5-Methyltetrahydrofolate Rapidly Improves Endothelial Function and Decreases Superoxide Production in Human Vessels

Effects on Vascular Tetrahydrobiopterin Availability and Endothelial Nitric Oxide Synthase Coupling

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**Background**—The circulating form of folic acid, 5-methyltetrahydrofolate (5-MTHF), may have beneficial effects on endothelial function; however, its mechanisms of action remain uncertain. Decreased nitric oxide (NO) bioavailability and increased vascular superoxide production in vascular disease states are due in part to endothelial NO synthase (eNOS) uncoupling related to deficiency of the eNOS cofactor tetrahydrobiopterin (BH4), but whether this mechanism is important in human atherosclerosis and represents a rational therapeutic target remains unclear. We hypothesized that 5-MTHF would improve endothelial function by decreasing superoxide and peroxynitrite production and by improving eNOS coupling, mediated by BH4 availability.

**Methods and Results**—Vascular superoxide/peroxynitrite production and vasomotor responses to acetylcholine and bradykinin were determined in saphenous veins and internal mammary arteries from 117 patients undergoing CABG. The effects of 5-MTHF were examined ex vivo (n=61) by incubating vessels with 5-MTHF (1 to 100 μmol/L) and in vivo by intravenous infusion of 5-MTHF or placebo before vessel harvest (n=56). 5-MTHF improved NO-mediated endothelium-dependent vasomotor responses and reduced vascular superoxide, both ex vivo and in vivo. These changes were not explained by direct superoxide scavenging by 5-MTHF in vitro or by changes in plasma total homocysteine in vivo. Rather, 5-MTHF was a strong peroxynitrite scavenger and increased vascular BH4 and the BH4/total biopterin ratio. Furthermore, 5-MTHF reversed eNOS uncoupling, as assessed by N^G-nitro-L-arginine methyl ester–inhibitable superoxide production, increased the eNOS dimer:monomer ratio, and enhanced eNOS activity.

**Conclusions**—5-MTHF has beneficial effects on endothelial function and vascular superoxide production in human atherosclerosis, by preventing peroxynitrite-mediated BH4 oxidation and improving eNOS coupling. (Circulation. 2006;114:1193-1201.)

**Key Words:** acetylcholine ■ antioxidants ■ coronary disease ■ endothelium ■ free radicals ■ nitric oxide ■ nitric oxide synthase
action of folic acid supplementation in human vascular disease is critical to guide future clinical studies.

Oxidative stress plays an important role in the pathogenesis of atherosclerosis, through effects of reactive oxygen species (ROS) on NO bioavailability and through interactions with numerous redox-sensitive signaling pathways. Although ROS scavenging has been proposed as a therapeutic strategy to target oxidative stress in atherosclerosis, the outcome of clinical trials using simple “antioxidants” have been disappointing. Indeed, a greater understanding of redox signaling in the vessel wall is required to target antioxidant therapies in a specific and rational manner. One such specific redox target in vascular disease is the regulation of eNOS by the cofactor BH4. Oxidation of BH4 by ROSs such as peroxynitrite appears to be an important mechanism linking oxidative stress to endothelial dysfunction. Indeed, the kinetics and progression of BH4 oxidation by peroxynitrite have been well defined, whereas the reaction rate between BH4 and superoxide is much smaller than that with peroxynitrite in vivo, which suggests that superoxide is not a major contributor of BH4 oxidation in vivo. Therefore, restoration of endothelial BH4 availability by scavenging peroxynitrite radicals in atherosclerosis may improve endothelial function and reduce atherosclerotic progression.

In the present study, we explored the effects of 5-MTHF on endothelial function and vascular superoxide/peroxynitrite production in human vessels both ex vivo and in vivo. We hypothesized that 5-MTHF acts as an effective intracellular antioxidant and sought to evaluate the effects of 5-MTHF on eNOS “coupling” and on vascular BH4 availability in human atherosclerosis.

