Tetrahydrobiopterin levels regulate endothelial cell proliferation

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Marinos, Rebecca S., Wei Zhang, Guoyao Wu, Katherine A. Kelly, and Cynthia J. Meininger. Tetrahydrobiopterin levels regulate endothelial cell proliferation. Am J Physiol Heart Circ Physiol 281: H482–H489, 2001.—Vascular abnormalities, including altered angiogenesis, are major factors contributing to the morbidity and mortality of diabetes. We hypothesized that impaired angiogenesis in diabetes results from decreased tetrahydrobiopterin (BH4)-dependent synthesis of nitric oxide (NO) by endothelial cells (EC). To test this hypothesis, we utilized EC from spontaneously diabetic BB (BBd) and nondiabetes-prone BB (BBn) rats to investigate the link between BH4 and EC proliferation. There were significant decreases in the proliferation rate and expression of proliferating cell nuclear antigen in BBd versus BBn EC, with no evidence of apoptosis in either group. Sepiapterin (a precursor of BH4 via the salvage pathway) increased BH4 synthesis and enhanced proliferation of BBd EC. The stimulating effect of sepiapterin on EC proliferation was attenuated by Nω-monomethyl-L-arginine, a NO synthase inhibitor. Reducing BH4 concentrations in BBn EC caused a decrease in proliferation, which was attenuated by a long-acting NO donor. Our results suggest that BH4 levels regulate proliferation of normal EC and that a BH4 deficiency impairs NO-dependent proliferation of BBd EC.

Tetrahydrobiopterin levels regulate endothelial cell proliferation. Endothelial cell proliferation

Diabetes mellitus is a common metabolic disorder, affecting more than 12 million people in the United States (29). Since the advent of insulin therapy in the 1920s, people with diabetes live longer and therefore develop the long-term complications associated with this disease. The most significant and dire complications of diabetes result from vascular abnormalities that are both microvascular and macrovascular in origin. Microvascular complications typically predominate in the retinal and glomerular vasculature but may affect all microvascular beds. Diabetic retinopathy is the leading cause of adult-onset blindness in the Western world, and diabetes-induced nephropathy is a leading cause of end-stage renal disease (29). Macrovascular complications include coronary artery disease, cerebrovascular disease, and peripheral vascular disease.

Common diabetes-induced vascular alterations thought to lead to vascular dysfunction include basement membrane thickening, changes in vascular permeability, and changes in cell proliferation (19, 22, 30). Alterations in cell proliferation vary by vascular bed (18), consistent with the observed differences in their angiogenic response. For example, the number of endothelial cells (EC) in retinal capillaries is increased with development of the microaneurysms and neovascularization seen in diabetic retinopathy (18, 19, 22). This increase in EC proliferation is proposed to be a response of the diabetic retinal vasculature to a hypoxic environment, with the intensity of vascularization dependent on the degree of ischemia. Tissue hypoxia is normally a potent stimulator of angiogenesis. However, diabetic patients with ischemic heart disease showed significantly lower postmortem myocardial capillary density when compared with either normoglycemic patients with ischemic heart disease or diabetic patients without ischemic heart disease (43). Thus an abnormality exists in the process leading to new vessel formation in diabetes. This decrease in angiogenesis will result not only in increased morbidity and mortality from heart disease but also poor wound healing and increased risk of lower extremity amputation.

Wound healing in diabetic patients is slow and often beset with complications (11). Unfortunately, there is little information available to explain the impaired ability of diabetics to vascularize wounds. Cameron et al. (2) studied basement membrane thickness in rats made diabetic with streptozotocin and found no difference in thickness at 4-wk duration of the disease. Yet wound vascularization was already significantly diminished by this time (7). Fahey et al. (7) investigated whether the defect in diabetic healing might be due to altered cytokine release at the wound site. They found that levels of tumor necrosis factor-α, a cytokine involved in the response to wounds and tissue repair,
were not different in diabetic animals compared with normal animals, although vascularization was significantly reduced in the diabetic animals. Other investigators (23) suggested that reduced vascularization might be due to a direct inhibitory effect of hyperglycemia on EC. It is noteworthy, however, that diabetic individuals with good glycemic control may still develop vascular complications of the disease.

