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Folic Acid Improves Endothelial Function in Coronary Artery Disease via Mechanisms Largely Independent of Homocysteine Lowering

Sagar N. Doshi, BSc, MBChB, MRCP; Ian F.W. McDowell, MD, MRCP, FRCPath; Stuart J. Moat, BSc, PhD; Nicola Payne, BSc; Hilary J. Durrant, PhD, MBChB, MRCP; Malcolm J. Lewis, PhD, DSc, FESC; Jonathan Goodfellow, BSc, MBBS, MRCP

Background—Homocysteine is a risk factor for coronary artery disease (CAD), although a causal relation remains to be proven. The importance of determining direct causality rests in the fact that plasma homocysteine can be safely and inexpensively reduced by 25% with folic acid. This reduction is maximally achieved by doses of 0.4 mg/d. High-dose folic acid (5 mg/d) improves endothelial function in CAD, although the mechanism is controversial. It has been proposed that improvement occurs through reduction in total (tHcy) or free (non–protein bound) homocysteine (fHcy). We investigated the effects of folic acid on endothelial function before a change in homocysteine in patients with CAD.

Methods and Results—A randomized, placebo-controlled study of folic acid (5 mg/d) for 6 weeks was undertaken in 33 patients. Endothelial function, assessed by flow-mediated dilatation (FMD), was measured before, at 2 and 4 hours after the first dose of folic acid, and after 6 weeks of treatment. Plasma folate increased markedly by 1 hour (200 compared with 25.8 nmol/L; \( P < 0.001 \)). FMD improved at 2 hours (83 compared with 47 \( \mu \)m; \( P < 0.001 \)) and was largely complete by 4 hours (101 compared with 51 \( \mu \)m; \( P < 0.001 \)). tHcy did not significantly differ acutely (4-hour tHcy, 9.56 compared with 9.79 \( \mu \)mol/L; \( P = 0.02 \)). fHcy did not differ at 3 hours but was slightly reduced at 4 hours (1.55 compared with 1.78 \( \mu \)mol/L; \( P = 0.02 \)). FMD improvement did not correlate with reductions in either fHcy or tHcy at any time.

Conclusions—These data suggest that folic acid improves endothelial function in CAD acutely by a mechanism largely independent of homocysteine.

Key Words: risk factors ■ plasma ■ coronary disease ■ endothelium

Elevated total plasma homocysteine (tHcy) is now an accepted and potentially modifiable risk factor for cardiovascular disease and death that appears to be largely independent of other conventional risk factors.1,2 However, it remains controversial as to whether the increased risk is mediated directly by homocysteine or whether homocysteine may simply be an epiphenomenon.3

Plasma homocysteine can be safely and inexpensively reduced by supplementation with oral B-group vitamins. Folic acid reduces total plasma homocysteine by 25%4 and is maximally achieved by doses of 0.4 to 0.5 mg daily.4,5 This has led to the proposal that folic acid treatment may reduce cardiovascular risk by reducing tHcy.

Endothelial dysfunction is a key process in atherosclerosis6 and independently predicts cardiovascular events.7 High-dose folic acid (5 mg daily), alone or in combination with other B-group vitamins, can improve endothelial function in patients with coronary artery disease (CAD). However, the data are limited, and whether this improvement is due to a reduction in homocysteine or some other effect of folic acid is uncertain.8–10

We sought to investigate whether high-dose folic acid supplementation would improve endothelial dysfunction in patients with significant CAD who were taking standard therapy, independent of changes in homocysteine.

Methods

Study Design

The study was a randomized, double-blind, placebo-controlled, parallel design in which 33 patients were allocated to receive either 5 mg daily folic acid (16 patients) or matched placebo (17 patients) for 6 weeks. Patients attended on 2 occasions separated by 6 weeks. All patients gave written informed consent, and the protocol conformed to the Declaration of Helsinki.
Subjects
Patients with CAD who were <70 years of age were recruited. We defined CAD as either angiographically proven coronary disease (≥50% luminal stenosis) or a history of myocardial infarction (creatinine kinase rise >2-fold normal with ECG changes). The level of plasma homocysteine was not an entry criterion. Patients were excluded if an acute coronary event had occurred <3 months before entry or if there was diabetes mellitus, uncontrolled hypertension, fasting plasma cholesterol >6.5 mmol/L, impaired renal function (creatinine >120 μmol/L), or clinically significant heart failure. Patients actively smoking or who had recently ceased smoking (<6 months), patients taking antioxidant vitamins (E or C), folic acid, or fish oils, and women taking hormone replacement were also excluded. All selected subjects were tested to exclude vitamin B12 deficiency before entry, which precludes folic acid treatment.

