Hyperhomocysteinemia After an Oral Methionine Load Acutely Impairs Endothelial Function in Healthy Adults

M.F. Bellamy, MB, MRCP; I.F.W. McDowell, MRCP, MRCPath; M.W. Ramsey, MB, MRCP; M. Brownlee, RGN; C. Bones, BSc; R.G. Newcombe, PhD, CStat; M.J. Lewis, MB, DSc

Background—Elevated plasma homocysteine is a risk factor for arteriosclerosis, but a cause-and-effect relationship remains to be fully established. Endothelial dysfunction, an early event in the atherogenic process, has been shown to be associated with hyperhomocysteinemia in experimental and human studies. To further establish a direct relationship between changes in plasma homocysteine and endothelial dysfunction, we investigated whether moderate hyperhomocysteinemia induced by an oral methionine load would acutely impair flow-mediated endothelium-dependent vasodilatation in healthy adults.

Methods and Results—Twenty-four healthy volunteers completed a randomized crossover study in which an oral methionine load (0.1 g/kg) was administered on 1 of 2 study days, 7 days apart. At each visit, plasma homocysteine and brachial artery endothelium-dependent and -independent dilatation were measured at baseline and at 4 hours. To further elucidate the temporal relationship between methionine, homocysteine, and endothelial function, an oral methionine load was administered in 10 subjects on a separate visit, and the time courses of plasma methionine, homocysteine, and flow-mediated brachial artery dilatation were measured at baseline and after 1, 2, 3, 4, and 8 hours. After oral methionine, plasma homocysteine increased from 7.9 ± 2.0 μmol/L at baseline to 23.1 ± 5.4 μmol/L at 4 hours (P < 0.0001, n = 24) and was associated with a decrease in flow-mediated brachial artery dilatation from 0.12 ± 0.09 to 0.06 ± 0.09 mm (P < 0.05). The time course of the impairment of flow-mediated vasodilatation mirrored the time course of the increase in homocysteine concentration.

Conclusions—Oral methionine loading raises plasma homocysteine and impairs flow-mediated endothelium-dependent vasodilatation. This supports the view that homocysteine may promote vascular disease by inducing endothelial dysfunction.

Key Words: homocysteine • endothelium • methionine • vasodilation

Homocysteine is a sulfur-containing amino acid that is derived from dietary methionine. An elevated plasma homocysteine level is associated with an increased risk of arteriosclerosis (coronary, cerebral, and peripheral) independent of other risk factors, but a causal role has not yet been fully established.

In homocystinuria, which is a rare inherited disorder (most often due to cystathionine β-synthase deficiency, 1 in 200,000), plasma homocysteine levels are markedly elevated (>50 μmol/L; normal range, 5 to 15 μmol/L), and patients have severe, widespread vascular disease. In the general population, mild to moderate elevations in plasma homocysteine (15 to 35 μmol/L) are common and may be due to inherited enzyme variants and/or a relative deficiency of folate, vitamin B12, or vitamin B6, which are required for the normal metabolism of homocysteine. Methionine taken orally is converted to homocysteine by demethylation, and the effect of an oral load can be used as a diagnostic test to identify individuals with enzyme defects who show an exaggerated rise in homocysteine levels.

Endothelial injury appears to be an early event in the promotion of atherogenesis and may be one mechanism whereby homocysteine leads to an increased risk of both arterial and venous disease. Studies in vitro have demonstrated that homocysteine may injure endothelium, but the mechanism for such an effect is not yet known. Dietary modification to increase homocysteine levels in monkeys, including augmenting methionine intake, impairs vascular function. In humans, endothelial dysfunction has been demonstrated in homocystinuria and in association with less markedly elevated plasma homocysteine levels more relevant to the general population.

We therefore hypothesized that moderate elevations of plasma homocysteine would be acutely injurious to endothelium. The objectives of the present study were to investigate the effect of an oral methionine load on plasma homocysteine and flow-mediated endothelial function in healthy subjects and to observe the time course of any such effects.