Angiogenesis is a tightly regulated process leading to the formation of new capillaries from preexisting microcirculatory beds, and it requires EC proliferation to be successful. Nitric oxide (NO) stimulates angiogenesis in vivo (44) and has been shown to stimulate EC proliferation in vitro (45). Pharmacological agents, which act as NO donors, also stimulate EC proliferation (44). Vascular endothelial cell growth factor, an angiogenic factor, stimulates EC proliferation via NO production (25, 26) and by upregulating NO synthase (NOS), the enzyme that catalyzes the synthesis of NO from L-arginine (13). Indeed, blocking the formation of NO with inhibitors of NOS prevents the proliferative response of EC to this mitogen (25). Wound healing studies (21, 42) performed in NOS-deficient mice have provided further evidence for the critical role of NO in angiogenesis. Taken together, these data suggest that NO may act as an endogenous proliferative signal for endothelial cells.

Wu and Meininger (39) demonstrated impaired NO synthesis by coronary EC from the spontaneously diabetic BB (BBd) rat (an animal model of insulin-dependent diabetes mellitus) compared with EC from non-diabetes-prone BB (BBn) rats. We also found a marked deficiency in tetrahydrobiopterin (BH4), a cofactor necessary for NOS activity, in BBd EC (24). We hypothesized that impaired angiogenesis in diabetes results from a decrease in BH4-dependent synthesis of NO by EC. The purpose of this study was to investigate the link between BH4 levels, NO production, and EC proliferation in vitro. Our results demonstrate that proliferation of BBd microvascular EC is impaired due to a BH4 deficiency and that this impairment can be ameliorated by treatment of the cells with sepiapterin or a long-acting NO donor. Conversely, proliferation of normal EC can be adversely affected by inhibiting their ability to produce BH4, further supporting a critical role for BH4 in NO-dependent EC proliferation.

**MATERIALS AND METHODS**

**Reagents.** Dulbecco’s phosphate-buffered saline, Dulbecco’s modified Eagle’s medium (DMEM), Joklik’s modified minimal essential medium, L-glutamine, penicillin-streptomycin-amphotericin B, gentamicin, sodium pyruvate, and trypsin-EDTA were obtained from Gibco-BRL (Gaithersburg, MD). Heparin sodium was purchased from Elkins-Sinn (Cherry Hill, NJ). Bovine serum albumin, ferricytochrome C, NADPH, monomethyl-L-arginine (L-NMA), 2,4-diamino-6-hydroxy-pyrimidine (DAHP), and GenElute Mammalian Genomic DNA purification miniprep were from Sigma (St. Louis, MO). N-acetyl serotonin (NAS) was obtained from Alexis (San Diego, CA). Diethylenetriamine NO adduct (DETA) and S-nitroso-N-acetyl penicillamine (SNAP) were obtained from Research Biochemical (Natick, MA). Acetylated low-density lipoprotein, labeled with 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate, was purchased from Bio-medical Technologies (Stoughton, MA), whereas fetal bovine serum (FBS) was obtained from Summit (Greeley, CO). Collagenase type II was purchased from Worthington Biochemical (Freehold, NJ). Catalase, superoxide dismutase (SOD), and horseradish peroxidase were obtained from Boehringer-Mannheim (Indianapolis, IN). Chromagen was obtained from ICN (Costa Mesa, CA). Protease inhibitor cocktail (Complete, Mini) was purchased from Roche (Indianapolis, IN), nitrocellulose (15 cm × 15 cm sheets) was from BioRad (Hercules, CA), mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody was from Santa Cruz (Santa Cruz, CA), peroxidase-conjugated donkey anti-mouse IgG was from Jackson (West Grove, PA), SuperSignal West Dura Extended Duration Substrate was from Pierce (Rockford, IL), Biomax ML film was from Kodak (Rochester, NY), and E-gels were from Invitrogen (Carlsbad, CA).

