Nitric Oxide Synthesis and Oxidative Stress in the Renal Cortex of Rats with Diabetes Mellitus

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Abstract. Experiments were performed to test the hypothesis that diabetes mellitus disrupts the balance between synthesis and degradation of nitric oxide (NO) in the renal cortex. Diabetes was induced by injection of streptozotocin, and sufficient insulin was provided to maintain moderate hyperglycemia for the ensuing 2 wk. Despite an 80% increase in total NO synthase activity measured by 1-citrulline assay, nicotinamide adenine dinucleotide phosphate–diaphorase staining was unaltered, and no changes in NO synthase isoform protein levels or their distribution were evident in renal cortex from diabetic rats. Superoxide anion production was accelerated twofold in renal cortical slices from diabetic rats, with an associated 50% increase in superoxide dismutase activity. Western blots prepared by use of a monoclonal antinitrotyrosine antibody revealed an approximately 70-kD protein in renal cortex from sham rats, the nitrotyrosine content of which was threefold greater in cortical samples from diabetic rats. These observations indicate that the early stage of diabetes mellitus provokes accelerated renal cortical superoxide anion production in a setting of normal or increased NO production. This situation can be expected to promote peroxynitrite formation, resulting in the tyrosine nitration of a single protein of unknown identity, as well as a decline in the bioavailability of NO. These events are consistent with the postulate that oxidative stress promotes NO degradation in the renal cortex during the early stage of diabetes mellitus.

In the early stages of insulin-dependent diabetes mellitus (IDDM), renal afferent arteriolar dilation elicits glomerular hyperfiltration, which engenders microalbuminuria and the ultimate development of more severe functional and morphologic glomerular injury. The literature is replete with reports attempting to identify the nature of the potentiated vasodilator influence or curtailed vasoconstrictor influence responsible for reduced preglomerular vascular resistance during the hyperfiltration stage of IDDM. The identification of nitric oxide (NO) as a physiologically relevant renal vasodilator has fueled efforts to detail its involvement in a variety of pathophysiologic states, including IDDM. Reports that NO synthase (NOS) inhibition normalizes GFR in diabetic rats suggest that increased renal NO levels contribute to diabetic hyperfiltration (1–3), whereas other studies have failed to discern any effect of IDDM on the renal hemodynamic response to NOS inhibition (4–6).

Studies focusing on the renal microvascular influence of NO in IDDM have yielded results that are more consistent. Renal microvessels from rats with streptozotocin (STZ)-induced IDDM exhibit suppressed agonist-induced endothelium-dependent relaxation (7), diminished NO-dependent basal tone (8), and loss of the modulatory influence of endogenous NO on agonist-induced constriction (4,9). Superoxide anion (O2−) production has been implicated as one factor that opposes the effects of NO on the renal microvasculature of diabetic rats (7–9). Because O2− decreases the half-life of NO, increased renal O2− levels in IDDM should reduce the bioavailability of NO and curtail its tonic impact on arteriolar tone. Although accumulating evidence suggests that renal oxidative stress accompanies IDDM, O2− production has not been compared in kidneys from normal and diabetic animals. Discrete aspects of the renal oxidant/antioxidant balance have been addressed in IDDM (10–12); however, these investigations have focused on alterations evident in severely hyperglycemic animals studied months after onset of the disease. The relative status of renal NO synthesis and its degradation by O2− has not been explored during the hyperfiltration stage of IDDM.

The present study was designed to evaluate the hypothesis that the hyperfiltration stage of IDDM is accompanied by an imbalance between NO synthesis and O2−-dependent NO degradation in the kidney. Attention was focused on the renal cortex, wherein lies the arteriolar vasculature that controls glomerular capillary hydrostatic pressure. Our approach was to quantify parameters related to NO synthesis (NOS; NOS activity, NOS protein levels and localization, and nicotinamide
Materials and Methods

