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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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Crabtree MJ, Smith CL, Lam G, Goligorsky MS, Gross SS. 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. *Am J Physiol Heart Circ Physiol* 294: H1530–H1540, 2008. First published January 11, 2008; doi:10.1152/ajpheart.00823.2007.—5,6,7,8-Tetrahydrobiopterin (BH₄) is an essential cofactor of nitric oxide synthases (NOSs). Oxidation of BH₄, in the setting of diabetes and other chronic vasoinflammatory conditions, can cause cofactor insufficiency and uncoupling of endothelial NOS (eNOS), manifest by a switch from nitric oxide (NO) to superoxide production. Here we tested the hypothesis that eNOS uncoupling is not simply a consequence of BH₄ insufficiency, but rather results from a diminished ratio of BH₄ vs. its catalytically incompetent oxidation product, 7,8-dihydrobiopterin (BH₂). In support of this hypothesis, [³H]BH₄ binding studies revealed that BH₄ and BH₂ bind eNOS with equal affinity ($K_d \approx 80$ nM) and BH₂ can rapidly and efficiently replace BH₄ in preformed eNOS-BH₄ complexes. Whereas the total biopterin pool of murine endothelial cells (ECs) was unaffected by 48-h exposure to diabetic glucose levels (30 mM), BH₂ levels increased from undetectable to 40% of total biopterin. This BH₂ accumulation was associated with diminished calcium ionophore-evoked NO activity and accelerated superoxide production. Since superoxide production was suppressed by NOS inhibitor treatment, eNOS was implicated as a principal superoxide source. Importantly, BH₄ supplementation of ECs (in low and high glucose-containing media) revealed that calcium ionophore-evoked NO bioactivity correlates with intracellular BH₄:BH₂ and not absolute intracellular levels of BH₄. Reciprocally, superoxide production was found to negatively correlate with intracellular BH₄:BH₂. Hyperglycemia-associated BH₄ oxidation and NO insufficiency was recapitulated *in vivo*, in the Zucker diabetic fatty rat model of type 2 diabetes. Together, these findings implicate diminished intracellular BH₄:BH₂, rather than BH₄ depletion per se, as the molecular trigger for NO insufficiency in diabetes.

nitric oxide; diabetes; endothelial dysfunction

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 (6*R*)-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). Electronparamagnetic 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-

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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 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 BH₂ 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-M Ω 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 polyoma 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 NO_x 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(6)]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(5,6)]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 μ l of ethanol-water (50%) and then twice with 200 μ l 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 μ l. 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 μ l) was added to a 90- μ l binding mixture including [³H]BH₄; after a 15-min preincubation period, dissociation was initiated by addition of unlabeled BH₄ at 100 μ M final concentration. Displacement binding assays were performed after preincubation of [³H]BH₄ with eNOS for 15 min, followed by incubation with desired concentrations of BH₂ or BH₄ for a further 30 min and then quantification of residual [³H]BH₄-NOS 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 μ l 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 $B = (B_{max} + T + K_d) / 2 - \sqrt{(B_{max} + T + K_d)^2 - B_{max}T}$, where B is the concentration of bound ligand, B_{max} is the total eNOS concentration, T is the total ligand concentration, and K_d is the concentration of [³H]BH₄ that gives half-maximal binding. This formula derives from the basic equilibrium equation: $[L][R]/[LR] = K_d$, when L (total ligand concentration) is significantly greater than the free ligand concentration (R).

Western blotting. Cells were suspended in RIPA lysis 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 μ l 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 μ l) were transferred into HPLC vials for the analysis of total biopterin, BH₄, the quinonoid isoform 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 (Sigma) 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 unmedicated vehicle (5% CM-cellulose) by gavage. After death, rat lungs were harvested for pterin analysis as described above, and plasma was

