Tubuloglomerular feedback-dependent modulation of renal myogenic autoregulation by nitric oxide

Ying Shi, Xuemei Wang, Ki H. Chon, and William A. Cupples.

Tubuloglomerular feedback-dependent modulation of renal myogenic autoregulation by nitric oxide. Am J Physiol Regul Integr Comp Physiol 290: R982–R991, 2006. First published November 17, 2005; doi:10.1152/ajpregu.00346.2005.—Nonselective inhibition of nitric oxide (NO) synthase (NOS) augments myogenic autoregulation, an action that implies enhancement of pressure-induced constriction and dilatation. This pattern is not explained solely by interaction with a vasoconstrictor pathway. To test involvement of the Rho-Rho kinase pathway in modulation of autoregulation by NO, the selective Rho kinase inhibitor Y-27632 and/or the NOS inhibitor Nω-nitro-ω-arginine methyl ester (L-NAME) were infused into the left renal artery of anesthetized rats. Y-27632 and L-NAME were also infused into isolated, perfused hydrenalineic kidneys to assess myogenic autoregulation over a wide range of perfusion pressure. In vivo, L-NAME reduced renal vascular conductance and augmented myogenic autoregulation, as shown by increased slope of gain reduction and associated phase peak in the pressure-flow transfer function. Y-27632 (10 μmol/l) strongly dilated the renal vasculature and profoundly inhibited autoregulation in the absence or presence of L-NAME in vivo and in vitro. Afferent arteriolar constriction induced by 30 mmol/l KCl was reversed (−92 ± 3%) by Y-27632. Phenylephrine caused strong renal vasoconstriction but did not affect autoregulation. Inhibition of neuronal NOS by Nω-(1-imino-3-butenyl)-L-ornithine (L-VNIO) did not cause significant vasoconstriction but did augment myogenic autoregulation. Thus vasoconstriction is neither necessary (L-VNIO) nor sufficient (phenylephrine) to explain the augmented myogenic autoregulation induced by L-NAME. The effect of L-VNIO implicates tubuloglomerular feedback (TGF) and neuronal NOS at the macula densa in regulation of the myogenic mechanism. This conclusion was confirmed by the demonstration that systemic furosemide removed the TGF signature from the pressure-flow transfer function and significantly inhibited myogenic autoregulation. In the presence of furosemide, augmentation of myogenic autoregulation by L-NAME was significantly reduced. These results provide a potential mechanism to explain interaction between myogenic and TGF-mediated autoregulation.

NONSELECTIVE INHIBITION of nitric oxide (NO) synthases (NOS) by Nω-nitro-ω-arginine derivatives causes an important and long-lasting rise in blood pressure accompanied by profound reduction of renal blood flow (RBF) (3, 10, 28, 47) so that renal vascular conductance typically is reduced by 50–60%. After NOS inhibition, autoregulation of RBF is enhanced, whether assessed by dynamic (10, 23, 24, 51, 52, 54) or steady-state experiments (14, 15, 28, 47). Using time-series analysis, we and others showed modulation of the myogenic component of autoregulation (23, 51, 52). Two changes in RBF dynamics were induced by NOS inhibition: 1) gain reduction by the myogenic system was increased so that RBF became more stable, and 2) RBF dynamics changed in a way that indicated emergence of a rate-sensitive component of the myogenic mechanism. This means that the myogenic mechanism responded not only to the level of perfusion pressure, but also to the rate of change of perfusion pressure. Effectively, it worked faster and provided better stabilization of RBF. It is important to remember that pressure fluctuation is not simply upward or downward but bidirectional and that autoregulatory adjustment of conductance occurs in both cases. Given that NOS inhibition results in increased stability of RBF in the face of upward and downward blood pressure fluctuations (52), it follows directly that pressure-induced constriction and pressure-induced dilatation are augmented by NOS inhibition. Any consideration of the effect of NOS inhibition on autoregulation must recognize this fact, which has been demonstrated recently (24). Interestingly, the modulation of myogenic autoregulation shows regional specificity and is not apparent in the gut (10) or the hindlimb (24). Thus modulation of myogenic autoregulation by NO seems clear, although the mechanism or mechanisms by which it occurs are not well understood. Two questions in particular need to be resolved: 1) What mechanism(s) explains enhancement of pressure-induced dilatation in the face of strong vasoconstriction? 2) What is the source of NO that modulates the myogenic mechanism?

