Quantification of nitric oxide synthase activity in microdissected segments of the rat kidney

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Wu, Feng, Frank Park, Allen W. Cowley, Jr., and David L. Mattson. Quantification of nitric oxide synthase activity in microdissected segments of the rat kidney. Am. J. Physiol. 276 (Renal Physiol. 45): F874–F881, 1999.—This study was designed to quantify nitric oxide synthase (NOS) activity in microdissected glomeruli (Glm), pars convoluta, pars recta, cortical collecting duct, cortical thick ascending limb, outer medullary collecting duct, medullary thick ascending limb and thin limb, inner medullary collecting duct (IMCD) and thin limb, and vasa recta (VR). Total protein from microdissected segments was incubated with L-[3H]arginine and appropriate cofactors, and the L-arginine and converted L-citrulline were separated by reverse-phase HPLC and radiochemically quantitated. NOS activity was found to be greatest in IMCD (11.5 ± 1.0 fmol citrulline·mm⁻¹·h⁻¹) and moderate in Glm (1.9 ± 0.3 fmol·glomerulus⁻¹·h⁻¹) and VR (3.2 ± 0.8 fmol·mm⁻¹·h⁻¹). All other renal structures studied exhibited significantly less NOS activity. The mRNA for NOS isoforms in the NOS activity-positive segments was then identified by RT-PCR. The IMCD contained mRNA for neuronal (nNOS), endothelial (eNOS), and inducible NOS (iNOS), but Glm and VR only expressed the mRNA for nNOS and eNOS. These experiments demonstrate that the greatest enzymatic activity for NO production in the kidney is in the IMCD, three- to sixfold less activity is present in the Glm and VR, and minimal NOS activity is found in other segments studied.

Sprague-Dawley rat; kidney tubules; high-pressure liquid chromatography; messenger ribonucleic acid

Nitric oxide (NO) is known to have a potent influence on renal function through both renal hemodynamic and tubular effects. Administration of NO synthase (NOS) enzyme inhibitors has been demonstrated to lead to retention of sodium and water in dogs (14, 26) and rats (10). The reduced renal excretory ability after NO inhibition may be a consequence of decreased renal blood flow (14) and glomerular filtration rate (4, 34) due to increased pre- and postglomerular vascular resistance (9). Other studies have shown that chronic NOS inhibition selectively reduces blood flow in the renal medullary circulation (21), which could secondarily influence sodium and water excretion by altering physical factors. The renal vascular resistance changes that occur after NOS inhibition are thought to be mediated by withdrawal of the effects of NO on vascular smooth muscle and possibly through potentiation of tubuloglomerular feedback (TGF) (33). In addition to the vascular and TGF effects of NO, a number of studies indicate that NO may influence sodium and/or water transport in proximal tubules (9, 11, 13, 32) and collecting ducts (12, 28).

To better understand the functional effects of NOS and its inhibition in the kidney, a variety of techniques and approaches have been used to identify both mRNA and immunoreactive protein for neuronal (nNOS), endothelial (eNOS), and inducible NOS (iNOS) in renal tissue. Immunohistochemical studies demonstrated the presence of NOS protein in the macula densa (3, 30, 33), and in situ hybridization and RT-PCR of microdissected renal vessels and tubules have shown NOS mRNA in the macula densa, inner and outer medullary collecting duct, glomerulus, arcuate artery, and renal nerves in perivascular connective tissue (3, 27, 29). Further RT-PCR studies detected eNOS mRNA in the glomeruli, arcuate arteries, interlobular arteries, and afferent arteriole (31), and eNOS protein was identified in the endothelium of pre- and postglomerular vessels by immunohistochemical techniques (3). The mRNA encoding iNOS has been found in arcuate and interlobular arteries, glomeruli, proximal tubules, thick ascending limbs, and collecting ducts by in situ hybridization and RT-PCR of microdissected segments (2, 19). Though these studies have localized the different NOS isoforms within renal vascular and tubular structures, quantitative information regarding the renal tubular and/or vascular localization of NOS enzymatic activity is lacking.