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Subjects

Crossover Study
Twenty-eight members of the hospital staff were invited to enter the study, but 3 volunteers later declined to participate after randomization. One subject developed a severe headache after sublingual nitroglycerin (NTG) and withdrew after completing the first visit. Twenty-four subjects (mean age, 32 years; range, 21 to 46 years; 14 men) completed the protocol.

Time-Course Study
Five of the 24 subjects who completed the crossover study volunteered to attend on a second occasion, together with 5 additional participants not studied in the first phase (see below). All were nonsmokers, were normotensive (blood pressure <150/90 mm Hg), had serum cholesterol <6.5 mmol/L, and were not taking vitamin supplements. Baseline characteristics are shown in Table 1. All subjects gave informed, written consent, and the study protocol was approved by the local Research Ethics Committee.

Study Design

Randomized Crossover Study of an Oral Methionine Load
Twenty-four subjects attended after an overnight fast on 2 separate days, 1 week apart. Subjects were randomized to receive oral l-methionine (0.1 g/kg, Scientific Hospital Supplies) on either day 0 or day 7 as a crossover study. Randomization to methionine on day 0 or day 7 was performed by a research nurse, who administered the oral methionine load in a room separate from the vascular laboratory to ensure that the investigators performing the vascular measurements remained blinded throughout the study. Due to the distinctive taste of methionine, subjects knew which day they had received methionine but were specifically instructed not to inform the ultrasound operators. At baseline, flow-mediated (endothelium-dependent) and NTG-related (endothelium-independent) brachial artery dilatation was measured and venous blood was sampled for total plasma homocysteine (with additional sampling for total cholesterol, triglycerides, glucose, folate, and B12 on the first visit). Total plasma homocysteine were measured at baseline and at 1, 2, 3, 4, and 8 hours. This was an open (nonblinded) study. In those subjects who had previously participated in the crossover study (5 of the 10 subjects), these measurements were performed on a separate visit at least 7 days later.

Noninvasive Measurement of Flow-Mediated, Endothelium-Dependent Vasodilatation
Endothelial function was assessed by comparing endothelium-dependent vasodilatation in response to flow with the endothelium-independent response to sublingual NTG. The increase in brachial artery flow was induced by release of a wrist cuff after a period of hand ischemia. This technique thereby avoids ischemia of the brachial artery itself.

Subjects lay supine in a temperature-controlled room (21°C to 23°C) with the left arm outstretched on a pneumatic cushion. As previously described,14 brachial artery end-diastolic diameter was measured by high-resolution vessel wall tracking (Vadirec, Medical Systems Arnhem, resolution ±3 μm), blood flow by continuous-wave Doppler ultrasound (SciMed Dupstation) derived from the mean velocity time integral corrected for Doppler angle and internal brachial artery diameter, and blood pressure and heart rate by finger photoplethysmography (Finapres, Ohmeda).

Measurements were made at baseline (after 15 minutes of supine rest), at 60 and 120 seconds of hand hyperemia (produced by releasing a pneumatic wrist cuff inflated for 5 minutes to suprasystolic pressure), and 3 minutes after 400 μg NTG. The maximum increase in end-diastolic brachial artery diameter from baseline was used as the measure of dilatation. All hemodynamic measurements were confirmed as having returned to baseline 15 minutes after each intervention.

Blood Samples and Assays
Venous blood was sampled into tubes containing EDTA (for homocysteine), lithium-heparin (for methionine), SST (gel and clot activator) (for lipids, B12, and folate), and fluoride-oxalate (for glucose). Samples for homocysteine were immediately placed on ice, plasma methionine by ion-exchange amino acid chromatography using SBD-F (ammonium 7-fluoro-2-oxa-1,3-diazole-4-sulfonate) derivatization (within-batch precision, 2.2%). Plasma methionine was measured by ion-exchange amino acid chromatography (Biotronik LC 5001).

Statistical Analysis
Data are presented in the text, tables, and figures as group mean±SD unless stated otherwise. Changes in hemodynamic data, brachial artery flow-mediated dilatation, and NTG-mediated dilatation from baseline at 4 hours during the methionine period were compared with those occurring during the control period by a paired analysis based on that of the 2-period crossover trial.14 Thus, the difference between changes occurring in the first and second periods was compared between the 2 treatment order groups by an unpaired t test, which obviates confounding with period differences introduced by unequal numbers in the 2 groups. In the time-course study, a Wilcoxon paired test was used to compare changes in methionine, homocysteine, and flow-mediated dilatation at each of the 5 time points relative to baseline.

