Endothelial Nitric Oxide Synthase Deficiency Produces Accelerated Nephropathy in Diabetic Mice

Hui John Zhao,* Suwan Wang,* Huifang Cheng,* Ming-zhi Zhang,* Takamune Takahashi,* Agnes B. Fogo,† Matthew D. Breyer,* and Raymond C. Harris*

*Division of Nephrology and Hypertension, Department of Medicine, and †Department of Pathology, Vanderbilt University, Nashville, Tennessee

Functionally significant polymorphisms in endothelial nitric oxide synthase (eNOS) and reduced vascular eNOS activity have been associated with increased human diabetic nephropathy (DN), but the pathogenic role of eNOS deficiency in the development of DN has not yet been confirmed. This study characterizes the severity of DN in eNOS<sup>−/−</sup> mice that were backcrossed to C57BLKS/J db/db mice. Although the severity of hyperglycemia was similar to C57BLKS/J db/db mice, by 26 wk, eNOS<sup>−/−</sup> C57BLKS/J db/db mice exhibited dramatic albuminuria, arteriolar hyalinosis, increased glomerular basement membrane thickness, mesangial expansion, mesangiolyis, and focal segmental and early nodular glomerulosclerosis. Even more remarkable, eNOS<sup>−/−</sup> C57BLKS db/db exhibited decreases in GFR to levels <50% of that in eNOS<sup>+/+</sup> C57BLKS db/db, as confirmed by increased serum creatinine. In summary, eNOS<sup>−/−</sup> db/db mice provide the most robust model of type II DN that has been described to date and support a role for deficient eNOS-derived NO production in the pathogenesis of DN.

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Measurement of GFR
GFR was measured by a single-bolus FITC-inulin injection method, as described previously (12).

Histologic Analysis
Renal histology was assessed in mice that were killed at 24 to 26 wk of age. The perfused kidneys were removed and fixed overnight in 10% formalin at 4°C, and 3-μm-thick sections were stained with periodic acid-Schiff. Histologic evaluation was performed without knowledge of the identity of the various groups. A semiquantitative index was used to evaluate the degree of glomerular mesangial expansion and sclerosis. Each glomerulus on a single section was graded from 0 to 4, where 0 represents no lesion, and 1, 2, 3, and 4 represent mesangial matrix expansion or sclerosis, involving ≤25, 25 to 50, 50 to 75, or >75% of the glomerular tuft area, respectively.

Immunohistochemistry
Immunohistochemical detection of fibronectin staining was performed using an anti-fibronectin antibody (Sigma, St. Louis, MO). The sections then were incubated using the avidin-biotin-horseradish peroxidase technique (Elite Vectastain ABC kit; Vector Laboratories, Burlingame, CA), and staining was visualized using 3,3′-diaminobenzidine.

Statistical Analyses
All values are presented as means ± SEM. Bonferroni t test corrected for multiple comparisons was used for statistical analysis, and differences were considered significant at P < 0.05.

Results
Development of Type 2 Diabetes in eNOS−/− C57BLKS/db/db Mice
We first investigated the timing of development of diabetes in this model. Hyperglycemia was evident by approximately 6 to 8 wk of age. The fasting blood glucose level in db/db mice was significantly higher than that in the lean controls at 26 wk of age (P < 0.05; Figure 1A). eNOS deletion did not alter fasting blood glucose in either db/db or control mice. As was described previously (13,14), moderate hypertension was observed in lean mice with genetic deletion of eNOS by age 24 to 28 wk (Figure 1B). SBP was significantly greater in eNOS−/− C57BLKS/J db/db mice (eNOS−/− db/db) than in db/db C57BLKS/J mice (db/db; 158 ± 10 versus 110 ± 3; n = 4; P < 0.05) but was not significantly higher than in nondiabetic eNOS−/− C57BLKS/J mice (eNOS−/−; 143 ± 7 mmHg; n = 5; NS).

Albuminuria in eNOS−/− C57BLKS/J db/db Mice
Albuminuria is a hallmark of DN. At 26 wk of age, moderate albuminuria was observed in db/db (262 ± 24 μg albumin/mg creatinine; n = 10) and eNOS−/− (387 ± 27; n = 19), compared with control mice (43 ± 9; n = 8; P < 0.05; Figure 2A). In contrast, eNOS−/− db/db exhibited a marked increase in spot urine ACR (1503 ± 176; n = 12; P < 0.001 compared with their age-matched controls, db/db, or eNOS−/−). Urine ACR was elevated by age 8 wk in eNOS−/− db/db (data not shown).

