D iabetes mellitus is associated with alterations in nitric oxide (NO)-mediated vasomotor function. As recently summarized by Pieper,1 diabetes and hyperglycemia impair endothelium-dependent vasodilation in various vascular beds, species, and experimental models. This phenomenon may contribute to increased susceptibility to the development of hypertension and vascular complications in diabetic patients. In contrast, early stages of some diabetic complications, such as nephropathy, are accompanied by local hyperperfusion, which has been shown to be, at least in part, NO dependent.2-4 The nature of these local hemodynamic changes has been well studied because of their role in the initiation of the process leading ultimately to organ failure.5

NO is synthesized as a by-product of conversion of its physiological precursor L-arginine to L-citrulline.6 This reaction is catalyzed by a family of enzymes known as NO synthase (NOS).7 In vascular smooth muscle cells, NO activates soluble guanylate cyclase to increase cGMP, resulting in vascular relaxation.8 Three NOS isoforms (neuronal [nNOS, NOS1], inducible [iNOS, NOS2], and endothelial [eNOS, NOS3]) have been identified in mammalian tissues, including the kidney.7 Most of the work exploring NO functions in the regulation of systemic and local hemodynamics has focused on endothelium-dependent vasodilation, which is presumably mediated by NO generated by NOS3. Unlike the endothelium-derived NO, hemodynamic roles of NO originating from other sources (such as NOS1) have been less well studied in normal physiology and remain completely unknown in the study of diabetes. Recent studies, using newly available selective NOS1 inhibitors such as 7-nitroindazole (7-NI)9 and S-methyl-L-thiocitrulline (SMTC),10,11 have demonstrated that this NO-generating enzymatic pathway is involved in the control of blood pressure. Moreover, NOS1, originally found in brain, has been recently studied by renal physiologists because of its expression in macula densa (MD) cells,12,13 and several groups have reported its role in the control of renal hemodynamics in normal animals.13,14

In the present study, we hypothesized that in addition to alterations in endothelium-derived NO generation, diabetic vascular dysfunction may be in part caused by changes in NOS1 activity. To explore this hypothesis, changes in systemic and renal hemodynamics were assessed before and after acute inhibition of NOS1 with SMTC, a specific water-soluble inhibitor of the enzyme, in control and strep-
tozotocin (STZ)-diabetic rats. Furthermore, the possible interaction of NOS1-derived NO and the renin-angiotensin system (RAS) was studied in a separate group of rats pretreated with the angiotensin AT1 receptor blocker losartan.

**Methods**

**Diabetic Rat Model**

Studies were conducted in adult male Sprague-Dawley rats (Harlan Sprague Dawley Inc, Indianapolis, Ind) with initial weights of ~300 g. The rats were made diabetic by intraperitoneal injection of STZ (65 mg/kg body wt, Sigma Chemical Co). Two days later, induction of diabetes was confirmed by measurements of tail blood glucose (BG) levels using a reflectance meter (One Touch II, Lifescan). Diabetic rats received daily evening injections of ultralente insulin (Iletin II, Eli Lilly) in doses individually adjusted to maintain BG levels between 200 and 300 mg/dL (11 to 17 mmol/L). BG levels were monitored at least weekly in all diabetic rats. Weight-matched nondiabetic rats served as controls. All rats were fed standard rat chow (Rodent Laboratory Chow 5001, Ralston Purina) ad libitum. Nondiabetic rats served as controls. All rats were fed standard rat chow (Rodent Laboratory Chow 5001, Ralston Purina) ad libitum. These studies were approved by the Portland Veteran Affairs Institutional Animal Care and Use Subcommittee.

To explore the acute systemic and renal hemodynamic effects of NOS1 inhibition, control and diabetic rats were studied before and after administration of a NOS1 inhibitor, SMTC (Alexis). In diabetic rats, experiments were performed after 3 to 5 weeks of diabetes. BG was measured before the experiment and before the completion of measurements. After surgical preparation as described below and after 75 minutes 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 (UV), urinary sodium (UnV), and nitrite/nitrate (NOx) excretion (UnO4V). Thereafter, rats received a continuous 10-minute infusion of SMTC (0.1 mg/kg body wt) or the same volume of vehicle (0.9% NaCl, vehicle 1), and all measurements were repeated to assess changes from baseline. After these measurements, the effects of a higher dose of SMTC (0.5 mg/kg body wt) were assessed in a similar manner, including the effects of vehicle alone (vehicle 2).