Induction of Diabetes Mellitus

The procedures used in this study were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee and conducted according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (SAS:VAF strain; Charles River Laboratories, Wilmington, MA) were anesthetized with methohexital sodium (50 mg/kg, intraperitoneally) to facilitate injection of STZ (65 mg/kg intravenously; Sigma Chemical, St. Louis, MO). Sham rats received vehicle treatment. The rats were allowed to recover from anesthesia and housed overnight with free access to food and water. On the following morning, blood glucose levels were measured (Accu-Check III model 766; Boehringer Mannheim Co., Indianapolis, IN), and the STZ rats were anesthetized again to allow either (1) intraperitoneal implantation of an osmotic minipump (model 2002; Alzet, Palo Alto, CA) prepared for delivery of 3.5 to 4.0 U kg⁻¹ day⁻¹ insulin (Iletin II; Eli Lilly, Indianapolis, IN) or (2) subcutaneous insertion via a 16-G needle of a 2.3 × 2.0-mm sustained-release insulin implant (Implant; Linshin Canada, Scarborough, Ontario, Canada). Both modes of insulin delivery maintained moderate hyperglycemia during the ensuing 2-wk period. Sham rats received either diluent infusion via a minipump or a microcrystallized palmitic acid (vehicle) implant. Animals that were subjected to minipump implantation received penicillin G procaine (60,000 U intramuscularly) immediately after surgery. Thereafter, blood glucose concentration and body weight were measured at 3- to 4-d intervals. Some animals were housed individually in metabolic cages (Nalgene; Nalge Nunc International, Rochester, NY) for the 2 d preceding the terminal experiment. The volume of urine collected (under oil) during the final 24-h period was determined gravimetrically. Urine samples were centrifuged, filtered, and stored at −70°C until measurement of nitrate + nitrite (NOₓ) and creatinine concentrations. Four groups of sham and STZ rats were used in this study, as detailed below.

Group 1

Two wk after injection with STZ or vehicle, rats were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally) and tracheotomized, to facilitate spontaneous respiration. Both kidneys were flushed with cold isotonic saline from the abdominal aorta and were removed quickly and weighed, and cortical samples were frozen in liquid N₂. Animals that were subjected to minipump implantation received penicillin G procaine (60,000 U intramuscularly) immediately after surgery. Thereafter, blood glucose concentration and body weight were measured at 3- to 4-d intervals. Some animals were housed individually in metabolic cages (Nalgene; Nalge Nunc International, Rochester, NY) for the 2 d preceding the terminal experiment. The volume of urine collected (under oil) during the final 24-h period was determined gravimetrically. Urine samples were centrifuged, filtered, and stored at −70°C until measurement of nitrate + nitrite (NOₓ) and creatinine concentrations. Four groups of sham and STZ rats were used in this study, as detailed below.

NOS Assay. Total NOS activity in renal cortex was determined on the basis of the rate of L-[¹H]citrulline formation from L-[¹H]arginine, as described previously (13). Briefly, the renal cortex was weighed, minced, and homogenized in ice-cold buffer containing 10 mM HEPES, 320 mM sucrose, 0.1 mM ethylenediaminetetraacetate, 1 mM dithiothreitol, 0.1 mM soybean trypsin inhibitor, 0.1 mM aprotinin, and 1 mM phenylmethylsulfonyl fluoride (pH 7.4). After 20 min of treatment of the homogenate with 0.2 vol/wt 200 mM CHAPS at approximately 4°C and subsequent centrifugation at 10,000 × g, DOWEX (50WX8-400; Sigma) was used to remove endogenous L-arginine from the supernatant (14). A 20-µl aliquot of the supernatant was added to 50 µl of incubation buffer (pH 7.2) composed of 20 mM HEPES, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM L-arginine, 2 µM tetrahydrobiopterin (BH₄), 1 mM β-NADPH, 0.5 µM calmodulin, 20 µM L-arginine, and 0.2 µM L-[2,3,4,5-³H]arginine (12.6 µCi/ml assay buffer, 63 Ci/mM specific activity; Amersham Life Science, Buckinghamshire, UK). Parallel reactions were performed in the presence of 300 µM N²-nitro-L-arginine (L-NNA), a NOS inhibitor (15). Samples were incubated for 30 min at 37°C, after which the reaction was terminated by DOWEX treatment to remove L-arginine. L-[¹H]citrulline that formed during the reaction was quantified by liquid scintillation counting. Total NOS activity of each background-corrected sample was determined as the difference between the radioactivity with and without 300 µM L-NNA and was expressed as picomoles of L-[¹H]citrulline formed per hour per milligram of protein. The conditions of this assay minimized any confounding impact of endogenous L-arginine (removed by DOWEX treatment) or arginase activity (inhibited by exogenous L-arginine). We could not control for the potential recycling of L-citrulline to L-arginine via endogenous argininosuccinate synthase and argininosuccinate lyase. However, because the Km for argininosuccinate lyase is 3.7 mM (16), the fact that L-NNA–dependent processes in renal cortical homogenates yield nanomolar concentrations of L-citrulline in the presence of micromolar concentrations of L-arginine under these conditions makes it unlikely that the recycling reaction significantly influences this widely accepted assay for NOS activity.

