Demonstration of Rapid Onset Vascular Endothelial Dysfunction After Hyperhomocysteinemia
An Effect Reversible With Vitamin C Therapy

John C. Chambers, MRCP; Andrew McGregor, RGN; Jeff Jean-Marie; Omar A. Obeid, PhD; Jaspal S. Kooner, MD, FRCP

**Background**—Hyperhomocysteinemia is a major and independent risk factor for vascular disease. The mechanisms by which homocysteine promotes atherosclerosis are not well understood. We hypothesized that elevated homocysteine concentrations are associated with rapid onset endothelial dysfunction, which is mediated through oxidant stress mechanisms and can be inhibited by the antioxidant vitamin C.

**Methods and Results**—We studied 17 healthy volunteers (10 male and 7 female) aged 33 (range 21 to 59) years. Brachial artery diameter responses to hyperemic flow (endothelium dependent), and glyceryl trinitrate (GTN, endothelium independent) were measured with high resolution ultrasound at 0 hours (fasting), 2 hours, and 4 hours after (1) oral methionine (L-methionine 100 mg/kg), (2) oral methionine preceded by vitamin C (1 g/day, for 1 week), and (3) placebo, on separate days and in random order. Plasma homocysteine increased (0 hours, 12.8±1.4; 2 hours, 25.4±2.5; and 4 hours, 31.2±3.1 μmol/l, P<0.001), and flow-mediated dilatation fell (0 hours, 4.3±0.7; 2 hours, 1.1±0.9; and 4 hours, −0.7±0.8%) after oral L-methionine. There was an inverse linear relationship between homocysteine concentration and flow-mediated dilatation (P<0.001). Pretreatment with vitamin C did not affect the rise in homocysteine concentrations after methionine (0 hours, 13.6±1.6; 2 hours, 28.3±2.9; and 4 hours, 33.8±3.7 μmol/l, P=0.27), but did ameliorate the reduction in flow-mediated dilatation (0 hours, 4.0±1.0; 2 hours, 3.5±1.2 and 4 hours, 2.8±0.7%, P=0.02). GTN-induced endothelium independent brachial artery dilatation was not affected after methionine or methionine preceded by vitamin C.

**Conclusions**—We conclude that an elevation in homocysteine concentration is associated with an acute impairment of vascular endothelial function that can be prevented by pretreatment with vitamin C in healthy subjects. Our results support the hypothesis that the adverse effects of homocysteine on vascular endothelial cells are mediated through oxidative stress mechanisms. (Circulation. 1999;99:1156-1160.)

**Key Words:** endothelium ▪ arteriosclerosis ▪ antioxidants ▪ nitric oxide ▪ blood flow

Hyperhomocysteinemia is a major and independent risk factor for vascular disease, and venous thrombosis. Homocysteine concentrations are elevated in up to 30% of patients with atherosclerosis, and levels only 12% above the upper limit of normal (15 μmol/L, mild hyperhomocysteinemia), are associated with a 3-fold increase in the risk of acute myocardial infarction. Homocysteine concentrations are determined by genetic and nutritional factors. Mutations in the genes for enzymes involved in homocysteine metabolism and deficiencies of vitamins B₆, B₁₂, and folic acid are associated with hyperhomocysteinemia. The mechanisms by which hyperhomocysteinemia promotes atherosclerosis are not fully understood. High homocysteine levels may cause endothelial damage, affect platelet function and coagulation factors, and promote LDL oxidation. Increasing evidence suggests that homocysteine may exert these effects through an action on the endothelium. In children with severe hyperhomocysteinemia and in adults with moderate hyperhomocysteinemia, chronically elevated homocysteine concentrations are associated with impaired flow-mediated endothelium-dependent vasodilatation. However, these studies have been unable to clarify whether endothelial dysfunction is caused by elevated homocysteine or occult atherosclerotic disease. To separate these effects, we studied the vascular endothelial responses to acutely elevated homocysteine concentrations in healthy human subjects. To investigate if homocysteine impairs endothelial function through increased oxidative stress mechanisms and can be inhibited by the antioxidant vitamin C.

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**Conclusions**—We conclude that an elevation in homocysteine concentration is associated with an acute impairment of vascular endothelial function that can be prevented by pretreatment with vitamin C in healthy subjects. Our results support the hypothesis that the adverse effects of homocysteine on vascular endothelial cells are mediated through oxidative stress mechanisms. (Circulation. 1999;99:1156-1160.)

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stress, we have studied the effects of homocysteine on vascular endothelial responses before and after treatment with vitamin C.

