Cardiovascular, Pulmonary and Renal Pathology

Deficiency of Endothelial Nitric-Oxide Synthase Confers Susceptibility to Diabetic Nephropathy in Nephropathy-Resistant Inbred Mice

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Recent studies have implicated dysfunctional endothelial nitric-oxide synthase (eNOS) as a common pathogenic pathway in diabetic vascular complications. However, functional consequences are still incompletely understood. To determine the role of eNOS-derived nitric oxide (NO) in diabetic nephropathy, we induced diabetes in eNOS knockout (KO) and wild-type (WT) mice on the C57BL/6 background, using low-dose streptozotocin injection, and we investigated their glomerular phenotype at up to 20 weeks of diabetes. Although the severity of hyperglycemia in diabetic eNOS KO mice was similar to diabetic WT mice, diabetic eNOS KO mice developed overt albuminuria, hypertension, and glomerular mesangiolysis, whereas diabetic WT and nondiabetic control mice did not. Glomerular hyperfiltration was also significantly reduced in diabetic eNOS KO mice. Electron micrographs from diabetic eNOS KO mice revealed an injured endothelial morphology, thickened glomerular basement membrane, and focal foot process effacement. Furthermore, the anionic sites at glomerular endothelial barrier estimated by cationic ferritin binding were reduced in diabetic eNOS KO mice. In aggregate, these results demonstrate that deficiency of eNOS-derived NO causes glomerular endothelial injury in the setting of diabetes and results in overt albuminuria and glomerular mesangiolysis in nephropathy-resistant inbred mice. The findings indicate a vital role for eNOS-derived NO in the pathogenesis of diabetic nephropathy. (*Am J Pathol* 2007, 170:1473-1484; DOI: 10.2353/ajpath.2007.060481)

Nitric oxide (NO) is a free radical that mediates diverse functions in a range of biological systems.¹ NO is produced by a family of nitric-oxide synthase (NOS) enzymes to catalyze the stoichiometric five-electron oxidation of the terminal guanidino group of L-arginine to produce NO and L-citrulline. Three NOS isoforms have been distinguished in mammalian species: neuronal NOS, inducible NOS, which is expressed in a variety of activated tissues, and endothelial NOS (eNOS).¹ In the vasculature, NO is mostly generated by eNOS, and the generated NO plays a crucial role in maintaining vascular homeostasis, including vascular tone, leukocyte adhesion to endothelium, proliferation of vascular smooth muscle cells, and platelet aggregation and adhesion to the vessel wall.^{1,2}

Diabetic micro- and macroangiopathy, including nephropathy and accelerated atherosclerosis, are the main causes of morbidity and mortality in patients with diabetes mellitus. A hallmark of diabetic vascular complications is endothelial cell dysfunction, which is characterized by loss of NO, including impaired endothelium-dependent vasorelaxation, enhanced leukocyte-endothelial interactions, and thrombosis.³ A large body of evidence has implicated dysfunctional eNOS and decreased NO availability in endothelium as a major pathogenic mechanism in diabetic vascular complications in humans and diabetic animals.³ Diabetic stimuli, including hyperglycemia and advanced glycation end products, induce superoxide overpro-

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duction, and chemical interactions of superoxide with NO reduce NO bioavailability and promote formation of peroxynitrite, the superoxide/NO reaction product. Peroxynitrite in turn oxidizes tetrahydrobiopterin, an essential cofactor for the eNOS enzyme. Loss of tetrahydrobiopterin availability causes eNOS uncoupling, which diverts electrons flowing from eNOS reductase domain to the oxygenase domain to molecular oxygen rather than to L-arginine, leading to excessive superoxide production and reduced NO synthesis.^{4,5} In addition, chronic exposure to hyperglycemia and accumulation of advanced glycation end products reduces eNOS expression and activity through multiple mechanisms and advances diabetic endothelial cell damage.^{6–8} Last, recent experimental studies have shown that gene therapy to restore eNOS uncoupling or expression effectively restores diabetic endothelial dysfunction.^{9,10} Thus, a dysfunctional eNOS/NO system is a major mediator of endothelial dysfunction in diabetes.

Similar changes have been found to occur in glomerular microvasculature in diabetes. Experimental studies have demonstrated up-regulated but dysfunctional (uncoupled) eNOS protein in the glomeruli of diabetic animals,^{11,12} and *in situ* detection of NO and superoxide has revealed decreased NO levels and excessive superoxide production in the diabetic glomeruli.¹² Cell culture studies using glomerular endothelial cells or mouse kidney slices have also shown that high glucose rapidly up-regulates eNOS protein expression but decreases NO synthesis in glomerular endothelial cells through superoxide overproduction and protein kinase C activation.^{13,14} Furthermore, it has been shown that NO release and NO-dependent cGMP production progressively declined in the glomeruli isolated from diabetic rats, indicating that glomerular NO synthesis and availability is further decreased in later stages of diabetic nephropathy, possibly because of increases in superoxide production and advanced glycation end product accumulation combined with an impaired antioxidant defense system.^{15,16} Finally, genetic studies in human diabetes have shown that eNOS gene polymorphisms that potentially reduce eNOS gene transcription and activity are associated with an increased risk of advanced diabetic nephropathy in type 1 and type 2 diabetic patients.^{17–19} Taken together, these findings suggest a critical role for the eNOS/NO system in the pathogenesis of diabetic nephropathy.²⁰ However, the precise role of eNOS-derived NO in mediating diabetic glomerular injury is still largely unknown.

