Early diabetes as a model for testing the regulation of juxtaglomerular NOS I

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Thomson, Scott C., Aihua Deng, Norikuni Komine, John S. Hammes, Roland C. Blantz, and Francis B. Gabbai. Early diabetes as a model for testing the regulation of juxtaglomerular NOS I. Am J Physiol Renal Physiol 287: F732–F738, 2004.—Dysregulation of kidney nitric oxide synthase (NOS) I may alter renal hemodynamics in diabetes. Four types of studies were performed in anesthetized 1- to 2-wk-streptozotocin diabetic rats. 1) Glomerular filtration rate (GFR) was measured before and during NOS I blockade. Subsequent addition of nonspecific NOS blocker tested for residual NO from other isoforms. Acute systemic NOS I blockade reduced GFR only in diabetics. Nonspecific NOS blockade had no additional effect on NOS I-blocked diabetics. 2) Renal blood flow (RBF) was monitored for evidence that tubuloglomerular feedback (TGF) resets during 1 h of continuous activation with benzamilide. NOS I blockade was added to test for the role of NOS I in TGF resetting. During 1 h of TGF activation in controls, RBF initially declined and then returned to baseline. In diabetic and NOS I-blocked rats, RBF declined and remained low. 3) The ability of NOS I blockade to increase the homeostatic efficiency of TGF in diabetes was tested by micropuncture in free-flowing nephrons. The addition of NOS I blocker to the tubular fluid increased TGF efficiency in control and diabetic rats. 4) The influence of distal salt delivery on local NOS I activity was tested by micropuncture. Henle’s loop was perfused at varying rates with NOS I blocker while single-nephron GFR (SNGFR) from the late proximal tubule was measured. In controls, NOS I blockade mainly reduced SNGFR when flow through Henle’s loop was high. In diabetics, NOS I blockade reduced SNGFR independently of Henle’s loop. In conclusion, normally, salt delivered to the macula densa (MD) exerts immediate control over MD NOS I activity. In diabetes, there is ongoing overactivity of NOS I that is not regulated by MD salt.

glomerular filtration; macula densa; micropuncture; nitric oxide; tubuloglomerular feedback

EARLY IN DIABETES glomerular filtration rate (GFR) and renal blood flow (RBF) are supranormal. It is reasonable to suspect that this results from an imbalance between the endogenous vasodilators and vasoconstrictors that set vascular tone in the kidney (reviewed in Ref. 14). The kidney expresses all three isoforms of nitric oxide synthase (NOS). A simple hypothesis to consider is that one or more of these NOS isoforms generate excess NO, which causes diabetic hyperfiltration. In fact, some have reported enhanced vasoconstriction of the hyperfiltering diabetic kidney in response to nonselective NOS blockers (7, 10, 18, 28, 31), although others have not (1, 11, 15). Because systemic administration of nonselective NOS blockers is too blunt an instrument to unravel the multiple roles of NO in normal kidney physiology (reviewed in Ref. 22), it is not surprising that these methods also fail to provide a coherent view of NO in the diabetic kidney.

In contrast, subtype-specific NOS blockers should be less encumbered by confounding effects. Two groups have used a selective NOS II antagonist, L-NIL, to determine whether the so-called “inducible” NOS II contributes to diabetic hyperfiltration. In neither case was a role demonstrated for the NOS II isoform in diabetic hyperfiltration (18, 31).

Given its prominence as a vasodilator, one might consider endothelial NOS III as a candidate mediator for diabetic hyperfiltration. Because there are no antagonists specific to NOS III, this issue can only be addressed indirectly. Reports on the expression of NOS III in diabetic kidneys or glomeruli are equally divided as to whether expression is increased (4, 31) or not increased (6, 18).

In the present study, we tested for abnormalities of renal hemodynamics related to the NOS I isoform in rats with early streptozotocin diabetes. Experiments were designed based on what is already known about NOS I in normal kidney physiology. For example, we know that NOS I is featured in the macula densa (MD) where it performs certain functions (32). One of these functions is to buffer the acute tubuloglomerular feedback (TGF) response (30). Another is to participate in the normal rightward resetting of the TGF response that occurs within 30–60 min when TGF is continuously stimulated (5, 23, 25, 26). Regarding the signals that regulate MD NOS I, it has been shown that MD NO production can increase rapidly when the apical MD is perfused with salt (9). Regarding tissue content of the enzyme itself, MD NOS I generally varies inversely with what the kidney sees as the effective volume state of the animal, although the amount of NOS I enzyme and the magnitude of its functional effect are often dissociated (reviewed in Ref. 22).

