Folic acid: a marker of endothelial function in type 2 diabetes?

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Objectives: Endothelial dysfunction is a common feature of type 2 diabetes. Recent studies suggest that the B-vitamin folic acid exerts direct beneficial effects on endothelial function, beyond the well known homocysteine lowering effects. Therefore, folic acid might represent a novel "biomarker" of endothelial function. We sought to determine whether plasma levels of folic acid determine endothelial-dependent vasodilation in patients with type 2 diabetes.

Methods: Forearm arterial blood flow (FABF) was measured at baseline and during intra-brachial infusion of the endothelial-dependent vasodilator acetylcholine (15 μg/min) and the endothelial-independent vasodilator sodium nitroprusside (2 μg/min) in 26 type 2 diabetic patients (age 56.5 ± 0.9 years, means ± SEM) with no history of cardiovascular disease.

Results: FABF ratio (ie, the ratio between the infused and control forearm FABF) significantly increased during acetylcholine (1.10 ± 0.04 vs 1.52 ± 0.07, $p < 0.001$) and sodium nitroprusside (1.12 ± 0.11 vs 1.62 ± 0.06, $p < 0.001$) infusions. After correcting for age, gender, diabetes duration, smoking, hypertension, body mass index, microalbuminuria, glycated hemoglobin, low-density lipoprotein cholesterol, and homocysteine, multiple regression analysis showed that plasma folic acid concentration was the only independent determinant ($p = 0.037$, $R^2 = 0.22$) of acetylcholine-mediated, but not sodium nitroprusside-mediated, vasodilatation.

Conclusions: Folic acid plasma concentrations determine endothelium-mediated vasodilatation in patients with type 2 diabetes. These results support the hypothesis of a direct effect of folic acid on endothelial function and the rationale for interventions aimed at increasing folic acid levels to reduce cardiovascular risk.

Keywords: folic acid, homocysteine, endothelium, type 2 diabetes

Introduction

Impaired endothelial function is a common feature in type 2 diabetes (McVeigh et al 1992; Enderle et al 1998; Hogikyan et al 1998; Chowienczyk et al 1999; Kawagishi et al 1999; Makimattila et al 1999; Rizzoni et al 2001). This might contribute to the increased cardiovascular morbidity and mortality observed in type 2 diabetes by accelerating the atherosclerotic process and enhancing the prothrombotic state (Stehouwer et al 2002; Landmesser et al 2004).

Several factors including co-existing hypertension, obesity, insulin resistance, hyperglycemia, hypercholesterolemia, and a proinflammatory state may account for endothelial dysfunction in type 2 diabetes (Guerci et al 2001). Folic acid, a B-vitamin, has recently gained considerable interest because of its potential to enhance endothelial function in several pathological conditions including coronary artery disease, smoking, familial hypercholesterolemia, and type 2 diabetes (Verhaar et al 1999; Chambers et al 2000; Mangoni et al 2002; van Etten et al 2002). Recent evidence supports the hypothesis that the effects of folic acid on endothelium may be "direct" (ie, independent) of the well known homocysteine lowering effects (Doshi et al 2002;

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Mangoni and Jackson 2002; Mangoni et al 2002). Folic acid levels might represent a "biomarker" of endothelial function, easily modifiable through safe, effective, and inexpensive dietary and/or pharmacological interventions. Therefore, we sought to determine whether folic acid plasma concentrations affected endothelial function in a group of patients with type 2 diabetes.

Methods

Subjects

Twenty-six patients with type 2 diabetes (age 56.5 ± 0.9 years, range 46–65; diabetes duration 5.5 ± 0.6 years, means ± SEM) were recruited from diabetic and general medical outpatient clinics and through local advertising. The subjects had no history of angina, myocardial infarction, stroke, or peripheral occlusive disease. Hypertension (previous sphygmomanometric blood pressure values > 130/80 mmHg and treatment with antihypertensive drugs) was present in 16 patients. Antihypertensive treatment included diuretics in 6 patients, angiotensin converting enzyme inhibitors in 7, angiotensin II receptor antagonists in 3, beta blockers in 7, Ca channel blockers in 4, and alpha blockers in 2 patients. Antidiabetic treatment included oral hypoglycemic agents and insulin in 4 patients, oral hypoglycemic agents alone in 19, insulin alone in 2, and diet alone in 1 patient. None of the subjects were on vitamin supplements or drugs known to significantly alter folic acid and/or homocysteine blood concentrations. Microalbuminuria, defined as urinary albumin–creatinine ratio ≥ 2.5 mg/mmol (men) or ≥ 3.5 mg/mmol (women), was present in 8 subjects. The study had been approved by the Local Research Ethics Committee. Each subject gave written informed consent before starting the study.