To determine the role of eNOS-derived NO in diabetic nephropathy, we here induced diabetes in eNOS knockout (KO) and wild-type (WT) mice with C57BL6 background by low-dose streptozotocin (STZ) injections and examined their glomerular phenotype at up to 20 weeks of diabetes. Our data demonstrate that a deficit of eNOS-derived NO causes glomerular endothelial injury in diabetic mice and leads to the development of overt albuminuria and glomerular mesangiolysis in nephropathy-resistant inbred mice. The findings demonstrate a

Figure 1. Induction of diabetes in eNOS KO mice. Diabetes (DM) was induced in eNOS KO and WT male mice at the age of 8 weeks by multiple low-dose STZ injections. Blood glucose (BG) was measured at 10 weeks of age, and the mice with hyperglycemia were used for subsequent study. Measurements and tissue sampling were performed before STZ injection and after the onset of DM as indicated.

pivotal role for eNOS-derived NO in the pathogenesis of diabetic nephropathy.

Materials and Methods

Animals

All animal experiments were performed under approval of the Institutional Animal Care and Use Committee at Vanderbilt University. The eNOS KO and WT strains with C57BL/6J background were purchased from The Jackson Laboratories (Bar Harbor, ME). Mice were housed in microisolator cages in a pathogen-free barrier facility and given standard chow (Lab Diet 5015; PMI Nutrition International, Richmond, IN) and water ad libitum.

Induction of Diabetes

Diabetes was induced in the mice at the age of 8 weeks by injecting STZ (50 mg/kg) intraperitoneally for 5 consecutive days.²¹ STZ (Sigma, St. Louis, MO) was prepared in 0.1 mol/L sodium citrate buffer (pH 4.5) immediately before injection. Control animals received intraperitoneal injections of citrate buffer alone. The development (onset) of diabetes was evaluated by measuring fed blood glucose 2 weeks after the first injection, and the mice with a blood glucose level higher than 300 mg/dl were considered diabetic and were used for the study. Forty mice were examined per group. Blood parameters ($n > 10$ per group), blood pressure ($n > 7$ per group), urinary albumin excretion ($n > 10$ per group), glomerular filtration rate ($n > 7$ per group), and renal histology (at least $n = 7$ per group at each time point) were assessed before STZ injection and 4, 12, and 20 weeks after the onset of diabetes (10 weeks of age) as illustrated in Figure 1.

Determination of Blood Glucose

Blood samples were obtained by saphenous vein puncture under anesthesia or by cardiac puncture at sacrifice. Blood glucose was measured on samples obtained after a 6-hour fast using Accu-Chek test strips (Roche Applied Science, Indianapolis, IN).²¹

Measurement of Blood Pressure

Systolic blood pressure was measured in conscious trained mice at room temperature using a tail-cuff monitor (model 65-12 manual scanner; IITC Life Science, Woodland Hills, CA).

Measurement of Urinary Albumin Excretion

Urinary albumin excretion was assessed by determination of spot urine albumin-to-creatinine ratio and 24-hour urinary albumin excretion. Spot urine was collected in the morning using a custom-made mouse urine collection station, and 24-hour urine was collected from individually caged mice using polycarbonate metabolic cages (Tecniplast, Buguggiate, Italy) as previously described.²¹ Urinary albumin was determined using the Albuwell M Murine Microalbuminuria enzyme-linked immunosorbent assay kit (Exocell Inc., Philadelphia, PA). Urinary creatinine was measured using the Creatinine Companion kit (Exocell Inc.).

Measurement of Glomerular Filtration Rate

Glomerular filtration rate (GFR) was measured by a single-bolus fluorescein isothiocyanate (FITC)-inulin injection method, as previously described.²² In brief, 5% FITC-inulin (Sigma) was injected (3.74 μ l/g body weight) retro-orbitally to mice, and $\sim$ 20 μ l of blood was collected via saphenous vein at 3, 7, 10, 15, 35, 55, and 75 minutes after the injection. Plasma concentrations of FITC-inulin were determined using a Fluoroscan Ascent FL (FIN-0081; Labsystems, Helsinki, Finland) at 485-nm excitation and 538-nm emission. GFR was calculated using the equation: $GFR = I/(A/\alpha + B/\beta)$, where I is the amount of injected FITC-inulin, A and B are the y -intercept values of the two decay rates, and α and β are the decay constants for the distribution and elimination phases.²²

