin-converting enzyme; SHR: spontaneously hypertensive rats.

Figure 8. Results of AGTR1/GAPDH levels using western blot for all groups of Wistar and SHR rats. Western blot revealed that compared to the Wistar groups the expression of AGTR1 in the corresponding SHR groups decreased. $^*P = 0.022$. AGTR1: angiotensin II receptor type 1; SHR: spontaneously hypertensive rats.

Figure 9. Expression of ACE mRNA (A) and AGTR1 mRNA (B) in fibroblasts stimulated by Hcy of 100 μmol/L for different times. Compared with the control group, $^*P < 0.05$. ACE: angiotensin-converting enzyme; AGTR1: angiotensin II receptor type 1; CTL: control.

Figure 10. The expression of ACE promoted by Hcy at different concentration of 0 μmol/L, 50 μmol/L, 100 μmol/L and 200 μmol/L. Control: the Hcy in the control group was 0 μmol/L. ACE: angiotensin-converting enzyme; Hcy: homocysteine.
Figure 11. With the increase of concentration (A) and treated time (B) of Hcy, the expression of collagen type 1 and AGTR1 increased, and the maximal effect appears at Hcy concentration of 100 μmol/L and at 48 h of stimulation. *P < 0.05. AGTR1: angiotensin II receptor type 1; Hcy: homocysteine.

the total expression levels of p-ERK1/2 and p-STAT3 across different groups were statistically significant (P < 0.001). As compared with the control group, p-ERK1/2 and p-STAT3 expression peaked at an Hcy concentration of 100 μmol/L (P < 0.05) and then gradually decreased (Figure 12). With an increased Hcy stimulation time, p-ERK1/2 and p-STAT3 expression gradually increased. When the Hcy stimulation time was 24 h, the expression of p-ERK1/2 reached its peak and the expression of p-STAT3 reached its peak at 48 h (Figure 13). In addition, the expression level of p-ERK1/2

Figure 12. With the increase of Hcy stimulation concentration, the expression levels of p-ERK1/2 and p-STAT3 were gradually increased, and the total expression of p-ERK1/2 (A) and p-STAT3 (B) in different groups were statistically significant. Compared with the control group, the expression of p-ERK1/2 and p-STAT3 reached the maximum at the Hcy concentration of 100 μmol/L and then gradually decreased. *P < 0.05. Hcy: homocysteine.
in the Hcy group was significantly higher than that found in the control group, and p-ERK1/2 expression in the U0126+ Hcy group was significantly lower than that found in both

the control ($P = 0.002$) and Hcy groups ($P = 0.006$; Figure 14). Expression of p-STAT3 in the Hcy group was clearly higher than that found in the control group. In the U0126+Hcy

**P** < 0.001). In U0126+Hcy group the expression of p-STAT3 was significantly lower ($^# P < 0.001$) than the Hcy group (panel B). Hcy: homocysteine.

Figure 14. The expression level of p-ERK1/2 in the Hcy group was significantly higher than that found in the control group, and the expression of p-ERK1/2 in the U0126+Hcy group was significantly lower than that in the control group ($^{**}P = 0.002$) and the Hcy group (panel A; $^*P = 0.006$); expression of p-STAT3 in Hcy group was obviously higher than that of the control ($^{***}P < 0.001$). In U0126+Hcy group the expression of p-STAT3 was significantly lower ($^# P < 0.001$) than the Hcy group (panel B). Hcy: homocysteine.
Figure 15. AGTR1 expression in the Hcy group was significantly higher than that in the control group, \( P < 0.05 \). The expression of AGTR1 in the U0126+Hcy group was not significantly different from the control group, but it was significantly lower than that in the Hcy group, \( P < 0.001 \). AGTR1: angiotensin II receptor type 1; Hcy: homocysteine.

In the Hcy group, the expression of p-STAT3 was significantly lower than found in the Hcy group (\( P < 0.001 \)). AGTR1 expression in the Hcy group was significantly higher than that in the control group. Finally, AGTR1 expression of in the U0126+Hcy group was not significantly different from that of the control group; however, it was significantly lower than that found in the Hcy group (Figure 15).

4 Discussion

Our study suggests that elevated level of Hcy may cause elevated blood pressure, hydrogen sulfide is involved in the process and this effect may be realized through the RAAS and ERK pathways. Through clinical observations we found that high levels of Hcy might be associated with increased blood pressure levels. A cross-sectional study of the northeastern region of China showed that adults in rural areas had a higher prevalence of HHcy; moreover, HHcy might represent a risk factor for hypertension, especially for male subjects.\(^{[25]}\) In a cross-sectional study that was reported by Cohen, et al.,\(^{[26]}\) significant differences were found for Hcy levels when comparing the data derived from 9237 male subjects and 4353 female subjects with a calculated mean value of 12.6 \( \mu \)mol/L and 9.6 \( \mu \)mol/L, respectively. In the HHcy group, 73.0% of the patients were male, which suggests that males might indeed present with a higher risk of HHcy. Analyses also revealed that HDL-C was significantly reduced in the HHcy group. HDL-C can promote nitric oxide synthesis, following which, endothelial function is enhanced. In this study, it was found that damaged renal function was related to elevation of Hcy levels. Depressed renal function revealed that patients with primary hypertension and poor renal function tended to develop HHcy, or that elevated levels of Hcy provoked early renal damage in hypertensive patients. In chronic kidney disease or renal insufficiency, reduced excretion of sulfur-containing amino acids can increase Hcy levels.\(^{[29]}\) In these conditions, early interventions are advised in an attempt to prevent early kidney damage and thereby lower the risk of HHcy.

