Folic Acid Improves Arterial Endothelial Function in Adults With Hyperhomocystinemia

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OBJECTIVES
To evaluate whether oral folic acid supplementation might improve endothelial function in the arteries of asymptomatic adults with hyperhomocystinemia.

BACKGROUND
Hyperhomocystinemia is an independent risk factor for endothelial dysfunction and occlusive vascular disease. Folic acid supplementation can lower homocystine levels in subjects with hyperhomocystinemia; however, the effect of this on arterial physiology is not known.

METHODS
Adults subjects were recruited from a community-based atherosclerosis study on healthy volunteers aged 40 to 70 years who had no history of hypertension, diabetes mellitus, hyperlipidemia, ischemic heart disease or family history of premature atherosclerosis (n = 89). Seventeen subjects (aged 54 ± 10 years, 15 male) with fasting total homocystine levels above 75th percentile (mean, 9.8 ± 2.8 μmol/liter) consented to participate in a double-blind, randomized, placebo-controlled and crossover trial; each subject received oral folic acid (10 mg/day) and placebo for 8 weeks, each separated by a washout period of four weeks. Flow-mediated endothelium-dependent dilation (percent increase in diameter) of the brachial artery was assessed by high resolution ultrasound, before and after folic acid or placebo supplementation.

RESULTS
Compared with placebo, folic acid supplementation resulted in higher serum folate levels (66.2 ± 7.0 vs. 29.7 ± 14.8 nmol/liter; p < 0.001), lower total plasma homocystine levels (8.1 ± 3.1 vs. 9.5 ± 2.5 μmol/liter, p = 0.03) and significant improvement in endothelium-dependent dilation (8.2 ± 1.6% vs. 6 ± 1.3%, p < 0.001). Endothelium-independent responses to nitroglycerin were unchanged. No adverse events were observed.

CONCLUSION
Folic acid supplementation improves arterial endothelial function in adults with relative hyperhomocystinemia, with potentially beneficial effects on the atherosclerotic process.

Recent epidemiologic studies have demonstrated a clear association between hyperhomocystinemia (HHC) and premature coronary, cerebral and peripheral vascular disease (1,2). HHC is found in approximately one in three subjects with premature coronary artery disease and is therefore considered a common as well as an independent risk factor for occlusive vascular disease (1). Homocystine (Hcy) is a highly reactive amino acid, damaging to arterial endothelial cells in both cell culture and experimental animal studies (3,4), and recently we (5) and others have demonstrated impaired arterial endothelial function in otherwise healthy adults with HHC (6) or homocystine levels at upper physiologic range (7).

Folic acid is a naturally occurring nutritional element that reduces homocystine levels by increasing the rate of recycling of homocystine to methionine (2). It is unknown, however, whether oral folic acid supplements can modify vascular disease in subjects with HHC. As arterial endothelial dysfunction is an important early event in atherogenesis (8), we assessed the effect of folic acid on endothelial physiology in adults with HHC, in a double-blind, randomized, placebo-controlled study.

METHODS

Subjects. Adult subjects were recruited from a community-based atherosclerosis study carried out in Hong Kong on healthy volunteers aged 40 to 70 years who had no history of hypertension, diabetes mellitus, hyperlipidemia, ischemic heart disease or family history of premature atherosclerosis (5). None were taking any cardiovascular medications.

HHC was defined as fasting total plasma Hcy (tHcy) above...
the 75th percentile for the 89 subjects studied to date; of the 22 adults approached, 17 gave informed consent and participated in the folic acid trial. None had been taking multivitamin supplements in the 12 months before enrollment. This study was approved by the institutional ethics committee.

**Study design.** After enrollment, each subject was randomized to receive either oral folic acid (10 mg/day) or placebo for eight weeks, in a double-blind fashion, followed by a “washout” period of four weeks, then crossed over to either placebo or folic acid for the final eight weeks. A 10–mg oral daily dose of folic acid is known to be safe and was chosen to maximize the Hcy-lowering effect within the time frame of the study. Subjects were reviewed at the end of each eight-week treatment period (but not after the washout phase). On each occasion, subjects attended after a 14-h fast (except for the study medication) for a blood test, measurement of resting supine blood pressure and an arterial ultrasound study (as described below). Ultrasound studies were analyzed by an observer “blinded” in every case to subject identity and to the stage of the experiment, as previously reported (9).

