Nitric Oxide Synthase Activity and Isoforms in Rat Renal Vasculature

David L. Mattson, Feng Wu

Abstract—Experiments were performed to quantify nitric oxide synthase (NOS) activity and identify the NOS isoforms present in the Sprague-Dawley rat renal vasculature. NOS enzymatic activity was measured by adding $[^3H]$arginine to microdissected renal blood vessels and quantifying the conversion to $[^3H]$citrulline by reverse-phase high-performance liquid chromatography. Total NOS activity was greatest in microdissected vasa recta ($123 \pm 41 \text{ pmol} \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$, n=5) and significantly less in glomeruli ($46 \pm 9 \text{ pmol} \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$, n=6) and afferent arterioles ($42 \pm 10 \text{ pmol} \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$, n=6) and averaged <5 pmol · mg⁻¹ · h⁻¹ in arcuate (n=8) and interlobular (n=9) arteries. Addition of 1.0 mmol/L EDTA to the reaction decreased NOS activity to <5 pmol · mg⁻¹ · h⁻¹ in afferent arterioles, glomeruli, and vasa recta (n=5 each), indicating that the NOS enzymatic activity in these segments is primarily a result of constitutive NOS. Both neuronal and endothelial NOS mRNA were identified in each vascular segment by reverse transcription—polymerase chain reaction, but inducible NOS mRNA was detected only in microdissected arcuate arteries. The present experiments indicate that the vasa recta, glomeruli, and arterioles contain large amounts of calcium-dependent NOS enzymatic activity and that neuronal NOS and endothelial NOS mRNA are present in these segments. (Hypertension. 2000;35[part 2]:337-341.)

Key Words: rats, Sprague-Dawley ■ kidney ■ nitric oxide synthase ■ RNA

Nitric oxide (NO) has potent effects on the renal vasculature. The inhibition of NO synthase (NOS) leads to increased renal vascular resistance,¹⁻⁵ whereas stimulation of endogenous NOS after L-arginine administration leads to decreased vascular resistance.⁶ A number of studies have demonstrated that inhibition or stimulation of NO can influence the diameter of large preglomerular vessels,⁷ the afferent and efferent arterioles,⁸⁻¹⁰ and vasa recta.¹¹ Therefore, NO is an important modulator of vascular tone throughout the renal vasculature.

Many biochemical and molecular studies have been performed to identify both mRNA and immunoreactive protein of neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) in renal vessels. The mRNA for nNOS has been localized in glomeruli and vasa recta;¹² nNOS protein has been identified in efferent arterioles;¹³ the mRNA encoding iNOS has been identified in glomeruli, arcuate arteries, interlobular arteries, and afferent arterioles;¹⁴; and eNOS protein has been identified in the endothelium of preglomerular and postglomerular vessels by immunohistochemical techniques.¹⁵ The mRNA encoding iNOS has also been found in arcuate and interlobular arteries and glomeruli by in situ hybridization and reverse transcription (RT)—polymerase chain reaction (PCR) of microdissected segments.¹⁶ Despite these types of studies, however, there is little information available regarding NOS enzymatic activity in the individual segments of the renal vasculature. The present study was designed to examine total and calcium-independent NOS enzymatic activity in dissected vessels obtained from the Sprague-Dawley rat kidney and to identify the NOS isoforms present in each vessel segment by RT-PCR.

Methods
Experiments were performed on 36 male Sprague-Dawley rats (250 to 300 g) obtained from Harlan Laboratories (Madison, Wis). 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 procedures were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

Microdissection of Renal Vessels
Microdissection was performed as described previously.¹²,¹⁶,¹⁷ 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, the left kidney was flushed with 10 mL ice-cold dissection solution containing (in mmol/L) NaCl 135, KCl 3, CaCl₂ 1.5, MgSO₄ 1, KH₂PO₄ 2, glucose 5.5, and HEPES 10 (pH 7.4) and then perfused with 1 mL of 1.0-µm Blue Dyed Polybeads (Polyscience, Inc) for isolation of the arcuate artery, interlobular artery, afferent arteriole, and glomerulus or with 0.1-µm Blue Dyed Polybeads for isolation of the vasa recta. After perfusion, the kidney was removed and cut into 1- to 2-mm-thick sections containing the entire corticomедullary axis. The sections were incubated at 37°C for 30 minutes in digestion solution that was prepared by adding 2 mg/mL collagenase (CLS 2, Worthington) to the dissection solution.

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From the Department of Physiology, Medical College of Wisconsin, Milwaukee.
Correspondence to David L. Mattson, PhD, Department of Physiology, Medical College of Wisconsin, 8701 Watertown Plank Rd, Milwaukee, WI 53226. E-mail dmatsson@mcw.edu
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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 MSZ stereomicroscope with dark-field illumination. The following segments were dissected: arcuate artery, interlobular artery, afferent arteriole, glomerulus, and vasa recta. The time period for dissection was limited to 1.5 hours.

