Effects of Folate Treatment and Homocysteine Lowering on Resistance Vessel Reactivity in Atherosclerotic Subjects

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ABSTRACT

Hyperhomocysteinemia is associated with arterial hypertension and endothelial dysfunction in healthy humans. Placebo-controlled vitamin intervention studies cannot distinguish intrinsic actions of homocysteine (tHcy) and folate concentrations on the endothelium. The present two-period crossover study investigates the effects of tHcy lowering through oral folic acid on antioxidant status and resistance vessel reactivity in patients with established coronary artery disease (CAD). We investigated 27 male patients with angiographically documented multivessel CAD aged 50 (range 46–56) years. Resistance vessel reactivity was assessed by measurement of postischemic reactive hyperemia (RH) in the forearm using venous occlusion plethysmography at baseline, after 6 weeks of treatment with 5 mg of oral folic acid, and after a washout period of another 6 weeks. Plasma folate increased 3.49-fold with a mean tHcy reduction of 21.3%. Peak reactivity of resistance vessels improved significantly (18.97–23.60 ml/min−1 per 100 ml; P = 0.01) with unchanged total antioxidant status (TAS; 0.912–0.944 μM; P = 0.4). This effect was limited to subjects (n = 14) with a tHcy reduction >2 μM (median reduction, 14.4–9.6 μM, P < 0.001). In the 13 subjects with a below-median reduction, tHcy remained unaltered (9.7–9.6 μM, P = 0.88) and TAS increased significantly (0.923–1.055 μM, P = 0.006), whereas RH peak flow was not affected (20.22–22.99 ml/min−1 per 100 ml, P = 0.28). Homocysteine lowering >2 μM through folic acid supplementation improves resistance vessel reactivity in patients with CAD. Our data support the hypothesis that homocysteine lowering may have intrinsic vasoprotective effects largely independent of folate.

Elevated total plasma homocysteine (tHcy) is an independent risk factor for peripheral vascular, cerebrovascular, and coronary artery disease (CAD) (Boushey et al., 1995). Even moderate hyperhomocysteinemia (>9 μM) is prospectively associated with increased risk of mortality in CAD patients (Nygaard et al., 1997; Anderson et al., 2000).

Homocysteine concentrations are determined by genetic and nutritional factors (Ueland and Refsum, 1989). Deficiencies of vitamins B6, B12, and folic acid in particular are associated with hyperhomocysteinemia (Ubbink et al., 1993). Consequently, vitamin supplementation, especially with folic acid, has been demonstrated to effectively lower homocysteine levels (Clarke et al., 1998).

Homocysteine is a highly reactive amino acid derived from methionine metabolism and is known to produce endothelial cell injury in experimental animals (Harker et al., 1983), in cell culture (Wall et al., 1980), and in humans (Tawakol et al., 1997). The vascular endothelium plays a critical role in the control of vascular tone and regulation of blood flow. Vascular dysfunction is an established early step in the development of vascular disease and contributes to the pathogenesis of atherosclerosis (Celermajer et al., 1992) and CAD (Zeiher et al., 1991). Furthermore, systemic endothelial dysfunction as assessed in the brachial artery is closely related to the extent of CAD (Neunteufl et al., 1997).

In healthy humans, impaired endothelial function of conduit vessels is found in hyperhomocysteinemia (Woo et al., 1997) and is even induced through physiological increments of plasma homocysteine (Chambers et al., 1999a). Vitamin C prevents these effects, suggesting a role for oxidant stress in homocysteine-induced impairment of vascular endothelial function (Chambers et al., 1999b). Furthermore, folic acid was recently demonstrated to improve endothelial function in healthy adults with hyperhomocysteinemia (Woo et al., 1999).

ABBREVIATIONS: tHcy, total plasma homocysteine; CAD, coronary artery disease; FMD, flow-mediated dilation; RH, reactive hyperemia; TAS, total antioxidant status; NO, nitric oxide.
All of these studies used flow-mediated vasodilation (FMD) of the brachial artery in healthy subjects to examine endothelial function of conduit vessels. Blood flow to target organs, in the absence of hemodynamically significant stenoses in conduit vessels, is regulated at the level of local resistance vessels (Chillian et al., 1986). Impaired reactivity of coronary resistance vessels has been shown to be associated with exercise-induced ischemia in subjects free of macrovascular coronary artery disease (Zeiher et al., 1995). Recently, endothelial dysfunction in patients with moderate CAD was found to be accompanied by myocardial perfusion defects (Hasdai et al., 1997). Both reports demonstrate the importance of resistance vessel reactivity in the regulation of myocardial blood flow.

