Improved Vascular Endothelial Function After Oral B Vitamins

An Effect Mediated Through Reduced Concentrations of Free Plasma Homocysteine

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***Background*—**Hyperhomocysteinemia is an independent risk factor for coronary heart disease (CHD). Dietary supplementation with B vitamins lowers plasma homocysteine by up to 30%. However, little is known about the potential beneficial effects of homocysteine lowering on vascular function in patients with CHD.

***Methods and Results*—**We investigated 89 men with CHD (aged 56 [range 39 to 67] years). Brachial artery flow–mediated dilatation (endothelium dependent) and nitroglycerin-induced dilatation (endothelium independent) were measured before and 8 weeks after treatment with either (1) folic acid (5 mg) and vitamin B_{12} (1 mg) daily (n=59) or (2) placebo (n=30). Total, protein-bound, and free plasma homocysteine, serum folate, and vitamin B_{12} were measured at baseline and at 8 weeks. Flow-mediated dilatation improved after treatment with B vitamins (2.5±3.2% to 4.0±3.7%, $P=0.002$) but not placebo (2.3±2.6% to 1.9±2.6%, $P=0.5$). Vitamin therapy lowered plasma concentrations of total homocysteine (from 13.0±3.4 to 9.3±1.9 μmol/L, $P<0.001$), protein-bound homocysteine (from 8.7±2.8 to 6.2±1.4 μmol/L, $P<0.001$), and free homocysteine (from 4.3±1.2 to 3.0±0.6 μmol/L, $P<0.001$) and raised concentrations of serum folate (from 10.3±4.3 to 31.2±10.8 ng/mL, $P<0.001$) and vitamin B_{12} (from 314±102 to 661±297 pg/mL, $P<0.001$). In regression analysis, improved flow-mediated dilatation correlated closely with the reduction in free plasma homocysteine ($r=-0.26$, $P=0.001$), independent of changes in protein-bound homocysteine, folate, and vitamin B_{12}. Nitroglycerin-induced dilatation was unchanged after both B vitamins and placebo.

***Conclusions*—**Folic acid and vitamin B_{12} supplementation improves vascular endothelial function in patients with CHD, and this effect is likely to be mediated through reduced concentrations of free plasma homocysteine concentrations. Our data support the view that lowering homocysteine, through B vitamin supplementation, may reduce cardiovascular risk. **(*Circulation*. 2000;102:2479-2483.)**

Key Words: endothelium ■ nutrition ■ arteriosclerosis

Hyperhomocysteinemia is an independent risk factor for coronary heart disease (CHD).[1–6] Elevated homocysteine concentrations are found in almost one third of all patients with atherosclerosis, and levels only 12% above the upper limit of normal (15 μmol/L) are associated with a 3-fold increase in the risk of acute myocardial infarction.[2,6] Homocysteine concentrations are determined by genetic and nutritional factors.[7] Vitamin B_{12} and folic acid are essential cofactors for the remethylation of homocysteine to methionine, and dietary supplementation with these vitamin lowers plasma homocysteine by up to 30%.[8] These observations have formed the basis of large-scale intervention trials that are seeking to determine whether lowering homocysteine concentrations through B vitamin supplementation can improve survival in patients with CHD.[9] However, at present, little is known about the beneficial effects of homocysteine lowering in patients with CHD.

Increasing evidence suggests that the adverse vascular effects of elevated homocysteine are mediated through endothelial dysfunction,[7,10–20] an early manifestation of atherosclerosis. Studies investigating the effects of lowering homocysteine concentrations on vascular endothelial function have yielded conflicting results.[21–27] In primates, folate supplementation is reported to reduce plasma homocysteine concentrations but not to affect vascular function.[21] In healthy human subjects, folate supplementation is associated with reduced

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homocysteine concentrations and improved vascular endothelial function,[22,23,26] although in these studies, there was no relationship between homocysteine concentrations and endothelial function, implying that the effects of B vitamins on endothelial function may not be mediated through homocysteine lowering.[26,27] However, a major limitation of previous studies is that they have used total plasma homocysteine concentrations as the sole index of homocysteine status.

We have studied the effects of dietary folate and vitamin B_{12} supplementation on vascular endothelial function in patients with CHD and examined the relationship between vascular endothelial function and concentrations of total, protein-bound, and free plasma homocysteine.

