Role of neuronal nitric oxide synthase (NOS1) in the pathogenesis of renal hemodynamic changes in diabetes

RADKO KOMERS, JESSIE N. LINDSLEY, TERRY T. OYAMA, KRISTEN M. ALLISON, AND SHARON ANDERSON
Division of Nephrology and Hypertension, Oregon Health Sciences University, and Portland Veterans Affairs Medical Center, Portland, Oregon 97201

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Komers, Radko, Jessie N. Lindsley, Terry T. Oyama, Kristen M. Allison, and Sharon Anderson. Role of neuronal nitric oxide synthase (NOS1) in the pathogenesis of renal hemodynamic changes in diabetes. Am J Physiol Renal Physiol 279:F573–F583, 2000.—Nitric oxide (NO) has been implicated in the pathogenesis of renal hemodynamic changes in diabetes mellitus. However, the contribution of nitric oxide synthase (NOS) isoforms to intrarenal production of NO in diabetes remains unknown. To explore the role of NOS1 in the control of renal hemodynamics in diabetes, we assessed renal responses to inhibition of NOS1 with L-NAME induced a further decrease in arterial pressure and renal hemodynamics in streptozotocin-diabetic rats. We found that the systemic administration of the specific, water-soluble, NOS1 inhibitor L-NMMA in SMTC-treated diabetic rats. The number of NOS1-positive cells in macula densa of D and C kidneys was also evaluated by immunohistochemistry. D rats demonstrated elevated glomerular filtration rate (GFR) compared with C. SMTC (0.05 mg/kg) normalized GFR in D but had no effect in C. SMTC-induced reduction of renal plasma flow (RPF) was similar in C and D. Normoglycemic diabetic rats demonstrated blunted renal hemodynamic responses to NOS1 inhibition compared with hyperglycemic animals. Mean arterial pressure was stable in all groups. L-NAME induced a further decrease in RPF, but not in GFR, in D rats treated with SMTC. Immunohistochemistry revealed increased numbers of NOS1-positive cells in D. These observations suggest that NOS1-derived NO plays a major role in the pathogenesis of renal hemodynamic changes early in the course of diabetes. NOS1 appears to be the most important isoform in the generation of hemodynamically active NO in this condition.

Address for reprint requests and other correspondence: S. Anderson, Div. of Nephrology and Hypertension, Oregon Health Sciences Univ., PP262, 3314 SW US Veterans Hospital Road, Portland, OR 97201-2940 (E-mail: anderssh@ohsu.edu).

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possible that the renal response to SMTC was modulated by extrarenal factors and did not reflect “pure” effects of NOS1 inhibition in the kidney. This phenomenon was previously described in studies with nonspecific NOS inhibitors (15), showing markedly different renal hemodynamic effects of systemic and local NOS inhibition.

These studies were designed to further explore the hypothesis that NOS1-mediated NO production in MD is elevated in diabetes, resulting in a decrease in preglomerular vascular resistance and ultimately in hyperfiltration. To address this issue, we assessed renal hemodynamic responses to inhibition of NOS1 with SMTC in moderately hyperglycemic diabetic rats and their nondiabetic, and normoglycemic diabetic counterparts. To diminish possible effects of systemic NOS1 inhibition, the inhibitor was administered into the abdominal aorta above the left renal artery. Furthermore, the possible contribution of other NOS isoforms to alterations in renal hemodynamics in diabetes was also evaluated by assessing the responses to nonspecific NOS inhibition in SMTC-treated diabetic rats. In addition to renal functional studies, the number of NOS1-positive cells in MD regions of diabetic and control kidneys was evaluated by immunohistochemistry.

STUDY DESIGN AND METHODS

The diabetic rat model. Studies were conducted in adult male Sprague-Dawley rats, with initial weights of ~300 g. The rats were made diabetic by intraperitoneal injection of streptozotocin (Sigma, St. Louis, MO), 65 mg/kg body wt. Two days later, induction of diabetes was confirmed by measurements of tail blood glucose (BG) level by using a reflectance meter (One Touch II; Lifescan, Milpitas, CA). Diabetic rats (D) received daily evening injections of ultralente insulin (Humulin II, Eli Lilly, Indianapolis, IN) in doses individually adjusted to maintain BG levels between 200 and 300 mg/dl (11–17 mmol/l). BG levels were monitored at least weekly in all diabetic rats. Weight-matched nondiabetic rats served as controls (C). All rats were fed standard rat chow (Rodent Laboratory Chow 5001, Ralston Purina, Richmond, IN) ad libitum. These studies were approved by the Portland Veterans Affairs Institutional Animal Care and Use Subcommittee.

