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Short communication

Tetrahydrobiopterin improves endothelium-dependent vasodilation by increasing nitric oxide activity in patients with Type II diabetes mellitus

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Abstract

Aims/hypothesis. Tetrahydrobiopterin is an essential cofactor of nitric oxide synthase, and its deficiency decreases nitric oxide bioactivity. Our aim was to find whether supplementation of tetrahydrobiopterin could improve endothelial dysfunction in diabetic patients.
Methods. Forearm blood flow responses to the endothelium-dependent vasodilator acetylcholine (0.75–3.0 $\mu g \cdot 100\ ml^{-1} \cdot min^{-1}$) and to the endothelium-independent vasodilator sodium nitroprusside (0.1–1.0 $\mu g \cdot 100\ ml^{-1} \cdot min^{-1}$) before and during concomitant intra-arterial infusion of tetrahydrobiopterin (500 μg/min) were measured by venous occlusion plethysmography in 12 control subjects and 23 patients with Type II (non-insulin-dependent) diabetes mellitus.
Results. In control subjects, tetrahydrobiopterin had no effect on the dose-response curves to acetylcholine and sodium nitroprusside. In contrast, in diabetic patients, the attenuated endothelium-dependent vasodilation to acetylcholine was considerably improved by concomitant treatment with tetrahydrobiopterin, whereas the endothelium-independent vasodilation was not affected. This beneficial effect of tetrahydrobiopterin in diabetic patients could be completely blocked by N^G-monomethyl-L-arginine.
Conclusion/interpretation. These findings suggest the possibility that endothelial dysfunction in Type II diabetes might be related to decreased availability of tetrahydrobiopterin. [Diabetologia (2000) 43: 1435–1438]

Keywords Diabetes mellitus, endothelium, nitric oxide, tetrahydrobiopterin, endothelium-dependent vasodilation, acetylcholine, N^G-monomethyl-L-arginine.

The endothelium has a pivotal role in maintaining vascular tone and function and is intimately involved in the pathogenesis of atherosclerosis. Experimental and clinical investigations have shown that diabetes mellitus is associated with endothelial dysfunction. The exact mechanisms are not known but decreased synthesis of nitric oxide (NO) or increased inactivation of NO by oxygen free radicals have both been proposed. Recent reports have shown that exogenous treatment with tetrahydrobiopterin improves impaired NO bioactivity in patients with hypercholesterolaemia [1] and heavy smokers [2]. Notably, in vitro studies with aortic rings of diabetic rats showed restoration of endothelial function after pre-incubation with tetrahydrobiopterin (BH4) [3]. Tetrahydrobiopterin is a critical cofactor for NO formation and seems to modulate nitric oxide synthase (NOS) activity. In the absence of sufficient BH4, NOS changes its functional profile: instead of oxidizing L-arginine, the

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Abbreviations: NO, Nitric Oxide; NOS, nitric oxide synthase; ACh, acetylcholine; SNP, sodium nitroprusside; L-NMMA, N^G-monomethyl-L-arginine; FBF, forearm blood flow; BH4, tetrahydrobiopterin.

Table 1. Clinical characteristics of study groups

	Diabetic patients	Control subjects
n	23	12
Sex (male/female)	16/7	8/4
Age (years)	52 ± 2	50 ± 3
Duration of diabetes (years)	5.3 ± 0.6	–
BMI (kg/m^2)	27.4 ± 0.4[a]	24.7 ± 0.6
HbA_{1c} (%)	7.8 ± 0.2[a]	5.4 ± 0.2
Fasting glucose (mmol/l)	9.4 ± 0.6[a]	4.9 ± 0.1
Total cholesterol (mmol/l)	5.3 ± 0.2	5.4 ± 0.4
LDL-cholesterol (mmol/l)	3.3 ± 0.2	3.4 ± 0.3
HDL-cholesterol (mmol/l)	1.1 ± 0.06	1.2 ± 0.05
Triglycerides (mmol/l)	2.1 ± 0.2[a]	1.4 ± 0.3
Mean blood pressure (mm Hg)	92 ± 3[a]	88 ± 4
Forearm volume (liter)	1.02 ± 0.03	1.08 ± 0.09
Forearm blood flow (ml × 100 ml^{-1} × min^{-1})	2.8 ± 0.1	2.9 ± 0.2