**Animals.** BBd and BBn rats were obtained from the Animal Resources Division of the Health Protection Branch (Ottawa, Ontario, Canada). All experiments using these animals were formally approved by the Texas A&M University Laboratory Animal Care Committee. Rats were housed in a light- and climate-controlled facility (12:12-h light-dark cycle). Animals were provided ad libitum with drinking water and laboratory rat chow (Hardland-Taklad; Bartville, IL). Diabetic rats were maintained initially by insulin injection and later via an implantable pump to prevent hyperglycemia, ketosis, and hypoinsulinemia. BBd rats (95–100 days old) 25–30 days after onset of diabetes and age-matched BBn rats were used to prepare coronary EC. Urine glucose tests were performed twice daily (8 AM and 4 PM) for insulin-injected BBd rats with the use of Chemstrip.uG (37). Glucosuria was not detectable. Daily blood glucose tests were not performed because of the stress inflicted on the animals. Before the animals were euthanized, blood was obtained from the tail vein of box-restrained, anesthetized rats with the use of a microhematocrit tube. Serum glucose concentrations were determined by an enzymatic method involving hexokinase and glucose-6-phosphate dehydrogenase (37) and were found to be 5.64 ± 0.43 and 6.17 ± 0.68 mM (means ± SE, n = 30) for BBn and BBd rats, respectively, at the time of death.

**Isolation of coronary EC.** Microvascular EC were isolated from BBd and age-matched BBn rats by collagenase perfusion, using the method of Ford and Rovetto (8), as described previously by our laboratories (24, 38, 39). Briefly, heparin sodium (130 U/100 g body wt) was injected intraperitoneally into rats 20 min before the rats were euthanized. Rats were anesthetized by intraperitoneal injection of pentobarbital sodium. The hearts were removed and placed in ice-cold Joklik’s modified minimal essential medium containing 60 mM taurine, 20 mM creatine, and 5 mM HEPES. The aortas were cannulated with stainless steel tubing (2-mm inner diameter) and perfused with the above medium from a static 40-mmHg hydrostatic pressure head. The oxygenated (100%) perfusate, which was supplemented with 0.1% diazoylated bovine serum albumin and heparin (1 U/ml), was passed through the hearts once. After a 10-min washout period, new medium with collagenase (0.7 mg/ml) was introduced, and the perfusate was allowed to recirculate until the aortic perfusion pressure decreased to below 40 mmHg (30–40 mmHg). The ventricles were cut from the hearts, minced, and placed in fresh collagenase-containing perfusate. The tissue was shaken at 250 rpm in a water bath for 10 min. CaCl2 (50 μM) was added to the minced tissue, and digestion with collagenase continued for an additional 10 min. The cells were dispersed, filtered through a double layer of cheese-
cloth, and diluted 1:4 with buffer containing 0.1% dialyzed bovine serum albumin. The resulting suspension was allowed to settle to separate myocytes (which are heavier) from EC. EC were further purified by centrifugation through cushions of 6% and 3% bovine serum albumin in Joklik’s modified minimal essential medium. The endothelial identity of the collected cells was confirmed by the uptake of modified low-density lipoprotein (34) or expression of factor VIII-related antigen.

Culture of EC. EC from three to four rat hearts were pooled into one gelatin-coated (1.5% in Dulbecco’s phosphate-buffered saline) 60-mm culture dish. EC were cultured at 37° under 10% CO₂ in 3 ml of DMEM containing 20% FBS, 2 mM l-glutamine, 20 mM d-glucose, 20 U/ml heparin, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. After the cells neared confluence, they were passaged with the use of 0.25% trypsin and 0.02% EDTA. No phenotypical changes were noted in the passages used.