GFR in eNOS−/− C57BLKS/J db/db Mice
HPLC serum creatinine determined at 26 wk was 0.10 ± 0.001 (n = 5) in the control lean mice, 0.11 ± 0.01 (n = 7) in db/db, 0.115 ± 0.001 (n = 9) in eNOS−/−, and 0.17 ± 0.02 (n = 5) in eNOS−/− db/db (P < 0.05). GFR was examined in diabetic mice and age-matched controls using FITC-inulin clearance. Given the disparity in body weights between the control (lean) and diabetic (obese) mice, GFR was calculated per mouse as well as per gram of body weight. At 26 wk of age, GFR determined per mouse was numerically increased in db/db (366 ± 39 μl/min per mouse; n = 8) but was not statistically significant compared with age-matched controls (331 ± 25; n = 10). In contrast, the GFR of eNOS−/− db/db (164 ± 27; n = 10) was significantly decreased compared with db/db (P < 0.001) and eNOS−/− (265 ± 21; n = 12; P < 0.05; Figure 2B). At 26 wk, the body weights of the obese mice were more than double those of the aged-matched controls (lean controls 23.4 ± 0.9 g; db/db 59.4 ± 2.4; eNOS−/− 26.5 ± 1.5; eNOS−/− db/db 58.1 ± 2.3). GFR per gram of body weight were as follows: lean controls 14.1 ± 1.1 μl/min per g; db/db 6.2 ± 0.6; eNOS−/− 9.9 ± 0.6; and eNOS−/− db/db 2.5 ± 0.4. Given that body fat represents approximately 50% of total body weight in db/db mice (15),
determination of GFR/body weight in the obese mice will underestimate the true GFR. Serum creatinines correlated with the GFR measurements in mice from all groups in which both measurements were obtained ($n = 23$; $r^2 = 0.699$, $P < 0.0001$; Figure 2C).

**Histologic Analysis**

Kidneys were assessed by light and electron microscopy (Figure 3). Compared with control (Figure 3A, a), moderate mesangial expansion was observed in glomeruli of $db/db$ (Figure 3A, b and e) and eNOS$^{-/-}$ (Figure 3A, c) at 26 wk of age. In contrast, marked mesangial expansion, focal nodular sclerosis, and mesangiolysis (Figure 3A, d and f) as well as arteriolar hyalinosis (Figure 3A, f) were noted in eNOS$^{-/-}$ $db/db$ glomeruli at 26 wk of age. There was only minimal tubulointerstitial fibrosis in eNOS$^{-/-}$ $db/db$ kidneys. Immunohistochemical examination also revealed minimal accumulation of fibronectin in the mesangial regions of control (Figure 3B, a), $db/db$ (Figure 3B, b), and eNOS$^{-/-}$ (Figure 3B, c), compared with the striking fibronectin accumulation that was observed in the eNOS$^{-/-}$ $db/db$ glomeruli (Figure 3B, d).

Glomerular injury that was assessed semiquantitatively was significantly increased in eNOS$^{-/-}$ $db/db$ at 24 to 26 wk of age, as compared with other groups ($n = 6$ to 9/group; $P < 0.05$; Figure 3C). The amount of albuminuria correlated with the glomerular injury index in mice from all groups in which both measures were obtained ($n = 19$; $r^2 = 0.58$, $P < 0.0002$; Figure 3D).

Glomerular ultrastructure was examined by electron microscopy at age 16 wk (Figure 3E). Compared with control mice (270 ± 47 nm; Figure 3E, a), at this age, there was no thickening of glomerular basement membrane (GBM) in $db/db$ mice (240 ± 76 nm; Figure 3E, b), in contrast to the markedly thickened GBM that was seen in eNOS$^{-/-}$ $db/db$ mice (322 ± 57 nm; Figure 3E, c). No electron-dense deposits were present.

**Discussion**

Human DN is a characteristic clinical syndrome that consists of albuminuria, progressively declining GFR, and defined histopathologic features that include thickening of the GBM and mesangial expansion, often with nodular glomerulosclerosis, arteriolar hyalinosis, and tubulointerstitial fibrosis (16,17). These features would be important features of a robust animal model of DN (18).

Previous animal models of diabetic kidney disease have manifested albuminuria and early renal pathologic changes such as GBM thickening and mesangial expansion but with only minimal or inconsistent expression of other characteristic histopathologic features such as arteriolar hyalinosis and nodular glomerulosclerosis. Furthermore, the failure of previous models to manifest a decline in renal function has called into question their utility as analogues of human DN (16,18,19).

eNOS$^{-/-}$ C57BLKS $db/db$ mice not only developed striking albuminuria and characteristic pathologic changes of DN but also exhibited remarkably decreased GFR on the basis of inulin clearance and serum creatinine. Furthermore, the onset and the progression of the pathologic features in eNOS$^{-/-}$ $db/db$ mice...
were rapid, which makes this DN model attractive as a potential platform for testing efficacy of new therapeutic agents.

