Posttranslational regulation of NO synthase activity in the renal medulla of diabetic rats

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Shear stress increases nitric oxide (NO) production by endothelial cells, inner medullary collecting duct cells, and thick ascending limb. We postulated that the osmotic diuresis accompanying type 1 diabetes is associated with increased NO synthase (NOS) activity and/or expression in the renal medulla. Diabetes was induced by injection of streptozotocin, with insulin provided to maintain moderate hyperglycemia (Hyp) or euglycemia (Eug) for 3 wk. Sham rats received vehicle treatments. A separate group of rats (Phz) received phlorizin to produce a glucose-dependent osmotic diuresis. Renal medullary NOS1 and NOS2 activities did not differ between groups, whereas NOS3 activity was significantly increased in Hyp. Neither NOS1 nor NOS3 protein levels differed significantly between groups. Reduced phosphorylation of NOS3 at Thr495 and Ser633 was evident in medullary homogenates from Hyp rats, with no difference apparent at Ser1177. Immunohistochemical analysis indicated prominent expression of pThr495/NOS3 in the thick ascending limb and collecting duct of Sham and Phz rats. Hyp rats displayed staining in the collecting duct but minimal thick ascending limb staining. Immunostaining with anti-pSer1177/NOS3 was evident only in the thick ascending limb, with no apparent differences between groups. In summary, glucose-dependent osmotic diuresis alone did not alter NOS activity or expression in the renal medulla. Diabetic hyperglycemia increased medullary NOS3 activity without a concomitant increase in NOS3 protein levels; however, NOS3 phosphorylation was reduced at Thr495 and Ser633. Thus changes in the phosphorylation of NOS at known regulatory sites might represent the primary mechanism underlying increased renal medullary NOS activity in diabetic hyperglycemia.

Streptozotocin; phosphorylation; phlorizin; nitric oxide synthase; NOS3; NOS1

AN IMPORTANT PHYSIOLOGICAL regulator of renal function, nitric oxide (NO) is synthesized as a by-product of the conversion of L-arginine to L-citrulline. The greatest capacity for renal NO synthesis resides in the medulla (23, 34), which expresses all three isoforms of NO synthase (NOS) (18). A paracrine NO system has been postulated to reside within the renal medulla, relying on cross-talk between epithelial and microvascular cells to match vasa recta blood flow to tubular metabolic transport activity (6). Accordingly, a decrease in NO production achieved by medullary interstitial infusion of NOS inhibitors leads to a reduction in medullary blood flow and salt retention, whereas infusion of NOS substrate increases medullary blood flow (15, 28). A previous report (3) from our laboratory revealed that NO production is modulated by shear stress in an inner medullary collecting duct cell line (IMCD-3), suggesting that tubular fluid flow may play a role in regulating sodium and water excretion by virtue of shear stress-mediated control of NO production. Moreover, Ortiz et al. (27) recently reported that flow stimulates translocation and phosphorylation of NOS3 and an associated increase in NO production by isolated thick ascending limb segments. However, it is unclear whether chronic increases in urine flow per se provoke an increase in NO synthesis and/or NOS expression in the renal medulla. One approach to examining this postulate is to determine whether pathophysiological states associated with increased urine flow are associated with increased NOS activity in the renal medulla. For example, urine flow is significantly increased in type 1 diabetes mellitus (T1D) because of a glucose-dependent osmotic diuresis. Thus, if shear stress represents an important determinant of NO production in the thick ascending limb and/or collecting duct, one would predict that renal medullary NOS activity would be increased in T1D.

Most of the literature concerning the renal complications of T1D focuses on alterations in glomerular structure/function or impaired regulation of arteriolar tone/reactivity. However, recent studies have revealed that the early stage of T1D is accompanied by altered expression of multiple renal medullary transport proteins: e.g., the bumetanide-sensitive Na+-K+-2Cl− cotransporter; epithelial Na+ channel subunits-α, -β, and -γ; aquaporins 2 and 3; urea transporter-A1 (16, 30). These changes should significantly influence salt and water balance, which also relies on appropriate NO regulation of medullary blood flow and transport in the distal nephron; however, little is known about the status of the medullary NO system in T1D. Shin et al. (29) detected increased NOS1 and NOS3 mRNA in the outer medulla of diabetic rats with no change evident in the inner medulla when compared with normal rats. These investigators also described a significant increase in NOS1 and NOS3 immunostaining in the proximal straight tubule and the medullary thick ascending limb of diabetic rats, with only a mild increase in NOS1 immunostaining observed in the outer medullary collecting duct and inner medullary collecting duct (29). Conversely, Choi et al. (4, 5) found no change in the protein level of any NOS isoform in the renal medulla of rats.
studied 28 days after streptozotocin injection, although expression of all three isoforms was elevated in the cortex. To date, no studies have quantified NOS activity in the renal medulla of diabetic animals. The need to quantify NOS activity per se in this setting is underscored not only by the dearth of information available concerning renal medullary NOS protein levels in T1D but also by the fact that the activity of each NOS isoform is regulated by a complex array of posttranscriptional and posttranslational events, any or all of which might be deranged in pathophysiological states. For example, the exposure of endothelial cells to high-glucose conditions results in O-glycosylation of NOS3 (7), which has the potential to impede serine/threonine phosphorylation of the enzyme at any of the numerous sites known to be important in regulating enzyme activity. Reduced NOS3 phosphorylation at Ser1177 has been documented in aortic endothelium from rats studied 16 wk after the onset of T1D (22); however, no information is available concerning the possibility that T1D alters NOS phosphorylation in the renal medulla.