Protocol 1: Effects of NOS1 (nNOS) inhibition in control and diabetic rats. These studies were performed to assess the acute renal hemodynamic effects of NOS1 inhibition with SMTC (Alexis, San Diego, CA), administrated into the abdominal aorta, in experimental diabetes. The diabetic rats were studied after 4–5 wk of diabetes. BG level was measured in the morning preceding the experiment (basal BG) and at midtime of the first clearance period after baseline measurements (experimental BG). After the surgical preparation described below, and after 75 min of equilibration, all rats underwent baseline measurements of mean arterial pressure (MAP), glomerular filtration rate (GFR), renal plasma flow (RPF), filtration fraction (FF), renal vascular resistance (RVR), urinary flow rate (UF), and urinary sodium (UNaV). Thereafter, control and diabetic rats were randomized to receive either SMTC (C-SMTC, D-SMTC; 0.05 mg/kg body wt into the abdominal aorta, in ~60 μl of 0.9% NaCl) or the same volume of vehicle (C-VE, D-VE; vehicle 1), and all measurements were repeated to assess changes from baseline. The dose of SMTC was determined during the pilot studies and represents the highest dose of the substance that is not associated with an increase in MAP. After completion of baseline measurements, we assessed whether further renal hemodynamic changes can be achieved with the higher dose of SMTC (0.5 mg/kg body wt) despite the rise in renal perfusion pressure. The measurements were performed in a similar manner as described above including the effects of vehicle alone (vehicle 2). Furthermore, an additional group of diabetic rats (D-SMTC-INS) was studied in a similar manner as above, to explore the role of hyperglycemia and/or insulinopenia in renal responses to NOS1 inhibition in diabetes. Preceding renal functional studies, BG levels in these rats were acutely normalized by intraperitoneal injection of 0.5–1.0 U of insulin. The dose of insulin was adjusted according to the morning BG level. During experimentation, BG levels were analyzed at least every 60 min, and only those rats that remained normoglycemic throughout the procedure were included in the analysis.

Protocol 2: Renal effects of nonspecific NOS inhibition in diabetic rats treated with SMTC. As already stated, the contribution of individual isoforms to altered renal hemodynamics and NO overproduction in diabetes is unknown. Analysis of further changes in renal hemodynamics imposed by nonspecific NOS inhibition on the changes induced by NOS1 blockade would provide information whether NOS1 is a major determinant of diabetic hyperfiltration or whether NOS1 activities of other NOS isoforms contribute to altered renal hemodynamics in this disorder. These experiments were designed to address this issue. Groups of diabetic rats underwent baseline measurements and measurements assessing the changes from baseline in response to the lower dose of SMTC (0.05 mg/body wt), as described in Protocol 1: Effects of NOS1 (nNOS) inhibition in control and diabetic rats. Thereafter, the rats were administered with a bolus intra-aortic injection of the nonspecific NOS inhibitor N^5-nitro-l-arginine methyl ester (l-NAME; D-SMTC-NAME; 0.02 mg/kg body wt in 60 μl of 0.9% NaCl, Sigma) or the same volume of vehicle (D-SMTC-VE). The dose of l-NAME was titrated during the pilot studies to induce no further changes in MAP compared with SMTC.

Surgical preparation and functional studies. Rats were anesthetized with Inactin (100 mg/kg ip; Research Biochemi- cals International, Natick, MA) and placed on a temperature-regulated table. The left femoral artery was catheterized with a PE-10 catheter fused to PE-50. The tip of the catheter was, under visual control, fed retrogradely to the abdominal aorta and inserted 1–2 mm above the left femoral artery. A baseline blood collection was obtained for measurements of hematocrit (Hct) and insulin and para-aminohippurate (PAH) “blanks.” This arterial catheter was used for subsequent periodic blood sampling and measurement of MAP via an electronic transducer connected to a direct-writing recorder. After tracheostomy, venous catheters were inserted for infusions of insulin, PAH, and plasma. Intravenous infusions of rat plasma and 10% insulin solution in 0.9% NaCl were started at rates of 6.0 and 1.2 ml/h, respectively. PAH was added to the insulin solution at a concentration of 0.8%. The left ureter was catheterized for urine collection. Euvolemia was maintained by infusing isooncotic rat serum at 6 ml/h in a total amount equal to 1% of body wt, followed by a reduction in infusion rate to 1.6 ml·kg⁻¹·h⁻¹ to maintain Hct constant. Diabetic rats received extra saline to match the excessive urinary losses during the procedure. Timed samples of urine (~15 min) were collected for determinations of flow rate and insulin and PAH concentrations. Arterial blood samples were taken at midtime of each period for determinations of Hct and plasma concentrations of insulin and PAH. These mea-
Table 1. General characteristics of control and diabetic rats