Protocol

Investigations were performed in a temperature-controlled laboratory (25–27 °C). The subjects were asked to abstain from cigarette smoking and alcohol consumption from the evening prior to the study. Each subject underwent 2 visits. During visit 1, a physical examination and an electrocardiogram were performed, and blood pressure (BP) (mean of three consecutive readings after the subject was resting for 5 min) and heart rate (HR) were recorded. During visit 2, a fasting blood sample was taken (serum lipids and glucose, glycated hemoglobin, full blood count, homocysteine, and folic acid). Then, endothelial function was assessed by the perfused forearm technique.

Forearm arterial blood flow

The brachial artery was cannulated using a 27-gauge cannula connected via an epidural catheter to a infusion pump. Forearm arterial blood flow (FABF) was measured simultaneously in both arms (infused and control forearm) by strain-gauge venous occlusion plethysmography (DE Hokanson Inc, Bellevue, WA, USA). Measurements were obtained at baseline after each subject rested supine for 20 min and during an 8-min intra-arterial infusion of the endothelium-dependent vasodilator acetylcholine (15 μg/min, Clinalfa, Switzerland). After a second baseline was obtained, the endothelium-independent vasodilator sodium nitroprusside (2 μg/min, David Bull Laboratories, Warwick, UK) was infused. The doses of acetylcholine and sodium nitroprusside used did not have any systemic effect on BP and HR. FABF measurements were taken during the final 2 min of each step. Circulation to the hands was excluded 1 min before FABF measurement by inflating a pediatric cuff around the wrist at 200 mmHg. Vasodilators were stopped 5 days before FABF assessment. This washout period was considered adequate, as the elimination half-life of vasodilators ranged between 11 and 22 hours.

Folic acid and homocysteine

Serum folic acid was measured from fresh samples by competitive protein binding enzyme immunoassays on the

Table 1 Baseline characteristics of the patients studied

Parameter	Mean ± SEM	(95% CI)
Age (years)	56.5 ± 0.9	(54.6–58.4)
Male:female		14:12
Hypertension		16/26
Smoking		6/26
Microalbuminuria		8/26
Diabetes duration (years)	5.5 ± 0.6	(4.3–6.7)
Body mass index (kg/m^2)	31.4 ± 1.1	(29.2–33.6)
Plasma glucose (mmol/L)	11.4 ± 0.8	(9.7–13.2)
Glycated hemoglobin (%)	8.3 ± 0.3	(7.6–8.9)
Serum folic acid (μg/L)	8.0 ± 0.6	(6.8–9.3)
Plasma homocysteine (μmol/L)	11.7 ± 0.4	(10.5–12.3)
Serum creatinine (mmol/L)	89 ± 3	(83–94)
Total cholesterol (mmol/L)	5.3 ± 0.1	(5.0–5.5)
HDL-cholesterol (mmol/L)	1.2 ± 0.1	(1.1–1.3)
LDL-cholesterol (mmol/L)	2.6 ± 0.1	(2.3–2.9)
Serum triglycerides (mmol/L)	3.3 ± 0.3	(2.7–4.0)
Systolic blood pressure (mmHg)	137 ± 3	(130–144)
Diastolic blood pressure (mmHg)	77 ± 2	(74–81)
Heart rate (beats/min)	74 ± 2	(69–79)

Abbreviations: CI, confidence interval; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Centaur analyzer (Bayer Diagnostics, Newbury, UK). The coefficient of variation was 5.2%. Fasting venous blood samples were collected into tubes containing disodium EDTA and tubes without anticoagulation. The samples were centrifuged at 1800 *g* within 30 min, and the plasma and serum separated and stored at –20 °C. Plasma homocysteine was determined using a fluorescence polarization immunoassay on an IMX analyser (Abbott Diagnostics, Maidenhead, UK) (Refsum et al 1989). Between-batch imprecision was assessed at homocysteine concentrations of 7.0, 12.5, and 25.5 μmol/L, and coefficients of variation of 2.4%, 2.3%, and 1.6% respectively were obtained (n = 19).

