Nitric Oxide Synthase Isoforms and Glomerular Hyperfiltration in Early Diabetic Nephropathy

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Abstract. This study tested the hypothesis that nitric oxide (NO)-mediated renal vasodilation due to the activity of the inducible nitric oxide synthase (iNOS) contributes to glomerular hyperfiltration in diabetic rats. Two weeks after induction of diabetes mellitus by streptozotocin, mean arterial BP (MAP), GFR (inulin clearance), and renal plasma flow (RPF) (para-aminophenylurate clearance) were measured in conscious instrumented rats. Diabetic rats had elevated GFR (3129 ± 309 μl/min versus 2297 ± 264 μl/min in untreated control rats, P < 0.05) and RPF (10526 ± 679 μl/min versus 8005 ± 534 μl/min), which was prevented by chronic insulin treatment. Intravenous administration of 0.1 and 1 mg of L-imino-ethyl-lysine (L-NIL), an inhibitor of iNOS, did not affect MAP, GFR, or RPF, either in diabetic or control rats. A higher L-NIL dose (10 mg) increased MAP and decreased RPF in diabetic rats significantly (n = 6, P < 0.05), but not in controls (n = 6). In addition, 0.1 mg of N[^6]-nitro-L-arginine methyl ester (L-NAME), a nonselective blocker of NOS isoforms, decreased GFR (2389 ± 478 μl/min) and RPF (7691 ± 402 μl/min) in diabetic animals to control levels, while renal hemodynamics in normoglycemic rats were not altered. Higher L-NAME doses (1 and 10 mg) reduced GFR and RPF in diabetic and control rats to identical levels. In glomeruli isolated from diabetic and control rats, neither iNOS mRNA nor iNOS protein expression was detected. In contrast, increased protein levels of endothelial constitutive NOS (ecNOS) were found in glomeruli of diabetic rats compared with controls. By immunohistochemistry, ecNOS but not iNOS staining was observed in the endothelium of preglomerular vessels and in diabetic glomeruli. These results support the notion that increased NO availability due to greater abundance of ecNOS contributes to the pathogenesis of glomerular hyperfiltration in early experimental diabetic nephropathy. In contrast, we found no functional or molecular evidence for increased glomerular expression and activity of iNOS in diabetic rats.

Diabetic nephropathy is the most common single cause of end-stage renal disease in the United States and in Europe (1,2). Increases of renal perfusion and GFR occur early in the course of diabetic nephropathy, a feature seen in experimental and clinical diabetes. Micropuncture studies demonstrated renal vasodilation predominantly of the preglomerular or afferent resistance vessels as a cause of diabetic hyperfiltration (3). This was reported to be due to increased formation of nitric oxide (NO) (4,5). NO originates from L-arginine in a reaction catalyzed by several different nitric oxide synthase (NOS) isoenzymes. The endothelial constitutive NOS (ecNOS) is found in the vascular endothelium, depends on Ca^{2+} and calmodulin, and releases small amounts (pM) of NO. The inducible NOS (iNOS) can be expressed by various cell types, including macrophages, vascular smooth muscle cells, and glomerular mesangial cells, leading to the formation of large amounts of NO (nM), and can be induced by endotoxins and cytokines (6). It has been proposed that stimulation of the inducible isoform of NOS due to hyperglycemia may lead to increased generation of NO, which in turn contributes to diabetic hyperfiltration and glomerular abnormalities in diabetes (7,8).

This study in rats was designed to test the hypothesis that induction of iNOS in glomeruli or preglomerular vessels contributes to glomerular hyperfiltration in diabetes mellitus caused by streptozotocin. We investigated the renal functional effects of L-iminoethyl-lysine (L-NIL), which is a 30-fold more potent inhibitor of iNOS than of ecNOS (9,10). For comparison, we also used different doses of N[^6]-nitro-L-arginine methyl ester (L-NAME), a nonselective blocker of all NOS isoforms. In addition, we investigated the glomerular expression of iNOS and ecNOS in kidney sections and isolated glomeruli.

