Impaired nitric oxide production in coronary endothelial cells of the spontaneously diabetic BB rat is due to tetrahydrobiopterin deficiency

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Endothelial cells (EC) from diabetic BioBreeding (BB) rats have an impaired ability to produce NO. This deficiency is not due to a defect in the constitutive isoform of NO synthase in EC (ecNOS) or alterations in intracellular calcium, calmodulin, NADPH or arginine levels. Instead, ecNOS cannot produce sufficient NO because of a deficiency in tetrahydrobiopterin (BH_4), a cofactor necessary for enzyme activity. EC from diabetic rats exhibited only 12% of the BH_4 levels found in EC from normal animals or diabetes-prone animals which did not develop disease. As a result, NO synthesis by EC of diabetic rats was only 18% of that for normal animals. Increasing BH_4 levels with sepiapterin increased NO production, suggesting that BH_4 deficiency is a metabolic basis for impaired endothelial NO synthesis in diabetic BB rats. This deficiency is due to decreased activity of GTP-cyclohydrolase I, the first and rate-limiting enzyme in the *de novo* biosynthesis of BH_4. GTP-cyclohydrolase activity was low because of a decreased expression of the protein in the diabetic cells.

Key words: diabetes, GTP-cyclohydrolase, nitric oxide synthesis, vascular disease.

INTRODUCTION

Insulin-dependent diabetes mellitus is associated with severe cardiovascular complications which are responsible for most of the morbidity and mortality associated with this disease. These complications include coronary heart disease, hypertension, cerebrovascular disease, nephropathy, retinopathy, thrombosis, ischaemia and, through the reduction in blood flow, neuropathy. Much research has been devoted to understanding diabetic vascular disease. Enhanced production of vasoconstrictors or reduction in the synthesis of vasodilators could theoretically contribute to many of the vascular complications of long-term diabetes. Recently it has been shown that there is an impaired endothelium-dependent relaxation in diabetic patients [1–3] and in the spontaneously diabetic BioBreeding (BBd) rat [4–6], an animal model of human insulin-dependent diabetes mellitus [7]. Although the mechanism for this defect in blood vessels is not understood, it has been hypothesized that the abnormal endothelial function in diabetic patients may be due to a defect in the synthesis of NO [8], the so-called endothelium-dependent relaxing factor.

The production of NO is catalysed by the various isoforms of NO synthase (NOS). The constitutively active form of NOS found in endothelial cells (EC), ecNOS, requires arginine as a substrate and several cofactors {e.g. NADPH, calcium and tetrahydrobiopterin [(6*R*)-5,6,7,8-tetrahydro-L-biopterin, or BH_4]} for normal activity. We have shown previously [9] that NO synthesis is impaired in coronary EC from the BBd rat. The defect responsible for this decreased NO synthesis was not elucidated.

Currently, there is little information available in the literature concerning NOS in diabetes mellitus. A few studies suggest normal or even enhanced levels of NOS protein and mRNA in diabetic animals or in EC exposed to high concentrations of glucose [10–12]. Indeed, we have shown normal NOS activity in EC homogenates [9], suggesting that there is no intrinsic defect in ecNOS from the BBd rat. The fact that NO production *in vivo* or *in vitro* is reduced in spite of normal NOS activity (measured under optimal conditions) suggests that substrate or cofactor availability may be limiting for NOS in EC. Therefore, the objective of the present study was to investigate whether a deficiency in one or more of the cofactors of NOS is responsible for impaired NO synthesis in intact EC of the BBd rat. We demonstrate a marked decrease in BH_4 concentration in EC from the diabetic animals compared with EC from the non-diabetic animals. The concentration of BH_4 could be increased by treatment of the cells with sepiapterin, suggesting an intact salvage pathway for BH_4 synthesis. The BH_4 deficiency in the diabetic cells is the result of a reduced *de novo* synthesis of BH_4, due to decreased GTP-cyclohydrolase activity.