NOS Enzyme Assay
The quantification of NOS enzymatic activity was performed as we have previously described. The dissected arcuate artery, interlobular artery, afferent arteriole, glomerulus, and vasa recta were incubated with 2 mmol/L CaCl₂, 1 mmol/L NADPH, 25 μmol/L flavin adenine dinucleotide, 1.25 μg/mL calmodulin, 10 μmol/L tetrahydrobiopterin, and 1 μCi [³H]arginine (=300 000 cpm, specific activity 68 Ci/mmol) in 50 μL of 20 mmol/L HEPES buffer, pH 7.2, at 37°C for 2 hours. Calcium-independent activity was measured after the addition of 1.0 mmol/L EDTA and the elimination of calcium calmodulin from the assay. The reaction mixture was centrifuged and separated into the supernatant and sediment. The l-arginine and converted l-citrulline in the supernatant were separated by isocratic reverse-phase high-performance liquid chromatography with a Supelco LC-18-DB column (mobile phase 11.5% methanol, 11.5% acetonitrile, 1% tetrahydrofuran, and 0.1 mol/L KH₂PO₄, pH 5.9). The amounts of l-¹[³H]arginine and converted l-¹[³H]citrulline were quantified by radiochemical detection (Packard). NOS activity was determined from the ratio of the total l-¹[³H]arginine converted to l-¹[³H]citrulline and the amount of total arginine in the reaction. The vessel sediment was washed once with dissection solution and then dissolved in Coomassie blue dye for the determination of total vessel protein. The total vessel length was not determined in these studies.

RNA Extraction and RT-PCR of NOS Isoforms
The extraction of RNA and RT-PCR identification of NOS isoforms in the dissected renal vessels was performed as we have previously described.

The dissected kidney vessels or vessel pieces were washed and placed in individual tubes containing 500 μL TRizol reagent (Life Technologies); after vortex mixing, chloroform was added to each tube (100 μL of reagent (Life Technologies); after vortex mixing, chloroform was added to each tube (100 μL). The mixtures were centrifuged at 12 000 g for 15 minutes, and the aqueous phase was removed. The extraction of RNA and RT-PCR identification of NOS isoforms

All nucleotide primers were purchased from Operon Technologies, Inc, as we have previously described. The primer pairs were chosen from the published cDNA sequences of rat nNOS, eNOS, and human β-actin. The primer sequence for nNOS corresponded to 5'-GGC ACT GCC ATC GCA CCC TT-3' (sense, base pairs 4096 to 4115) and 5'-CTT TGG CCT GTC CGG TTC CC-3' (antisense, base pairs 4308 to 4289); the final PCR product was 213 bp in size. The primers for eNOS corresponded to 5'-CTG CTG CCC GAG ATA TCT CT-3' (sense, base pairs 235 to 274) and 5'-CAG GTA CTG CAG TCC TTC CT-3' (antisense, base pairs 482 to 465); the final PCR product was 228 bp in size. The primers for iNOS corresponded to 5'-AGC ATC ACC CCT GTG TAC CC-3' (sense, base pairs 1592 to 1613) and 5'-TGG GCC AGT CTC CAT TGC CA-3' (antisense, base pairs 1979 to 1990); the final PCR product was 388 bp in size. Oligonucleotide primers for β-actin (sense, 5'-AAC CGC GAG AAG ATC CAG ATG TT-3'; antisense, 5'-AGC AGC CGT GGC CAT TTG CTC CAA GT-3') were used as positive controls in each on the basis of previous work by Briggs et al involving microdissected tubular and vascular segments. The PCR products were separated on a 1.5% agarose gel in 1x tris-borate EDTA electrophoresis buffer (10 V/cm, gel length for 1 hour), stained with ethidium bromide (0.5 μg/mL), visualized under UV light, and photographed.

The RT-PCR products for nNOS, eNOS, and iNOS were ligated into pCR2.1 vector (Invitrogen), and the subsequent plasmid DNA was purified by use of ion-exchange columns (QIAGEN). To confirm the authenticity of the RT-PCR product, each insert was sequenced by ThermoSequenase with the use of the dideoxynucleotide chain termination reaction (Amersham). The samples were resolved on a DNA sequencer (model 725, Molecular Dynamics).

Statistical Methods
Data are presented as mean±SE. The significance of differences was evaluated by ANOVA and Student-Newman-Keuls post hoc tests. A confidence level of P<0.05 was considered significant.