Methods

Patients

We studied 117 patients with coronary artery disease undergoing routine CABG surgery at the John Radcliffe Hospital, Oxford, United Kingdom. Exclusion criteria were the existence of any inflammatory, infective, liver, or renal disease or malignancy. Patients receiving nonsteroidal antiinflammatory drugs or any diuretics, proinflammatory, infective, liver, or renal disease or malignancy. Patients were divided into 2 groups: 5-MTHF treatment group and the control group. The study was approved by the local Research Ethics Committee, and each patient gave written informed consent.

Vessel Harvesting and Ex Vivo Studies

Samples of saphenous vein (SV; n = 38) and internal mammary artery (IMA; n = 46) from a total of 61 patients were harvested during CABG surgery. Saphenous vessels were harvested from the lower calf, and IMA segments were transferred to the laboratory within 30 minutes in ice-cold Krebs-Henseleit buffer. Segments of SV and IMA were incubated with 5-MTHF (0 to 100 μmol/L) in Krebs-Henseleit buffer for 45 minutes before assays of endothelial function, superoxide/peroxynitrite production, radiolabeled arginine/citrulline conversion, Western blotting, or tissue 5-MTHF and biopterin levels, as described below.

In Vivo Studies

Patients (n = 56) undergoing CABG participated in a double-blind, placebo-controlled study in which they received an intravenous infusion of either the natural diastereoisomer of 5-MTHF (Merck Eprova AG, Schaffhausen, Switzerland) or placebo, administered before the CABG. 5-MTHF was administered at a dose of 0.13 mg/kg body weight, which in preliminary studies achieved a plasma concentration of 2 to 3 μmol/L immediately after administration and 1 to 2 μmol/L at 45 minutes after infusion. This concentration was chosen on the basis of the dose-response analysis performed in the ex vivo experiments. Samples of SV and IMA were harvested 45 minutes after the infusion of 5-MTHF (mean time of harvesting 45.7 ± 2.9 minutes after infusion) and assayed for NO-mediated vasomotor function, vascular superoxide production, tissue 5-MTHF levels, and biopterin levels, as described below.

Vasomotor Studies

Endothelium-dependent and -independent dilatation were assessed with isometric tension studies, as we have described previously. Vessel rings were equilibrated and passively pretensioned to 3 g, an optimal resting tension that was determined in baseline studies of contractile response to KCl. After precontraction with phenylephrine (3 × 10−6 mol/L), vasomotor responses to the endothelium-mediated agonists acetylcholine (ACH; 10−7 to 10−3 mol/L) and bradykinin (BK; 10−7 to 10−5 mol/L) were quantified in 4 equally sized segments from the same vessel. In the ex vivo experiments, vasomotor responses were repeated after incubation for 45 minutes with 0 (control), 1, 10, or 100 μmol/L 5-MTHF added to the organ chambers. Finally, relaxations to the NO donor sodium nitroprusside (SNP; 10−10 to 10−5 mol/L), were evaluated in the presence of the NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L).

Superoxide/Peroxynitrite Measurements

Vascular superoxide production was measured from fresh human vessels with lucigenin (5 μmol/L)–enhanced chemiluminescence as described previously. Samples of SV and IMA from the same patient were opened longitudinally to expose the endothelial surface and then equilibrated for 20 minutes in oxygenated (95% O2/5% CO2) Krebs-HEPES buffer (2 mL, pH 7.4) at 37°C. NOS-derived superoxide was measured by adding L-NAME (100 μmol/L) to the equilibrating buffer and calculating the difference in superoxide signal compared with basal conditions. Vascular peroxynitrite was determined with luminol (100 μmol/L) instead of lucigenin and by subtracting the remaining signal after the addition of the specific peroxynitrite scavenger uric acid (1 mmol/L), as described previously.

Oxidative Fluorescent Microtopography

In situ superoxide production was determined in vessel cryosections with the oxidative fluorescent dye dihydroethidium (DHE). Paired cryosections (30 μmol/L) from the same vessel were incubated with DHE (2 μmol/L) in PBS, with or without L-NAME (100 μmol/L). Fluorescence images (×40, Zeiss LSM 510 META laser scanning confocal microscope, Carl Zeiss GmbH, Jena, Germany) were obtained from each vessel quadrant, with the luminal side of the vessel incorporated to quantify endothelial cell fluorescence. In each case, segments of vessel rings (with and without L-NAME) were analyzed in parallel with identical imaging parameters. DHE image analysis was performed in a blinded fashion by 2 independent investigators using Image-Pro Plus software (Media Cybernetics, Silver Spring, Md).

