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Ratio of 5,6,7,8-tetrahydrobiopterin to 7,8-dihydrobiopterin in endothelial cells determines glucose-elicited changes in NO vs. superoxide production by eNOS

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NITRIC OXIDE (NO) is a biological messenger that is produced by enzymes of the nitric oxide synthase (NOS) gene family, comprising endothelial (eNOS), inducible (iNOS), and neuronal (nNOS) isoforms. In the vasculature, eNOS-derived NO plays a pivotal role in physiological regulation of vessel tone and inflammatory status (58). Diminished availability of eNOS-derived NO is common to chronic vascular disorders that share endothelial dysfunction as a hallmark, e.g., diabetes (11, 37, 60), hypertension (28), and atherosclerosis (28, 46). While the mechanistic basis for this attenuated NO bioavailability is uncertain, both slowed NO synthesis and accelerated NO scavenging by reactive oxygen species (ROS) have been implicated as causes (16). In contrast, levels of eNOS protein are typically unchanged or paradoxically increased. Oxidative stress, imposed by excessive ROS production, constitutes a unifying feature and likely generic trigger for endothelial dysfunction in chronic vascular conditions (1).1

The redox-sensitive NOS cofactor (68)-5,6,7,8-tetrahydrobiopterin (BH4) is required for NO synthesis by all NOS isoforms. Whereas fully reduced tetrahydropterins support catalysis by NOSs, oxidized pterin species are catalytically incompetent (e.g., 7,8-dihydrobiopterin, BH2) (14, 27, 47). Electron paramagnetic resonance (EPR) studies showed that in the absence of BH4 (or presence of excess BH2), superoxide is the sole in vitro product of recombinant eNOS (51). In the absence of BH4, electron transfer within eNOS becomes “uncoupled” from L-arginine oxidation and ferrous dioxygen releases superoxide with a finite probability (51).

BH4 is prone to oxidation in vitro, readily occurring in laboratory solutions unless suppressed by chemical reductants and low temperature (10, 25). BH4 oxidation has also been found to occur in vascular cells, in the setting of oxidative stress associated with hypertension (28), atherosclerosis (29), and diabetes (33). Depletion of BH4 in oxidatively stressed endothelial cells (ECs) can result in product switching from NO to O2•−. Moreover, uncoupled eNOS may initiate a futile feed-forward cascade whereby the reaction product of NO and O2•−, ONOO−, elicits further BH4 oxidation (26, 34), progressively more eNOS uncoupling (61), and a downward spiral in levels of vascular NO bioactivity.

Oxidant stress, such as that associated with hyperglycemia, can potentially overwhelm the natural antioxidant defense mechanisms that serve to maintain BH4 in its reduced form, resulting in endothelial dysfunction. Glutathione (GSH), vita-
min C, and vitamin E are key cellular antioxidants that preserve BH4 and diminished levels of these antioxidants are evident in diabetic patients (57, 59). Vitamin C treatment was shown to increase eNOS activity in ECs specifically via chemical stabilization of BH4 (9, 21). Augmentation of endothelial BH4 levels by adenovirus-mediated overexpression of the rate-limiting enzyme for BH4 synthesis, GTP cyclohydrolase 1 (GTPCH), was similarly found to restore eNOS activity in high-glucose-treated human ECs in culture (7) in rodent blood vessels of ApoE-null (42) and streptozotocin models (36) of atherosclerosis and diabetes, respectively. BH4 supplementation was also shown to acutely improve endothelial dysfunction in chronic smokers (20) and patients with hypercholesterolemia (46), diabetes (37, 44), or ischemia-reperfusion injury (48). In aortas of mice with deoxycorticosterone acetate salt-induced (DOCA-salt) hypertension, production of NOS-derived ROS increased (48). In aortas of mice with deoxycorticosterone acetate salt-induced (DOCA-salt) hypertension, production of NOS-derived ROS is markedly increased and BH4 oxidation is evident (28). Treatment of DOCA-salt mice with oral BH4 attenuated vascular ROS production, increased NO levels, and blunted hypertension compared with non-hypertensive control mice (28). Thus multiple lines of evidence implicate BH4 oxidation as a basis for eNOS uncoupling in vascular conditions associated with oxidative stress.

We hypothesized that the accumulation of BH2 in ECs may bind eNOS with significant avidity and hence directly suppress eNOS activity, rather than being an inert product of BH4 oxidation. If so, the intracellular ratio of BH4 to BH2, rather than the level of intracellular BH4 per se, could be the key determinant of eNOS-derived NO vs. O2•− production. To test this possibility, we examined the influence of BH4 oxidation on binding to eNOS and the extent to which BH4 oxidation occurs in ECs and animal tissues after exposure to diabetic levels of glucose. We show that eNOS binds BH4 and BH2 with equal affinity and that BH3 displaces eNOS-bound BH4 in vitro. We also demonstrate a glucose-induced switch from NO to superoxide production through eNOS uncoupling in ECs, determined by the BH4-to-BH2 ratio. Our findings implicate the intracellular BH4-to-BH2 ratio, not simply BH4 amount, as a critical in vivo determinant of eNOS product formation. Accordingly, diminished BH4:BH2 is likely to be the fundamental molecular link between oxidative stress and endothelial dysfunction in diabetes and other chronic vasoinflammatory conditions.