Results
Fasting plasma total homocysteine and basal vascular measurements were similar on each of the 2 study days, and there was no evidence of an order effect. Administration of the oral methionine load increased total homocysteine from 7.9±2.0 μmol/L at baseline to 23.1±5.4 μmol/L at 4 hours (P<0.0001) (Table 2). Flow-mediated brachial artery dilatation decreased from 0.12±0.09 to 0.06±0.09 mm (P=0.045), despite similar hyperemic blood flow (67±66 versus 78±81 mL/min). NTG-induced dilatation was similar at baseline and

TABLE 1. Subject Characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Crossover</th>
<th>Time Course</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>n=24</td>
<td>n=10</td>
</tr>
<tr>
<td>Age, y</td>
<td>32 (7)</td>
<td>29 (6)</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>14/10</td>
<td>8/2</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>23 (2)</td>
<td>23 (3)</td>
</tr>
<tr>
<td>Total homocysteine, μmol/L</td>
<td>7.5 (2.2)</td>
<td>7.2 (1.4)</td>
</tr>
<tr>
<td>Folate, μg/L</td>
<td>6.9 (2.3)</td>
<td>7.5 (3.0)</td>
</tr>
<tr>
<td>Vitamin B12, ng/L</td>
<td>447 (107)</td>
<td>505 (90)</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.7 (0.8)</td>
<td>4.5 (0.5)</td>
</tr>
<tr>
<td>Triglyceride, mmol/L</td>
<td>0.9 (0.4)</td>
<td>1.1 (0.4)</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>4.8 (0.4)</td>
<td>4.5 (0.3)</td>
</tr>
</tbody>
</table>

Data are mean (SD).
at 4 hours (0.48±0.17 versus 0.40±0.11 mm, P=0.32) (Figure 1). There was no significant correlation between change in flow-mediated dilatation and basal, postload, or increase in plasma homocysteine. Five subjects reported mild nausea after oral methionine, which was most pronounced in the first hour after administration.

In the group of 10 subjects who participated in the time-course study, plasma methionine increased from 27±5 μmol/L at baseline to a maximum of 714±155 μmol/L at 1 hour and declined thereafter but remained above baseline in all subjects at 8 hours (P=0.002) (Figure 2). Plasma total homocysteine increased from 7.2±1.4 μmol/L at baseline to 23.5±5.2 μmol/L at 4 hours (P<0.001) and remained elevated at 25.9±7.0 μmol/L at 8 hours (P=0.002 versus baseline). Flow-mediated brachial artery dilatation decreased in all subjects (P=0.002) from 0.12±0.04 mm at baseline and remained impaired up to 8 hours.

### Discussion

The principal finding of this study is that an oral methionine load, which produces an increase in plasma homocysteine, acutely impairs flow-mediated brachial artery dilatation in healthy subjects. These findings provide evidence to support the hypothesis that homocysteine is a risk factor for vascular disease by a mechanism that involves injury to the endothelium. However, these data do not exclude some other possible interpretation, such as a direct effect of methionine or an alteration in methylation reactions, which are dependent on methionine-homocysteine interconversion. Inspection of the time-course data is relevant to this point. The impairment of endothelial function from 0 to 4 hours mirrored in all subjects the increase in plasma homocysteine, which is consistent with a direct toxic effect of homocysteine. However, the tendency for flow-mediated responses to improve at 8 hours when methionine is falling but homocysteine continues to rise is more indicative of a direct methionine effect. Different experimental approaches will be necessary to distinguish these possibilities.

The absence of any carryover effect in those subjects who received methionine on day 0 suggests that the effect on endothelium-dependent dilatation is reversible within 7 days. This is consistent with the return of plasma homocysteine to baseline at ~24 hours after an oral methionine load.17 The effect of methionine loading was repeatable. In the 5 subjects who received methionine twice, similar increases in homocysteine were measured at 4 hours in association with similar decreases in flow-mediated vasodilatation on both occasions.