EXPERIMENTAL PROCEDURES

Reagents

BSA, Dulbecco's PBS, Joklik's modified minimal essential medium, Dulbecco's modified Eagle's medium, L-glutamine and penicillin/streptomycin/amphotericin B were obtained from Gibco-BRL (Gaithersburg, MD, U.S.A.). Acetylated low-density lipoprotein labelled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate was from Biomedical Technologies (Stoughton, MA, U.S.A.); fetal bovine serum was from Summit (Greeley, CO, U.S.A.); collagenase (type II) was from Worthington Biochemical (Freehold, NJ, U.S.A.); calmodulin and mouse anti-calmodulin antibody were from Upstate Biotechnology (Lake Placid, NY, U.S.A.); sepiapterin was from

Abbreviations used: BH_4, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; EC, endothelial cells; NOS, nitric oxide synthase; ecNOS, constitutive isoform of NOS in EC; BBd, diabetic BioBreeding; BBn, non-diabetes-prone BioBreeding.

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Research Biochemicals (Natick, MA, U.S.A.) and heparin was from Elkins-Sinn (Cherry Hill, NJ, U.S.A.).

Animals

Diabetic (BBd), diabetes-prone and non-diabetes-prone (normal; BBn) BB rats were obtained from the Animal Resources Division of the Health Protection Branch (Ottawa, ON, Canada). Diabetic rats were maintained with daily subcutaneous injections of 2–4 units of Ultralente insulin (Eli Lilly, Indianapolis, IN, U.S.A.) to prevent hyperglycaemia, ketosis and hypoinsulinaemia as described previously [13]. BBd rats (95–100 days of age) 25–30 days post onset of diabetes and age-matched BBn rats were used as a source of coronary microvascular EC. Serum glucose concentrations were determined by an enzymic method involving hexokinase and glucose-6-phosphate dehydrogenase [13] and were found to be 5.64 ± 0.43 and 6.17 ± 0.68 mM (means $\pm$ S.E.M., $n = 30$) for BBn and BBd rats, respectively, at the time of death. Diabetes-prone animals not developing diabetes within the usual time frame (70–100 days) were maintained until 150 days of age to verify the absence of disease.

Isolation and culture of microvascular EC

Microvascular EC were isolated from BBd, age-matched BBn, and diabetes-prone but normal BB rats (i.e. those not developing the disease) by collagenase perfusion, using the method of Ford and Rovetto [14], as described previously by our laboratories [9,15]. EC were obtained free of smooth-muscle cells and myocytes. The endothelial identity of the cultured cells was confirmed by the uptake of modified low-density lipoprotein [16]. EC from three or four rat hearts were pooled into one gelatin-coated (1.5 % in PBS) 60-mm culture dish. EC were cultured at 37 °C under 10 % CO_2 in Dulbecco's modified Eagle's medium containing 20 % fetal bovine serum, 2 mM L-glutamine, 20 mM D-glucose, 20 units/ml heparin, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. After the cells neared confluence, they were passaged using 0.25 % trypsin and 0.02 % EDTA and used at passages 5–15. No phenotypical changes were noted in the cells in this range of passage levels.

NO synthesis by EC

For the measurement of NO synthesis, EC were rinsed with PBS and cultured for 48 h in serum-free, Phenol-Red-free Dulbecco's modified Eagle's medium containing 0.2 mM L-glutamine, 0.4 mM L-arginine, 20 mM D-glucose, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. At the end of the 48-h culture period, the conditioned media were used for the determination of nitrite and nitrate (two major stable end products of NO oxidation [17]) with $NaNO_2$ and $NaNO_3$ as standards, respectively, as described previously [18], using a microplate reader. In all experiments, culture medium incubated without cells was used as the blank. Cells, harvested from the culture dish, were used for metabolite analysis and measurements of enzyme activity, as described below.

HPLC analysis of cellular BH_4, arginine and NADPH

The cellular content of BH_4 was determined using a modification of the HPLC method of Fukushima and Nixon [19] in which EC (3×10^6) were lysed in 0.3 ml of 0.1 M phosphoric acid containing 5 mM dithioerythritol (an antioxidant) and 35 μl of 2 M trichloroacetic acid. Arginine levels in EC were measured by HPLC as described previously [9]. Extraction of NADPH from EC and its analysis by HPLC were done as described previously [20], except that 1 mM bathophenanthroline-disulphonic acid (a bivalent metal chelator) was used to prevent oxidation of NADPH by iron [21] and NADPH was detected by a fluorimeter (excitation 340 nm, emission 460 nm) to improve sensitivity.