To date, the quantitation of NOS enzymatic activity has only been performed on a whole tissue basis. Experiments in which cGMP formation was measured after NOS stimulation suggested that the renal medulla may have a greater capacity to synthesize NO than the renal cortex (5). Further studies have since demonstrated that the renal medulla contains greater amounts of immunoreactive protein and total NOS enzymatic activity than the renal cortex when normalized to whole tissue protein (16, 20). The present study was designed to quantitate NOS activity in microdissected renal segments by measuring the amount of radiolabeled L-arginine converted to L-citrulline. The L-arginine and L-citrulline were separated by reverse-phase HPLC and radiochemically quantitated. We then conducted a further analysis in the NOS enzymatic activity-positive renal segments to determine the presence of individual NOS isoform mRNA by RT-PCR.

**METHODS**

Experiments were performed on male Sprague-Dawley rats (250–300 g) obtained from Harlan Laboratories (Madison, WI). The rats were housed in the Animal Resource Center at the Medical College of Wisconsin, with normal rat chow and tap water provided ad libitum. All animal proce-
dures were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

Protocol 1: NOS Activity in Microdissected Renal Segments

In this initial protocol, total NOS enzymatic activity was determined in microdissected glomeruli (Glm), pars convoluta (PC), pars recta (PR), cortical collecting duct (CCD), cortical thick ascending limb (CTAL), outer medullary collecting duct (OMCD), medullary thick ascending limb (MTAL) and thin limb (OMTl), inner medullary collecting duct (IMCD) and thin limb (IMtL), and vasa recta (VR). Total tissue protein was incubated with L-[3H]arginine and appropriate cofactors, and the L-arginine and converted L-citrulline were separated by reverse-phase HPLC and radiochemically quantitated. Experimental details are outlined below.

Microdissection of renal segments. Microdissection was performed as described previously (22, 23). Rats were anesthetized with pentobarbital sodium (50 mg/kg body wt ip), and the aorta, below the left renal artery, was isolated and cannulated. After ligating the aorta at a site between the origin of the left and right renal arteries, we perfused the left kidney with 10 ml ice-cold dissection solution containing (in mmol/l) 135 NaCl, 3 KCl, 1.5 CaCl2, 1 MgSO4, 2 KH2PO4, 5.5 glucose, and 20 HEPES (pH 7.4). The kidney was then perfused with 10 ml of the digestion solution, which we prepared by adding 2 mg/ml collagenase (243 U/mg) to the dissection solution. After perfusion, the kidney was removed and cut into 1- to 2-mm-thick sections containing the entire corticomedullary axis. The sections were incubated at 37°C for 30 min in the digestion solution, with gentle shaking and bubbling with 100% O2. The slices were then rinsed twice with collagenase-free dissection solution and transferred into a Petri dish filled with ice-cold dissection solution containing 1% BSA. The Petri dish was then mounted on the cooling microscope stage and maintained at 4°C during dissection. Microdissection was performed under a LEICA M3Z stereomicroscope with dark-field illumination. The dissected renal segments were measured with a calibrated eyepiece micrometer and the time period for dissection was limited to 1.5 h. In general, 20 glomeruli and 20-mm total length renal segments were pooled for use as one sample.

NOS enyme assay. For tissue homogenate preparation, rat kidneys were removed, and the renal cortex, outer medulla, and inner medulla were separated. We homogenized the whole tissue using a Potter-Elvejem tissue grinder at 3,000 rpm in a solution containing (in mmol/l) 250 sucrose, 1 EDTA, 0.1 phenylmethylsulfonyl fluoride, and 5 potassium phosphate, pH 7.7. The homogenate was centrifuged at low speed (15,000 g, 4°C, 20 min), and the protein concentration of the supernatant was determined using a Coомassie dye assay, with albumin as a standard. The samples of the supernatant was determined using a Coomassie dye assay, with albumin as a standard. The samples of the supernatant were separated by reverse-phase HPLC and fluorometrically quantitated by previously described methods (17). Before HPLC separation, the samples were deproteinized with an equal volume of 0.14 mol/l sulfosalicylic acid containing a known concentration of L-β-alanine, which served as an internal standard. The samples were mixed, centrifuged at 10,000 g for 15 min to remove the precipitated protein, and derivatized with an equal volume of o-phthalaldehyde (1 mg/ml). The individual samples were separated by reverse-phase HPLC with a system consisting of a Bio-Rad AS-100 Auto Sampler, Hitachi L-7100 gradient pump, Waters Resolve column (15 cm x 3.9 mm, 5 µm), Waters 474 fluorescent detector (excitation 338 nm; emission 425 nm) and a Hitachi D-2500 integrator.