Immunoblot Analysis

Glomerular expression of NOS isoforms was assessed by Western blot analysis. Mice in each group ($n = 4$ per group) were anesthetized, and a cannula was inserted in the left ventricle. After the renal vein was cut, the whole body was perfused with phosphate-buffered saline (PBS) using an infusion pump (flow rate, 5 ml/minute). The glomeruli were isolated by differential sieving (150-, 106-, 90-, and 53- μ m sieves)²³ and lysed in Laemmli sodium dodecyl sulfate sample buffer, and 28 μ g of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 8.5% gels under reducing condition and transferred onto a polyvinylidene difluoride membrane. After blocking, the membrane was reacted with: 1) rabbit anti-mouse eNOS polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), 2) rabbit anti-mouse inducible NOS polyclonal antibody (BD Transduction Laboratories, Lexington, KY), 3) rabbit anti-neuronal NOS polyclonal antibody (CAYMAN Chemical, Ann Arbor, MI), and 4) rabbit anti- γ -tubulin antibody (Santa Cruz Biotech-

nology). After washing, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Amersham, Piscataway, NJ). The reaction was visualized using an enhanced chemiluminescence system (Amersham).

Examination of Renal Histopathology

Mice were anesthetized by isoflurane inhalation, and the kidneys were perfused with PBS via the left ventricle, followed by incision of renal vein. The kidneys were removed and weighed, and right kidney was processed for histological examinations. For examination by light microscopy, 4% paraformaldehyde-fixed kidney tissues were embedded in paraffin, and 4- μ m-thick sections were stained with periodic acid-Schiff (PAS), Masson trichrome (Masson), and periodic acid-methenamine. For electron microscopy, kidneys were cut into small tissue blocks (1 mm³) and fixed in 2.5% glutaraldehyde fixative with 0.1 mol/L cacodylate buffer, pH 7.4, overnight at 4°C. After postfixation with 1% osmium tetroxide, tissues were dehydrated in a series of graded ethanol preparations and embedded in epoxy resin (Poly/Bed 812 Embedding Media; Polysciences, Warrington, PA). Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were observed by transmission electron microscopy (H-7000; Hitachi, Tokyo, Japan) at 75 kV.

Evaluation of Morphological Changes

The PAS mesangial matrix area was measured as previously described.^{24,25} In brief, the glomeruli were photographed at a magnification of $\times$ 600 by light microscopy (AX 80; Olympus, Tokyo, Japan) with a CCD camera (Olympus DC 70). Mesangial area was selected using Adobe Photoshop (version 5.0; Adobe Systems, San Jose, CA) and measured using Lumina Vision (version 2.04; Mitani Co., Toyama, Japan). Mesangial matrix fraction was calculated as the ratio of mesangial area to glomerular tuft area. Seven mice were analyzed per group, and 35 glomeruli that did not accompany mesangiolysis were measured per mouse. The glomeruli in focal renal scars or infarctions were excluded from the measurements. The frequency of glomerular mesangiolysis (percentage) was also determined on PAS-stained sections. At least 100 glomeruli were evaluated per mouse. The thickness of glomerular basement membrane (GBM) was determined by the orthogonal intercept method as described previously.^{26,27} In brief, the shortest distance between endothelial cytoplasmic membrane and the outer lining of the lamina rara externa underneath the cytoplasmic membrane of the epithelial foot processes was measured with a logarithmic ruler where gridlines transected the GBM. The true harmonic mean thickness (Th) was estimated from the apparent harmonic mean thickness (Ih) using the following equation: $Th = 8/3\pi \times 10^6/M \times Ih$, where M represents the final print magnification.²⁶ At least five glomeruli (total of 700 intercepts) were examined per mouse. The glomeruli that accompanied

mesangiolysis were excluded from the measurements. Six mice were analyzed per group.

In Situ Detection of NO

Intracellular NO production in the glomeruli was assessed as previously described.¹² In brief, mice ($n = 4$ per group) were anesthetized, and a cannula was inserted in the left ventricle. After the right atrium was cut, whole body was perfused with PBS (37°C) using an infusion pump (flow rate, 5 ml/minute). Once blood had been removed, the mice were perfused with PBS containing 0.01 mmol/L 4,5-diaminofluorescein (Alexis Biochemicals, San Diego, CA), 0.1 mmol/L L-Arg, and 2 mmol/L CaCl_2 for an additional 10 minutes at a flow rate of 1 ml/minute. Unreacted 4,5-diaminofluorescein was removed by postperfusion with PBS for 10 minutes. After perfusion with 4% paraformaldehyde, the glomeruli were isolated from the kidney by differential sieving as described previously.²³ Fluorescent images of NO were taken using confocal microscopy (LSM510; Carl Zeiss, Thornwood, NY) with excitation at 495 nm and emission at 515 nm.

