Homocysteine impairs coronary artery endothelial function by inhibiting tetrahydrobiopterin in patients with hyperhomocysteinemia

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He L, Zeng H, Li F, Feng J, Liu S, Liu J, Yu J, Mao J, Hong T, Chen AF, Wang X, Wang G. Homocysteine impairs coronary artery endothelial function by inhibiting tetrahydrobiopterin in patients with hyperhomocysteinemia. Am J Physiol Endocrinol Metab 299: E1061–E1065, 2010. First published September 21, 2010; doi:10.1152/ajpendo.00367.2010.—Hyperhomocysteinemia (HHcy) has been associated with impaired vascular endothelial function. Our previous study demonstrated significantly higher secretion of the chemokine monocyte chemoattractant protein-1 from monocytes in response to lipopolysaccharide in patients with HHcy. In the present study, we investigated whether coronary endothelial function was damaged in patients with chronic HHcy (plasma level of homocysteine >15 μmol/l) and, if so, whether this impaired endothelial function is induced by the uncoupling of endothelial nitric oxide synthase (eNOS). When tetrahydrobiopterin levels are inadequate, eNOS is no longer coupled to L-arginine oxidation, which results in reactive oxygen species rather than nitric oxide production, thereby inducing vascular endothelial dysfunction. The 71 participants were divided into two groups, control (n = 50) and HHcy (n = 21). Quantification of coronary flow velocity reserve (CFVR) was after rest and after adenosine administration done by noninvasive Doppler echocardiography. Plasma levels of nitric oxide and tetrahydrobiopterin were significantly lower in patients with HHcy than in controls (37.68 ± 13.79 vs. 50 ± 15.60 μmol/l, all P < 0.05). Furthermore, CFVR was significantly lower in the HHcy than the control group (2.76 ± 0.49 vs. 3.09 ± 0.52, P < 0.05). In addition, plasma level of homocysteine was negatively correlated with CFVR. Chronic HHcy may contribute to coronary artery disease by inducing dysfunction of the coronary artery endothelium. The uncoupling of eNOS induced by HHcy in patients with chronic HHcy may explain this adverse effect in part.

nitric oxide

Hyperhomocysteinemia (HHcy) is an independent risk factor for coronary artery disease (CAD) (2). The possible mechanism of accelerated vascular disease in HHcy includes endothelial cell damage, vascular endothelial dysfunction, and enhanced oxidative stress (4, 17). Increasing evidence suggests that HHcy may adversely affect CAD by decreasing vascular endothelial function. In healthy individuals, homocysteine (Hcy) has been associated with impaired coronary microvascular dilator function (16). In models of acute methionine-induced HHcy, endothelium-dependent dilation was significantly inhibited in parallel with elevated plasma Hcy levels (1). HHcy induces NADPH oxidase activity, thus contributing to increased reactive oxygen species (ROS) production in rat coronary vessels (19).

Nitric oxide (NO) protects against CAD through anti-inflammatory and antioxidant effects. Loss of NO bioavailability is a key feature of endothelial dysfunction preceding the appearance of atherosclerosis. Tetrahydrobiopterin (BH4) is an essential cofactor for NO synthase (NOS). When BH4 levels are inadequate, endothelial NOS (eNOS) is no longer coupled to L-arginine oxidation, which results in ROS rather than NO production, thereby inducing vascular endothelial dysfunction (15). Administration of BH4 improves endothelial function in diabetic patients and patients with or without CAD (9–11). Hattori et al. (6) showed that oral administration of BH4 slowed the progression of atherosclerosis in apolipoprotein E (apoE<sup>−/−</sup>) knockout mice. The association of Hcy and endothelial dysfunction depends largely on its damaging effect on eNOS coupling (12). HHcy induces eNOS uncoupling in cultured endothelial cells (8). Topal et al. (18) reported that the oxidative stress and inhibition of NO release induced by Hcy depend on eNOS uncoupling due to reduced BH4 availability. Acute HHcy induced by methionine load impaired coronary circulation in diabetic patients (3). However, the long-term effects of chronic HHcy on coronary artery endothelial function remain uncertain.

Our previous study demonstrated that Hcy promoted the expression of monocyte chemoattractant protein-1 (MCP-1) and enhanced the production of ROS and the activity of NADPH oxidase in primary cultured human monocytes. MCP-1 secretion from isolated monocytes was significantly enhanced in response to lipopolysaccharide in patients with HHcy (20, 22). We also observed that Hcy-induced ROS upregulated the expression and translocation of redox factor 1 via NADPH oxidase, which accelerated the development of atherosclerosis in HHcy apoE<sup>−/−</sup> mice (4). Our clinical study showed that plasma levels of Hcy and MCP-1 were significantly increased, and superoxide dismutase (SOD) levels were reduced in patients with acute coronary syndrome (21). Coronary flow velocity reserve (CFVR) assessed by transthoracic Doppler echocardiography is an effective method to evaluate CFVR and indicate coronary endothelial function (7). HHcy may induce endothelial dysfunction in animals and patients; however, the long-term effects of chronic HHcy on coronary
artery endothelial function and its potential mechanism remain uncertain.