It was determined that the concentration of endogenous L-arginine in the whole tissue homogenates averaged 22.5 ± 2.3 nmol/mg total protein in the renal cortex, 6.2 ± 1.1 nmol/mg in the outer medulla, and 7.9 ± 2.2 nmol/mg in the renal inner medulla. The total concentration of L-arginine in the NOS reaction mixture of the homogenized renal cortex, outer medulla, and inner medulla was adjusted to 23 µmol/l by the addition of exogenous cold L-arginine to equalize the starting substrate concentration in each tube and permit the comparison of enzymatic activity. In contrast to the homogenized whole tissue, endogenous L-arginine was undetectable in the isolated segments after the dissection protocol. The initial concentration of L-arginine in the enzymatic reaction with the microdissected segments was therefore solely accounted for by the amount of L-[3H]arginine added (60 nmol/l).

Protocol 2: Identification of mRNA for NOS Isoforms in Activity-Positive Segments

After identification of the renal tubular and vascular segments with the greatest NOS enzymatic activity, RT-PCR was performed on RNA extracted from the microdissected segments that possessed significant amounts of NOS activity. This study was therefore performed to identify which NOS isoforms were present in the activity-positive Glm, VR, and IMCD; the PR was used as a negative control because that tubular segment demonstrated minimal enzymatic activity.

RNA extraction and RT-PCR of NOS isoforms. Microdissected segments were transferred into individual tubes and washed with dissection solution containing 1% BSA. The segments were then resuspended with 450 µl TRIzol reagent (GIBCO-BRL, Life Technologies); 90 µl of chloroform was added to separate the mixture into an aqueous and an organic phase. The aqueous phase was removed after centrifugation and the RNA was precipitated with 270 µl of isopropanol. The RNA (which was not visible) was washed with 75% ethanol and allowed to dry at room temperature, and the total RNA was resuspended in 8 µl of RNase-free diethyl pyrocarbonate (DEPC) water. A first-strand cDNA synthesis kit (Pharmacia Biotech) was used to reverse transcribe RNA extracted from the renal segments. The total RNA (in 8 µl)
was heated at 65°C for 10 min and rapidly chilled on ice, then mixed with 7 µl of the reagents supplied with the kit. The reaction mixture contained 0.2 µg random hexadeoxynucleotides, 45 mmol/l Tris (pH 8.3), 68 mmol/l KCl, 15 mmol/l dithiothreitol, 9 mmol/l MgCl2, 0.08 mg/ml BSA, 1.8 mmol/l 3′-deoxythymidine 5′-triphosphate set (dNTPs), and 100 U MMLV RT. The reaction mixture was incubated at 37°C for 60 min and then heated to 65°C for 10 min to inactivate the RT.

The PCR reactions were performed in a total volume of 50 µl using a PCR Supermix kit (GIBCO-BRL) containing 22 mmol/l Tris-HCl (pH 8.4), 55 mmol/l KCl, 1.65 mmol/l MgCl2, 200 µmol/l dNTPs, 3 µl RT reaction mixture (1 µl for β-actin), 22 U recombinant Taq DNA polymerase, and 40 pmol of the specific primer pairs for eNOS, nNOS, iNOS, or β-actin. The PCR reactions were cycled 35 times between 94°C (denaturation) for 1 min; 66°C (annealing) for nNOS, 64°C (annealing) for eNOS and iNOS, 60°C (annealing) for β-actin and 1 min; and 72°C (extension) for 1 min. Samples were incubated at 72°C for an additional 7 min after the last cycle was completed. Negative control PCR reactions with a substitution of water for the RT reaction were performed in parallel.