Assessment of the Anionic Sites at Glomerular Wall

The anionic sites at glomerular wall were assessed by cationic ferritin binding as previously described.²⁸ In brief, mice ($n = 6$) in each group were anesthetized, and a cannula was inserted in the left ventricle. After the right atrium was cut, the whole body was perfused with PBS using an infusion pump (flow rate, 5 ml/minute; perfusion pressure, 140 mm Hg). Once blood had been removed, the mice were perfused with PBS containing 2 mg of cationic ferritin (Sigma). After a brief washout with PBS (flow rate, 5 ml/minute; perfusion pressure, 140 mm Hg), kidneys were removed, and small tissue blocks (1 mm³) of renal cortex were fixed in 2% glutaraldehyde with 0.1 mol/L cacodylate buffer (pH 7.4) overnight at 4°C. After postfixation with 1% osmium tetroxide, tissues were dehydrated in a series of graded ethanol preparations and embedded in epoxy resin (Poly/Bed 812 Embedding Media; Polysciences). Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were observed by transmission electron microscopy (H-7000; Hitachi) at 75 kV. The anionic sites at glomerular endothelium was measured as described previously.²⁸ In brief, 300-point grid was superimposed on enlarged glomerular micrographs. The fraction covered by cationic ferritin (CF) was then estimated as % CF = F/T , where F is the number of points hitting areas of CF deposition and T is the total of points hitting the surface of glomerular endothelium. Six mice were analyzed per group, and at least five glomeruli that did not develop mesangiolysis (total of 20 capillaries) were measured per mouse.

Statistical Analysis

All data are expressed as means $\pm$ SEM. Analysis of variance with Bonferroni test was used for comparisons between multiple groups. Unpaired t -test was used to assess statistical significance between two experimental groups. $P < 0.05$ was considered significant.

Results

Hyperglycemia and Systolic Blood Pressure

To mitigate nonspecific cytotoxicity of STZ, diabetes was induced in 8-week-old males (total $n = 40$ per group) by multiple low-dose STZ injections (Figure 1). Approximately 80% of the animals developed hyperglycemia within 2 weeks, and there was no difference in the incidence of hyperglycemia between eNOS KO and WT mice. As shown in Figure 2A, the mice that developed hyperglycemia were diabetic throughout the entire study period, and there was no difference in the severity of hyperglycemia between diabetic eNOS KO (STZ-eNOS KO) and diabetic WT (STZ-WT) mice. Hyperglycemia was not observed in the control mice (Buffer-eNOS KO, Buffer-WT) that received citrate buffer alone. Significant differences were not observed in levels of fasting plasma cholesterol and triglyceride among the groups, although diabetic eNOS KO mice showed higher levels of plasma cholesterol compared with the other groups (data not shown). Systolic blood pressure (SBP) was measured in each group of mice. As shown in Figure 2B, induction of diabetes elevated SBP in both eNOS KO and WT mice, and diabetic eNOS KO mice exhibited significantly higher SBP at 4, 12, and 20 weeks of diabetes compared with diabetic WT mice. The nondiabetic eNOS KO mice also exhibited higher SBP when compared with nondiabetic WT mice. There was no difference in SBP between eNOS KO and WT mice before STZ injection. All diabetic eNOS KO ($n = 17$) and diabetic WT ($n = 17$) mice survived until sacrifice 20 weeks after induction of diabetes.

Development of Albuminuria in Diabetic eNOS KO Mice

Albuminuria was assessed by determination of albumin-to-creatinine ratio on morning spot urine and urinary albumin excretion in 24-hour urine collections. As shown in Figure 3, induction of diabetes remarkably increased urinary albumin excretion in eNOS KO mice as early as 4 weeks of diabetes, whereas albuminuria did not develop in diabetic WT mice and nondiabetic controls. Albuminuria in diabetic eNOS KO mice progressively increased as the duration of diabetes increased, exceeding 200 $\mu\text{g/day}$ at 20 weeks of diabetes (Figure 3B).

GFR and Kidney Weight-to-Body Weight Ratio

GFR in diabetic and nondiabetic mice was assessed by FITC-inulin clearance.²² As shown in Figure 4A,

Figure 2. Blood glucose and systolic blood pressure. Fasting blood glucose (**A**) and systolic blood pressure (**B**) were measured in the four groups of mice at the indicated time points. Data are presented as means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, STZ-eNOS KO versus STZ-WT mice. *** $P < 0.01$, STZ-eNOS KO versus Buffer-eNOS KO mice. † $P < 0.05$, †† $P < 0.01$, Buffer-eNOS KO versus Buffer-WT mice. §§ $P < 0.01$, STZ-WT versus Buffer-WT mice. The diabetic eNOS KO (STZ-eNOS KO) mice are significantly hypertensive compared with diabetic WT (STZ-WT) mice, although there was no difference in the severity of hyperglycemia between the two groups.