Calcium measurement *in situ*

EC grown on coverslips were co-loaded with SNARF-1 and Fura-2 (Molecular Probes, Eugene, OR, U.S.A.) to simultaneously measure intracellular pH and calcium concentration as we described previously [22,23]. Simultaneous measurements of these two ions is necessary because the K_d of Fura-2 is affected by pH. We corrected for this interaction but, for the purposes of discussion, present only the Fura-2 data.

Immunoblot procedure

Cellular protein from BBd and BBn EC was loaded on to 9.5–16 % polyacrylamide gels. Separated proteins were blotted on to nitrocellulose and the blots were blocked with 5 % non-fat dried milk in Tris-buffered saline with 0.1 % Tween 20 (blocking buffer). For analysis of calmodulin, the primary antibody, mouse anti-bovine calmodulin (Upstate Biotechnology), was used at 1 μg/ml in blocking buffer and the secondary antibody, horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson Laboratories, West Grove, PA, U.S.A.), was used at 1:75000 in blocking buffer. For analysis of GTP-cyclohydrolase I, primary antibody (prepared against purified rat recombinant GTP-cyclohydrolase I [24]) was diluted 1:10000 in blocking buffer and the secondary antibody, peroxidase-conjugated donkey anti-rabbit IgG (Jackson Laboratories) was used at 1:100000 in blocking buffer. Bands were visualized using SuperSignal™ West Dura Extended Duration Substrate (Pierce, Rockford, IL, U.S.A.) according to the manufacturer's directions with Kodak Biomax ML film (Kodak, Rochester, NY, U.S.A.). Blots were scanned using a UMAX S6E scanner (UMAX Data System, Hsinchu, Taiwan) with VistaScan software (Kodak) and analysed using Multi-Analyst software (BioRad, Hercules, CA, U.S.A.).

Determination of GTP-cyclohydrolase activity

GTP-cyclohydrolase activity in EC was determined as described by Viveros et al. [25]. The product of this enzymic reaction was converted into neopterin by alkaline phosphatase (1 unit/μl in 50 mM $MgCl_2$; 10 units/tube). Neopterin was then analysed by HPLC as described by Fukushima and Nixon [19].

Statistical analysis

Data were analysed by paired or unpaired t test using the SAS program [26]. Probability values of $P < 0.05$ were taken to indicate statistical significance.

RESULTS AND DISCUSSION

There are many pharmacological studies demonstrating impaired dilator responses to agonists in conduit and resistance blood vessels from experimental diabetic animals (see [27] for review). We have shown previously that coronary EC from the BBd rats are unable to produce as much NO as EC from non-diabetic rats [9]. The defect in the ability of these cells to produce NO, however, was not in the NOS enzyme itself. Instead, our earlier work suggested that it might be a deficiency in the amount of a cofactor necessary for full activity of ecNOS [9].

Table 1 Calcium, NADPH and arginine concentrations in BB rat EC

EC from BBn and BBd rats were analysed for calcium, NADPH and arginine concentrations. Data are means ± S.E.M., with the number of experiments given in parentheses. *$P < 0.01$, BBd versus BBn, as analysed by unpaired t test.

EC	Calcium (nM)	NADPH (pmol/10^6 cells)	Arginine (nmol/10^6 cells)
BBn	13.3 ± 0.96 (24)	66.3 ± 5.4 (8)	2.88 ± 0.23 (10)
BBd	15.2 ± 0.95 (29)	117.9 ± 10.8 (8)*	4.37 ± 0.31 (10)*

Table 2 BH_4 concentrations in BB rat EC

EC from BBn and BBd rats were analysed for BH_4. Data are means ± S.E.M., with the number of experiments given in parentheses. *$P < 0.01$, BBd versus BBn, as analysed by unpaired Student's t test. †$P < 0.01$, group with sepiapterin versus group with no sepiapterin, as analysed by paired t test.