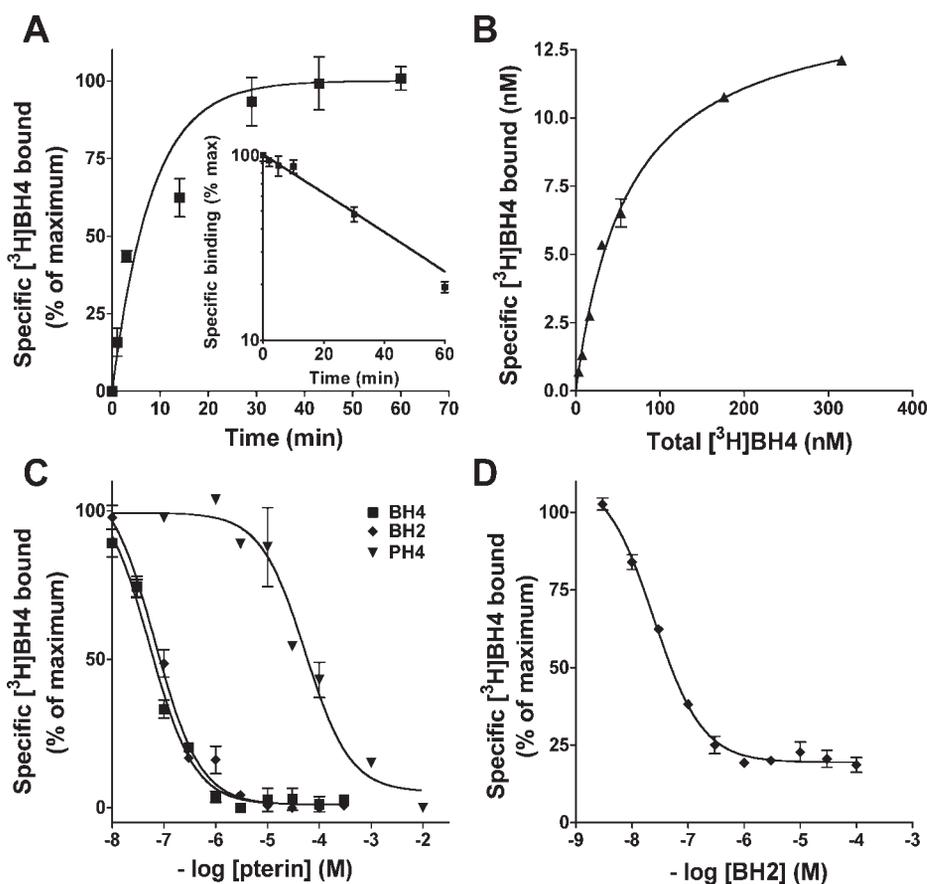
assayed for glucose content. Plasma glucose was quantified with a kit based on the modified Trinder color reaction, according to the manufacturer's protocol (Raichem, San Diego, CA). The animal study protocol was approved by the Institutional Animal Care and Use Committee.

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 \pm 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 (*T*_{1/2}) = 28.1 \pm 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 ^{BH₄}*K*_d = 82.1 \pm 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 \pm 19.0 and 67.4 \pm 11.1 nM, respectively. In contrast, tetrahydropterin

Fig. 1. Characterization of ³H-labeled 5,6,7,8-tetrahydrobiopterin (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 ^{BH₄}*K*_d = 82.1 \pm 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-dihydrobiopterin (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 preincubating 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 \pm SE of triplicate determinations.