Superoxide and Peroxynitrite Scavenging Assays

The direct superoxide scavenging effect of 5-MTHF was evaluated with the use of the xanthine/xanthine oxidase system. Superoxide production was induced by the addition of xanthine oxidase (6.67 MU/mL) in Krebs HEPES buffer (2 mL, pH 7.4; 37°C) containing xanthine (0.133 mmol/L) and lucigenin (5 μmol/L) in the presence of 5-MTHF (0 to 100 μmol/L) or equal concentrations of ascorbic acid as positive control. Peroxynitrite production was induced by addition of 3-morpholinosydnonimine (SIN-1, 1 μmol/L) in Krebs HEPES buffer (2 mL, pH 7.4; 37°C) containing luminol (100 μmol/L) in the presence of different concentrations of 5-MTHF (0 to 100 μmol/L) or equal concentrations of uric acid as positive control, as described previously. The scavenging effects of 5-MTHF,
vitamin C, or uric acid were calculated as the percent inhibition of chemiluminescence compared with control.

**Determination of 5-MTHF and tHcy**

Blood samples were collected before administration of 5-MTHF or placebo and at the time of vessel harvesting. tHcy was measured by a fluorescence polarization immunoassay adapted to the IMx analyzer (Abbott Diagnostics, Abbott Park, Ill). Plasma and tissue levels of 5-MTHF were determined by high-performance liquid chromatography (HPLC), as described previously.17

**Determination of Biopterin Levels**

Biopterin levels in human vessels were determined by HPLC, as described previously,18 and expressed as pmol/g of tissue. To examine the effect of 5-MTHF on peroxynitrite-induced oxidation of BH₄, we used SIN-1 (2 μmol/L) to oxidize BH₄ (0.1 μmol/L) in the presence or absence of 5-MTHF (1 μmol/L) at 37°C for 14 minutes.19

**Evaluation of eNOS Activity in Intact Vessels**

The activity of eNOS was estimated by HPLC quantification of radiolabeled arginine to citrulline conversion from intact vessel rings, as described recently.20

**Western Blot Analysis**

The eNOS dimer:monomer ratio in paired IMA samples incubated for 45 minutes in the presence or absence of 5-MTHF 1 μmol/L was measured by Western blotting as described previously.21 Bands were visualized with chemiluminescence and quantified with NIH Image software (National Institutes of Health, Bethesda, Md).

**Statistical Analysis**

Analyses were performed with the SPSS 12.0 statistical package for Windows (SPSS Inc, Chicago, Ill). Normally distributed data are presented as mean±SEM, whereas nonnormally distributed variables (such as vascular superoxide) are presented as median (25th to 75th percentile values). Baseline comparisons between groups were performed with 1-way ANOVA for multiple comparisons, followed by Bonferroni correction. The effects of 5-MTHF on vasomotor responses in each vessel ring were assessed by 2-way ANOVA for repeated measurements. The effects of 5-MTHF incubations on superoxide production and levels of 5-MTHF or tHcy were assessed by Mann-Whitney U tests, Wilcoxon signed rank tests, or t tests for unpaired or paired data, as appropriate. A 2-tailed P<0.05 was considered statistically significant.