Assessment of cell proliferation. EC were seeded in gelatin-coated, 24-well trays at 5,000 cells/cm². EC were scraped from the dish, collected, and extracted with lysis buffer (1% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate, 10 mM Tris, pH 8.0, 140 mM NaCl, and protease inhibitor cocktail). Protein concentration was determined by using the bicinchoninic acid protocol with bovine serum albumin as a standard. Protein from 20,000 cells was eluted by an isocratic mobile-phase solution consisting of 5% methanol and 7.5 mM sodium phosphate buffer (pH 6.35) at a flow rate of 1.0 ml/min and was detected by a fluorimeter (excitation 350 nm, emission 440 nm). The amount of BH₄ in the EC extracts was calculated from the difference between the amount of bipterin formed by oxidation under acidic conditions and the amount of bipterin formed by oxidation under alkaline conditions.

Statistical analysis. Statistical analysis was performed with the use of the StatView program for Macintosh. Data are presented as means ± SE, and statistical comparisons among groups were performed by using unpaired t-test or one-way ANOVA, followed by a post hoc test (Fisher’s protected least significant differences test). Differences were considered significant at P ≤ 0.05.

RESULTS

To evaluate angiogenesis, the proliferation of coronary microvascular EC from age-matched BBn and BBd rats was compared. As shown in Fig. 1, the proliferation of the BBd EC was significantly less than
that of the BBn EC. When PCNA expression was evaluated, BBd EC exhibited lower PCNA levels than BBn EC (Fig. 2). Expression of PCNA was not evident in BBd EC by day 4 of the experiment, whereas PCNA expression remained high for BBn EC throughout the experiment. DNA was extracted from BBd and BBn EC on day 5. No evidence of DNA laddering was found in either group of cells (Fig. 3).

BBd EC cultured in the presence of 10 μM sepiapterin exhibited an increase in NO production (24) and proliferation (Fig. 4). The ability of sepiapterin to stimulate proliferation was attenuated by the addition of L-NMMA, an arginine analog that inhibits NOS activity (40). Sepiapterin also increased proliferation of BBn EC as well as EC from Sprague-Dawley rats, indicating that the effect is not limited to BBd rats (data not shown).

To investigate the role of BH₄ in EC proliferation without the potential confounding factors linked to the disease state, the effect of inhibition of BH₄ synthesis on cellular BH₄ concentration and subsequent cell proliferation was examined in BBn EC. Inhibition of the de novo pathway of BH₄ synthesis was accomplished with the use of DAHP, which inhibits GTP cyclohydrolase I by acting as an analog of the first pyrimidine intermediate formed in the reaction, thereby acting as a direct competitive inhibitor (35, 41). Inhibition of both the de novo and the salvage pathway was achieved by using NAS, which inhibits the action of sepiapterin reductase (35). Concentrations of the inhibitors used in this study were derived from previously published studies (1, 16, 17, 35, 36). Table 1 shows the decrease in the cellular level of BH₄ in BBn EC in response to 24 h of treatment with either 10 mM DAHP or 2 mM NAS. Cells treated with DAHP contained only 32% of the BH₄ found in the control cells. The cells treated with NAS contained only 24% of the BH₄ found in the nontreated cells.

Table 1. BH₄ concentrations in BBn EC treated with BH₄ synthesis inhibitors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BH₄ pmol/10⁶ cells</th>
<th>DAHP (10 mM)</th>
<th>NAS (2 mM)</th>
</tr>
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<tbody>
<tr>
<td>BBn Ec</td>
<td>1.40 ± 0.32</td>
<td>0.45 ± 0.07*</td>
<td>0.34 ± 0.07*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 experiments. BBn EC, nondiabetes-prone BB rat endothelial cells; BH₄, tetrahydrobiopterin; DAHP, 2,4-diamino-6-hydroxypyrimidine; NAS, N-acetyl serotonin. BBn EC were cultured for 24 h in the presence of 0 or 10 mM DAHP or 2 mM NAS and then used for analysis of BH₄. *P < 0.05, treated cells vs. untreated control cells, as analyzed by one-way analysis of variance. There was no significant difference (P > 0.05) between DAHP and NAS groups.
Consistent with BH₄ levels, EC proliferation was significantly decreased in the cultures treated with the inhibitors (Fig. 5). The numbers of DAHP-treated and NAS-treated EC were 37% and 35% of control, respectively, after 48 h of treatment. Cell viability, analyzed with the use of trypan blue exclusion, was no different in treated cells versus untreated cells. The ability of the inhibitors to reduce cell proliferation was concentration dependent (data not shown).