Study Protocol
Biochemical Parameters
Lipids, glucose, creatinine, and B12 were analyzed on the day of sampling; other samples were separated and the serum/plasma stored at −70°C until analysis. EDTA plasma for fHcy (non–protein bound) assay was deproteinized within 15 minutes of venepuncture before storage. EDTA plasma for tHcy assay was immediately separated by centrifuging before storage. At the first visit, venous blood was drawn before and at each hour for 4 hours after the first tablet for assay of plasma folate, fHcy, and tHcy. 5-Methyltetrahydrofolate (5-MTHF) was assayed in the folate group at baseline, 1 to 4 hours, and 6 weeks. Blood (creatinine kinase rise (≥2-fold normal with ECG changes)). The level of plasma homocysteine was not an entry criterion. Patients were excluded if an acute coronary event had occurred <3 months before entry or if there was diabetes mellitus, uncontrolled hypertension, fasting plasma cholesterol >6.5 mmol/L, impaired renal function (creatinine >120 μmol/L), or clinically significant heart failure. Patients actively smoking or who had recently ceased smoking (<6 months), patients taking antioxidant vitamins (E or C), folic acid, or fish oils, and women taking hormone replacement were also excluded. All selected subjects were tested to exclude vitamin B12 deficiency before entry, which precludes folic acid treatment.

Noninvasive Measurement of Endothelial Function
Endothelial vasomotor function was assessed by flow-mediated dilatation (FMD), a nitric oxide–mediated process. FMD was measured by means of high-resolution ultrasound and wall-tracking, as previously described by us, after release of a cuff placed at the wrist, inflated for 5 minutes at 250 mm Hg. FMD was taken as the greatest absolute increase in vessel end-diastolic diameter (EDD) during the first 3 minutes after cuff release. Vascular studies were performed by a single experienced operator in a temperature-controlled room (21°C to 24°C) at the same time of day on patients fasted overnight. Medications were omitted on the morning of the visit, and nitrates were withheld for 24 hours before studies. Endothelium-independent dilation, in response to 400 μg glyceryl trinitrate, was recorded at baseline, 4 hours, and 6 weeks. Blood pressure was measured continuously in the study arm by means of photoplethysmography (Finapres). Blood flow was calculated as the product of the Doppler time-velocity integral, heart rate, and brachial artery diameter measured by wall-tracking at that time. FMD was recorded before, at 2 and 4 hours after the first dose, and after 6 weeks of daily treatment.

Biochemical Assays
Lipids, glucose, and creatinine were assayed routinely. fHcy was measured by enzymatic immunoassay (Abbott IMX, Abbott Diagnostics) and B12 was measured by competitive protein binding assays on an Elecsys 2010 analyzer (Roche Diagnostics). Folate was measured by competitive binding assay with an Immx analyzer (Abbott Diagnostics). fHcy was measured by HPLC with fluorescence detection, with a modification of the technique described by Araki and Sako. 5-MTHF was measured by HPLC.16

Withdrawals, Medication Changes, and Compliance
All randomized patients completed the study. Other medications remained unchanged during the study period. No side effects were reported, and compliance assessed by a tablet count was >98%.

TABLE 1. Clinical Characteristics of Study Subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Placebo (n=17)</th>
<th>Folate (n=16)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>56±7</td>
<td>55±7</td>
<td>NS</td>
</tr>
<tr>
<td>Male:female</td>
<td>16/1</td>
<td>14/2</td>
<td>NS</td>
</tr>
<tr>
<td>Height, m</td>
<td>1.73±0.05</td>
<td>1.71±0.07</td>
<td>NS</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>81.2±12</td>
<td>89.9±13</td>
<td>NS</td>
</tr>
<tr>
<td>Angiographic CAD</td>
<td>16 (94.1)</td>
<td>16 (100)</td>
<td>NS</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>10 (58.8)</td>
<td>9 (56.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Revascularization (CABG or PTCA)</td>
<td>15 (88.3)</td>
<td>13 (81.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension</td>
<td>7 (41.2)</td>
<td>5 (31.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Family history &lt;60 y</td>
<td>11 (64.7)</td>
<td>9 (56.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking history</td>
<td>13 (81.3)</td>
<td>10 (58.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Cerebrovascular event</td>
<td>1 (5.9)</td>
<td>0 (0)</td>
<td>NS</td>
</tr>
<tr>
<td>Peripheral vascular disease</td>
<td>1 (5.9)</td>
<td>1 (6.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Angina</td>
<td>2 (11.8)</td>
<td>2 (12.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Antplatelet therapy</td>
<td>15 (88.3)</td>
<td>16 (100)</td>
<td>NS</td>
</tr>
<tr>
<td>Lipid-lowering therapy</td>
<td>16 (94.1)</td>
<td>16 (100)</td>
<td>NS</td>
</tr>
<tr>
<td>Statin</td>
<td>14 (82.4)</td>
<td>14 (87.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Fibrate</td>
<td>3 (17.6)</td>
<td>3 (18.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Other drug therapy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Blocker</td>
<td>10 (58.8)</td>
<td>6 (37.5)</td>
<td>NS</td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>1 (5.9)</td>
<td>1 (6.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Calcium channel blocker</td>
<td>2 (11.8)</td>
<td>3 (18.8)</td>
<td>NS</td>
</tr>
<tr>
<td>ATII receptor blocker</td>
<td>0 (0)</td>
<td>1 (6.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Nitrate</td>
<td>2 (11.8)</td>
<td>1 (6.3)</td>
<td>NS</td>
</tr>
</tbody>
</table>