Statistical analysis

Data are presented as means ± SEM and 95% confidence intervals. FABF values at baseline and during acetylcholine infusion are expressed as ratio between the infused and control forearm. FABF ratio differences between baseline and acetylcholine infusion were assessed by paired Student *t* test. Univariate analysis was performed by calculating the correlation coefficient *r* between different parameters. Determinants of endothelium-dependent vasodilatation were identified by backward stepwise regression analysis. (SPSS for Windows 11.0, SPSS Inc, Chicago, IL, USA). The factors included in the model were age, gender, diabetes duration, hypertension, smoking, glycated hemoglobin, body mass index, microalbuminuria, low-density lipoprotein (LDL) cholesterol, folic acid, and homocysteine concentrations. A p-value < 0.05 indicated statistical significance.

Results

Baseline characteristics are illustrated in Table 1. No patient had biochemical or clinical evidence of folic acid deficiency. A significant increase in FABF ratio was observed during acetylcholine (1.10 ± 0.04 baseline vs 1.52 ± 0.07 during acetylcholine, $p < 0.001$) and sodium nitroprusside (1.12 ± 0.11 baseline vs 1.62 ± 0.06 during sodium nitroprusside, $p < 0.001$), indicating significant vasodilatation in the infused arm. Univariate analysis of baseline clinical and biochemical parameters did not show any significant relationship apart from a negative correlation between homocysteine and glycated hemoglobin concentrations (Table 2). After correcting for age, gender, diabetes duration, smoking, hypertension, body mass index, microalbuminuria, glycated hemoglobin, LDL-cholesterol, and homocysteine, multivariate regression analysis showed that folic acid concentration was the only significant and independent determinant of acetylcholine-mediated endothelium-dependent, but not of sodium nitroprusside-mediated endothelium-independent, vasodilatation ($p = 0.037$, $R^2 = 0.22$; Tables 3 and 4).

Discussion

Folic acid levels significantly and independently determined forearm endothelium-dependent, but not endothelium-independent, vasodilatation in a group of stable patients with type 2 diabetes. Therefore, blood concentrations of this B-vitamin might represent a biologically and clinically useful marker of endothelial function in these patients.

Table 2 Univariate analysis with correlation coefficients

Parameter	Folate	BMI	LDL-Chol	Hcy	Age	Diab dur	HbA1c
Folate	–	–0.011	–0.068	–0.170	–0.058	–0.288	–0.273
p		0.956	0.754	0.438	0.780	0.153	0.178
BMI	–0.011	–	0.352	–0.009	0.000	0.252	–0.059
p	0.956		0.091	0.969	1.000	0.215	0.776
LDL-Chol	–0.068	0.352	–	0.039	0.021	0.182	–0.089
p	0.754	0.091		0.864	0.923	0.394	0.678
Hcy	–0.170	–0.009	0.039	–	0.345	0.204	–0.498
p	0.438	0.969	0.864		0.107	0.351	0.016
Age	–0.058	0.000	0.021	0.345	–	0.160	–0.219
p	0.780	1.000	0.923	0.107		0.435	0.282
Diab dur	–0.288	0.252	0.182	0.204	0.160	–	0.304
p	0.153	0.215	0.394	0.351	0.435		0.132
HbA1c	–0.273	–0.059	–0.089	–498	–0.219	0.304	–
p	0.178	0.776	0.678	0.016	0.282	0.132	

Abbreviations: BMI, body mass index; LDL-Chol, low-density lipoprotein cholesterol; Hcy, homocysteine; Diab dur, diabetes duration; HbA1c, glycated hemoglobin; p, probability.

Table 3 Backward multiple regression analysis (last 6 steps) of changes in maximal endothelial-dependent vasodilatation during acetylcholine infusion

Model	Beta	t	Sig	R	R^2
Gender	−0.312	−1.251	0.233	0.72	0.52
Body mass index	−0.328	−1.411	0.182		
Smoking	0.378	1.806	0.094		
LDL-cholesterol	−0.298	−1.452	0.170		
Homocysteine	0.244	1.193	0.254		
Folic acid	0.645	3.3037	0.010		
Gender	−0.356	−1.424	0.176	0.68	0.47
Body mass index	−0.335	−1.420	0.178		
Smoking	0.329	1.580	0.136		
LDL-cholesterol	−0.288	−1.380	0.189		
Folic acid	0.608	2.850	0.013		
Gender	−0.316	−1.234	0.236	0.63	0.40
Body mass index	−0.413	−1.750	0.101		
Smoking	0.325	1.518	0.150		
Folic acid	0.600	2.730	0.015		
Body mass index	−0.261	−1.275	0.220	0.58	0.33
Smoking	0.238	1.159	0.264		
Folic acid	0.493	2.402	0.29		
Body mass index	−0.244	−1.185	0.252	0.53	0.28
Folic acid	0.465	2.260	0.037		
Folic acid	0.469	2.253	0.037	0.47	0.22

NOTE: Dependent variable = changes in FABF ratio during acetylcholine infusion. **Abbreviations:** Beta, regression coefficient; t, regression coefficient; Sig, significance; R, R statistic; R^2, R squared; FABF, forearm arterial blood flow.

