Physiological Increments in Plasma Homocysteine Induce Vascular Endothelial Dysfunction in Normal Human Subjects

John C. Chambers, Omar A. Obeid, Jaspal S. Kooner

Abstract—We hypothesized that physiological increments in plasma homocysteine after low-dose oral methionine or dietary animal protein induce vascular endothelial dysfunction and that there is a graded, inverse relationship between homocysteine concentration and endothelial function. We studied 18 healthy volunteers aged 18 to 59 years. Brachial artery flow-mediated and glyceryltrinitrate-induced dilatation were measured after 1) oral L-methionine (10, 25, and 100 mg/kg), 2) dietary animal protein (lean chicken 551 ± 30 g, comprising 3.2 ± 0.2 g methionine), and 3) methionine-free amino acid mix (100 mg/kg). Methionine (10, 25, and 100 mg/kg) induced a dose-related increase in homocysteine (9.4 ± 1.3 to 12.2 ± 2.1, 17.6 ± 2.6, and 26.1 ± 4.2 μmol/L, respectively; P < 0.001) and a reduction in flow-mediated dilatation (4.1 ± 0.8 to 2.1 ± 0.8, 0.3 ± 0.8, and −0.7 ± 0.8%, respectively; P < 0.001) at 4 hours. Compared with usual meal, animal protein increased plasma homocysteine (9.6 ± 0.8 to 11.2 ± 0.9 μmol/L, P = 0.005) and reduced flow-mediated dilatation (4.5 ± 0.7% to 0.9 ± 0.6%, P = 0.003). Methionine-free amino acid mix did not induce any changes. Glyceryltrinitrate-induced dilatation was unchanged throughout.

In this study, small physiological increments in plasma homocysteine after low-dose methionine and dietary animal protein induced vascular endothelial dysfunction. We propose that protein intake–induced increments in plasma homocysteine may have deleterious effects on vascular function and contribute to the development and progression of atherosclerosis. (Arteriosclerosis and Thrombosis. 1999;19:2922–2927.)

Key Words: homocysteine ■ endothelium ■ methionine ■ dietary protein

Hyperhomocysteinemia is an independent risk factor for peripheral vascular,1 cerebrovascular,2 and coronary artery3 disease. Elevated homocysteine concentrations are found in almost one-third of all patients with atherosclerosis4 and levels only 12% above the upper limit of normal (15 μmol/L) are associated with a 3-fold increase in the risk of acute myocardial infarction.3

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.5 Homocysteine is derived from the metabolism of the essential amino acid methionine. Methionine is found at greatest concentration in animal protein. In humans, dietary animal protein results in a transient rise in plasma homocysteine levels, which peaks at 8 hours and may persist for up to 24 hours.6 Previous studies have suggested animal protein intake may account for the day-to-day variation of up to 25% in plasma homocysteine.6

Increasing evidence suggests that the effects of elevated homocysteine are mediated through endothelial dysfunction. In children with cystathionine-β-synthase deficiency and severe hyperhomocysteinemia7 and in adults with moderate hyperhomocysteinemia,8,9 chronically elevated homocysteine concentrations are associated with impaired endothelium-dependent vasodilation. In primates, elevated homocysteine concentrations following a methionine-enriched diet for 4 weeks are associated with vascular endothelial dysfunction.10 Similarly, in normal human subjects, high-dose oral methionine (100 mg/kg), which increases plasma homocysteine by 3- to 4-fold, is accompanied by a reciprocal fall in brachial artery flow-mediated dilatation,11–13 an effect likely to be mediated through oxidative stress mechanisms.13

However, the major limitations of previous studies investigating the relationship between endothelial function and hyperhomocysteinaemia is that the homocysteine concentrations achieved have been at least 2- to 3-fold higher than normal.11–13 The vascular effects of physiological and diet-induced increments in plasma homocysteine have not been investigated previously. In this study, we have measured the effects of graded concentrations of homocysteine on brachial artery endothelium-dependent dilatation. To examine the physiological relevance of our findings, we have assessed vascular responses after dietary animal protein.
Methods

Eighteen healthy volunteers (11 male and 7 female), mean age 33 (range 18 to 59) years, were recruited from hospital staff. All were normotensive, with normal serum cholesterol, and with no previous history of diabetes or vascular disease. Six of the 18 subjects were active cigarette smokers, although their smoking pattern did not change during the course of the study. Smoking was not permitted on the study days. None were taking medications (including vitamin supplements or the oral contraceptive pill). All subjects gave informed and written consent. The study was approved by the local ethics committee.