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>BWT, g</th>
<th>LKW, g</th>
<th>LKW/BWT, %</th>
<th>BG Basal, mg/100 ml</th>
<th>BG III, mg/100 ml</th>
<th>HBA1c, %</th>
<th>Hct</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-VE</td>
<td>8</td>
<td>347 ± 11</td>
<td>1.23 ± 0.05</td>
<td>0.35 ± 0.01</td>
<td>89 ± 5</td>
<td>4.8 ± 0.7</td>
<td>0.44 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>C-SMTC</td>
<td>12</td>
<td>343 ± 4</td>
<td>1.24 ± 0.04</td>
<td>0.36 ± 0.01</td>
<td>86 ± 4</td>
<td>5.5 ± 0.4</td>
<td>0.44 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>D-VE</td>
<td>8</td>
<td>351 ± 11</td>
<td>1.79 ± 0.13*</td>
<td>0.51 ± 0.03*</td>
<td>282 ± 26*</td>
<td>11.6 ± 0.9*</td>
<td>0.43 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>D-SMTC</td>
<td>8</td>
<td>333 ± 6</td>
<td>1.62 ± 0.07*</td>
<td>0.46 ± 0.02*</td>
<td>279 ± 15*</td>
<td>10.4 ± 0.9*</td>
<td>0.44 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>D-SMTC-INS</td>
<td>5</td>
<td>323 ± 10</td>
<td>1.64 ± 0.12*</td>
<td>0.50 ± 0.02*</td>
<td>107 ± 5*</td>
<td>13.1 ± 1.7*</td>
<td>0.43 ± 0.01</td>
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<tr>
<td>D-SMTC-VE</td>
<td>8</td>
<td>337 ± 7</td>
<td>1.87 ± 0.06*</td>
<td>0.55 ± 0.02*</td>
<td>260 ± 21*</td>
<td>11.8 ± 1.7*</td>
<td>0.43 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>D-SMTC-NAME</td>
<td>8</td>
<td>350 ± 6</td>
<td>1.70 ± 0.08*</td>
<td>0.50 ± 0.03*</td>
<td>268 ± 21*</td>
<td>10.8 ± 0.9*</td>
<td>0.43 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of rats; C-VE, C-SMTC, control rats treated with vehicle or S-methyl-l-thiocitrulline (SMTC); D-VE, D-SMTC, diabetic rats treated with vehicle or SMTC; D-SMTC-INS, diabetic rats treated with vehicle or SMTC and insulin; D-SMTC-VE, D-SMTC-NAME, diabetic rats treated with SMTC (0.05 mg/kg body wt) followed by vehicle or N⁰-nitro-l-arginine methyl ester (l-NAME); BWT, body weight; LKW, left kidney weight; LKW/BWT, left kidney weight-to-body weight ratio; BG, blood glucose; BG III, blood glucose level determined during the 3rd clearance period; Hct, hematocrit. *P < 0.001 vs. control groups. †P < 0.01 vs. diabetic groups.
Table 2. Effects of local NOS1 inhibition on mean arterial pressure, renal excretory functions, and plasma renin concentration in control and diabetic rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>MAP, mmHg</th>
<th>UF, µl/min</th>
<th>UNaV, µmol/min</th>
<th>PRC, ng ANG 1·ml⁻¹·h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-VE Basal</td>
<td>119 ± 6</td>
<td>9.4 ± 1.7</td>
<td>1.15 ± 0.37</td>
<td></td>
</tr>
<tr>
<td>Vehicle 1</td>
<td>121 ± 6</td>
<td>9.3 ± 1.4</td>
<td>1.18 ± 0.36</td>
<td></td>
</tr>
<tr>
<td>Vehicle 2</td>
<td>119 ± 6</td>
<td>9.3 ± 1.5</td>
<td>1.25 ± 0.37</td>
<td>124 ± 20</td>
</tr>
<tr>
<td>C-SMTC Basal</td>
<td>121 ± 3</td>
<td>13.6 ± 2.1</td>
<td>1.84 ± 0.43</td>
<td></td>
</tr>
<tr>
<td>SMTC 0.05</td>
<td>123 ± 3</td>
<td>12.4 ± 1.8</td>
<td>1.77 ± 0.38</td>
<td></td>
</tr>
<tr>
<td>SMTC 0.5</td>
<td>129 ± 4</td>
<td>11.5 ± 1.6</td>
<td>1.68 ± 0.37</td>
<td>86 ± 13</td>
</tr>
<tr>
<td>D-VE Basal</td>
<td>116 ± 6</td>
<td>22.3 ± 7.5</td>
<td>1.69 ± 0.47</td>
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</tr>
<tr>
<td>Vehicle 1</td>
<td>115 ± 6</td>
<td>24.0 ± 7.5</td>
<td>1.94 ± 0.51</td>
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</tr>
<tr>
<td>Vehicle 2</td>
<td>117 ± 6</td>
<td>23.7 ± 7.3</td>
<td>1.96 ± 0.43</td>
<td>74 ± 11</td>
</tr>
<tr>
<td>D-SMTC Basal</td>
<td>120 ± 4</td>
<td>23.6 ± 3.3</td>
<td>1.21 ± 0.31</td>
<td></td>
</tr>
<tr>
<td>SMTC 0.05</td>
<td>120 ± 4</td>
<td>18.5 ± 1.9</td>
<td>1.07 ± 0.24</td>
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</tr>
<tr>
<td>SMTC 0.5</td>
<td>125 ± 4</td>
<td>16.2 ± 2.1</td>
<td>1.14 ± 0.24</td>
<td>67 ± 9</td>
</tr>
<tr>
<td>D-SMTC-INS Basal</td>
<td>112 ± 4</td>
<td>9.0 ± 1.9</td>
<td>0.94 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>SMTC 0.05</td>
<td>114 ± 4</td>
<td>10.1 ± 1.9</td>
<td>1.02 ± 0.37</td>
<td></td>
</tr>
<tr>
<td>SMTC 0.5</td>
<td>121 ± 6†</td>
<td>10.7 ± 3.3</td>
<td>1.00 ± 0.33</td>
<td>113 ± 38</td>
</tr>
</tbody>
</table>