**EXPERIMENTAL PROCEDURES**

**Materials.** Pterin analogs were purchased from B. Schircks (Jona, Switzerland). Additional chemicals and solvents, unless otherwise stated, were purchased from Sigma (St. Louis, MO). HPLC mobile phase and samples were prepared with water with >18-MΩ resistance water (Millipore, MA).

**Cell culture.** Murine ECs (sEnd.1) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco Life Technologies) supplemented with 10% fetal bovine serum. This cell line was a gift from Dr. Patrick Vallance (University College, London) and originally established from a mouse skin capillary endothelioma induced by infection with a retrovirus harboring an insert that encodes polyclonal middle T antigen (56). Notably, sEnd.1 cells have not been reported to display features inconsistent with their EC origin. Cells were grown to confluence in T75 flasks or six-well plates and harvested immediately before use. RFL-6 fibroblasts were a gift from Dr. Ferid Murad (University of Texas at Houston) and were grown in Ham’s F-12 medium (Invitrogen) containing 10% fetal calf serum. All cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO2. All culture media were supplemented with 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Invitrogen).

**Purification of recombinant eNOS.** Bovine eNOS was purified from BL21 E. coli harboring both pGroELS and pCW-eNOS expression plasmids (31, 32). Purified eNOS was assayed for enzyme activity based on NO2 accumulation with the Griess assay method (55) and shown to be >90% pure by protein staining of polyacrylamide gels with Coomassie blue (data not shown).

**[3H]BH4 synthesis.** To quantify and characterize BH4 binding (6R·[3H]BH4) ([3H]BH4) was custom synthesized by complete reduction of 7,8-BH2 with sodium borotritide (New England Nuclear/Perkin Elmer). Although the initial product was (6R,6S)-[3H]BH4, exchange of N5 tritium on the biopterin ring with solvent protons, followed by HPLC purification by cation exchange chromatography on a Partisil 10 SCX column, allowed isolation of [3H]BH4 stereoisomers labeled at the 6 position. The R stereoisomer was used in the present study and is designated [3H]BH4 throughout this report. [3H]BH4 was stored as a 1 mM stock solution in equimolar HCl at −70°C.

**[3H]BH4 binding to eNOS.** [3H]BH4 binding assays were performed with polyvinylidene difluoride membrane-bottom 96-well filtration plates (Millipore). Before the assay, filtration membranes were sequentially washed under vacuum once with 100 μl of ethanol-water (50%) and then twice with 200 μl of Tris (50 mM) pH 7.6. All binding reactions contained Tris (50 mM) pH 7.6, DTT (1 mM), eNOS (10 pmol), the desired concentration of [3H]BH4, and other specified additions, comprising a final volume of 100 μl. Pseudoequilibrium binding was analyzed after sample incubation for 20 min at 23°C. Binding reactions were initiated by the addition of eNOS. For measurements of association rate, binding was initiated by addition of [3H]BH4. In dissociation experiments, eNOS (10 μg) was added to a 90-μl binding mixture including [3H]BH4: after a 15-min preincubation period, dissociation was initiated by addition of unlabeled BH4 at 100 μM final concentration. Displacement binding assays were performed after preincubation of [3H]BH4 with eNOS for 15 min, followed by incubation with desired concentrations of BH4 or BH2 for a further 30 min and then quantification of residual [3H]BH4–eNOS complexes. All binding reactions were terminated by rapid filtration of the 96-well filter plates, followed by three washes with iced Tris buffer (50 mM, pH 7.6). Plates were air dried for 30 min, followed by the addition of 25 μl of scintillation cocktail (Optiphase, Wallac) and radioactivity counting in a MicroBeta plus scintillation counter (Perkin Elmer).

**Analysis of [3H]BH4 binding.** Equilibrium binding data, as well as association and dissociation kinetics, were analyzed with Prism (Graphpad Software) and Ligand (Biosoft, Cambridge, UK) programs. Binding isotherms were calculated based on the equation B = (Bmax + T + Kd)2 − √[(Bmax + T + Kd2)2 − 4BmaxT], where B is the concentration of bound ligand, Bmax is the total eNOS concentration, T is the total ligand concentration, and Kd is the concentration of [3H]BH4 that gives half-maximal binding. This formula derives from the basic equilibrium equation: [L][B]/[LR] = Kd, where L (total ligand concentration) is significantly greater than the free ligand concentration (R).

**Western blotting.** Cells were suspended in RIPA lysing buffer (in mM: 20 Tris·HCl, 150 NaCl, 1 Na2EDTA, and 1 EGTA, with 1% Triton, 0.1% SDS, and 0.1 sodium deoxycholate, pH 7.4) containing a cocktail of protease inhibitors and subjected to four cycles of freezing-thawing in liquid nitrogen. Western blotting was carried out by standard techniques with anti-eNOS (Santa Cruz Biotechnology), anti-GTPCH, and anti-GTPCH feedback regulatory protein (GFRP) antibodies.