With these points borne in mind, experiments were performed to evaluate whether dysregulation of NOS I in the diabetic kidney contributes to glomerular hyperfiltration, reduced TGF efficiency (29), or abnormal resetting of TGF. Findings suggest that the tonic influence of NOS I over glomerular filtration is enhanced in diabetes and that the normal dependence of NOS I activity on MD salt is lost such that the diabetic kidney generates excess, and functionally significant NO, even when not stimulated to do so by variations in MD salt.

METHODS

Experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Adult...
male Wistar rats were made diabetic with streptozotocin (65 mg/kg ip; Sigma). Those with blood glucose >300 mg/dl after 24 h were carried into studies. Insulin (1.0–1.5 U/day sc; PZI, Blue Ridge Pharmaceuticals, Memphis, TN) was administered daily to maintain blood glucose concentration at 300–400 mg/dl. Blood glucose was measured with a glucometer in the midafternoon daily or QOD, and daily insulin was increased by 0.5 U for values >400 mg/dl and decreased by 0.5 U for values <300 mg/dl. Whole kidney and micropuncture studies were performed under Inactin anesthesia (100 mg/kg ip; Byk-Gulden, Constance, Germany) after 1–2 wk of diabetes. Animals were prepared with vascular and bladder catheters. GFR was determined as [1H]inulin clearance. Diabetic animals received Ringer saline at 3.5 ml/h and nondiabetic rats received 2 ml/h to compensate for the point of infusing L-NMMA in NOS I-blocked animals. If a prolonged TGF stimulus is imposed, TGF and this fraction constitutes an index of TGF efficiency, the fractional compensation for small nephrons (30). Therefore, MD NOS I may activate NOS I to buffer that response.

Effects of NOS I Blockers on Diabetic Hyperfiltration

GFR was measured in control and diabetic rats pretreated with placebo, with the water-insoluble NOS I blocker 7-nitroindazole (7-NI), or with the water-soluble NOS I blocker S-methyl thioctyrline (l-SMTC), n = 5–7 for each group. 7-NI was given as a single dose after anesthesia, 25 mg/kg ip in ~150 μl DMSO. This dose was chosen to inhibit NOS I in the juxtaglomerular apparatus (JGA) without affecting blood pressure (2, 27). Controls for 7-NI were injected with DMSO vehicle. SMTC was administered in Ringer saline at 5.1 μmol·kg⁻¹·h⁻¹ by continuous infusion to avoid sudden changes in blood pressure. This dose of SMTC inhibits NOS I in the kidney while minimally affecting the blood pressure (20).

GFR was determined twice more from serial 15-min timed urine collections. The point of infusing l-NMMA in NOS I-blocked rats was to test for any residual NO, presuming that this would be due to NOS III.

Effect of NOS I Blockade and Diabetes on Resetting of TGF

The JGA normally calibrates the TGF response to be steepest near the ambient tubular flow. If a prolonged TGF stimulus is imposed, then the system normally adapts by shifting the TGF response rightward within 60 min (23, 25). At the same time, RBF normally increases back toward baseline by a mechanism that requires NOS I (5). To investigate whether this function of MD NOS I is preserved in diabetes, we monitored RBF with a perivascular ultrasonic transit time optical technique (videometric flow velocimetry) as described (21). Perturbations ranged from ~8 to +8 ml/min in 4-ml/min increments and were done in variable order. SMTC was added (2 nM at 4 ml/min) using a second pump positioned slightly downstream from the perturbation pump. The perturbation pump was adjusted, in turn, to compensate for the additional flow of the drug pump. Fractional compensation was calculated by linear regression of the changes in measured flow against the applied perturbations.