The goal of the present study was to evaluate the hypothesis that increased flow through the distal nephron (as would occur in the polyuric states such as T1D) provokes an increase in renal medullary NOS activity. The strategy was to quantify NOS activity in rats with either 1) streptozotocin-induced T1D or 2) polyuria resulting from phlorizin treatment of normal rats. Although increased medullary NOS3 activity was demonstrated in rats with T1D, phlorizin-induced osmotic diuresis yielding comparable urine flow was insufficient to provoke an increase in NOS activity. Accordingly, further experiments evaluated the contribution of increased NOS protein levels and/or posttranslational events (phosphorylation at known regulatory sites) as mechanisms that might underlie increased renal medullary NOS activity in T1D.

METHODS

Animals

All animal procedures were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee and conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experiments were performed using male Sprague-Dawley rats (SAS:VAF strain) purchased from Charles River Laboratories (Wilmington, MA). At the beginning of the study (day 0), blood glucose levels were measured (Accu-Check III model 766; Boehringer Mannheim, Indianapolis, IN) and each rat was weighed and assigned randomly to one of four groups: normal rats receiving vehicle treatments (Sham; n = 17), diabetic rats with partial insulin replacement sufficient to maintain modest hyperglycemia (Hyp; n = 16), diabetic rats with insulin replacement to achieve euglycemia (Eug; n = 7), and normal rats receiving phlorizin treatment to induce a glucose-dependent osmotic diuresis (Phz; n = 10).

Beginning on day 0, rats in the Phz group received 0.4 g·kg⁻¹·day⁻¹ phlorizin delivered in twice daily subcutaneous injections of 40% phlorizin in propylene glycol. These animals were housed in pairs until the end of the study with free access to food and water containing 5% sucrose (to prevent weight loss due to chronic glucosuria).

Rats in the Sham, Hyp, and Eug groups were anesthetized on day 0 with methohexital sodium (50 mg/kg ip) to facilitate intravenous injection of either 65 mg/kg streptozotocin (Sigma; St. Louis, MO; Hyp and Eug rats) or vehicle (Sham rats). These rats were allowed to recover from anesthesia and housed in pairs overnight with free access to food and water. The next day (day 1), blood glucose levels were measured and the rats were anesthetized with methohexital sodium (50 mg/kg ip) to facilitate subcutaneous insertion via a 16-G needle of either a 2.3 × 2.0 mm sustained-release insulin implant (Hyp rats), a 5.25 × 2.0 mm sustained-release insulin implant (Eug rats) or a 2.3 × 2.0 mm microencrystallized palmitic acid implant (vehicle; Sham rats). The insulin and vehicle implants were purchased from Linshin Canada (Scarborough, Ontario, Canada). After recovery from anesthesia, the animals were housed in pairs with free access to food and water until the end of the study.

Blood glucose concentration and body weight were measured in all rats at 3- to 4-day intervals for 19 ± 1 days, and most animals were housed individually in metabolic cages (Naïge Nuc International, Rochester, NY) for 2 days just before termination of the study. The volume of urine collected (under oil) during the final 24-h period was determined gravimetrically, and urine samples were centrifuged, filtered, and stored at −80°C until analysis. At the end of the study, rats were anesthetized with pentobarbital sodium (50 mg/kg ip) and tracheotomized to facilitate spontaneous respiration. A blood sample was obtained from the abdominal aorta, centrifuged, and the plasma was stored at −80°C until analysis. Both kidneys were flushed with cold isotonic saline via the aorta, harvested, weighed, and the medulla was snap frozen in liquid N₂ and stored at −80°C until analysis.

