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Folate Improves Endothelial Function in Coronary Artery Disease

An Effect Mediated by Reduction of Intracellular Superoxide?

Sagar N. Doshi, Ian F.W. McDowell, Stuart J. Moat, Derek Lang, Robert G. Newcombe, Mahmud B. Kredan, Malcolm J. Lewis, Jonathan Goodfellow

Abstract—Homocysteine is a risk factor for coronary artery disease (CAD). Folic acid lowers homocysteine and may improve endothelial function in CAD, although the mechanism is unclear. We investigated the effect of folic acid on endothelial function, homocysteine, and oxidative stress in patients with CAD. We also examined the acute effect of 5-methyltetrahydrofolate (5-MTHF), the principal circulating folate, on endothelial function in vivo and on intracellular superoxide in cultured endothelial cells. A randomized crossover study of folic acid (5 mg daily) for 6 weeks was undertaken in 52 patients with CAD. Ten further patients were given intra-arterial 5-MTHF. Endothelial function was assessed by flow-mediated dilatation (FMD). Folic acid increased plasma folate ($P<0.001$), lowered homocysteine by 19% ($P<0.001$), and improved FMD ($P<0.001$). FMD improvement did not correlate with homocysteine reduction. Malondialdehyde and total plasma antioxidant capacity, markers of oxidative stress, were unchanged. 5-MTHF acutely improved FMD ($P<0.001$) without altering homocysteine ($P=0.47$). In vitro, 5-MTHF abolished homocysteine-induced intracellular superoxide increase ($P<0.001$); this effect was also observed with folic acid and tetrahydrobiopterin. Our data support the beneficial effect of folic acid on endothelial function in CAD but suggest that the mechanism is independent of homocysteine. Reduction of intracellular endothelial superoxide may have contributed to the effect. (*Arterioscler Thromb Vasc Biol.* 2001;21:1196-1202.)

Key Words: folic acid ■ homocysteine ■ coronary artery disease ■ endothelial function ■ 5-methyltetrahydrofolate

Elevated total plasma homocysteine (tHcy) is associated with an increased risk of cardiovascular disease and appears to be largely independent of other conventional risk factors.¹ However, whether the increased risk is mediated directly by homocysteine or whether it may simply be a marker remains controversial.²

The metabolism of folate and homocysteine are interrelated, and increasing folate intake augments remethylation of homocysteine, leading to a reduction of up to 25% in its plasma concentration.³ This effect occurs despite normal plasma folate and can be achieved by folic acid in doses of 400 μg to 5 mg/d.^{3,4} This has led to the proposal that folic acid treatment may reduce cardiovascular risk by reducing tHcy.

In homocystinuria, a rare inborn error with markedly elevated tHcy concentrations ($>100 \mu\text{mol/L}$), folic acid and pyridoxine (vitamin B₆) lower tHcy and reduce cardiovascular events, the major cause of mortality.⁵ This benefit is observed despite residual tHcy concentrations that are often above the upper limit of "normal," which suggests a benefit from B group vitamins that is independent of homocysteine.⁶ However, in the general population, in which tHcy concen-

trations are much lower (5 to 15 $\mu\text{mol/L}$),⁷ there are, as yet, only limited data on the effect of folic acid treatment on cardiovascular outcome.^{8,9}

Endothelial dysfunction is a key process in atherosclerosis¹⁰ and has been reported in chronic mild fasting hyperhomocysteinemia in subjects free of vascular disease¹¹ and in experimental hyperhomocysteinemia, which is induced by methionine loading in normal subjects.¹² Precisely how homocysteine may promote endothelial dysfunction is unclear; however, generation of reactive oxygen species is proposed to be an important mechanism.¹³ Recent studies suggest that B group vitamins enhance endothelial function in coronary artery disease (CAD) or hyperhomocysteinemia. However, the data are limited, and the mechanism underlying this improvement has not been established.¹⁴⁻¹⁷

5-Methyltetrahydrofolate (5-MTHF), the main circulating folate, can improve endothelial function in subjects with hypercholesterolemia who are free of vascular disease and not receiving lipid-lowering treatment.¹⁸ However, the effect of 5-MTHF in subjects with severe CAD on lipid-lowering treatment is unknown.