A potential drawback of the study was that a matched placebo was not possible because of the distinctive taste of methionine. The study did, however, incorporate a randomized crossover design, and particular attention was paid to ensure that the observers performing vascular measurements were unaware of the order of methionine administration.

Flow-mediated brachial artery dilatation reflects endothelium-dependent vasodilatation. It can be largely blocked by inhibitors of nitric oxide synthase (eg, the nonmetabolized L-arginine analogue Nω-nomonomethyl-L-arginine [L-NMMA]) and is therefore attributable predominantly to nitric oxide activity.18 In this study, flow-mediated dilatation of a conduit artery was measured in response to reactive hyperemia of the antecubital fossa. These differences in methodology may account for differences in “normal” values for flow-mediated brachial artery dilatation compared with some previously published data11,19,20 but are consistent with results obtained by other investigators using the same technique.

Endothelial dysfunction, when present, tends to be generalized and therefore is likely to be of clinical significance.
when demonstrated in an artery that is usually spared of atheroma. Indeed, a close relation has been demonstrated between endothelial function in the human coronary and peripheral circulation, suggesting that even in the absence of overt atheroma, these findings have relevance to vascular beds that are more commonly affected by atherosclerosis.

A clear association now exists between mild to moderate increases in plasma homocysteine and arteriosclerotic vascular disease. Homocysteine could promote atheroma formation by various mechanisms, including endothelial dysfunction, smooth muscle cell proliferation, alteration of coagulation factors, and lipoprotein oxidation. At the molecular level, there is some evidence that homocysteine may promote oxidant stress, thereby decreasing nitric oxide bioavailability.

In vitro studies have shown evidence of cellular injury in the presence of homocysteine, but the effect is nonspecific at the high concentrations used. The effect of more clinically relevant levels of homocysteine on vascular reactivity has been reported in a primate model in which dietary modification with methionine feeding and folate restriction impaired relaxation to endothelium-dependent vasodilators. Responses to endothelium-independent dilators were also impaired, although to a lesser extent.

In human studies, impaired endothelium-dependent, flow-mediated dilatation of the brachial and femoral arteries has been demonstrated in young patients with homocystinuria, in whom homocysteine concentrations would be markedly elevated. Endothelial dysfunction has also been reported in association with mild hyperhomocysteinemia and in subjects with hyperhomocysteinemia secondary to low vitamin B12 concentrations. This study demonstrates a temporal relationship between a rise in homocysteine and changes in flow-related endothelial function, but a cause-and-effect relationship remains to be established.

In a typical western diet, postprandial increases in plasma methionine and homocysteine are usually small, the latter on the order of 1 to 2 μmol/L, and therefore unlikely to have any effect on vascular reactivity. However, the findings of this study may be relevant to the general population, because mild to moderate elevations in plasma homocysteine may com-

Figure 1. Changes in brachial artery end-diastolic diameter induced by flow-mediated vasodilation (top) and NTG (bottom) expressed as absolute change in diameter (mm) from basal measurement. Flow-mediated, endothelium-dependent vasodilation was impaired at 4 hours after oral methionine compared with control (no methionine), while response to NTG was unchanged.

Figure 2. Changes in plasma methionine (top), total homocysteine (middle), and flow-mediated brachial artery dilatation (bottom) with time. Data are shown as group mean ± SEM (* P<0.002 vs baseline).
commonly occur as a result of inherited enzyme variants and/or suboptimal vitamin status or in association with disease states such as renal failure.27 Subjects homozygous for the thermolabile variant of methylene tetrahydrofolate reductase, the frequency of which is \( \approx 12\% \) in healthy subjects in our region (Z. Clark, BSc, written communication, March 1998), are prone to develop raised homocysteine concentrations28,29 that may be similar to those achieved here after an oral methionine load. Endothelial dysfunction arising from mild to moderate hyperhomocysteinemia could thereby contribute to the atherogenic process in these individuals and could potentially be ameliorated by lowering plasma homocysteine with vitamin supplementation, particularly folic acid.

Acknowledgments

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References

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