Hcy inactivates proteins by homocysteinylation at an elevated level, including that of CSE,\(^{[30]}\) which catalyzes the production of \( \text{H}_2\text{S} \) in the kidney and cardiovascular system.\(^{[6,7]}\) A key reason for increased expression levels of Hcy is thought to be an overloaded intake of methionine or an inability to metabolize.\(^{[31]}\) A high-methionine diet of approximately 1.7% methionine is utilized in the induction of HHcy in rats, and a diet supplemented with 0.008% folate is effective in decreasing free levels of Hcy.\(^{[19,22]}\) In our study on SHR rats, SBP in the control group was already at a set-point higher level at base-line, and thus the Hcy group failed to exhibit any further elevation in SBP. While SBP for the folate group significantly decreased, which was concordant with the results obtained for the folate group of Wistar rats, this outcome suggested that HHcy might exert a pathogenic effect in the setting of hypertension.

Hydrogen sulfide is an endogenous vasorelaxant that activates \( \text{K}_{\text{ATP}} \) channels, resulting in smooth muscle relaxation.\(^{[26]}\) Zhao, et al.\(^{[26]}\) investigated blood pressure changes in rats, and provided persuasive evidence for a vasorelaxant effect of \( \text{H}_2\text{S} \) following intravenous bolus injection. Our results showed that production of \( \text{H}_2\text{S} \) was suppressed following heightened levels of Hcy. Moreover, serum \( \text{H}_2\text{S} \) levels increased after folate treatment. Given these observations, we can assume that down-regulation of CSE expression in the kidney is possible following reduced serological levels of \( \text{H}_2\text{S} \) in HHcy rats. In a research reported by Perna, et al.,\(^{[32]}\) it was concluded that CSE expression was significantly down-regulated and \( \text{H}_2\text{S} \) was negatively correlated with Hcy and cysteine levels. In this study, elevated Hcy levels suppressed mRNA expression of CSE and decreased serum \( \text{H}_2\text{S} \) that resulted in gradually increased blood pressure that could be counteracted by folate treatment. Hydrogen sulfide also acts directly on ion channels of smooth muscle cells and reacts with metal ions to implement vasorelaxation. In an ex-vivo study of human endothelial cells, observations showed that \( \text{H}_2\text{S} \) interacted with the active zinc center of ACE and inhibited enzymic activity.\(^{[33]}\)
Results from our study found that elevated H2S levels in the Wistar folate group together with depressed ACE expression, served to support the notion of the above-mentioned idea. Reduced activation of the RAAS system that was induced by HHcy, might decrease synthesis of angiotensin hormones and dampen the increase in blood pressure, which was manifested as decreased arterial systolic blood pressure in the folate group.

Vascular remodeling is an important pathophysiological process in the development of hypertension, involving vascular smooth muscle cell proliferation and increases in the extracellular matrix. In the current study, pathological sections of rat aortic rings showed that vascular smooth muscle layers in both Hcy groups were significantly thickened. Following folic acid intervention, the thickness of the vascular smooth muscle layer decreased. Furthermore, high level of Hcy inhibited H2S generation, and provoked a weakened inhibitory effect of H2S on vascular smooth muscle cells proliferation. A prior study found that CSE expression in human aortic smooth muscle cells (HASMCs) increased significantly after adenoviral transduction of HASMCs, and found increased H2S production, inhibited cell growth, and promotion of cellular apoptosis. Authors concluded that CSE induced apoptosis by activating the ERK and p38 MAPK signaling pathways. However, in previous work, our team found that in a balloon injury rat model, HHcy significantly upregulated AGTR1 expression in the impaired carotid artery. Thus, we carried out further cell biological studies and found increased AGTR1, p-STAT3 and p-ERK1/2 expression in arterial fibroblasts that was both Hcy concentration- and time-dependent. U0126 could significantly suppress AGTR1, p-STAT3 and p-ERK1/2 expression, which indicated that Hcy might enhance fibroblastic AGTR1 expression in the tunica adventitia of the rat aorta, and did so via the ERK1/2 - STAT3 signaling pathway.

In conclusion, in this study, we found that high levels of Hcy might be associated with increased blood pressure levels. Elevated Hcy levels suppressed mRNA expression of CSE and decreased serum H2S that resulted in gradually increased blood pressure that could be counteracted by folate treatment. Activation of the RAAS system and vascular remodeling that was induced by HHcy, may lead to increase of blood pressure. Hcy might enhance fibroblastic AGTR1 expression in the tunica adventitia of the rat aorta, and did so via the ERK1/2 - STAT3 signaling pathway.

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