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We sought to determine whether high-dose folic acid supplementation can improve endothelial dysfunction, a surrogate of cardiovascular risk, in patients with significant CAD on standard therapy, and we endeavored to correlate this effect, if any, with changes in plasma homocysteine, plasma folate, and oxidant stress. To further investigate mechanisms, we examined the acute effect of 5-MTHF on endothelial function in a similar group of 10 patients and its effect on intracellular levels of superoxide in cultured porcine aortic endothelial (PAE) cells.

Methods

Subjects

Patients with CAD aged <70 years were recruited. We defined CAD as either angiographically proven coronary disease ($\geq 50\%$ luminal stenosis) or a history of myocardial infarction (creatinine kinase rise >2-fold normal with ECG changes). Plasma homocysteine was not an entry criterion, and levels were not made known to the investigators during the course of the study. 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 $\mu\text{mol/L}$), 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 on hormone replacement were also excluded.

The present study was designed to recruit a minimum of 46 subjects, to achieve 80% power to detect an improvement in flow-mediated dilatation (FMD) from 40 μm on placebo to 80 μm on folic acid at the $\alpha=0.05$ level. Of 534 patients screened, 52 were eligible for entry to the folic acid study, and 10 similar subjects were identified for the intra-arterial study. All selected subjects were tested to exclude vitamin B₁₂ deficiency, which precludes folic acid treatment, before entry.

Study Design

Folic Acid Study

The study was a randomized, double-blind, placebo-controlled cross-over design. It involved two 6-week treatment periods of folic acid (5 mg daily) or matched placebo separated by a washout period of 4 months.

Intra-Arterial 5-MTHF Study

The study was open label and investigated the acute effects of 5-MTHF on brachial artery FMD and its NO component.

All patients gave written informed consent, and both protocols were approved by the Local Research Ethics Committee.

Study Protocol

Folic Acid Study

Each patient was studied at week 0, week 6, week 22, and week 28. At each visit, venous blood was collected into Vacutainers. Lipids, glucose, and creatinine were analyzed on the day of sampling; other samples were separated; and the serum/plasma was stored at -70°C until analysis. 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 the studies. At each visit, FMD was measured. Vascular measurements were made at baseline, at 1-minute intervals for 6 minutes, and at 8 and 10 minutes after cuff release to establish the time course of vessel diameter change. The nitroglycerin (NTG) response was then recorded.

Intra-Arterial 5-MTHF Study

After baseline venous blood sampling, baseline vascular measurements and the NTG response were recorded, and a period of at least 45 minutes was allowed to elapse. A 27-gauge needle was then inserted into the brachial artery of the nondominant arm, and

saline was infused at 0.5 mL/min, and this infusion rate was kept constant throughout. The brachial artery was imaged 7- to 10-cm distal to the puncture site, and FMD was measured. After this, 5-MTHF (Sigma Chemical Co) was then infused at 50 $\mu\text{g}/\text{min}$ to achieve a plasma concentration of at least 457 $\mu\text{g}/\text{L}$ (1 $\mu\text{mol}/\text{L}$), which has been shown to improve endothelial function in untreated hypercholesterolemia.¹⁸ After 30 minutes of infusion, venous blood samples were drawn from the ipsilateral antecubital vein, and FMD was reassessed. *N*^G-Monomethyl-L-arginine (L-NMMA), an inhibitor of endothelial NO synthase (eNOS), was then coinfused (3 mg/min) with 5-MTHF to assess the NO component of the observed FMD. The L-NMMA infusion was then stopped, and during infusion with 5-MTHF, the response to NTG was reassessed.

Noninvasive Measurement of Endothelial Function

FMD was measured by using high-resolution ultrasound and wall tracking, as previously described by us¹⁹ in response to increased flow in the brachial artery induced by 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. Endothelium-independent dilatation in response to NTG (400 μg) was measured after return of the vessel diameter to baseline and reported as the greatest absolute increase in EDD. Blood pressure was measured continuously in the study arm by using 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.