Materials and Methods

Animals

Studies were performed in male Sprague Dawley rats (Charles River, Sulzfeld, Germany) of 230 to 290 g body wt. All experiments in animals were performed according to the guidelines of the American Physiological Society and were approved by the local government’s animal research ethics committee (Regierung von Mittelfranken, AZ 621-2531.31-19/96). Diabetes was induced by intraperitoneal injection of streptozotocin (Sigma, Deisenhofen, Germany) (65 mg/kg body wt) dissolved in 0.1 M sodium citrate buffer, pH 4.5. Two days later, blood was drawn from the retro-orbital plexus, and the diabetic state was confirmed by measurement of blood glucose, using a reflectance meter (Glucometer Elite II; Bayer, Leverkusen, Germany).
Only rats with blood glucose levels >250 mg/dl were included. Diabetic and control rats were followed for 2 wk; in addition, some diabetic rats were also treated with insulin to achieve good metabolic control (blood glucose <150 mg/dl). For insulin treatment, diabetic rats received insulin pellets (Møllegaard, Ejby, Denmark) in doses adjusted to maintain glucose levels in the previously defined range. Blood glucose was monitored weekly (at 8 a.m.) in all diabetic rats. All rats received standard chow (no. 1320; Altromin, Lage, Germany) and tap water ad libitum. Rats used for renal hemodynamic measurements were placed in metabolic cages for 2 d to determine urinary albumin excretion rates.

Renal Hemodynamics

Two weeks after successful streptozotocin injection, measurements of GFR and renal plasma flow (RPF) were performed as described previously (11). Briefly, femoral arterial and venous catheters, as well as a bladder fistula, were implanted under intraperitoneal methohexital (50 mg/kg) anesthesia. The animals were then placed in a restrainer and allowed to recover for at least 4 h. In conscious rats, continuous infusions of inulin and para-aminohippurate in saline were started, and MAP was monitored by pressure transducers connected to a polygraph (Grass Medical Instruments, Quincy, MA). The infusion rate was set at 62.5 μl/min in control and normoglycemic diabetic animals. To account for polyuria, extra saline was administered to diabetic animals to match the urinary losses during the procedure. After 2 h, urine samples were collected for determination of flow rate. Urine volume was measured gravimetrically. Steady-state conditions were assumed when diuresis matched infusion rate during three subsequent 10-min intervals. After reaching steady-state conditions, arterial blood was drawn for measurements of hematocrit, sodium, potassium, chloride, as well as inulin and para-aminohippurate to determine GFR and RPF.

Subsequently, diabetic rats received 0.1, 1, or 10 mg (six rats per dose) of l-NAME (Alexis, Grünberg, Germany) (9,10,12). Six nondiabetic control rats received 10 mg of l-NAME. Measurements of GFR and RPF were performed before and after intravenous bolus injection of the putative iNOS inhibitor at the following time points: −20 min, −10 min, +10 min, +20 min, +40 min, +60 min, and +120 min.

In addition, 0.1, 1, and 10 mg of the nonselective NOS inhibitor l-NAME (Sigma) were administered to six diabetic and six nondiabetic control rats, as described for l-NAME-treated animals. GFR and RPF were measured before the injection of each dose as well as 15, 45, and 120 min after administration.

Measurements of para-aminohippurate and inulin, as well as calculation of GFR and RPF, were performed as described previously (11).

Extraction of RNA and Protein from Isolated Glomeruli

Nine diabetic and six nondiabetic control animals that did not undergo renal hemodynamic studies were sacrificed for the assessment of glomerular NOS RNA and protein. Rats were killed in deep thiobarbital anesthesia (100 mg/kg body wt, intraperitoneally), and the left kidney was snap-frozen in liquid nitrogen for immunohistochemistry. The right kidney was used for isolation of glomeruli as described previously (13,14). Glomerular RNA and protein were extracted using TRI-Reagent (Molecular Research Center, Inc., Cincinnati, OH) (15). Two different positive controls were used for iNOS detection. First, three rats were sacrificed 4 h after an intraperitoneal injection of lipopolysaccharides (LPS) (3 mg/kg body wt; Sigma), which induces iNOS expression in the kidney (16). Second, we used six rats that were treated orally with a 3.7 nmol/L (10 mg%) solution of l-NAME (17) and had undergone surgery (arterial and venous catheters) 1 d before sacrifice. Others had previously reported induction of renal iNOS by surgery (18), and increased expression of iNOS in gut mucosa after l-NAME treatment (19). We have confirmed renal glomerular induction of iNOS RNA and protein after surgery in l-NAME-treated rats (see Results).

