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# Endothelial Nitric Oxide Synthase Deficiency Produces Accelerated Nephropathy in Diabetic Mice

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## Abstract

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$^{-/-}$ 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$^{-/-}$ C57BLKS/J *db/db* mice exhibited dramatic albuminuria, arteriolar hyalinosis, increased glomerular basement membrane thickness, mesangial expansion, mesangiolysis, and focal segmental and early nodular glomerulosclerosis. Even more remarkable, eNOS$^{-/-}$ C57BLKS *db/db* exhibited decreases in GFR to levels <50% of that in eNOS$^{+/+}$ C57BLKS *db/db*, as confirmed by increased serum creatinine. In summary, eNOS$^{-/-}$ *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.

Diabetic hyperglycemia causes microvascular dysfunction, which contributes to the development of ESRD (1–3). In recent years, studies have focused first on the mesangial cell and more recently on the podocyte as both initiators and targets of diabetic nephropathy (DN) (4–6). Although these cells undoubtedly are involved in the development of DN, there is equally compelling reason to consider a role for endothelial cell dysfunction in the initiation and propagation of the characteristic glomerular lesions that are seen in DN.

Endothelial cell–derived vasodilators, such as nitric oxide (NO), seem to be important modulators of permeability in the vasculature. Inhibition or genetic deletion of endothelial NO synthase (eNOS, NOS III) induces opening of the interendothelial junctions and increases vascular permeability in microvascular beds, suggesting that NO production may play an important role in regulating the endothelial barrier function (7,8).

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Functionally significant polymorphisms in eNOS have been recognized in human DN (9). In addition, vascular eNOS activity is altered in diabetes. NO products have been reported to be increased early after the onset of diabetes and may be involved in mediating renal vasodilation and hyperfiltration; however, with more prolonged diabetes, renal eNOS production decreases (10). The exact function of eNOS in the development of DN remains undetermined. These studies were designed not only to understand better the role of eNOS-mediated events in the development and progression of DN but also to determine whether eNOS deficiency results in a mouse model of DN that more closely approximates the functional and structural changes that are seen in human DN.

## Materials and Methods

### Animals

eNOS$^{+/-}$ mice initially on the C57/B6 background and *db* heterozygous mice on the C57BLKS/J (BKS) background were purchased from The Jackson Laboratory (Bar Harbor, ME). The eNOS$^{+/-}$ mice were backcrossed for 10 generations to the C57BLKS/J background and then crossed with *db* heterozygous mice. Genotyping was performed by PCR. Whenever possible, the same mice underwent all physiologic assessments (BP measurement, GFR, and albumin/creatinine ratio [ACR] measurement) and procurement of kidney tissue for histologic analysis.

### BP Measurement

Systolic BP (SBP) was measured in conscious, trained mice at room temperature using a tail-cuff monitor (BP-2000 BP Analysis system, Visitech System, NC).

### Determination of Blood Glucose and Creatinine

Blood glucose was determined using the OneTouch glucometer and test strips (LifeScan, Milpitas, CA). Serum creatinine was measured by a previously described HPLC method (11).

### Urine Albumin and Creatinine

Spot urine was collected from individually caged mice using polycarbonate metabolic cages. Urinary albumin and creatinine excretion was determined using Albuwell-M kits (Exocell Inc., Philadelphia, PA).