MD Salt as Determinant of NOS I Activity

Micropuncture in wax-blocked nephrons. In addition to initiating the TGF response, MD salt may activate NOS I to buffer that response (9). If this is true, then blocking NOS I should reduce single-nephron GFR (SNGFR) to a greater extent when there is more salt being delivered to the MD. To test this theory, we performed micropuncture to manipulate MD salt while blocking NOS I. Two microperfusion pipettes were placed in the most downstream visible segment of the proximal tubule. One pipette contained ATP and the other contained ATP + SMTC (1 μM). A wax block was injected immediately upstream from the perfusion pipettes. A series of four timed collections was made upstream from the wax block to determine SNGFR by [1H]inulin clearance. The first collection was made during perfusion with ATP at 38 ml/min, the second during ATP at 8 ml/min, the third during ATP at 30 ml/min + SMTC at 8 ml/min, and the fourth during SMTC at 8 ml/min. Collections were made for 3 min. Outcome measures included the range of the TGF response (difference in SNGFR during high- and low-microperfusion rates) and the influence of TGF stimulation on the effect of SMTC.

Statistics

Effects were tested by ANOVA with a design for repeated measures and Tukey’s test for post hoc intergroup comparisons where appropriate. Significance is defined as P < 0.05.

RESULTS

GFR During Systemic Administration of NOS Blockers

Response to NOS I inhibitors (7-NI or SMTC) was measured after 2 wk of diabetes (Table 1 and Figs. 1 and 2). General characteristics are shown in Table 1, confirming that diabetic kidneys were larger and that there was no important confounding by body weight, blood pressure, or glycemic control.

GFR was greater in diabetes (P = 2 × 10⁻¹¹ by 2-way ANOVA incorporating diabetes and NOS I blockade). GFR correlated with kidney weight within and across all groups (r = 0.7, P = 8 × 10⁻⁴). Correcting for the effect of kidney weight eliminated diabetes as a determinant of GFR. The effects of 7-NI and SMTC on GFR were similar in all respects. NOS I blockade reduced GFR in diabetics to a significantly greater degree than in controls (P = 0.035). The effect of NOS I blockade on GFR in diabetes is more apparent by intergroup comparisons (see Figs. 1 and 2).

l-NMMA significantly reduced GFR in those diabetics in which NOS I had not previously been blocked. l-NMMA had no significant effect on GFR in any other group (Figs. 1 and 2). Because increased blood pressure confounds the direct effects of l-NMMA on renal function (reviewed in Ref. 22), the
present data do not imply absence of NOS III activity in the normal kidney.

RBF Recovery During Activation of TGF With Benzolamide

Hemodynamic evidence for TGF resetting (5) was sought during a 1-h infusion of benzolamide, with or without SMTC, in 26 animals. In these experiments, SMTC caused arterial blood pressure to increase by ~10 mmHg in nondiabetics (P < 0.05) and ~5 mmHg in diabetics (P = not significant). Over the subsequent 60-min period of benzolamide infusion, blood pressure gradually declined by ~5 mmHg. There was no apparent effect of diabetes or SMTC on the minor drift in blood pressure during benzolamide (Fig. 3). By multiway ANOVA, RBF was greater among diabetic rats (P = 0.001); initial activation of TGF reduced RBF (P < 0.0005); SMTC reduced basal RBF (P < 0.0005) by similar amounts in diabetes and controls. SMTC tended to enhance the initial response to benzolamide and this effect appeared greater in diabetes, but this effect of diabetes was not statistically significant (P ~0.15). Those diabetic rats that went on to receive SMTC began with greater RBF than those diabetic rats that did not receive SMTC. This was unintentional but did result in similar RBF among both groups of diabetic rats at the time benzolamide was started. After initially declining, RBF gradually increased toward normal during continuous infusion of benzolamide in controls. This restoration of RBF was prevented with SMTC, as previously reported (5). In diabetic rats, RBF remained depressed throughout 1 h of benzolamide and was unaffected by SMTC (Fig. 4).

TGF efficiency was measured by perturbation analysis before and during SMTC microperfusion in 14 control nephrons, 14 nephrons from 1-wk-old diabetic rats, and 12 nephrons from 2-wk-old diabetic rats (Fig. 5). The 2-wk data were obtained after the discovery that TGF efficiency was not reduced as much as expected in 1-wk diabetic rats. That expectation was based on past experience with rats after 5–6 wk of diabetes (29). Consistent with prior experience, neither diabetes nor NOS I blockade affected ambient late proximal flow. In 2-wk diabetic rats, fractional compensation was 50% less than in control (P = 0.005). The addition of SMTC to nephrons caused TGF efficiency to increase (P = 0.007 by repeated-measures ANOVA applied to all groups). The effect of SMTC appeared to be greatest in 2-wk diabetic rats. However, this difference could not be made statistically significant without an untenable increase in sample size.