**Detection of endothelium-derived NO with RFL-6 cell cGMP reporter bioassay.** Endothelium-derived NO bioactivity was measured based on the increase in cGMP elicited in RFL-6 reporter cells, after exposure to preconditioned media from sEnd.1 endothelial cells, as previously described (24).
Pterin quantification by HPLC and electrochemical detection. Cellular pterin levels were quantified with a modified HPLC method that utilizes sequential electrochemical and fluorescence detectors in series (15). Cells were harvested in PBS (pH 7.4) and pelleted by centrifugation (2,000 g, 1 min). Supernatants were discarded, and cells were resuspended in 300 μl of ice-cold acid precipitation buffer (0.1 M phosphoric acid, 0.23 M trichloroacetic acid), followed by centrifugation (12,000 g at 4°C) for 1 min. Two aliquots of supernatant (120 μl) were transferred into HPLC vials for the analysis of total biopterin, BH4, the quinonoid isomer of BH2 (qBH2), and 7,8-BH2, as described previously (15). Quantitation of BH4 and 7,8-BH2 was done by comparison with external standards after normalization for total protein content.

GSH measurement. For quantitation of GSH, a modified microtiter plate enzymatic recycling assay was used, adapted from the standard spectrophotometric assay (13).

Superoxide quantitation by lucigenin chemiluminescence. The production of ROS in response to elevated levels of glucose was measured by lucigenin-dependent chemiluminescence, as previously described (2).

Experimental animals. Studies used Zucker diabetic fatty (ZDF) and nondiabetic lean control (ZL) rats (Charles River Laboratories, Wilmington, MA), aged 8, 16, and 22 wk. Animals were allowed free access to rat chow and water throughout the study and housed in animal quarters maintained at 22°C with a 12:12-h light-dark cycle. ZDF rats were randomly divided into two groups. One group was treated daily with ebselen (Daiichi) dissolved in 5% CM-cellulose and administered by gavage in two daily doses of 5 mg/kg body weight commencing at 8 wk of age. A second group of ZDF and ZL rats received a similar amount and dosing schedule of unmediated vehicle (5% CM-cellulose) by gavage. After death, rat lungs were harvested for pterin analysis as described above, and plasma was assayed for glucose content. Plasma glucose was quantified with a kit based on the modified Trinder color reaction, according to the manufacturer’s protocol (Raichem, San Diego, CA). The animal study protocol was approved by the Institutional Animal Care and Use Committee.

RESULTS

Characterization of [3H]BH4 binding to eNOS. Studies were performed to define the kinetics of [3H]BH4 binding to purified recombinant bovine eNOS and the relative ability of unlabeled pterins to compete for binding. All binding assays were performed in the presence of 0.1 mM DTT to minimize [3H]BH4 oxidation. As shown in Fig. 1A, [3H]BH4 rapidly associates with eNOS; under the study conditions tested (50 nM [3H]BH4 and 10 pmol eNOS, at 22°C) half-maximal occupancy was obtained in 5.3 ± 1.4 min and binding was >95% complete by 20 min (n = 5). The dissociation of preformed [3H]BH4-eNOS complexes occurred with monophasic kinetics and was 50% complete at (T1/2) = 28.1 ± 2.5 min (see Fig. 1A, inset).

Pseudoequilibrium binding of [3H]BH4 to eNOS was analyzed after incubation of purified eNOS (10 pmol) with indicated concentrations of [3H]BH4 for 20 min at 22°C (Fig. 1B). Binding was found to be saturable and reconciled by a single class of sites with apparent KM = 82.1 ± 17.8 nM. Competition binding studies were performed to compare the ability of nonradiolabeled pterins to vie for [3H]BH4 binding to eNOS. As shown in Fig. 1C, BH4 and BH2 bound eNOS with indistinguishable affinities; EC50 values were 59.3 ± 19.0 and 67.4 ± 11.1 nM, respectively. In contrast, tetrahydropterin

![Fig. 1. Characterization of 3H-labeled 5,6,7,8-tetrahydrobioperin (BH4) binding to purified recombinant bovine endothelial nitric oxide synthase (eNOS) and competition by pterin analogs. A: kinetics of association and dissociation (inset) of [3H]BH4 binding (50 nM) to eNOS (10 pmol). For dissociation studies, [3H]BH4-eNOS complexes were formed during a 15-min preincubation, and residual complexes were analyzed at varying times after addition of a 2,000-fold molar excess of unlabeled BH4. B: pseudoequilibrium binding of [3H]BH4 to eNOS (10 pmol) after 15-min incubation. Calculated apparent KM = 82.1 ± 17.8 nM (n = 3). C: competition of unlabeled pterins for [3H]BH4 binding to eNOS. [3H]BH4 (50 nM) and eNOS (10 pmol) were incubated with indicated concentrations of 7,8-dihydrobioperin (BH2), BH4 or tetrahydropterin (PH4); binding was terminated and analyzed after 15 min. D: displacement by BH2 of [3H]BH4 from preformed eNOS-[3H]BH4 complexes. Complexes were formed by preculturing eNOS (10 pmol) with [3H]BH4 (50 nM) for 15 min, and residual complexes were quantified 30 min after addition of indicated concentrations of BH2. All binding reactions were conducted at 22°C. Points are means ± SE of triplicate determinations.](http://ajpheart.physiology.org/)