Brachial artery flow-mediated dilatation (endothelium dependent) and glyceryltrinitrate-induced dilatation (glyceryltrinitrate [GTN], endothelium independent) were measured before and 4 hours after 1) oral L-methionine 10 mg/kg, 2) methionine 25 mg/kg, and 3) methionine 100 mg/kg. The measurements were extended to 8 and 24 hours during the high-dose methionine study. As a control, we performed studies after, 1) methionine-free amino-acid mix (100 mg/kg) and after, 2) diluted fruit juice alone (vehicle). To examine the physiological role of elevated homocysteine, we compared vascular responses after intake of lean chicken (551 ±30 g), comprising 3.2 ±0.2 g methionine, with changes after the usual meal. All studies were performed on separate days, in random order, and at least 14 days apart.

Brachial Artery Diameter

Studies were performed after an overnight fast with subjects supine and at rest. Room temperature ranged from 21°C to 24°C. Brachial artery flow-mediated dilatation was measured using a 7.0 MHz linear array transducer, an Acuson 128XP/10 system, and high resolution ultrasonic vessel wall tracking system (Vadirec), as described by Celermajer et al. The brachial artery was scanned longitudinally, and a stereotactic clamp 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 taken from the anterior to posterior “m” line at end diastole, using the R-wave on the electrocardiogram. 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 measured by identifying a clear section of the vessel on B-mode. The vessel 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 mcg) was then administered and the fourth scan of the brachial artery undertaken. The vessel diameter was measured by 2 independent observers unaware of the subjects clinical details and the type and stage of the study. The technique for measurement of brachial artery flow-mediated dilatation is reproducible in our laboratory. There was a close correlation between the observers for brachial artery measurements (diameter 0.99, dilatation 0.90). The coefficient of variation for flow-mediated dilatation was 2%, based on measurements taken from the same subjects on separate days, under resting conditions. This compares favorably with other centers. Flow-mediated dilatation of conduit arteries is endothelium-dependent and largely mediated by nitric oxide.

Nutritional Products

Oral methionine was administered as L-methionine (Scientific Hospital Supplies) in diluted fruit juice (25 mg methionine per mL orange juice). Methionine-free amino acid mix (Scientific Hospital Supplies) was used as an amino acid control to exclude a nonspecific effect of amino acid ingestion on vascular responses. Methionine-free amino acid mix was administered in diluted fruit juice at the same concentration as L-methionine (methionine free mix comprises L-Alanine 0.04 g, L-Arginine 0.07 g, L-Aspartic acid 0.06 g, L-Carnitine 0.001 g, L-Cystine 0.03 g, L-Glutamine 0.01 g, L-Histidine 0.04 g, L-Isoleucine 0.06 g, L-Leucine 0.10 g, L-Lysine 0.07 g, L-Phenylalanine 0.05 g, L-Proline 0.07 g, L-Serine 0.04 g, L-Threonine 0.05 g, L-Tryptophan 0.02 g, L-Tyrosine 0.05 g, L-Valine 0.07 g, and Taurine 0.001 g per g of mix).

Lean chicken was used as the dietary source of methionine. Subjects consumed 551 ±30 g chicken. This was chosen to provide a mean intake of 2 g methionine per subject. The intake of chicken was timed as a single evening meal between 8 PM and 10 PM and vascular measurements performed 12 hours later to coincide with the expected time of peak homocysteine concentration. Chicken was purchased fresh as breast meat and grilled to cook. No dietary restrictions were applied for the usual meal. All recipes were recorded as weighed intakes and analyzed using the commercially available package Microdiet.