Biochemical Assays

Lipids, glucose, and creatinine were assayed routinely. tHcy was measured by enzymatic immunoassay (Abbot IMx, Abbot Diagnostics). Plasma malondialdehyde (MDA) and 5-MTHF were measured by high-performance liquid chromatography.^{20,21} Plasma total antioxidant capacity (TAOC) was measured by using a commercially available kit (Randox Laboratories) according to the method of Miller et al.²² Vitamin B₁₂ and folate were measured by competitive protein binding assays on an Elecys 2010 analyzer (Roche Diagnostics).

Effect of 5-MTHF, Folic Acid, and BH₄ on Intracellular Superoxide In Vitro

Experiments were performed on cultured porcine endothelial cells to assess the effects of 5-MTHF, folic acid, and tetrahydrobiopterin (BH₄) on intracellular superoxide in cells exposed to homocysteine. Free reduced L-homocysteine was prepared from L-homocysteine thiolactone, as described previously.²³ PAE cells were isolated and cultured as previously described,²⁴ and all experiments were carried out on first-passage cells. PAE cells were incubated at 37°C for 24 hours with buffer or homocysteine alone (1 mmol/L) or were cocubated with homocysteine (1 mmol/L) and 5-MTHF, folic acid, or BH₄ (all at 0.5 mmol/L). Intracellular PAE cell superoxide levels were then measured as previously described.²⁵ Briefly, cells were washed with sterile saline (0.9% [wt/vol]) before being trypsin (0.05% [wt/vol])–digested and isolated. The resulting cell pellet was resuspended in HEPES-buffered physiological saline, and the cell number measured with a Coulter Counter. Cells were added to an aliquot of buffer, to which lucigenin was added to a final concentration of 500 $\mu\text{mol}/\text{L}$. Cells were then placed into the warmed chamber of a luminometer with output measured in millivolts. Intracellular superoxide was measured after the addition of a lysing agent (Triton X-100, 1% [vol/vol]), calculated from the integral for the response, and normalized for cell number.

Withdrawals, Medication Changes, and Compliance

Two subjects were withdrawn from the folic acid study: 1 with a nonfatal myocardial infarction and 1 with atrial fibrillation. During the study, all efforts were made to hold medication constant, but careful consideration for medication changes in 6 patients. Treatment with

folic acid was well tolerated, and no side effects were reported. Compliance assessed by a tablet count was 96%.

Statistical Analysis

The main statistical analyses of the folic acid study results were based on 50 subjects after removal of the 2 withdrawals. Prefolate to postfolate changes in biochemical and vascular measurements were compared with corresponding preplacebo to postplacebo changes by using the Hills-Armitage method.²⁶ The relationships between changes in FMD, homocysteine, and other parameters on folate administration were characterized by the Spearman rank correlation, with 95% CIs calculated by the \tanh^{-1} method. In the intra-arterial study, paired *t* tests were used. The main outcome variables of FMD, folate, and homocysteine were little changed after removal of the 6 subjects in whom medication changes took place.

For the *in vitro* experiments, data are expressed as mean \pm SEM ($n=10$) and compared by ANOVA, followed by an appropriate multiple range test. All differences were considered significant at $P<0.05$.

Results

Baseline Characteristics

The folic acid study group consisted of 52 patients aged 57 ± 8 years (males 57 ± 8 years, females 58 ± 8 years). The 5-MTHF study group consisted of 10 similar subjects, aged 55 ± 9 years (all male). Baseline characteristics were similar in the 2 populations (Table 1).

Folic Acid Study

Effects on Biochemical Parameters

Biochemical parameters are shown in Table 2. Folic acid significantly decreased tHcy (9.3 ± 2.4 versus 10.8 ± 2.4 [placebo] $\mu\text{mol/L}$, $P<0.001$) and markedly increased plasma folate (310 ± 234 versus 9.1 ± 3.4 [placebo] $\mu\text{g/L}$, $P<0.001$). MDA and plasma TAOC were not significantly altered by folic acid. Vitamin B₁₂, lipids, glucose, and creatinine were unchanged by folic acid compared with placebo.