Values are means ± SE. NOS1, neuronal nitric oxide synthase; MAP, mean arterial pressure; UF, urinary flow; UNaV, urinary sodium excretion; PRC, plasma renin concentration; SMTC 0.05, SMTC 0.5; 0.05 mg/kg body wt and 0.5 mg SMTC/kg body wt, respectively. See text for intergroup comparisons. *P < 0.05 vs. basal. †P < 0.01 vs. basal and SMTC 0.05. ‡P < 0.01 vs. SMTC 0.05.

A significant rise in MAP was observed in response to the higher dose of SMTC in the controls (P < 0.001 vs. baseline and SMTC 0.05 mg/kg) and in both groups of diabetic animals (P < 0.01 vs. baseline and SMTC 0.05 mg/kg), suggesting a significant spillover of the substance into the systemic circulation. As shown in Fig. 1, administration of SMTC at a dose of 0.05 mg/kg resulted in a reduction of GFR in diabetic rats (P < 0.001), whereas the higher dose of SMTC induced no further significant changes in this parameter. In C-SMTC, a significant decrease in GFR, compared with basal values, was observed only after the higher dose of the inhibitor (P < 0.05 vs. basal). The GFR response was significantly more pronounced in D-SMTC compared with C-SMTC (F = 4.5, P < 0.05). Unlike the GFR, SMTC-treated control and diabetic rats demonstrated similar decreases in RPF in response to the lower dose of the inhibitor (P < 0.01). The higher dose of SMTC induced further reduction of RPF in diabetic (P < 0.05) but not in control rats. The RVR increased in a dose-dependent manner both in control and diabetic animals (C-SMTC, P < 0.01 vs. basal; D-SMTC, P < 0.001 vs. basal). The lower dose of SMTC increased FF in control rats. In contrast, the FF was stable in diabetic rats in response to the lower dose of SMTC, and a significant increase in FF was, in D-SMTC, observed only after the pressor dose of the inhibitor (P < 0.01 vs. baseline and SMTC 0.05 mg/kg). The normoglycemic diabetic rats demonstrated blunted renal hemodynamic responses to NOS1 inhibition compared with both their moderately hyperglycemic counterparts or control animals. The GFR remained stable throughout the studies, whereas significant reductions in RPF, and increases in RVR, were observed only in response to the pressor dose of SMTC (P < 0.05 vs. baseline). The FF rose in response to both doses of SMTC (P < 0.05). Unlike the SMTC-treated control and diabetic rats, no changes in renal hemodynamics were noted in vehicle-treated control and diabetic animals.

As presented in Table 2, both groups of hyperglycemic diabetic rats had polyuria compared with control and euglycemic diabetic animals (P < 0.05). In D-SMTC, NOS1 inhibition had an antidiuretic effect that was observed after both doses of the inhibitor (P < 0.05). A similar, although not significant, trend was apparent also in C-SMTC rats. In other groups of rats, the FF remained stable throughout the experiments. There were no differences in UNaV between the groups, and no changes in this parameter were observed after administration of SMTC. Plasma renin concentration determined after the completion of renal hemodynamic studies was not different between control and diabetic rats and was not affected by treatment with SMTC.