Analyses

NOS activity. Total NOS activity in the renal medulla was determined on the basis of the rate of L-[3H]citrulline formation from L-[3H]arginine, as described previously (13). Briefly, the medulla of the left kidney was weighed, minced, and homogenized in ice-cold buffer containing 50 mmol/l Tris, 0.1 mmol/l EDTA, 10% glycerol, 0.1% β-mercaptoethanol, 1 mmol/l aprotinin, 1 mmol/l pepstatin A, 2 mmol/l leupeptin, and 1 mmol/l PMSF (pH 7.4). The homogenate was centrifuged at 100,000 × g and separated into cytosolic and particulate fractions. The particulate fraction was resuspended in half of the original homogenization buffer along with the original concentration of inhibitors and homogenized. Aliquots of cytosolic and particulate fractions were incubated with [3H]arginine (71 Ci/mmol, 10 μmol/l final arginine concentration; Perkin-Elmer Life and Analytical Sciences, Shelton, CT) in the presence of 1 mmol/l NADPH, 30 mmol/l calmodulin, 3 μmol/l tetrahydrobiopterin, 2 mmol/l CaCl₂, 3 mmol/l valine, 1 μmol/l FAD, and 1 μmol/l flavin mononucleotide in a final volume of 50 μl for 30 min at room temperature. NOS activity was normalized to the milligram of protein (pmol/30 min/total protein). Total NOS activity was defined as [3H]arginine-to-[3H]citrulline conversion that was inhibited by the nonselective NOS inhibitor Nω-nitro-L-arginine (1-NNa; 1 μmol/l). The activity of each NOS isoform was determined based on the actions of isoform-specific inhibitors: Nω-(1-imino-3-butenyl)-L-ornithine (1 μmol/l) was employed as a specific NOS1 inhibitor, and 1400W dihydrochloride (1400W, 100 mmol/l) was used as a NOS2-specific inhibitor. NOS1 and NOS2 activities were defined as the Nω-(1-imino-3-butenyl)-L-ornithine-sensitive and 1400W-sensitive components of total NOS activity, respectively. NOS3 activity was estimated as total NOS activity − (NOS1-specific activity + NOS2-specific activity).

Western blot analysis. The Western blot protocol was performed as described previously (13, 31). Briefly, proteins (300 μg) were separated on 7.5% SDS-PAGE and transferred to nitrocellulose by wet blotting (Bio-Rad Laboratories, Hercules, CA) for 55 min. The blots were allowed to air-dry for 20 min and blocked with 5% nonfat dry milk diluted in Tris-buffered saline (blocking buffer) for 1 h at room temperature. The blots were incubated overnight with primary antibody against NOS isoforms (NOS3; 1:1,000; NOS1; 1:2,500; Transduction Laboratories, Lexington, KY) or phosphorylation site-specific primary antibody (pSer1177NOS3, 1:500, Cell Signaling Technologies, Beverly, MA; pSer495NOS3, 1:500, Upstate Biotechnology, Lake Placid, NY; pThr495NOS3, 1:500; Upstate). The blots were...
incubated with the secondary antibody (horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit; 1:2,000; Amersham Biosciences, Piscataway, NJ) for 1 h at room temperature, followed by 5 washes with Tris-buffered saline. The specific bands were detected with the use of enhanced chemiluminescence (Supersignal Chemiluminescent Substrate; Pierce Biotechnology, Rockford, IL.). Densitometry was used to quantify the bands with an Alpha Innotech Imaging System (San Leandro, CA). The Bradford assay was used to assay protein concentrations. Equal protein loading was verified by stripping with ReBlot Plus Stripping Solution (Chemicon International, Temecula, CA) and reprobing the immunoblots for β-actin (1:5,000; Sigma-Aldrich).

Immunohistochemical analysis. In some rats, kidneys were cleared of blood under pentobarbital anesthesia with heparinized saline via an aortic cannula and then perfused with 4% paraformaldehyde in 100 mmol/l dibasic sodium phosphate buffer. Kidneys were removed and immersed in 4% paraformaldehyde solution overnight at room temperature, transferred to 20% sucrose for 24 h at 4°C and paraffin embedded. The kidneys were sectioned at a thickness of 4 μm onto Superfrost plus slides. Endogenous peroxidase was blocked by exposure to 3% H2O2 for 15 min, followed by washing successively in deionized distilled H2O and phosphate-buffered saline for 5 min. Tissues were processed for specific staining by first utilizing Target Retrieval Solution (DakoCytomation, Carpinteria, CA), rinsing in phosphate-buffered saline, and incubating in the absence or presence of primary antibody (pThr147/148NOS3, 1:100; or pSer1177/1179NOS3, 1:50) in humidity chambers overnight at 4°C. Slides were rinsed with phosphate-buffered saline, followed by incubation with peroxidase-conjugated donkey anti-rabbit IgG (1:300; Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min at room temperature. Specific staining was detected with diaminobenzamidine (liquid DAB substrate; DakoCytomation), counterstained with Mayer’s hematoxylin, and coveredslipped with Cytoseal 60 mounting medium (Richard-Allan Scientific, Kalamazoo, MI). The stained sections were viewed with a microscope (model BX40, Olympus America, Melville, NY) on brightfield setting fitted with a digital camera (model DP12, Olympus America).

Plasma and urine analyses. Electrolyte concentrations were determined by ion-selective electrodes (Synchron EL-ISE; Beckman Instruments, Brea, CA). Urinary cGMP concentration was measured by a scintillation proximity method, as described previously (14). ELISA instruments, Brea, CA). Urinary cGMP concentration was measured by (model DP12, Olympus America).