Biochemical Measurements

Fasting glucose, total cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides, total plasma homocysteine, red-cell folate, and serum B12 were measured for each subject. Samples for plasma homocysteine were collected before each vascular study. Additional samples for glucose and lipids were taken before and after the meal studies. Aliquots were placed on ice, centrifuged within 1 hour, and the separated plasma stored at −20°C before assays. Lipid profiles were determined using an Olympus AU800 multichannel analyzer; B12 and red cell folate by MEIA (Abbott IMX system) and total plasma homocysteine by high pressure-liquid chromatography. For each subject, samples were analyzed in 1 batch. The within assay coefficient of variation for homocysteine was 4%.

Data Processing and Statistical Analysis

Data were analyzed using Statistical Products and Service Solutions (SPSS) version 8.0 statistical package. Continuous data were expressed as mean±SEM. The effects of methionine 10, 25, and 100 mg/kg, compared with placebo on flow-mediated dilatation, GTN-induced dilatation, brachial artery diameter, and plasma homocysteine, were investigated by repeated measures ANOVA. When significant effects were identified, the paired samples t test was used post-hoc to localize differences, with Bonferroni’s adjustment for multiple comparisons. Measurements after animal protein meal were compared with those after usual meal, using the paired samples t test. For each study, the linear regression equations relating flow-mediated dilatation to plasma homocysteine were calculated for each
subject and used to examine the presence of a relationship between flow-mediated dilatation and homocysteine in 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 in Table 1. All subjects were normotensive, with normal serum cholesterol, fasting blood glucose, red cell folate, and serum vitamin B12. Baseline flow-mediated dilatation was 4.2±0.7% and was reproducible in individual subjects (coefficient of variation 2%). Flow-mediated dilatation was not significantly lower in smokers compared with nonsmokers (4.6±0.7% versus 4.1±1.1%, $P=0.72$).

**Effects of Oral Methionine**

Oral methionine (100 mg/kg) induced a significant rise in plasma homocysteine at 2 hours (9.5±0.9 versus 26.1±4.2 μmol/L, $P=0.001$; Figure 1). The peak homocysteine response occurred at 8 hours (37.9±6.2 μmol/L), and levels returned towards normal, although they were significantly higher than baseline, at 24 hours (19.6±5.1 μmol/L, $P=0.05$). After methionine, flow-mediated dilatation was impaired by 2 hours (4.2±0.7% to 1.8±1.1%, $P=0.05$). The maximal fall in flow-mediated dilatation occurred at 4 hours (0.1±0.9%, $P=0.001$), and responses returned towards normal at 24 hours. There was an inverse relationship between plasma homocysteine and flow-mediated dilatation ($P=0.018$). For each subject, a regression equation describing their 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 following equation: flow-mediated dilatation = 5.05 – (0.14 × plasma homocysteine), where the standard error of the intercept = 0.99, and the standard error of the regression slope = 0.05. There were no changes in GTN-induced, endothelium-independent dilatation (20.0±1.8% to 18.6±1.4%, $P=0.41$) before and after methionine. Baseline brachial artery diameter (4.1±0.2 to 4.1±0.2 mm, $P=0.37$), basal brachial arterial flow (103±15 to 111±15 mL/min, $P=0.32$), and the hyperemic increase in brachial arterial flow (138±19% to 143±19%, $P=0.86$) were also unchanged before and after methionine.

Compared with baseline, methionine at doses of 10 mg/kg and 25 mg/kg increased plasma homocysteine (9.4±1.3 to 12.2±2.1 μmol/L, $P=0.007$ and 9.6±1.4 to 17.6±2.6 μmol/L, $P=0.001$, respectively) and reduced flow-mediated dilatation (4.1±0.8% to 2.1±0.8%, $P=0.05$ and 3.6±0.9% to 0.3±0.8%, $P=0.008$, respectively; Figure 2). Similar to after high-dose methionine study, vascular function was related to plasma homocysteine in the low-dose methionine studies. Flow-mediated dilatation was impaired after methionine in the 11 subjects in whom the increments in plasma homocysteine did not exceed the upper limit of normal (15 μmol/L, homocysteine 7.6±0.9 to 9.7±0.6 μmol/L, $P<0.001$; flow-mediated dilatation 5.3±1.1 to 19.8±0.8, $P=0.04$).