In the present study, we investigated whether coronary endothelial function was damaged in patients with chronic HHcy and, if so, whether this impaired endothelial function is induced by the uncoupling of eNOS.

**MATERIALS AND METHODS**

**Subjects.** Patients were selected from the cardiovascular internal medicine department at Peking University Third Hospital from October 2007 to March 2009. We enrolled 71 participants, including 50 healthy controls, and 21 patients with HHcy (plasma level of Hcy >15 μmol/l). Patients with acute myocardial infarction, CAD (>50% stenosis as shown on angiography), heart failure, renal function impairment, liver function impairment, systemic inflammatory disease, infectious disease, cancer, or a serious illness or who were undergoing nitrates or vitamin treatment that would affect their participation were excluded.

**Study design.** The study participants were divided into two groups, healthy controls (n = 50) and patients with HHcy (n = 21). All patients underwent coronary Doppler echocardiography after rest and after adenosine treatment. Fasting blood samples were drawn for analysis of clinical chemistry and Hcy, NO, BH4, and SOD levels. Plasma samples were stored at −70°C for further analysis.

All subjects gave their written informed consent. This study was approved by the Ethics Committee of the Health Science Center, Peking University.

**BH4 measurement.** For the measurement of total biopterin, 50 μl of 1 M trichloroacetic acid was added to 200 μl plasma, and the mixture was centrifuged at 20,000 g for 15 min at 4°C. The supernatant (170 μl) was transferred to an amber tube, and 25 μl of iodine solution was added. After mixing and standing for 60 min in the dark at room temperature, excess iodine was reduced by the addition of 10 μl of 1% ascorbic acid solution. For the indirect measurement of BH4, 10 μl of 6 M sodium hydroxide and 25 μl of iodine solution were added to the deproteinized plasma. Samples were mixed and incubated for 60 min in the dark. The reaction was terminated by adding 10 μl of 6 M hydrochloric acid and 10 μl of 1% ascorbic acid solution. Samples oxidized under acidic or alkaline conditions were centrifuged at 20,000 g for 10 min at 4°C; the supernatant, at 90 μl, was injected into the column by use of a high-performance liquid chromatography system with an autosampler and a fluorescence detector (Agilent 1100). A Hypersil C18 column (4.6 × 250 mm, 5 μm) was used for separation of biopterin with a mobile phase of ratio of methanol to water (5:95, vol/vol) running at a flow rate of 1.0 ml/min. The retention time of biopterin was ~8 min, and the excitation and emission wave lengths were 350 and 440 nm, respectively. Compounds were quantitated by their peak height compared with external standards. BH4 concentration, expressed as picomols per milliliter, was calculated by subtracting BH2 + B from total biopterin.

**The assessment of coronary artery endothelial function.** Assessment by transthoracic Doppler echocardiography (TTDE) is a effective method to evaluate CFVR (7). Coronary artery endothelial function was assessed by the measurement of CFVR, which was evaluated by TTDE (Vivid 7 Dimension; GE). The peak coronary flow velocities in the distal left anterior descending coronary artery were recorded at rest and during hyperemia after intravenous infusion of adenosine (0.14 mg·kg⁻¹·min⁻¹) for 2 min in all subjects. All patients were fasting and in resting condition during the examination. CFVR was calculated by the formula CFVR = peak coronary flow velocity during hyperemia/peak coronary flow velocity at rest.