Data presented as means ± SEM. [a] $p < 0.05$ vs control subjects

enzyme reduces molecular oxygen to superoxide anion, even in the setting of adequate substrate concentration [4]. An abnormal metabolism of tetrahydrobiopterin has recently been found to be the major cause of impaired endothelium-dependent relaxation through nitric oxide/O_2^- imbalance in aorta of insulin-resistant rats [5].

The intention of our study was therefore to assess whether BH4 could improve the endothelial dysfunction of patients with Type II (non-insulin-dependent) diabetes mellitus.

Subjects and methods

Subjects. We studied twelve control subjects and 23 patients with Type II diabetes (Table 1). Glycaemic control was achieved by diet alone ($n = 7$) or diet plus oral hypoglycaemic agents ($n = 16$) (metformin or sulphonylureas or both). The criteria for exclusion of both diabetic and control subjects included hypertension (> 145/90 mmHg), increased LDL-cholesterol (> 75th centile for age and sex), cardiac or cerebrovascular disease, renal impairment and the habit of smoking. Further exclusion criteria were current use of insulin, antioxidants or hormone replacement therapy. All female subjects were postmenopausal. Forearm volume was measured according to the water displacement method. Informed consent was obtained from all subjects, and the study was approved by the local ethics committee.

Study protocol. Forearm blood flow (FBF) was measured by venous occlusion plethysmography as described recently [2, 6]. Endothelium-dependent vasodilation was assessed by infusing acetylcholine (ACh, Farmigea, Pisa, Italy) in increasing concentrations of 0.75, 1.5 and 3.0 μg · 100 ml forearm tissue^{-1} · min^{-1} into the brachial artery. Sodium nitroprusside (SNP; Schwarz Pharma, Monheim, Germany) was infused to assess endothelium-independent vasodilation (0.1, 0.3 and 1.0 μg · 100 ml forearm tissue^{-1} · min^{-1}). During coinfusion of NO-synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA 8 μmol/min, Calbiochem, Bad Soden, Germany), the acetylcholine (ACh) response curve was repeated. After a rest of 30 min, tetrahydrobiopterin (BH4 500 μg/min, Schircks laboratories, Iona, Switzerland) was given to all study participants to test the effects of BH4 on FBF response to ACh and sodium nitroprusside (SNP). This dose of BH4 was chosen to reach plasma concentrations that have been shown to achieve maximum NO production by NOS in vitro [7] and to improve endothelium-dependent vasodilation in patients with hypercholesterolaemia [1] and heavy smokers [2]. Subsequently, the dose-response curve to ACh was repeated during co-infusion of BH4 and L-NMMA.

Statistical analysis. All values are reported as means ± SEM. Group comparisons with baseline characteristics were done by unpaired *t* test. Responses to ACh, SNP and L-NMMA, with and without BH4, were analysed by ANOVA for repeated measures and Scheffe's test was applied for multiple comparison testing. A value of $p < 0.05$ was considered statistically significant.

Results

The clinical characteristics of the study groups are provided in Table 1. The diabetic patients and control subjects were matched for age. The serum cholesterol concentrations were similar in both groups. Diabetic subjects had statistically significantly higher fasting glucose, glycated haemoglobin, body mass index, mean blood pressure and triglyceride concentrations. Forearm volume and basal forearm blood flow were similar between diabetic and control subjects.