eNOS KO mice before STZ injection showed significantly lower GFR when compared with wild-type mice. Induction of diabetes increased GFR significantly in wild-type mice; however, an increase in GFR was less evident in diabetic eNOS KO mice. Consistent with these results, eNOS KO (diabetic and nondiabetic) mice revealed significantly higher plasma creatinine levels compared with wild-type counterparts (STZ-eNOS KO, 0.089 ± 0.004 mg/dl, $P < 0.05$ versus STZ-WT; Buffer-eNOS KO, 0.104 ± 0.012 mg/dl, $P < 0.05$ versus Buffer-WT; STZ-WT, 0.071 ± 0.003 mg/dl; Buffer-WT, 0.080 ± 0.002 mg/dl; DM 20w, $n = 8$ per group, HPLC method). Renal blood flow was also as-

sessed using a small animal blood flow meter (Supplemental Figure S1, see <http://ajp.amjpathol.org>). The data demonstrated significantly reduced renal blood flow in diabetic eNOS KO mice compared with the diabetic wild-type and nondiabetic eNOS KO mice. Renal hypertrophy was evaluated by determination of kidney weight-to-body weight ratio in each group of mice. As shown in Figure 4B, induction of diabetes significantly increased the kidney weight-to-body weight ratio in both eNOS KO and WT mice as early as 4 weeks of diabetes. There was no difference in the kidney weight-to-body weight ratio between diabetic eNOS KO and diabetic WT mice.

Figure 3. Development of albuminuria in diabetic eNOS KO mice. Urinary albumin excretion in the four groups of mice was evaluated by determination of albumin-to-creatinine ratio on morning spot urine (**A**) and 24-hour urinary albumin excretion (**B**). Data are presented as means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ versus STZ-WT mice. † $P < 0.05$, †† $P < 0.01$ versus Buffer-eNOS KO mice.

Figure 4. Renal changes in diabetic eNOS KO mice. **A:** GFR was determined in conscious mice by FITC-inulin clearance method as described in Materials and Methods. **B:** Renal hypertrophy was assessed by measurements of kidney weight-to-body weight ratio. Data are presented as means $\pm$ SEM. $^{*}P < 0.01$ versus WT mice. $^{*}P < 0.05$, $^{**}P < 0.01$ versus STZ-WT mice. $^{*}P < 0.05$, $^{**}P < 0.01$ versus Buffer-eNOS KO mice. $^{*}P < 0.05$, $^{**}P < 0.01$ versus Buffer-WT mice.

Renal Histopathology

Development of albuminuria in diabetic eNOS KO mice led us to investigate glomerular pathology in the mice. Kidney sections were prepared and examined by light and electron microscopy. As shown in Figure 5, A and B, diabetic eNOS KO mice exhibited focal renal scars and infarctions, affecting $1.7 \pm 0.4\%$ ($n = 12$, DM 20w) of renal cortex area, whereas distinct pathological phenotype including increased medial thickening and disruption of medial layers was not observed in the renal vasculature. The glomerular tufts in diabetic eNOS KO mice showed mild mesangial cell proliferation and matrix expansion (Figure 5F), and some glomeruli displayed prominent mesangiolysis, often accompanied by intraluminal thrombosis (Figure 5, D and E). Glomerular microaneurysm, ballooning of capillaries, was not observed in the mice. Global glomerulosclerosis with hypocellularity and dissociated matrices was also observed in $4.6 \pm 0.7\%$ ($n = 12$, DM 20w) of the glomeruli. Prominent tubulointerstitial fibrosis was not observed in the kidney other than the region of renal infarction. The frequency of glomerular mesangiolysis in diabetic eNOS KO mice increased as diabetes progressed (Figure 5J), and apoptotic endothelial cells were detected in mesangiolytic glomeruli by terminal deoxynucleotidyl transferase dUTP nick-end labeling staining (data not shown). Glomerular mesangiolysis was also observed in nondiabetic eNOS KO mice; however, the frequency was significantly lower than that in diabetic eNOS KO mice and was not increased as the mice aged (Figure 5J). Focal renal scars and infarctions and global glomerulosclerosis were also noted in nondiabetic eNOS KO mice, but there was no difference in the frequency or the extent of these lesions between diabetic and nondiabetic eNOS KO mice (data not shown). Renal infarction, mesangiolysis, and glomerulosclerosis were not observed in diabetic WT mice (Figure 5, G and H), whereas the mice showed similar mesangial expansion (Figure 5I). As revealed by semiquantitative analysis (Figure 5J), significant difference was not observed in mesangial ma-

trix expansion between diabetic eNOS KO and diabetic WT mice.

Electron micrographs from diabetic eNOS KO mice disclosed mild mesangial expansion, thickening of GBM, and local foot process effacement (Figure 6, D and E). As shown in Figure 6A, prominent mesangiolysis was observed in some glomeruli. The mesangiolysis was accompanied by dissociation of mesangial matrix and injured endothelial cell morphology, which is illustrated by endothelial cell detachment (Figure 6A). Glomerular endothelial injury was also evidenced by subendothelial leukocyte infiltration (Figure 6B), subendothelial insudation (Figure 6C), and increased platelets (Figure 6C). The anchoring of the mesangium to the GBM was scarcely involved (Figure 6, A and B). Morphometric analysis revealed a significant increase in the GBM thickness in diabetic eNOS KO mice compared with diabetic wild-type mice (Figure 6F).