Given the known targets of cGMP and, thus, NO (46) in vascular smooth muscle cells, it is not difficult to understand how NOS inhibition could cause strong renal vasoconstriction by, for instance, inhibition of large-conductance, Ca2+-sensitive K+ channels or voltage-sensitive K+ channels or by increased availability of L-type Ca2+ channels. All these channels have been implicated in responses to NOS inhibition in other beds (12, 40–42). However, it is unclear how NOS inhibition could affect any of these targets in a way that would augment myogenic vasodilatation. Another potential target of NO that could account for enhanced myogenic dilatation is Ca2+ sensitivity (4, 7). Regulation of Ca2+ sensitivity provides a plausible way in which NO could modulate myogenic autoregulation. When Ca2+ sensitivity is increased, smaller and, thus, faster excursions of intracellular Ca2+ concentration are...
required to achieve the same change in developed tension. Such an effect would be most apparent in vascular smooth muscle having rapid kinetics and dynamics, exactly the attributes of the afferent arteriole (10, 29, 32, 39). Recent studies have shown that activation of Rho kinase is the principal pathway leading to Ca2+ sensitization in vascular smooth muscle and that this occurs by phosphorylation and inactivation of myosin light chain phosphatase (43). Similarly, activation of myosin light chain phosphatase contributes importantly to vasodilatation induced by NO (4, 7).

The second issue, the source of NO that modulates myogenic autoregulation, has functional consequences, because all three NOS isoforms are constitutively present in kidney (34), so that any or all could contribute to autoregulation. A vascular source for NO, presumably produced by endothelial NOS (eNOS), is perhaps the simplest to explain and predicts a source for NO, presumably produced by endothelial NOS so that any or all could contribute to autoregulation. A vascular three NOS isoforms are constitutively present in kidney (34), genic autoregulation, has functional consequences, because all to vasodilatation induced by NO (4, 7).

METHODS

The studies were approved by the Animal Care Committees of the SMBD-Jewish General Hospital (in vivo studies) and the University of Calgary (in vitro studies) and conducted under the guidelines promulgated by the Canadian Council on Animal Care. Experiments were performed in 10- to 14-wk-old male Wistar rats (in vivo studies) or Sprague-Dawley rats (in vitro studies) obtained from Charles River (St. Hyacinthe, QC, Canada). Dynamic autoregulation and renal vascular responses to NO inhibition are identical in these strains (51), and the dynamics of renal autoregulation in the hydronephrotic kidney match those of the myogenic mechanism in vivo (9). Rats had free access to water and food at all times before experiments.

Procedures. Anesthesia was induced by 5% isoflurane in inspired gas (30% O2-70% air). After induction, the anesthetic concentration was reduced to ~2%. The animal was transferred to a servo-controlled, heated table to maintain body temperature at 37°C, intubated, and ventilated by a pressure-controlled small animal respirator (RSP 1002, Kent Scientific, Litchfield, CT) operating in respiratory-assist mode. During the 1-h post-surgical equilibration period, inspired anesthetic concentration was titrated to the minimum concentration that precluded a blood pressure response when the tail was pinched (i.e., ~1%).

Cannulas were placed in the right femoral artery (PE-90 with narrowed tip) and vein (PE-50). A constant intravenous (iv) infusion delivered 1% of body weight per hour throughout the experiment and contained 2% charcoal-washed bovine serum albumin in normal saline. A fine Teflon cannula was placed in the left femoral artery and advanced into the abdominal aorta for subsequent placement in the left renal artery. The left kidney was approached by a left subcostal abdominal incision, and the left renal artery. The left kidney was approached by a left subcostal incision, and the distal end of the Teflon cannula was manipulated into the renal artery. It received several separate lines for drug infusion and one line connected to a magnetic motor that was driven at 1.2 Hz. This provided alternating negative and positive pressure in the Teflon infusion cannula and served to mix the drugs with renal arterial blood, as shown by Parekh (37) and Cavarape et al. (6). Pilot experiments established that placement of the cannula tip into the renal artery or operation of the mixing pump had no discernible effect on blood pressure or RBF. The flow probe was placed around the renal artery as previously described (50–52); the probe head was fixed in place, and acoustic coupling was ensured by application of a gel based on surgical lubricant and NALCO 1181 as recommended by Transonic Systems. Femoral arterial pressure was measured by a pressure transducer (model TRN050; Kent) driven by an amplifier (model TRN005; Kent). RBF was measured by a transit time ultrasonic flowmeter [model T401 (model 1PRB probe); Transonic Systems, Ithaca, NY].