To verify that the effect of BH₄ suppression was the result of an inability to make NO, DAHP- or NAS-treated cultures were provided with exogenous NO contributed by NO donors (10). In this experiment, the number of EC in the presence of 2 mM NAS was only 43% of control, but a significant improvement occurred with the addition of 10 μM DETA (Fig. 6). The same improvement did not occur with addition of 10 μM SNAP. While the number of EC in the presence of 10 mM of DAHP was only 40% of the number in control cultures, the addition of 10 μM DETA increased proliferation by 28% (Fig. 7). Again, no significant improvement was noted with the addition of SNAP.

Because NOS may synthesize superoxide anions when BH₄ is deficient (40), we evaluated whether the decrease in cell proliferation was due to the production of either superoxide anion or hydrogen peroxide. Culturing BBn EC in the presence of either DAHP + catalase or DAHP + SOD did not attenuate the decrease in cell proliferation that occurred with inhibition of BH₄ synthesis. Concentrations of 200–1,000 U/ml of catalase and 1,000 U/ml of SOD were tested with no effect. Figure 8 shows a representative experiment utilizing 10 mM DAHP and 1,000 U/ml each of SOD and catalase.

**DISCUSSION**

Little is known about the mechanisms responsible for EC dysfunction in microvessels involved in diabetic vascular complications. Our laboratory has shown that EC from the BBd rat have a reduced ability to produce NO (24, 39). We have also shown that there was no difference in NOS activity in EC from the diabetic and nondiabetic rats (when all cofactors are supplied at optimal concentrations) and that L-arginine and NADPH levels were actually higher in the BBd EC compared with the BBn EC (39). In addition, intracellular calcium and calmodulin concentrations were not significantly different between the BBd and BBn EC and were sufficient for full NOS activity (24). However, BH₄ levels in the BBd EC were significantly decreased compared with the BBn EC (39). In addition, intracellular calcium and calmodulin concentrations were not significantly different between the BBd and BBn EC and were sufficient for full NOS activity (24). However, BH₄ levels in the BBd EC were significantly decreased compared with the BBn EC as a result of significantly lower expression and activity of GTP cyclohydrolase I (the first- and rate-controlling enzyme for de novo synthesis of BH₄) in the diabetic EC (24).

EC proliferation is an integral part of the process of angiogenesis. In this study, we have shown that proliferation of microvascular EC from BBd rats is significantly impaired, and this impairment can be ameliorated with the administration of sepiapterin. The improvement seen with sepiapterin can be attenuated with the addition of L-NMMA, suggesting that the increased proliferation is a direct result of increased...
production of NO brought about by increased synthesis of BH₄. This finding is consistent with published reports (21, 25, 26, 42, 44, 45) showing the importance of NO in angiogenesis. Thus inhibiting NO production by inhibiting BH₄ synthesis significantly reduces proliferation of BBn EC, but proliferation can be rescued by exogenous NO supplied by long-acting NO donors.

BH₄ is an essential cofactor for all NOS isoforms, yet its exact mechanism of action remains unclear. It has been suggested that l-arginine and BH₄ induce conformational changes in NOS through a positive cooperative interaction that increases the affinities of the binding domains for each ligand (27, 35). BH₄ is synthesized either via the de novo pathway or the salvage pathway. In the de novo pathway, GTP is converted to 7,8-dihydroneopterin triphosphate. This rate-limiting step is catalyzed by GTP cyclohydrolase I. 7,8-Dihydroneopterin triphosphate is then converted to 6-pyruvoyl tetrahydropterin by 6-pyruvoyl tetrahydropterin synthase. Finally, two NADPH-dependent reductions, catalyzed by sepiapterin reductase, convert 6-pyruvoyl tetrahydropterin to BH₄ (35). In the salvage pathway, BH₄ can be synthesized from either preexisting dihydrobiopterins or exogenously administered sepiapterin utilizing sepiapterin reductase and dihydrofolate reductase (35). Because NO stimulates angiogenesis and because BH₄ is a necessary cofactor for the production of NO, it follows that impairment of the BH₄ synthesis pathway would result in altered angiogenesis.

