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Improvement of endothelial nitric oxide synthase activity retards the progression of diabetic nephropathy in db/db mice

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Impaired endothelial nitric oxide synthase (eNOS) activity may be involved in the pathogenesis of diabetic nephropathy. To test this, we used the type 2 diabetic *db/db* mouse (BKS background) model and found impaired eNOS dimerization and phosphorylation along with moderate glomerular mesangial expansion and increased glomerular basement membrane (GBM) thickness at 34 weeks of age. Cultured murine glomerular endothelial cells exposed to high glucose had similar alterations in eNOS dimerization and phosphorylation. Treatment with sepiapterin, a stable precursor of the eNOS cofactor tetrahydrobiopterin, or the nitric oxide precursor L-arginine corrected changes in eNOS dimerization and phosphorylation, corrected permeability defects, and reduced apoptosis. Sepiapterin or L-arginine, administered to *db/db* mice from weeks 26 to 34, did not significantly alter hyperfiltration or affect mesangial expansion, but reduced albuminuria and GBM thickness, and decreased urinary isoprostane and nitrotyrosine excretion (markers of oxidative stress). Although there was no change in glomerular eNOS monomer expression, both sepiapterin and L-arginine partially reversed the defect in eNOS dimerization and phosphorylation. Hence, our results support an important role for eNOS dysfunction in diabetes and suggest that sepiapterin supplementation might have therapeutic potential in diabetic nephropathy.

Kidney International (2012) **82,** 1176–1183; doi:10.1038/ki.2012.248; published online 11 July 2012

KEYWORDS: *db/db* mice; diabetic nephropathy; eNOS; glomerular endothelial cells; sepiapterin

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Received 7 February 2012; revised 20 April 2012; accepted 22 May 2012; published online 11 July 2012

Multiple mechanisms contribute to the development of diabetic nephropathy, and there is increasing evidence that all three types of glomerular cells are potential targets for injury.[1] Specifically, there is increasing evidence that dysfunction of the fenestrated glomerular endothelial cells (GEnCs) is an important contributory factor to progressive diabetic glomerular injury.[2–5] Nitric oxide (NO) is an important endothelium-derived mediator, with multiple actions, including vasodilation and antiplatelet, antiproliferative, antiadhesive, permeability-decreasing, and anti-inflammatory properties.[3] Previous studies have examined whether there are alterations in NO production and/or bioavailability in diabetes but have provided conflicting results. *In vitro* studies have shown that high glucose (HG) medium could either induce[4,5] or inhibit[6] endothelial nitric oxide synthase (eNOS) expression in cultured endothelial cells. In animal model of diabetes mellitus, eNOS gene expression has been described to be either unchanged,[7] diminished,[8,9] or increased.[10,11] Hohenstein *et al.*[12] recently reported upregulated eNOS expression in glomeruli from type 2 diabetic patients with nephropathy. In contrast, we and others have found that eNOS insufficiency will accelerate nephropathy in mouse models of both type 1 and type 2 diabetes.[13–19] Therefore, the underlying mechanisms and potential roles of impaired eNOS in glomerular endothelium during the development of diabetic nephropathy still remain incompletely understood.[14]

RESULTS

HG induced GEnC impairment and eNOS dysfunction

Pilot studies indicated that incubation of cultured mouse GEnCs with HG for 48 h demonstrated significant alterations. At that time, GEnCs in HG increased bovine serum albumin (BSA) clearance compared with normal glucose (NG) medium or mannitol ($9.9 \pm 0.6\%$ in NG and $11.0 \pm 1.0\%$ in mannitol vs. $40.8 \pm 1.7\%$ in HG after 40 min, $n = 5$, $P < 0.05$) (Figure 1a) without significantly altering proliferation (data not shown). In addition, HG induced profound apoptosis in GEnCs ($1 \pm 0.2\%$ in NG vs. $7.9 \pm 0.4\%$ in HG, $n = 4$, $P < 0.05$)