To assess RBF dynamics in vivo, a motorized clamp was placed on the aorta between the right and left renal arteries and was used to increase the amplitude of pressure fluctuations, that is, to “force” blood pressure. The motor was driven by a program implemented in DT-VEE (Data Translation). Briefly, the program operates as a negative-feedback controller to maintain downstream pressure [renal perfusion pressure (RPP)] 15–20% below the spontaneous level of blood pressure. It iterates at 2 Hz, and at each iteration the target pressure is randomly changed within a predefined range, typically ±5% (50). In each period, the pressure drop across the occluder was adjusted so that drug actions could be compared at the same mean RPP.

Normally, experimental drugs were infused into the renal artery (ira) to minimize confounding systemic effects. Nonselective inhibition of NOS was achieved by Nω-nitro-l-arginine methyl ester (l-NAME; Sigma), which was delivered at 10 μg/min for 20 min and then at 3 μg/min for the remainder of the experiment. This dose duplicates the renal vascular effects of 10 mg/kg l-NAME delivered as an intravenous bolus, a dose that is saturating on mean arterial pressure (3). The selective Rho kinase inhibitor Y-27632 (19, 43, 48), a generous gift from Welfide, Japan, was delivered to achieve a nominal concentration of 10 μmol/l in renal arterial blood, with the assumption of an RBF of 6 ml/min. This concentration of Y-27632 provides essentially complete and highly selective blockade of Rho kinase (43). Preliminary studies showed that a delivered concentration of 20 μmol/l resulted in RPP too low for an autoregulation study and that 5 μmol/l Y-27632 produced some pressure reduction, indicating drug recirculation, but caused incomplete blockade of renal vascular responses to l-NAME. Two NOS isoform-selective inhibitors were used: N-[3-(aminomethyl)benzyl]acetamidine (1400W; Tocris Bioscience, Ellisville, MO), a highly selective inhibitor of inducible NOS (iNOS) (13), and the nNOS inhibitor Nω-(1-imino-3-butenyl)-l-ornithine [l-VNIO; Alexis Biochemicals (Axxora), San Diego, CA], which is highly selective for nNOS vs. iNOS and moderately selective for nNOS vs. eNOS (2). Both are slow-onset inactivating inhibitors of their respective isoforms. 1400W was delivered for 30 min to achieve a nominal concentration of 5 μmol/l in renal arterial blood (13); then the infusion was stopped and data acquisition was begun. l-VNIO was infused to achieve a nominal concentration of 1 μmol/l in renal arterial blood together with 2 μmol/l l-arginine to minimize inhibition of eNOS. Data acquisition began 30 min after the start of the infusion, which continued until the end of the experiment. Similar doses and infusion protocols have been used previously (11).

In vivo experiments. Two experiments were performed to demonstrate that renal vasoconstriction, per se, has no effect on RBF dynamics and that the route of administration of the constrictor (iv or ira) also did not affect the result. In five rats, after a control recording was acquired, phenylephrine was infused at 10 μg·kg⁻¹·min⁻¹ iv. In another five rats, the control recording was followed by ira infusion of phenylephrine (1 μg·kg⁻¹·min⁻¹).

Two experiments were performed to assess the responses of renal vascular conductance and myogenic autoregulation to l-NAME in the absence and presence of selective Rho kinase inhibition by Y-27632. The first experiment employed seven rats. A 25-min control period (ira saline vehicle) was followed by ira of Y-27632 for 55 min and finally by 55 min of Y-27632 + l-NAME infusion. In each period, RPP was forced and data were acquired for the last 25 min. In the second experiment, the order of drug administration was reversed, so
that l-NAME was given alone followed by l-NAME + Y-27632; eight rats were used.

Two experiments assessed the renal vascular responses to isoform-specific NOS blockade. In each case, a control period was followed by isoform-specific NOS inhibition and, subsequently, by infusion of l-NAME to provide a positive control. Eight rats received 1400W ira, and six rats received l-VNIO. Because the results of these experiments suggested an involvement of nNOS at the macula densa in myogenic autoregulation, a further experiment was performed using eight rats in which the initial control period was followed by iv furosemide infusion (5 mg/kg + 5 mg·kg⁻¹·h⁻¹ and 4 ml/h saline added to the infusion) and then by l-NAME (10 mg/kg iv).