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(PH₄), a BH₄ analog that differs only in the lack of a 6-position dihydroxypropyl side chain, bound eNOS with >1,000-fold lower affinity (EC₅₀ = 112 μM) versus BH₄ or BH₂. These results demonstrate that partial oxidation of the biopterin ring, from tetrahydro- to dihydro-, does not diminish the affinity for eNOS binding, whereas the 6-position side chain of biopterins is essential for high-affinity binding to eNOS.

Given our findings that BH₂ binds eNOS with nanomolar affinity (equivalent to that of BH₄) and BH₄ dissociates from eNOS complexes in minutes at 22°C, we hypothesized that BH₂ could efficiently replace BH₄ when complexed with eNOS. To test this possibility, [³H]BH₄-eNOS complexes were formed and remaining complexes were quantified 30 min after the addition of specified concentrations of BH₂. As shown in Fig. 1D, [³H]BH₄-eNOS complexes were progressively lost with increasing BH₂ concentration, to a maximum extent of 80%; half-maximal [³H]BH₄ displacement was observed when the concentrations of BH₂ and [³H]BH₄ approached equivalence (50 nM). Together, these binding studies indicate that if BH₂ were to accumulate in ECs, it should effectively compete with BH₄ for eNOS occupancy. Since BH₂ binding to eNOS is known to cause enzyme uncoupling, this association would predictably result in a decrease in eNOS-derived NO and increase in eNOS-derived superoxide.

Attenuation of EC-derived NO production by elevated glucose. NO bioactivity was measured in the culture medium of murine endothelial cells (sEnd.1 line) after 20-min incubation with calcium ionophore (A-23187; 5 μg/ml). Quantification of NO bioactivity was determined based on the extent of increase in cGMP content following a 5-min incubation of phosphodiesterase-inhibited RFL-6 reporter cells (a soluble guanylyl cyclase-rich cell line) with EC-conditioned medium. As shown in Fig. 2, treatment of ECs for 48 h with progressively increasing glucose concentrations (from 5 to 30 mM) resulted in a concentration-dependent decrease in ionophore-elicited release of NO bioactivity. A ~50% decrease in released NO bioactivity was observed in cells pretreated for 48 h with 30 mM relative to 5 mM glucose (P < 0.05).

eNOS-dependent BH₄ oxidation occurs in ECs after exposure to elevated glucose. The BH₄ redox status in ECs was analyzed by HPLC, with combined electrochemical and fluorescence detection (15). Total pterin (BH₄ + BH₂ + biopterin) was indistinguishable in high (30 mM)- and low (5 mM)-glucose-treated ECs. This notwithstanding, high glucose was found to decrease intracellular BH₄ by 40–50% in association with a reciprocal increase in BH₂ content (P < 0.01; Fig. 3). The accumulation of BH₂ was almost exclusively as 7,8-BH₂; a significant contribution of the quinonoid tautomer, qBH₂ (also known as 5,6-BH₂), was not detected (not shown). Also, fully oxidized BH₄ (i.e., biopterin) and its side chain cleaved product (pterin) were not detected in ECs after 48-h incubation in high-glucose medium (not shown).

BH₂ accumulation in EC increased progressively with an increasing duration of glucose exposure (Fig. 3A) and with increasing glucose concentrations for a fixed duration (Fig. 3B). High-glucose-elicited oxidation of BH₂ was prevented by >50% in the presence of a NOS-specific inhibitor, 3 mM Nω-nitro-L-arginine methyl ester (L-NAME), and abolished by diphenyleneiodonium (DPI), an agent that inhibits superoxide production by NOS and other flavoproteins (including NADPH oxidase) (Fig. 3C). These findings suggest a key role for superoxide and/or derived species in the oxidation of BH₄ and implicate uncoupled eNOS as a key contributor. Accordingly, treatment of ECs with high glucose (30 mM) was associated with a significant increase in O₂− release (200%, Fig. 3D). The authenticity of this apparent superoxide was confirmed by its disappearance when cells were treated with 100 U of CuZn-SOD (Fig. 3D). High-glucose-induced superoxide formation was also blocked by treatment with a selective NOS inhibitor (L-NAME; Fig. 3D), identifying uncoupled eNOS as the source. Moreover, an identical degree of suppression of superoxide formation was observed in cells treated with either L-NAME or the general flavoprotein inhibitor DPI. Thus products of un-coupled eNOS are necessary for the increases in BH₄ oxidation and O₂− production that we observe in high-glucose-treated ECs.