### Demographic Characteristics of the Participants

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<th>Intravenous 5-MTHF Study</th>
<th>Placebo</th>
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<td>12 (37.5)</td>
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<td>Plasma 5-MTHF, nmol/L</td>
<td>26.4±4.68</td>
<td>32.4±9.01</td>
<td>35.4±8.10</td>
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**Angiographic extent of CAD**

- 2-Vessel disease: Ex Vivo 12 (20%), Intravenous 5-MTHF 4 (17%), Placebo 6 (19%)
- 3-Vessel disease: Ex Vivo 45 (80%), Intravenous 5-MTHF 20 (83%), Placebo 26 (81%)

**Medication**

- Statins: Ex Vivo 51 (84%), Intravenous 5-MTHF 18 (75%), Placebo 28 (88%)
- ACE inhibitors: Ex Vivo 44 (72%), Intravenous 5-MTHF 18 (75%), Placebo 20 (63%)
- Calcium channel blockers: Ex Vivo 18 (30%), Intravenous 5-MTHF 6 (24%), Placebo 9 (28%)
- Angiotensin receptor blockers: Ex Vivo 3 (5%), Intravenous 5-MTHF 2 (8%), Placebo 2 (6%)
- β-Blocker: Ex Vivo 53 (87%), Intravenous 5-MTHF 20 (83%), Placebo 28 (88%)
- Nitrates: Ex Vivo 28 (46%), Intravenous 5-MTHF 11 (46%), Placebo 13 (41%)
- Aspirin: Ex Vivo 47 (79%), Intravenous 5-MTHF 19 (79%), Placebo 24 (75%)
- Clopidogrel: Ex Vivo 18 (30%), Intravenous 5-MTHF 8 (33%), Placebo 10 (31%)

CAD indicates coronary artery disease.

Values are mean±SEM or n (%). There were no significant differences between groups in clinical characteristics or medication.
The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Patient Characteristics

Vessels were obtained from 117 patients (61 included in the ex vivo part of the study and 56 in the in vivo part). There were no significant differences in demographic characteristics or baseline plasma tHcy levels between the treatment groups (Table).

Effects of 5-MTHF on Endothelial Function and on Superoxide and Peroxynitrite Production in Human Vessels Ex Vivo

We first investigated the effects of 5-MTHF on vasomotor responses to ACh and BK in vessel segments at baseline and after 45-minute incubation in organ chambers with 0 to 100 μmol/L 5-MTHF. Relaxations in response to ACh and BK were similar between the 4 rings from the same vessel at baseline. Maximum relaxations to ACh and BK were similar between the 4 rings from the same vessel at baseline. Maximum relaxations to either ACh or BK. The maximum relaxations to ACh were significantly correlated with the maximum relaxations to either ACh or BK. The maximum relaxations to ACh were significantly increased after 45 minutes of incubation with 5-MTHF. Absolute contractions in response to phenylephrine were 7.9±0.8g at baseline and remained unchanged after incubation. *P<0.05, **P<0.01 vs baseline.

Vascular superoxide and peroxynitrite production from SV and IMA were determined after 45 minutes’ incubation with either buffer alone or with 5-MTHF at 1, 10, or 100 μmol/L. Incubation with 1 μmol/L 5-MTHF significantly reduced both vascular superoxide and peroxynitrite production in SV and IMA, with no further reductions at higher 5-MTHF concentrations (Figure 2).

Effects of 5-MTHF on Endothelial Function and Superoxide Production in Human Vessels In Vivo

On the basis of our findings of improved endothelial function and reduced superoxide and peroxynitrite production after ex vivo incubation with 1 μmol/L 5-MTHF, but no further effects at higher concentrations, we aimed to test the effects of similar 5-MTHF concentrations in vivo. In pilot studies, 0.13 mg/kg body weight 5-MTHF administered intravenously increased plasma 5-MTHF from 74.1±22.2 nmol/L to 2.48±0.47 μmol/L (n=4) 5 minutes after infusion and to 1.71±0.82 μmol/L at 45 minutes.