Immunohistochemical analysis showed decreased immunoreactivity for vascular endothelial cadherin in diabetic eNOS KO glomeruli, indicating impaired endothelial junctional integrity in the glomeruli (Supplemental Figure S2, see <http://ajp.amjpathol.org>). Distinct difference was not noted in immunostaining for vascular endothelial growth factor receptor 2, laminin, vascular endothelial growth factor-A, nephrin, or vimentin between diabetic eNOS KO and diabetic wild-type mice (Supplemental Figure S2, see <http://ajp.amjpathol.org>).

Glomerular NO Synthesis and NOS Isoform Expression

Because compensatory up-regulation of NOS isoforms has been demonstrated in retinal vasculature in eNOS KO mice,^{29,30} we next asked whether glomerular lesions in diabetic eNOS KO mice were the result of reduced glomerular NO synthesis. Glomerular NO synthesis and NOS isoform expression were evaluated in isolated glomeruli as described in Materials and Methods. As shown in Figure 7A, *in situ* evaluation of glomerular NO synthesis demonstrated remarkable reduc-

Figure 5. Renal histopathology in diabetic eNOS KO mice. **A–F:** Representative pathological changes in the kidneys of diabetic eNOS KO mice (at 20 weeks of diabetes). Diabetic eNOS KO mice display focal renal scar and infarction (**A**, yellow arrows, and **B**) and mild mesangial expansion (**F**). Some glomeruli show prominent mesangiolysis (**C**, red arrow; **D**; and **E**) and intraluminal thrombosis (**D**). **G–I:** Representative renal histopathology in diabetic WT mice (at 20 weeks of diabetes). **J:** The frequency of glomerular mesangiolysis (%) and the PAS mesangial matrix fraction were assessed as described in Materials and Methods. Data were presented as means $\pm$ SEM. $^{**}P < 0.01$ versus Buffer-eNOS KO mice. $^{SS}P < 0.01$ versus Buffer-WT mice. $n = 7$ per group. Original magnifications: $\times 40$ (**A** and **G**); $\times 100$ (**B**, **C**, and **H**); $\times 400$ (**D** and **E**); $\times 600$ (**E** and **I**). **A** and **G**, Masson trichrome stain; **B**, **C**, **D**, **F**, **H**, and **I**, PAS stain; **E**, periodic acid methenamine silver (PAM) stain.

Figure 6. Ultrastructural features of diabetic eNOS KO glomeruli. **A–E:** Representative electron micrographs from diabetic eNOS KO mice (at 20 weeks of diabetes) are shown. Diabetic eNOS KO glomeruli show mesangiolysis (**A**) and injured endothelial morphology including endothelial cell detachment (**arrows** in **A**), migration of leukocytes into subendothelial space (**arrow** in **B**), subendothelial insudation (**asterisks** in **C**), and increased platelets (**C**). An **arrowhead** in **B** indicates disruption of anchoring of GBM to mesangium. Local foot process effacement is also observed (**arrows** in **D**). A highly magnified image shows irregularly thickened GBM (**E**). Original magnifications: $\times 10,000$ (**A** and **B**); $\times 20,000$ (**D**); $\times 30,000$ (**C**); and $\times 50,000$ (**E**). Mes, mesangial cells; M Φ , macrophage; En, endothelial cells; Plt, platelet. **F:** The thickness of GBM was assessed by morphometric analysis as described in Materials and Methods. Data are presented as means $\pm$ SEM. ****** $P < 0.01$ versus STZ-WT mice. ******* $P < 0.01$ versus Buffer-eNOS KO mice. $n = 6$ per group.

tion in glomerular NO synthesis in both diabetic and nondiabetic eNOS KO mice. Furthermore, diabetic WT mice exhibited decreased glomerular NO synthesis compared with nondiabetic WT mice. Similar results

were obtained at 20 weeks of diabetes (data not shown). Consistent with these findings, only minimal up-regulation of NOS isoforms (inducible NOS and neuronal NOS) were observed in diabetic and nondia-

Figure 7. NO synthesis and NOS expression in the diabetic eNOS KO glomeruli. Glomerular NO synthesis (**A**) and NOS expression (**B**) were assessed in diabetic and nondiabetic control mice at 4 weeks of diabetes as described in Materials and Methods. Representative results in each group of mice ($n = 4$) are shown. Anti- γ -tubulin immunoblot was performed as a loading control. Remarkably reduced NO synthesis is seen in the eNOS KO glomeruli (glom).

betic (data not shown) eNOS KO glomeruli by immunoblot analysis of isolated glomeruli (Figure 7B) and immunohistochemistry (data not shown).

Evaluation of Glomerular Endothelial Charge Barrier

The endothelial cell surface coat (known as the glycocalyx), which is composed of negatively charged proteoglycans and glycosaminoglycan, has been implicated as an important contributor to regulation of glomerular macromolecule permeability by acting as a charge barrier.³¹ Therefore, we evaluated anionic sites on the glomerular endothelial cell surface coat by cationic ferritin perfusion, as previously described.²⁸ As shown in Figure 8, binding of cationic ferritin to glomerular endothelial cell surface and GBM (lamina rara interna) was obviously reduced in diabetic eNOS KO mice compared with the other three groups. Semiquantitative analysis of the extent of cationic ferritin deposition revealed a significant decrease in the density of anionic sites at the glomerular endothelial surface layer in diabetic eNOS KO mice (Figure 8E), indicating impaired glomerular charge barrier in the mice.