Effects on Flow-Mediated Dilatation and Vascular Measurements

Vascular data involving folic acid treatment are shown in Table 3. The coefficient of variation for the measurement of FMD in our laboratory was 5.6%. FMD was impaired in the folic acid group at baseline compared with published normal values (50 ± 33 μm and $1.2\pm 0.97\%$ baseline EDD).²⁷ FMD significantly improved after folic acid compared with placebo (110 ± 43 versus 47 ± 35 μm , respectively; $P<0.001$; Figure 1), and in addition, the time course of vessel diameter change after cuff release was significantly altered (Figure 2). Heart rate, blood pressure, baseline brachial artery EDD, peak hyperemic flow, and NTG response did not differ significantly after folic acid.

Intra-Arterial 5-MTHF Study

Effects on Biochemical Parameters

After 30 minutes of infusion, plasma 5-MTHF markedly increased (from 20.2 to 1595 $\mu\text{g/L}$, $P<0.001$), whereas no change in plasma homocysteine was observed (from 10.50 ± 2.46 to 10.53 ± 2.52 $\mu\text{mol/L}$, $P=0.47$).

Effects on Flow-Mediated Dilatation and Vascular Measurements

Vascular data during control, 5-MTHF infusion, and 5-MTHF/L-NMMA coinfusion are shown in (Table 4). FMD

TABLE 1. Clinical Characteristics of Study Subjects

Characteristic	Folic Acid Study (n=50)	5-MTHF Intra-Arterial Study (n=10)
Age, y	57 ± 8	55 ± 9
Male, n (%)	44 (88)	10 (100)
Female, n (%)	6 (12)	0
Body mass index	28.5 ± 4.4	28.3 ± 2.2
Angiographic CAD, n (%)	48 (96)	9 (90)
Myocardial infarction, n (%)	33 (66)	3 (30)
Revascularization, n (%)	39 (78)	9 (90)
CABG	32 (64)	7 (70)
PTCA	7 (14)	2 (20)
Hypertension	20 (40)	4 (40)
Family history <60 y, n (%)	26 (52)	6 (60)
Smoking history, n (%)		
Exsmoker (>6 mo)	36 (72)	6 (60)
Never smoked	14 (28)	4 (40)
Cerebrovascular event, n (%)	4 (8)	1 (10)
Peripheral vascular disease, n (%)	1 (2)	0
Angina, n (%)	7 (14)	0
Antiplatelet therapy, n (%)		
Aspirin	46 (92)	9 (90)
Clopidogrel	2 (4)	1 (10)
None	2 (4)	0
Lipid-lowering therapy, n (%)		
Statin	44 (88)	10 (100)
Fibrate	5 (10)	0
None	1 (2)	0
Other drug therapy, n (%)		
β -Blocker	33 (66)	9 (90)
ACE inhibitor	7 (14)	1 (10)
Calcium channel blocker	12 (24)	2 (20)
ATII receptor blocker	3 (6)	0
Nitrate	4 (8)	0

CABG indicates coronary bypass grafting; PTCA, percutaneous coronary angioplasty; and ATII, angiotensin II. Values are mean \pm SD or patient numbers (with percentages in parentheses).

was impaired in the 10 subjects at baseline compared with published normal values (FMD 43 ± 15 μm , $0.96\pm 0.34\%$ baseline EDD).²⁷ 5-MTHF acutely improved FMD compared with control (80 ± 20 versus 43 ± 15 μm , respectively; $P<0.001$), an effect that was completely suppressed by coinfusion with L-NMMA (Figure 3). Heart rate, blood pressure, baseline brachial artery EDD, peak hyperemic flow, and NTG response did not differ significantly during 5-MTHF infusion compared with control.