GH levels determine extent of BH₄ oxidation by glucose in ECs. Since GSH is the major EC reservoir of reduced thiols, we investigated whether glucose-elicited BH₄ oxidation is concomitant with GSH oxidation and whether intracellular GSH levels determine the extent of BH₄ oxidation. As shown in Fig. 4, A and B, 48-h treatment with 30 mM glucose resulted in a 35–40% relative decrease in both intracellular GSH and BH₄ relative to levels observed in cells grown in 5 mM glucose. Intracellular GSH levels in ECs in 5 mM glucose medium were increased by 220% after incubation in medium containing 2 mM GSH ester (Fig. 4A). Notably, this level of GSH repletion in ECs afforded complete protection against both high-glucose-elicited BH₄ oxidation and GSH depletion (Fig. 4B). Reciprocally, depletion of GSH to 20% of basal levels found in cells cultured in 5 mM glucose was obtained after pretreatment with a selective γ-glutamylcysteinyln-thase inhibitor, buthionine sulfoximine (BSO; Fig. 4C). This level of GSH depletion sensitized ECs to high-glucose-induced BH₄ oxidation (from 40% BH₄ oxidation without prior GSH
BH₄ oxidation to BH₂ ratio determines extent of eNOS coupling in high-glucose-treated EC. If BH₄ oxidation is the primary basis for eNOS uncoupling, one would predict that BH₄ supplementation would rapidly reinstate NO synthesis by uncoupled eNOS. This prediction is supported by results from multiple in vitro and in vivo studies showing that administration of BH₄ acutely enhances NO bioactivity and suppresses eNOS-derived superoxide generation (1). Nonetheless, the possibility exists that progressive oxidation of administered BH₄ would ultimately result in intracellular BH₂ buildup, leading to increased binding of BH₂ to eNOS and a consequent long-term worsening of eNOS uncoupling. To evaluate the more long-lived consequences of biopterin supplementation, we investigated the extent to which eNOS coupling and biopterin oxidation in EC were influenced by 24-h incubation with either BH₄ or BH₂ (Fig. 5).

As shown in Fig. 5A, incubation of ECs with a 10 µM concentration of either BH₄ or BH₂ in both low- and high-glucose-containing medium (5 and 30 mM, respectively), resulted in a similar 10-fold increase in total intracellular biopterin (BH₄ + BH₂), compared with ECs grown in non-biopterin-supplemented medium. Whereas total biopterin in low-glucose-grown ECs was found to be exclusively BH₄ in non-biopterin-supplemented medium (i.e., BH₂ was undetectable), in both BH₄- and BH₂-supplemented ECs BH₄ levels constituted 60% of total biopterin (with BH₂ as the remainder). In high-glucose medium, BH₄ supplementation of ECs was associated with markedly greater levels of intracellular BH₂ than in ECs in low-glucose medium (85% and 40% of total biopterin as BH₂, respectively). Despite the enhanced accumulation of BH₂ in ECs maintained in BH₄-supplemented high-glucose medium, it is notable that the absolute level of BH₄ in these cells was more than twofold that measured in high-glucose-treated ECs that were not BH₄ supplemented (see Fig. 5A).

Treatment of non-biopterin-supplemented ECs with high glucose (30 mM) vs. low glucose (5 mM) resulted in a 40–50% decrease in A-23187-elicited NO bioactivity (Fig. 5B) and a 500% increase in superoxide generation that was fully prevented by addition of a selective NOS inhibitor (L-NAME) to the superoxide assay mix (Fig. 5C). Whereas supplementation of ECs with BH₄ had no significant effect on NO bioactivity elicited in low-glucose medium, in high-glucose medium a paradoxical 40% decrease in NO bioactivity was observed, relative to non-biopterin-supplemented ECs (Fig. 5B). Notably,
this apparent increase in eNOS uncoupling was concomitant with a paradoxical doubling of absolute levels of intracellular BH4 (Fig. 5A). Despite a BH4 supplementation-evoked doubling of BH4 levels, it is notable that a far greater decrease in the intracellular ratio of BH4 to BH2 was observed in non-supplemented vs. BH4-supplemented ECs (1:1 vs. 1:6, respectively). These findings reveal that the extent of eNOS coupling correlates inversely with the ratio of intracellular BH4 to BH2, but not absolute levels of intracellular BH4.

In contrast to findings with BH4-supplemented ECs in high-glucose medium, supplementation with BH2 resulted in a similar extent of total biopterin accumulation, but substantially greater accumulation as BH4 (BH4:BH2 ≈ 1:6 vs. 1:1, respectively). Accordingly, BH2 supplementation of ECs was associated with a twofold increase in absolute BH4 levels, relative to levels observed in ECs supplemented with an identical concentration of BH4. The relative increase in accumulation of BH4 in BH2-supplemented vs. BH4-supplemented ECs was associated with a modestly enhanced extent of eNOS coupling, as evidenced by a 45% increase in evoked NO bioactivity and a 25% decrease in superoxide generation (Fig. 5, B and C).