Superoxide Dismutase Assay. Renal cortical tissue was weighed, minced, and homogenized in ice-cold 250 mM sucrose. The homogenate was centrifuged (10 min at 8500 × g, 4°C), and the supernatant was stored at −70°C until assay for SOD activity, as described previously (13). Briefly, the thawed sample was subjected to ethanol:chloroform extraction, mixed, and centrifuged (10 min at 3000 × g, 4°C). A commercially available kit (BIOXYTECH SOD-525; OXIS International, Portland, OR) was used to measure SOD activity in the aqueous upper layer of the extract.

Group 2

Two wk after injection with STZ or vehicle, rats were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally). The right renal artery and vein were ligated, and the kidney was removed quickly and weighed, and cortical samples were frozen in liquid N₂. These samples were stored at −70°C until protein isolation and Western blot analysis (NOS-1, NOS-2, NOS-3, and nitrotyrosine). The distal aorta was cannulated and a 1-ml blood sample was collected into a heparinized syringe. Plasma from this sample was stored at −70°C until measurement of NOₓ concentration. The left kidney was flushed with saline, perfused with 40 to 50 ml of 4% paraformaldehyde in phosphate buffer (pH 7.4), and removed from the rat. After overnight postfixation in paraformaldehyde at 20°C, the kidney was maintained in 20% sucrose at 4°C until processing for NADPH-diaphorase staining.

Protein Isolation. Tissue lysates were prepared by standard homogenization techniques. Briefly, frozen cortical tissue was pulverized to a powder and solubilized in homogenization buffer (vol/wt ratio of 10; 50 mM Tris-HCl [pH 7.4]; 10 mM ethylenediaminetetraacetate, 0.1% 2-mercaptoethanol) in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 µM leupeptin, 1 µM pepstatin A, and 0.1% aprotinin). The preparation was homogenized for 20 strokes in a Dounce glass/Teflon homogenizer on ice. Treatment with CHAPS (20 mM, 20 min, 4°C) was used to separate further
the homogenate into a detergent-soluble fraction and detergent-insoluble fraction. The supernatant collected after centrifugation \((10,000 \times g, 45 \text{ min}, 4^\circ C)\) was considered the “soluble fraction.” The pellet was resolubilized in half the original homogenization buffer with 20 mM CHAPS \((20 \text{ min at } 4^\circ C)\). The resolubilized pellet was then centrifuged at 100,000 \(g\) \((30 \text{ min at } 4^\circ C)\); this is considered the “resolubilized pellet fraction.” The resulting detergent-insoluble pellet was rehomogenized in half the original homogenization buffer volume and stored at \(-80^\circ C\) until analysis. For detection of NOS-1 and NOS-3, the soluble fraction and resolubilized pellet fraction were purified further by 2',5'-adenosine diphosphate-Sepharose column chromatography as described previously \((17)\).

**Western Blot Analysis.** Proteins were separated on 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose by wet blotting \((Bio-Rad Laboratories, Hercules, CA)\) for 45 min. The blots were allowed to air-dry for 30 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 with the primary antibody diluted in blocking buffer overnight at 4°C, followed by two washes with blocking buffer at room temperature. The blots then were incubated with the secondary antibody (horse radish peroxidase–conjugated goat anti-mouse or anti-rabbit antibody; Amersham) for 1 h at room temperature, followed by four washes with Tris-buffered saline. The specific bands were detected by use of the enhanced chemiluminescence system \((Amersham)\). Densitometry was used to semiquantify the bands with the Alpha Innotech Imaging System \((San Leandro, CA)\). Primary antibodies used were anti–NOS-1 \((1:1000, SA-227; Biomol Research Laboratories, Plymouth Meeting, PA)\), anti–NOS-2 \((1:3000, SA-200; Biomol)\), anti–NOS-3 \((1:1000; N30020: Transduction Laboratories, Lexington, KY)\), and nitrotyrosine-specific antibody \((1:50 (18))\). The antinitrotyrosine mouse monoclonal antibody has high affinity for nitrotyrosine-containing proteins, is specific for nitrotyrosine residues, and recognizes a variety of nitrated proteins with similar affinity \((18)\). Molecular weights were estimated by use of rainbow \((32 to 216 \text{ kD}; Amersham)\) and enhanced chemiluminescence markers \((32 to 97 \text{ kD}; Amersham)\).

**NADPH-Diaphorase Histochemistry.** Histochemical localization of the catalytic activity of NOS was assessed by use of the NADPH-diaphorase reaction. This method has been exploited to localize *in situ* NOS activity in a variety of tissues, including kidney. Briefly, postfixed kidneys in 20% sucrose were blocked in the coronal plane and sectioned in a cryostat. The sections were collected in phosphate buffer \((\text{pH 7.4})\) containing 0.3% Triton X-100, 0.1 mg/ml nitro blue tetrazolium, and 1.0 mg/ml \(\beta\)-NADPH. The sections were warmed to 37°C for 75 min, rinsed in phosphate buffer, mounted on chrome-alum coated slides, and dried, and coverslips were mounted directly with Permount \((Fisher Scientific, Fairlawn, NJ)\). NADPH-diaphorase staining in sections from sham and STZ rats was quantified by computerized assessment of blue saturation density \((19)\). Animal weights were calculated on the basis of 20 to 30 measurements per rat \((10 \text{ regions per slide; 2 to 3 slides per rat})\), and statistical analyses were performed by use of these animal means.