(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

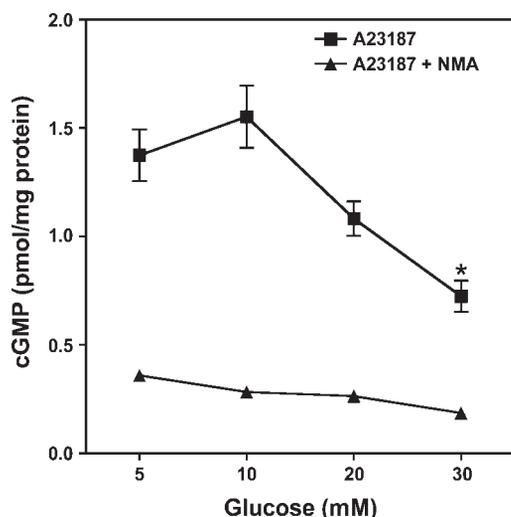


Fig. 2. Glucose-induced attenuation of nitric oxide (NO) production by endothelial cells. sEnd.1 cells were exposed to indicated concentrations of glucose for 48 h, and NO bioactivity was quantified in culture medium based on ability to elicit cGMP accumulation in the RFL-6 reporter cell line. sEnd.1 endothelial cells were incubated in the absence or presence of *N*^ω-methyl-L-arginine (L-NMA, 3 mM) for 20 min before activation with A-23187 (5 μg/ml). High levels of glucose (30 mM) were found to markedly attenuate endothelial cell-derived NO bioactivity (**P* < 0.05). Symbols are means ± SE of 3 replicate measurements.

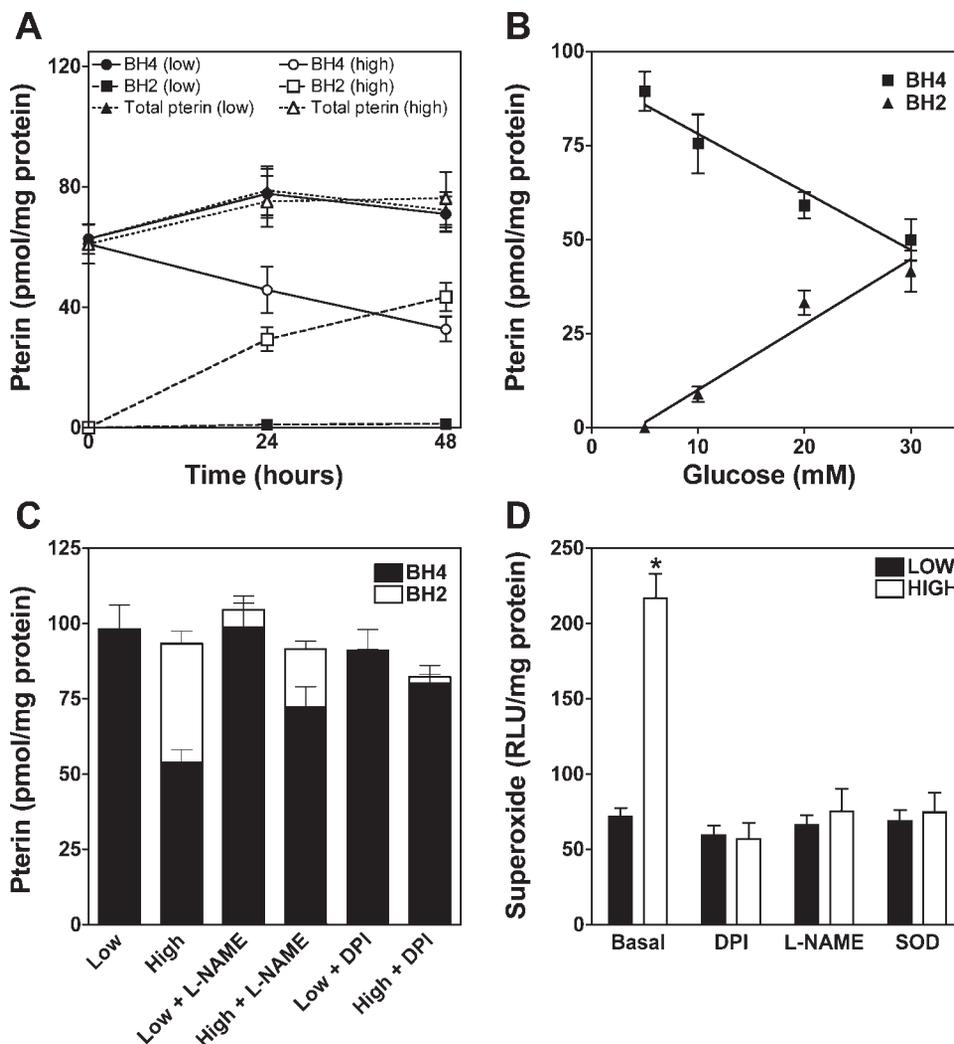
in a concentration-dependent decrease in ionophore-elicited release of NO bioactivity. A ≈50% decrease in released NO bioactivity was observed in cells pretreated for 48 h with 30 mM relative to 5 mM glucose (*P* < 0.05).