All nucleotide primers were purchased from Operon Technologies (Alameda, CA). The primer pairs were chosen from the published cDNA sequences of rat nNOS (6; GenBank accession no. X59949), rat eNOS (19; U02543), rat iNOS (1; D12520), and human β-actin (7; X00351). All primer pairs spanned at least one intron in the genomic DNA sequence. The primer sequence for nNOS corresponded to 5′-GGC ACT GGC ATC GCA CCC TT-3′ (sense, bp 4096–4115) and 5′-CTT TGG CCT CTC CCG TTC CC-3′ (antisense, bp 4308–4289); the final PCR product was 230 bp in size. The primers for eNOS corresponded to 5′-CTG CTC CCC AGA ATG TCT TC-3′ (sense, bp 255–274) and 5′-CAG GTA CGT CAG TCC CTC CT-3′ (antisense, bp 482–465); the final PCR product was 213 bp in size. The primer for iNOS corresponded to 5′-AGC ATC ACC CCT GTG TCC CAC CC-3′ (sense, bp 1592–1613) and 5′-TGG GCC AGT CTC CAT TGC CA-3′ (antisense, bp 1979–1960); the final PCR product was 388 bp in size. Oligonucleotide primers for β-actin (sense, 5′-AAC CGC GAG AAG ATG ACC CAG ATC ATG TTT-3′; and antisense, 5′-AGC AGC GCA CCC TT-3′) were used as a positive control in each sample, based on previous work by Briggs et al. (7) in microdissected tubular and vascular segments. The PCR products were separated on a 1.5% agarose gel in 1 Tris-borate-EDTA (TBE) buffer (10 mmol/l Tris, 0.05 mmol/l EDTA and 1 mmol/l L-NAME were added to the incubation buffer (Fig. 1C), the citrulline peak was nearly completely eliminated (0.0059 ± 0.001 fmol·mm−1·h−1; n = 6).

Time dependence and tubular length dependence of citrulline formation by microdissected tubular segments. To validate the use of the NOS assay for use with microdissected renal segments, we performed a set of experiments to demonstrate the time and length dependence of L-citrulline formation from L-arginine by the microdissected tubular segments. A series of IMCD segments with lengths varying from 5 to 30 mm was dissected from the same rat kidney and incubated with L-[3H]arginine and the above cofactors for 2 h. Figure 2 presents tubular length-dependent changes in L-citrulline formation by IMCD. Correlation analysis showed a linear relationship between the IMCD length and NOS activity, with a correlation coefficient of 0.98, a slope of 0.02 pmol·h−1·mm−1, n = 6, and a y-intercept of 0.025 pmol/h. We performed further experiments to determine the time dependence of L-citrulline formation by microdissected tubule segments. Twenty-segment IMCD dissected from the same kidney were incubated with L-[3H]arginine simultaneously, and the reactions were terminated separately at 1, 2, and 4 h for HPLC analysis and radiochemical quantitation. As expected, L-citrulline formation increased with time (Fig. 2A), and 4 h was considered significant.
shown in Fig. 2, inset, the amount of L-citrulline formation increased linearly with time from 1 to 4 h. An incubation time of 2 h was then used for the NOS activity assay in renal segments.

NOS activity in microdissected renal segments. Total NOS enzymatic activity was quantitated in microdissected Glm, PC, PR, CTAL, MTAL, CCD, OMCD, IMCD, OMTL, IMtL, and VR. The NOS activity in these segments is expressed in either femtomoles L-citrulline formed per millimeter length per hour (fmol·mm⁻¹·h⁻¹) or femtomoles L-citrulline formation per nanogram of protein per hour (fmol·ng⁻¹·h⁻¹); the results are summarized in Fig. 3. The IMCD (11.5 ± 1.0 fmol·mm⁻¹·h⁻¹) was found to have the greatest NOS activity in the segments examined; the VR (3.2 ± 0.8 fmol·mm⁻¹·h⁻¹) and Glm (1.9 ± 0.3 fmol·glomerulus⁻¹·h⁻¹) possessed moderate NOS activity, which was significantly lower than in IMCD, but significantly higher than in the other renal structures. The other renal structures studied had minimal NOS activity. NOS activity along the renal segments exhibited approximately the same distribution manner regardless of the method of normalization. The protein content in the microdissected nephron segments was examined using a Coomassie protein microassay protocol. Glm, PC, PR, CTAL, MTAL, CCD, OMCD, and IMCD were determined to contain 70.6 ± 10.5, 159 ± 16.0, 143.3 ± 10.9, 50.5 ± 7.7, 50.6 ± 4.5, 74.3 ± 4.4, 58.4 ± 6.3, and 88.3 ± 5.7 ng protein/mm tube length (or glomerulus), respectively (n = 6). The protein content of OMTL, IMtL, and VR was not determined because of the small amount of protein in these segments, which was undetectable with our protein assay.