Northern Blot Analysis

Total RNA was size-fractionated on a 1% agarose formaldehyde gel and transferred onto Hybond nylon membranes (Amersham, Braunschweig, Germany). The blot was baked at 80°C for 2 h, prehybridized with 5× Denhardt’s solution, 5× SSC, 50% formamide, 50 mM Na2PO4, 0.1% SDS, and 0.25 mg/ml salmon sperm DNA at 40°C for 2 h. DNA hybridization probes were labeled with α-[32P]-dCTP, using a random-primed labeling kit (Boehringer, Mannheim, Germany). Blots were hybridized in prehybridization solution containing 2×106 cpm/ml of probe at 40°C overnight. Blots were washed twice for 15 min with 2× SSC containing 0.1% SDS and then 30 min with 0.1× SSC containing 0.2% SDS. Blots were exposed to Kodak XAR-2 films with intensifying screens at −80°C. For hybridization with iNOS cDNA, a 611-bp EcoRI/PstI cut insert of the murine iNOS cDNA clone piNOS B2 was used, as described previously (20). As a probe for GAPDH, a 470-bp fragment corresponding to nucleotides 510 to 980 of the rat GAPDH sequence (GenBank accession no. X022331) was amplified using the primers and PCR conditions specified below.

Reverse Transcription-PCR

For reverse transcription (RT), 1 μg of total glomerular RNA was reverse-transcribed by Moloney murine leukemia virus reverse transcriptase using oligo-dT. Five microliters of a 20-μl RT reaction were used for subsequent PCR. PCR for iNOS was performed by 35 PCR cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min in a Touchdown thermocycler (Hybaid, London, United Kingdom). The primers 5′-CCG GAT CCT CTT TGC TAC TGA GAC AGC-3′ and 5′-CCG ATT TCG GGA TCT GAA TGC AAT GTT-3′, corresponding to nucleotides 1613–1634 and 2057–2078 of the full-length rat iNOS cDNA, yielded a 465-bp product (21). To exclude amplification of genomic DNA, primers were selected to be localized in different exons. Because the genomic organization of the rat iNOS gene was not available, primer selection was based on the assumption of structural homology between rat and human iNOS genes. If this assumption holds true, the sense primer would be localized in exon 14, and the antisense primer in exon 18 (22), approximately 7 kb distant from each other. For GAPDH, the primers 5′-AAT GCA TCC TGC ACC ACC AA-3′ and 5′-GTC ATT GAG AGC AAT GCC AGC-3′ were used in 22 PCR cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, as described previously (20).

Western Blot Analysis

Protein samples containing 20 μg of total protein were denatured by boiling for 5 min and were separated on an 8% denaturing SDS-PAGE gel. After electrophoresis, the gels were electroblotted onto polyvinylidene difluoride membranes (Pall Filtron, Karlstein, Germany), blocked with 3% bovine serum albumin (BSA)/Tris-buffered saline (TBS)/0.2% NaN3/0.1% Tween 20 overnight, and incubated with the primary antibodies for 2 h. Antibodies N 32030 and N 30020 from Transduction Laboratories (Lexington, KY) were used to detect iNOS and ecNOS, respectively. NOS protein was visualized with a
secondary horseradish peroxidase-conjugated anti-rabbit or antimouse IgG antibody, respectively, using the enhanced chemiluminescence system (Amersham).

**Immunolocalization**

Immunohistochemical analysis for iNOS and ecNOS in renal tissue sections was performed using the APAAP technique as described (23). Five-micrometer cryostat kidney sections were air-dried for 10 min, fixed in cold acetone for 10 min, washed twice in TBS/1% BSA, and incubated with 100% fetal calf serum for 30 min at 37°C. Sections were then layered with the primary antibodies and incubated at 4°C overnight. As a negative control, equimolar concentrations of preimmune rabbit IgG was used. After rinsing in TBS/1% BSA, the sections were incubated with secondary antibodies mouse anti-rabbit and subsequently rabbit anti-mouse (M 737 and Z 456; both from Dako, Hamburg, Germany) for 1 h at 37°C each. Sections were then layered with the APAAP complex (D 651; Dako) for 30 min at room temperature. A Neufuchsin kit (K 698; Dako) was used as a chromogen. Finally, sections were counterstained with hematoxylin and embedded in Aquatex (Merck, Darmstadt, Germany). For immunofluorescence detection of ecNOS, an analogous protocol was used, except that sections were incubated with CY2-labeled goat anti-mouse IgG secondary antibody from Dianova (Hamburg, Germany) for 2 h. Washed sections were then covered with Tris-buffered mowiol, pH 8.6 (Hoechst, Frankfurt am Main, Germany). Microscopy was performed using a Leitz Aristoplan microscope with a ×40 Fluotar objective (Leica Instruments, Nussloch, Germany). For CY2 fluorescence detection, 520-nm emission filters were used.