In vitro experiments. Hydromephonic kidneys were created by ~6 wk of ureteral occlusion, harvested, and perfused as previously described with a DMEM-based perfusate plus control of PO₂, temperature, and pH (9, 26, 31, 32, 46). Perfusion pressure, which was monitored within the renal artery, was maintained at ±80 mmHg. Afferent arteriolar diameter was sampled at 3 Hz over a 10- to 20-μm segment near the midpoint of the vessel and determined by online image processing. Mean diameter values were then averaged over the plateau of the response. All agents were added directly to the perfusate. A control period was followed by inclusion in the perfusate of l-NAME (100 μmol/l, n = 6) or Y-27632 (10 μmol/l, n = 6) and then by a third period in which both drugs were included in the perfusate. In each period, perfusion pressure was increased in 20-mmHg steps at 1-min intervals from 60 to 180 mmHg. An additional five hydromephonic kidneys were perfused at 80 mmHg under control conditions after perfusate K⁺ concentration ([K⁺]) was increased isotonically to 30 mmol/l and then with 10 μmol/l Y-27632 added to the high-[K⁺] perfusate.

Data acquisition and analysis. The blood pressure and RBF signals were low-pass filtered at 20 Hz and sampled at 100 Hz online. Data segments of 1,311 s (2^{17} points) were band-pass filtered (0.004 and 1 Hz) using a fast Fourier transform (FFT)-inverse FFT filter with a rectangular window and subsampled to 3.125 Hz. Power spectra, Hz) using a fast Fourier transform (FFT)-inverse FFT filter with a

### Table 1. Effects of l-NAME and Y-27632 on RBF dynamics

<table>
<thead>
<tr>
<th>PE</th>
<th>RPP, mmHg</th>
<th>G, ml·min⁻¹·mmHg⁻¹</th>
<th>Slope, dB/decade</th>
<th>Phase Peak, rad</th>
<th>Coh05–08</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intravenous</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>89±3</td>
<td>0.063±0.007</td>
<td>30±3</td>
<td>0.97±0.08</td>
</tr>
<tr>
<td>PE</td>
<td></td>
<td>103±2*</td>
<td>0.047±0.005†</td>
<td>34±5</td>
<td>1.12±0.12</td>
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<tr>
<td>Intrarenal arterial</td>
<td>5</td>
<td>100±4</td>
<td>0.072±0.009</td>
<td>21±2</td>
<td>0.72±0.08</td>
</tr>
<tr>
<td>PE</td>
<td></td>
<td>102±2</td>
<td>0.042±0.009†</td>
<td>19±3</td>
<td>0.58±0.15</td>
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<tr>
<td>t-NAME–Y-27632</td>
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<td>91±3</td>
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<td>22±4</td>
<td>0.80±0.16</td>
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<tr>
<td>Control</td>
<td></td>
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<td>0.042±0.005†</td>
<td>43±8*</td>
<td>1.41±0.21*</td>
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<tr>
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<td>0.092±0.007†</td>
<td>11±1†</td>
<td>0.48±0.06†</td>
</tr>
<tr>
<td>Y-27632–t-NAME</td>
<td>7</td>
<td>92±1</td>
<td>0.073±0.006</td>
<td>23±2</td>
<td>0.87±0.08</td>
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<tr>
<td>Y-27632</td>
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<td>84±2</td>
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<td>7±1†</td>
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<tr>
<td>Y-27632 + t-NAME</td>
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<td>86±2</td>
<td>0.100±0.009</td>
<td>10±2</td>
<td>0.42±0.05*</td>
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</tbody>
</table>

Values are means ± SE. RPP, renal perfusion pressure; G, renal vascular conductance; coh05–08, coherence at 0.05–0.08 Hz; slope and phase peak, slope of gain reduction and phase peak due to myogenic mechanism; PE, phenylephrine; l-NAME, N²-nitro-l-arginine methyl ester. Statistical analysis tested change in each variable between consecutive periods (1st vs. 2nd and 2nd vs. 3rd): *P < 0.05; †P < 0.01.
As shown in Table 1, ira infusion of L-NAME induced a marked reduction of conductance ($P < 10^{-5}$); RPP did not differ between the two periods. Infusion of Y-27632 reduced blood pressure, and thus RPP, in the absence and presence of L-NAME (Table 1; $P < 0.01$ in both cases); it also caused substantial vasodilatation with or without L-NAME (Table 1; $P < 10^{-4}$ in both cases). In addition, there was no constrictor response when L-NAME was infused during infusion of Y-27632.