Patients were randomized to receive either intravenous 5-MTHF or placebo in a double-blind fashion. Baseline plasma levels of 5-MTHF were not significantly different between patients who received intravenous 5-MTHF (32.4±9.01 nmol/L) and those who received placebo (26.4±4.68 nmol/L, P=NS). In contrast, plasma 5-MTHF levels were increased significantly at the time of vessel harvesting in the 5-MTHF–treated group (2.28±0.21 μmol/L; P<0.001 versus baseline) but remained unchanged in the placebo-treated group (25.8±6.85 nmol/L, P=NS versus baseline, P<0.001 versus 5-MTHF–treated group). Correspondingly, tissue 5-MTHF levels in SV (9.35±1.72
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Figure 3. Segments of SVs obtained ~45 minutes after intravenous infusion of 5-MTHF (0.13 mg/kg body weight, n=15) had significantly greater vasomotor responses to both ACh (A) and BK (B) than segments from patients who received placebo (n=17). There was no significant difference in vasomotor responses to nitroprusside (SNP) between patients who received intravenous 5-MTHF and those who received placebo. Absolute precontractions to phenylephrine in these vessels were 8.20±1.26g and 7.72±1.31g in the 5-MTHF- and placebo-treated groups, respectively (P=NS). *P<0.05 vs placebo.

nmol/g) and IMA (12.7±2.58 nmol/g) were increased significantly in the 5-MTHF–treated group compared with the placebo group (1.19±0.25 nmol/g for SV and 1.54±0.38 nmol/g for IMA, respectively; P<0.001 for both), which confirms that intravenous administration of 5-MTHF substantially increases vascular tissue levels. Plasma tHcy was modestly decreased in patients who received 5-MTHF (from 11.0±0.62 to 9.42±0.46 μmol/L, P<0.05) 45 minutes after infusion but remained unchanged in the placebo group (from 12.0±1.23 to 11.6±1.26 μmol/L, P=NS).

We evaluated the effects of in vivo administration of 5-MTHF on endothelial function by quantification of vasomotor responses to ACh and BK in vessel segments from patients who received either placebo or 5-MTHF. Intravenous 5-MTHF had striking effects on maximal vasorelaxations to ACh (B) than segments from patients who received placebo (n=25, shaded bars). Measurements were performed in paired samples of both SVs and IMAs from the same patients using 5 μmol/L lucigenin-enhanced chemiluminescence. Values are expressed as median (horizontal line), 25th to 75th percentile (box), and range (whiskers). *P<0.01 vs placebo. RLU/sec/mg indicates relative light units per second per milligram.

Direct Superoxide/Peroxynitrite Scavenging Capacity of 5-MTHF

To investigate whether direct superoxide scavenging by 5-MTHF could account for its effects on vascular superoxide production and endothelial function, we assessed the ability of 5-MTHF to scavenge superoxide in comparison with a known superoxide scavenger, ascorbic acid. When added to a xanthine/xanthine oxidase system that generated superoxide at similar levels to those observed in vascular tissues, ascorbic acid had a potent scavenging effect, reducing measurable superoxide by 50% at 1 μmol/L (Figure 5). In contrast, low concentrations of 5-MTHF (1 to 10 μmol/L) had no detectable effect on measurable superoxide (Figure 5), with only modest superoxide scavenging observed even at very high 5-MTHF concentrations (100 μmol/L).

The direct peroxynitrite scavenging capacity of 5-MTHF was assessed in comparison with a known peroxynitrite scavenger, uric acid. When added to an SIN-1 system, 5-MTHF had a potent scavenging effect, reducing measurable peroxynitrite by 75% at 1 μmol/L (Figure 5), comparable to the effect of uric acid at the same concentration. These observations suggest that 5-MTHF, at concentrations used in

Figure 4. Superoxide production from intact vessel segments was significantly lower in patients ~45 minutes after intravenous infusion of 5-MTHF (0.13 mg/kg body weight, n=15, open bars) than in placebo patients (n=25, shaded bars). Measurements were performed in paired samples of both SVs and IMAs from the same patients using 5 μmol/L lucigenin-enhanced chemiluminescence. Values are expressed as median (horizontal line), 25th to 75th percentile (box), and range (whiskers). *P<0.01 vs placebo. RLU/sec/mg indicates relative light units per second per milligram.

5-MTHF had a strong direct peroxynitrite scavenging effect at concentrations as low as 1 μmol/L, comparable to that of equal concentrations of uric acid. Values are mean±SEM of 3 separate experiments. *P<0.01 vs vitamin C; †P<0.05 and ‡P<0.01 vs 0 μmol/L.