In Vitro Effect of 5-MTHF, Folic Acid, and BH₄ on Intracellular Superoxide Levels

Intracellular superoxide content was 21.0 ± 2.4 mV \cdot s per 10^6 cells in the control. Exposure to homocysteine resulted in a significant ($P<0.001$) increase in superoxide levels to 53.4 ± 2.9 mV \cdot s per 10^6 cells. This increase was abolished ($P<0.001$) by coinubation with either 5-MTHF, folic acid, or BH₄.
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TABLE 2. Biochemical Parameters at Baseline, Before Placebo and Folic Acid, and After 6-wk Administration

Variable	Baseline (n=50)	Before Placebo (n=50)	After Placebo (n=50)	Before Folic Acid (n=50)	After Folic Acid (n=50)	P*
Homocysteine, $\mu\text{mol/L}$	11.2 \pm 2.7	10.5 \pm 2.5	10.8 \pm 2.4	11.1 \pm 2.8	9.3 \pm 2.4	<0.001
Vitamin B ₁₂ , ng/L	432 \pm 116	430 \pm 125.75	428 \pm 128	435 \pm 123	411 \pm 128	0.23
Plasma folate, $\mu\text{g/L}$	8.8 \pm 3.4	9.3 \pm 2.9	9.1 \pm 3.4	8.9 \pm 3.5	310 \pm 235	<0.001
Cholesterol, mmol/L	4.7 \pm 0.8	4.6 \pm 0.7	4.6 \pm 0.7	4.8 \pm 0.7	4.7 \pm 0.7	0.87
HDL cholesterol, mmol/L	1.2 \pm 0.3	1.1 \pm 0.3	1.1 \pm 0.4	1.2 \pm 0.4	1.2 \pm 0.4	0.18
LDL cholesterol, mmol/L	2.8 \pm 0.7	2.8 \pm 0.6	2.8 \pm 0.7	2.8 \pm 0.6	2.8 \pm 0.6	0.95
Triglycerides, mmol/L	1.6 \pm 0.9	1.7 \pm 0.9	1.6 \pm 1.0	1.7 \pm 0.9	1.8 \pm 1.1	0.06
Glucose, mmol/L	5.3 \pm 0.6	5.3 \pm 0.6	5.2 \pm 0.6	5.3 \pm 0.7	5.3 \pm 0.7	0.46
Creatinine, $\mu\text{mol/L}$	98.6 \pm 13.2	98.7 \pm 13.6	98.8 \pm 14.8	98.9 \pm 13.7	98.0 \pm 13.3	0.47
MDA, nmol/mL	0.49 \pm 0.12	0.50 \pm 0.13	0.47 \pm 0.10	0.50 \pm 0.12	0.49 \pm 0.11	0.57
TAOC, mmol/L	1.56 \pm 0.13	1.56 \pm 0.11	1.56 \pm 0.12	1.56 \pm 0.13	1.54 \pm 0.11	0.13

Values are mean \pm SD.

*Comparing changes with placebo vs changes with folic acid.

Correlates of Improved FMD

Although homocysteine was significantly decreased by folic acid, a positive correlation between improvement in FMD and reduction in plasma homocysteine was not found. To the contrary, there was a trend toward a negative correlation ($r_s = -0.21$, $P = 0.15$, 95% CI -0.46 to 0.08). FMD improvement was weakly and negatively correlated with baseline homocysteine ($r_s = -0.23$, $P = 0.11$, 95% CI -0.48 to 0.06). No correlation was found between improvement in FMD and increase in plasma folate ($r_s = 0.09$, $P = 0.54$, 95% CI -0.20 to 0.36) or with changes in MDA or TAOC.

Discussion

The present study demonstrated a significant improvement in endothelial function after 6 weeks of treatment with folic acid (5 mg daily) in subjects with significant CAD on standard therapy and with good lipid control (mean cholesterol 4.7 mmol/L). The majority (88%) of patients were taking statins, which have previously been shown to improve endothelial function,²⁸ indicating additive benefit.

The oral folate study, statistically the most powerful to our knowledge, confirms and extends the findings of recent parallel group studies of folic acid (5 mg daily) alone or in combination with other B group vitamins on endothelial

function in CAD.^{14,15} In previous studies,^{14,15} mean baseline tHcy concentration was higher than that in the present study: 13 $\mu\text{mol/L}$ ¹⁴ and 12.3 $\mu\text{mol/L}$ ¹⁵ compared with 11.2 $\mu\text{mol/L}$ in the present study. Furthermore, improvement in endothelial function in the present study was not confined to those with higher baseline homocysteine concentrations. Indeed, there was a trend toward greater FMD enhancement in patients with tHcy <9 $\mu\text{mol/L}$ (n=14) compared with tHcy >9 $\mu\text{mol/L}$ (n=36); change in FMD was 73 \pm 43 versus 47 \pm 43 μm , respectively ($P = 0.07$). This suggests that the benefit is independent of pretreatment tHcy level.