Figures 1A and 2A show the RPP power spectra acquired in the experiments that assessed responses of RBF dynamics to L-NAME and Y-27632. RPP power spectra did not differ among periods and were also very similar in the two experiments, indicating that very similar inputs were achieved under all conditions. As shown in Figs. 1B and 2B, coherence between RPP and RBF was uniformly high at frequencies faster than 0.08 Hz and, during the control period, tended to decline at slower frequencies in both experiments. L-NAME alone reduced Coh$_{0.05-0.08}$ ($P = 0.014$), and Y-27632 significantly increased Coh$_{0.05-0.08}$ in the absence ($P = 0.004$) or presence ($P = 0.001$) of L-NAME. Admittance gains are shown in Figs. 1C and 2C, and admittance phases are shown in Figs. 1D and 2D. In the control periods of each experiment, there were discrete regions of gain reduction by the myogenic and TGF mechanisms, respectively. The slope of gain reduction by the myogenic system was significantly enhanced by L-NAME, and this enhancement was, as expected, accompanied by an increased phase peak, as summarized in Table 1. Autoregulation was markedly impaired by Y-27632 in the absence and presence of L-NAME. It is apparent in Figs. 1C and 2C that the discrete regions of gain reduction were much less obvious during infusion of Y-27632. This effect is summarized in Table 1, which shows that the slope of gain reduction by the myogenic mechanism and its associated phase peak were strongly reduced during Y-27632 infusion (all $P < 0.001$). Responses to L-NAME were almost completely inhibited by prior Y-27632 infusion, although L-NAME induced a small rise of phase peak in the presence of Y-27632.
To exclude potential effects of hypotension on autoregulatory mechanisms, studies were performed in the hydronephrotic kidney in vitro. In this preparation, perfusion pressure is determined solely by the experimenter (9) and can be varied over a wide range. Under control conditions, strong pressure-dependent vasoconstriction was observed when perfusion pressure was increased in steps from 80 to 180 mmHg \((P < 10^{-6})\). When l-NAME was added to the perfusate, autoregulatory vasoconstriction was not significantly altered. However, when l-NAME and Y-27632 were present in the perfusate, autoregulation was completely inhibited (Fig. 3A; drug × pressure interaction, \(P < 10^{-6}\)). Figure 3B illustrates the results that were obtained when l-NAME was administered after Y-27632. As expected, during the control period, there was effective and progressive pressure-dependent reduction of arteriolar diameter at all pressures \(>80\) mmHg \((P < 10^{-6})\). In this experiment, the pressure-dependent constriction was replaced after Y-27632 by modest, but significant, pressure-induced dilatation \((160\) vs. \(80\) mmHg \((P = 0.012)\) and \(180\) vs. \(80\) mmHg \((P < 0.001)\)). After l-NAME was added to the perfusate, no pressure-dependent change of arteriolar diameter was observed. In the third experiment of this series, increasing perfusate \([K^+]_o\), at \(80\) mmHg perfusion pressure, reduced afferent arteriolar diameter from \(19.6 \pm 0.6\) to \(4.1 \pm 0.2\) \(\mu m\) \((P = 8 \times 10^{-5})\). Subsequent addition of Y-27632 to the perfusate markedly blunted the constriction \((18.4 \pm 0.8\) \(\mu m\), \(P = 6 \times 10^{-5}\) vs. high \([K^+]_o\)). The results of the experiment employing the iNOS inhibitor 1400W are illustrated in Fig. 4 and summarized in Table 2. The positive control, l-NAME, had all the expected effects, including strongly reduced conductance, increased slope of gain reduction by the myogenic mechanism and the associated phase peak, and reduced Coh05–08. However, 1400W had no effect on any of these variables. The response to l-VNIO was somewhat more complex (Table 2, Fig. 5). l-VNIO did not significantly reduce conductance \((P = 0.072)\) and increased the slope of gain reduction by the myogenic mechanism and the height of the associated phase peak, but did not affect Coh05–08. Subsequent l-NAME infusion caused the expected strong vasoconstriction but had no further effect on slope, although the phase peak was increased further, while Coh05–08 was significantly reduced. In this experiment there was clearly some leakage of l-NAME into the systemic circulation as RPP was significantly higher during l-NAME infusion. It is a routine finding in this and other studies \((10, 51, 52)\) that nonselective NOS inhibition results in increased positive gain in the pressure-passive region of the spectrum above \(0.2\) Hz. It is thus notable that l-VNIO did not alter gain in the \(0.4\)- to \(0.8\)-Hz band: \(3.8 \pm 0.1\) dB for control, \(4.6 \pm 0.4\) dB with l-VNIO (not significant vs. control), and \(8.0 \pm 0.3\) dB with l-NAME \((P = 0.008\) vs. l-VNIO).
The effects of furosemide and subsequent L-NAME are shown in Table 2 and Fig. 6. Although in this experiment drugs were given systemically, RPP was well matched during the control, furosemide, and L-NAME periods. Furosemide grossly inhibited TGF (Fig. 6, C and D). The local maximum of gain between 0.03 and 0.04 Hz and the phase peak below 0.04 Hz that were present during the control period were absent in the subsequent experimental periods. Furosemide alone significantly, but modestly, reduced conductance (Table 2). Furosemide also resulted in reduced slope of gain reduction by the myogenic mechanism and the associated phase peak. All re-