Methods

Subjects

We investigated 89 men with CHD (aged 56 [range 39 to 67] years) identified from the cardiology outpatient unit, coronary care unit, and coronary angiography records of 2 West London hospitals. Criteria for CHD were as follows: (1) myocardial infarction (chest pain associated with ECG evidence of myocardial infarction and/or elevated cardiac enzymes) or (2) angiographically proven coronary artery disease (>50% stenosis in ≥1 major epicardial vessel). Patients were not studied within 3 months of myocardial infarction or coronary intervention. All subjects were receiving regular medication for CHD; this remained unchanged during the course of the study, and cardiovascular drugs were omitted on the day of investigation. The present study was approved by the local ethics committee, and all subjects gave written informed consent to participate.

Procedures

Brachial artery flow–mediated dilatation (endothelium dependent) and nitroglycerin (NTG)-induced dilatation (endothelium independent) were measured before and 8 weeks after either (1) folic acid (5 mg) and vitamin B_{12} (1 mg) daily (n=59) or (2) matched placebo (n=30). Treatment allocation was randomized and double blind.

Brachial Artery Diameter

Brachial artery flow–mediated dilatation was measured by using a 7.0-MHz linear array transducer, an Acuson 128XP/10 system, and a high-resolution ultrasonic vessel wall tracking system (Vadirec, Ingenious Systems) as previously described.[17,18] In brief, the brachial artery was scanned longitudinally, and brachial artery diameter was measured at end diastole. After the baseline resting scan, a pneumatic cuff placed at the level of the mid forearm was inflated to 300 mm Hg for 4.5 minutes. The second scan was performed 55 to 65 seconds after cuff deflation. Fifteen minutes was allowed for vessel recovery, after which the second baseline scan was performed. NTG (400 μg) was then administered, and the fourth scan of the brachial artery was undertaken. The vessel diameter was measured by 2 independent observers unaware of the clinical details of the subjects and the type and stage of the study. The technique for measurement of brachial artery flow–mediated dilatation is reproducible in our laboratory. The intraindividual between-day coefficient of variation for flow-mediated dilatation is 3%, which compares favorably with that in other centers.[28] Flow-mediated dilatation of conduit arteries is endothelium dependent and largely mediated by NO.[29]

Biochemical Measurements

For each subject, concentrations of total plasma homocysteine, free (unbound) plasma homocysteine, serum folate, vitamin B_{12}, glucose, total cholesterol, HDL cholesterol, and triglycerides were measured at baseline and at 8 weeks. All samples were collected in the fasting state (overnight). For measurement of homocysteine species, blood was drawn into lithium heparin tubes and immediately centrifuged at 10 000*g* for 1 minute. The plasma was then divided into 2 aliquots. The first aliquot was deproteinized with sulfosalicylic acid, and the supernatant was used for measurement of free homocysteine. The second aliquot was used for measurement of total plasma homocysteine. Concentrations of total and free plasma homocysteine were determined by high-pressure liquid chromatography with fluorescence detection,[30] and the concentration of protein-bound homocysteine was calculated as the difference between the 2 concentrations. Serum folate and B_{12} were measured by MEIA (Abbott IMX system), and lipid profiles were determined by use of an Olympus AU800 multichannel analyzer. For each subject, homocysteine and vitamin samples were analyzed in one batch.

TABLE 1. Baseline Clinical and Biochemical Characteristics of Subjects

	Placebo Group	Vitamin Group	*P*
n	30	59	
Age, y	56±7	56±6	0.69
Current smokers, n (%)	0 (0)	7 (12)	0.05
Hypertension, n (%)	19 (63)	31 (53)	0.33
Diabetes, n (%)	5 (17)	6 (10)	0.40
Body mass index, kg/m^2	26.9±2.9	27.0±3.6	0.88
Systolic BP, mm Hg	138±17	138±21	0.85
Diastolic BP, mm Hg	86±10	87±11	0.75
Total cholesterol, mmol/L	5.0±0.9	4.9±0.9	0.57
HDL cholesterol, mmol/L	1.1±0.3	1.1±0.2	0.12
Fasting triglycerides, mmol/L	2.1±1.1	1.9±0.9	0.44
Fasting glucose, mmol/L	6.1±2.4	5.6±1.5	0.27
Creatinine, μmol/L	107±20	108±19	0.79

Values are mean±SD. BP indicates blood pressure.