Results
NOS Enzymatic Activity in Isolated Renal Blood Vessels
Total NOS enzymatic activity in the dissected renal microvascular segments is illustrated in Figure 1. When normalized to total tissue protein, the greatest NOS enzymatic activity was measured in the isolated vasa recta and averaged 123±36 pmol ⋅ mg⁻¹ ⋅ h⁻¹ (n=5 vessel preparations on tissue obtained from 5 rats). Significantly less activity was found in isolated afferent arterioles (n=6 from 4 rats) and glomeruli (n=6 from 5 rats), which averaged 42±10 and 46±8 pmol ⋅ mg⁻¹ ⋅ h⁻¹, respectively. Total enzymatic activity in the arcuate (n=8 from 7 rats) and interlobular (n=9 from 6 rats) arteries was much less, averaging <5 pmol ⋅ mg⁻¹ ⋅ h⁻¹. Further studies were then performed to determine the potential contribution of calcium-independent iNOS to total enzymatic activity in the afferent arteriole, glomeruli, and vasa recta (n=5 from 5 rats for each). When calcium was eliminated and EDTA was added to the reaction with these segments, the total activity was reduced to <5 pmol ⋅ mg⁻¹ ⋅ h⁻¹ in each segment, indicating minimal calcium-independent NOS activity.

RT-PCR Identification of NOS Isoforms in Renal Microvessels
After the measurement of total NOS enzymatic activity in different vascular segments, further studies were performed...
to identify the NOS isoforms present in each vessel. A representative ethidium bromide–stained gel with RT-PCR products for nNOS, eNOS, iNOS, and β-actin from RNA obtained from arcuate arteries, interlobular arteries, afferent arterioles, glomeruli, and vasa recta is illustrated in Figure 2. RT-PCR products for nNOS and eNOS were identified in all segments studied, but an iNOS product was found only in the arcuate artery. The β-actin RT-PCR product was used as a control for each reaction. This figure is representative of 4 separate experiments for each segment performed on tissue obtained from 4 separate animals.

**Figure 2.** Ethidium bromide–stained gel demonstrating RT-PCR products for nNOS, eNOS, iNOS, and β-actin in RNA isolated from ARC, IL, AA, Glm, and VR. The lower size marker on the upper 2 panels is 100 bp, and the lower size marker on the lower panels is 200 bp.

The present data demonstrate in individual segments of the rat renal vasculature that NOS enzymatic activity is greatest in the vasa recta, afferent arterioles, and glomeruli, with lesser NOS activity observed in the arcuate and interlobular arteries. The enzymatic activity in the vasa recta, afferent arterioles, and glomeruli was almost completely inhibited after the addition of EDTA and deletion of calcium from the reaction, indicating that the majority of the activity in these vessels is due to constitutive NOS. The enzymatic activity measurements in isolated segments of the preglomerular vasculature are the first that we are aware of, although we previously reported the NOS activity in isolated glomeruli and vasa recta. Further studies identified RT-PCR products for nNOS and eNOS in all segments examined, whereas an iNOS RT-PCR product was detected only in RNA isolated from the arcuate artery. These data begin to address the potential role of NOS/NO in the control of renal segmental vascular resistance.

The present NOS activity data determined from isolated vessels were normalized to total protein of each vascular segment; total length was not determined, so NOS activity per unit length was not calculated. In addition, there is no way to distinguish between NOS in the endothelium and that in vascular smooth muscle in the different vessels. If, as is commonly assumed, most of the NOS in vessels is found in the endothelium, this method of normalization may skew the data, because there are proportionately more nonendothelial cells, such as vascular smooth muscle cells, in the larger arteries than are found in smaller arteries. To normalize the present NOS enzymatic activity data to the amount of endothelium in the larger cortical vessels, the morphometric data of Smeda et al21 were used to calculate that the endothelium accounted for 3.8%, 5.2%, and 22.7% of total cross-sectional area in the arcuate arteries, interlobular arteries, and afferent arterioles, respectively. If it is assumed that 95% of vasa recta mass is endothelial cells and that the protein content of each component of the vascular wall is proportionate to surface area, the calculated NOS enzymatic activity/endothelial protein in these segments is 37, 90, 186, and 130 pmol/mg endothelial protein per hour in the arcuate artery, interlobular artery, afferent arteriole, and vasa recta, respectively. The present data, when normalized to total tissue protein or total estimated endothelial protein, may therefore indicate the potential capacity of these different vessels to produce and respond to NO. Indeed, it has been previously shown in the isolated perfused split kidney preparation that the afferent arterioles exhibit a greater constriction to Nω-nitro-L-arginine7 than do the arcuate or interlobular vessels. The diameter of the efferent arteriole has also been demonstrated to be extremely sensitive to NOS inhibition,8–10 although we were unable to obtain sufficient tissue to determine NOS enzymatic activity in efferent arterioles in the present study.