Table 2. Effects of 1400W and L-NAME, L-VNIO and L-NAME, and furosemide and L-NAME on RBF dynamics

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>RPP, mmHg</th>
<th>G, ml·min⁻¹·mmHg⁻¹</th>
<th>Slope, dB/decade</th>
<th>Phase Peak, rad</th>
<th>Coh05–08</th>
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<td></td>
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<tr>
<td>Control</td>
<td></td>
<td>100±3</td>
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<td>28±5</td>
<td>0.92±0.10</td>
<td>0.83±0.05</td>
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<td>1400W</td>
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<tr>
<td>Control</td>
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<td>41±4†</td>
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<td></td>
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<tr>
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<td>0.078±0.013†</td>
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<tr>
<td>Furosemide + L-NAME</td>
<td>8</td>
<td>99±3</td>
<td>0.036±0.006†</td>
<td>38±5†</td>
<td>1.18±0.13†</td>
<td>0.78±0.05†</td>
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</table>

Values are means ± SE. L-VNIO, N⁵-(1-imino-3-butenyl)-L-ornithine. *P < 0.05; †P < 0.01.

The effects of furosemide and subsequent L-NAME are shown in Table 2 and Fig. 6. Although in this experiment drugs were given systemically, RPP was well matched during the control, furosemide, and L-NAME periods. Furosemide grossly inhibited TGF (Fig. 6, C and D). The local maximum of gain between 0.03 and 0.04 Hz and the phase peak below 0.04 Hz that were present during the control period were absent in the subsequent experimental periods. Furosemide alone significantly, but modestly, reduced conductance (Table 2). Furosemide also resulted in reduced slope of gain reduction by the myogenic mechanism and the associated phase peak. All re-

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Fig. 5. RPP and RBF dynamics during control, N⁵-(1-imino-3-butenyl)-L-ornithine (L-VNIO) and L-VNIO + L-NAME treatment. A: RPP power spectra, which were similar in the 3 periods. B: coherence between RPP and RBF. C and D: admittance gain and phase. RBF dynamics were significantly affected by L-VNIO and L-NAME. Average slopes of gain reduction by the myogenic mechanism are shown by dashed lines. Slope of gain reduction by the myogenic mechanism was increased by L-VNIO (P = 0.002), as was the associated phase peak (P = 0.004); L-NAME had no additional effect on the slope of gain reduction but did increase further the associated phase peak (P = 0.014).

Fig. 6. RPP and RBF dynamics during control, furosemide, and furosemide + L-NAME periods. A: RPP power spectra, which were similar in the 3 periods. B: coherence between RPP and RBF. C and D: admittance gain and phase. Average slopes of gain reduction by the myogenic mechanism are shown by dashed lines. RBF dynamics were altered by furosemide infusion, as shown by reduced slope of gain reduction and phase peak (both P < 0.01), while L-NAME increased the slope of gain reduction and the amplitude of the associated phase peak (both P < 0.002).
sponses to L-NAME were preserved in the presence of furosemide: conductance was strongly reduced, while the increase in the slope of gain reduction and the phase peak of the myogenic response were highly significant, as was the reduction of \(C_{\text{H2O}}\).