Data Processing and Statistical Analysis

Data were analyzed with the use of SPSS version 8.0 statistical package and are expressed as mean±SD. Continuous data were analyzed by the independent-samples *t* test or the paired-samples *t* test for comparisons between groups and within subjects, respectively. The χ^2 test was used for categorical data. Linear regression analysis was conducted to examine the relationships between flow-mediated dilatation and concentrations of plasma homocysteine, serum folate, and vitamin B_{12}. Statistical significance was inferred at a value of $P<0.05$.

Results

Clinical and Biochemical Characteristics

The baseline clinical and biochemical measurements of subjects are summarized in Table 1. Age, body mass index, blood pressure, glucose, and lipid profile were similar in the vitamin and placebo groups.

Brachial Artery Measurements

There were no significant differences between the vitamin and placebo groups in flow-mediated dilatation at baseline (Table 2, Figure). At the 8-week follow-up visit, flow-mediated dilatation was improved in the vitamin group (1.5±3.5% change compared with baseline, $P=0.002$). The increase in flow-mediated dilatation after B vitamins was evident in the 13 subjects with initially elevated homocysteine (>15 μmol/L; 2.2±2.3% change, $P=0.01$) as well as in the 46 subjects with baseline homocysteine within the reference range (1.2±3.7% change, $P=0.03$). In contrast, flow-

TABLE 2. Brachial Artery and Biochemical Measurements at Baseline and at 8-wk Follow-Up

	Placebo Group			Vitamin Group		
	Baseline	8 wk	*P*	Baseline	8 wk	*P*
Flow-mediated dilatation, %	2.3±2.6	1.9±2.6	0.50	2.5±3.2	4.0±3.7	0.002
NTG-induced dilatation, %	20.3±8.2	17.7±5.5	0.20	20.0±6.9	19.0±7.2	0.20
Brachial artery diameter, mm	4.48±0.62	4.53±0.59	0.28	4.50±0.64	4.48±0.59	0.74
Basal blood flow, mL/min	82±47	88±43	0.53	85±44	87±50	0.90
Hyperemic flow, mL/min	375±120	401±128	0.22	392±113	416±159	0.21
Free plasma homocysteine, μmol/L	4.9±1.8	5.0±2.0	0.76	4.3±1.2	3.0±0.6	0.001
Protein-bound homocysteine, μmol/L	9.6±3.7	10.0±4.6	0.29	8.7±2.8	6.2±1.4	0.001
Total plasma homocysteine, μmol/L	14.5±5.4	14.9±6.5	0.38	13.0±3.4	9.3±1.9	0.001
Serum folate, ng/mL	10.4±5.1	10.2±4.5	0.86	10.3±4.3	31.2±10.8	0.001
Serum vitamin B_{12}, pg/mL	290±79	291±73	0.96	314±102	661±297	0.001

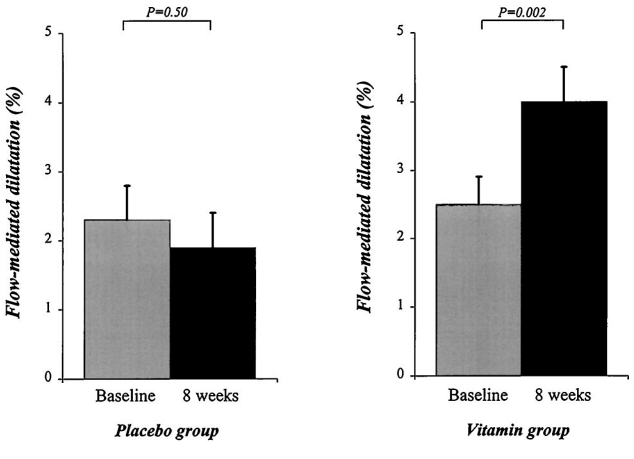
Values are mean±SD.

mediated dilatation was not altered by placebo (−0.3±2.5% change compared with baseline, P=0.50). At 8 weeks, flow-mediated dilatation was now significantly higher in the vitamin compared with the placebo group (P=0.008; Table 2, Figure).

NTG-induced dilatation, brachial artery diameter, and brachial artery blood flow were similar in the vitamin and placebo groups at baseline. These measures were unchanged after 8 weeks treatment with either vitamins or placebo (Table 2).