NOS I and Range of TGF Response

Serial micropuncture data were obtained from 45 nephrons in 2-wk diabetic rats and 25 nephrons from nondiabetic controls (Table 2 and Figs. 6 and 7). Diabetic hyperfiltration was confirmed, and there was a TGF response in each nephron. We refer to SNGFR during minimal TGF stimulation as SNGFR<sub>max</sub> and SNGFR during maximal stimulation of TGF

Table 1. General characteristics of animals studied for effects on GFR of systemic NOS inhibition

<table>
<thead>
<tr>
<th>n</th>
<th>Basal Mean Arterial Pressure, mmHg</th>
<th>During L-NMMA Mean Arterial Pressure, mmHg</th>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con + DMSO 6</td>
<td>92±5</td>
<td>355±13</td>
</tr>
<tr>
<td>Con + 7NI 6</td>
<td>95±5</td>
<td>350±7</td>
</tr>
<tr>
<td>DM + DMSO 6</td>
<td>328±22*</td>
<td>286±19*</td>
</tr>
<tr>
<td>DM + 7NI 6</td>
<td>312±26*</td>
<td>307±8*</td>
</tr>
<tr>
<td>Con 5</td>
<td>93±5</td>
<td>382±17</td>
</tr>
<tr>
<td>Con + SMTC 5</td>
<td>97±2</td>
<td>374±13</td>
</tr>
<tr>
<td>DM 6</td>
<td>288±8*</td>
<td>307±3*</td>
</tr>
<tr>
<td>DM + SMTC 7</td>
<td>299±13*</td>
<td>325±13*</td>
</tr>
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</table>

Values are means ± SE. n. No. of rats; GFR, glomerular filtration rate; NOS, nitric oxide synthase; Con, control; 7NI, 7-nitroindazole; SMTC, S-methyl thiocitrulline; DM, diabetic; Wt, weight, L-NMMA, L-ω-monomethyl-ω-arginine. *P < 0.05 vs. respective control. †P < 0.05 vs. baseline period.

Fig. 1. Glomerular filtration rate (GFR) measured before and during infusion of nonspecific nitric oxide synthase (NOS) blocker Nω-monomethyl-L-arginine (L-NMMA) ± NOS I blocked [7-nitroindazole (7NI)] control (Con) or diabetic (DM) rats. †P < 0.005 for effect of L-NMMA by paired t-test. Those effects of L-NMMA marked not significant (ns) are by paired t-test. Other P values shown are by ANOVA with Tukey test for multiple groups. BW, body weight.

Fig. 2. GFR measured before and during infusion of nonspecific NOS blocker (L-NMMA) ± NOS I blocked [S-methyl thiocitrulline (SMTC)] control or DM rats. †P < 0.005 for effect of L-NMMA by paired t-test. NS refers to effect of L-NMMA by paired t-test. Other P values shown are by ANOVA with Tukey test for multiple groups.
as SNGFR\textsubscript{min}. The addition of SMTC to nondiabetic nephrons reduced SNGFR\textsubscript{max} by 9\% (\(P = 0.07\)) and SNGFR\textsubscript{min} by 24\% (\(P < 0.0005\)). In diabetic rats, adding SMTC reduced SNGFR\textsubscript{max} by 24\% (\(P < 0.0005\)) and SNGFR\textsubscript{min} by 31\% (\(P < 0.0005\)). SNGFR\textsubscript{max} was more sensitive to NOS I blockade in diabetic rats than in nondiabetic rats (\(P = 0.006\)). SNGFR\textsubscript{min} was similarly sensitive to NOS I blockade in diabetic and nondiabetic rats. The range of the TGF response (SNGFR\textsubscript{max}−SNGFR\textsubscript{min}) was increased by NOS I blockade in controls (\(P < 0.05\)) but not diabetic rats (\(P = \text{not significant}\)). In other words, imposing a large salt signal on the MD increased the sensitivity of SNGFR to NOS I blockade in normal rats. In diabetic rats, SNGFR was highly sensitive to NOS I blockade regardless of the amount of salt being delivered to the MD.