The eNOS$^{-/-}$ db/db mice had significant obesity and hypertension, which typically is seen in humans with type 2 diabetes (20,21). The mutated leptin receptor in db/db mice leads to defective signaling of leptin in the hypothalamus and results in persistent hyperphagia and obesity and development of peripheral insulin resistance (19). In addition, when the db/db mutation is on the C57BLKS background, mice develop a late (4 mo) insulitis so that insulin levels are inappropriately low for the level of hyperglycemia (22). eNOS$^{-/-}$ db/db mice were previously reported to develop moderate systemic hypertension (13,14). This increased SBP was slightly greater in diabetic eNOS$^{-/-}$ db/db mice, although the results did not reach statistical significance.

There is growing evidence that endothelial cell dysfunction contributes to hypertension and microvascular complications of diabetes (2,23). A major defense of endothelial cells against vascular injury is eNOS, which generates NO in the presence of the substrate l-arginine, and the co-factor (6R)-5,6,7,8-tetrahydrol-\(\text{L}\)-biopterin (BH4). NADPH oxidases are major sources of reactive oxygen species in endothelium and are activated in

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**Figure 3.** Glomerular histopathology. (A) Representative glomerular lesions of diabetic mouse kidneys at 24 to 26 wk: control (a); db/db (b and e); eNOS$^{-/-}$ (c); eNOS$^{-/-}$ db/db (d and f). (f) Arteriolar hyalinosis (big arrow) and early nodular glomerulosclerosis (small arrow) in eNOS$^{-/-}$ db/db mice (periodic acid-Schiff). (B) Glomerular fibronectin expression in control (a), db/db (b), eNOS$^{-/-}$ (c), and eNOS$^{-/-}$ db/db mice (d). (C) Glomerular injury scores in diabetic mice. Mesangial expansion and sclerosis were scored on a scale from 0 to 4 as described in The Materials and Methods section. *P < 0.05, eNOS$^{-/-}$ db/db (24 to 26 wk) mice versus age-matched controls, db/db, and eNOS$^{-/-}$ mice. (D) Correlation of glomerular injury index with albuminuria. (E) Electron micrographs of glomeruli (16 wk). Opposed arrows indicate GBM. GBM thickening was notable in eNOS$^{-/-}$ db/db mice (c) compared with control (a) and db/db mice (b). Magnifications: ×400 in A, a through d, and B; ×200 in A, e and f.
animal models of hypertension and diabetes. Superoxide reacts avidly with vascular NO to form peroxynitrite, leading to BH4 oxidation and subsequent promotion of superoxide production by eNOS itself, so-called “eNOS uncoupling” (24,25). Uncoupled eNOS is detected in conditions that are associated with oxidant stress, hypertension, and diabetes (23).

Human DN progresses through several pathophysiologic stages, initially characterized by early hyperfiltration and hypertrophy followed by microalbuminuria and mesangial expansion, and then overt proteinuria, sclerosis, and a progressive decline of GFR (16,17). Early in diabetes, increased endothelial NO production may contribute to the observed hyperfiltration and microalbuminuria (10), but with advancing nephropathy, there is progressive NO deficiency. Hyperglycemia, advanced glycosylation end products, increased oxidant stress, endogenous inhibitors of NO such as asymmetric dimethylarginine, activation of protein kinase C, and TGF-β all are potential contributors to decreased NO production and/or eNOS uncoupling (26,27).

In humans, the eNOS gene is found on chromosome 7q. Genome-wide scans have indicated that regions on 7q, 18q, and 22q may influence proteinuria and/or development of DN in type 2 diabetes (27). Of note, evidence for a region on 7q overlapped all studies (28). Recent studies have reported an association between eNOS polymorphisms that lead to decreased eNOS expression and the development of advanced DN in both patients with type 1 (29–31) and with type 2 diabetes (32,33). Other studies also have found an association of these polymorphisms with nondiabetic causes of ESRD (9,34). However, not all studies have detected an association of disease with these eNOS polymorphisms (35–39).

Although short-term studies have examined the effect of administration of NOS inhibitors in experimental models of diabetes (10,40), we are not aware of any published studies to date that have examined long-term chronic administration (4 to 6 mo). Whereas administration of NOS inhibitors alone produces renal lesions that are consistent with ischemic nephropathy with minimal glomerulosclerosis (41), chronic administration of nonspecific NOS inhibitors does accelerate glomerulosclerosis in the remnant model of rat glomerulopathy (42), although similar studies are yet to be performed in mouse models. Therefore, it is possible that NOS inhibitors may impart a similar acceleration of injury in experimental diabetic models, although a potential advantage of the current genetic model in dissecting pathophysiologic mechanisms of DN is that eNOS is selectively deleted while other NOS isoforms remain active.

**Conclusion**

Our results demonstrate that eNOS−/− db/db mice exhibit significant albuminuria and glomerular pathology that parallel the later phase of DN in patients with type 2 diabetes and include arteriolar hyalinosis, mesangial expansion, thickening of GBM, and focal segmental and early nodular glomerulosclerosis. This model should prove useful for studying the role of endothelial dysfunction in development of DN and in facilitating the development of new diagnostic and therapeutic interventions.

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