Although the NOS1 specificity of SMTC has been previously reported,10,11 we performed measurements of systemic vasodilatory responses to the endothelium-dependent (NOS3) vasodilator acetylcholine (ACh, 10 μg/kg in 50 μL saline) in a separate group of control rats (n=5). ACh was administered after increasing doses of SMTC. These experiments showed that profound decreases in MAP caused by ACh were not altered by pretreatment with SMTC at doses of 0.1 and 0.5 mg/kg, suggesting that the inhibitor had negligible effects on NOS3 activity. An attenuated response to ACh was observed after only 5 mg/kg SMTC, suggesting a possibility of concomitant NOS3 inhibition at this dose.

Further studies were performed to explore whether changes in systemic and renal hemodynamics induced by NOS1 inhibition may be mediated by angiotensin II (Ang II). In additional groups of control and diabetic rats, a bolus dose of the AT1 receptor blocker losartan (3 mg/kg in 50 μL of 0.9% NaCl, Merck) was given after completion of baseline measurements; at the same time, the lower-dose SMTC infusion was started. A group of diabetic rats treated with losartan alone was also studied to obtain time-control measurements for comparison with the diabetic groups treated either with SMTC alone or in combination with losartan. In these experiments, SMTC was omitted in vehicle infusions after the basal measurements and losartan bolus. The efficacy of the losartan dose to prevent the pressor response to Ang II has been previously shown in our laboratory.15 In addition, new experiments showing complete prevention of the pressor response to Ang II (1 μg/kg bolus) were performed before these studies.

After all experiments, aortic blood was taken in a chilled syringe and then subdivided into tubes containing EDTA (for plasma renin concentration [PRC]) and heparin coating (for glycosylated hemoglobin [HBA1c]). The left kidney was rapidly excised and weighed.

**Functional Studies**

Surgical preparation and renal function studies were performed as described previously.14 Rats were anesthetized with thiobutabarbital (100 mg/kg IP). GFR was measured by inulin clearance, and RPF was measured by PAH clearance. FF and RVR were calculating by standard formulas. Inulin concentrations were measured spectrophotometically. HBA1c was determined by affinity column chromatography (Glyco-Gel B, Pierce Chemical). PRC was measured by radioimmunoassay (NEN Dupont). Urinary NOx was determined by the Greiss method,16 with the use of a commercial kit (Cayman Chemical).

**Statistical Analysis**

Data are expressed as mean±SEM. All analyses were performed by ANOVA followed by the Scheffé test, with use of Statview SE and Graphics software (Brainpower) on a Macintosh PowerBook 165c computer. A value of P<0.05 was viewed as statistically significant.

**Results**

General characteristics of control and diabetic rats are shown in Table 1. Body weights were comparable in all groups. As expected, diabetic animals demonstrated renal hypertrophy (as assessed by kidney weight and kidney/body weight ratio), moderate hyperglycemia, and increased HBA1c. Values for hematocrit did not differ among groups.

Effects of SMTC on MAP are shown in Figures 1 and 2. Baseline MAP values did not differ among the groups. SMTC
induced sustained dose-dependent increases in MAP (P<0.01), with almost identical pressor responses in the control and diabetic rats (Figures 1 and 2). A significant increase in MAP (P<0.05 vs minute 0) was detected by 2 minutes of infusion of the lower dose of SMTC, and further increases occurred with the higher dose. Sustained elevations of MAP persisted for the duration of the hemodynamic studies.

When compared with control rats, diabetic rats had elevated baseline values for GFR (P<0.05) and FF (P<0.001). No changes in renal hemodynamics were observed in control rats after low-dose SMTC. Significant changes in RPF, RVR, and FF in control animals were seen only with the higher dose of SMTC (all P<0.01 versus basal and SMTC at 0.1 mg/kg body wt). In contrast, diabetic rats responded with statistically significant renal vasoconstriction, even at the low dose of SMTC (P<0.01 versus basal RPF, RVR, and FF). The high dose of SMTC caused further increases in RVR (P<0.01 versus SMTC at 0.1 mg/kg body wt) and in FF (P<0.01 versus SMTC at 0.1 mg/kg body wt) but not in RPF. There were no changes in GFR in any group.

In the control rats, coadministration of losartan did not modulate the renal responses to SMTC. In contrast, in the diabetic animals, losartan abolished the renal vasoconstriction response to the lower dose of SMTC. Similar to responses in the control rats, the renal hemodynamic responses to the higher dose of SMTC remained unchanged by concomitant AT1 receptor blockade. Losartan alone in the diabetic rats decreased MAP (P<0.05, vehicle 2 versus basal). In contrast to diabetic rats receiving SMTC (with or without losartan), rats receiving losartan alone exhibited significant renal vaso-dilation, as suggested by increases in RPF (P<0.05 versus basal) and reduction in RVR (P<0.01 versus basal). These responses were accompanied by stable GFR values and a marked reduction in FF (P<0.01 versus basal).