**Statistical Analyses**

Statistical analyses were performed using the SAS software package (SAS Institute, Cary, NC). For normally distributed data, t test was used; otherwise nonparametric methods were applied. Data are given as mean ± SEM. A P value < 0.05 was considered significant.

**Results**

After 2 wk of diabetes, baseline characteristics differed significantly between the investigated groups. Diabetic rats receiving no insulin had greater kidney weights and a higher kidney:body weight ratio (Table 1). Blood glucose, diuresis, and albuminuria were markedly elevated in diabetic animals, whereas MAP did not differ between the groups (114 ± 7 mmHg versus 118 ± 6 mmHg) (Figure 1). In accordance with previous reports, diabetic rats exhibited renal hyperperfusion and glomerular hyperfiltration (Table 1, Figures 1 and 2) that was abolished by insulin treatment.

Administration of the iNOS inhibitor L-NIL in doses of 0.1 and 1 mg caused no changes of systemic or glomerular hemodynamics in diabetic rats (Figure 1). Only use of the highest dose of L-NIL (10 mg) induced a mild yet significant increase of MAP and significantly reduced RPF from 10360 to 7260 ml/min (Figure 1). This maximal dose of L-NIL had no effect on glomerular hemodynamics in control rats.

L-NAME treatment caused significant changes of GFR, RPF, and MAP (Figure 2). In diabetic rats, even the lowest dose of L-NAME (0.1 mg) decreased GFR and RPF significantly, reducing renal hemodynamics to control values. This low dose of L-NAME had no effect in control rats. After the two higher doses of L-NAME, GFR and RPF (Figure 2) de-
clined in diabetic and control rats, showing no further differences between the groups. One milligram of L-NAME increased MAP significantly by 15 ± 6 mmHg in diabetic animals and by 13 ± 5 mmHg in controls, whereas 0.1 mg of L-NAME had no effect on MAP in either group. Ten milligrams of L-NAME increased MAP significantly by 25 ± 4 mmHg in diabetic animals and by 30 ± 4 mmHg in controls.

In RNA extracted from isolated glomeruli of diabetic and control rats, iNOS mRNA could not be detected either by Northern blot (data not shown) or by RT-PCR (Figure 3). Control hybridization or RT-PCR amplification for the housekeeping gene GAPDH was positive in all glomerular RNA samples used (data not shown), excluding RNA degradation as a reason for the negative results on iNOS. In contrast, iNOS mRNA was detectable by RT-PCR in glomeruli from control rats treated with LPS 24 h before sacrifice. Positive results were also obtained from chronically L-NAME-treated animals that had undergone surgery the day before (Figure 3). A second, slightly larger amplicon (approximately 550 bp) was also detected in positive control samples. Contamination with genomic DNA cannot explain this finding, as evidenced by the lack of an amplicon in reactions without RT (Figure 3, lane 6) and the fact that genomic DNA should yield a 7-kb product (22). We can only speculate that alternative splicing might be responsible for a larger iNOS transcript (24).

Similarly, by Western blot, iNOS protein could not be de-

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**Figure 1.** Mean arterial pressure (MAP), GFR, and renal plasma flow (RPF) in streptozotocin (STZ) diabetic and control animals before and after treatment with three different doses of L-arginine-ethyl-lysine (L-NIL) (intravenous bolus). Only the highest dose of L-NIL decreased RPF and increased MAP slightly in diabetic animals. Data are given as mean ± SEM.
tected in diabetic and control rats but was detected in positive controls (Figure 4). Furthermore, immunohistochemistry revealed no iNOS staining in diabetic and control rat glomeruli, whereas marked glomerular iNOS staining was present in both types of positive control, i.e., in rats pretreated with LPS or L-NAME (Figure 5). Here, glomerular immunostaining for iNOS was uniformly diffuse in a predominantly mesangial pattern.