Figure 5. Superoxide scavenging by 5-MTHF was assessed with a xanthine/xanthine oxidase system (A). 5-MTHF had a weak superoxide scavenging effect, but only at concentrations >10 μmol/L, whereas vitamin C had the expected scavenging effect even at low concentrations. Peroxynitrite scavenging by 5-MTHF and uric acid was assessed with SIN-1 (1 μmol/L; B). 5-MTHF had a strong direct peroxynitrite scavenging effect at concentrations as low as 1 μmol/L, comparable to that of equal concentrations of uric acid. Values are mean±SEM of 3 separate experiments. *P<0.01 vs vitamin C; †P<0.05 and ‡P<0.01 vs 0 μmol/L.
the present in vivo studies, exerts a significant peroxynitrite but not superoxide-scavenging effect.

**Effects of 5-MTHF on eNOS-Derived Vascular Superoxide Production and eNOS Activity**

Because the direct superoxide scavenging capacity of 5-MTHF did not account for the observed reduction in vascular superoxide production, we next examined whether the antioxidant effect of 5-MTHF was due to an improvement in eNOS coupling. NOS-derived superoxide production was estimated in paired samples of SV and IMA by quantifying the effects of NOS inhibition with L-NAME. Vascular superoxide production was decreased by L-NAME in both SV and IMA in the placebo group, which suggests a net contribution to vascular superoxide production by NOS. In contrast, L-NAME increased superoxide production in vessels from 5-MTHF–treated patients, which suggests net NO production (Figure 6). Importantly, L-NAME–inhibitable superoxide production in SV or IMA was correlated with plasma 5-MTHF ($r=0.511$, $P=0.006$ and $r=0.690$, $P=0.0001$, respectively) but not with plasma tHcy levels ($r=0.040$, $P=0.829$ and $r=−0.286$, $P=0.106$, respectively).

To visualize vascular superoxide production and to specify the changes in eNOS-mediated endothelial superoxide production, we used oxidative fluorescent microtopography with DHE (Figure 6). L-NAME decreased endothelial DHE fluorescence in vessels from placebo patients, which indicates eNOS uncoupling. Conversely, LNAME increased endothelial DHE fluorescence in vessels from 5-MTHF–treated patients, which suggests an improvement in eNOS coupling in these vessels (Figure 6). Importantly, DHE fluorescence in other regions of the vessel wall was unaffected by 5-MTHF, which provides a within-section control and demonstrates the endothelium-specific effect of NOS inhibition.

To further evaluate the effects of 5-MTHF on eNOS activity, we used HPLC to measure the conversion of radiolabeled arginine to citrulline in samples of IMA from 12 patients, incubated with or without 5-MTHF 1 µmol/L for 45 minutes. We observed a significant increase in citrulline production in vessels incubated with 5-MTHF (0.20±0.03%/g tissue) compared with paired control vessels from the same patients (0.14±0.02%/g, $P<0.05$), which suggests that 5-MTHF directly increases eNOS activity in human vessels. Taken together, these observations suggest that 5-MTHF reduces vascular superoxide production and improves NO-mediated endothelial function through effects on eNOS coupling.