Acetylcholine dose-dependently increased FBF in both groups (Fig. 1). The vasodilator responses to ACh were statistically significantly attenuated in diabetic patients (maximum FBF: 9.8 ± 1.0 ml · 100 ml^{-1} · min^{-1}) compared with control subjects (maximum FBF: 18.3 ± 1.1 ml · 100 ml^{-1} · min^{-1}). Co-infusion of the NOS-inhibitor, L-NMMA, blunted the ACh-induced vasodilation statistically significantly in control subjects. In diabetic patients, L-NMMA had, however, no significant effect. Infusion of BH4 did not change basal FBF in either group. In control subjects, co-infusion of BH4 had no effect on ACh-induced vasodilation. In diabetic patients, ACh-induced vasodilation was, however, statistically significantly enhanced by concomitant BH4 treatment (maximum FBF: 15.1 ± 1.2 ml · 100 ml^{-1} · min^{-1}). When the effect of L-NMMA was re-tested in the presence of BH4, the NOS-inhibitor completely abolished the vasodilatory effect of BH4 in diabetic patients (maximum FBF: 8.9 ± 1.1 ml × 100 ml^{-1} · min^{-1}). In control subjects, BH4 did not alter the inhibitory effect of L-NMMA on the ACh dose-response curve.

The FBF response to SNP was no different between diabetic patients (maximum FBF: 14.3 ± 1.1 ml · 100 ml^{-1} · min^{-1}) and control subjects (maximum FBF: 14.7 ± 1.2 ml · 100 ml^{-1} · min^{-1}) and was not modified by BH4 (diabetic patients: maximum FBF 14.7 ± 1.2 ml · 100 ml^{-1} · min^{-1}; control subjects: maximum FBF: 14.4 ± 1.1 ml · 100 ml^{-1} · min^{-1}).

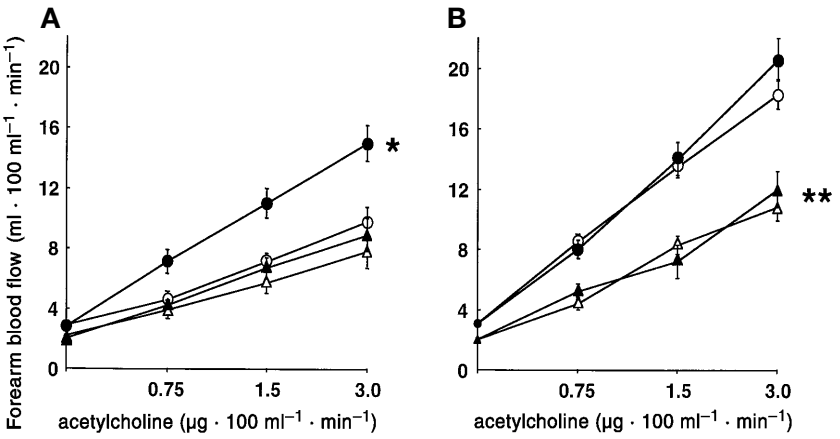

Fig. 1 A, B. Effect of tetrahydrobiopterin (BH4) on acetylcholine-induced vasodilation in diabetic patients (**A**) and control subjects (**B**) with and without co-infusion of L-NMMA. Tetrahydrobiopterin significantly improved acetylcholine-induced vasodilation in diabetic patients, but did not change the dose-response curve in control subjects. In diabetic patients, co-infusion of L-NMMA had no effect under control conditions (saline) but completely blocked the BH4-induced increase in forearm blood flow. In control subjects, the effect of L-NMMA on the dose-response curve was not altered by BH4. Data are shown as means ± SEM. * significant difference in the overall dose response vs saline and L-NMMA-co-infusion ($p < 0.05$). ** significant difference between infusions with and without L-NMMA ($p < 0.05$). –○– Saline; –●– BH_4; –△– Saline + L-NMMA; –▲– BH_4 + L-NMMA

Discussion

The salient findings of this study are that treatment with the endothelial NOS cofactor, BH4, improves endothelium-dependent vasodilation in patients with Type II diabetes and that this beneficial effect of BH4 could be blocked by the NOS-inhibitor, L-NMMA.