Materials and Methods

Subjects
Seventeen healthy volunteers (10 male and 7 female) mean age 33 (range 21 to 59) years were recruited from hospital staff. All were normotensive with normal serum cholesterol and had no previous history of diabetes or vascular disease. Eight of the 17 subjects were cigarette smokers. All subjects abstained from smoking on the day of the study. None were taking medications. All subjects gave informed and written consent. The study was approved by the local ethics committee.

Methods
Studies were performed on 3 separate days, in random order, and at least 2 weeks apart. Brachial artery diameter responses to hyperemic flow (endothelium dependent) and glyceryltrinitrate (GTN, endothelium independent) were measured at 0 hours (fasting), 2 hours, and 4 hours after (1) oral methionine (L-methionine 100 mg/kg, the metabolic precursor of homocysteine), (2) oral methionine preceded by vitamin C (1 g/day orally for 1 week), and (3) placebo (methionine-free fruit juice). L-methionine (Scientific Hospital Supplies) was administered in diluted, chilled fruit juice (25 mg methionine per mL orange juice) to mask its flavor.

Brachial Artery Diameter
Studies were performed after an overnight fast in subjects supine and at rest. Room temperature ranged from 21°C to 24°C. Brachial artery flow-mediated dilatation was measured with a 7.0 MHz linear array transducer, an Acuson 128XP/10 system, and a high resolution ultrasonic vessel wall tracking system (Vadirec, Ingenious Systems) as described by Celermajer et al.13 The brachial artery was scanned longitudinally and a stereotactic clamp was used to hold the transducer in the same position throughout the procedure. The transmit (focus) zone was set to the depth of the near wall of the artery. Depth and gain settings were set to optimize images of the lumen-arterial wall interface. The images were magnified by a resolution box function and measurements were taken from the anterior to posterior "m" line at end diastole by the use of the R wave on the ECG. Brachial artery diameter was measured by identifying a clear section of the vessel on B-mode. The M-mode cursor was then placed over this point at right angles to the vessel wall. A 5-second segment of the A-mode signal was then routed to the wall tracking system designed to track vessel wall movement on a beat to beat basis. The minimal arterial diameter was calculated from the distance between opposite lumen-arterial interfaces, as identified by manual selection of the maximal change in recorded radio frequency amplitude.

After the baseline resting scan, a pneumatic cuff, placed at the level of the wrist, was inflated to 300 mm Hg for 4.5 minutes. The second scan was performed 55 to 65 seconds after cuff deflation. Fifteen minutes were allowed for vessel recovery, after which the second baseline scan was performed. GTN (400 µg) was then administered and the fourth scan of the brachial artery was undertaken. The vessel diameter was measured by 2 independent observers unaware of the subjects’ clinical details, the type, and stage of the study. Repeat measurements in individuals by the use of this technique are consistent and reproducible.14 Flow-mediated dilatation of conduit arteries is endothelium dependent and largely mediated by nitric oxide.15

Biochemical Measurements
Blood samples for glucose, total cholesterol, HDL cholesterol, triglycerides, total plasma homocysteine, red-cell folate, and serum B12 were collected at 0 hours. Additional samples for plasma homocysteine were collected at 2 hours and 4 hours during the study. Aliquots were placed on ice, centrifuged within 1 hour, and the separated plasma stored at −20°C before assays. Lipid profiles were determined by the use of an Olympus AU800 multichannel analyzer, vitamin B12, and red cell folate by MEIA (Abbott IMX system), and total plasma homocysteine by high pressure liquid chromatography.16

Data Processing and Statistical Analysis
Data were analyzed by the use of SPSS version 8.0 statistical package. Continuous data were expressed as mean±SEM. Repeated measures ANOVA was used to examine the fixed effects of administration of methionine, vitamin C, and time on flow-mediated dilatation, GTN-induced dilatation, and plasma homocysteine. Linear regression equations relating flow-mediated dilatation to plasma homocysteine were calculated for each subject and were used to predict the mean flow-mediated dilatation for any homocysteine level and its 95% CI for the study population. Statistical significance was inferred at a P value of <0.05.

Results
Clinical and Biochemical Characteristics
Baseline clinical and biochemical measurements are summarized (Table 1). All subjects had normal blood pressure, fasting blood glucose, lipid profile, red cell folate, and serum B12.

Brachial Artery Flow-Mediated Dilatation and Methionine Loading
Plasma homocysteine concentrations increased after oral methionine compared with placebo (P<0.001, Table 2). Mean brachial artery flow-mediated dilatation fell rapidly after oral methionine, but not placebo (P<0.001, Figure 1, Table 2). Flow-mediated dilatation was strongly related to plasma homocysteine (P<0.001) with no independent effect of time. For each subject, a regression equation describing the relationship between flow-mediated dilatation and plasma homocysteine was generated. The fitted model predicting mean flow-mediated dilatation for the study population was described by the equation: flow-mediated dilatation=1.579−0.245×(plasma homocysteine−20.86), where the standard error of the intercept=0.638, standard error of the regression slope=0.045, and residual SD=8.607. In contrast, GTN-induced brachial artery dilatation after methionine and after placebo were not significantly different. There were no
significant differences in baseline brachial artery diameter between the 2 sets of studies.