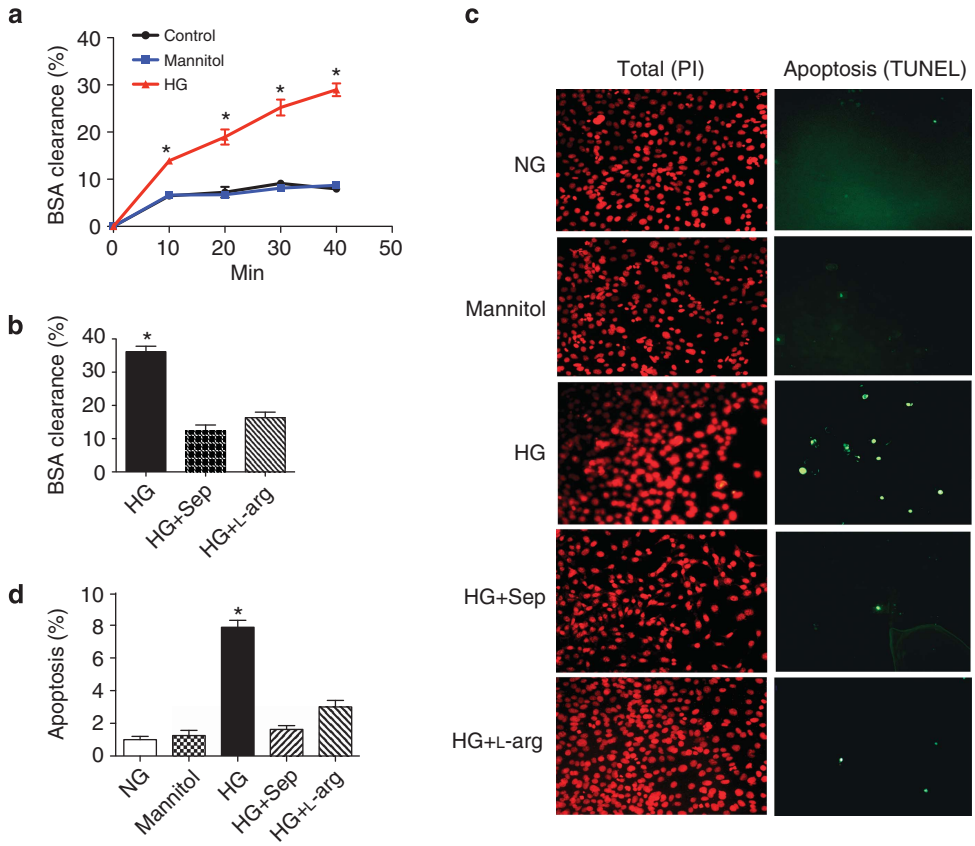

Figure 1 | High glucose increased bovine serum albumin (BSA) permeability and apoptosis in glomerular endothelial cells (GEnCs); these effects were inhibited by sepiapterin or L-arginine. (**a**) High glucose (HG), but not mannitol, increased permeability in GEnCs, indicated by BSA clearance at 5–40 min. *$P<0.05$ HG versus normal medium (NG) or osmolality control (mannitol at same concentration). (**b**) Sepiapterin (Sep) and L-arginine (L-arg) attenuated HG increases in permeability, *$P<0.05$ HG versus normal medium or HG with Sep or L-arg coincubation. (**c**) Effect of HG on apoptosis in GEnCs. Representative images of terminal transferase dUTP nick-end labeling (TUNEL) assay from four independent experiments. (**d**) Sep and L-arg prevented HG-induced apoptosis. *$P<0.05$ HG versus normal medium or HG with Sep or L-arg coincubation.

(Figure 1b and c). Incubation in the high-glucose medium decreased eNOS dimerization (Figure 2a) and phosphorylation at Ser1179 (Figure 2b) without changing eNOS monomer expression or phosphorylation at Thr479, whereas incubation with mannitol as an osmolar control did not have the same effects (Supplementary Figure S1a and b online). HG also decreased NOS activity, indicated by decreased levels of the stable end products of NO (nitrate/nitrite) (to 0.53 ± 0.07 fold of NG. $n=4$, $P<0.05$ compared with NG or mannitol control) (Figure 2c and Supplementary Figure S1c online).