	BH_4 (pmol/10^6 cells)	
EC	No sepiapterin	+10 μM Sepiapterin
BBn	1.42 ± 0.22 (7)	32.1 ± 2.9 (4)†
BBd	0.17 ± 0.01 (5)*	29.9 ± 2.6 (4)†

Figure 1 GTP-cyclohydrolase I immunoblot

Cellular protein from Sprague–Dawley rat liver (lane 1, 5 μg/lane; positive control), BBn EC (lanes 2 and 4, 30 μg/lane), and BBd EC (lanes 3 and 5, 30 μg/lane) was separated on a polyacrylamide gradient gel, blotted on to nitrocellulose and probed using a rabbit anti-rat GTP-cyclohydrolase I antibody. The arrow indicates a protein band corresponding to a molecular mass of approx. 29000 Da.

We investigated the intracellular concentrations of calcium, NADPH and calmodulin, all required for full activity of ecNOS [28]. Steady-state intracellular calcium concentrations in unstimulated EC from BBd and BBn rats were not significantly different (Table 1) and thus would not account for the differences in NO production. The enzyme ecNOS oxidizes arginine to produce citrulline and NO in a process that consumes NADPH [28]. It is therefore possible that decreased NO production is due to decreased availability of NADPH in BBd EC. However, NADPH levels were significantly higher in the BBd EC compared with BBn EC (Table 1). This greater concentration of NADPH in BBd EC is consistent with increased activity of the pentose cycle pathway for NADPH provision in these cells (G. Wu and C. Meininger, unpublished work). Finally, calmodulin levels in BBd and BBn EC were not significantly different. Calmodulin levels were 2.80 ± 0.13 ng/μg of protein in the BBn EC (means ± S.E.M., $n = 7$) and 2.78 ± 0.13 ng/μg of protein in the BBd EC ($n = 6$; calculated by comparison of absorbance of samples with that of a standard curve). These data suggest that adequate amounts of calcium, NADPH and calmodulin exist in the EC of diabetic animals to support NO production.

Because decreased availability of substrate for ecNOS could also result in lowered production of NO, we measured arginine levels in the BBd and BBn EC. Arginine uptake was not different in the two cell types [9], yet intracellular arginine concentrations were significantly higher in the EC from diabetic animals (Table 1). We have shown previously that arginase activity is decreased in the EC from BBd rats [9], which would account for the increase in intracellular arginine concentrations and would theoretically provide more substrate for NO production by ecNOS. Thus it is unlikely that the arginine substrate was limiting for NO production in the BBd EC.

BH_4 plays an essential role in EC NO synthesis [29]. To determine whether the defect in NO production was due to a deficiency in this cofactor for ecNOS, we measured BH_4 levels in the EC. BBd EC exhibited only 12% of the BH_4 level found in BBn EC (Table 2). The levels found in the BBn EC were similar to those previously reported for EC from human umbilical veins [30]. Importantly, EC isolated from diabetes-prone BB rats that *did not* develop disease exhibited BH_4 levels equivalent to non-diabetes prone (i.e. normal) BB rats (1.45 ± 0.36 pmol/10^6 cells, mean ± S.E.M., $n = 6$). Equivalent levels were found in cells analysed immediately upon isolation from the animals without being cultured (1.51 ± 0.27 pmol/10^6 BBn EC versus 0.22 ± 0.03 pmol/10^6 BBd EC, $n = 4$, $P < 0.01$). These findings imply that the BH_4 deficiency is not simply an inbred defect in the diabetes-prone animals or the result of cell culturing but is somehow linked to the disease state.

BH_4 is produced in EC by two pathways: a salvage pathway which makes BH_4 from dihydrobiopterin in the cell and a *de novo* synthetic pathway which generates BH_4 from GTP [30]. Sepiapterin is taken up by EC and utilized in the salvage pathway to generate BH_4. Treating EC from either the normal or diabetic rats with 10 μM sepiapterin resulted in a significant increase in BH_4 levels in the cells (Table 2). This result suggests that there is

Table 3 Effect of sepiapterin on NO production in BB rat EC (pmol/h per 10^6 cells)

EC from BBd and BBn rats were cultured in growth medium containing 0.2 mM L-glutamine for 48 h in the presence or absence of 10 μM sepiapterin. Conditioned media were analysed for nitrite and nitrate concentrations as a measure of NO production. Data are means ± S.E.M., $n = 8$. *$P < 0.01$, group with sepiapterin versus group with no sepiapterin, as analysed by paired t test. †$P < 0.01$, BBd versus BBn group, as analysed by unpaired t test.