Streptozotocin-Treated Rats

Table 1. Characteristics of Sham, euglycemic, hyperglycemic, and phlorizin-treated rats

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Eug</th>
<th>Hyp</th>
<th>Phz</th>
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<tbody>
<tr>
<td>BW, g</td>
<td>385±13 (n=17)</td>
<td>397±5 (n=7)</td>
<td>346±9* (n=16)</td>
<td>385±6 (n=10)</td>
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<tr>
<td>Left KW, mg</td>
<td>1,381±62 (n=12)</td>
<td>1,342±63 (n=7)</td>
<td>1,689±66* (n=12)</td>
<td>1,428±44 (n=10)</td>
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<tr>
<td>Left KW/BW, mg/g</td>
<td>3.38±0.09 (n=12)</td>
<td>3.38±0.15 (n=7)</td>
<td>4.66±0.16* (n=12)</td>
<td>3.71±0.09 (n=10)</td>
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<tr>
<td>Plasma [insulin], μg/l</td>
<td>2.75±0.33 (n=9)</td>
<td>2.96±0.64 (n=7)</td>
<td>1.09±0.12* (n=8)</td>
<td>1.88±0.30 (n=10)</td>
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<tr>
<td>Blood [glucose], mg/dl</td>
<td>86±2 (n=17)</td>
<td>100±11 (n=7)</td>
<td>355±12* (n=16)</td>
<td>78±1 (n=10)</td>
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<tr>
<td>Urine flow, ml/h</td>
<td>0.8±0.1 (n=9)</td>
<td>1.1±0.1 (n=7)</td>
<td>3.8±0.6* (n=8)</td>
<td>3.2±0.3* (n=10)</td>
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<tr>
<td>Glucose excretion, mg/h</td>
<td>0.11±0.04 (n=9)</td>
<td>20±9 (n=7)</td>
<td>331±67* (n=8)</td>
<td>188±18* (n=10)</td>
</tr>
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<td>Na+ excretion, mM/h</td>
<td>66±4 (n=9)</td>
<td>78±4 (n=7)</td>
<td>92±10* (n=8)</td>
<td>54±5 (n=6)</td>
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<td>K+ excretion, mM/h</td>
<td>217±8 (n=9)</td>
<td>227±8 (n=8)</td>
<td>323±28* (n=8)</td>
<td>223±11 (n=10)</td>
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<tr>
<td>Microalbumin excretion, μg/l</td>
<td>55±15 (n=9)</td>
<td>70±18 (n=9)</td>
<td>113±53 (n=5)</td>
<td>97±21 (n=7)</td>
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<tr>
<td>cGMP excretion, pmol/h</td>
<td>2,720±470 (n=7)</td>
<td>2,786±484 (n=7)</td>
<td>5,369±425* (n=8)</td>
<td>3,028±647 (n=10)</td>
</tr>
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</table>

Values are means±SE; n, no. of rats. Sham, rats receiving vehicle treatments; Eug, streptozotocin-treated rats receiving partial insulin replacement; Hyp, streptozotocin-treated rats receiving insulin replacement sufficient to restore euglycemia; Phz, rats receiving chronic phlorizin treatment; BW, body weight on date of death; KW, kidney weight. Blood [glucose] values represent averages obtained from measurements made every 3 to 4 days from day 1 until termination of the study. Excreta data were derived from 24-h urine collections in metabolic cages within 48 h of termination of the study. Plasma samples were obtained under anesthesia immediately before termination of the study. *P < 0.05 vs. Sham.

Statistics

All values are expressed as means±SE (n = number of animals). Statistical comparisons were performed using one-way ANOVA, followed by the Holm-Sidak test for multiple comparisons. If the data failed a normality test, the Kruskal-Wallis one-way ANOVA on ranks was applied in concert with Dunn’s method for multiple comparisons. P values <0.05 were considered significant.

RESULTS

Animal Characteristics

On day 0 (before any experimental manipulation), the rats utilized in this study weighed 317±4 g and had blood glucose levels averaging 84±2 mg/dl (n=50). Twenty-four hours after streptozotocin injection, blood glucose levels were significantly elevated in Hyp and Eug rats (332±14 mg/dl; n=23), confirming the onset of T1D before implantation of sustained release insulin pellets. The efficacy of the two insulin replacement regimens is indicated by significantly reduced plasma insulin levels and increased blood glucose concentration averaging ~350 mg/dl in Hyp rats (Table 1), whereas Eug rats exhibited only a minimal increase in blood glucose concentration with plasma insulin levels that did not differ significantly from those observed in Sham rats. Table 1 also summarizes other characteristics of the animals as evident ~3 wk after induction of T1D or initiation of phlorizin treatment. Relative to Sham rats, animals in the Hyp group displayed renal hypertrophy, polyuria, natriuresis, kaliuresis, and increased urinary excretion of cGMP. None of these parameters differed significantly between Sham and Eug rats. The ability of the Phz rat model to mimic the diuresis of T1D is evidenced by the fact that urine flow did not differ significantly in Phz vs. Hyp rats, averaging 4~5 times that observed in Sham rats. In addition, urinary glucose excretion was markedly increased in the Phz group, although not to the extent observed in Hyp rats (P = 0.025). However, Phz rats did not differ from Sham rats with regard to any other of these basic characteristics.