We found no correlation between tHcy reduction and improvement in endothelial function and, indeed, observed a trend toward the reverse. This contrasts with the findings of a recent study in which a significant positive correlation between tHcy reduction and improvement in endothelial function was reported.¹⁵ It has recently been suggested that improved endothelial function seen with B group vitamins (which included folic acid) in CAD is mediated by a reduction in free (unbound) but not total homocysteine, in accordance with the observation that FMD improvement is correlated with a reduction in free but not total homocysteine.¹⁴ A number of factors would appear to argue against this correlation being a causal relationship. First, all of the

TABLE 3. Vascular Data at Baseline, Before Placebo and Folic Acid, and After 6-wk Administration

Variable	Baseline (n=50)	Before Placebo (n=50)	After Placebo (n=50)	Before Folic Acid (n=50)	After Folic Acid (n=50)	P*
Vessel EDD, mm	4.37 \pm 0.72	4.36 \pm 0.73	4.38 \pm 0.72	4.39 \pm 0.70	4.39 \pm 0.70	0.89
FMD, μm	50 \pm 33	46 \pm 33	47 \pm 35	52 \pm 34	110 \pm 43	<0.001
NTG diameter change, μm	333 \pm 78	340 \pm 72	340 \pm 72	340 \pm 76	340 \pm 77	0.79
Baseline blood flow, mL/min	41 \pm 21	40 \pm 20	40 \pm 19	40 \pm 19	40 \pm 18	0.79
Peak hyperemic flow, mL/min	198 \pm 73	196 \pm 68	196 \pm 71	202 \pm 67	198 \pm 66	0.51
Heart rate, bpm	59 \pm 11	59 \pm 10	60 \pm 10	59 \pm 10	59 \pm 10	0.64
Systolic blood pressure, mm Hg	133 \pm 16	132 \pm 16	133 \pm 14	133 \pm 17	133 \pm 14	0.11
Diastolic blood pressure, mm Hg	73 \pm 9	73 \pm 9	71 \pm 8	74 \pm 9	73 \pm 9	0.49

Values are mean \pm SD.

*Comparing changes with placebo vs changes with folic acid.

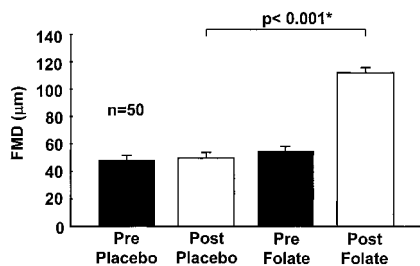


Figure 1. FMD before and after 6 weeks of placebo and folic acid (5 mg daily). Data are presented as mean±SEM. FMD was defined as the greatest (absolute) increase in EDD during the first 3 minutes after cuff release. *Comparing change with placebo vs change with folic acid.

observational data linking homocysteine and cardiovascular risk has been based on total, not free, homocysteine. Second, of the free component, only ≈1% is in the reduced form (ie, possesses a free sulfhydryl group), which can support auto-oxidation and therefore superoxide generation, with the remaining component being oxidized.^{29,30} The pathological significance of such low levels of free reduced homocysteine on oxidative burden in the plasma has been questioned recently.³¹

The lack of a positive correlation between the enhancement in endothelial function and tHcy reduction suggests that the beneficial effect of folic acid is unlikely to be mediated principally via tHcy lowering. This proposal is supported by the intra-arterial study, which demonstrated acute improvement in endothelial function after 30 minutes of infusion with 5-MTHF independent of a change in tHcy. This effect has not been previously reported in subjects with CAD. The improvement was abolished by coinfusion with the NO synthase inhibitor L-NMMA, indicating that it was mediated by an increase in NO bioavailability.