Discussion

The present study demonstrates that a deficit of eNOS-derived NO causes albuminuria, accelerated hypertension, lower GFR, mesangiolysis, and GBM thickening in the C57BL6 strain diabetic mice, which is known to be

resistant to nephropathy.^{21,32} The findings clearly demonstrate that reduced eNOS activity enhances glomerular injury in the setting of diabetes, supporting human studies suggesting that eNOS polymorphism and reduced eNOS activity are associated with accelerated diabetic nephropathy.

Endothelial dysfunction and damage is a hallmark of diabetic vascular complications.³ Our data illustrate enhanced endothelial dysfunction and glomerular endothelial damage in diabetic eNOS KO mice. These include accelerated hypertension, reduced renal blood flow, glomerular mesangiolysis accompanied by injured endothelial morphology and thrombosis, subendothelial insudation, and down-regulation of vascular endothelial cadherin. NO is known to play a pivotal role in vasorelaxation, inhibition of platelet aggregation and leukocyte adhesion to endothelium, and inhibition of vascular smooth muscle cell proliferation.² NO also inhibits endothelial activation induced by cytokines.^{33,34} Furthermore, it has been shown that eNOS-derived NO is a critical regulator of endothelial junctional integrity and serves to maintain the low basal permeability of endothelium.³⁵ Importantly, a recent study has demonstrated that NO prevents high glucose-induced endothelial apoptosis by suppressing nuclear factor- κ B activity.³⁶ In addition, it has been shown that NO prevents aldose reductase activation and sorbitol accumulation and potentially suppress vascular complications during diabetes.³⁷ Taken together, it is conceivable that a deficit of these protective effects of NO on vasculature may enhance glomerular endothelial damage in diabetic eNOS KO mice. It is

Figure 8. Cationic ferritin binding to glomerular wall. The anionic sites of glomerular wall were assessed using cationic ferritin (CF) as described in Materials and Methods. Representative electron micrographs of each group of mice ($n = 6$ per group) at 20 weeks of diabetes are shown. Dense CF depositions are seen at the endothelial surface (arrows) and lamina rara interna (white arrowheads) in nondiabetic WT (A), diabetic WT (B), and nondiabetic eNOS KO (C) glomeruli. Fewer cationic ferritin depositions are observed in the diabetic eNOS KO glomeruli (D). **E:** The density of CF depositions (% CF) at glomerular endothelial walls was assessed by morphometry as described in Materials and Methods. Data are presented as means $\pm$ SEM. $**P < 0.01$ versus STZ-WT mice. $***P < 0.01$ versus Buffer-eNOS KO mice. $n = 6$ per group. Original magnification, $\times 30,000$. CL, capillary lumen; Pod, podocyte foot process.

noteworthy that albuminuria and advanced glomerular lesions were not observed in nondiabetic eNOS KO mice, whereas glomerular NO synthesis was remarkably reduced in the mice. The finding indicates that a higher level of NO is required to maintain homeostasis of glomerular microvasculature in diabetes.

A large body of work has indicated a critical role of hypertension to induce diabetic renal injury.³⁸ Because diabetic eNOS KO mice showed elevated blood pressure, glomerular injury in the mice may be explained by systemic hypertension and reduced renal blood flow. In fact, mesangiolytic was more frequently observed in the outer half of renal cortex (data not shown). Clearly, further

studies controlling blood pressure in diabetic eNOS KO mice are required to determine the impact of hypertension on their renal consequences. However, it is noteworthy that morphological changes of renal vasculature in diabetic eNOS KO mice are quite mild compared with the glomerular lesions. Furthermore, Chu et al¹⁴ have demonstrated that glomeruli are the major renal structures, showing strong NO production and abundant eNOS expression. Therefore, it is likely that glomerular pathology in diabetic eNOS KO mice is mostly caused by a deficit of eNOS-derived NO in the glomerulus.

Prominent mesangiolytic was observed in the glomeruli of diabetic eNOS KO mice. Mesangiolytic is divided into three types in their mode of origin and morphological features.³⁹ The first type is severe mesangiolytic with glomerular microaneurysm, which is caused by mesangial cell injury (eg, anti-Thy1 model). The second type is mesangiolytic associated with extensive widening of the subendothelial space, which is thought to follow endothelial injury (eg, thrombotic microangiopathy). The third type is mesangiolytic with lamellated mesangial nodules, which is suggested to result from mild but persistent or recurrent mesangial and endothelial damage (eg, diabetic nephropathy). It is obvious that morphological features of the mesangiolytic in diabetic eNOS KO mice are identical to the second type of mesangiolytic. The finding indicates that endothelial injury in diabetic eNOS KO mice is more severe than that in human diabetic nephropathy.