Protocol 2: Effects of nonspecific NOS inhibition in diabetic rats treated with SMTC. There were no differences in basal MAP, renal hemodynamics, and urinary excretions of sodium between the two groups of diabetic rats (Table 3). As in protocol 1, MAP remained stable (Table 3 and Fig. 2) and GFR and RPF decreased (P < 0.01), whereas RVR increased (P < 0.01) in response to SMTC (0.05 mg/kg) in both groups of diabetic rats (P < 0.01), without a significant change in FF (Fig. 2). Administration of vehicle after the NOS1 inhibition induced no further changes in MAP and renal hemodynamics in D-SMTC-VE. MAP remained stable also when the administration of SMTC was followed by L-NAME. In contrast to D-SMTC-VE, nonspecific NOS inhibition caused further decreases in RPF (P < 0.01) and an increase in RVR (P < 0.01). There were, however, no further significant decreases in GFR in D-SMTC-L-NAME rats resulting in an increase in FF compared with baseline values (P < 0.01) and values after treatment with SMTC (P < 0.05). The baseline UF and UNaV (Table 3) were similar in both groups of rats and remained stable throughout the experiments. The PRC did not differ between the groups (Table 3).

Protocol 3: Renal immunohistochemical expression of NOS1 in control and diabetic rats. The control and diabetic rats demonstrated similar general characteristics as in previous protocols. The body weight was not different between the groups (control: 355 ± 24; diabetic: 345 ± 27 g; P = not significant). Diabetic rats had increased BG levels (268 ± 21 mg/dl), HBA1c (9.7 ± 0.9%), left kidney weight (1.75 ± 0.12 g), and left kidney weight-to-body weight ratio (0.55 ± 0.04%) compared with controls (101 ± 9 mg/dl; 3.3 ± 0.4%; 1.29 ± 0.07 g; and 0.35 ± 0.05%, respectively; all P < 0.01). Abundant immunoreactivity for NOS1 was detected both in control and diabetic rats in the cells of the MD. Representative photomicrographs are presented in Fig. 3. NOS1 immunoreactivity was also...
found in some cells of thick ascending limbs, and diffuse, less intensive staining was detectable in inner medullary collecting ducts. In some glomeruli, NOS1 was weakly detectable in the parietal epithelium of Bowman’s capsule. Paired comparisons revealed that diabetic kidneys had a higher proportion of glomeruli with adjacent NOS1-positive MD regions of all glomeruli in the section than control kidney in four of five cases. The average number of NOS1 positive cells in MD was also higher in diabetic kidneys in four of five pairs, whereas the number of NOS1-positive cells expressed per number of all glomeruli was higher in diabetic kidneys in all pairs. Statistical analysis of pooled data showed significantly higher numbers of MD regions and NOS1-positive cells expressed per total number of glomeruli in diabetic rats compared with controls ($P < 0.05$) (Table 4).
DISCUSSION

Increased production of NO during the early stages of diabetes has been implicated in the pathogenesis of renal hemodynamic changes in diabetes (6, 20, 24, 49). However, the contribution of individual NOS isoforms in the pathogenesis of this process remains incompletely defined. In the present study, NOS1 inhibition with the specific NOS1 inhibitor SMTC, administered to the abdominal aorta, near-normalized GFR in hyperfiltering diabetic rats. In contrast, control, nondiabetic rats demonstrated a significant decrease in GFR only after a 10-fold higher dose of the inhibitor. Different responses in GFR between the diabetic and control rats were present despite similar vasoconstrictor responses to NOS1 inhibitor, as assessed by its effects on RPF and RVR. These observations indicate that NOS1 substantially contributes to altered renal NO production and hemodynamics in experimental diabetes. Furthermore, the renal hemodynamic effects of SMTC were attenuated in normoglycemic diabetic rats, suggesting that alterations in NOS1 activity are related to the metabolic control of diabetes.

Micropuncture studies by Hostetter et al. (17), as well as later studies by Zatz et al. (59), identified the major renal hemodynamic alteration in diabetes as disproportionately decreased afferent arteriolar resistance resulting in elevated glomerular capillary pressure. Our experiments were not designed to directly assess SMTC-induced changes in glomerular microcirculation. Despite that, whole kidney hemodynamic responses, as observed in the present study, can provide some insight into the effects of SMTC on glomerular arteriolar tone and possible differences between the control and diabetic rats. As described by Ichihara et al. (19) in an in vitro model of blood-perfused juxtamedullary nephron, NOS1 inhibition with SMTC causes constriction of both afferent and efferent arterioles. Our observations in control animals are in accordance with these findings. However, despite renal vasoconstriction similar to that in the control animals, diabetic rats responded to the SMTC with a marked decrease in GFR, which was in contrast to the lack of change in this parameter in control rats. In keeping with our hypothesis, such a whole kidney hemodynamic pattern suggests a greater effect of NOS1 inhibition on preglo-

**Table 3. Effects of local nonspecific NOS inhibition on MAP, renal excretory functions, and PRC in diabetic rats treated with SMTC**