At present, the role for homocysteine on resistance vessel function is less clear. In particular, the effect of folic acid supplementation on resistance vessel function in patients with atherosclerotic disease has not been investigated before. The extent of homocysteine lowering required for a beneficial effect on vascular dysfunction is unknown.

This study was designed to investigate the intrinsic effects of folate supplementation and homocysteine lowering on resistance vessel reactivity in patients with angiographically documented multivessel CAD.

Materials and Methods

Study Protocol and Subjects. Twenty-seven male patients with angiographically documented CAD, mean age 50.2 ± 6.2 (range 46.0–56.6) years, were enrolled in this two-period crossover study. CAD was defined as >50% stenosis of the lumen diameter in at least one of the major epicardial vessels. All subjects gave written informed consent, and the study was approved by the Ethics Board of the Karl-Franzens University. The investigation conforms with the principles outlined in the Declaration of Helsinki.

Patients were excluded if they had myocardial infarction, unstable angina or coronary intervention within the past 6 months, diabetes mellitus, dysfunction of the thyroid gland, or impaired liver or kidney function. Patients with prior intake of nitrates, angiotensin-converting enzyme inhibitors, vitamins, and other medication known to interfere with homocysteine metabolism, or change of medication during the time of investigation, were also excluded. Blood pressure was recorded (after 15 min of supine rest) on each day of the study. Hypertension was defined as diastolic blood pressure >90 mm Hg and/or systolic blood pressure >140 mm Hg. Subjects were normotensive during the 12-week period of investigation. All participants received standard medication for CAD (low-dose acetylsalicylic acid [100 mg] and β-blockers) and were asked to refrain from intake of lipid-lowering drugs 6 weeks before the first measurement. This remained unchanged during the entire course of investigation.

After the baseline investigation, each patient received 5 mg of folic acid orally per day for a period of 6 weeks, followed by a washout period of another 6 weeks. At baseline (0) and after 6 and 12 weeks, fasting blood samples were taken and reactivity of resistance vessels was assessed as measured by postischemic reactive hyperemia (RH) by venous occlusion plethysmography. All subjects were tested on all three visits after an 8-h overnight fast and nonsmoking period.

Laboratory Assays. Fasting (F) plasma folate, cholesterol, triglycerides, vitamins B₉ (B₉) and B₁₂, lipoprotein (a), and total antioxidant status (TAS) were measured for each subject on each day of investigation.

All blood samples were taken from an antecubital vein between 7:00 and 8:00 AM after an overnight fasting period of at least 8 h. Samples were processed immediately, centrifuged at 4°C (3000g for 10 min) within 15 min, and stored at −70°C until analysis. All serological analyses were carried out without prior knowledge of clinical data. Measurements of plasma homocyst(e)ine in EDTA plasma were performed using high-performance liquid chromatography and fluorescence detection according to the method of Araki and Sako (1987) with modifications by Ubbink et al. (1991) and Vester and Rasmussen (1991). Briefly, after reduction with tri-N-butylphosphine, the free thiol groups were derivatized with SBD-F (7-fluoro-benzofurazane-4-sulfonic acid). Separation was performed under isocratic conditions on a reversed phase column at pH 2.1 with mercaptopyrropropionylglycine as internal standard. The intra-assay variability of the method was between 1.3% (27 μM) and 2.5% (10 μM).

Because this procedure involved a reducing step, the method did not distinguish between homocysteine and its oxidized analogs. Therefore, the measured moeity may be referred to as homocyst(e)ine.

Plasma folate and vitamin B₁₂ concentrations were determined with an Abbott AxSYM analyzer by commercially available Ion Capture Assay and Microparticle Enzyme Immunoassay, respectively (Abbott Diagnostics, Wiesbaden, Germany). Standard methods were used for serum lipids.

TAS of plasma was measured with the TAS assay (Troxol equivalent antioxidant capacity) commercially available in a modified form (Randox Laboratories, Crumlin, Antrim, UK). With this assay, antioxidants in the plasma inhibit formation of 2,2’-azinobis (3-ethylbenzothiazoline-6-sulfonate) radical cations detected photometrically at a wavelength of 600 nm. Absorptions obtained 3 min after start of the reaction are compared with those of Trolox, and results are given in millimoles per liter Trolox equivalents.