### 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-$\mu$m-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$^{-/-}$ mice were reported previously 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 (6*R*)-5,6,7,8-tetrahydro-L-biopterin ($BH_4$). NADPH oxidases are major sources of reactive oxygen species in endothelium and are activated in animal models of hypertension and diabetes. Superoxide reacts avidly with vascular NO· to form peroxynitrite, leading to $BH_4$ 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-$\beta$ 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 de creased 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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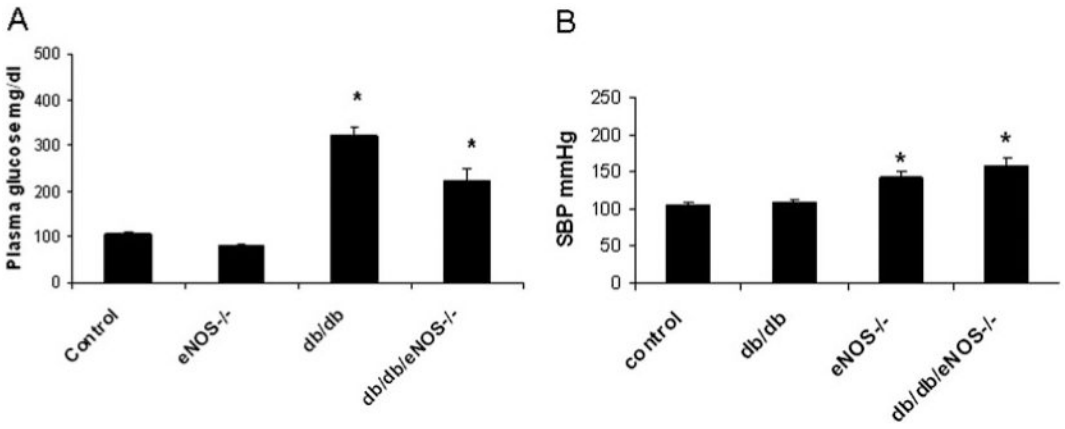


**Figure 1.**

Metabolic and physiologic parameters. (A) Fasting blood glucose at 24 to 26 wk in *db/db* mice and age-matched controls ± eNOS$^{-/-}$. *$P < 0.05$ *versus* control and eNOS$^{-/-}$. Values are means ± SE of at least eight mice. (B) Systolic BP. *$P < 0.05$ eNOS$^{-/-}$ or eNOS$^{-/-}$ *db/db versus* control or *db/db*. Values are means ± SE of at least four mice.

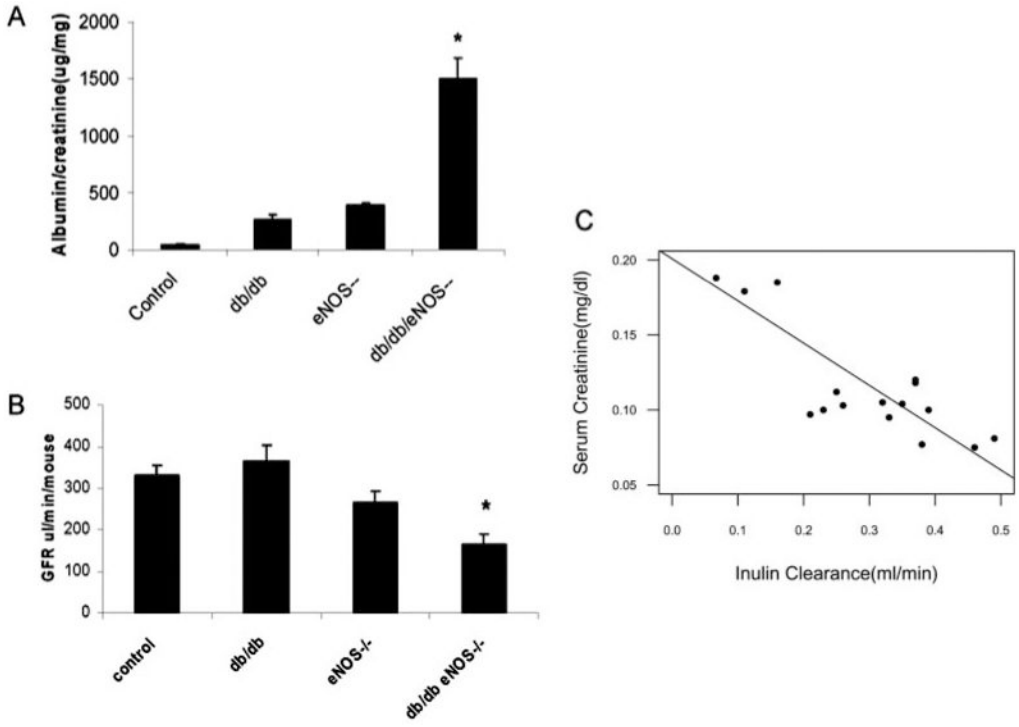


**Figure 2.**

Functional renal parameters. (A) Urinary albumin/creatinine ratio (ACR) at 24 to 26 wk of age. Values are means ± SE of at least eight mice. **P* < 0.05 *versus* respective controls, *db/db*, and eNOS$^{-/-}$. (B) GFR in diabetic mice. **P* < 0.05 *versus* control, *db/db*, and eNOS$^{-/-}$. Values are means ± SE of at least eight mice. (C) Correlation of serum creatinine with GFR.