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 BH₄ was prevented by >50% in the presence of a NOS-specific inhibitor, 3 mM *N*^ω-nitro-L-arginine methyl ester (L-NAME), and abolished by diphenyleioidonium (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 uncoupled eNOS are necessary for the increases in BH₄ oxidation and O₂⁻ production that we observe in high-glucose-treated ECs.

GSH 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-glutamylcysteinyl synthase 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

Fig. 3. Glucose elevation increases oxidation of BH₄ to BH₂ in a time-, concentration- and eNOS-dependent manner. sEnd.1 endothelial cells were exposed to glucose at low (5 mM), intermediate (10 and 20 mM), or high (30 mM) levels for 0, 24, or 48 h. **A**: time course of changes in levels of total pterins (BH₄ and more oxidized species, circles), BH₄ (squares), and BH₂ (triangles) in cells exposed to low (open symbols) vs. high (closed symbols) levels of glucose. Whereas total pterin was unaffected, high glucose elicited significant oxidation of BH₄ by 24 h ($P < 0.05$), and this was potentiated at 48 h. **B**: glucose concentration dependence for oxidation of BH₄ to BH₂ after 48-h exposure. BH₄ oxidation in cells was not evident with low glucose medium but accelerated progressively as levels of medium glucose were increased. **C**: influence of the NOS-selective inhibitor *N*^ω-nitro-L-arginine methyl ester (L-NAME, 3 mM) and the flavoprotein-selective inhibitor diphenyleneiodonium (DPI, 10 μM) on high glucose-elicited BH₄ oxidation to BH₂ after 48-h exposure. Note that L-NAME significantly attenuated ($P < 0.05$) and DPI abolished high-glucose-elicited BH₄ oxidation. **D**: rate of superoxide production, as determined by lucigenin chemiluminescence. High-glucose treatment significantly accelerated O₂^{•-} production ($P < 0.01$), and this acceleration was abolished by treatment with DPI (10 μM), L-NAME (3 mM), or SOD (10,000 U). All indicated values are means ± SE of pterin levels as determined by HPLC-EC/fluorescence detection ($n = 4-5$). RLU, relative light unit.



depletion to 85% BH₄ oxidation with GSH depletion) and was sufficient to elicit BH₄ oxidation even in low-glucose medium (20%), where BH₄ oxidation was not otherwise detected (Fig. 4D).