**Table 1. Baseline characteristics and metabolic parameters of control subjects and patients with HHcy**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Group (n = 50)</th>
<th>HHcy Group (n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>55.12 ± 12.71</td>
<td>52.90 ± 13.23</td>
</tr>
<tr>
<td>Body mass index</td>
<td>25.64 ± 2.80</td>
<td>26.23 ± 3.08</td>
</tr>
<tr>
<td>Sex (males/females)</td>
<td>33/17</td>
<td>15/6</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>Hypertension (no.)</td>
<td>23</td>
<td>11</td>
</tr>
<tr>
<td>Diabetes (no.)</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Metabolic parameters, mmol/l</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>5.34 ± 0.88</td>
<td>5.45 ± 1.00</td>
</tr>
<tr>
<td>HDL</td>
<td>1.06 ± 0.24</td>
<td>1.04 ± 0.22</td>
</tr>
<tr>
<td>LDL</td>
<td>2.75 ± 0.71</td>
<td>3.03 ± 0.80</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.95 (1.40−3.86)</td>
<td>1.86 (1.38−3.93)</td>
</tr>
<tr>
<td>Fasting plasma glucose</td>
<td>5.90 ± 1.00</td>
<td>5.60 ± 0.70</td>
</tr>
<tr>
<td>PWV, cm/s</td>
<td>1.550 ± 272</td>
<td>1.463 ± 267</td>
</tr>
<tr>
<td>hs-CRP, mg/l</td>
<td>1.24 (0.60−2.88)</td>
<td>1.41 (0.49−2.24)</td>
</tr>
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<td>Hypertension (no.)</td>
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Data are means ± SD unless indicated otherwise. HHcy, hyperhomocysteinemia; PWV, pulse wave velocity; hs-CRP, high-sensitivity C-reactive protein. Triglyceride and hs-CRP levels are given as median and range (range is in parentheses).
Pulse wave velocity measurement. Arterial stiffness was assessed by measurement of automatic brachial-ankle pulse wave velocity (PWV) by use of the Colin VP-1000 apparatus. The basic principle of PWV assessment is that pressure pulse generated by ventricular ejection is propagated along the arterial system at a speed determined by the elasticity of the arterial wall. Knowing the distance and pulse transit time, the velocity can be calculated. Subjects were placed in a recumbent position and, after a 10-min rest, underwent brachial-ankle PWV measurement.

Laboratory measurements. Blood samples were taken in the morning after an overnight fast and collected into vacuum tubes containing EDTA for the measurement of plasma lipid and lipoprotein levels. Total cholesterol, high-density lipoprotein cholesterol, and triglyceride levels were analyzed by colorimetric enzymatic assays with use of an autoanalyzer (Hitachi 7170). Low-density lipoprotein cholesterol level was calculated. Fasting plasma Hcy, glucose, high-sensitivity C-reactive protein (hs-CRP), fasting insulin, and hemoglobin A1c levels were determined at the central chemistry laboratory of Peking University Third Hospital.

Level of NO was measured by the use of ELISA kits according to the manufacturer’s protocols (Jiamay Biotech). The plasma level of NO in the HHcy group and the control group was significantly lower in the HHcy group than in the control group (99.54 ± 32.23 vs. 119.50 ± 36.68 μmol/l, P < 0.05; Fig. 1B). Furthermore, we found that plasma Hcy level was negatively correlated with plasma NO level (r = −0.28, 95% confidence interval −0.51 to −0.011, P < 0.05; Fig. 2).

Plasma level of BH4 in the HHcy group and the control group. BH4 is an essential cofactor for NOS. When BH4 levels are inadequate, eNOS is no longer coupled to L-arginine oxidation, thereby inducing vascular endothelial dysfunction. Plasma BH4 level was significantly lower in the HHcy group than in the control group (1.43 ± 0.41 vs. 1.73 ± 0.52 pmol/ml, P < 0.05; Fig. 1C).

RESULTS
Baseline clinical characteristics of the study participants. The clinical characteristics of study participants are summarized in Table 1. The control and HHcy groups did not differ significantly in age, sex, body mass index, smoking, or prevalence of hypertension and hypercholesterolemia. The plasma level of Hcy was significantly higher in the HHcy group than in the control group (23.15 ± 10.12 vs. 11.30 ± 2.24 μmol/l, P < 0.0001; Fig. 1A).

Plasma level of NO in the HHcy group and the control group. Plasma NO level was significantly lower in the HHcy group than in the control group (99.54 ± 32.23 vs. 119.50 ± 36.68 μmol/l, P < 0.05; Fig. 1B). Furthermore, we found that plasma Hcy level was negatively correlated with plasma NO level (r = −0.28, 95% confidence interval −0.51 to −0.011, P < 0.05; Fig. 2).

Plasma level of BH4 in the HHcy group and the control group. Plasma NO level was significantly lower in the HHcy group than in the control group (99.54 ± 32.23 vs. 119.50 ± 36.68 μmol/l, P < 0.05; Fig. 1B). Furthermore, we found that plasma Hcy level was negatively correlated with plasma NO level (r = −0.28, 95% confidence interval −0.51 to −0.011, P < 0.05; Fig. 2).

Coronary flow velocity reserve (CFVR) was decreased significantly in HHcy patients compared with the control group. Values are expressed as means ± SD; n = 46 in control group and 15 in HHcy group.