<table>
<thead>
<tr>
<th>Group</th>
<th>MAP, mmHg</th>
<th>UF, μl/min</th>
<th>( U_{\text{NaV}}, \mu \text{mol/min} )</th>
<th>PRC, ng ANG ( \text{l}^{-1} \text{ml}^{-1} \text{h}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-SMTC-VE</td>
<td>Basal</td>
<td>110 ± 4</td>
<td>30 ± 8</td>
<td>1.45 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>SMTC</td>
<td>112 ± 3</td>
<td>28 ± 8</td>
<td>1.54 ± 0.28</td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td>111 ± 5</td>
<td>28 ± 8</td>
<td>1.65 ± 0.59</td>
</tr>
<tr>
<td>D-SMTC-NAME</td>
<td>Basal</td>
<td>118 ± 3</td>
<td>27 ± 8</td>
<td>1.15 ± 0.48</td>
</tr>
<tr>
<td></td>
<td>SMTC</td>
<td>118 ± 4</td>
<td>26 ± 8</td>
<td>1.28 ± 0.54</td>
</tr>
<tr>
<td></td>
<td>L-NAME</td>
<td>121 ± 3</td>
<td>24 ± 7</td>
<td>1.44 ± 0.70</td>
</tr>
</tbody>
</table>

Values are means ± SE. See text for intergroup comparisons.

**Fig. 2. Effects of \( \text{N}^\text{G} \)-nitro-\( \text{l} \)-arginine methyl ester \( (\text{l}-\text{NAME}; 0.2 \text{ mg/kg}) \) or vehicle on GFR, RPF, FF, and RVR in SMTC-treated \( (0.05 \text{ mg/kg}) \) diabetic rats. *P < 0.05 vs. basal. †P < 0.01 vs. basal. ¶P < 0.05 vs. SMTC.**
merular vascular tone in diabetic rats. In addition to this mechanism, we cannot exclude a possibility that a decrease in GFR observed in diabetic rats occurred, in part, due to SMT-induced reduction in ultrafiltration coefficient. As suggested by Bachmann et al. (5), MD-derived NO diffuses into the extraglomerular mesangium and its inhibition may therefore result in contraction of these contractile elements. Furthermore, this phenomenon was demonstrated after local NOS inhibition with nonspecific inhibitors (15).

We can only speculate about the underlying mechanism causing enhanced NOS1 activity in diabetes. Under physiological conditions, sodium delivery to the distal tubule is the major determinant of NOS1 activity in the MD cells (19, 54). However, on the basis of micropuncture studies by Vallon et al. (50), showing a decrease in solute content in early distal tubular fluid in diabetic rats, stimulation of NOS1 by increased solute delivery to the MD is unlikely. In this context, it is important to note that diabetes and/or hyperglycemia is associated with blunted TGF activity (7, 50, 56) that cannot be fully accounted for by changes in tubular reabsorption (50). Whether this phenomenon reflects increased NOS1 activity or provides an environment facilitating NOS1 actions on the glomerular vasculature resulting in hyperfiltration remains to be established. In any case, previous reports suggesting attenuation of TGF activity in hyperglycemia and/or diabetes are consistent with involvement of NOS1.

Welch and Wilcox (53) reported that blunting of TGF responses by NO could be limited by L-arg availability in the tubular lumen and by its uptake via a y^{+} transport system. To our knowledge, availability of L-arg in diabetic, compared with normal kidneys, remains unknown. Indirect clues, which could help to elucidate this issue, are rather conflicting. Plasma L-arg levels are decreased in diabetes (34). However, tubular concentrations may be influenced by the increased filtered load of L-arg in hyperfiltering rats, and

Table 4. Quantification of immunohistochemical expression of NOS1 in control and diabetic kidneys

<table>
<thead>
<tr>
<th>%NOS1-positive</th>
<th>Average Number of NOS1-Positive Cells/Section^{1}</th>
<th>NOS1-Positive Cells/MD^{1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD-All</td>
<td></td>
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<tr>
<td>Glomeruli</td>
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</tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>21.9 ± 1.0</td>
<td>5.17 ± 0.30</td>
</tr>
<tr>
<td>Diabetic</td>
<td>25.2 ± 1.2*</td>
<td>5.48 ± 0.17</td>
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</tbody>
</table>

Values are means ± SE. MD, macula densa. *P < 0.05 vs. control.

Fig. 3. Representative photomicrographs showing macula densa (MD) regions (arrows) in kidney sections of control (top) and diabetic (bottom) rats incubated with a nonimmune control serum (A and C) or with an anti-neuronal nitric oxide synthase (NOS1) antibody. Magnification: ×200.
proximal solute and water reabsorption. Moreover, urinary L-arg excretion was reported to be markedly increased in diabetes (35).