**DISCUSSION**

These data permit four main conclusions about the role of NOS I in the control of renal function by the JGA. These will be stated here and then discussed one at a time. First, ambient NO from the JGA exerts a greater tonic influence over GFR in the diabetic kidney. Second, NOS I activity in the normal JGA responds, from minute to minute, to changes in distal salt delivery, whereas NOS I activity in the diabetic JGA does not depend on distal salt delivery. Third, the effect of MD NOS I on SNGFR cannot be fully explained by autocrine inhibition of MD transport. NO from the JGA must also affect the glomerular microvessels. Fourth, when TGF is continuously activated for 1 h, RBF normally relaxes back toward baseline. For this to occur, NOS I activity in the JGA must be free to change. If NO from the JGA is clamped in the “off position” as with NOS I blockers or in the “on position” as in diabetes, then RBF remains depressed by initial TGF activation and does not recover.

The first point mentioned above is that the diabetic kidney is under increased influence from NOS I. This confirms the recent report of another group (8) who observed remarkably similar effects of NOS blockers in whole kidney clearance studies. The most obvious utility of such data might be to supplement the list of putative causes of diabetic hyperfiltration (14). However, these data may be equally useful for considering the role of MD NOS I in normal physiology. This is because there are unlikely to be any regulatory pathways that are unique to diabetes or uniquely absent in diabetes. Therefore, if the diabetic kidney responds differently to NOS I inhibition, this should be due to quantitative differences in the activities of mechanisms that exist in nondiabetic and diabetic subjects alike. In this way, nuances in the diabetic response to NOS I blockade could reveal details of a mechanism that happens to be confounded or concealed under normal conditions.

The second point to be made from the present data relates to salt delivery as a determinant of NOS I activity in the MD. So far, apical salt entry is the only proximate stimulus that has been directly observed to activate NOS I in MD cells (9). The present data from control rats confirm that this mechanism can be physiologically relevant. Apical entry of salt is also the first step toward eliciting a vasoconstrictor TGF response (17) and it is well known that NO made in the MD normally offsets the TGF response (30). Based on this situation, one might imagine that the osmotic effect of glucose in the proximal tubule leads to increased distal delivery of salt, continuous overproduction of NO by the MD, and glomerular hyperfiltration. This would make NO the link between hyperglycemia and glomerular hyperfiltration, which is a cardinal feature of early diabetes. Such a simple scheme may be appealing, but it is fatally flawed for at least two reasons. First, increased distal salt delivery is not a prerequisite for diabetic hyperfiltration. In fact, due to a

![Fig. 3. Arterial blood pressure before and during 1-h infusion of benzolamide, with or without NOS I blocker (SMTC) in control and diabetic rats. Animals were equilibrated with perivascular flow probe around the left renal artery for 2 h before. Corresponding renal blood flow (RBF) measurements are shown in Fig. 4.](http://ajprenal.physiology.org/)