In contrast to iNOS, ecNOS protein was detectable in glomeruli from diabetic as well as control animals (Figure 6). Compared with controls, ecNOS protein was clearly elevated in diabetic rat glomeruli (Figure 6). Western blotting was performed in three groups of diabetic rats and controls, and the increase of ecNOS levels in diabetic rat glomeruli was averaged 174% over control, determined by densitometry. By immunohistochemistry and immunofluorescence microscopy, ecNOS protein in glomeruli appeared to be localized to the endothelium of preglomerular vessels and glomerular tufts. Positive ecNOS staining was more abundant in diabetic than in control rat glomeruli (Figure 7).

**Discussion**

The results of our combined functional and molecular experiments suggest that early glomerular hyperfiltration in streptozotocin diabetic rats is dependent on increased NO generation due to greater expression and activity of ecNOS in glomeruli and afferent arterioles. The data of our in vivo investigations strongly argue against the assumption that the inducible form of NO synthases is of importance for the glomerular hyperfiltration observed in the streptozotocin model of diabetes mellitus. If our data are confirmed in other models of diabetes mellitus, selective iNOS inhibitors, which are currently developed for experimental and potential clinical
use (10), will be of no use to normalize renal hemodynamics in early diabetic nephropathy. It may prove useful to investigate factors that regulate the expression and/or activity of ecNOS (25), in addition to its well known activation by the calcium-calmodulin pathway (26). We confirmed that 2 wk of streptozotocin diabetes led to renal hypertrophy, increased albuminuria, renal hyperperfusion, and glomerular hyperfiltration, as described previously (9). These changes were clearly due to the diabetic state and not to streptozotocin itself, as shown by insulin treatment, which normalized GFR and RPF.

Our data confirm the important role of NO in the pathogenesis of glomerular hyperfiltration in early diabetes mellitus: Acute inhibition of NOS with a low dose of the nonselective NOS inhibitor (l-NAME) decreased GFR and RPF significantly in diabetic animals back to normal control values, whereas this dose did not affect renal hemodynamics in control rats. This finding indicates that glomerular hyperfiltration is markedly dependent on increased NO generation and action at this early stage of experimental diabetic nephropathy. These results agree with previous reports, which postulated a significant role of NO in diabetic hyperfiltration (4,5,27,28), even though the involvement of specific NOS isoforms in this process remains unclear.

To test the potential contribution of iNOS to NO-mediated glomerular hyperfiltration, we performed experiments with a relatively selective iNOS blocker, l-NIL. We found that in awake rats, selective iNOS inhibition with doses up to 1 mg (approximately 4 mg/kg body wt) had no effect on renal

Figure 5. Immunohistochemistry of cryostat kidney sections for iNOS protein; positive staining in red, counterstain with hematoxylin. Staining for iNOS was evident in positive controls: lipopolysaccharide-treated rats (A) and l-NAME-treated rats 24 h after surgery (B). Glomerular staining pattern was diffuse with apparent mesangial predominance. In contrast, glomerular iNOS staining was not observed in glomeruli of diabetic (C) or control (D) rats. Magnification, ×400.
hemodynamics and BP (Figure 1). Studies of others had shown that this dose was sufficient to completely block iNOS activity (9). However, the highest dose of L-NIL (10 mg) significantly decreased RPF (with no statistically significant change of GFR) and increased BP. This effect of the highest dose of L-NIL is most likely explained by nonselective inhibition of all NOS isoforms, because L-NIL is only an approximately 30 times more specific inhibitor of iNOS than of eNOS (10). A 100-fold lower dose of the nonselective inhibitor L-NAME (0.1 mg L-NAME versus 10 mg L-NIL), however, exerted similar or greater effects on renal hemodynamics than 10 mg of L-NIL. Recently, Schwartz et al. reported that in conscious LPS-treated rats, the glomerular effects of increased NO production by iNOS could be prevented when the rats had been pretreated with 3 mg/kg L-NIL intraperitoneally (16). BP and heart rate were not affected. These observations indicated that doses of L-NIL, which correspond to the middle dose of our dose–response curve established after intravenous application of the inhibitor, are able to block the effects of iNOS in the rat kidney.