Effects of sepiapterin and L-arginine on HG-induced GEnC injury

We treated the GEnCs exposed to high-glucose medium with the NO precursor, L-arginine, or the tetrahydrobiopterin (BH_4) cofactor, sepiapterin. Either L-arginine or sepiapterin partially corrected the HG–induced defects in eNOS dimerization and phosphorylation (Figure 2a and b), as well as eNOS activity (to 0.86 ± 0.05- and 0.83 ± 0.08-fold control by L-arginine or sepiapterin, respectively, $n=4$, $P<0.05$ compared with untreated) (Figure 2c). In addition, both L-arginine and sepiapterin significantly blunted GenC alterations in permeability and apoptosis induced by incubation in the high-glucose medium (Figure 1b–d).

eNOS dysfunction in db/db mice

db/db mice are a well-established type 2 diabetic model, but some strains are relatively resistant to diabetic renal injury despite the significant hyperglycemia.[15,16] *db/db* mice on the BKS background have been shown to develop hyperglycemia and diabetic renal injury.[17,18] We found that at 34 weeks, *db/db* (BKS) mice had developed glomerulopathy characterized by mesangial matrix accumulation (Figure 3a), foot process effacement, and increased glomerular basement membrane (GBM) thickness (154 ± 6 in BKS control vs. 335 ± 23 nm in untreated *db/db* mice) (Figure 3a and b) and had significant albuminuria (alb/cre: 18 ± 3 in BKS control vs. 759 ± 281 in untreated *db/db*, $n=8$, $P<0.05$) (Figure 3c) and hyperfiltration (glomerular filtration rate: 206 ± 33 µl/min in BKS control; 403 ± 36 in untreated *db/db*, $n=6$–12, $P<0.05$) (Figure 3d). Furthermore, urine F2-isoprostanes, a marker of oxidative stress, increased in the diabetic mice (5.7 ± 0.9 in *db/db* mice vs. 1.4 ± 0.1 ng 8-iso-PGF2α/mg Cr in BKS

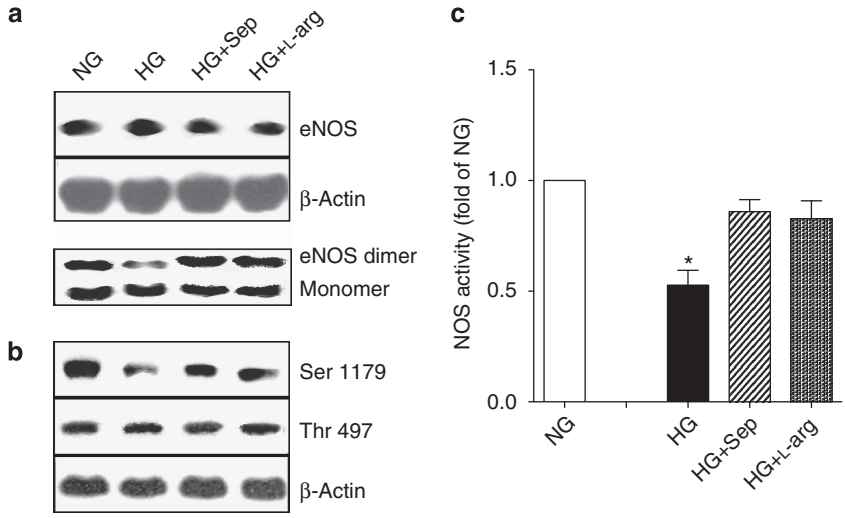

Figure 2 | Sepapterin (Sep) and L-arginine (L-arg) reversed high glucose–induced endothelial nitric oxide synthase (eNOS) impairment in glomerular endothelial cells (GEnCs). (**a**) High glucose did not alter eNOS expression, but decreased eNOS dimerization in GEnCs, which could be prevented by Sep or L-arg. Representative photo from three independent experiments. (**b**) High glucose decreased eNOS phosphorylation at Ser1179, without significant change at Thr479. Sep or L-arg coincubation reversed phosphorylation at Ser1179. Representative photo from three independent experiments. (**c**) High glucose reduced nitrate/nitrite production by GEnCs, which was corrected by supplementation with Sep or L-arg. *$P<0.05$ compared with normal medium or high glucose with Sep or L-arg. HG, high glucose; NG, normal glucose.

control, $n=6$–11, $P<0.05$) (Figure 3e). Increased oxidative stress in *db/db* mice was also confirmed by the accumulation of nitrotyrosine, another superoxide marker (Figure 3f).

To investigate the potential role of eNOS in the diabetic renal injury, we determined glomerular eNOS expression. There were no differences in eNOS monomer expression between control and diabetic mice (Figure 4a), which was further confirmed by immunohistochemical staining (data not shown). However, glomerular eNOS dimerization (Figure 4b) and phosphorylation at Ser1179 (Figure 4c) decreased by ~50% in *db/db* mice, whereas phosphorylation at Thr497 was unchanged (Figure 4c). HG–mediated alterations in endothelial function have been reported to be associated with reduced intracellular BH_4.[19,20] In agreement with these findings, we found that glomerular BH_4 in *db/db* mice (0.7 ± 0.1 ng/mg pro.) was significantly lower than BKS control (2.3 ± 0.2 ng/mg pro., $n=6$, $P<0.05$).