Biochemical Measurements

At baseline, concentrations of total and free plasma homocysteine and of serum folate and vitamin B_{12} were similar in the vitamin and placebo groups (Table 2). After the treatment period, concentrations of total, protein-bound, and free plasma homocysteine fell, and serum folate and vitamin B_{12} rose compared with baseline concentrations, in the vitamin but not the placebo group (Table 2). There were no significant changes in blood pressure, fasting glucose, or lipid profile between baseline and 8 weeks in either treatment group (data not shown).



Flow-mediated dilatation (mean±SEM) at baseline and at 8 weeks after B vitamin/placebo in patients with established CHD.

Determinants of Flow-Mediated Dilatation

In univariate analysis, flow-mediated dilatation was inversely correlated with concentrations of free homocysteine (r=−0.26, P=0.001), protein-bound homocysteine (r=−0.20, P=0.008), and total homocysteine (r=−0.24, P=0.002) and positively correlated with levels of folate (r=0.17, P=0.03) and vitamin B_{12} (r=0.12, P=0.05). Free, protein-bound, and total plasma homocysteine, folate, and vitamin B_{12} concentrations were also closely intercorrelated (Table 3).

In multivariate analysis, the inverse relationship between flow-mediated dilatation and free homocysteine remained significant after adjustment for concentrations of protein-bound homocysteine, folate, and vitamin B_{12} (Table 4) and after further adjustment for age, blood pressure, total and HDL cholesterol, fasting glucose, and cigarette smoking (P=0.02). In contrast, the relationships between flow-mediated dilatation and protein-bound homocysteine, folate, and vitamin B_{12} that were evident in univariate analysis became nonsignificant after adjustment for free plasma homocysteine concentrations (Table 4).

Discussion

We have found that dietary supplementation with folic acid and vitamin B_{12} improves vascular endothelial function in patients with CHD, an effect likely to be mediated through reduced concentrations of free plasma homocysteine. Our

TABLE 3. Correlation Coefficients (Pearson) Between Concentrations of fHcy, pHcy, tHcy, Folate, and Vitamin B_{12}

	fHcy	pHcy	tHcy	B_{12}	Folate
fHcy	...	0.81	0.91	−0.55	−0.63
pHcy	0.81	...	0.98	−0.42	−0.53
tHcy	0.91	0.98	...	−0.48	−0.58
B_{12}	−0.55	−0.42	−0.48	...	0.63
Folate	−0.63	−0.53	−0.58	0.63	...

fHcy indicates free homocysteine; pHcy, protein-bound homocysteine; and tHcy, total homocysteine. Placebo and vitamin subjects were combined. All P<0.001.

TABLE 4. Relationships Between Flow-Mediated Dilatation and Concentrations of Homocysteine, Folic Acid, and Vitamin B_{12} in Multivariate Analysis

	Partial Correlation Coefficient	*P*
Free plasma homocysteine	−0.16	0.03
Protein-bound homocysteine	−0.02	0.76
Folate	−0.03	0.68
Vitamin B_{12}	−0.001	0.99

results provide direct evidence that dietary supplementation with B vitamins may reduce cardiovascular risk in patients with established atherosclerosis.

In the present study, folic acid and vitamin B_{12} supplementation was associated with a significant improvement in brachial artery flow–mediated dilatation. This effect was seen in patients with moderately raised homocysteine (>15 μmol/L) as well as in patients whose homocysteine concentrations were within the reference range. Regression analysis demonstrated an inverse relationship between flow-mediated dilatation and concentrations of plasma homocysteine, suggesting that the effect of B vitamins on endothelial function may be mediated through reduced homocysteine concentrations. The precise mechanisms underlying the relationship between plasma homocysteine and vascular endothelial dysfunction are not well understood. Because flow-mediated dilatation is endothelium dependent and largely mediated by the release of NO,[29] our observations suggest an increase in the availability of NO after homocysteine lowering by B vitamins. The present study does not exclude a direct effect of folate or its related metabolites or an effect of vitamin B_{12} on endothelial function. However, the absence of a significant relationship between the levels of these vitamins and flow-mediated dilatation, after adjustment for plasma homocysteine concentrations, argues against this hypothesis.