**Contribution of mitochondrial-derived superoxide to high-glucose-elicited BH4 oxidation in ECs.** Having found that eNOS-derived superoxide is necessary for sustained BH4 oxidation in high-glucose-treated ECs, we questioned whether mitochondrial-derived superoxide is required to initiate BH4 oxidation and eNOS uncoupling. Notably, the mitochondrial electron transport chain is considered to be the predominant source of superoxide in normally respiring cells, and elevated glucose is known to increase mitochondrial respiration and thereby accelerate mitochondrial-derived superoxide generation (35, 45). To test whether mitochondrial-derived superoxide plays a role in high-glucose-induced BH4 oxidation, we assessed whether selective inhibitors of the mitochondrial electron transport chain afford protection against high-glucose-induced BH4 oxidation. As shown in Fig. 6, glucose-elicited BH4 oxidation was markedly and significantly prevented by coincubation of ECs with selective inhibitors of mitochondrial electron transport complexes I or II [2 μM rotenone and 5 μM thenoyltrifluoroacetone (TTFA), respectively]. These findings implicate a role for mitochondrion-derived superoxide in the genesis of high-glucose-induced BH4 oxidation, leading to eNOS uncoupling.

**BH4 oxidation in vivo.** To assess whether the high-glucose-evoked BH4 oxidation that we observed in EC culture studies has relevance in vivo, we sought to determine the relationship between plasma glucose and tissue BH4 oxidation in a rodent model of type II diabetes and metabolic syndrome, the Zucker diabetic fatty (ZDF) rat. Unlike Zucker lean (ZL) control rats, ZDF rats develop moderate hyperglycemia by 8 wk of age, with glucose levels of 197.9 ± 11.7 mg/dl vs. 144.8 ± 26.2 mg/dl in age-matched ZL controls (Fig. 7A). By 16 wk of age, ZDF rats become severely hyperglycemic, with resting glucose levels of 341.6 ± 35.9 mg/dl (vs. 162.8 ± 8.2 mg/dl in ZL).
These increases in BH4 oxidation were observed in heart, kidney, and brain (not shown). This aging-associated decrease in the BH4-to-BH2 ratio in ZDF rat lungs (but not ZL controls) was apparent by 16 wk (Fig. 7C; \( P < 0.05 \)). Notably, we previously reported that at 16 wk and beyond, ZDF rats exhibit marked NO insufficiency, loss of endothelium-dependent vasorelaxation, and accumulation of 3-nitrotyrosine (3-NT) in tissue proteins, and that each of these measures of endothelial dysfunction was protected by cotreatment with the peroxynitrite scavenger ebselen (30). As shown in Fig. 7B, ebselen similarly protected against BH4 oxidation in 22-wk ZDF rats, consistent with a role for peroxynitrite or a related oxidant in the mediation of glucose-elicited BH4 oxidation.

**DISCUSSION**

Diminished NO bioactivity is a significant predictor of cardiovascular risk (4, 40) and a hallmark of endothelial dysfunction (18). NO insufficiency has been implicated in the etiology and progression of major chronic vasoinflammatory conditions, including diabetic vasculopathy (54). Mitochondrial superoxide overproduction is considered to provide a trigger for metabolic derangements that mediate diabetic complications (6). While scavenging of NO by superoxide offers a simple explanation for consumption of NO bioactivity in diabetic blood vessels, the peroxynitrite product of this reaction can further compromise NO bioactivity by promoting the oxidation of BH4, leading to eNOS uncoupling. Oxidation of BH4 and eNOS uncoupling has previously been observed in genetic models of type 1 and type 2 diabetes (3, 41). An earlier report by Vasquez-Vivar and colleagues (52) provided the first evidence of BH2 binding to recombinant eNOS in vitro, based on an EPR-detectable increase in superoxide formation. Here we extend this finding with the first direct quantitative analysis of bipterin binding to eNOS.

Using an EC model of hyperglycemia-elicited eNOS uncoupling, we provide evidence for in vivo binding of BH2 to

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Fig. 5. Pterin supplementation (24 h) increases intracellular BH4 levels in low- and high-glucose-treated ECs; however, this is associated with BH2 accumulation and failure to improve or worsened eNOS coupling. sEnd.1 cells were grown in high (30 mM) or low (5 mM) glucose-containing media, and after 24 h cells were either supplemented with BH4 (10 \( \mu \)M) or BH2 (10 \( \mu \)M) or left unsupplemented (basal). After a further 24 h, assays were performed to quantify intracellular bipterins (BH4 and BH2; A), release of NO (B), and production of superoxide (C). Intracellular levels of BH4 (filled bars) and BH2 (open bars) were quantified by HPLC, and release of NO bioactivity was assessed based on cGMP accumulation in RFL-6 reporter cells. Superoxide production was quantified based on the difference in lucigenin chemiluminescence in the absence and presence of 3 mM l-NAME. Values are means + SE (n = 5). *P < 0.05.