Measurement of Forearm Blood Flow. For measurement of resistance vessel endothelial function, forearm blood flow was determined by venous strain-gauge plethysmography (ECS5; Hokanson Inc., Bellevue, WA) with electrically calibrated mercury-in-Silastic strain gauges (Wascher et al., 1998). In brief, the experiments were started at 8:00 AM after an overnight fast in a temperature-controlled room (22–23°C). Subjects were in a supine position for 30 min before the measurements of resting blood flow to assure stable baseline conditions. Measurements were performed on the right arm; the strain gauge was positioned at the widest part of the forearm. For occlusion of venous outflow, the upper arm cuff pressure was set at 50 mm Hg; for induction of ischemia a pressure of 200 mm Hg was chosen. During measurements of forearm perfusion, the hand was occluded from the circulation with a second cuff positioned at the wrist, inflated immediately before the measurements. Resting blood flow was determined by calculation of the mean of three measurements within 1 min. RH was induced by 5 min of ischemia. After release of the upper arm cuff, forearm blood flow was measured every 10 s for up to 60 s. Peak blood flow values, termed peak postischemic RH, are observed thereby 10 s after the onset of reperfusion.

Statistical Analysis. The MacLab package (AD Instruments Pty Ltd., Castle Hill, Australia) on an Apple Macintosh computer was used to acquire and analyze data from the measurements of resistance vessel endothelial function. The mean slope of the volume curve from the second to the fifth pulsewave at each measurement was taken as the respective blood flow. Blood flow data are given as milliliters of perfusion per minute in 100-ml forearm tissue.

Data were analyzed with use of SYSTAT Version 5.1 (SYSTAT Inc., Richmond, CA) on an Apple Macintosh computer. The effects of folic acid on resistance vessel function were compared with analysis of variance for two-way repeated measurements. An unpaired Student’s t test was used for group comparison. All data are expressed as mean ± S.E., unless otherwise stated. The level of significance was "P < 0.05."

Results

All 27 male subjects (mean age 50.3 years) completed the crossover trial according to protocol. No side effects of the
vitamin medication were observed or reported by the participants. The baseline clinical and biochemical measurements are summarized in Table 1. In linear regression analysis, folate and homocyst(e)ine levels were not significantly related at baseline \( (P = 0.1, r^2 = 0.11) \).

At baseline, subjects with above median tHcy reduction had higher homocysteine levels \((14.4 \pm 1.2 \text{ versus } 9.7 \pm 0.7 \mu M; P = 0.002)\) and lower folate levels \((5.1 \pm 0.6 \text{ versus } 8.5 \pm 1.4 \text{ ng/ml; } P = 0.03)\) than subjects responding with below median tHcy reduction. After 6 weeks without folic acid supplementation, plasma folate and tHcy reversed but not quite to baseline values, indicating a carryover effect (Table 2).

**Biochemical Measurements.** Oral folic acid \((5 \text{ mg/day})\) induced a 3.49-fold increase of plasma folate \((6.7 \pm 0.8 \text{ to } 23.4 \pm 2.5 \text{ ng/ml; } P < 0.0001)\) and lowered homocysteine levels \(21.3\% (12.2 \pm 0.8 \text{ to } 9.6 \pm 0.5 \mu M; P < 0.001)\). TAS increased significantly after 6 weeks of folic acid supplementation and returned to baseline at day 12. Throughout the observation period, vitamin supplementation was not seen to have any effects on vitamin B\(_6\) and B\(_{12}\) levels, plasma cholesterol, triglycerides, and lipoprotein(a).

After 6 weeks of folic acid supplementation, the median change of plasma homocysteine was \(2 \mu M\). Fourteen patients had responded with above median reduction \( (>2 \mu M)\) of plasma homocysteine levels \((14.4 \pm 1.1 \text{ versus } 9.6 \pm 0.8 \mu M; P < 0.0001)\), whereas homocysteine concentrations were not altered in 13 patients with individual reductions below median \((9.7 \pm 0.7 \text{ to } 9.6 \pm 0.8 \mu M; P = 0.88)\) (Table 2). TAS did not change in the 14 subjects with above median reduction of plasma homocysteine levels \((0.912 \pm 0.037 \text{ versus } 0.944 \pm 0.037 \text{ mM; } P = 0.4)\) but increased significantly in the remaining 13 subjects \((0.923 \pm 0.045 \text{ versus } 1.055 \pm 0.045 \text{ mM; } P = 0.006)\) between study days 0 and 6.