NOS activity in renal cortex, outer medulla, and inner medulla. After adjustment of total L-arginine in the reaction mixture to 23 µmol/l, the total NOS activity in the renal inner medulla and outer medulla was 3.31 nmol·mg protein⁻¹·h⁻¹ and 0.52 nmol·mg protein⁻¹·h⁻¹, respectively, which was 26 and 4 times greater than NOS activity in the renal cortical homogenate (0.13 nmol·mg protein⁻¹·h⁻¹; Fig. 4). The addition of 1 mmol/l EDTA into the NOS reaction buffer inhibited the NOS activity by 62% in cortex, 87% in outer medulla, and 82% in inner medulla, indicating that calcium-dependent constitutive NOS isoforms contributed, to a great extent, to the basal level of NOS activity in the normal kidney.

Protocol 2: Identification of mRNA for NOS Isoforms in Activity-Positive Segments

Figure 5 is an ethidium bromide-stained gel containing RT-PCR products of nNOS, eNOS, iNOS, and β-actin mRNA from Glm, IMCD, VR, and PR. PR served as a negative control because of the minimal NOS enzymatic activity in this segment. Each band on the gel represents the RT-PCR products from RNA extracted from a 4.5-mm segment of IMCD, VR, or PR or from five glomeruli. The mRNA for all three NOS isoforms was expressed in IMCD, whereas only nNOS and eNOS were detected in Glm and VR. RT-PCR products were not observed for any of the NOS isoforms.
in RNA extracted from PR. As a positive control for RNA extraction and the RT reaction, the β-actin product was detected in all isolated segments with the predicted size of 350 bp. The addition of RNA extracted from the dissected renal segments without the RT reaction did not produce any signal, demonstrating that the RT-PCR products were not derived from genomic DNA. The 213-, 230-, and 388-bp RT-PCR products were confirmed to be nNOS, eNOS, and iNOS cDNA by sequencing of the subcloned PCR products.

**DISCUSSION**

The present studies demonstrate the distribution of NOS enzymatic activity among the different segments of the rat kidney. Of the renal tubular and vascular segments examined, the IMCD is the segment with the greatest NOS enzymatic activity when expressed per unit length or per amount of total protein. Compared with the IMCD, the VR and Glm had moderate NOS activity, whereas the other renal tubular segments demonstrated minimal NOS enzymatic activity. Further studies then identified nNOS, eNOS, and iNOS mRNA in the IMCD, and nNOS and eNOS mRNA in Glm and VR. Finally, it was determined that ~7% of the total NOS activity in IMCD is calcium independent, indicating that a small portion of the basal activity in this segment is due to iNOS.

On a whole tissue basis, the present data qualitatively correspond with previous studies, which have indicated that the renal medulla is enriched in NOS enzyme. Biondi and Romero (5) originally demonstrated, in slices of canine kidney, that the renal medullary tissue will produce more cGMP in vitro than slices of renal cortical tissue. Further work by Moridani and Kline (20) demonstrated that NOS catalytic activity in homogenates of both the rat renal inner medulla and outer medulla is nearly 10-fold greater than activity in the renal cortex. Western blotting data from our laboratory have shown a large amount of nNOS, eNOS, and iNOS immunoreactive protein in the inner and outer medulla, with much less in the renal cortex (16). Moreover, it has recently been shown in vivo that NO levels are much higher in the renal medullary interstitium than in the renal cortex (35). Taken together, these studies provide strong evidence that the renal medulla is enriched in NOS enzyme compared with the renal cortex.