### Group 3

Two wk after injection with STZ or vehicle, rats were anesthetized with pentobarbital sodium \((50 \text{ mg/kg, intraperitoneally})\). The abdominal aorta was cannulated, allowing the kidneys to be flushed with heparinized saline. Each kidney was excised and weighed. Two near-medial slices were obtained from each kidney with a Stadie-Riggs microtome \((Thomas Scientific, Swedesboro, NJ)\). The medullary portion of each slice was removed carefully and discarded. The cortical slices thus obtained were bisected and used for measurement of \(\text{O}_2^-\) production.

**\(\text{O}_2^-\) Production Assay.** \(\text{O}_2^-\) production was measured on the basis of its ability to reduce ferricytochrome c, according to established methods \((20)\). Renal cortical slices were incubated at 37°C in Hank’s balanced salt solution containing 80 \(\mu\text{M}\) cytochrome c and either 5 mM glucose \((right\ kidney)\) or 20 mM glucose \((left\ kidney)\). To control for nonspecific reduction of ferricytochrome c, slices from each kidney were incubated in cytochrome c solution containing 500 U/ml SOD. As a positive control, slices from each kidney also were incubated in the presence of 250 \(\mu\text{g/ml}\) heat-aggregated bovine IgG, a known stimulant of renal \(\text{O}_2^-\) production \((20)\). The supernatant was removed at 5, 30, 60, or 90 min after initiating the cytochrome c incubation, centrifuged \((10,000 \times g)\) to remove any cellular debris, and absorbance was measured at 550 nm. The extinction coefficient of cytochrome c was assumed to be 2.1 \(\times 10^4 \text{ M}^{-1} \text{ cm}^{-1}\). SOD-sensitive \(\text{O}_2^-\) production was expressed as nanomoles of reduced cytochrome c per milligram of protein.

### Group 4

Two wk after injection with STZ or vehicle, rats were decapitated and tissues were harvested for studies relating to another project \((21)\). A 3-mm-thick midcoronal section of the left kidney was reserved for immunohistochemistry.

**NOS-1 and NOS-3 Immunohistochemistry.** Tissue was immersed overnight in 10% buffered formalin, dehydrated, embedded in paraffin blocks, sectioned \((4 \mu\text{m})\) onto super-frost plus slides, and stained by the Labeled Streptavidin-Biotin method for rat \((DAKO, Carpinteria, CA)\). Endogenous peroxidase was blocked by 3% \(\text{H}_2\text{O}_2\) for 15 min, followed by washing successively in water and Tris-buffered saline. Sections were incubated in humidity chambers with diluted primary antibody or control solution \((anti–NOS-1: Transduction N31020, 1:500, 3 \text{ h}; anti–NOS-3: N30020, 1:800, 1 \text{ h})\). The slides were washed with Tris-buffered saline, followed by incubation with biotinylated horse universal secondary antibody according to the kit instructions for 10 min. Staining was detected with streptavidin peroxidase and dianimobenzidine according to the kit instructions. The sections were counterstained with hematoxylin, rinsed with water, dehydrated with increasing percentages of alcohol, and mounted with cytoseal 60. The stained sections were viewed with a Zeiss Axioskop microscope on bright field setting fitted with a digital camera \((Spot 2; Diagnostic Instruments, Sterling Heights, MI)\) and scanned with Adobe Photoshop imaging software \((Adobe Systems, San Jose, CA)\).

**Creatinine, NO\textsubscript{X}, and Protein Assays**

Protein concentrations were determined by a standard Bradford assay \((Bio-Rad)\). Creatinine concentration in urine samples was measured by use of a picric acid–based microplate assay \((22)\). The ozone-chemiluminescence method \((Model 280 NO Analyzer; Sievers Instruments, Boulder, CO)\) was used to measure \(\text{NO}_x\) concentration in urine and plasma \((deproteinized by cold ethanol precipitation, according to the manufacturer’s instructions)\).

**Statistical Analyses**

Data were analyzed by ANOVA for repeated measures and Newman-Keuls tests, or unpaired \(t\) tests, as appropriate. \(P < 0.05\) was considered significant. All data are reported as the mean ± SD.