Sepiaterin or L-arginine partially restored eNOS function and attenuated renal injury in db/db mice

To examine the impact of L-arginine or sepiapterin *in vivo*, we administered these agents to 26-week-old *db/db* mice for 8 weeks. Sepiapterin treatment significantly restored glomerular BH_4 levels (to 1.9 ± 0.3 ng/mg pro., $n=6$, $P<0.05$ compared with untreated *db/db*). Consistent with the *in vitro* results, both L-arginine and sepiapterin improved glomerular eNOS function in *db/db* mice, indicated by restoration of eNOS dimerization (Figure 4b) and phosphorylation at Ser1179 (Figure 4c). In addition, oxidative stress, indicated by nitrotyrosine accumulation and urinary F2-isoprostane levels, was significantly decreased (3.1 ± 0.2 ng 8-iso-PGF2α/mg Cr for L-arginine and 2.7 ± 0.2 for sepiapterin, $n=6$–11, $P<0.05$ compared with untreated) (Figure 3e and f).

Neither L-arginine nor sepiapterin treatment markedly affected the increased mesangial expansion, but both of them reduced GBM thickness and foot-process effacement in *db/db* mice (Figure 3a and b). They also significantly reduced albuminuria (sepiapterin: 40 ± 7 and L-arginine: 163 ± 37 μg alb/mg Cr, respectively, $n=6$–8, $P<0.05$ vs. untreated *db/db*) (Figure 3c) without affecting glomerular filtration rate (398 ± 16 in sepiapterin and 425 ± 50 μl/min in L-arginine, respectively, $n=6$, $P<0.05$ compared with BKS control; not significantly different vs. untreated *db/db*) (Figure 3d).

DISCUSSION

Our studies showed that HG induced dysfunction of GEnCs *in vitro*, as indicated by the increased permeability to BSA and apoptosis, as well as decreased eNOS dimerization, impaired eNOS phosphorylation at Ser 1179, and decreased NO production. All of these abnormalities were ameliorated by coincubation with either the NO precursor, L-arginine, or with sepiapterin, a stable precursor of the eNOS cofactor, tetrahydrobiopterin. These *in vitro* findings were confirmed in *db/db* (BKS) mice, in which supplementation with either sepiapterin or L-arginine attenuated albuminuria and GBM thickness and decreased oxidative stress.

NO has an important role in controlling vascular tone and modulating platelet activation. Decreased NO bioactivity contributes to arterial thrombosis and endothelial dysfunction.[21] Chronic kidney diseases, including diabetic nephropathy, are often characterized by relative NO deficiency,