**Effects of Methionine-Free Amino Acid Mix and Fruit Juice Alone**

After methionine-free amino acid mix or following fruit juice alone (vehicle), there were no changes in plasma homocysteine (9.8±1.6 to 9.9±1.7 μmol/L, $P=0.81$ and 9.4±0.8 to 10.0±1.0 μmol/L, $P=0.18$, respectively) or flow-mediated dilatation (3.3±0.9% to 4.4±0.7%, $P=0.34$ and 4.4±1.1% to 4.4±1.0%, $P=0.98$, respectively), excluding a nonspecific effect of amino acids or fruit juice on the observed vascular changes.
Effects of Usual Meal and Intake of Animal Protein

Compared with usual meal, intake of animal protein, comprising 3.2±0.2 g methionine, was associated with an increase in plasma homocysteine (from 9.6±0.8 to 11.2±0.9 μmol/L, P=0.005; Figure 3) and fall in flow-mediated dilatation (4.5±0.7% to 0.9±0.6%, P=0.003; Figure 4). Flow-mediated dilatation was related to plasma homocysteine (P=0.05). Intake of animal protein was not accompanied by any significant change in total, LDL or HDL cholesterol, or triglycerides, although blood glucose was lower compared with usual meal (Table 2). Univariate analysis did not show any relationship between flow-mediated dilatation and blood glucose. Animal protein did not affect GTN induced dilatation, baseline brachial arterial diameter, basal brachial arterial blood flow, or the hyperemic increase in brachial artery blood flow (Table 2).

Nutritional analysis of meals confirmed that intake of total protein and methionine was higher in animal protein compared with usual meal (Table 2). Intakes of total energy and fat were similar and carbohydrate lower in animal protein than usual meal.

Discussion

We have shown that physiological increments of 2 to 3 μmol/L in plasma homocysteine after low-dose oral methionine and dietary animal protein induce vascular endothelial dysfunction. In this study, we observed an inverse and dynamic relationship between plasma homocysteine and endothelial function.

We found evidence of impaired flow-mediated dilatation within 2 hours of high dose oral methionine (100 mg/kg), the dietary precursor of homocysteine. Regression analysis showed an inverse relationship between homocysteine concentration and flow-mediated dilatation. Previous studies show that brachial artery flow-mediated dilatation is endothelium dependent and is largely mediated by the release of nitric oxide. Our findings therefore imply that vascular endothelial nitric oxide activity may be impaired during acutely elevated homocysteine concentrations. Previous stud-
ies have shown that high dose methionine, which elevates plasma homocysteine concentrations to more than 2- to 3-fold normal, is associated with endothelial dysfunction. However, in these studies, concentrations of plasma homocysteine have exceeded those encountered in the majority of patients with premature vascular disease. The vascular effects of physiological increments in plasma homocysteine have not been investigated previously. In this study, we administered methionine at lower doses to examine the effects of small increments in plasma homocysteine on the vascular endothelium. In our subjects, methionine 25 mg/kg resulted in an 1.6 μmol/L increment and methionine 10 mg/kg resulted in a 2.8 μmol/L increment in plasma homocysteine. These levels of plasma homocysteine were accompanied by a rapid and dose-related impairment of brachial artery endothelium-dependent dilatation. Our observations lend support to the hypothesis that the vascular effects of homocysteine are graded as in the case of cholesterol and present even during small increments in plasma homocysteine.

To examine the physiological relevance of our observations, we studied the vascular effects of animal protein, the major dietary source of methionine. Mean homocysteine concentration rose by 1.6 μmol/L after animal protein and was accompanied by a reduction in endothelium-dependent dilatation, compared with responses after the usual meal. These are the first data to provide evidence of a deleterious effect of dietary animal protein on vascular endothelial function. Previous studies have suggested a relationship between animal protein intake and atherosclerosis and have proposed a role for disturbances in lipid metabolism. In this study, endothelial dysfunction induced by animal protein was not accompanied by changes in blood lipids. Instead, our data support a key role for homocysteine in the mechanisms underlying the association between dietary animal protein and endothelial dysfunction. Metabolic studies in humans show that high animal protein intake may affect homocysteine levels for at least 8 hours and may contribute to intraindividual day-to-day variation in fasting homocysteine of up to 25%. Based on the results of the present study, physiological changes in homocysteine of this magnitude may impair vascular endothelial function, and in susceptible subjects, contribute to the development and progression of atherosclerosis and thrombosis.