**Methods.** At baseline study, venous blood from fasting subjects was assayed for total cholesterol, triglycerides, HDL-cholesterol, glucose and creatinine levels by standard techniques. At baseline and also after each of the eight-week treatment periods, vitamin B12 and folate levels in serum were measured by immunoassay (Abbott IMX Analyzer; Abbott Park, Illinois) (10). The coefficient of variation of this assay is 3.4% at plasma homocystine concentrations of 7.7 to 29.1 μmol/liter. On repeated measurement, we have been unable to reproduce the very high homocystine levels previously reported by our group, using high performance liquid chromatography (5). This may have been due to problems with sample preparation, or the analytical technique itself, where measurement of total plasma homocystine level along with all sulfated groups may have accounted for a significant and systematic overestimation of the true level. On reanalyzing all samples, by the enzymatic immunoassay method, the “high tHcy” group (top 25%) had levels of 10.5 ± 3.0 μmol/liter and the “low tHcy” group had levels of 8.5 ± 1.1 μmol/liter, and there remains a significant difference between groups (p < 0.05) (11). Therefore, the current study still enrolled a group of relatively HHC subjects, from our population.

Endothelial function of the brachial artery was studied at baseline using high resolution ultrasound, as described previously (5,9). In brief, the diameter of the brachial artery was measured on B-mode ultrasound images, using a (median frequency, 7.5 MHz) linear array transducer (L 10–5) and a standard Advanced Technology Laboratories 3000 system. Scans were acquired at rest, during posttour- niquet reactive hyperemia (to induce endothelium-dependent dilation, EDD) and after sublingual glyceryl- trinitrate (GTN, an endothelium-independent dilator). EDD is predominantly due to endothelial nitric oxide release (12), and endothelial responses of the brachial artery measured by this method correlate significantly well with both coronary endothelial function in the same subjects (13) and with the extent of coronary atherosclerosis (14). The accuracy, reproducibility and low interobserver error for this measurement of arterial physiology have been demonstrated previously (9).

**Statistical analysis.** Descriptive data are expressed as mean ± SD. The primary study end point was the comparison between EDD on folic acid or on placebo; this was performed using the paired Student t test. Other outcome variables after folic acid or placebo were also compared using the Student t test, adjusted for multiple comparisons (15). Determinants of the folic acid-related improvement in EDD (calculated for each subject) were explored by standard univariate and multivariate regression analyses, with age, gender, degree of reactive hyperemia, vessel size, total cholesterol and change in serum folate and homocystine levels as the independent variables. Statistical significance was inferred at a two-tailed p value <0.05.

**RESULTS**

All 17 subjects completed the crossover protocol. No adverse events were reported, and tablet counts revealed >90% compliance with all study medications in each case. Subjects were aged 54 ± 10 (41 to 69) years; 15 were male and 2 were regular smokers. At baseline, the fasting tHcy measured was 9.8 ± 2.8 (8 to 20) μmol/liter, total cholesterol was 5.7 ± 0.8 (3.6 to 6.7) mmol/liter, mean serum folate level was 34.4 ± 10.4 nmol/liter, and glucose, creatinine and vitamin B12 levels were within normal limits in all subjects. EDD (percentage increase in brachial artery diameter) was 5.7 ± 1.2%, and GTN-induced dilation was 17.8 ± 5.0%.

Compared with placebo, folic acid supplementation resulted in a higher serum folate level (66.2 ± 7 after folate vs 29.7 ± 14.8 nmol/liter after placebo, p < 0.001), lower tHcy levels (8.1 ± 3 vs. 9.5 ± 2.5 μmol/liter, p = 0.03), but no significant changes in cholesterol or vitamin B12 levels (p > 0.10) (Table 1). There was a significant improvement (relative increase of 36.7 ± 21.8%) in EDD after folic acid
There was no significant change, however, in the GTN response (16.6 ± 2.9% vs. 18.0 ± 2.4%, p = 0.30). These data are consistent with improved endothelial function after folic acid supplementation.