Generation of reactive oxygen species is proposed to be an important mechanism of homocysteine-induced endothelial

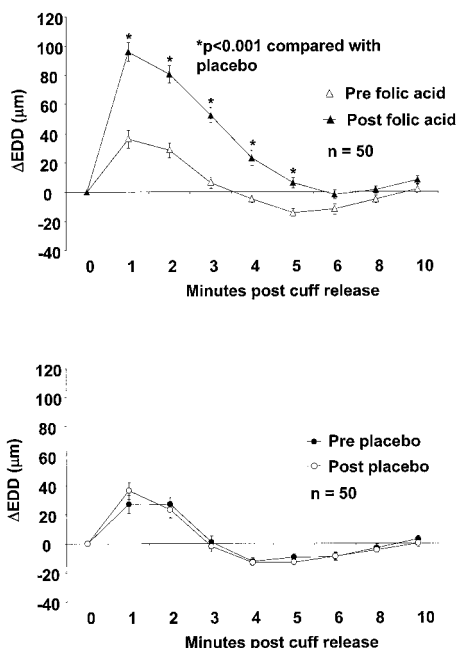


Figure 2. Time course of EDD change (ΔEDD, mean±SEM) after cuff release, before and after placebo and folic acid.

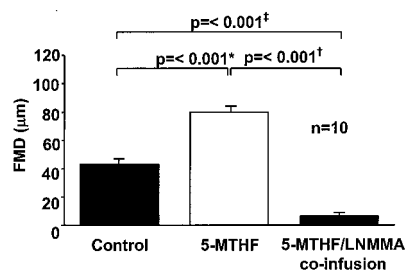


Figure 3. FMD during control (saline), 5-MTHF infusion (50 µg/min), and 5-MTHF/L-NMMA coinfusion (mean±SEM). FMD was defined as the greatest (absolute) increase in EDD during the first 3 minutes after cuff release. *Comparing 5-MTHF vs control. †Comparing 5-MTHF/L-NMMA coinfusion vs 5-MTHF alone. ‡Comparing control vs 5-MTHF/L-NMMA coinfusion. All comparisons were made by 2-tailed paired t test.

injury.¹³ This view is supported by methionine loading in normal subjects, which raises tHcy to ≈30 to 35 µmol/L and which acutely impairs endothelial function.¹² The mechanism is believed to involve oxidant stress³² and is abrogated by treatment with antioxidant agents.^{33,34} However, it cannot be assumed that moderately elevated homocysteine levels in the general population exert oxidative stress, because methionine loading results in large unphysiological increases in free reduced homocysteine and methionine.³⁵ Although some observational studies have shown a correlation between tHcy levels and markers of oxidant stress in the general population,^{36,37} a causal relationship has not been established. In the present study, although tHcy was significantly reduced by 19%, there was no reduction in MDA or any increase in TAOC, a marker of the antioxidant capacity of plasma. This suggests either that homocysteine does not directly exert oxidative stress in the plasma or that measurement of MDA and TAOC are not sensitive enough indicators. However, MDA and TAOC were selected because methionine loading in normal subjects is associated with an increase in MDA and decrease in TAOC, indicating that under these conditions, they can detect changes in plasma oxidant stress.^{38,39}

The in vitro study used concentrations of homocysteine that were far higher than those experienced in vivo (1 mmol/L versus 10 to 15 µmol/L, respectively). The high concentration of homocysteine used was similar to previously published reports of in vitro work. Higher pharmacological concentrations are sometimes required in vitro to reproduce the in vivo situation, in part, because the exposure times used are much shorter than those experienced in hyperhomocysteinemia in humans. The main aim of the present study was to establish homocysteine-induced endothelial dysfunction and investigate possible mechanism(s) by which folate may reverse this and not to mimic the in vivo situation exactly. We have recently reported that exposure of cultured endothelial cells to homocysteine (≥30 µmol/L) stimulates intracellular generation of superoxide.²⁵ The in vitro study confirmed this finding and further revealed that 5-MTHF can reduce levels of intracellular superoxide, suggesting a possible explanation for improvement in FMD observed in the human study. A number of mechanisms may account for this: (1) intracellular homocysteine was decreased, (2) 5-MTHF scavenged superoxide directly, and/or (3) 5-MTHF reduced superoxide production. Given that it takes some weeks before levels of