	No sepiapterin			+10 μM Sepiapterin		
	Nitrite	Nitrate	Nitrite + nitrate	Nitrite	Nitrate	Nitrite + nitrate
BBn	53.9 ± 4.1	238.9 ± 15.8	292.8 ± 18.9	107.4 ± 6.7*	406.3 ± 20.9*	514.9 ± 24.2*
BBd	17.3 ± 1.9†	35.1 ± 5.0†	52.3 ± 6.6†	46.5 ± 3.8†*	206.1 ± 11.0†*	253.8 ± 13.5†*

no defect in BH_4 synthesis via the salvage pathway in BBd EC. To determine whether there may be a defect in *de novo* synthesis of BH_4 from GTP in BBd EC, we measured the activity of GTP-cyclohydrolase I, the first and rate-limiting enzyme in this *de novo* synthetic pathway [29]. Enzyme activity was higher ($P < 0.01$) for BBn cells (0.624 ± 0.2 pmol/mg per 90 min, mean $\pm$ S.E.M., $n = 5$) than for BBd cells (0.072 ± 0.04 pmol/mg per 90 min, $n = 5$). When GTP-cyclohydrolase I levels were analysed by immunoblot, the amount of protein was found to be significantly higher in the EC from the non-diabetic animals (Figure 1). This decrease in enzyme expression would explain the deficiency in enzyme activity. Interestingly, BH_4 levels in the BBd EC were decreased to the same extent as the decrease in GTP-cyclohydrolase activity, consistent with the role of GTP-cyclohydrolase as the rate-limiting enzyme for *de novo* BH_4 synthesis. Thus the decrease in GTP-cyclohydrolase I activity is sufficient to account for the BH_4 deficiency in EC of diabetic rats.

To investigate whether increasing intracellular BH_4 levels could enhance NO production in BBd EC, we cultured EC in the presence and absence of sepiapterin. We found that 10 μM sepiapterin increased the production of NO by both BBn and BBd EC (Table 3). In the absence of sepiapterin, EC from the diabetic animals made only 18% of the NO produced by EC from the non-diabetic animals. The addition of 10 μM sepiapterin increased NO production by BBd EC to a level approximately equal to that of BBn EC in the absence of sepiapterin. Sepiapterin increased NO production by BBn EC an additional 76%. Whereas sepiapterin treatment was able to normalize BH_4 levels in EC from diabetic animals (Table 2), the nitrite/nitrate accumulation by these cells was less than that of EC from non-diabetic animals treated with sepiapterin over the same time period (Table 3). This difference in nitrite/nitrate levels was probably the result of the time necessary for BH_4 levels to increase in order to augment NO production in the BBd EC. The BBn EC did not start with a deficit in BH_4 and therefore accumulated more nitrite/nitrate during the treatment period.

In conclusion, we have shown a BH_4 deficiency in coronary EC from the diabetic BB rat due to a decreased expression of GTP-cyclohydrolase, the rate-limiting enzyme for *de novo* synthesis of BH_4. With insufficient BH_4 the cells are unable to make sufficient NO. This BH_4 deficiency may be the metabolic basis for the heretofore unexplained observation that endothelium-dependent relaxation is impaired in diabetic animals as well as diabetic patients, and may contribute significantly to the pathogenesis of diabetic vascular complications.

Funding for this study was received from the American Heart Association, National Office and Texas Affiliate, Dallas, TX, U.S.A., as well as from Interdisciplinary Research Initiative Funds from Texas A&M University. G.W. is an Established Investigator of the American Heart Association. We thank Tony E. Haynes for his technical expertise in isolating these cell lines and Wene Yan for technical assistance with HPLC measurements.

Received 13 March 2000; accepted 17 April 2000

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