On regression analyses, the folic acid-related improvement in EDD was not associated with subject age, gender, vessel size, degree of reactive hyperemia or serum total cholesterol, but associated marginally with changes in tHcy (r = 0.35, p = 0.17) and folate levels (r = 0.39, p = 0.12).

**DISCUSSION**

A large number of epidemiologic studies have now identified HHC as a common and independent risk factor for premature vascular events (1,2). Cellular, experimental animal and human studies have recently implicated endothelial injury as a key early event in Hcy-related arterial disease (3–5). For example, we have previously documented impaired endothelial function in children with homozygous homocystinuria (16) and in adults in the highest quartile range of homocysteine levels within our population (5,11). The relationship of hyperhomocystinemia and endothelial dysfunction has been reported to be concentration-dependent and present even at physiologic levels of homocysteine (7). This may be due to the chemical effects of the free thiol group on Hcy, interacting with nitric oxide or reducing disulfide bonds in endothelial cell molecules (3,17), and/or due to the local generation of reactive oxygen species (18). The pathophysiologic consequences of such endothelial injury may include impaired release of nitric oxide by the vessel wall (3), with significant alterations in arterial reactivity and abnormal interactions among the vessel wall, platelets and leukocytes (19). All of these vascular changes associated with arterial endothelial dysfunction are known to be important events in atherogenesis (8).

**Homocysteine, folate and arterial endothelial function.** Recent studies have demonstrated that oral folic acid in a daily dose of 1 to 10 mg consistently reduces Hcy levels by 10 to 20% (19–21). As Hcy itself has toxic effects on arterial endothelial cells, and folic acid has an excellent safety profile, this agent may therefore have therapeutic applications in lowering vascular risk in subjects with HHC. In this study, we now demonstrate that folic acid supplementation improves nitric oxide-mediated arterial endothelial function.

### Table 1. Biochemical Parameters and Vascular Study Results at Baseline, After Eight Weeks of Placebo and Eight Weeks of Folic Acid Supplementation, in 17 Adults With Hyperhomocystinemia

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Baseline</th>
<th>Placebo</th>
<th>Folate</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate (nmol/liter)</td>
<td>34.4 ± 10.4</td>
<td>29.7 ± 14.8</td>
<td>66.2 ± 7.0</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>tHcy (µmol/liter)</td>
<td>9.8 ± 2.8</td>
<td>9.5 ± 2.5</td>
<td>8.1 ± 3.0</td>
<td>0.03</td>
</tr>
<tr>
<td>Vitamin B12 (pmol/liter)</td>
<td>372.1 ± 168.8</td>
<td>422.6 ± 183.3</td>
<td>391.7 ± 148.5</td>
<td>0.64</td>
</tr>
<tr>
<td>Cholesterol (mmol/liter)</td>
<td>5.7 ± 0.8</td>
<td>5.4 ± 1.0</td>
<td>5.4 ± 0.8</td>
<td>0.90</td>
</tr>
<tr>
<td>Vessel size (mm)</td>
<td>4.1 ± 0.6</td>
<td>4.1 ± 0.6</td>
<td>4.1 ± 0.6</td>
<td>0.90</td>
</tr>
<tr>
<td>EDD (%)</td>
<td>5.7 ± 1.2</td>
<td>6.0 ± 1.3</td>
<td>8.2 ± 1.6</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>GTN (%)</td>
<td>17.8 ± 5.0</td>
<td>16.6 ± 2.9</td>
<td>18.0 ± 2.4</td>
<td>0.30</td>
</tr>
<tr>
<td>Hyperemia (%)</td>
<td>618 ± 503</td>
<td>772 ± 402</td>
<td>752 ± 403</td>
<td>0.90</td>
</tr>
</tbody>
</table>

*Comparing the postplacebo and postfolic acid results: see “Methods” section.

B-12 = Vitamin B-12; EDD = endothelium-dependent dilation; GTN = glyceryltrinitrate-induced dilation; tHcy = total plasma homocyst(e)ine.