BH₄-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 bio-

pterin (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,

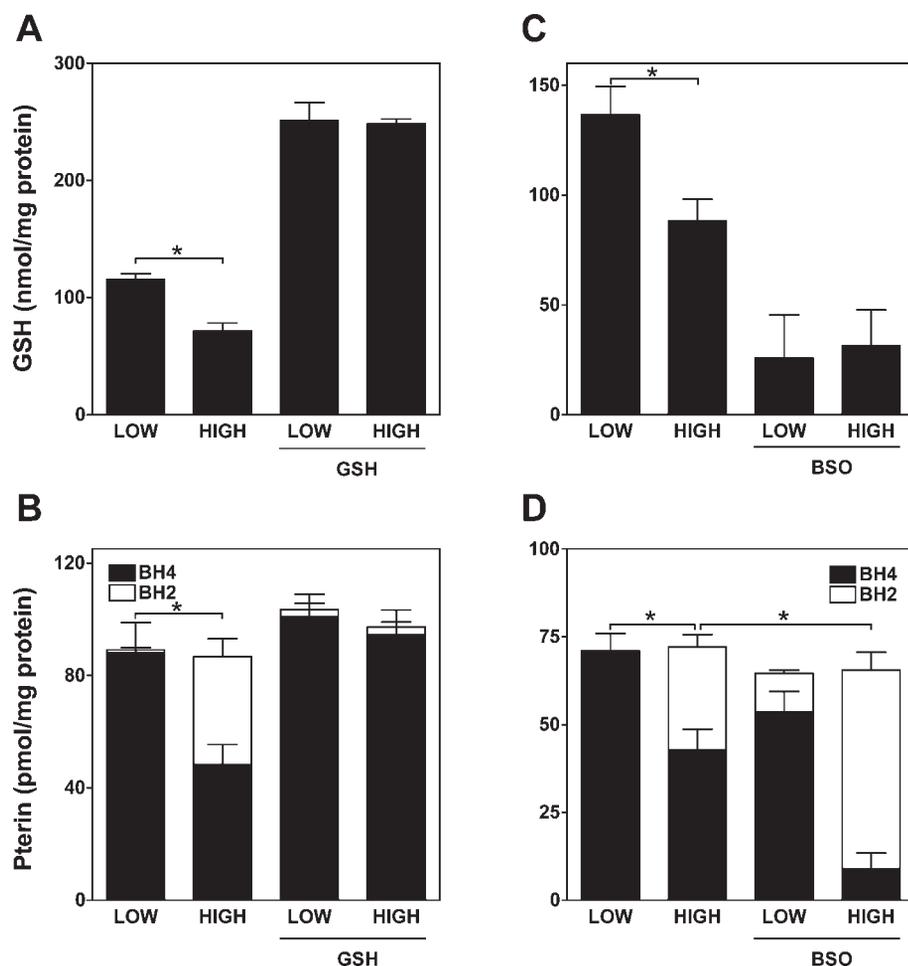


Fig. 4. Intracellular glutathione (GSH) protects against high-glucose-elicited BH₄ oxidation. sEnd.1 cells were cultured with medium containing either low (5 mM) or high (30 mM) levels of glucose. Cells were then harvested after 48 h, and both GSH and pterin content were measured. **A** and **C**: impact of high glucose on GSH levels and effects of GSH repletion (by cotreatment with 1 mM GSH ethyl ester) and depletion [by block of GSH synthesis with 100 μ M buthionine sulfoximine (BSO)]. Results show that high glucose exposure significantly diminishes GSH content in endothelial cells (ECs) on its own ($*P < 0.05$), and this is prevented by GSH supplementation and potentiated by inhibition of GSH synthesis. **B** and **D**: impact of GSH repletion and depletion on high glucose-elicited BH₄ oxidation. Results show that BH₄ oxidation is prevented when GSH levels in ECs are enhanced and accentuated ($*P < 0.01$) when GSH is depleted. All indicated values are means \pm SE ($n = 5$).

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 \approx 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 mitochondrion-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 mitochondrion-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 mitochondrion-derived superoxide generation (35, 45). To test whether mitochondrion-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 in ECs. 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 μ M rotenone and 5 μ M 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 \pm 11.7 mg/dl vs. 144.8 \pm 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 \pm 35.9 mg/dl (vs. 162.8 \pm 8.2 mg/dl in ZL) that