NO exerts antioxidant effects by direct scavenging of superoxide.⁴⁰ The endothelial damage in diabetic eNOS KO mice may be caused by increased cellular oxidative stress. However, we think this mechanism is less likely. Growing evidence demonstrates that uncoupled eNOS is a major source of superoxide generation in diabetic endothelium. Therefore, it is more likely that a lack of uncoupled eNOS may actually reduce oxidative stress in diabetic eNOS KO mice. Indeed, immunohistochemistry for 8-hydroxydeoxy-guanosine and malon-dialdehyde, markers of cellular oxidative stress and DNA damage, revealed decreased oxidative stress in the diabetic eNOS KO glomeruli (data not shown). On the other hand, STZ is known to have nonspecific toxic effects on a variety of tissues, including renal tissues,⁴¹ so it is possible that eNOS deficiency may enhance toxicity of STZ to endothelial cells and cause glomerular endothelial damage. However, we think this is less likely, because eNOS-deficient spontaneously diabetic mice (PDX1^{PB}-HNF6 mice)⁴² showed similar outcomes on C57BL/6 background (data not shown). The finding suggests that STZ toxicity is not the major cause of the glomerular injury in diabetic eNOS KO mice. It is noteworthy that STZ-induced diabetic eNOS KO mice do not exhibit progressive diabetic kidney disease as observed in db/db eNOS KO mice,⁴³ which is accompanied by a decline in GFR, prominent mesangial expansion, and focal nodular glomerulosclerosis. Because the C57BL/6 strain eNOS KO mice, whose genetic background is identical to db/db eNOS KO mice, develops more advanced nephropathy that is similar to db/db eNOS KO mice on STZ treatment (data not shown), we think it is more likely that the dis-

crepancies between these two models result from their genetic backgrounds rather than the mode of diabetes.

Microalbuminuria is the earliest detectable sign of renal damage in human diabetic nephropathy, which is recognized as a hallmark of endothelial dysfunction.^{44,45} The glomerular filtration barrier comprises three layers: glomerular endothelial cells, GBM, and podocytes. The glomerular barrier restricts the passage of anionic macromolecules relative to uncharged molecules of similar size and configuration.⁴⁶ Previous studies showed that the charge selectivity resides mainly at endothelial cells or podocytes rather than the GBM,^{47,48} and a more recent study has shown that endothelial cell glycocalyx, a negatively charged surface layer of membrane-associated proteoglycans and glycosaminoglycans, is a critical determinant in glomerular charge selectivity.³¹ Furthermore, Arcos et al²⁸ showed that chronic NO inhibition using an NO inhibitor impairs glomerular endothelial charge barrier and causes albuminuria and GBM thickening. The present study demonstrates that a deficit of eNOS-derived NO in diabetes may impair the glomerular endothelial charge barrier (possibly by decreasing endothelial glycocalyx) and result in albuminuria. The finding agrees with recent studies of human diabetes that endothelial glycocalyx damage coincides with microalbuminuria, endothelial dysfunction, and coagulation activation in diabetes.^{49,50}

Glomerular hyperfiltration and hypertrophy are early functional renal alterations in diabetes. NO has been suggested as a candidate mediator of these diabetic renal changes.⁵¹ However, the present study indicates that eNOS-derived NO may not be required for diabetic renal hypertrophy. Our data support a recent experimental study demonstrating markedly decreased NO synthesis in the glomeruli of early-phase diabetes.¹² On the other hand, reduced glomerular hyperfiltration in diabetic eNOS KO mice is consistent with previous experimental studies, demonstrating inhibition of diabetic glomerular hyperfiltration by nonspecific NOS inhibitors.⁵² However, this might be due to prominently reduced renal blood flow in diabetic eNOS KO mice. Further studies will be required to determine the precise role of eNOS-derived NO in diabetic glomerular hyperfiltration.

In human diabetes, there is a clear-cut genetic susceptibility to the development of nephropathy.^{53,54} Cohorts susceptible to nephropathy are characterized by progressively increasing albuminuria and development of characteristic renal lesions, including mesangial expansion and glomerulosclerosis. In contrast, cohorts resistant to nephropathy do not develop albuminuria and severe glomerular lesions despite comparable levels of hyperglycemia. Furthermore, recent studies have demonstrated that genetic factors determine susceptibility to diabetic nephropathy in mice as well as in humans. Among the inbred strains tested, C57BL/6 strain mice were shown to be resistant to nephropathy (albuminuria).^{21,32} Importantly, the present study has demonstrated that insufficient eNOS activity may alter susceptibility of C57BL/6 mouse to diabetic glomerular injury, indicating a vital role of eNOS in the development of diabetic nephropathy. Our results in mice support the

finding in human diabetes that a predisposition for development of nephropathy is closely associated with eNOS polymorphisms.^{19,55,56} Determination of the molecular pathways through which eNOS deficiency causes glomerular endothelial damage in the setting of diabetes may provide new insights into pathogenesis of diabetic nephropathy and offer better treatment strategies.

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