An additional important observation of these studies is the presence of relatively large amounts of total NOS enzymatic activity as well as the mRNA for nNOS and eNOS in the vascular structures of the renal cortex, particularly in the afferent arterioles and glomeruli. A direct comparison of
NOS activity measured in renal cortical tissue homogenates and isolated tubules and vessels is not possible because of experimental limitations. It can be inferred from these studies, however, that renal cortical blood vessels possess a level of NO synthetic capacity similar to that found in glomeruli and vasa recta. Because we previously demonstrated that glomeruli and vasa recta have significantly greater total NOS enzymatic capacity than do renal cortical tubular structures, renal cortical blood vessels appear to be relatively enriched in NOS. This conclusion, which is based on biochemical evidence, is consistent with results of large number of functional studies that have demonstrated the effects of NOS in the control of renal vascular resistance. It should also be noted that the vasa recta of the renal medulla has been identified as an additional vascular segment containing large amounts of NOS enzymatic activity. This finding may help explain previous observations in our laboratory demonstrating that systemic administration of the NOS inhibitor L-NAME had a preferential effect on the renal medullary vasculature.22

In the expression studies, RT-PCR products for the constitutive NOS isoforms, nNOS and eNOS, were found in all vessels segments examined, whereas iNOS was detected only in the large arcuate artery. Both eNOS and nNOS have been observed in renal blood vessels in studies from other laboratories,13,14,23 and we previously reported both nNOS and eNOS mRNA in glomeruli and vasa recta.12 iNOS, however, has not been as consistently detected in renal vessels. Whereas one previous study24 did not detect iNOS mRNA in the vasculature of the normal rat, another study15 has indicated that iNOS is present in glomeruli and in arcuate and interlobular arteries. In the present study, iNOS mRNA was detected in the arcuate artery, although only minimal calcium-independent NOS activity was detected in any of the vessels, indicating that iNOS protein is minimally expressed in the renal vasculature. The reason for the differences in iNOS expression found between different studies is unclear.

The potential role of the different NOS isoforms in the control of renal hemodynamics remains to be determined. It is generally accepted that eNOS is the primary source of NO in vessels, although the other isoforms could potentially play a significant role in the regulation of renal vascular resistance. Previous studies have indicated that nNOS may indeed play a role in the control of renal vascular resistance. In 2 different studies,25,26 selective inhibition of nNOS with 7-nitroindazole had no effect on renal vascular resistance in normal rats, although it decreased renal blood flow in furosemide-treated rats or those maintained on a low sodium diet. More recently, chronic 7-nitroindazole administration has been demonstrated to lead to a decrease in renal blood flow in rats maintained on a low or a high sodium diet.27 These pharmacological studies indicate that nNOS participates in the control of renal vascular resistance, although the role of nNOS expressed in the vasculature and that expressed in the macula densa, operating through the tubuloglomerular feedback mechanism,28 is unclear at this time. Other studies29,30 have examined the effects of iNOS inhibition with aminoguanidine. Acute aminoguanidine did not alter whole-kidney blood flow29 or cortical blood flow30 in anesthetized rats, indicating that this isoform has minimal effects on renal vascular resistance. Interestingly, renal medullary blood flow was slightly increased after aminoguanidine administration.30 It is important to note that the renal epithelial segments also contain plentiful amounts of NOS activity12 and that NO produced by different nephron segments may also influence renal vascular resistance.

The changes that occur in NOS enzymatic activity in renal vessels during alterations in dietary sodium intake, changes in hormonal status, and changes during the developmental and maintenance phase of hypertension remain to be determined. Interestingly, changes in tissue NOS activity in hypertensive and normotensive rats are not predictable. Constitutive NOS activity was reduced in the renal medulla of the Dahl salt-sensitive rat made hypertensive by high sodium intake compared with the normotensive Dahl salt-sensitive rat maintained on a low sodium diet.31 In contrast, NOS activity was observed to be increased in the renal medulla of the spontaneously hypertensive rat compared with the Wistar-Kyoto rat.31 It is not presently clear whether these changes occur as a buffering mechanism in response to the elevation in arterial blood pressure, whether they occur because of tissue damage caused by elevated arterial pressure, or whether they are triggered by other factors. In addition, it is not known whether the changes occur in the vasculature or in the renal tubules. Further experiments will be required to begin to understand these changes.

In summary, the present data indicate that a large amount of NOS enzymatic activity is found in the vasa recta, afferent arterioles, and glomeruli, with lesser NOS activity observed in the arcuate and interlobular arteries. The majority of this activity is calcium dependent, indicating that constitutive NOS isoforms produce most of the NO in these vessels. Furthermore, RT-PCR products for nNOS and eNOS were identified in all segments studied. Data from these studies begin to address the potential role of different NOS isoforms in the control of renal segmental vascular resistance.

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References


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