NOS Activity

Total NOS activity in the renal medulla was measured as l-[[3H]citrulline formation from l-[3H]argin-
ine in the presence of NOS cofactors. In tissue from Sham rats \( (n = 9) \), total NOS activity in the cytosolic fraction averaged \( 17.9 \pm 2.8 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1} \), whereas activity in the particulate fraction averaged \( 16.1 \pm 2.6 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1} \) (Fig. 1, A and B). On the basis of the effects of isoform-specific inhibitors, \(~75\%\) of total NOS activity in both the cytosolic and particulate fractions could be attributed to NOS3, with \(~20\%\) of total activity reflecting NOS1 and \(~5\%\) attributed to NOS2. This general trend was apparent in each of the animal groups.

Total NOS activity in the cytosolic fraction of renal medulla from Hyp rats was twice that observed in samples from Sham rats \( (P < 0.05) \), whereas values observed in tissue from Eug and Phz rats did not differ significantly from Sham (Fig. 1A). This phenomenon was mirrored by an increase in NOS3 activity in the cytosolic fraction prepared from Hyp rats (approximately double that observed in Sham rats; \( P < 0.05 \)), while Phz and Eug rats displayed NOS3 activities similar to Sham. NOS1 activity in the cytosolic fraction tended to be greater in Hyp than in Sham; however, its value was numerically similar to Eug, and ANOVA failed to verify a significant difference in this parameter between the four treatment groups \( (P = 0.22) \). Moreover, NOS2 activity in the cytosolic fraction did not differ between the treatment groups \( (P = 0.59) \). In the particulate fraction, none of the treatment groups differed with regard to either total NOS activity or the activity of any single NOS isoform (Fig. 1B; all \( P \) values >0.22). Thus Hyp (but not Phz) rats displayed increased total NOS activity in the cytosolic fraction of renal medulla and this effect could be attributed primarily to an increase in NOS3 activity.

**NOS Protein Levels**

Renal medullary NOS1 and NOS3 protein levels were assessed by Western blot analysis in the four groups of rats. Two immunoreactive bands for NOS1 were detected in the immunoblots of renal medullary tissue. Additional immunoblots were performed with two other NOS1-specific primary antibodies with the same results (data not shown). In all four groups, NOS1 protein levels were markedly higher in the cytosolic fraction than in the particulate fraction (Fig. 2). There was a tendency toward increased NOS1 levels in medullary cytosolic fractions from Hyp and Phz rats; however, the densitometric data were quite variable. Accordingly, ANOVA failed to verify a significant difference between the four treatment groups with regard to NOS1 levels in either the cytosolic fraction \( (P = 0.09) \) or the particulate fraction \( (P = 0.19) \).

In contrast with NOS1 distribution, NOS3 levels tended to be greater in the particulate fraction than in the cytosolic fraction (Fig. 3). Sham, Hyp, Eug, and Phz rats did not differ significantly with regard to NOS3 protein levels in either fraction (cytosolic, \( P = 0.07 \); particulate, \( P = 0.50 \)). Thus the immunoblotting data failed to provide convincing evidence that an increase in NOS3 protein levels underlies the elevated NOS3 activity apparent in the renal medulla of Hyp rats.

**NOS Phosphorylation**

Phosphorylation of NOS3 at known regulatory sites was evaluated in medullary homogenates (without separation into cytosolic and particulate fractions) from Sham and Hyp rats by Western blot analysis. As shown in Fig. 4, pThr\(^{495}\)NOS3/total-NOS3 in medullary tissue from Hyp rats was only 30% of that detected in Sham rats \( (P = 0.002, n = 8) \). pSer\(^{633}\)NOS3/total-NOS3 in Hyp tissue also averaged 30% of values evident in medullary homogenates from Sham rats \( (P = 0.02, n = 8) \). However, this analysis failed to reveal a significant difference between Sham and Hyp rats with regard to pSer\(^{1177}\)NOS3/total-NOS3 \( (P = 0.16, n = 8) \). Thus decreased NOS3 phosphorylation at Thr\(^{495}\) and Ser\(^{633}\) is apparent in the renal medulla of Hyp rats.