Endothelial dysfunction is an independent predictor of mortality in patients with type 2 diabetes followed up for 9 years, even after correcting for urinary albumin excretion and markers of inflammation (Stehouwer et al 2002). Therefore, enhancement of endothelial function might provide significant cardiovascular protection.

The ameliorative effects of folic acid supplementation on endothelial function have been traditionally ascribed to its homocysteine lowering effects (Mangoni and Jackson 2002). It is well established that homocysteine acutely and chronically impairs endothelial function by inhibiting the synthesis and release of nitric oxide and enhancing the production of superoxide (Zhang et al 2000; Mangoni and Jackson 2002; Weiss et al 2003). Therefore, lowering of homocysteine levels might explain the enhancement of endothelial function following folic acid treatment (Bellamy et al 1999; Woo et al 1999).

Recent studies, however, support the hypothesis that folic acid exerts direct effects on the endothelium (Doshi et al 2002; Mangoni et al 2002). There is in vitro evidence that 5-methyltetrahydrofolate, the active form of folic acid, interacts with the enzyme endothelial nitric oxide synthase (eNOS) in a fashion analogous, yet independent, of the co-factor tetrahydrobiopterin to enhance endothelial function (Hyndman et al 2002).

The results of our study suggest that folic acid, by directly interacting with eNOS, directly modulates NO production and endothelial function, independently of established markers of endothelial dysfunction and cardiovascular disease in type 2 diabetes. Of note, none of the study subjects had folic acid deficiency, suggesting that a "relative" rather than an "absolute" deficiency may already provide adverse effects on endothelial function. This might have important clinical implications, as folic acid levels can be easily and safely increased by dietary intervention and/or vitamin supplementation (Lucock 2004).

Our patients were on several antihypertensive and hypoglycaemic drugs affecting endothelial function, thus potentially limiting data interpretation. Although antihypertensive vasodilators were stopped at least 5 half-lives before the study day, we ran another regression analysis

Table 4 Backward multiple regression analysis (last 6 steps) of changes in maximal endothelial-independent vasodilatation during sodium nitroprusside infusion

Model	Beta	t	Sig	R	R^2
Smoking	0.415	1.844	0.088	0.68	0.47
Microalbuminuria	−0.383	−1.691	0.115		
LDL-cholesterol	−0.301	1.424	0.178		
Age	0.467	1.954	0.073		
Gender	0.258	1.170	0.263		
Hypertension	0.443	1.793	0.096		
Smoking	0.442	1.952	0.071	0.64	0.41
Microalbuminuria	−0.384	−1.676	0.116		
LDL-cholesterol	−0.369	−1.797	0.094		
Age	0.408	1.724	0.107		
Hypertension	0.396	1.604	0.131		
Smoking	0.342	1.493	0.156	0.55	0.30
Microalbuminuria	−0.222	−1.025	0.321		
LDL-cholesterol	−0.367	−1.701	0.110		
Age	0.258	1.129	0.277		
Smoking	0.329	1.439	0.169	0.50	0.25
LDL-cholesterol	−0.356	−1.647	0.119		
Age	0.263	1.148	0.268		
Smoking	0.242	1.111	0.282	0.44	0.19
LDL-cholesterol	−0.367	−1.687	0.110		
LDL-cholesterol	−0.367	−1.676	0.111	0.36	0.13

NOTE: Dependent variable = changes in FABF ratio during sodium nitroprusside infusion. **Abbreviations:** Beta, regression coefficient; t, regression coefficient; Sig, significance; R, R statistic; R^2, R squared; LDL, low-density lipoprotein.

to study the effect of antihypertensive and hypoglycemic treatment. None of these drugs affected the relationship between folic acid levels and endothelial-dependent vasodilatation (data not shown).

The limitations of our study are related to the relatively small sample size, the lack of data on oxidative stress markers to further support a beneficial effect of folic acid on eNOS activity, and the absence of "hard" end points such as cardiovascular morbidity and mortality. Larger randomized controlled studies are urgently needed to demonstrate whether folic acid reduces cardiovascular risk in type 2 diabetes.

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