![Fig. 4. RBF left kidney blood flow expressed per 300 g body wt. Points before \(-20\) min reflect equilibrated preblockade values for those receiving NOS I blocker SMTC. Those receiving SMTC were reequilibrated before benzolamide was started. Benzolamide started at time 0. \(* P < 0.05\) for effect of benzolamide. \(**P < 0.05\) for effect of SMTC on slope of tubuloglomerular feedback (TGF) resetting.](http://ajprenal.physiology.org/)
marked increase in reabsorptive capacity of the proximal tubule, delivery of salt to the MD falls well below normal in diabetes, notwithstanding the increased reabsorptive burden imposed by hyperfiltration (16, 19, 29). Therefore, the initial requirement for increased salt to stimulate NOS I is not met. Second, the present data reveal that the diabetic JGA can produce abundant NO independently of MD salt. The relevance of this finding should extend beyond diabetes because, again, it is unlikely that any regulatory pathway is unique to diabetes. Henceforth, any theory to explain the regulation of MD NO primarily affects SNGFR by blocking MD transport and there must be salt transport available for NO to inhibit. Our main clue regarding how much NO is present comes from the decrement in SNGFR when NOS I is acutely blocked with SMTC. However, the amount of NO may not be the only determinant of the response to SMTC. For example, if MD NO primarily affects SNGFR by blocking MD transport then, for a given amount of NO, there will be a lesser effect of SMTC when there is less transport available to inhibit. There are two opportunities to observe this among the present findings. The first, and most obvious, is that MD transport is eliminated as a target for NO when SNGFR is measured without flow through Henle’s loop. The finding that SMTC reduced SNGFR in diabetic nephrons under this circumstance eliminates MD salt thereby blunting the sensory limb of the TGF system. NO from the MD might also impact glomerular hemodynamics by a paracrine vasodilatory effect on the glomerular microcirculation. However, demonstrating that NO is capable of these autocrine and paracrine effects does not reveal to what degree each of these impacts kidney function. The present data help to sort this out. For there to be an autocrine effect of MD NO on the TGF response, there must be NO available to inhibit salt transport and there must be salt transport available for NO to inhibit. Our main clue regarding how much NO is present comes from the decrement in SNGFR when NOS I is acutely blocked with SMTC. However, the amount of NO may not be the only determinant of the response to SMTC. For example, if MD NO primarily affects SNGFR by blocking MD transport then, for a given amount of NO, there will be a lesser effect of SMTC when there is less transport available to inhibit. There are two opportunities to observe this among the present findings. The first, and most obvious, is that MD transport is eliminated as a target for NO when SNGFR is measured without flow through Henle’s loop. The finding that SMTC reduced SNGFR in diabetic nephrons under this circumstance eliminates MD salt.

Table 2. Effects of NOS blockade, diabetes, and TGF activation on SNGFR

<table>
<thead>
<tr>
<th>LOH Perfusate and TGF</th>
<th>ATP</th>
<th>ATP + NOS I Blockade</th>
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<tr>
<td>TGF activated</td>
<td></td>
<td></td>
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<tr>
<td>Nondiabetic (n = 25)</td>
<td>21.0 ± 2.5</td>
<td>32.4 ± 2.5</td>
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<tr>
<td>Diabetic (n = 45)</td>
<td>27.8 ± 1.7*</td>
<td>45.9 ± 1.6*</td>
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Values are means ± SE. TGF, tubuloglomerular feedback; SNGFR, single-nephron GFR; ATP, artificial tubular fluid; LOH, loop of Henle. TGF activated: LOH perfused at 38 nl/min. TGF not activated: LOH perfused at 8 nl/min. Four measurements were made in each nephron according to a temporal sequence represented by reading the table from left to right. *P < 0.05 vs. nondiabetic. †P < 0.05 for effect of NOS I blockade.
transport as the sole target of MD NO in diabetes. Therefore, NO made in the JGA must be able to act on the glomerular microvessels. Although it is unlikely that the mechanism involved is unique to diabetes, this conclusion could only be reached by considering the results in diabetic rats, because flow dependence of the SMTc effect in nondiabetic rats makes it impossible to rule out MD transport as the sole target of NO in those animals. Thus a physiological mechanism has been confirmed by studying pathophysiology. Second, MD salt content is less in diabetes (13, 16, 19, 29). Therefore, if MD salt were both the main originator and the main target of MD NO in diabetes, there would be a lesser effect of NOS I blockade on GFR. Because this is contrary to the present finding, the role of NOS I as a modulator of TGF cannot be limited to inhibiting MD transport.

The fourth point to be made from the present data pertains to the role of MD NO in the temporal adaptation, or resetting, of TGF. Most research on TGF assumes there to be a static relationship between SNGFR and MD salt. However, it is obvious that the TGF relationship cannot be static because the role of MD NOS I as a modulator of TGF cannot be limited to inhibiting MD transport.

As a final caveat, overactive NOS has received attention as a potential cause of diabetic hyperfiltration. From the present data, it is clear that ambient NO excess does not fully explain diabetic hyperfiltration. This is not surprising given that diabetic kidneys are large and that, all else being equal, GFR should vary in proportion to kidney mass (24). Even had acute NOS I blockade completely normalized GFR in diabetes, it would not have been appropriate to invoke this as proof that NO “caused” hyperfiltration in the first place. Whether continuous exposure to excess NO is necessary for the diabetic kidney to become large is a subject distinct and separate from the present study.

REFERENCES