**Effects of 5-MTHF on BH$_4$ Levels and eNOS Dimer:Monomer Ratio in Human Vessels**

To further investigate the mechanisms by which 5-MTHF improves eNOS coupling, we examined the effects of 5-MTHF on vascular BH$_4$ levels. Incubation of SV for 45 minutes ex vivo with 1 µmol/L 5-MTHF significantly increased vascular BH$_4$ and the BH$_4$/total biopterin (tBio) ratio (Figure 7). More strikingly, both the absolute levels of BH$_4$ and the BH$_4$/tBio ratio were significantly elevated in patients who received intravenous infusion of 5-MTHF in vivo compared with placebo-treated patients (Figure 7). Because we observed that 5-MTHF is a strong peroxynitrite scavenger, we then examined the effects of 5-MTHF on peroxynitrite-induced BH$_4$ oxidation. 5-MTHF significantly reduced the decrease of both BH$_4$ and BH$_4$/tBio ratio after exposure to peroxynitrite generated by SIN-1 (Figure 7). Furthermore, the dimer:monomer ratio was also increased after exposure of IMA rings to 5-MTHF 1 µmol/L (Figure 7). We also found that the vascular BH$_4$/tBio ratio was significantly correlated with the L-NAME–induced change in superoxide production (Figure 8) in both SV ($r=0.495$, $P=0.002$) and IMA ($r=0.621$, $P=0.001$) from individual patients. Finally, the BH$_4$/tBio ratio in SV and IMA was significantly correlated with plasma 5-MTHF ($r=0.498$, $P=0.01$ and $r=0.656$, $P=0.0001$, respectively) but not with plasma tHcy ($r=0.033$, $P=0.836$ and $r=−0.165$, $P=0.374$).
respectively), which suggests that 5-MTHF itself rather than plasma tHcy is the critical parameter that prevents the oxidation of BH4 in human vessels in vivo. These observations suggest a direct functional relationship between vascular BH4 availability and eNOS coupling in human vessels that is a major determinant of both NO-mediated endothelial function and vascular superoxide production.

**Discussion**

In the present study, we show that 5-MTHF, the circulating form of folic acid, has striking direct effects on vascular function. 5-MTHF acutely improves NO-mediated endothelial function and decreases superoxide production, through mechanisms independent of direct superoxide scavenging. Rather, the effects of 5-MTHF on the vascular endothelium appear to be mediated through restoration of the enzymatic coupling of eNOS due to increased vascular BH4 availability. This effect may be due to the protective effect of 5-MTHF on peroxynitrite-induced oxidation of BH4. Taken together, these findings demonstrate that BH4-mediated regulation of eNOS is an important determinant of endothelial function and superoxide production in human atherosclerosis. More generally, they provide proof-of-principle that targeting specific redox mechanisms in human atherosclerosis can provide a rational basis for therapeutic intervention.

Plasma levels of tHcy and folate are related to cardiovascular risk and endothelial function. Early clinical trials suggested that lowering tHcy with folic acid may retard progression of atherosclerosis, but these findings were not confirmed by more recent studies. Whereas high-dose folic acid treatment may be beneficial specifically in patients with high tHcy levels, benefits in other patient groups have been inconsistent. Three recent larger trials in patients with stroke, myocardial infarction, or stable coronary artery disease found that folic acid treatment did not improve clinical outcome. In part, the continued uncertainty over the effects of folate status and tHcy-lowering relates to incomplete understanding of the mechanistic relationships between folate, homocysteine, and vascular disease. Evidence suggests that mechanisms other than homocysteine-lowering may be important factors in the relationship between folate and atherosclerosis. Recent studies have reported beneficial effects of folic acid treatment on endothelial function in patients with advanced atherosclerosis that are discordant with changes in plasma tHcy. More specifically, 5-MTHF appears to be most strongly associated with endothelial function, independent of tHcy levels. Indeed, direct intra-
arterial infusion of 5-MTHF improved flow-mediated dilation in patients with coronary artery disease before any detectable reduction in plasma tHcy. Because 5-MTHF is a reduced form of folic acid that does not require conversion by dihydrofolate reductase, some direct effects may be attributable to redox mechanisms that are not seen when oral folic acid is used to increase plasma folate levels.

We now demonstrate that 5-MTHF has a rapid, direct effect on the human vascular endothelium in a concentration range that is readily achievable in vivo. Specifically, incubation of vessels with 1 μmol/L 5-MTHF for 45 minutes improved vasomotor responses to both ACh and BK, with no further improvement at higher concentrations. Accordingly, we used an intravenous infusion of 5-MTHF in patients undergoing CABG that was sufficient to increase circulating 5-MTHF levels to 1 to 3 μmol/L. These concentrations also improved vasomotor responses to both ACh and BK in SV segments, which suggests an improvement in NO bioavailability in these vessels. The present study adds further weight to the notion that the salutary effects of 5-MTHF on vascular function and a decrease of superoxide production at 5-MTHF concentrations as low as 1 μmol/L, both ex vivo and in vivo, with no evidence of significant direct superoxide scavenging. The assays of superoxide production used in clinical material have limited quantitative specificity and could be improved, for example, by HPLC analysis of DHE oxidation. However, we also found that 5-MTHF was a strong scavenger for peroxynitrite in vitro and reduced peroxynitrite production from vessel rings. These observations now add major new clinical importance to the previous key finding that 5-MTHF can interact with purified eNOS in vitro, preventing its “uncoupling.” We observed that 5-MTHF could enhance eNOS activity and qualitatively restore eNOS coupling in human vessels, from a net producer of superoxide (as revealed by inhibition of superoxide production by L-NAME) to a net producer of NO (leading to increased superoxide release by L-NAME). We also observed that 5-MTHF increased the dimer:monomer ratio in human vessels, which suggests an improvement in eNOS protein dimerization.