Changes in urinary excretion rates and PRC levels are depicted in Table 2. All diabetic groups had higher baseline UVs than those in control rats (P<0.05). No significant changes in UV were observed in any group treated with vehicle, SMTC, or SMTC+losartan. Baseline UNaV was similar in all groups of rats. In control rats, there were no changes in UNaV with either vehicle or any drug. In the diabetic rats, there were similar and statistically significant increases in UNaV in the vehicle-treated and SMTC-treated groups. However, changes in UNaV in other the other groups of diabetic rats did not achieve statistical significance.

PRC levels were similar in control rats treated with vehicle or SMTC. No significant differences were noted between vehicle-treated and SMTC-treated diabetic rats. The SMTC+losartan–treated control and diabetic rats and the diabetic rats treated with losartan alone had similar elevations of PRC (both P<0.01 versus groups not receiving losartan). UNOxV, as an indicator of renal NO production,17 was analyzed at baseline and after administration of the higher dose of SMTC or vehicle 2. This parameter remained stable in vehicle-treated control rats. Excretion increased significantly in the SMTC-treated control rats (P<0.01) but not in rats pretreated with losartan. Similar trends were apparent in the diabetic groups. There were no changes in the vehicle group, the SMTC+losartan group, or the group receiving losartan alone, whereas UNOxV increased significantly in the SMTC-treated diabetic rats (P<0.05).
The role of NOS1 in diabetes-induced changes in renal hemodynamics, our data also provide new evidence regarding the physiological role of NOS1 in the normal kidney. Previous in vivo studies exploring the effects of NOS1 inhibition relied mainly on inhibition with 7-NI (see below). However, 7-NI is inadequately soluble in water and requires intraperitoneal application in oil or in large amounts of vehicle when administered parenterally. Therefore, we performed these studies with SMTC, which is devoid of these disadvantages and has been shown to exert potent effects in the glomerular vasculature. Because SMTC has not been previously reported in in vivo renal studies, we calculated the doses that approximately match the 10 mmol/L concentrations of SMTC in a perfusate used in the above-mentioned study, assuming that the inhibitor is freely distributed in the extracellular compartment.

Studies exploring the effects of systemic NOS1 inhibition on blood pressure have not provided unequivocal evidence. The effect of SMTC on blood pressure seen in the present study was similar to that reported by Gozal et al. Our data show that a slight pressor effect can be observed, even with much smaller doses of the inhibitor than reported in that study. In contrast, others did not find a rise in blood pressure in response to acute NOS1 inhibition with 7-NI,19–21 It is possible that the different pharmacology of the 2 NOS1 inhibitors is responsible for these disparate findings. Supporting this explanation is the fact that Ollerstam et al.18 did find increases in blood pressure after chronic 7-NI administration. Two recent studies failed to detect NOS1 in components of the normal vascular wall. However, NOS1 activity has been identified in perivascular nerves and associated with sympathetic innervation. This suggests the physiological importance of nitroxidergic innervation in the control of blood pressure, which has been reported in studies with nonspecific NOS1 inhibitors24 and more recently in studies showing the ability of 7-NI to inhibit rat hindlimb arterial vasodilation produced by electric stimulation of the superior laryngeal nerve. This intervention causes vasodilation by releasing NO or NO-containing factors from axonal terminals of postganglionic sympathetic cell bodies. In control rats receiving high-dose SMTC, the lack of effect of losartan on the SMTC-induced pressor response suggests that the neural nitroxidergic system may, at least in part, control vascular tone independent from traditional regulators such as the RAS.

The role of NOS1- or MD-derived NO in the control of the renal microcirculation has been previously studied mainly in association with tubuloglomerular feedback (TGF) activity. These studies have shown that MD-derived NO counteracts increases in afferent arteriolar tone mediated by TGF and thus contributes to the control of glomerular capillary pressure. Several studies have also explored the effect of NOS1 inhibition on whole-kidney hemodynamics. Ollerstam et al. reported lower GFR in rats after acute intraperitoneal application of 7-NI compared with vehicle. However, these authors did not provide baseline measurements before admin-
istration of 7-NI. Furthermore, the GFR was not affected by chronic 7-NI administration, and renal perfusion was not measured. Beierwaltes\textsuperscript{20} reported an acute decrease in renal blood flow after 7-NI in rats on a low-sodium diet, ie, with activation of the RAS. Recently, Tan et al\textsuperscript{28} found no changes in GFR and, in contrast to our findings, no changes in RPF after a 5-day infusion of 7-NI. Lack of effect of 7-NI on RPF was present even in salt-resistant animals on a high-sodium intake, which demonstrated a significant rise in blood pressure. Because those experiments were performed in conscious rats, it is possible that anesthesia, as used in our studies, increases the sensitivity of the renal vascular tree to NOS1 inhibition. An important piece of evidence regarding the effects of NOS1 inhibition in the glomerular microcirculation has been provided by Ichihara et al.\textsuperscript{11} Using SMTC in an in vitro model of the blood-perfused juxtamedullary nephron, they found potent vasoconstriction of both afferent and efferent arterioles. Changes in whole-kidney hemodynamics in the present study are in accordance with these direct measurements reported in that study.