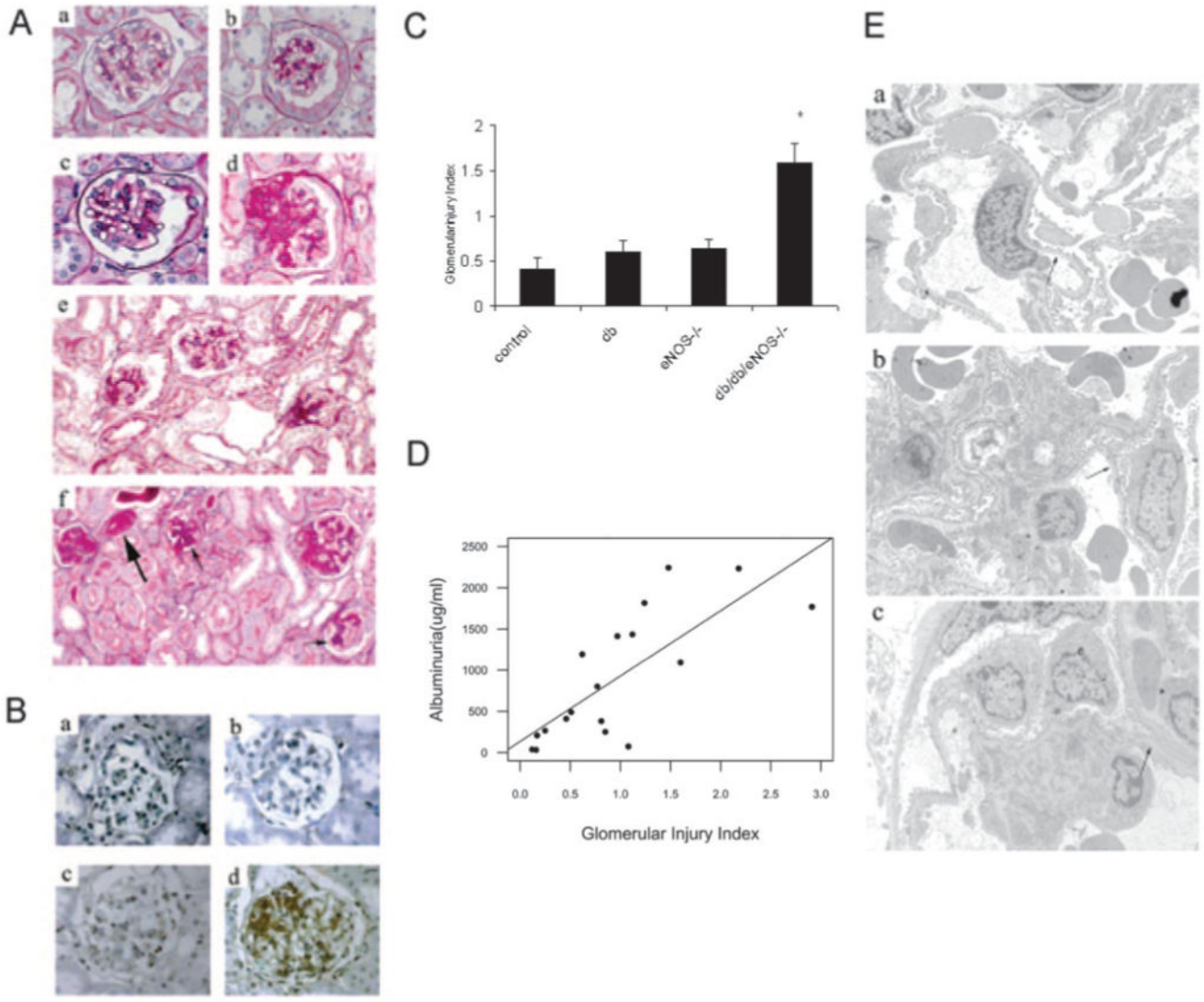


**Figure 3.**

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.