Previous studies involving healthy volunteers[22–24] and patients with familial hypercholesterolemia[26,27] have not shown a significant relationship between the improvement in endothelial function and the change in plasma homocysteine after B vitamin supplementation. The precise reasons for this are not known; however, in all studies, total plasma homocysteine concentrations were used as the sole index of homocysteine status. In plasma, homocysteine exists in protein-bound (≈70%) and free (≈30%) forms; the latter includes reduced homocysteine and homocysteine disulfides.[31] We found an independent relationship between flow-mediated dilatation and concentrations of free, but not protein-bound, homocysteine. Our results suggest that free plasma homocysteine concentrations may be a more accurate index of the biological activity of homocysteine in vivo. This is an important, yet unrecognized, confounding factor that may limit interpretation of previous experimental and clinical studies investigating the relationship between homocysteine and endothelial function. Evidence to support this assertion also comes from in vitro data, which show that free homocysteine species inactivate NO, promote the generation of oxygen-derived free radicals, induce tissue factor release, and cause endothelial cell injury.[10,12,13,32]

Our observations of improved vascular function after B vitamin supplementation in patients with established atherosclerosis are in contrast with previous findings in patients with end-stage renal disease[25] and in animal models of atherosclerosis.[21,33] The lack of improvement in endothelial function despite reduced homocysteine concentrations in these studies may be explained by the failure of folate supplementation to lower plasma homocysteine concentrations <20 μmol/L in patients with end-stage renal disease[25] and the concurrent administration of an atherogenic diet in primates,[21] factors that may have a continuing effect on endothelial function. In the present study, the doses of folate and vitamin B_{12} were identical to those currently being used in the largest prospective intervention trial, the Study of Additional Reductions in Cholesterol and Homocysteine (SEARCH), and were selected to provide maximal homocysteine reduction.[9] However, folate doses as low as 400 μg/d have been shown to have a similar homocysteine-lowering effect. The results of the present study lend support to the hypotheses that elevated homocysteine concentrations may have a key role in the development of atherosclerosis and that B vitamin supplementation may reduce cardiovascular risk in patients with CHD.

In summary, we have found that supplementation with folic acid and vitamin B_{12} improves brachial artery endothelium-dependent dilatation in patients with CHD and that this action may be mediated through reduced concentrations of free plasma homocysteine. These data provide evidence that dietary supplementation with B vitamins may reduce cardiovascular risk in patients with established atherosclerosis.

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References

1. Boushey CJ, Beresford SA, Omenn GS, et al. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease: probable benefits of increasing folic acid intakes. *JAMA*. 1995;274:1049–1057.
2. Graham IM, Daly LE, Refsum HM, et al. Plasma homocysteine as a risk factor for vascular disease: the European Concerted Action Project. *JAMA*. 1997;277:1775–1781.
3. Nygard O, Nordrehaug JE, Refsum H, et al. Plasma homocysteine levels and mortality in patients with coronary artery disease. *N Engl J Med*. 1997;337:230–236.
4. Chambers JC, Obeid OA, Refsum H, et al. Plasma homocysteine concentrations and coronary heart disease risk in UK Indian Asian and European white men. *Lancet*. 2000;355:523–527.
5. Clarke R, Daly L, Robinson K, et al. Hyperhomocysteinemia: an independent risk factor for vascular disease. *N Engl J Med*. 1991;324:1149–1155.
6. Stampfer MJ, Malinow MR, Willett WC, et al. A prospective study of plasma homocyst(e)ine and risk of myocardial infarction in US physicians. *JAMA*. 1992;268:877–881.
7. Welch GN, Loscalzo J. Homocysteine and atherothrombosis. *N Engl J Med*. 1998;338:1042–1050.