The clinical correlates of fasting and postload homocysteine and of flow-mediated dilatation are presented in Table 3. Fasting homocysteine was associated with male sex and serum creatinine, and postload homocysteine with body mass index (BMI), fasting triglycerides, and diastolic blood pressure. Baseline flow-mediated dilatation was correlated with age and inversely correlated with baseline brachial artery diameter. Postload flow-mediated dilatation was inversely correlated with age, male sex, baseline brachial artery diameter, and creatinine, and positively correlated with vitamin B12.

Pretreatment with vitamin C did not significantly affect the increase in homocysteine concentrations after oral methionine (Table 2). However, the fall in flow-mediated dilatation after methionine was prevented by pretreatment with vitamin C (P<0.02, Figure 2).

The major findings of this study are that an acute elevation in homocysteine concentration is associated with rapid onset endothelial dysfunction, which can be prevented by pretreatment with vitamin C. Our results support the hypothesis that the adverse effects of homocysteine on vascular endothelial cells are mediated through oxidative stress mechanisms.

In this study, brachial artery flow-mediated dilatation was impaired within 2 hours of oral methionine. Previous studies indicate that brachial artery flow-mediated dilatation is endothelium dependent and is largely mediated by the release of nitric oxide. Our results therefore imply that endothelial nitric oxide activity may be impaired during acute hyperhomocysteinemia in normal human subjects. Regression analysis showed an inverse relationship between homocysteine concentration and flow-mediated dilatation. These findings are consistent with previous reports of a dose- and time-

**Discussion**

Homocysteine and Endothelial Dysfunction

**Table 2. Homocysteine and Brachial Artery Responses After Methionine, Methionine Preceded by Vitamin C, and Placebo**

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Methionine</th>
<th>Methionine + vitamin C</th>
<th>Placebo</th>
<th>Methionine</th>
<th>Methionine + vitamin C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocysteine, μmol/l</td>
<td>13.4±1.3</td>
<td>12.8±1.4</td>
<td>13.6±1.6</td>
<td>13.0±1.5</td>
<td>25.4±2.5</td>
<td>28.3±2.9</td>
</tr>
<tr>
<td>Flow-mediated dilatation, %</td>
<td>4.3±1.0</td>
<td>4.3±0.7</td>
<td>4.0±10</td>
<td>5.1±1.1</td>
<td>1.1±0.9</td>
<td>3.5±1.2</td>
</tr>
<tr>
<td>GTN-induced dilatation, %</td>
<td>19.9±1.36</td>
<td>20.5±1.7</td>
<td>21.4±2.0</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Baseline brachial diameter, mm</td>
<td>4.0±0.1</td>
<td>4.1±0.2</td>
<td>4.2±0.2</td>
<td>4.1±0.2</td>
<td>4.1±0.2</td>
<td>4.1±0.2</td>
</tr>
</tbody>
</table>

* indicates methionine compared to placebo; †, methionine compared to methionine preceded by vitamin C; repeated measures ANOVA.

**Figure 1.** Flow-mediated and GTN-induced dilatation at baseline and 4 hours after oral methionine.
dependent effect of homocysteine on endothelial cellular function and may help to explain the incremental risk of vascular events with increasing homocysteine concentrations. In this study, endothelial dysfunction was detected at homocysteine concentrations similar to those associated with increased risk of myocardial infarction and stroke. Our results extend previous observations of impaired endothelial function in children with cystathionine β-synthase deficiency and in elderly patients with low serum folate who have chronically elevated homocysteine concentrations. However, in both studies, it was not possible to distinguish whether endothelial dysfunction was caused by homocysteine or established atherosclerosis. Our findings of acute onset endothelial dysfunction after oral methionine support a role for homocysteine (and not structural arterial disease) in the observed vascular responses. Our results contrast with findings of preserved endothelial function in the obligate heterozygote parents of cystathionine β-synthase–deficient children, although total plasma homocysteine concentrations were not assessed in the latter study.