**Immunohistochemical Analysis**

Immunohistochemical analysis was performed to localize pThr\(^{495}\)NOS3 and pSer\(^{1177}\)NOS3 in the renal medulla of Sham, Hyp, and Phz rats. As illustrated in Fig. 5, pThr\(^{495}\)NOS3 immunostaining is evident in the thick ascending limb and in both the outer and inner medullary segments of the collecting duct in kidneys from Sham rats. Anti-pThr\(^{495}\)NOS3 immunostaining of the collecting duct cells appears cytosolic and...
diffuse, in striking contrast with the predominant apical localization of staining in the thick ascending limb. Immunostaining for pThr495NOS3 is also apparent in the inner medullary vasa recta, as well as the endothelium of intrarenal arteries. In kidneys from Hyp rats, pThr495NOS3 immunostaining is obvious in the outer and inner medullary segments of the collecting duct; however, minimal staining is evident in the thick ascending limb. Phz rats demonstrated renal medullary pThr495NOS3 immunostaining patterns similar to that observed in Sham rats (data not shown). The sections processed in the absence of primary antibody showed no immunostaining (Fig. 5E).

Immunostaining of pSer1177NOS3 in the renal medulla of Sham and Hyp rats (Fig. 6) showed no demonstrable differences between groups. Immunostaining was present in the apical membrane of the thick ascending limb, but little or no staining was evident in the collecting duct. Phz rats displayed similar patterns of medullary immunostaining for pSer1177NOS3 (data not shown).

**DISCUSSION**

The roles of NO and NOS in the diabetic kidney are controversial. A number of studies have produced contradictory findings, reviewed recently by Komers and Anderson (17), most likely reflecting differing levels of glycemic control and the phase of nephropathy. This report focuses on the early, hyperfiltration phase of T1D in a rat model with moderate hyperglycemia maintained by exogenous insulin therapy, thus resembling the clinically relevant state that ultimately engenders diabetic nephropathy. The major findings from the present study are the following: 1) NOS activity is increased in the cytosolic fraction of the renal medulla of diabetic rats; 2) this phenomenon can be attributed primarily to an increase in NOS3 activity; 3) the increased NOS activity accompanying T1D is prevented by insulin therapy sufficient to maintain euglycemia but is not mimicked by a phlorizin-induced osmotic diuresis; 4) medullary NOS1 and NOS3 protein levels are not significantly altered in T1D or phlorizin-induced osmotic diuresis; 5) kidneys of diabetic rats exhibit decreased phosphorylation of renal medullary NOS3 at Thr495 and Ser633 but not at Ser1177; and 6) the reduced phosphorylation of NOS3 at Thr495 appears most evident in the thick ascending limb.

Shear stress or flow can increase NO production by cultured IMCD-3 and isolated thick ascending limbs, suggesting that tubular fluid flow might influence sodium and water excretion via alterations in NO-dependent events in these nephron segments (3, 27). However, no previous studies have explored the possibility that NOS activity in these (or other) medullary structures might be increased in response to a chronic in vivo change from conditions of normal tubular fluid flow to conditions of high flow. Accordingly, the present study addressed the hypothesis that the glucose-dependent osmotic diuresis associated with T1D, with the attendant chronic increase in

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**Fig. 2.** NOS1 protein levels in cytosolic (C) and particulate (P) fractions of renal medulla from Sham, Eug, Hyp, and Phz rats. A: representative Western blots. B: summary of densitometric data, normalized to values observed in cytosolic fraction from Sham rats. Values are means ± SE. No significant between-group difference in NOS1 protein was apparent in either the cytosolic (C) or the particulate (P) fraction.

**Fig. 3.** NOS3 protein levels in cytosolic and particulate fractions of renal medulla from Sham, Eug, Hyp, and Phz rats. A: representative Western blots. B: summary of densitometric data, normalized to values observed in cytosolic fraction from Sham rats. Values are means ± SE. No significant between-group difference in NOS3 protein was apparent in either the cytosolic or the particulate fraction.
tubular fluid flow through the thick ascending limb and collecting duct, would evoke changes in renal medullary NOS activity. Changes in medullary NOS activity in T1D might result not only from polyuria per se but also as a direct effect of hyperglycemia and/or hypoinsulinemia on medullary structures. Hence, our approach was to determine whether chronic glucose-dependent osmotic diuresis, with the attendant increase in tubular fluid flow through the loop of Henle and collecting duct, is insufficient to account for the increase in renal medullary NOS (mainly NOS3) activity accompanying T1D.

The present study demonstrates that the NOS3 activity and expression in the renal medulla are cytosolic as well as associated with membrane structures. In contrast, NOS3 is localized to plasma membrane caveolar domains and Golgi membranes in endothelial cells due to posttranslational fatty acylation, specifically myristoylation and palmitoylation, at the NH$_2$ terminus of the protein (32). Future experiments are needed to clarify whether or not NOS3 in the kidney is regulated by fatty acylation similar to aortic endothelial cells. This process might have functional implications with respect to the pool of enzyme available for stimulation or the bioavailability of NO produced by NOS3 associated with cytosolic or membrane structures in the renal medulla.