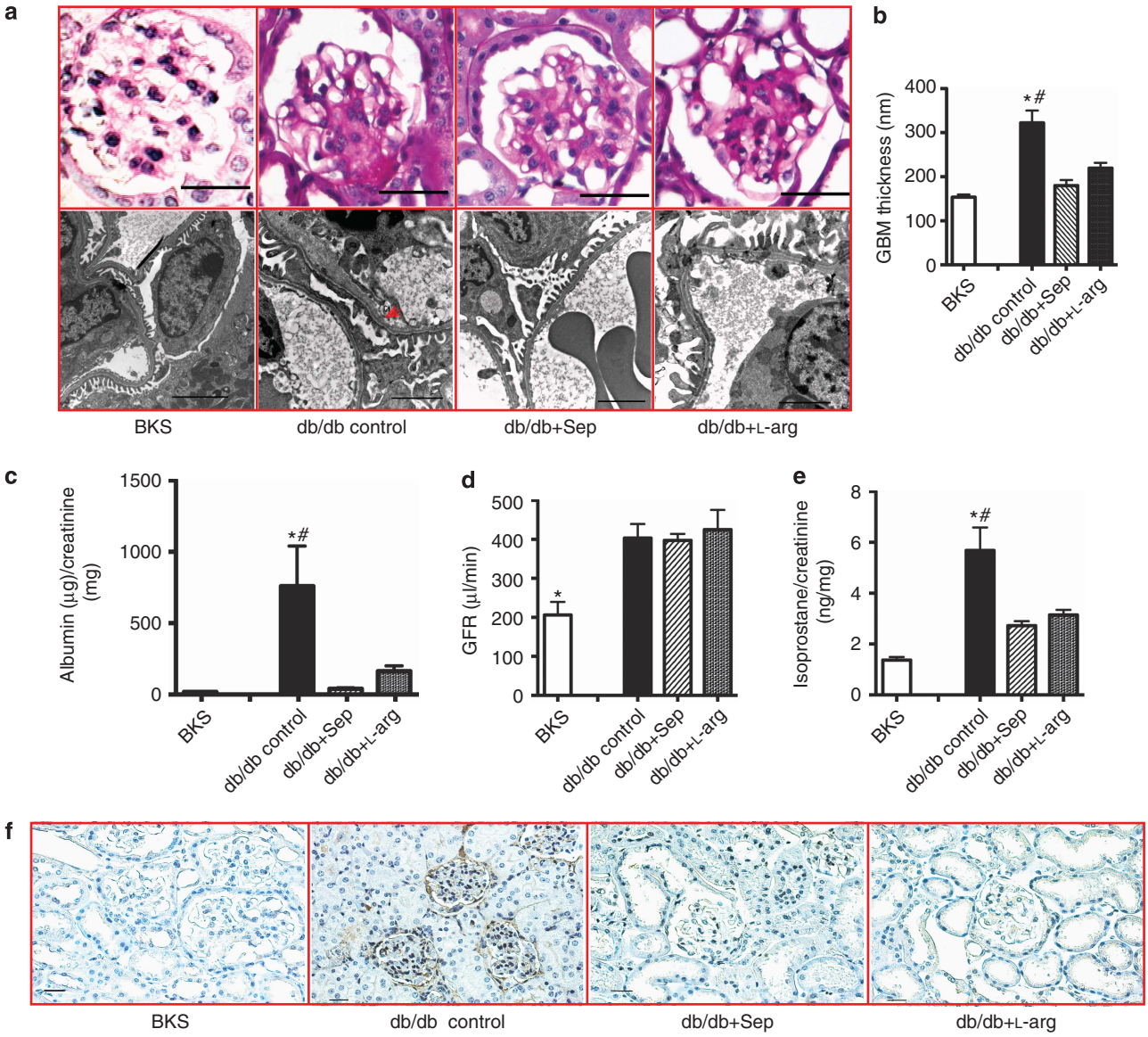

Figure 3 | Sepiapterin (Sep) and L-arginine (L-arg) attenuated renal injury in *db/db* mice. (**a**) The upper panel indicates glomerular mesangial expansion in *db/db* mice at 34 weeks by Periodic Acid Schiff staining, Bars: 20 μm; the lower panel indicates glomerular basement membrane (GBM) thickness and foot process effacement (red arrow) in *db/db* mice with or without treatment with Sep/L-arg, bars: 2 μm. (**b**) GBM thickness. $^{*}P<0.05$, compared with wild-type BKS; $^{\#}P<0.05$, versus each treatment. (**c**) Sep or L-arg decreased albuminuria in *db/db* mice at 34 weeks of age. $^{*}P<0.05$, compared with wild-type BKS; $^{\#}P<0.05$, versus each treatment. (**d**) Sep or L-arg had no effect on hyperfiltration seen in *db/db* mice, determined by fluorescein isothiocyanate-inulin clearance. $^{*}P<0.05$, BKS versus *db/db* mice regardless of treatments. (**e**) Sep or L-arg decreased elevations in urine isoprostane excretion in *db/db* mice. $^{*}P<0.05$, compared with wild-type BKS; $^{\#}P<0.05$, versus each treatment. (**f**) Increased nitrotyrosine accumulation in *db/db* mice was attenuated by Sep or L-arg treatment. Representative photo from three independent experiments. GFR, glomerular filtration rate.

which may contribute to disease progression.[22] NO is synthesized from L-arginine by its synthases. eNOS is the major source of NO production in vascular endothelial cells, including the endothelium of preglomerular vessels and glomeruli.[23]