There is convincing evidence suggesting that Ang II significantly contributes to the effects of systemic NOS inhibition in anesthetized rats.\textsuperscript{29,30} However, not all reports have confirmed these observations, most likely because of different experimental settings and differing states of activity of the RAS.\textsuperscript{31–33} Although our results were strongly suggestive of a role of Ang II in the response to SMTC, coadministration of losartan in control rats did not modulate renal changes induced by SMTC. Therefore, it is likely that some additional vasoactive system(s) interacts with NOS1-derived NO in the process of controlling the renal circulation.

Blood pressure responses to SMTC in diabetic rats were similar to those in control rats. In contrast, the renal hemodynamic studies indicated increased susceptibility of the vascular tree in diabetic rats to NOS1 inhibition. This finding corresponds to previous studies with nonspecific NOS inhibitors, indicating a role for NO in the renal hemodynamic changes in diabetes.\textsuperscript{2–4} However, systemic administration of \(N^G\text{-monomethyl-L-arginine} \) or \(N^G\text{-nitro-L-arginine methyl ester} \) reduced GFR and urinary excretion of NO\textsubscript{\textit{x}} in those studies.\textsuperscript{2–4} Because we saw no effect on GFR in the present study, it is possible that some other NOS isoform is also involved in the renal hemodynamic changes in diabetes. Unexpectedly, urinary excretion of NO\textsubscript{\textit{x}} increased after the administration of SMTC in both control and diabetic groups. We can only speculate about the causes of this finding. An increase in NO synthesis by NOS3 in response to shear stress may be part of the counterregulatory response to the heightened activity of the local RAS. This concept is further supported by the opposite effects of losartan compared with SMTC on renal hemodynamics.

However, more recent studies have demonstrated that NOS1 enzyme activity (as distinguished from protein expression) is decreased in nonclipped kidneys of 2-kidney, 1-clip Goldblatt hypertensive rats\textsuperscript{40} and that afferent arteriolar responses to SMTC are blunted in rats with hypertension induced by chronic Ang II administration.\textsuperscript{41} Although the renal microvasculature in the STZ-diabetic rat model is presumably also under influence of locally elevated Ang II levels, the diabetic model differs from the other models by the absence of hypertension. This may be an important phenomenon responsible for the disparate findings between the present study, showing an enhanced renal response to NOS1 inhibition, and prior studies, which have suggested decreased NOS1 activity in models with an activated RAS. Beierwaltes\textsuperscript{20} has recently suggested another link between the 2 systems. In his studies, chronic NOS1 inhibition with 7-NI reduced renin secretion induced by salt restriction, although no such changes were observed after acute 7-NI administration. We also did not observe any changes in PRC after acute SMTC administration. Conceivably, chronic administration is required to induce a detectable change in renal renin secretion.

NO is a natriuretic factor, acting via intrarenal hemodynamic changes\textsuperscript{42} or via direct tubular actions.\textsuperscript{33,44} Furthermore, nonspecific NOS inhibition attenuates pressure natriuresis, causing a right shift in the pressure-natriuresis curve.\textsuperscript{45,46} Mattson and Bellehumeur\textsuperscript{47} have reported that normal rats on high-salt diets develop hypertension after chronic specific NOS1 inhibition in the renal medulla. This observation suggests that NOS1 is involved in the natri-
uretic actions of the NO system and that shifts in the pressure-natriuresis curve after nonspecific NOS inhibition are, at least in part, due to NOS1 blockade. In our SMTC-treated control rats, UNaV remained stable despite increases in MAP. It is possible that this phenomenon reflects a shift to the right of the pressure-natriuresis curve, in response to nonspecific NOS inhibition. In contrast, responses in the diabetic rats were attenuated by AT1 receptor blockade, suggesting increased basal renal responses to NOS1 inhibition, suggesting increased basal renal NOS1 and the RAS in the diabetic kidney.

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**References**


Effects of Systemic Inhibition of Neuronal Nitric Oxide Synthase in Diabetic Rats
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