ATII receptor blocker indicates angiotensin II receptor antagonist. Data are presented as mean±SD or patient numbers and percentages in parentheses.

Statistical Analysis
Results are expressed as mean±SD unless otherwise stated. The main statistical analysis of the folic acid study was analyzed by ANCOVA. The associations between changes in FMD and fHcy, tHcy, and 5-MTHF were assessed by Spearman rank correlation analysis. A value of P<0.05 was considered statistically significant. We calculated that 18 subjects would be required to detect an improvement in FMD from 40 μm (SD, 20 μm) to 80 μm (SD, 30 μm) in the intervention group, with 90% power at the 5% significance level.

Results
Baseline Characteristics
The study comprised 33 patients (30 men and 3 women). There were no significant differences in baseline clinical or biochemical parameters (Tables 1 and 2).

Effects on FMD and Vascular Measurements
FMD was impaired in both groups at baseline compared with published normal values (folic acid, 1.26±0.75% EDD; placebo, 1.14±0.62% EDD) (Table 3). After folic acid, FMD improved markedly at 2 hours after the first dose (Figure 1), with small additional improvement after 6 weeks of daily treatment compared with the change at 4 hours (FMD, 111 compared with 101 μm; P=0.04). There was no significant difference between the glyceryl trinitrate response.
TABLE 2. Biochemical Parameters at Baseline and 6 Weeks

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline (n=17)</th>
<th>Folate (n=16)</th>
<th>P*</th>
<th>Baseline (n=17)</th>
<th>Folate (n=16)</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma folate, nmol/L</td>
<td>26.09±6.8</td>
<td>22.37±8.7</td>
<td>NS</td>
<td>26.8±5.6</td>
<td>604.2±429</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total homocysteine, µmol/L</td>
<td>10.8±2.2</td>
<td>10.6±2.6</td>
<td>NS</td>
<td>10.8±2.1</td>
<td>8.3±1.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Free homocysteine, µmol/L</td>
<td>1.99±0.47</td>
<td>2.05±0.49</td>
<td>NS</td>
<td>1.84±0.38</td>
<td>1.46±0.34</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vitamin B12, pmol/L</td>
<td>312±105</td>
<td>105±306</td>
<td>NS</td>
<td>326±97</td>
<td>327±81</td>
<td>NS</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>4.47±0.57</td>
<td>4.36±0.87</td>
<td>NS</td>
<td>4.3±0.58</td>
<td>4.6±0.84</td>
<td>0.01</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>1.16±0.36</td>
<td>1.08±0.2</td>
<td>NS</td>
<td>1.16±0.42</td>
<td>1.09±0.23</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>2.65±0.48</td>
<td>2.7±0.77</td>
<td>NS</td>
<td>2.41±0.47</td>
<td>2.74±0.83</td>
<td>NS</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>5.49±0.75</td>
<td>5.6±0.77</td>
<td>NS</td>
<td>5.52±0.72</td>
<td>5.61±0.93</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine, µmol/L</td>
<td>95.1±12.1</td>
<td>98.7±18.1</td>
<td>NS</td>
<td>92.8±11.4</td>
<td>95.2±14.5</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD. Comparing changes on folic acid with changes on placebo at baseline* and after 6 weeks of treatment †.

Effects on Biochemical Parameters

Plasma folate in the treatment group increased markedly by 1 hour and remained elevated at 2, 3, and 4 hours and at 6 weeks (Figure 2). tHcy fell in both groups during the initial 4 hours. There were no significant differences between tHcy levels during the 4 hours after the first dose of folic acid, but tHcy was significantly decreased after 6 weeks of treatment (Figure 3). fHcy (non–protein bound) fell in both groups but was not significantly altered during the first 3 hours after folic acid. tHcy was significantly reduced at 4 hours and 6 weeks (Figure 4). 5-MTHF increased from a baseline of 22.1 to 132.1 at 4 hours and 305.7 nmol/L at 6 weeks in the folate group (Figure 5). Vitamin B12, HDL, LDL, triglycerides, glucose, and creatinine were unchanged by folic acid after 6 weeks. Cholesterol was marginally higher in the folic acid group and lower in the placebo group at 6 weeks (Table 2).