Fig. 6. Prevention of BH4 oxidation by mitochondrial electron transport chain inhibitors. Cells were grown in low (5 mM; black bars) or high (30 mM; red bars) glucose-containing media for 48 h. Rotenone (2 \( \mu \)M) and thenoyltrifluoroacetone (TFFA, 5 \( \mu \)M), inhibitors of complexes I and II of the mitochondrial electron transport chain, respectively, were added after the initial 24 h, and cells were harvested for assay of BH4 and BH2; BH4 (filled bars) oxidation to BH2 (open bars) was significantly attenuated by both TFFA and rotenone treatment (*P < 0.01). Values are means + SE (n = 3).
eNOS, implicating BH2-eNOS assembly as a key effector of diabetic vasculopathies. Analysis of [3H]BH4 binding revealed that catalytically incompetent BH2 competes for eNOS occupancy with an affinity identical to that of the active cofactor, BH4. Furthermore, BH2 exchanges rapidly with BH4 on preformed eNOS complexes in vitro, achieving half-maximal substitution within 20 min at 22°C—this exchange rate is likely to be still more rapid at 37°C in cells. Importantly, levels of glucose known to be common in diabetic patients (30 mM) were found to elicit oxidant stress in ECs in culture to an extent that markedly perturbs EC pterin redox balance in favor of BH2 accumulation. Accumulated BH2 in ECs increases with increasing concentrations of glucose in the extracellular milieu, is progressive with time (for a given glucose concentration), and is coupled to levels of intracellular GSH. The accumulated BH2 in high-glucose-treated ECs was implicated as a trigger for eNOS uncoupling. Notably, high-glucose-elicited superoxide production was eradicated within minutes of exposure to a NOS-selective inhibitor, confirming eNOS as the dominant source. Accelerated peroxynitrite formation, inferred from accumulated 3-NT modification of proteins, provides further support for a switch in eNOS toward oxidant generation, rather than NO.

Our findings argue for a revised mechanistic view regarding the role of BH4 oxidation in endothelial dysfunction. The results suggest that the fundamental determinant of NO bioactivity conveyed by ECs in blood vessels is the balance between intracellular BH4 and its primary two-electron oxidation product BH2—not absolute quantities of BH4 as has generally been thought (1). This conclusion is supported by in vitro analyses of [3H]BH4 binding to purified recombinant eNOS and cell culture studies of the consequences of biopterin supplementation on total biopterin levels, BH4:BH2 redox balance, and associated changes in eNOS function. Notably, despite a two-fold increase in intracellular BH4 in 24-h BH4-supplemented, high-glucose-treated ECs, an even greater accumulation of BH2 was observed (12-fold), accompanied by hallmark features of increased eNOS uncoupling, i.e., diminished NO bioactivity (40%) and increased superoxide generation (200%). Thus eNOS uncoupling was found to worsen with BH4 supplementation of ECs, despite an increase in absolute levels of BH4. In contrast, while BH2 supplementation of high-glucose-treated ECs also resulted in a substantial increase in total biopterin (equal to that observed with BH4 supplementation), this was not associated with a decrease in the BH4-to-BH2 ratio vs. non-biopterin-supplemented ECs (BH4:BH2 ≈ 1:1 in each case) and resulted in a modest improvement in eNOS coupling (enhanced release of NO bioactivity and diminished superoxide production). The opposite consequences of BH4 and BH2 supplementation on eNOS coupling are best reconciled by a model in which BH4-to-BH2 ratios are the primary determinant of eNOS coupling in EC, rather than absolute levels of BH4. Predictably, intracellular BH4:BH2 would determine eNOS coupling in all biological settings where eNOS approaches saturation with biopeterin cofactor (BH4 or BH2). Thus, with cofactor saturation, any perturbation in BH4:BH2 balance, up or down, would be expected to modulate the extent of eNOS coupling in the same direction. The condition of BH4 sufficiency would appear to be met in the present BH4 supplementation studies, where eNOS coupling was apparently diminished despite a doubling of BH4 content (owing to a >10-fold...
increase in BH2 and hence an overall decrease in BH4:BH2). In contrast, under conditions in which eNOS is subsaturated with its biopterin cofactor, administered biopterins could potentially improve eNOS coupling even under circumstances in which the balance of BH4:BH2 is somewhat diminished. Detailed modeling studies will be needed to define boundary conditions that predict the consequences of changing intracellular levels of BH4, BH2, and eNOS on levels of [eNOS-BH4] versus [eNOS-BH2] and hence eNOS coupling efficiency. In any case, it is evident that BH2 binding to eNOS can constitute a major contributor to hyperglycemia-induced eNOS uncoupling, as observed for ECs in the present study.

Concomitant increases in plasma glucose and tissue levels of BH2 in ZDF diabetic rats provide in vivo validation of results obtained with ECs in culture. Notably, we previously showed (5) that the peroxynitrite scavenger ebselen, administered to ZDF rats in the same regimen as in the present study, inhibits peroxynitrite production (evidenced by protection against protein 3-NT accumulation in plasma and blood vessels). In the present study, we show that ebselen similarly attenuates the progressive accumulation of tissue BH2. Protection against BH2 accumulation provides a likely explanation for ebselen's effectiveness in limiting the progressive hyperglycemia-associated loss of endothelium-dependent vasodilatation and diminished NO bioactivity in ZDF rat blood vessels (5, 8).