A major determinant of eNOS function and enzymatic coupling is the cofactor BH₄. Recent studies in both transgenic mice and endothelial cells have shown that an imbalance between intracellular eNOS and BH₄ availability levels leads to uncoupling of eNOS, which results in superoxide production. BH₄ deficiency in vascular disease states appears to result principally from its increased intracellular oxidation by peroxynitrite. The present findings provide evidence that although 5-MTHF is a rather weak superoxide scavenger, it may be acting to increase intracellular BH₄ by preventing intracellular oxidation by peroxynitrite. Previous observations suggested that 5-MTHF could directly increase NO production by recombinant eNOS in the presence of BH₄, and it may also interact with the pterin-binding site of eNOS in a fashion analogous to BH₄.

In summary, we describe a striking effect of 5-MTHF on NO-mediated endothelial function and on superoxide production in human vessels from patients with atherosclerosis, both in vivo and ex vivo. We propose that 5-MTHF may prevent the peroxynitrite-induced oxidation of BH₄, leading to an improvement in eNOS coupling and an enhancement of eNOS activity. These findings show, in general, that targeting of specific redox mechanisms that regulate endothelial function in human atherosclerosis is a rational therapeutic approach. Specifically, BH₄ availability and eNOS function may be a key mechanism that can be targeted with 5-MTHF, or other therapies, to improve endothelial function in patients with vascular disease. Finally, the present study adds mechanistic insights to enable us to further understand the complex relationships between homocysteine, folate, and vascular disease. Identifying the mechanisms of action of folate in vascular disease states will allow more appropriate selection of patient groups and therapeutic regimens for future clinical trials.

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Disclosures
None.

References
The importance of homocysteine-lowering treatment with folic acid remains controversial, because initial reports that suggest a beneficial effect of folic acid in human atherosclerosis have not been confirmed by recent large-scale clinical trials. Understanding the mechanisms by which folic acid affects vascular function is critical to the design of future therapeutic strategies. We evaluated the effects of 5-methyltetrahydrofolate (5-MTHF), the circulating form of folic acid, in patients with coronary artery disease. We first studied 5-MTHF incubation of vessel rings ex vivo, then conducted a double-blind, placebo-controlled clinical trial of intravenous 5-MTHF. We demonstrate that 5-MTHF rapidly improves endothelial function and decreases superoxide production in vessels from patients with coronary artery disease by mechanisms that appear independent of homocysteine lowering. The effects of 5-MTHF are due in part to direct scavenging of the oxidant radical peroxynitrite. By this mechanism, 5-MTHF improves bioavailability of the endothelial nitric oxide synthase (eNOS) cofactor, tetrahydrobiopterin (BH4), which leads to an improvement in eNOS “coupling” and a decrease in eNOS-derived superoxide production. Thus, 5-MTHF has direct effects on vascular endothelium by improving BH4-mediated eNOS coupling in vessels from patients with coronary artery disease. Future clinical studies are required to evaluate how these novel effects of 5-MTHF, rather than homocysteine-lowering alone, may be related to clinical benefits in vascular disease.
5-Methyltetrahydrofolate Rapidly Improves Endothelial Function and Decreases Superoxide Production in Human Vessels: Effects on Vascular Tetrahydrobiopterin Availability and Endothelial Nitric Oxide Synthase Coupling

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