Because BH₄ levels and NO production were significantly lower in the EC from the BBd rat compared with the BBn rat (24, 39), the effect of a BH₄ deficiency on proliferation of BBn EC was investigated to avoid the potential confounding effects of diabetes mellitus. The de novo pathway for BH₄ synthesis was blocked using DAHP or NAS. Consistent with findings in other animal models and cell types (1, 16, 17, 35, 36), the use of these agents resulted in a significant reduction in synthesis of BH₄ in the BBn EC. More importantly, blocking BH₄ synthesis with these agents in the BBn EC resulted in a significant decrease in cell proliferation.

DAHP and NAS treatment decreased EC proliferation without altering cell viability, consistent with the findings of others (17, 36). There was no significant difference in untreated control cultures versus those in vehicle solution or those treated with the BH₄ synthesis inhibitors. It is possible, however, that the BH₄ deficiency results in accumulation of reactive oxygen species (40). This occurs because either BH₄ is not available to participate in free radical scavenging (14, 31–33) or because there is an actual increase in production of reactive oxygen species by NOS due to a lack of BH₄ (4–6, 12, 15, 20, 28, 32). Clearly, an accumulation of reactive oxygen species by either mechanism would be cytotoxic to the cells and likely would result in impaired proliferation and EC dysfunction. To that end, the role of hydrogen peroxide and superoxide anions in the DAHP-altered proliferation of the BBn EC was evaluated. Treatment of the BBn EC cultured in the presence of DAHP with SOD or catalase had no effect on cell proliferation, suggesting that the altered proliferation in these cultures was not the result of...
accumulation of superoxide anions or hydrogen peroxide.

The rate at which a population of cells increases in number is a function of the rate at which they proliferate and the rate at which they undergo apoptosis, or programmed cell death. It is possible that the increased slope of the growth curve of BBn EC vs. that of the BBd EC (Fig. 1) is due to a higher rate of apoptosis in the BBd EC than in the BBn EC. The biochemical hallmark of apoptosis is the degradation, or fragmentation, of genomic DNA. Thus we used DNA laddering as a means of assessing apoptosis in BBd and BBn cells cultured for proliferation studies. No apparent DNA laddering was visible for either the BBd or BBn EC, suggesting that a difference in the rate of apoptosis was not responsible for the differences in the growth curves for these cell populations. Instead, the data on PCNA expression suggested that the rate of proliferation was greater for the BBn EC than for the BBd EC. A smaller proportion of the BBd EC population expressed PCNA and no PCNA was expressed after day 3 in culture. In contrast, BBn EC expressed high levels of PCNA for longer periods of time.

We propose that the decrease in proliferation of BBd EC results from a decrease in NO production because of low BH4 levels, thus altering the cell signaling pathways for angiogenesis. We have previously shown that addition of sepiapterin to cultures of BBd EC markedly increased cellular BH4 concentration and NO production (24). We now show that sepiapterin increases BBd EC proliferation. When BBn EC were treated with the BH4 synthesis inhibitors, the reduction in proliferation could be significantly attenuated with DETA, which provides the cells with an exogenous source of NO. Both NAS-treated and DAHP-treated BBn EC showed a significant increase in cell proliferation in response to DETA. Interestingly, the inhibition of BH4 synthesis, and this effect could be attenuated by treatment with an NO donor. Thus NO production by microvascular EC depends on sufficient levels of BH4 and alterations in NO production modulate the rate at which these EC proliferate. It may be possible, in a clinical setting, to administer sepiapterin, BH4, and/or long-acting NO donors to increase microvascular EC proliferation, thereby preventing the altered angiogenesis associated with diabetes mellitus.

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