Accelerated glomerular injury in diabetic mice with eNOS deficiency strongly suggests that deficient eNOS activity may have an important role in the pathogenesis of diabetic nephropathy,[13,24,25] although there have been conflicting results about eNOS regulation in the diabetic kidney, which may relate to whether studies were conducted in early or late injury.[7–12,26] Previous studies in isolated blood vessels[27,28] and cultured umbilical vein cells[29,30] have been used as *in vitro* systems to examine the role of eNOS on endothelial function. Notably, GEnCs represent a microvascular endothelial cell with unique characteristics[31,32] and have a critical role in the pathogenesis of diabetic nephropathy. In the current study, we focused on the impact of hyperglycemia on eNOS impairment in cultured mouse GEnCs. In cultured GEnCs, we found that HG increased both permeability to

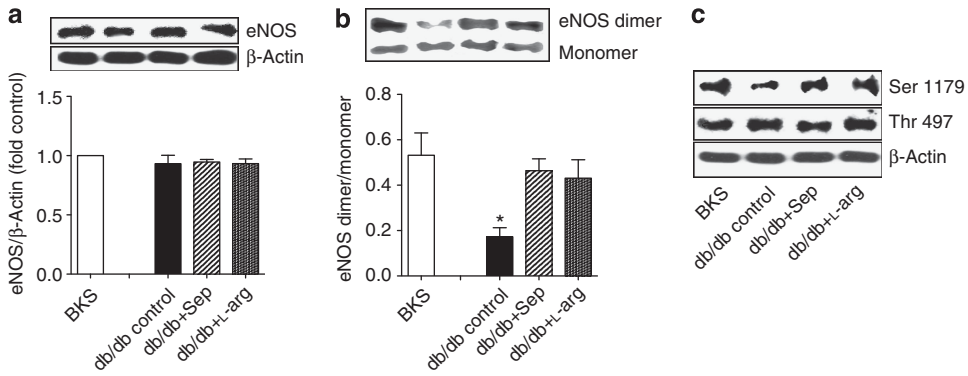

Figure 4 | Sepiapterin (Sep) and L-arginine (L-arg) reversed endothelial nitric oxide synthase (eNOS) impairment in *db/db* mice. (**a**) No significant differences in glomerular eNOS expression were detected between BKS and *db/db* mice with or without treatment. ($n = 4$; NS). (**b**) There was decreased eNOS dimerization in glomeruli from *db/db* mice, which was corrected by administration of Sep or L-arg ($n = 4$, $^*P < 0.05$, compared with wild-type or treated groups). (**c**) There was decreased eNOS phosphorylation at Ser1179, but not at Thr479, in glomeruli from *db/db* mice and normalization by Sep/L-arg treatment. Representative photo was from three independent experiments.

BSA and apoptosis, in association with endothelial eNOS dysfunction.

There is evidence that activation of eNOS requires formation of a homodimer through a linkage between the N-terminal oxygenase domains.[33] Although we did not find any significant alteration in eNOS monomer expression in glomeruli from *db/db* mice at 34 weeks or in GEnCs in response to HG, there was decreased eNOS dimerization. We also found that in both diabetic glomeruli and high-glucose GEnCs, eNOS phosphorylation was impaired at Ser1179 without significant alteration at Thr497. A crucial role for posttranslational control of eNOS bioactivity has been suggested, owing to its long half-life at baseline (10–35 h).[34,35] Akt-dependent phosphorylation of eNOS at Ser1179 is essential for endothelium-dependent relaxation,[36] whereas phosphorylation at Thr497 is considered inhibitory.[37] Reduced phosphorylation of eNOS at Ser1179 has previously been reported in moderately hyperglycemic diabetic rats.[38]

In addition to decreased eNOS expression and decreased eNOS activity due to uncoupling by reactive oxygen species,[22] alterations in the availability of substrate and/or essential cofactors may lead to decreased NO production. When either the essential substrate, L-arginine, or the essential cofactor, BH_4, is limited, electron transfer from eNOS flavins becomes uncoupled from L-arginine oxidation, and superoxide is produced from the oxygenase domain,[39–41] which may further reduce NO bioactivity and increase oxidative stress within endothelial cells by scavenging NO and forming peroxynitrite.[42] Reduced levels of both L-arginine and BH_4 have been observed in diabetic rats,[20,43] with more profound depletion in endothelial cells than in plasma.[20]