This study also demonstrates that the hyperglycemic condition increases cytosolic NOS3 activity without a concomitant increase in protein expression. Insulin treatment that lowers blood glucose levels normalized the increase in NOS activity. We (13) previously reported an increase in total NOS activity in renal cortical homogenates in T1D without a corresponding upregulation in any of the three NOS isoforms; however, that study did not evaluate medullary NOS or the subcellular localization of NOS isoforms. Omer et al. (24) reported an increase in Ca$^{2+}$-dependent NOS activity measured in whole kidney homogenates of female rats studied 2 wk after streptozotocin-induced T1D. Choi et al. (4, 5) reported unaltered expression of all three NOS isoforms in the renal medulla after 7 and 28 days of streptozotocin-induced T1D, a result similar to the present study. On the other hand, Shin et al. (29) demonstrated increased NOS1 and NOS3 mRNA in the outer medulla of rats with streptozotocin-induced T1D. Furthermore, these investigators reported that NOS1 and NOS3 immunoreactive staining was substantially increased in the proximal straight tubule and medullary thick ascending limb of diabetic rats, with slight increases evident in the collecting duct (29). The work by Shin et al. (29) was conducted 6 wk post streptozotocin, which may account for the difference with our study performed after 3 wk of diabetes.

Posttranslational phosphorylation/dephosphorylation of NOS3 plays an active role in the stimulation of NO production in aortic endothelial cells by shear stress, insulin, and various
other agonists (2, 9). The five sites on NOS3 known to be
regulated by serine/threonine phosphorylation in aortic endo-
thelial cells are Ser⁶¹⁶, Ser⁶¹⁷, Ser⁶³³, Ser¹¹⁷⁷, and Thr⁴⁹⁵
(human amino acid sequence numbering). Relatively little is
known about the roles of Ser¹¹⁶ and Ser⁶¹⁷ in regulating NOS3
activity; however, studies of aortic endothelial cells suggest
that NOS3 is activated by phosphorylation of Ser⁶³³ and
Ser¹¹⁷⁷ and/or dephosphorylation of Thr⁴⁹⁵ (1). Ortiz and
coworkers (27) recently provided the first evidence that NOS3
phosphorylation at Ser¹¹⁷⁷ is associated with increased NO
production in the kidney, specifically in the thick ascending
limb. The results of the present study provide further evidence
that NOS3 phosphorylation at known regulatory sites is altered
in the renal medulla under conditions associated with changes
in NOS3 activity.

The immunoblotting analysis revealed decreased NOS3
phosphorylation at Ser⁶³³ and Thr⁴⁹⁵ in the renal medulla of
Hyp rats, with no significant change apparent at Ser¹¹⁷⁷.
Dephosphorylation at Thr⁴⁹⁵ would favor NOS3 activation,
whereas dephosphorylation at Ser⁶³³ would favor a decline in
NOS3 activity (1). Given that NOS3 activity was increased in
Hyp rats (Fig. 1), one might speculate that the phosphorylation
state of Thr⁴⁹⁵ might be more predominant than that of Ser⁶³³
as a determinant of renal medullary NOS3 activity. Moreover,
different pools of NOS3 (e.g., localized in specific medullary
structures) could exist, with each potentially subject to distinct
phosphoregulatory mechanisms. In an initial attempt to explore
this postulate, immunostaining was employed to localize the
renal medullary structures that display NOS3 phosphorylation
at specific regulatory sites. Because immunostaining with anti-
pSer⁶³³NOS3 was unsuccessful with the use of paraffin-em-
bedded kidneys in this study, further research is necessary to
localize renal medullary structures exhibiting NOS3 phosphor-

Fig. 5. Representative immunohistochemical localization of pThr⁴⁹⁵NOS3 in the renal medulla from Sham rats (A and B) and Hyp rats (C–F). A. and C: outer

Fig. 6. Immunohistochemical localization of pSer¹¹⁷⁷NOS3 in the renal me-
dulla from Sham rats (A and C) and Hyp rats (B and D). A and B: outer
medulla. C and D: inner medulla. Arrowheads, collecting duct; Arrows, thick
ascending limb. Original magnification ×100.
ylation at Ser$^{633}$, particularly those subject to dephosphorylation in diabetic rats. However, immunohistochemical analysis of pThr$^{495}$NOS3 and pSer$^{1177}$NOS3 proved to be informative.

Strong pThr$^{495}$NOS3 immunostaining was apparent at the apical membrane of the thick ascending limb in kidneys from Sham rats, with diffuse staining evident in both outer and inner medullary segments of the collecting duct. In the Hyp group, pThr$^{495}$NOS3 immunostaining was reduced only in the thick ascending limb, with staining maintained in the collecting duct. Thus the reduced pThr$^{495}$NOS3 levels evident in the immunoblotting data are likely a reflection of events occurring in the thick ascending limb. Localization of pThr$^{495}$NOS3 in the apical membrane of the thick ascending limb in Sham rats and the corresponding loss of phosphorylation at this site in the Hyp rat kidneys should translate to an increase in NOS3 activity (Fig. 1) without a concomitant increase in expression (Fig. 3). Recently, Lin et al. (20) utilized mutant NOS3 transfectants to demonstrate that the dephosphorylation of Thr$^{495}$ may be a “switch” that determines whether NOS3 produces NO or superoxide. At the present time, it is unknown whether dephosphorylation at Thr$^{495}$ causes increased NO or superoxide production by NOS3 in the thick ascending limb.