Aminoguanidine, an inhibitor of nitric oxide synthase (NOS) and of advanced glycation end products, has been described to attenuate the progression of renal disease in experimental diabetes (29). The results of this study showed that the protective effects of aminoguanidine were not due to inhibition of iNOS but rather to inhibition of advanced glycation end product formation. These observations and our results would argue against a significant involvement of iNOS in the functional changes of the kidney in early and late diabetes mellitus.

We did not detect iNOS mRNA or protein in glomeruli of diabetic rats. Although we cannot completely exclude the possibility that the methods we used were not sufficiently sensitive to detect iNOS in diabetic animals, we consider this quite unlikely for the following reasons: (1) We used several independent methods (Northern blot, RT-PCR, Western blot, immunohistochemistry) on tissue of diabetic animals and positive control tissue of LPS-treated rats and after iNOS induction by L-NAME; (2) We were able to detect iNOS mRNA and protein with each method in positive control tissue of the same organ (kidney) that had undergone the same extraction procedures (isolation of glomeruli, RNA and protein, or snap-freezing for immunohistochemistry). This finding is in agreement with previous reports showing the inducing effects of LPS treatment (16) or surgery (18) on iNOS expression and the effects of L-NAME on gut mucosa iNOS expression (19). The induction of iNOS under these experimental circumstances has substantial relevance, because both nonsterile surgery and L-NAME treatment are commonly performed by researchers concerned with NOS, and the results obtained may be confounded by artifactual iNOS induction. In a recent study, iNOS and ecNOS protein expression was reported to be increased in kidneys of rats with streptozotocin-induced diabetes mellitus (30). These investigations, however, were limited to Western blot analyses of total renal cortex and no data were provided on isolated glomeruli. Furthermore, it is unclear whether the observed increase of iNOS protein expression was effected by nonsterile surgery in anesthetized rats.

In contrast to iNOS, we found increased amounts of ecNOS in glomeruli of diabetic rats. Besides its well known regulation by calcium, ecNOS can be regulated by phosphorylation (26) or translocation (31). In addition, expression control of ecNOS has been recently outlined in various tissues (25). For example, vascular endothelial growth factor, which is increased in the hyperglycemic state (32), can stimulate the expression of ecNOS (33). By immunohistochemistry, we could localize ecNOS to the endothelium of preglomerular arteries and arterioles as well as to the glomerular tuft. In light of the hemodynamic effects of L-NAME observed by us (Figure 2) and others (27), our findings suggest a key role for ecNOS in glomerular hyperfiltration observed in early streptozotocin-induced diabetes mellitus. During the preparation of this manuscript, it was reported that increased ecNOS production was detected in the afferent arteries of glomeruli in rats with experimental diabetes mellitus in the first 4 wk of disease development (34). It should be noted, however, that the importance of NO for glomerular hemodynamics may decrease over time in experimental diabetes mellitus (35). It has been suggested that this phenomenon could be due to progressive decline of NO-dependent cGMP generation (36).

In conclusion, the present results confirm that NO plays an important role for the development of glomerular hyperfiltra-

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**Figure 6.** Western blot for endothelial constitutive NOS (ecNOS) protein. (Top Panel) Western blot. (Bottom Panel) Densitometric evaluation. Lanes 1 through 3, glomerular protein from individual diabetic animals; lanes 4 and 5, glomerular protein from control rats; lane 6, positive ecNOS control (protein from cultured rat aortic endothelial cells).

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tion in early diabetic nephropathy of rats. Our findings did not reveal evidence for the induction of iNOS in glomeruli of diabetic rats. Instead, we observed that greater glomerular abundance of ecNOS causing increased generation of NO contributed to glomerular hyperfiltration. The mechanisms leading to increased levels of ecNOS in glomeruli of diabetic rats and their potential relevance for the initiation and/or progression of diabetic nephropathy require further study.

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**References**


*Figure 7.* Immunofluorescence microscopy (A and B) and immunohistochemistry (C and D) for ecNOS protein in cryostat kidney sections. (A and C) Diabetic animals. (B and D) Control animals. Positive staining in preglomerular and glomerular vessels was more extensive in diabetic than in control rats. Usually, ecNOS staining extended further into glomerular tufts of diabetic animals (A and C) compared with controls (B and D). The askerisk denotes the vascular pole of glomeruli. Dotted lines in A and B indicate the circumference of Bowman’s capsule. Magnification, ×400.


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