Statistical analysis. The differences between groups were analyzed by Student’s t-test. Proportions were analyzed by chi-square test. PWV and CFVR values and plasma levels of Hcy, NO, BH4, SOD, cholesterol, and glucose are expressed as means ± SD. Because triglyceride and hs-CRP values do not follow a normal distribution, plasma levels of triglycerides and hs-CRP are given as medians and ranges. P < 0.05 (2-tailed) was considered significant.
Positive correlation between plasma levels of NO and BH4. NO is generated mainly by eNOS, and BH4 is a cofactor for eNOS. We tested the correlation between plasma level of NO and BH4 in patients with or without HHcy. Plasma NO level was positively correlated with plasma BH4 level ($r = 0.33, 95\%$ confidence interval $0.05–0.561, P < 0.05$; Fig. 3).

**Plasma level of SOD in the HHcy group and the control group.** Plasma SOD may serve as an indicator of the balance between the damaging effects of HHcy and the clear capability of superoxide. However, plasma level of SOD did not differ between the HHcy and control groups ($5.06 \pm 0.94$ vs. $5.29 \pm 1.25$ U/ml).

**PWV values in the HHcy group and the control group.** Arterial stiffness can be assessed by measuring PWV. To investigate whether arterial stiffness was damaged by HHcy, PWV was measured in all subjects. The HHcy and control groups did not differ in PWV values ($1,463 \pm 267$ vs. $1,550 \pm 272$ cm/s; Table 1).

**CFVR values in the HHcy group and the control group.** CFVR is an effective method to evaluate coronary endothelial function. In total, 61 patients underwent assessment of CFVR by TTDE. CFVR value was significantly impaired in the HHcy group compared with the control group ($2.76 \pm 0.49$ vs. $3.09 \pm 0.52, P < 0.05$; Fig. 4). These results suggest that HHcy may contribute to CAD by inducing dysfunction of endothelium.

Negative correlation between plasma level of Hcy and CFVR values. To test whether Hcy levels were related to impaired coronary endothelial function, we tested the correlation between plasma level of Hcy and CFVR in patients with or without HHcy. Hcy level was negatively correlated with CFVR values ($r = -0.34, P < 0.01, 95\%$ confidence interval $-0.54$ to $-0.096$; Fig. 5).

**DISCUSSION**

HHcy has been associated with impaired vascular endothelial function. Our study demonstrates that coronary artery endothelial function was significantly impaired in patients with chronic HHcy. In addition, the plasma levels of NO and BH4 were positively correlated, and both of them were significantly decreased in HHcy patients compared with controls. Finally, plasma levels of NO and CFVR were negatively correlated with plasma level of Hcy. Chronic HHcy may induce dysfunction of the coronary artery endothelium through uncoupling eNOS, as seen by the low levels of NO and BH4.

Although the precise mechanisms are still not well understood, a number of studies of humans and animals have shown HHcy to cause vascular endothelial dysfunction that leads to CAD mainly by increasing oxidative stress and attenuating NO bioavailability. In acute methionine-induced HHcy, endothelium-dependent dilation was significantly inhibited in parallel with elevated plasma Hcy level (1). Hcy exposure impaired NO bioavailability and agonist-stimulated NO release through oxidant stress (5, 14). A recent study showed that the association of Hcy with endothelial dysfunction depends largely on its damaging effect on eNOS coupling (12). The oxidative stress and inhibition of NO release induced by Hcy depend on eNOS uncoupling because of reduced BH4 availability (18). Furthermore, Hcy could inhibit platelet eNOS activity, which results in the reduction of platelet NO level and thus stimulates platelet aggregation (13). Our previous study showed significantly enhanced activity of NADPH oxidase and MCP-1 secretion from isolated monocytes in patients with HHcy (20, 22). Hcy-induced ROS upregulate the expression and translocation of redox factor 1 via NADPH oxidase, which accelerates the development of atherosclerosis in HHcy apoE$^{-/-}$ mice (4). Our clinical study showed that plasma level of Hcy was significantly increased and SOD level reduced in patients with acute coronary syndrome (21).

We hypothesized that chronic HHcy could decrease coronary artery endothelial function by inducing eNOS uncoupling. We demonstrated that Hcy significantly decreased plasma levels of NO and BH4 and impaired coronary artery endothelial function, as measured by CFVR in patients with chronic HHcy. Moreover, the plasma level of Hcy was negatively correlated with CFVR in these patients. Given that CFVR is important in assessing coronary artery endothelial function, inhibiting CFVR by Hcy might have disadvantageous effects in patients with chronic HHcy. A previous study showed that acute HHcy induced by methionine load significantly impaired coronary circulation in patients with type 2 diabetes (3). Our present study demonstrated the long-term disadvantageous effects of chronic HHcy on coronary artery endothelial function in patients. However, patients and controls did not differ in plasma level of SOD. Perhaps plasma level of SOD cannot completely reflect oxidase stress in the local vasculature.

In conclusion, HHcy may contribute to coronary artery endothelial dysfunction and atherosclerosis by decreasing the activity of NO and inducing uncoupling of eNOS in patients with chronic HHcy.

**GRANTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

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