Our results do not exclude a direct effect of methionine on endothelial function, although previous studies that have examined the vascular effects of high dose oral methionine have not shown a convincing relationship between plasma methionine concentrations and flow-mediated dilatation. In our subjects, impaired endothelium-dependent dilatation after methionine was not due to a nonspecific effect of amino acid ingestion because there was no change in flow-mediated dilatation after the methionine-free amino acid mix. Both methionine and methionine-free preparations were isocaloric, isotonic, and isovolaemic, which suggests that factors such as fluid balance shifts or gastric emptying were unlikely to have influenced endothelium-dependent dilatation. In the present study, methionine (100 mg/kg) induced a maximal rise in plasma homocysteine at 8 hours and fall in flow-mediated dilatation at 4 hours. Precise reasons for the recovery in flow-mediated dilatation despite rising plasma homocysteine concentrations are not clear but may include the formation and release of protective vasodilator substances such as S-nitrosothiols and S-nitrosohomocysteine, substances with potent vasodilator and platelet inhibitor properties. However, a different study design will be necessary to determine the separate effects of methionine, homocysteine, and related species.

In summary, our results show that vascular endothelial dysfunction can be induced by small increments in plasma homocysteine after low dose oral methionine. Similar distur-

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### Table 2. Homocysteine Concentrations and Brachial Artery Vascular Responses 12 Hours After Usual Meal and Animal Protein Meal

<table>
<thead>
<tr>
<th></th>
<th>Usual Meal</th>
<th>Animal Protein Meal</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocysteine (μmol/L)</td>
<td>9.6±0.8</td>
<td>11.2±0.9</td>
<td>0.005</td>
</tr>
<tr>
<td>Flow-mediated dilatation (%)</td>
<td>4.5±0.7</td>
<td>0.9±0.6</td>
<td>0.003</td>
</tr>
<tr>
<td>GTN-induced dilatation (%)</td>
<td>20.0±1.3</td>
<td>18.7±1.4</td>
<td>0.17</td>
</tr>
<tr>
<td>Baseline brachial artery diameter (mm)</td>
<td>4.0±0.1</td>
<td>4.1±0.1</td>
<td>0.06</td>
</tr>
<tr>
<td>Basal brachial artery blood flow (mL/min)</td>
<td>99±16</td>
<td>99±11</td>
<td>1.00</td>
</tr>
<tr>
<td>Hyperemic increase in brachial blood flow (%)</td>
<td>144±19</td>
<td>132±14</td>
<td>0.54</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.9±0.2</td>
<td>4.7±0.1</td>
<td>0.05</td>
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<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.1±0.3</td>
<td>5.3±0.3</td>
<td>0.42</td>
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<td>HDL cholesterol (mmol/L)</td>
<td>1.4±0.1</td>
<td>1.4±0.1</td>
<td>1.00</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>3.2±0.3</td>
<td>3.3±0.3</td>
<td>0.49</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.2±0.1</td>
<td>1.3±0.1</td>
<td>0.86</td>
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<tr>
<td>Energy (kJ)</td>
<td>840±80</td>
<td>1020±100</td>
<td>0.47</td>
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<tr>
<td>Total protein (g)</td>
<td>36±4</td>
<td>141±7</td>
<td>0.001</td>
</tr>
<tr>
<td>Methionine (g)</td>
<td>0.8±0.1</td>
<td>3.3±0.2</td>
<td>0.001</td>
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<tr>
<td>Total fat (g)</td>
<td>35±6</td>
<td>28±5</td>
<td>0.31</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>86±6</td>
<td>43±11</td>
<td>0.004</td>
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</table>
bances in endothelial function are also evident after physiological increments in plasma homocysteine (2 to 3 μmol/L) after dietary animal protein. Protein intake–related increments in plasma homocysteine may therefore have deleterious effects on vascular function and contribute to the development and progression of atherosclerosis in man.

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References
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