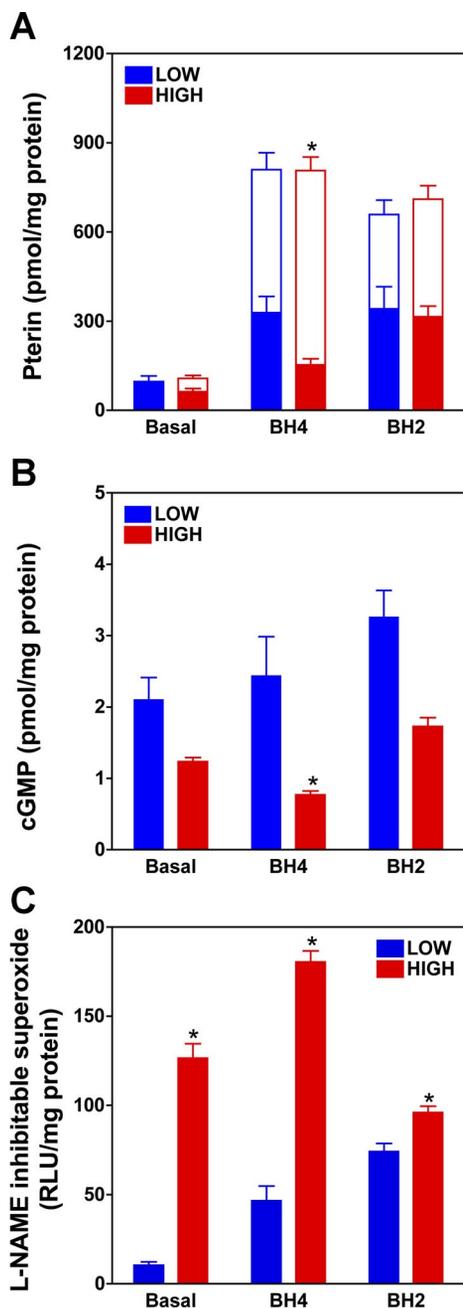


Fig. 5. Pterin supplementation (24 h) increases intracellular BH₄ levels in low- and high-glucose-treated ECs; however, this is associated with BH₂ accumulation and failure to improve or worsened eNOS coupling. sEnd.1 cells were grown in high (30 mM) or low (5 mM) glucose-containing media, and after 24 h cells were either supplemented with BH₄ (10 μ M) or BH₂ (10 μ M) or left unsupplemented (basal). After a further 24 h, assays were performed to quantify intracellular bioterins (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 luciferin chemiluminescence in the absence and presence of 3 mM L-NAME. Values are means \pm SE ($n = 5$). * $P < 0.05$.

reach 400.2 ± 33.6 mg/dl by 22 wk (vs. 180.9 ± 17.8 in ZL). These increases in plasma glucose are mirrored by a progressive oxidation of BH₄ without any detected change in total pterin content. This is shown for lung tissue in Fig. 7B; similar

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 biopterin binding to eNOS.

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

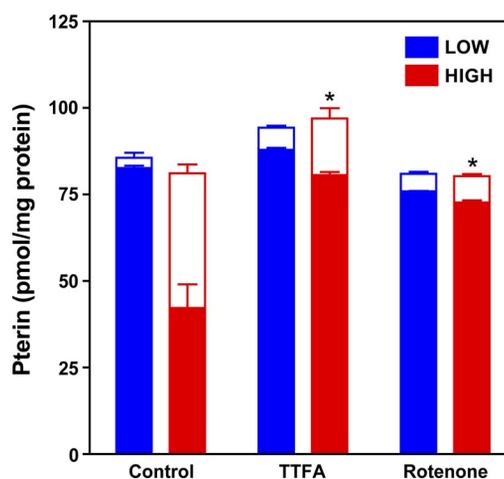


Fig. 6. Prevention of BH₄ oxidation by mitochondrial electron transport chain inhibitors. Cells were grown in low (5 mM; black bars) or high (30 mM; red bars) glucose-containing media for 48 h. Rotenone (2 μ M) and thenoyltrifluoroacetone (TTFA, 5 μ M), inhibitors of complexes I and II of the mitochondrial electron transport chain, respectively, were added after the initial 24 h, and cells were harvested for assay of BH₄ and BH₂ by HPLC-EC. BH₄ (filled bars) oxidation to BH₂ (open bars) was significantly attenuated by both TTFA and rotenone treatment (* $P < 0.01$). Values are means \pm SE ($n = 3$).