(8.2 ± 1.6% vs. 6 ± 1.3% with placebo, mean absolute difference 2.2 ± 1.3%, p < 0.001) (Fig. 1). There was no significant change, however, in the GTN response (18 ± 2.4% vs. 16.6 ± 2.9%, p = 0.30). These data are consistent with improved endothelial function after folic acid supplementation.

**Figure 1.** EDD at baseline and after eight weeks of therapy with either oral folic acid, 10 mg/day (left panel) or placebo (right panel). For each condition, the mean and SD for the group are presented.
in healthy adults with relatively high levels of Hcy (for the population studied). After eight weeks of oral folic acid, average EDD improved from 6 ± 1.3% to 8.2 ± 1.6% (36.7 ± 21.8% relative improvement) (whereas average EDD in control subjects from our population with lower range Hcy levels is 10.8 ± 1.7%) (5). Folic acid supplementation for a brief period of 8 weeks was associated with 15% lowering of Hcy level, which conforms to the responses previously reported, but may be underestimating the potential achievable after more prolonged supplementation. The mechanism whereby folic acid improves arterial EDD is uncertain. It could be due to reduction of Hcy levels, elevation of folate levels or another mechanism (20–22). We have observed a trend of association between changes in Hcy levels and improvement in EDD, which was not statistically significant however, possibly due to the relatively small sample size. An alternative explanation for improved arterial endothelial function is an independent vascular effect of folic acid, as suggested by a recent report demonstrating that 5-methyltetrahydrofolate (an active form of folic acid) restored endothelial function in familial hypercholesterolemia (23). However, to our knowledge, there have been no data on folate supplementation given to hypercholesterolemic, smoking patients with low Hcy levels.

**Studies of reversibility of endothelial dysfunction.** In the current study, we have investigated endothelial function in the brachial artery using high resolution vascular ultrasound, an accurate and reproducible test (9) that relates mainly to endothelial nitric oxide release in response to shear stress (12). Endothelial function in the brachial artery correlates significantly with that in the coronary vasculature (13) as well as with the extent of coronary disease assessed angiographically (14). As this method is noninvasive, we and others have reported the effects of certain interventions on serial measurements of endothelial function, both in the setting of primary prevention (for example, the use of L-arginine to improve EDD in asymptomatic hypercholesterolemic young adults) (24) and in secondary prevention (for example, vitamin C in patients with established coronary disease) (25). In the case of cholesterol lowering, there is evidence that this therapy improves both brachial and coronary endothelial function as well as cardiovascular outcomes (26–28). The recent demonstration that coronary endothelial function testing may have prognostic value (29) suggests that interventions that improve EDD may have clinical relevance, and this possibility awaits further prospective study. On the basis of our current observation that oral folic acid both lowers homocystine levels in relatively HHC subjects and improves EDD, clinical trials of this well-tolerated therapy may be indicated.

**Study limitations.** The current findings of improved arterial endothelial function after oral folic acid (10 mg/d) may be applicable only to asymptomatic subjects with higher range baseline Hcy levels, and may not necessarily be seen in patients with HHC in the setting of coronary artery disease or in subjects with low homocystine levels. It is also possible that lower doses of folic acid might result in similar benefit; however, the daily 10-mg dose of folic acid used in this trial was well tolerated by all subjects and not associated with any adverse events. Further studies will be required to assess if the improved endothelial function will be sustained for periods longer than eight weeks, or whether this arterial improvement will translate into a decrease in clinical event rates in subjects with HHC. As the beneficial effects on endothelial function were no longer present 12 weeks after treatment cessation (in those receiving folic acid then placebo, and in whom the folate levels had returned to baseline values), this suggests that it may be necessary to continue long-term supplementation to maintain the observed significant improvement in vascular physiology.

**CONCLUSIONS**

An emerging literature suggests an important link among HHC, arterial endothelial dysfunction and premature vascular events. Given the high prevalence of atherosclerosis in developed nations, strategies for intervention in this sequence of arterial damage may have important public health implications. By lowering homocystine levels and improving arterial endothelial function in humans, oral folic acid may have therapeutic potential as a vascular protective agent, in HHC subjects.

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