### Results

**Animal Characteristics**

On the initial day of study \((before injection with STZ or vehicle)\), blood glucose concentration averaged 4.5 ± 0.1 mM.
(n = 91). During the ensuing 2-wk period, blood glucose concentration averaged 18.1 ± 0.4 mM in STZ rats and 4.6 ± 0.1 mM in sham rats (P < 0.001 versus STZ). Thus, rats that received STZ were moderately hyperglycemic relative to sham (vehicle-treated) rats. STZ rats were larger than sham rats on the initial day of study (Table 1) but gained less weight (16 ± 4 g) than sham rats (66 ± 4 g) during the 2 wk after induction of IDDM (P < 0.001). Accordingly, body weight was similar in both groups of rats on the day of the terminal experiment, although kidney weight was greater in STZ rats than in sham rats (Table 1). Thus, the ratio of right kidney weight to body weight in STZ rats (5.2 ± 0.1 mg/g; n = 40) exceeded that observed in sham rats (3.7 ± 0.1 mg/g; n = 41; P < 0.001), indicating renal hypertrophy in the diabetic animals. Table 1 also provides excretory data based on 24-h urine collection from conscious sham and STZ rats housed in metabolic cages. In association with elevated urine flow, STZ rats displayed polydipsia, as evidenced by an average water intake of 7.7 ± 1.1 ml/h (sham, 1.8 ± 0.1 ml/h; P < 0.001). Urinary excretion of creatinine was increased by 30% in STZ rats, and, although plasma [NO\textsubscript{X}] did not differ between groups, urinary NO\textsubscript{X} excretion was diminished by 60% in STZ rats.

**NOS Activity, NOS Protein Levels and Localization, and NADPH-Diaphorase Staining**

Total NOS activity in renal cortex of sham and STZ rats was measured as l-NAME-sensitive l-[\textsuperscript{3}H]citrulline formation from l-NAMEarginine in the presence of 1 mM Ca\textsuperscript{2+}. NOS activity in cortical tissue from sham rats averaged 15.9 ± 2.0 pmol/h per mg protein (n = 11) and was increased by 80% in STZ rats (Figure 1A). Western immunoblots were prepared to determine the relative contributions of each NOS isoform to the increase in NOS activity evident in STZ kidneys (Figure 2). NOS-1 protein was detectable in renal cortex only after adenosine diphosphate purification of the soluble fraction. Densitometry protein was detectable in renal cortex only after adenosine diphosphate purification of the soluble fraction. Densitometry (540 6 1.1 ml/h (sham, 1.8 

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<th>Body Weight</th>
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<th>Urine Flow</th>
<th>Creatinine Excretion</th>
<th>NO\textsubscript{X} Excretion</th>
<th>Urinary [NO\textsubscript{X}]/[Cr]</th>
<th>Plasma [NO\textsubscript{X}]</th>
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<td>Sham</td>
<td>258 ± 5 (n = 44)</td>
<td>322 ± 4 (n = 44)</td>
<td>1154 ± 25 (n = 41)</td>
<td>5.9 ± 0.4 (n = 9)</td>
<td>254 ± 25 (n = 9)</td>
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<td>STZ</td>
<td>304 ± 2\textsuperscript{c} (n = 47)</td>
<td>320 ± 4 (n = 47)</td>
<td>1656 ± 38\textsuperscript{c} (n = 40)</td>
<td>7.9 ± 0.9\textsuperscript{b} (n = 7)</td>
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\textsuperscript{a} Values are mean ± SEM. Excretory data derived from 24-h urine collections in metabolic cages. NO\textsubscript{X}, nitrate + nitrite; Cr, creatinine; STZ, streptozotocin.

\textsuperscript{b} P < 0.05 versus sham.

\textsuperscript{c} P < 0.001 versus sham.
responses. As evident in Figure 4B, incubation of renal cortical tissue from sham/normal rats in 20 mM glucose tended to increase $\text{O}_2^\bullet \text{-}$ production from $1.83 \pm 0.35$ to $3.79 \pm 0.83$ nmol/mg protein per 90 min ($n = 11$); however, this effect did not achieve statistical significance ($P = 0.098$). Moreover, aggregated IgG failed to stimulate $\text{O}_2^\bullet \text{-}$ production by sham/normal tissue in the presence of 20 mM glucose ($3.16 \pm 0.63$ nmol/mg protein per 90 min; $P = 0.494$ versus sham 20 mM glucose).