The sodium/chloride-independent mechanisms resulting in NOS1 activation are also conceivably relevant. Blunted responses to NOS1 inhibition in diabetic rats rendered normoglycemic by additional insulin suggest direct effects of glucose on NOS1. The capability of glucose or glycosylation products to increase enzymatic activity has been shown previously in other NOS isoforms in various experimental settings (1, 10, 11, 43, 46), although other studies reported opposite findings (40). Influence of glucose on NOS1 activity may also involve activation of protein kinase C (22, 27).

Another theoretical explanation for activation of NOS1 in diabetes is based on observations that renal NOS1 is activated in parallel with the renin-angiotensin system (RAS) in such pathophysiological states as two-kidney, one-clip hypertension, furosemide treatment, or low-sodium diet (8). Our group has demonstrated that RAS is activated in kidneys from diabetic rats and implicated this alteration in the pathogenesis of diabetic nephropathy (3). With respect to close links between the two systems, it is possible that similar to the conditions mentioned above, NOS1 is activated in parallel with RAS also in the diabetic kidney. Indeed, in our recent work, renal responses to systemic NOS1 inhibition were, in diabetic rats, attenuated by coadministration of losartan, an AT1 angiotensin receptor blocker (21).

Having established renal hemodynamic responses to NOS1 inhibition, we attempted to determine whether NOS1 activity is the major determinant of hemodynamically active NO in diabetes or whether other NOS isoforms also significantly contribute to the pathogenesis of hyperfiltration. We addressed the issue by assessing the renal responses to the nonspecific inhibitor L-NAME in diabetic rats pretreated with SMTC. The lack of effect of L-NAME on GFR in SMTC-treated animals suggests that NOS1 is the isoform responsible for generation of NO, which is involved in pathogenesis of hemodynamic alterations specific to diabetes. However, the further decrease in RPF induced by L-NAME in SMTC-treated rats suggests that the other isoforms act in concert with NOS1 in the control of renal perfusion in diabetes.

The topography of NOS1 in kidneys of both control and diabetic rats was similar to that in previous studies (5, 36, 47, 55). Although the NOS1 immunostaining was abundant in both control and diabetic rats, quantification of histochemical findings revealed that the number of NOS1-positive cells in MD was enhanced, albeit modestly, in diabetic kidneys. This finding is complementary to our hemodynamic observations and provides further support for the pathophysiological roles of NOS1, as proposed in this paper. To our knowledge, two studies have previously explored NOS1 renal expression in diabetes. Supporting our findings, Choi et al. (9) found a marked increase in cortical NOS1 in STZ-diabetic rats, as assessed by Western blot analysis. In contrast, Yagihashi et al. (57) reported less intense immunohistochemical staining for eNOS (presumably NOS1) in MD and glomerular arterioles in rats with various durations of diabetes. A possible explanation for opposite findings in those studies, which were performed in a rat model of diabetes without insulin treatment, is the timing of the experiments. The former study measured NOS1 expression 7 days after induction of diabetes, whereas the latter studies were performed at least 6 wk after induction of diabetes. The degree of catabolism and dehydration, resulting in absence of the customary hemodynamics seen with more moderate hyperglycemia, may explain those disparate findings. Two other studies explored NOS1 expression in extrarenal neuronal tissue in diabetes. In accordance with our findings, Serino et al. (39) found increased hypothalamic NOS1 gene expression in STZ-diabetic rats. Corresponding to our observations, the authors also reported normalization of NOS1 expression in insulin-treated diabetic rats with near-normal BG levels. In contrast, Roufail et al. (37) found decreased protein NOS1 immunohistochemical expression and enzymatic activity in retinal neurons of diabetic rats. This abnormality was also normalized by insulin treatment. The level of hyperglycemia was almost identical in these studies and is not, therefore, a likely explanation for the contrast in their findings. It is possible that, in addition to the diabetic milieu, some other specific regulatory mechanisms, such as local perfusion or osmolality, influence NOS1 expression and activity in various regions of the nervous system.

Involvement of NOS3 (eNOS) in the pathogenesis of diabetic hyperfiltration has been suggested. Choi et al. (9) found an increase in NOS3 protein in renal cortex. Sugimoto et al. (42) found enhanced immunohistochemical expression and NADPH diaphorase staining in the endothelia of afferent, but not in efferent, arterioles, associated with increased afferent diameter and glomerular hypertrophy. These changes were corrected by insulin treatment and by treatment with L-NAME. That study however, did not provide persuasive hemodynamic data. Enhanced NOS2 immunostaining was observed in diabetic rats with significantly lower absolute GFR values compared with controls, and the GFR was not affected by L-NAME treatment. Furthermore, a decrease in NADPH diaphorase staining in response to L-NAME may reflect inhibition of other NOS isoforms, because this method is not specific for NOS3 (5).