Modulation of the arginine–NO pathway by supplementation with arginine may have protective effects on diabetic rats[44] and patients.[20] In a previous study, administration of BH_4 to db/db mice did not affect glucose, triglyceride, and cholesterol levels or body weight, but enhanced the reactivity of small mesenteric arteries to phenylephrine.[45] Acute intra-arterial infusion of BH_4 leads to short-term improvements of forearm blood flow responses to endothelium-dependent vasodilators in patients with type 2 diabetes.[46] Sepiapterin, the immediate precursor of BH_4 via the biopterin salvage pathway, is less sensitive to oxidative stress than BH_4.[47] It is noteworthy that administration of either L-arginine or sepiapterin not only corrected the defects in eNOS dimerization and phosphorylation but also decreased oxidative stress and partially corrected functional and structural glomerular abnormalities. Previous studies have also reported beneficial effects of L-arginine on renal dilatation in animal models[48] and humans.[49] The current *in vitro* study provided evidence for a direct role of both sepiapterin and arginine on GEnCs, independent of vasodilation.

In summary, we found that providing either a precursor for NO production or a stable precursor of tetrahydrobiopterin significantly improved the function of cultured GEnCs and decreased progression of glomerular injury in a model of type 2 diabetes. Recently, a more stable preparation of oral BH_4, sapropterin dihydrochloride (Kuvan, BioMarin, Tiburon, CA), has been developed and approved by the Food and Drug Administration for treatment of patients with phenylketonuria.[50] Our results provide evidence that BH_4 supplementation may have therapeutic potential in diabetic nephropathy.

MATERIALS AND METHODS

Materials

Sepiapterin, eNOS polyclonal antiserum, and Nitrate/Nitrite Colorimetric Assay Kits were from Cayman Chemical Co (Ann Arbor, MI). Rabbit anti-phospho-serine-1179 eNOS (bovine) and anti-phospho-Thr-497 polyclonal antibody were from Cell Signaling Technology (Danvers, MA); anti-nitrotyrosine antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The TUNEL (terminal transferase dUTP nick-end labeling) apoptosis detection kit was from Upstate (Lake Placid NY); other reagents were purchased from Sigma Chemical (St Louis, MO).

Experimental animals

We used *db/db* mice on the nephropathy-susceptible BKS background[51,52] as a model of type 2 diabetes. Age-matched (26 weeks old)

male mice were divided into the following groups: (1) Wild type (BKS); (2) *db/db* untreated (*db/db* control); (3) *db/db* mice treated with sepiapterin, in which the mice were administered fresh sepiapterin solution (10 mg/kg/day by gavage) for 8 weeks (from 26 to 34 weeks); (4) *db/db* mice with L-arginine, in which L-arginine (100 mg/kg/day in the drinking water) was administered for the same period. Mice were killed at the end of 34 weeks. All animal procedures were approved by the Animal Care and Use Committee of Vanderbilt University Medical Center.

Assessment of glomerular injury

Renal injury was assessed histologically with light microscopy by hematoxylin and eosin, Periodic Acid Schiff, and Jones staining and by electron microscopy. GBM thickness was randomly measured as the distance between the endothelial cell and epithelial cell plasma membranes $\times 20$ for each sample and evaluated by the renal pathologist. Urinary albumin levels were determined by enzyme-linked immunosorbent assay (ELISA) using a murine microalbuminuria ELISA kit, AlbuwellM (Exocell, Philadelphia, PA). The urine creatinine concentration was measured with a microplate assay kit, Creatinine Companion (Exocell). All measurements were recorded in duplicate, and albuminuria was determined as the ratio of urinary albumin (μg/ml) to creatinine (mg/ml). Renal function was evaluated by glomerular filtration rate as described previously.[13]

F2-isoprostane assay

F2-isoprostanes were measured in urine using stable isotope dilution methods and gas chromatography negative ion chemical ionization mass spectrometry,[53,54] and expressed as ng/mg urine creatinine.

Isolation of glomeruli

Glomeruli were isolated immediately after killing, using the Dynabeads method modified from Takemoto *et al.*[55] Briefly, mice were anesthetized by an injection of nembutal (0.05 mg/g BW) and perfused through the descending aorta with 8×10^7 Dynabeads diluted in phosphate-buffered saline. The kidneys were removed, minced into 1-mm^3 pieces, and digested in collagenase (1 mg/ml collagenase A, 100 U/ml deoxyribonuclease I in Hank's Balanced Salt Solution (HBSS)) at 37 °C for 30 min with gentle agitation. The collagenase-digested tissue was gently pressed through a 100-μm cell strainer using a flattened pestle, and the cell strainer was then washed with 5 ml of cold HBSS. The filtered cells were passed through a new cell strainer without pressing and the cell strainer washed with 5 ml of cold HBSS. The cell suspension was then centrifuged at 200 *g* for 5 min at 4 °C. The supernatant was discarded and the cell pellet was resuspended in 2 ml of cold HBSS. Finally, glomeruli containing Dynabeads were gathered by a magnetic particle concentrator and washed three times with cold HBSS. During the procedure, kidney tissues were kept at 4 °C, except for the collagenase digestion, which was kept at 37 °C. The preparation consisted of >90% glomeruli.