When renal cortical tissue harvested from STZ rats was incubated in media containing 20 mM glucose (to simulate in vivo ambient glucose levels), cumulative $\text{O}_2^\bullet \text{-}$ production during the 90-min incubation period was more than double that measured in tissue from sham/normal rats studied in the normal glucose environment ($P < 0.05$ versus sham 5 mM glucose; Figure 4B). Enhanced $\text{O}_2^\bullet \text{-}$ production by STZ tissue remained evident after acute restoration of ambient glucose concentration to 5 mM ($P < 0.05$ versus sham 5 mM glucose). Under these conditions, $\text{O}_2^\bullet \text{-}$ production by renal cortex of STZ rats remained 1.5-fold higher than that observed in sham/normal tissue. Aggregated IgG failed to augment further the $\text{O}_2^\bullet \text{-}$ production by STZ tissue, regardless of whether the tissue was incubated in media containing 5 or 20 mM glucose (data not shown). In this setting of markedly increased $\text{O}_2^\bullet \text{-}$ production in STZ rats, renal cortical SOD activity was increased by 50% relative to sham rats ($P < 0.001$; $n = 11$ per group; Figure 1B).

**Nitrotyrosine Levels**

Diabetes-induced alterations in renal cortical protein tyrosine nitration are summarized in Figure 5. Western blot analysis revealed an approximately 70-kD nitrotyrosine-containing protein in tissue from sham rats. The nitrated protein was evident in both the detergent-soluble (supernatant) and detergent-insoluble (pellet) fractions of the cortical homogenate. Immunostaining of this protein averaged $655 \pm 340$ densitometric units (supernatant + pellet; $n = 3$) in sham kidneys and was increased threefold in STZ kidneys ($2773 \pm 553$; $n = 5$; $P < 0.05$). STZ kidneys also contained a faint nitrotyrosine-positive protein with a molecular weight of approximately 40 kD. These results were confirmed by use of a commercially available antibody against nitrotyrosine (Cayman Chemical, Ann Arbor, MI; data not shown). Blots run in the absence of the antinitrotyrosine antibody confirmed that no immunoreactive products were detected by the secondary antibody alone.

**Discussion**

The present study was performed to quantify the status of the renal NO system and indices of oxidative stress during the hyperfiltration stage of IDDM. Rats were studied approximately 2 wk after onset of IDDM, during which partial insulin replacement was provided to maintain moderate hyperglycemia. The STZ rats were polydipsic and polyuric and continued to gain weight, albeit at a slower rate than sham rats. We previously documented that glomerular hyperfiltration is evident in this model of IDDM (23). The results of the present study provide evidence of renal cortical oxidative stress during this stage of IDDM. In this setting of normal or increased NO production, excess $\text{O}_2^\bullet \text{-}$ generation is evident, thereby establishing a situation favoring a decline in NO availability by...
Figure 3. Representative immunohistochemical localization of NOS-1 (A, C, and E) and NOS-3 (B, D, and F) in renal cortical sections from sham (A, B, and E) and STZ (C, D, and F) rats. In both sham and STZ kidneys, strong positive (brown) staining for NOS-1 is evident throughout the cytoplasm in the macula densa (arrows). NOS-1 staining is not manifested in other tubular segments or in vascular structures, including the glomerular capillary tuft. Preadsorption with the antigenic peptide abrogated NOS-1 immunostaining (E). In both sham and STZ kidneys, positive NOS-3 staining is evident at the luminal (endothelial) aspect of vascular structures, including glomerular arterioles (arrows) and large arterioles/small arteries (double arrowheads). Glomerular arterioles (*) are enlarged in the inset panels of B and D, in which endothelial staining is more readily apparent (arrowheads). Positive staining of glomerular capillary endothelium also is apparent in the inset panel of B. NOS-3 staining is absent in sections prepared in the absence of the primary antibody (F; inset = small artery from the same section). Sections were counterstained with hematoxylin. Bar = 25 μm.
ONOO$^-$ formation. This scenario is indicated by increased protein tyrosine nitration in the renal cortex of STZ rats.

Conflicting evidence exists concerning whether IDDM increases (2,24,25) or decreases (26,27) renal NOS protein levels, with alterations in this parameter typically interpreted as indicative of alterations in NOS activity. The present study assessed NOS activity in renal cortical homogenates on the basis of L-NNA-sensitive L-citrulline production in the presence of exogenous substrate and required co-factors. The data indicate an increase in NOS activity in tissue harvested from male STZ rats. Although no specific attempt was made to measure Ca$^{2+}$-independent NOS activity, the absence of NOS-2 in Western blot analyses of the soluble fractions from sham and STZ kidneys suggests that the measured increase in NOS activity in the post-CHAPS cortical homogenate represents primarily Ca$^{2+}$-dependent activity (NOS-1 and/or NOS-3). In accord with this contention, Omer et al. (28) reported a 50% increase in Ca$^{2+}$-dependent NOS activity measured in whole kidney homogenates from female rats studied 2 wk after STZ treatment. In contrast, Keynan et al. (26) reported that renal cortical Ca$^{2+}$-dependent NOS activity is reduced 7 d after STZ treatment, raising the possibility that IDDM alters this parameter in a time-related manner.