As summarized in the introduction, a great deal of evidence has demonstrated the presence of mRNA and immunoreactive protein for each of the NOS isoforms in a number of renal tubular and/or vascular segments. The distribution of NOS enzymatic activity, however, has not been demonstrated at the level of the isolated tubular or vascular segment. Earlier attempts to localize the NOS activity in the kidney were based on indirect methods such as the measurement of nitrite, nitrate, and cGMP levels, or the histochemical detection of NADPH diaphorase. Strong NADPH diaphorase...
in situ hybridization (2). Immunoreactive iNOS mRNA in IMCD of normal rat kidneys by RT-PCR is also consistent with previous work that detected mRNA and calcium-independent NOS activity in IMCD lary tissue homogenates (15, 17). The presence of iNOS in IMCD cells (24), as well as in total renal inner medullary protein has been demonstrated in cultured mouse IMCD (27, 29), and nNOS and eNOS mRNA and protein have been observed in the vascular segments of the normal rat kidney (3, 29, 31).

The distribution of nNOS and eNOS in the renal tubular segments appears to be consistent with previous reports, but the distribution of iNOS observed in the present study does not completely agree with previous studies. In the present study, iNOS mRNA was not detected in Glm, VR, or PR, which is in direct conflict with previous reports that identified iNOS mRNA in arcuate and interlobular arteries, Glm, PR, CTAL, and MTAL (2, 19). Although the presence of iNOS mRNA was not examined in CTAL and MTAL in the present study, minimal NOS enzymatic activity was observed in these segments. In support of the present data, the tubular segments with significant NOS enzymatic activity are the same segments in which NOS mRNA was identified by RT-PCR. This indicates that the present data are internally consistent but does not resolve the differences in iNOS expression between different studies. One possible explanation for the observed differences are the animals studied. Outbred rats obtained from different suppliers, purchased at different times of the year, and housed in different environments are likely to demonstrate variability in the expression of gene products known to be inducible. Nonetheless, the explanation for the differences in iNOS expression between the different studies is presently unclear.

An additional concern with the microdissection and RT-PCR experiments is the possibility of contamination between different segments. The adherence of minimal amounts of tissue from adjacent structures could easily lead to false positives when using an extremely sensitive technique such as RT-PCR. We do not believe that to be a problem in this preparation for two reasons. First, we previously demonstrated that microdissected VR are free of contamination by tubular structures because vasopressin V2 receptor mRNA was not detected in isolated VR, but was found in IMCD and OMCD (22, 23). Second, we have recently performed RT-PCR on RNA obtained from isolated VR and IMCD and identified different cationic amino acid transporters in these two structures (F. Wu and D. Mattson, unpublished observations). These data indicate that minimal cross-contamination occurs in our microdissection procedure. Nonetheless, there is always the possibility of contamination when using a technique of such extreme sensitivity as RT-PCR, and data obtained using these techniques must be carefully interpreted.
The results of the present studies may help explain some of the functional data we have previously obtained in our laboratory (21). Chronic infusion of the nonspecific NOS inhibitor L-NAME to conscious rats led to a selective decrease in renal medullary blood flow, retention of sodium, and development of hypertension. The presence of relatively high amounts of NOS in the renal medulla indicates that this region of the kidney, particularly the IMCD and VR, may be a preferential target of NOS enzyme inhibition. Although the role of each of the NOS isoforms in the control of renal function is not clear, other functional studies from our laboratory have indicated that selective blockade of nNOS (15) or iNOS (17) in the renal medulla leads to a decrease in renal medullary blood flow, retention of sodium, and development of hypertension.

The present study did not attempt to examine NOS in renal cortical blood vessels, which have been demonstrated to possess mRNA and protein for all NOS isoforms. We did, however, examine glomerular capillarities, which possessed significant amounts of NOS enzyme activity and both nNOS and eNOS mRNA. This result is consistent with the functional observations that acute NOS inhibition leads to a decrease in the glomerular ultrafiltration coefficient (4, 8, 25, 34).

In summary, the present studies demonstrate the quantitative distribution of NOS enzymatic activity among the different renal segments of the rat kidney. Of the renal tubular and vascular segments examined, the IMCD is the segment with the greatest NOS enzymatic activity when normalized to segment length or protein content. Compared with the IMCD, the VR and Glm had moderate NOS activity, and the other segments examined demonstrated minimal NOS enzymatic activity. Based on mRNA detection by RT-PCR, nNOS, eNOS, and iNOS are all expressed in the IMCD, and nNOS and eNOS are found in the Glm and VR. The present data suggest that these segments may be those in which NOS-derived NO exerts the greatest biological effect.

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