Peroxynitrite is likely to be the biologically relevant oxidant of BH4 in high-glucose-treated ECs. Although superoxide reacts with BH4 in vitro, the rate constant is 10,000-fold slower (3.9 \times 10^7 \text{ mol}^{-1}\text{s}^{-1}) (53) than its near-diffusion limited reaction with NO (6 \times 10^9 \text{ mol}^{-1}\text{s}^{-1}) (22). Accordingly, NO would predictably outcompete BH4 for reaction with superoxide. Peroxynitrite formed by the NO/superoxide reaction could then oxidize BH4 as previously described (29, 34) and thereby promote eNOS uncoupling. Notably, the reaction of peroxynitrite with BH4 occurs via the intermediacy of the BH4 radical cation and with a first-step rate constant that is several times faster than the reaction with thiols (6 \times 10^3 \text{ mol}^{-1}\text{s}^{-1}) (26). Inasmuch as intracellular thiol levels (millimolar) far exceed the estimated levels of BH4 in ECs (0.05–0.2 \mu M), thiol oxidation is expected to predominate over BH4 oxidation. This competition between thiols and BH4 for peroxynitrite-mediated oxidation provides one explanation for our observation that the extent of glucose-elicited BH4 oxidation in ECs is inversely related to GSH levels (Fig. 4).

Our finding that BH2 avidly binds eNOS and engenders uncoupling has important implications for possible uses of BH4 for therapy of endothelial dysfunction. Prior studies suggest a therapeutic potential of BH4 for reversal of endothelial dysfunction. While administration of high doses of BH4 has been shown to acutely restore endothelium-dependent (NO mediated) vasoactivity (12, 17, 19, 43, 46), studies have not yet addressed the more long-term consequences of BH4 administration in the setting of oxidative stress. The results reported here suggest that ongoing oxidative and nitrosative stress may elicit significant BH2 accumulation in ECs that opposes the desired NO-restoring action of administered BH4. Thus desensitization to the benefits of BH4 administration, or frank worsening, would result if BH2 was to progressively accumulate in ECs after repeated BH4 treatments. Accumulation of BH2 and consequent eNOS uncoupling also provides a likely explanation for paradoxical reports that BH4 treatment of vessel segments ex vivo (49) or animals (50) can worsen, rather than improve, endothelial dysfunction.

While BH4 is generally considered to be antioxidant, it can also be prooxidant. Indeed, BH4 undergoes autoxidation, yielding the quinonoid isoform of BH2 (qBH2, an isomer of 7,8-BH2) via reaction with molecular oxygen, generating superoxide in this process that can lead to oxidation of another molecule of BH4 (25). Once formed, qBH2 is nonenzymatically recycled to BH4, at the expense of extracellular thiols or other available reductants, creating a cycle of extracellular BH2 oxidation/reductant consumption. Oxidant stress imposed by this autooxidation of BH4 is a likely explanation for the paradoxical finding that high-glucose-treated ECs accumulate more BH4 when grown in BH4-supplemented medium compared with BH2-supplemented medium. Notably, an intracellular autoxidation chain reaction would predictably operate for BH4, but not BH2. Inasmuch as BH4 accumulation in tissues was also shown to be more efficient in mice treated with BH2, as opposed to BH4 (39), BH4 oxidation is likely to be important in vivo. Once in the cell, enzymatic regeneration of BH4 from BH2 will further consume pools of reducing potential (in the immediate form of reduced pyridine nucleotides) for support of the combined actions of dihydrofolate reductase (for substrate 7,8-BH2) and dihydropteridine reductase (for substrate qBH2). In contrast to extracellular redox cycling of BH4, intracellular redox cycling of BH4 would predictably impose an equivalent degree of oxidative stress in ECs supplemented with either BH2 or BH4. Thus, owing to the above-mentioned oxidative processes, BH4 supplementation therapy may have limited long-term benefit in improving eNOS coupling, despite the promise of reports suggested from the results of studies showing acute benefits.

Together, our findings recommend the following model for the initiation and progression of endothelial dysfunction in the setting of chronic vasoinflammation: Exposure of vascular endothelium to a prooxidative stimulus, including but not limited to diabetic levels of plasma glucose, triggers superoxide overproduction. This superoxide may derive from electron transport “leak” in mitochondria driven by high-glucose-accelerated metabolism in diabetes (as indicated by results depicted in Fig. 6) or other cell sources, such as inflammation-associated activation of NADPH oxidase (28). Reaction of superoxide with eNOS-derived NO will result in increased peroxynitrite synthesis that promotes BH4 oxidation and hence accumulation of BH2. Replacement of BH4 with BH2 in eNOS complexes would result in sustained eNOS-derived oxidant formation, perpetuating BH4 oxidation. At this stage, even after full resolution of the initiating oxidative insult, uncoupled eNOS could sustain the production of peroxynitrite, promote BH4 oxidation, and self-limit NO biosynthesis. According to this view, therapeutic approaches that can transiently recouple eNOS would provide a preferred means to interrupt the vicious cycle of endothelial dysfunction, engendering a sustained restoration of eNOS-derived NO production and a restoration of vascular health.

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