Cultured mouse GEnCs

We used early-passage GEnCs from Michael Madaio,[56] grown in DMEM/F12 medium with 10% FBS, at 37 °C in humidified air with 5% CO_2. After quiescence for 16–24 h, subsets of cells were exposed to high-glucose (30 mM) medium or osmolality control medium (30 mM mannitol) for 48 h before experimentation. Subsets of cells grown in high-glucose medium were coincubated with L-arginine (3 mM) or sepiapterin (100 μM).[57]

Immunoblotting

Cultured cells or isolated glomeruli were homogenized as described previously.[58] Proteins were resuspended in SDS sample buffer, diluted in SDS buffer containing 2-mercaptoethanol (Sigma Chemical), and boiled for 10 min before loading. The samples were run on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels under reducing conditions and transferred onto polyvinylidene fluoride membrane (Immobilion-P; Millipore, Bedford, MA). After blocking with 5% nonfat milk in Tris-buffered saline, the membranes were exposed to the primary antibody overnight at 4 °C, followed by horseradish peroxidase–conjugated secondary antibodies. The horseradish peroxidase signal was enhanced using the enhanced chemiluminescence method, and the images were developed on high-performance autoradiography film, Hyperfilm MP (Amersham Biosciences, Buckinghamshire, UK). Membranes were rehybridized with goat anti-β-actin antibody (Santa Cruz Biotechnology) to normalize protein loading. For detection of eNOS dimerization, we used nondenaturing, low-temperature sodium dodecyl sulfate polyacrylamide gel electrophoresis to run gels with 50 μg protein loading in each well without boiling, and calculated eNOS dimer/monomer ratios.

Apoptosis detection

A TUNEL apoptosis detection kit (Upstate) was used for measurements. Propidium iodide was used for counterstaining. The percentage of apoptotic cells in 400 total cells from the same field was determined for quantification.

Glomerular BH_4 (tetrahydrobiopterin)

Glomerular BH_4 was evaluated with the Tetrahydrobiopterin ELISA Kit (MyBioSource, LLC, San Diego, CA) using the company's instructions. BH_4 was normalized to protein content, expressing as ng/mg pro.

Endothelial cell permeability

Permeability was detected by clearance of albumin across endothelial monolayers as described previously.[59,60] Briefly, monolayers were grown to confluence on the inserts of Transwell (3.0 μm; Becton Dickinson Labware, Franklin Lake, NJ, 8×10^5 cells/insert). After washing, the monolayers were covered immediately with HBSS containing a trypan blue–albumin complex (trypan blue 0.035% and BSA 0.8%) and incubated at 37 °C with continuous agitation. Samples were collected every 5 min from the bottom well up to 40 min and were replaced by an equal volume of HBSS. Absorbance at 590 nm was measured from both the insert and the lower well fluids. Results were expressed as clearance of BSA, i.e., the amount of TB-BSA detected in the lower chamber, expressed as a percentage of the total TB-BSA initially added to the upper chamber.

NOS activity from cultured GEnCs

Activity was measured with a Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical) from stable end products of the reaction of NO with molecular oxygen, according to the manufacturer's instructions, normalized by the protein content and expressed as fold control (cells gown in normal medium).

Statistical analyses

All values are presented as mean ± s.e.m. ANOVA and Bonferroni *t*-tests were used for statistical analysis, and differences were considered significant when $P < 0.05$.

DISCLOSURE

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by funds from the Department of Veterans Affairs, the Vanderbilt George O'Brien Kidney and Urologic Diseases Center (DK DK79341), DK51265, DK62794, and Vanderbilt Diabetes Research and Training Center (DK20593).

SUPPLEMENTARY MATERIAL

Figure S1. High glucose induced eNOS dysfunction independent of its osmolality.

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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