Ortiz et al. (27) described a doubling of Ser$^{1177}$ phosphorylation at the apical membrane when isolated thick ascending limbs were introduced to flow conditions (27). The immunohistochemical results of the present study confirm the prominent localization of pSer$^{1177}$NOS3 at the apical membrane of the thick ascending limb under conditions of normal flow (Sham), as well as during chronic elevations in tubular fluid flow (Hyp), with minimal pSer$^{1177}$NOS3 apparent in the collecting duct. However, the immunoblotting analyses of the present study failed to reveal a difference between Sham and Hyp with regard to phosphorylation at Ser$^{1177}$. Thus NOS3 phosphorylation at Ser$^{1177}$ in the thick ascending limb may be differentially regulated within varied flow ranges (no flow-to-normal flow vs. normal flow-to-high flow conditions) and/or in response to acute vs. chronic flow alterations.

Dephosphorylation of Thr$^{495}$ and Ser$^{633}$ may occur directly through activation of phosphatases and/or inactivation of kinase pathways under hyperglycemic conditions. Protein phosphatases 1 and 2A have been shown to be active in the thick ascending limb, where they may play a role in regulating Na$^+$/K$^+$-ATPase activity (19). Although protein phosphatase 2A has been implicated in the control of NOS3 dephosphorylation in endothelial cells (2, 9), no data are available concerning whether or not this process contributes to regulation of NOS3 activity in the thick ascending limb. Inhibition of protein phosphatase 2B (calcineurin) blocks bradykinin-mediated dephosphorylation of Thr$^{495}$NOS3 and NO production in cultured aortic endothelial cells (11). Moreover, protein phosphatase 2B activation is evident in the renal cortex during the early phase of T1D and is implicated in the renal and glomerular hypertrophy in diabetic rats (10). An inhibitory effect of hyperglycemia on activation of Akt kinase and subsequent NOS3 phosphorylation at Ser$^{1177}$ has been reported to occur in endothelial cells (22); however, no previous reports have determined the impact of hyperglycemia on the phosphorylation status of Ser$^{1177}$ in the kidney or the phosphorylation status of Thr$^{495}$ or Ser$^{633}$ in any cell type during hyperglycemia. Our observations implicate NOS3 dephosphorylation at Ser$^{633}$ and Thr$^{495}$ as a potentially important mechanism contributing to the renal complications of T1D; however, further studies are warranted to clarify how hyperglycemia alters the posttranslational modifications of NOS3 in the renal medulla.

The physiological relevance of renal medullary NOS3 activation could have implications in the regulation of sodium and water transport and medullary blood flow. Renal medullary NOS3 activation in T1D likely involves, at least in part, dephosphorylation of Thr$^{495}$NOS3 residing in the apical membrane of the thick ascending limb. Increased NO production by the thick ascending limb reduces Na$^+$ reabsorption by that nephron segment (25), which would increase Na$^+$ delivery to more distal segments of the nephron, thereby promoting natriuresis. However, the increased solute delivery to the macula densa would tend to suppress glomerular filtration rate via the tubuloglomerular feedback mechanism, a phenomenon that might temper the magnitude of diabetic hyperfiltration. Indeed, alterations in distal tubular fluid/solute delivery have been proposed to contribute to the hyperfiltration state through a tubuloglomerular feedback-dependent mechanism (33). On the other hand, if dephosphorylation at Thr$^{495}$ acts as a molecular switch (20), it could increase superoxide production. An increase in superoxide production by the thick ascending limb would reduce Na$^+$ delivery to the macula densa and other distal nephron segments (i.e., changes that are the opposite of those evoked by NO). These changes might arise as a consequence of decreased NO bioavailability (due to formation of peroxynitrite) and/or the direct effect of superoxide to increase Na$^+$ reabsorption by the thick ascending limb (26). Moreover, it is also possible that increased NO production by the thick ascending limb would exert a paracrine influence on neighboring cells, altering transport function in the collecting duct and/or increasing blood flow through outer medullary vasa recta (6, 15, 28). As an additional caveat, the lack of information concerning the medullary structure(s) exhibiting dephosphorylation of NOS3 at Ser$^{633}$ during T1D makes it impossible to speculate on the functional consequences of this phenomenon. Given the complexity and interactive nature of NO-dependent parameters in the renal medulla, having both epithelial and vascular effectors, it is evident that a thorough investigation of the biochemical and functional consequences of changes in NOS3 phosphorylation at specific regulatory sites will be required to unveil the impact of these processes on solute and volume homeostasis during T1D.

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