More recently, Veelken et al. (51) reported increased cortical NOS3 expression and the lack of effect of a selective NOS2 (iNOS) inhibitor, contrasting to the effect of L-NAME, on renal hemodynamics in conscious STZ-diabetic rats. On the basis of these observations, the authors suggested the role of NOS3 in the pathogenesis of hyperfiltration. However, these findings do not exclude the possibility that NOS1 is involved in the process, because the reduction in GFR was achieved with a nonspecific NOS inhibitor.

Unlike NOS1 and NOS3, the contribution of NOS2 to the pathogenesis of hyperfiltration seems to be negligible. Except for one study (9), NOS2 cortical protein and mRNA expression have been found to be barely
detectable during the hyperfiltering state (43, 51). Furthermore, in an aforementioned study, selective NOS2 inhibition failed to alter renal hemodynamics in hyperfiltering diabetic rats (51). However, renal topography and expression of NOS isoforms in diabetic rats with moderate hyperglycemia, as used in our studies, remain undefined, and are presently being explored in our laboratory.

Although this discussion has so far focused on evidence related to the role of NO in mediating early renal hemodynamic changes in diabetes, it should be noted that the issue of NO pathophysiology in diabetic kidney disease is more complex. There is substantial evidence suggesting decreased, rather than increased, activity of the renal NO system in diabetes. For example, similar to peripheral vascular beds (33), endothelium-dependent vasodilation was found to be decreased in the renal artery (14), and NO-dependent cGMP generation in response to cholinergic stimuli was blunted in diabetic glomeruli (13, 52). Such findings, suggesting a defect in NOS3 activity, do not contradict the proposed role of NOS1 in mediating diabetic hyperfiltration, and are, in fact, compatible with the lack of effect of L-NAME in SMTC-treated rats.

In contrast to our present findings, as well as to previous findings by us and other groups (20, 21, 24, 49, 51), however, are studies showing blunted renal arteriolar and hemodynamic responses to nonspecific NOS inhibition (29–32). Most recently, Schoonmaker et al. (38) found normal afferent and blunted efferent responses to N\textsuperscript{G}-nitro-L-arginine in insulin-treated diabetic rats. There are no clear explanations for these conflicting findings. These phenomena cannot be explained by the presence or absence of insulin treatment or by the levels of metabolic control in rat models of diabetes used in these studies. Blunted or normal responses to NOS inhibitors were found also in insulin-treated rats with moderate hyperglycemia (29, 38), and enhanced responses in insulin-untreated animals (51). Furthermore, even insulin-untreated STZ-diabetic animals can demonstrate moderate hyperglycemia, when studied early after onset of diabetes. As suggested in an aforementioned recent report (38), the afferent response to N\textsuperscript{G}-nitro-L-arginine was preserved in diabetic rats pretreated with angiotensin-converting enzyme (ACE) inhibitor (ACEI). This finding is in contrast to previous studies by the same group, conducted in the same experimental model, suggesting a decrease in afferent arteriolar responses to NOS inhibition in diabetic rats that were not, however, treated with ACEI (29). Thus different degrees of activation of the RAS in various models could, at least in part, explain the above-mentioned conflicting findings. A complex interplay between the NO and RAS systems in the control of renal hemodynamics has been well established (16, 18, 30, 41, 44), as well as the already mentioned role of the RAS in the pathogenesis of diabetic nephropathy (2).

Several studies have suggested a nephroprotective role of NO in the course of development of nephropathy in diabetic rats. Reyes et al. (35) found a decrease in proteinuria and amelioration of hyperfiltration in STZ-diabetic rats after l-arg treatment. In that study, however, the measurements were performed 14 wk after onset of diabetes, and the authors failed to provide evidence that beneficial effects of l-arg were mediated via increased NO production. Convincing evidence for a renoprotective role of NO in diabetes was more recently provided by Craven et al. (12) in a study showing acceleration of nephropathy in STZ-diabetic rats after long-term inhibition with a nonpressor dose of the nonspecific NOS inhibitor. Our present results do not challenge those findings. We believe that the majority of the above-mentioned studies, including our present data, describe different episodes in one scenario. Early increases in activity and synthesis of NO contribute to the development of renal hemodynamic changes, with a consequent cascade of events resulting from the increase in glomerular pressure (4). At this stage, alteration in NOS1 activity seems to be an important factor. However, with increasing duration of diabetes, NO production, in particular mediated by NOS3, decreases, or is neutralized by accumulating superoxide radicals and glycosylation products. This leads to a gradual loss of protection against other factors implicated in the pathogenesis of nephropathy, such as the RAS, endothelin, vasoconstrictor prostanoids, various growth cytokines, and adhesion molecules. Renal pathophysiology of the NO system in more advanced stages of diabetic nephropathy has been far less studied and remains a challenge for future investigation in this area.

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