The mechanisms by which hyperhomocysteinemia evokes endothelial dysfunction are not well understood. In vitro studies show that initial exposure of cultured endothelial cells to homocysteine leads to the formation and release of nitric oxide, S-nitrosothiols, and S-nitrosohomocysteine, substances with potent vasodilator and platelet-inhibitor properties. However, with continued exposure the oxidative effects of homocysteine predominate (with the resultant generation

### Table 3: Clinical Correlates of Fasting and Post-Methionine Homocysteine Concentrations and Flow-Mediated Dilatation

<table>
<thead>
<tr>
<th></th>
<th>Correlation With Fasting Homocysteine</th>
<th>Correlation With Post-Load Homocysteine</th>
<th>Correlation With Baseline FMD</th>
<th>Correlation With Post-Load FMD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.20</td>
<td>0.46</td>
<td>0.07</td>
<td>0.79</td>
</tr>
<tr>
<td>Male gender</td>
<td>0.52</td>
<td>0.04</td>
<td>0.21</td>
<td>0.44</td>
</tr>
<tr>
<td>Smoking</td>
<td>−0.03</td>
<td>0.91</td>
<td>0.25</td>
<td>0.36</td>
</tr>
<tr>
<td>BMI</td>
<td>0.50</td>
<td>0.06</td>
<td>0.64</td>
<td>0.01</td>
</tr>
<tr>
<td>SBP</td>
<td>−0.05</td>
<td>0.85</td>
<td>0.19</td>
<td>0.49</td>
</tr>
<tr>
<td>DBP</td>
<td>0.05</td>
<td>0.84</td>
<td>0.58</td>
<td>0.02</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>0.26</td>
<td>0.33</td>
<td>0.38</td>
<td>0.15</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>0.30</td>
<td>0.25</td>
<td>0.46</td>
<td>0.07</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>−0.23</td>
<td>0.39</td>
<td>−0.41</td>
<td>0.11</td>
</tr>
<tr>
<td>Fasting triglycerides</td>
<td>0.37</td>
<td>0.17</td>
<td>0.57</td>
<td>0.02</td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>0.45</td>
<td>0.14</td>
<td>0.45</td>
<td>0.14</td>
</tr>
<tr>
<td>Fasting homocysteine</td>
<td>...</td>
<td>...</td>
<td>0.65</td>
<td>0.007</td>
</tr>
<tr>
<td>Red cell folate</td>
<td>−0.20</td>
<td>0.58</td>
<td>−0.14</td>
<td>0.71</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>−0.43</td>
<td>0.22</td>
<td>−0.58</td>
<td>0.09</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.63</td>
<td>0.01</td>
<td>0.40</td>
<td>0.13</td>
</tr>
<tr>
<td>Baseline FMD</td>
<td>−0.42</td>
<td>0.10</td>
<td>−0.03</td>
<td>0.91</td>
</tr>
<tr>
<td>Baseline brachial diameter</td>
<td>0.44</td>
<td>0.09</td>
<td>0.34</td>
<td>0.19</td>
</tr>
</tbody>
</table>

FMD indicates flow-mediated dilatation; other abbreviations as in Table 1.
of superoxide anion radicals and hydrogen peroxide leading to reduced production and/or inactivation of nitric oxide. Impaired availability of nitric oxide leaves the endothelium vulnerable to unopposed homocysteine-mediated oxidative damage. In the present study, pretreatment with vitamin C, an antioxidant that scavenges superoxide anion-radicals, prevented the decrease in flow-mediated dilatation after methionine. This finding suggests that oxidative stress mechanisms mediate endothelial dysfunction during hyperhomocysteinemia. Oxidative stress is a key factor in atherosclerosis. Generation of free-radical superoxide anions activates nitric oxide. Deactivation of nitric oxide, the major endothelium derived vasodilator, may lead to vasoconstriction, platelet aggregation, and monocye adhesion, all of which promote atherosclerosis. Our study does not exclude a direct effect of methionine or its related metabolites on endothelial function in our subjects. However, metabolites such as cysteine, present in plasma at a 3- to 4-fold greater concentration than homocysteine and capable of generating such radicals, prevented the decrease in flow-mediated dilatation after methionine. This finding suggests that oxidative stress mechanisms may lead to vasoconstriction, platelet aggregation, and monocyte adhesion, all of which promote atherosclerosis. Our study does not exclude a direct effect of methionine or its related metabolites on endothelial function in our subjects. However, metabolites such as cysteine, present in plasma at a 3- to 4-fold greater concentration than homocysteine and capable of generating superoxide, do not inhibit glutathione peroxidase, suggesting that homocysteine may have unique effects in mechanisms generating oxidative stress.

In summary, our results have shown that an elevation in homocysteine concentration is associated with an acute impairment of endothelial function that can be prevented by pretreatment with vitamin C. Our results support the hypothesis that the adverse effects of homocysteine on vascular endothelial cells are mediated through oxidative stress mechanisms.

Acknowledgments

This work was supported by a grant from the British Heart Foundation (PG/96193). We are grateful to Caroline Doré for statistical analysis.

References

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Circulation. 1999;99:1156-1160
doi: 10.1161/01.CIR.99.9.1156

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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