**Resistance Vessel Reactivity.** Resting blood flow remained almost unchanged in both groups over the entire 12 weeks (Table 2). Homocysteine lowering above median \((>2 \mu M)\) significantly increased peak reactive hyperemic blood flow after 6 weeks \((18.97 \pm 1.12 \text{ versus } 23.60 \pm 1.41 \text{ ml/min }^{-1} \text{ per } 100 \text{ ml; } P = 0.01)\), but subjects with below median reduction showed no improvement \((20.22 \pm 2.96 \text{ versus } 22.99 \pm 1.81 \text{ ml/min }^{-1} \text{ per } 100 \text{ ml; } P = 0.28)\). At the 6-week follow-up visit, the change of total blood flow \((1 \text{ min})\) was borderline significant in above median subjects \((11.09 \pm 0.98 \text{ versus } 13.03 \pm 1.09 \text{ ml/min }^{-1} \text{ per } 100 \text{ ml; } P = 0.058)\) and insignificant in below median subjects \((10.13 \pm 1.32 \text{ versus } 13.12 \pm 1.37 \text{ ml/min }^{-1} \text{ per } 100 \text{ ml; } P = 0.09)\). After 6 weeks of washout, resting flow, peak flow \((10 \text{ s})\), and total flow \((1 \text{ min})\) in responders were not significantly different from baseline.

**TABLE 1**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>All ((n = 27))</th>
<th>tHcy Reduction above Median ((n = 14))</th>
<th>tHcy Reduction below Median ((n = 13))</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr (range)</td>
<td>50.3 ± 6.2</td>
<td>48.5 ± 6.5</td>
<td>52.1 ± 5.5</td>
<td>N.S.</td>
</tr>
<tr>
<td>Body mass index, kg/m(^2)</td>
<td>27.0 ± 2.9</td>
<td>26.5 ± 3.5</td>
<td>27.4 ± 2.1</td>
<td>N.S.</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>12 (44.4%)</td>
<td>6 (42.8%)</td>
<td>6 (46.1%)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Former</td>
<td>15 (55.6%)</td>
<td>8 (57.1%)</td>
<td>7 (53.8%)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Arterial hypertension</td>
<td>14 (51.8%)</td>
<td>6 (42.8%)</td>
<td>8 (61.5%)</td>
<td>N.S.</td>
</tr>
<tr>
<td>TAS, mM</td>
<td>0.918 ± 0.028</td>
<td>0.912 ± 0.037</td>
<td>0.923 ± 0.045</td>
<td>N.S.</td>
</tr>
<tr>
<td>Plasma homocyst(e)ine, (\mu M)</td>
<td>12.2 ± 0.8</td>
<td>14.4 ± 1.1</td>
<td>9.7 ± 0.7</td>
<td>0.002</td>
</tr>
<tr>
<td>Plasma folate, ng/ml</td>
<td>6.7 ± 0.8</td>
<td>5.1 ± 0.6</td>
<td>8.5 ± 1.4</td>
<td>0.03</td>
</tr>
<tr>
<td>Plasma vitamin B(_{12}), ng/l</td>
<td>377 ± 26</td>
<td>377 ± 37</td>
<td>377 ± 35</td>
<td>N.S.</td>
</tr>
<tr>
<td>Serum vitamin B(_{6}), ng/l</td>
<td>16.8 ± 4.5</td>
<td>18.8 ± 7.1</td>
<td>13.7 ± 3.7</td>
<td>N.S.</td>
</tr>
<tr>
<td>Plasma cholesterol, mM</td>
<td>255 ± 9</td>
<td>223 ± 13</td>
<td>184 ± 8</td>
<td>0.03</td>
</tr>
<tr>
<td>Plasma triglycerides, mM</td>
<td>161 ± 25</td>
<td>171 ± 47</td>
<td>150 ± 11</td>
<td>N.S.</td>
</tr>
<tr>
<td>Lipoprotein(a), mM</td>
<td>21.4 ± 3.8</td>
<td>17.4 ± 4.7</td>
<td>24.8 ± 5.8</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

N.S., not significant.
fusion and reflects the vasodilatatory capacity of a vascular bed upon a predefined stimulus (Iwatsubo et al., 1997; Wacher et al., 1998).

Impairment of FMD through methionine loading was prevented by oral administration of antioxidant vitamin C, suggesting a role for oxidative stress in homocysteine-induced vascular (endothelial) dysfunction (Chambers et al., 1999a).