Our finding of reduced response to ACh is consistent with other reports of impaired endothelium-dependent vasodilation in patients with Type II diabetes [6]. Furthermore, using the NOS inhibitor, L-NMMA, to block the ACh-induced vasodilation, we found a considerably attenuated effect of L-NMMA in diabetic patients compared with control subjects, implying reduced bioactivity of NO.

Several potential mechanisms have been proposed to explain abnormal nitric oxide-mediated vasodilation in patients with diabetes. These include decreased synthesis of nitric oxide by the endothelium and increased inactivation of endothelium-derived nitric oxide by reactive oxygen species. Oxygen radicals can be produced in diabetes by a number of reactions, including auto-oxidation of glucose, stimulation of the polyol pathway and the formation of advanced glycation end products. Indeed, recent studies showed increased formation of free radical-catalysed products of arachidonic acid such as 8-epi-PGF2a in patients with diabetes [8]. Furthermore, endothelium-dependent vasodilation could be restored in patients with non-insulin-dependent diabetes by short-term treatment wtih the antioxidant vitamin C [6].

Tetrahydrobiopterin is an important cofactor for NOS and seems to determine whether the electron flow in the enzyme can be directed to L-arginine. Biochemical investigations showed that BH4 deficiency results in the uncoupling of oxygen reduction and arginine oxidation, thereby generating superoxide anions instead of nitric oxide [4]. These observations are confirmed by the finding of increased production of superoxide anions in insulin-resistant rat aorta which was restored to normal by pre-incubation with BH4 [5]. In this particular study, superoxide production was increased by stimulation of NOS with calcium ionophore A23187 and blocked by the NOS-inhibitor L-NAME, indicating that NOS rather than other sources produced most of the superoxide anions in aorta of diabetic rats. The concept of dysfunctional NOS due to BH4-deficiency in diabetes is further supported by organ chamber studies showing that treatment with BH4 to diabetic blood vessels could restore abnormal endothelium-dependent relaxation to normal [3].

Our study extends observations made in these experimental models of diabetes to patients with diabetes. Short-term supplementation of BH4 considerably improved endothelium-dependent vasodilation to ACh, whereas the endothelium-independent vasodilator response to SNP was not altered. Moreover, this improvement in endothelial function could be blocked by co-infusion of the NOS-inhibitor L-NMMA, indicating that the beneficial effect of BH4 is due to enhanced NO-bioactivity. The exact mechanisms by which BH4 improves NO activity in diabetic patients are not known. Tetrahydrobiopterin has antioxidant activity like other reduced pteridines and could have improved endothelial function by merely acting as a radical scavenger like vitamin C. The concentration of BH4 used in this study is, however,

about 100-fold less than the concentration of vitamin C used in a similar study in diabetic patients [6]. In addition, we have previously shown that tetrahydroneopterin, another pterin with equipotent antioxidant activity in vitro, failed to improve endothelium-dependent vasodilation in vivo [2]. These findings support the concept that BH4 improves endothelial function by acting as an intracellular cofactor of NOS rather than merely as a radical scavenger.

We question what could cause decreased availability of BH4 in diabetes? During catalytic activity of NOS, BH4 is continuously recycled into its reduced state. It has been suggested that enhanced oxidative stress can change the intracellular redox state, thereby accelerating oxidation of BH4 to 7,8-dihydrobiopterin, an inactive form of biopterin [4]. The BH4 content of aorta of diabetic rats were found to be decreased, whereas the concentrations of 7,8-dihydrobiopterin were increased compared with controls [5]. Recent biochemical studies showed that peroxynitrite rapidly oxidizes BH4 to cofactor-inactive pterins [9]. Because peroxynitrite is formed by the nearly diffusion limited reaction between superoxide and NO, it could be generated in higher amounts during dysfunctional NOS-activity. Nitrotyrosine residues, considered as a marker of peroxynitrite action, have been found in increased concentrations in diabetic tissue [10]. These data suggest that endothelial dysfunction in Type II diabetes are related, at least in part, to an absolute or relative deficit in BH4 availability.

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