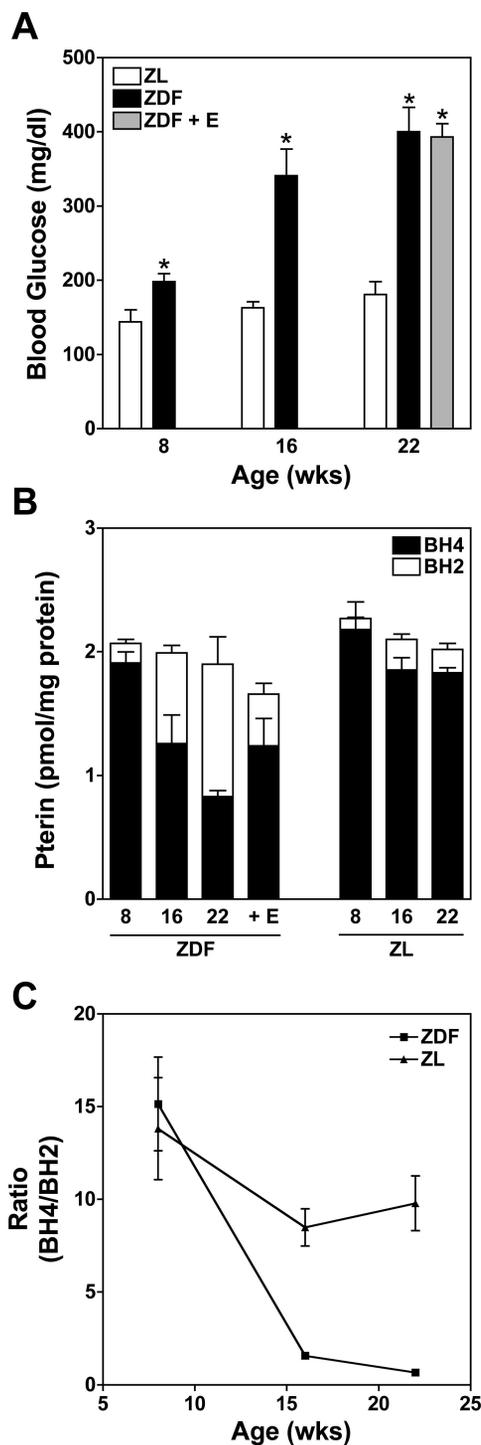


Fig. 7. Oxidation of BH₄ in association with increasing plasma glucose levels in the Zucker diabetic fatty (ZDF) rat model of type 2 diabetes and metabolic syndrome. Comparison between age-dependent changes in plasma glucose and pterin redox status in ZDF, ZDF + ebselen treatment (E; 5 mg/kg by gavage twice daily) and Zucker nondiabetic lean (ZL) control rats. **A:** progressive increase in plasma glucose levels as ZDF rats age, while no change is observed in age-matched ZL rats. Increased plasma glucose in ZDF rats is unaffected by ebselen, a peroxynitrite scavenger and antioxidant (**P* < 0.05). **B:** progressive oxidation of BH₄ in lungs of aging ZDF rats but not ZL control rats. Ebselen affords significant protection against BH₄ oxidation in ZDF rats. **C:** relationship between age and ratio of BH₄ to BH₂ in ZDF compared with ZL control rats. At 22 wk the BH₄-to-BH₂ ratio is significantly decreased in ZDF compared with ZL rats (*n* = 6). Values are means ± SE (*n* = 6).

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 biopterin 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 bipterin 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).

Peroxyntirite 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 ($3.9 \times 10^5 \text{ mol} \cdot \text{l}^{-1} \cdot \text{s}^{-1}$) (53) than its near-diffusion limited reaction with NO ($6.7 \times 10^9 \text{ mol} \cdot \text{l}^{-1} \cdot \text{s}^{-1}$) (22). Accordingly, NO would predictably outcompete BH₄ for reaction with superoxide. Peroxyntirite formed by the NO/superoxide reaction could then oxidize BH₄ as previously described (29, 34) and thereby promote eNOS uncoupling. Notably, the reaction of peroxyntirite with BH₄ occurs via the intermediacy of the BH₃ radical cation and with a first-step rate constant that is several times faster than the reaction with thiols ($6 \times 10^3 \text{ mol} \cdot \text{l}^{-1} \cdot \text{s}^{-1}$) (26). Inasmuch as intracellular thiol levels (millimolar) far exceed the estimated levels of BH₄ in ECs (0.05–0.2 μM), thiol oxidation is expected to predominate over BH₄ oxidation. This competition between thiols and BH₄ for peroxyntirite-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 autooxidation, 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 BH₄ 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 BH₂-supplemented medium compared with BH₄-supplemented medium. Notably, an extracellular autooxidation 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 peroxyntirite 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 peroxyntirite, 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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