In our study, plasma folate increased 349% in all subjects, but TAS increased only in subjects with no change in tHcy levels. Infusion of 5-methyltetrahydrofolate, the biologically active form of folic acid, improved endothelial function in patients with familial hypercholesterinemia but normal homocysteine concentrations (Verhaar et al., 1998). The response was immediate and did not affect homocysteine levels, suggesting a scavenging capacity of folate with an effect on endothelial function. This is supported by the finding that pretreatment with oral folic acid prevents the lipid-induced decrease in FMD as well as the lipid-induced increase in urinary radical-damage end products in healthy volunteers (Wilminck et al., 2000). Folic acid may therefore increase TAS by adding to antioxidative capacity, especially when homocysteine concentrations are unchanged. Furthermore, unchanged TAS in subjects with above median lowering of homocysteine may reflect a decrease in SH-(thiol) groups with simultaneous decrease of antioxidative capacity.

Homocysteine has been demonstrated to disturb NO bioavailability (Upchurch et al., 1997) and increase oxidative stress through autoxidation of homocysteine yielding hydrogen peroxide (Loscalzo, 1996; Kanani et al., 1999). Under these conditions, folic acid may improve NO bioavailability indirectly through decrease of homocysteine concentration leading to decreased oxidative stress and less impairment of NO-induced vasodilatation, with possibly additional antioxidative effect, however, consumed during homocysteine lowering. This is supported by our observation that in CAD patients nonresponders showed no improvement in endothelial function, although there was an increase in folate (and TAS). Because folate increased in both groups, but peak flow reactivity improvement was limited to subjects responding with a homocysteine-lowering effect of at least 2 μM, this effect is therefore most likely to be due to lower homocysteine concentrations and not to folate itself.

It must be noted that this effect has been found in CAD patients with presumably impaired NO metabolism. The findings are important because they demonstrate a vasoprotective effect through lowering homocysteine in atherosclerotic patients.

**Potential Study Limitations.** Potential study limitations comprise intake of medication that may influence endothelial function measurements. All participants refrained from intake of lipid-lowering drugs but received the same standard medication for CAD (only acetylsalicylic acid and β-blockers), as discontinuation would have been considered unethical.

Using postischemic RH in the human forearm to measure resistance vessel function does not allow discrimination between such mechanisms involved as NO, adenosine, prostaglandins, or myogrene relaxation.

It was recently suggested that reduced concentrations of free homocysteine may be responsible for improved vascular endothelial function of conduit vessels (Chambers et al., 2000). Most investigators measure total plasma homocysteine concentrations, but this does not permit discrimination between free and protein-bound homocysteine. For technical reasons, determination of free homocysteine is limited to experimental investigations. Measurement of total plasma homocysteine is most likely to continue universally, and our study indicates that it is suitable for clinical use.

**Implications.** In our study, supplementation with 5 mg of folic acid was associated with 21% lowering of homocysteine concentrations, which confirms previous reports that found similar lowering effects using dosages between 0.4 and 10 mg (Clarke et al., 1998). Importantly, Chambers et al. (1999b) have recently shown that a physiologic homocysteine increment of only 2 to 3 μM may induce endothelial dysfunction in healthy humans.

These data are in agreement with our finding that a reduction of >2 μM is required to produce a beneficial effect on endothelial function. Furthermore, a mean difference of −2 to 3 μM generally discriminates cohorts of vascular patients.
from age- and sex-matched healthy subjects in case-control studies (Kang et al., 1992). It appears that a deviation of as little as 2 to 3 μM homocysteine may produce a measurable effect on human vascular endothelium and distinguishes cases from healthy subjects. In meta-analyses, increased risk for CAD is found above 8 μM without threshold (Boushey et al., 1995), suggesting that this is the lower limit that should be targeted for maximum risk reduction. Currently, most references use a homocysteine plasma concentration of ~12 to 15 μM to define hyperhomocysteinemia. Our findings suggest that it is not the absolute value that is important for maximum vasoprotection, but the ability to further decrease homocysteine concentrations by >2 μM, which can be achieved through low-dose vitamin supplementation. Whether long-term homocysteine lowering through vitamin supplementation will not only improve endothelial function but also prevent cardiovascular complications must be determined in prospective studies currently in progress.

In conclusion, our findings demonstrate that lowering of total plasma homocysteine concentration improves resistance vessel reactivity in patients with established CAD. Our results support the hypothesis that intake of folic acid is vasoprotective through lowering plasma homocysteine.

References


References


References