TABLE 4. Vascular Data During Control (Saline), 5-MTHF Infusion, and 5-MTHF/L-NMMA Coinfusion

Variable	Control (n=10)	5-MTHF (n=10)	P*	5-MTHF/L-NMMA (n=10)	P†
Vessel EDD, mm	4.53±0.36	4.53±0.37	0.99	4.50±0.37	0.98
FMD, μ m	43±15	80±20	<0.001	7±6	<0.001
NTG diameter change, μ m	430±30	440±50	0.35		
Baseline blood flow, ml/min	32±12	31±11	0.67	24±9	<0.001
Peak hyperemic flow, mL/min	234±96	233±88	0.8	227±95	0.27
Heart rate, bpm	55±7	55±8	0.47	55±7	0.43
Systolic blood pressure, mm Hg	136±12	135±12	0.32	136±12	0.06
Diastolic blood pressure, mm Hg	71±11	70±11	0.11	71±10	0.13

Values are mean±SD.

*Comparing control vs 5-MTHF infusion (50 μ g/min).

†Comparing 5-MTHF infusion vs 5-MTHF/L-NMMA (3 mg/min) coinfusion. All comparisons were made by paired *t* test (2-tailed).

homocysteine fall significantly with folic acid, a reduction in homocysteine after 24 hours seems unlikely, and other direct actions of 5-MTHF more likely explain this observation. In vitro, 5-MTHF has recently been demonstrated to be capable of directly scavenging superoxide, increasing NO production by eNOS, and also reducing superoxide generation by eNOS.⁴⁰

The in vitro study also demonstrated that BH₄ is capable of inhibiting the homocysteine-induced increases in endothelial superoxide. BH₄ is an essential cofactor for eNOS, and its depletion results in uncoupling of eNOS activity and a switch from production of NO, from L-arginine, to generation of superoxide.⁴¹ 5-MTHF is essential in the redox cycling of the inactive quinonoid BH₂ (qBH₂) back to the active form, BH₄.⁴² Furthermore, oral BH₄ supplementation can improve endothelial function in CAD.⁴³ Thus, the ability of 5-MTHF to regenerate intracellular BH₄ from qBH₂ may, at least in part, explain the improvement in NO bioavailability observed in the human studies.

Implications of the Present Study

The oral folic acid study supports the finding that high-dose supplementation improves endothelial function in patients with CAD. Furthermore, this improvement is observed in subjects already treated with statins and is independent of baseline tHcy or its reduction. In contrast to earlier reports, the present data do not support the view that improvement is mediated by tHcy reduction but point rather to direct actions of folic acid, possibly mediated by reduction in intracellular but not plasma oxidant stress. The dose of folic acid used was

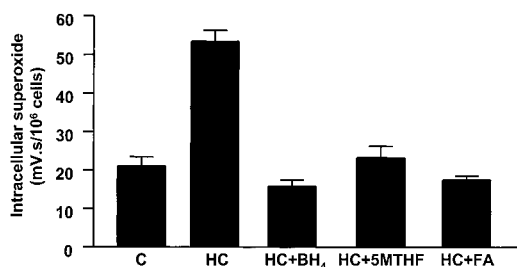


Figure 4. Intracellular superoxide levels in PAE cells exposed to control (C), homocysteine (HC) alone (1 mmol/L), or homocysteine (1 mmol/L) with either 5-MTHF (0.5 mmol/L), BH₄ (0.5 mmol/L), or folic acid (FA, 0.5 mmol/L).

pharmacological, with plasma levels far in excess of the normal range, and it cannot be assumed that the effects demonstrated will apply to low-dose folic acid (400 μ g/d) or improved dietary intake. In conclusion, folic acid is safe and offers a simple, well-tolerated, and inexpensive therapeutic option for improving endothelial function in subjects with ischemic heart disease.

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