The literature contains reports of increased NADPH-diaphorase staining in afferent arterioles and glomerular capillaries of rats studied 1 to 4 wk after induction of IDDM (24) and of reduced macula densa staining in rats studied 1 or 6 wk after STZ treatment (26,27). Although NOS activity was increased in cortical homogenates from diabetic rats in the present study, neither the distribution nor the intensity of NADPH-diaphorase staining differed between sham and STZ kidneys. It is possible that the NADPH-diaphorase staining method lacks the sensitivity required to distinguish reliably the modest alterations in NOS activity. However, we cannot discount the possibility that in vivo NOS activity is tempered by decreased substrate or co-factor availability in IDDM (29,30) and that this phenomenon explains why increased NADPH-diaphorase staining was not evident in kidneys from STZ rats.

The increase in cortical NOS activity measured in kidneys from STZ rats in the presence of exogenous substrate and required co-factors might be expected to mirror alterations in NOS protein levels. Accordingly, Western blot analysis was
performed to identify the NOS isoform responsible for the increase in L-citrulline production. All three NOS isoforms were evident in purified samples of rat renal cortex. An unanticipated observation emanating from the Western blot analysis was that renal cortical NOS-2 immunoreactive protein demonstrated a lower molecular weight than mouse macrophage NOS-2 immunoreactive protein (Figure 2). NOS-2 has been shown to bind calmodulin tightly, even under the denaturing conditions of sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis (31). We speculate that the lower molecular weight of renal NOS-2 may result from release of bound calmodulin by the detergent treatment used in the present study, although further studies are needed to address this possibility. In any case, Western blot analysis failed to reveal a change in any NOS isoform in cortical tissue from STZ rats, relative to tissue from sham rats. Directionally opposite alterations in NOS protein levels might occur within discrete cortical structures, a situation that could have substantial functional relevance without altering NOS protein levels evident by Western blot. However, immunohistochemical assessment revealed no apparent difference between sham and STZ rats regarding the staining intensity or distribution of NOS-1 or NOS-3 within the renal cortex. Hence, although no ideal indicator of in situ NO production is available, our accumulated measures of NO synthesis suggest that normal or increased NO production occurs in the renal cortex of STZ rats in the absence of any alteration in NOS protein levels or localization. In addition to the widely recognized Ca²⁺ dependence of NOS-1 and NOS-3 activities, emerging evidence indicates that a variety of additional factors can evoke posttranslational alterations in NOS activity, e.g., Hsp-90 (32), Akt-kinase (33,34), AMP-activated kinase (35). Further studies are required to address the potential involvement of these factors in the increased renal cortical NOS activity evident during diabetic hyperfiltration, although it is at least apparent that the factor and/or its impact on enzyme activity is retained in the tissue homogenate utilized in the L-citrulline assay.

Numerous studies have provided indirect evidence that O₂⁻⁻ production is accelerated in IDDM, primarily on the basis of measured increases in renal thiobarbituric acid reactive substances or antioxidant enzyme activity (10–12). The present study confirms these observations by documenting increased renal cortical SOD activity in STZ rats studied 2 wk after onset of IDDM. The suggestion of renal cortical oxidative stress was validated further by measurement of renal O₂⁻⁻ production in moderately hyperglycemic STZ rats. These data reveal a marked increase in O₂⁻⁻ production by renal cortical slices harvested during the hyperfiltration stage of IDDM. Although attempts to localize O₂⁻⁻ production within the various renal cortical structures were beyond the scope of the present study, increased O₂⁻⁻ production was demonstrated recently in glomeruli isolated from rats with poorly controlled IDDM (36). Increased O₂⁻⁻ production in IDDM could involve any of a variety of mechanisms, some of which are triggered by elevated extracellular glucose levels. Although we were unable to document a significant acute effect of 20 mM glucose on O₂⁻⁻ production by normal renal cortex, chronic elevations in glucose concentration may be sufficient to drive elevated renal O₂⁻⁻ production in IDDM. Autoxidation of glucose, formation of advanced glycation end products, and activation of NADPH-oxidase have been suggested to provoke oxidative stress in IDDM. O₂⁻⁻ production in IDDM also may arise as a result of increased NOS-1 or NOS-3 activity during conditions of limited substrate or co-factor availability (37,38). Indeed, reduced l-arginine availability has been reported in IDDM (29), and we note that our O₂⁻⁻ production experiments were performed in media devoid of l-arginine. Such a shift in renal NOS action from NO production to O₂⁻⁻ production could underlie our observation of decreased NOX excretion in IDDM, although we note that urinary NOX excretion is a crude indicator of renal NO production (39).