8. Homocysteine Lowering Trialists' Collaboration. Lowering blood homocysteine with folic acid based supplements: meta-analysis of randomised trials. *BMJ*. 1998;316:894–898.
9. Clarke R, Collins R. Can dietary supplements with folic acid or vitamin B6 reduce cardiovascular risk? Design of clinical trials to test the homocysteine hypothesis of vascular disease. *J Cardiovasc Risk*. 1998;5:249–255.
10. Starkebaum G, Harlan JM. Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine. *J Clin Invest*. 1986;77:1370–1376.
11. Lentz SR, Sadler JE. Inhibition of thrombomodulin surface expression and protein C activation by the thrombogenic agent homocysteine. *J Clin Invest*. 1991;88:1906–1914.
12. Stamler JS, Osborne JA, Jaraki O, et al. Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. *J Clin Invest*. 1993;91:308–318.
13. Fryer RH, Wilson BD, Gubler DB, et al. Homocysteine, a risk factor for premature vascular disease and thrombosis, induces tissue factor activity in endothelial cells. *Arterioscler Thromb*. 1993;13:1327–1333.
14. Celermajer DS, Sorensen K, Ryalls M, et al. Impaired endothelial function occurs in the systemic arteries of children with homozygous homocystinuria but not in their heterozygous parents. *J Am Coll Cardiol*. 1993;22:854–858.
15. Tawakol A, Omland T, Gerhard M, et al. Hyperhomocyst(e)inemia is associated with impaired endothelium-dependent vasodilation in humans. *Circulation*. 1997;95:1119–1121.
16. Chambers JC, McGregor A, Jean Marie J, et al. Acute hyperhomocysteinaemia and endothelial dysfunction. *Lancet*. 1997;351:36–37.
17. Chambers JC, McGregor A, Jean Marie J, et al. Demonstration of rapid onset vascular endothelial dysfunction after hyperhomocysteinemia: an effect reversible with vitamin C therapy. *Circulation*. 1999;99:1156–1160.
18. Chambers JC, Obeid OA, Kooner JS. Physiological increments in plasma homocysteine induce vascular endothelial dysfunction in normal human subjects. *Arterioscler Thromb Vasc Biol*. 1999;19:2922–2927.
19. Bellamy MF, McDowell IF, Ramsey MW, et al. Hyperhomocysteinemia after an oral methionine load acutely impairs endothelial function in healthy adults. *Circulation*. 1998;98:1848–1852.
20. Kanani PM, Sinkey CA, Browning RL, et al. Role of oxidant stress in endothelial dysfunction produced by experimental hyperhomocysteinaemia in humans. *Circulation*. 1999;100:1161–1168.
21. Lentz SR, Malinow MR, Piegors DJ, et al. Consequences of hyperhomocyst(e)inemia on vascular function in atherosclerotic monkeys. *Arterioscler Thromb Vasc Biol*. 1997;17:2930–2934.
22. Bellamy MF, McDowell IF, Ramsey MW, et al. Oral folate enhances endothelial function in hyperhomocysteinaemic subjects. *Eur J Clin Invest*. 1999;29:659–662.
23. Woo KS, Chook P, Lolin YI, et al. Folic acid improves arterial endothelial function in adults with hyperhomocystinemia. *J Am Coll Cardiol*. 1999;34:2002–2006.
24. Usui M, Matsuoka H, Miyazaki H, et al. Endothelial dysfunction by acute hyperhomocyst(e)inaemia: restoration by folic acid. *Clin Sci (Colch)*. 1999;96:235–239.
25. Kunz K, Petitjean P, Lisri M, et al. Cardiovascular morbidity and endothelial dysfunction in chronic haemodialysis patients: is homocyst(e)ine the missing link? *Nephrol Dial Transplant*. 1999;14:1934–1942.
26. Verhaar MC, Wever RM, Kastelein JJ, et al. Effects of oral folic acid supplementation on endothelial function in familial hypercholesterolemia: a randomized placebo-controlled trial. *Circulation*. 1999;100:335–338.
27. Verhaar MC, Wever RM, Kastelein JJ, et al. 5-Methyltetrahydrofolate, the active form of folic acid, restores endothelial function in familial hypercholesterolemia. *Circulation*. 1998;97:237–241.
28. Sorensen KE, Celermajer DS, Spiegelhalter DJ, et al. Non-invasive measurement of human endothelium dependent arterial responses: accuracy and reproducibility. *Br Heart J*. 1995;74:247–253.
29. Joannides R, Haefeli WE, Linder L, et al. Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries in vivo. *Circulation*. 1995;91:1314–1319.
30. Fiskerstrand T, Refsum H, Kvalheim G, et al. Homocysteine and other thiols in plasma and urine: automated determination and sample stability. *Clin Chem*. 1993;39:263–271.
31. Ueland PM. Homocysteine species as components of plasma redox thiol status. *Clin Chem*. 1995;41:340–342.
32. Dudman NP, Temple SE, Guo XW, et al. Homocysteine enhances neutrophil-endothelial interactions in both cultured human cells and rats In vivo. *Circ Res*. 1999;84:409–416.
33. Ambrosi P, Rolland PH, Bodard H, et al. Effects of folate supplementation in hyperhomocysteinemic pigs. *J Am Coll Cardiol*. 1999;34:274–279.