Correlates of Improved FMD

In univariate analysis, the change in FMD did not correlate with either tHcy or fHcy reduction or with folate or 5-MTHF increase at any point of the intervention (2 or 4 hours or 6 weeks; P=NS).

Discussion

The present study confirms and extends the findings of recent studies demonstrating improved endothelial function in patients with CAD taking high-dose folic acid (5 mg daily for 1.5 to 4 months) alone or in combination with other B-group vitamins.

The mechanism underlying this improvement is controversial and has been previously attributed to reductions in either tHcy or fHcy that occur with folate therapy. The present study confirms these findings and extends them to include improved FMD and endothelial function in patients taking high-dose folic acid.
study, however, provides new evidence indicating that homocysteine reduction is unlikely to account for the acute and possibly the chronic improvement observed with folic acid. Improvement in endothelial function was observed acutely after the first oral dose of folic acid and before any significant reduction in tHcy. tHcy fell in both groups in the acute phase, an effect that has been previously noted and due to reduction in plasma albumin induced by supine posture.\textsuperscript{18} Interestingly, despite a large fall in tHcy in the placebo group (9.8 ± 3.9% at 4 hours), no change in endothelial function was observed. This observation strengthens the conclusion that improved endothelial function in the folate group was not mediated by the similar reduction in tHcy in the acute phase. Furthermore, no correlation was found between FMD improvement and tHcy reduction at any time point. At 6 weeks, tHcy was significantly reduced by folic acid, with only a small further improvement in FMD compared with the effect at 4 hours (111 compared with 101 μm; \(P=0.042\)). This finding is consistent with some additional improvement conferred by tHcy reduction at 6 weeks but may be explained by enhancement after long-term folic acid treatment.

Improvement in FMD also occurred before a significant fall in fHcy, indicating that enhancement was also not explained by reduction in fHcy. Furthermore, no correlation was found between improvement in FMD and reduction in fHcy at any time.

Levels of plasma folate were increased to supraphysiological levels (>45.3 nmol/L) by 1 hour and remained elevated thereafter. Under normal conditions, the majority (>80%) of circulating folate exists as the metabolically active form, 5-MTHF. Plasma 5-MTHF is increased acutely after folic acid loading.\textsuperscript{19} Levels were supraphysiological by 1 hour after folic acid and were further increased at 6 weeks compared with the 4-hour level. The mechanism behind this increase is unclear but in part may be explained by induction of dihydrofolate reductase, the enzyme responsible for the initial step in the conversion of folic acid to 5-MTHF. Such induction has been observed with methotrexate in vitro after 48 hours.\textsuperscript{20} Whatever the mechanism, the increased level of 5-MTHF at 6 weeks may account for the further improvement in endothelial function observed at 6 weeks.

It is likely that the majority of improvement in endothelial function observed in the present study is due to direct pharmacological actions of folic acid rather than reductions in tHcy or fHcy. It is important to stress that plasma folate levels observed in this study cannot be achieved by dietary fortification with folic acid (up to 0.665 mg/d)\textsuperscript{21} or low-dose (0.4 mg) folic acid supplementation.\textsuperscript{5} Plausible mechanisms exist.
to explain how folic acid may acutely enhance endothelial function independent of homocysteine. In vitro, 5-MTHF has intrinsic antioxidant actions, can increase nitric oxide production by endothelial nitric oxide synthase, and, furthermore, can reduce superoxide production by endothelial nitric oxide synthase in the setting of reduced cofactor tetrahydrobiopterin (BH₄) bioavailability. In vivo, the intra-arterial infusion of 5-MTHF will acutely improve endothelial function in familial hypercholesterolemia and CAD without lowering homocysteine. Similarly, the effects of methionine loading (100 mg/kg), which increases plasma homocysteine and acutely impairs endothelial function, is abrogated by simultaneous administration of folic acid (20 mg) without alteration of homocysteine increase.

**Implications of the Study**

These data show that improved endothelial function observed with high-dose folic acid in CAD occurs acutely and is independent of changes in either tHcy or fHcy. It seems probable that this occurs largely by direct mechanisms other than through homocysteine reduction. The homocysteine-reducing effect of folic acid in CAD is maximal at 0.4 mg in patients with normal renal function. However, if homocysteine lowering is not the major mechanism of benefit of high-dose folic acid, on-going outcome studies with moderate doses of folate in patients with cardiovascular disease (0.8 to 2.5 mg) may not reveal benefit despite lowering homocysteine. This may reflect the lower doses of folate used, and this point should be borne in mind when interpreting the results of these studies.

**Acknowledgments**

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**References**