The reaction of NO with O₂⁻⁻ (to form ONOO⁻) occurs six times faster than the Cu/Zn-SOD reaction with O₂⁻⁻ (40). Thus, the 50% increase in SOD activity detected in kidneys from STZ rats would not be expected to compensate for the twofold increase in O₂⁻⁻ production. Peroxynitrite is more stable than NO and exerts multiple cytotoxic effects, some of which involve formation of protein-associated nitrotyrosine. Tyrosine nitration occurs in a number of diseases associated with oxidative stress and increased NOS activity (40,41), and increased protein tyrosine nitration is viewed widely as an indicator of peroxynitrite formation. Although the vast majority of previous reports of nitrotyrosine formation associated with disease have relied on immunohistochemical methods (41), the present study used Western blot analysis to provide a semiquantitative measure of protein nitration in renal tissue. The data reveal a significant increase in renal cortical nitrotyrosine levels during the early stage of IDDM. The formation of protein nitrotyrosine residues in renal cortex during IDDM is not promiscuous but, rather, involves primarily an approximately 70-kD protein. A modest increase in nitration of an approximately 40-kD protein also is evident. Increased nitration of proteins with similar molecular weights also occurs in association with endotoxin-induced kidney injury (42) and in the renal cortex of the spontaneously hypertensive rat (43). Cytoskeletal proteins have been suggested as prominent targets of tyrosine nitration in pathophysiologic states, as have a variety of enzymes involved in signal transduction (41). For example, nitration of intramitochondrial Mn-SOD results in a decline in its enzymatic activity during chronic rejection of renal allografts (44), although our results do not likely reflect increased nitration of Mn-SOD, because its molecular weight is <30 kD. An important goal of future studies will be to identify and localize the renal cortical protein(s) nitrated during the hyperfiltration stage of IDDM. Regardless of the identity of the nitrated protein, the trapping of nitro groups on protein tyrosine residues may contribute to the decline in NOX excretion observed in STZ rats in the present study. Thuraisingham et al. (45) recently provided immunohistochemical evidence of increased proximal tubular nitrotyrosine staining in biopsies obtained from patients with established diabetic nephropathy. Our observations indicate that renal nitrotyrosine levels are increased very early in the course of IDDM, before the onset of proteinuria.
Pieper (46) argued that disease duration is a critical determinant of the impact of IDDM on endothelium-dependent relaxation, and Craven et al. (47) suggested that IDDM provokes a period of increased renal NO synthesis followed by a period of diminished NO action. The results of the present study indicate that renal cortical oxidant stress and resulting ONOO− formation (which would diminish NO action as well as cause tissue damage) is evident during the period of normal or increased NO synthesis that occurs early in the course of IDDM. The functional consequences of these events are evidenced by our observation that the suppressed arteriolar constrictor response to NOS inhibition is restored by acute exposure of STZ kidneys to exogenous SOD (8) and our recent report that SOD treatment unmasks the normal modulatory impact of endogenous NO on afferent arteriolar responses to angiotensin II in this same model (9). Both of these observations indicate that O2−/O2•− production curbs the functional impact of NO on the renal microvasculature in IDDM, presumably via ONOO− formation. The reduced bioavailability of NO should exert a constrictor influence on the renal vasculature, which seems paradoxical in the setting of hyperfiltration. In light of the pleiotropic determinants of renal arteriolar tone, we surmise that the persistence of diabetic hyperfiltration under these conditions must reflect an overriding impact of other (NO-independent) vasodilator influences. Indeed, previous studies from our laboratory (48,49) suggest that decreased functional expression of voltage-gated Ca2+ channels and/or a decreased K+ conductance in preglomerular microvascular smooth muscle could engender diabetic hyperfiltration in the face of the reduced NO bioavailability that accompanies IDDM.

On the basis of our accumulated observations, we propose that the following scenario may accompany the early stage of IDDM in the rat. Normal or increased renal cortical NO synthesis in the setting of accelerated O2•− production results in rapid ONOO− formation, with at least two consequences. First, rapid ONOO− production establishes an imbalance between NO synthesis and degradation, promoting a decline in the bioavailability of NO that, in turn, diminishes the impact of endogenous NO on renal function. A second result of ONOO− production is the formation of nitrotyrosine residues on specific proteins, which also may engender specific functional consequences by interfering with tyrosine phosphorylation (50). Further experimental scrutiny is required to establish the validity of each aspect of this postulate.

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