**DISCUSSION**

Inhibition of NOS leads to profound renal vasoconstriction in this report as in all others. It is also well known that NOS inhibition leads to augmentation of renal autoregulation that can be observed in several different experiments. In a standard pressure ramp, the effect is seen as a leftward shift (i.e., to lower \(RPP\)) of the lower limit of autoregulation (28, 47). Time series analysis has shown a change in the dynamics of the myogenic system and, often, enhanced stabilization of RBF (10, 23, 51, 52). Consistent with this interpretation, individual pressure steps have been used to show increased energy in the faster, myogenic component of autoregulation (54) and increased velocity and autoregulatory efficiency of the myogenic mechanism in response to upward and downward pressure steps (24). Thus it is clear that the renal myogenic autoregulatory mechanism is modulated by NO. The present studies address the mechanism(s) by which this modulation occurs. The results indicate that the Rho-Rho kinase pathway is a potential mediator of the modulation of myogenic autoregulation by NO. They demonstrate that vasoconstriction is neither necessary nor sufficient to explain the augmentation of myogenic autoregulation that is seen after NOS inhibition. Finally, they demonstrate a role for TGF and for macula densa nNOS in regulation of the myogenic system.

The phenylephrine experiments were included to assess the effect on RBF dynamics of reduced conductance per se and to provide a quality control study for intrarenal infusions. Intrarenal infusions with a mixing device (6, 37) were used to reduce the number and degree of confounding systemic effects and, importantly, to minimize changes in blood pressure and blood pressure fluctuation induced by vasoactive agents. The observation that use of an intrarenal arterial cannula reduces the efficiency of myogenic autoregulation is probably a consequence of the invasiveness of the procedure and is not unexpected. It is commonly observed that myogenic mechanisms are more susceptible than pharmacological responses to mechanical or other disruption. The major constraint imposed by the difference in autoregulatory efficiency is that it limits comparisons across experiments, particularly if infusion routes differ.

We hypothesized that the major target of NOS inhibition is activation of Rho kinase, resulting in inhibition of myosin light chain phosphatase and increased \(Ca^{2+}\) sensitivity. To test this hypothesis, the Rho kinase inhibitor Y-27632 was infused i.a. before or during i.a. infusion of L-NAME, both drugs being administered at blocking doses. We chose to use blocking rather than submaximal doses because of concerns that the dynamic range and resolution of the experiment would be insufficient to permit statistically reliable results. RBF dynamics during infusion of Y-27632, in the absence or presence of L-NAME, showed strong inhibition of vascular tone and myogenic and TGF-mediated autoregulation. There may be a small remnant of the L-NAME response after Y-27632: the phase peak increased significantly, and there was a borderline increase in slope \((P = 0.068)\). However, the inhibition of autoregulation remained profound, if not technically complete, and the remaining response to L-NAME was trivial.

In the course of the in vivo experiments employing Y-27632, we were unable to find a concentration of the drug that, when infused directly into the kidney, did not reduce systemic blood pressure. The unavoidable reduction of RPP complicated interpretation of the results and led to the in vitro studies employing the hydronephrotic kidney. In this preparation, perfusion pressure is determined solely by the investigator, thus excluding pressure as an uncontrolled variable. Consequently, the effects of L-NAME and Y-27632 on myogenic autoregulation were examined over a wide range of perfusion pressures. Although little effect of NOS inhibition was apparent in this preparation, the effects of Y-27632 were striking. Autoregulation was paralyzed over the entire range of perfusion pressures tested from 80 to 180 mmHg by a concentration of Y-27632 (10 \(\mu\)mol/l) that is generally agreed to provide effective blockade of Rho kinase and to be selective for Rho kinase (43).

These results are consistent with the hypothesis that the major target of NOS inhibition is activation of Rho kinase but do not adequately test it, because autoregulation was almost paralyzed in the absence and presence of L-NAME, and the response to L-NAME was prevented and reversed. We therefore considered the extent to which Y-27632 affects processes mediated by depolarization and \(Ca^{2+}\) entry. It has been reported that 10 \(\mu\)mol/l Y-27632 caused only modest inhibition (−35 ± 5%) of afferent arteriolar vasoconstriction induced by 30 mmol/l \(K^+\) (36). This would be expected only to blunt responses due to depolarization and, if confirmed, would permit one to draw conclusions concerning NO targets that affect membrane potential in vascular smooth muscle cells. However, in our hands, there was essentially complete inhibition (−92 ± 3%) of vasoconstriction induced by 30 mmol/l \(K^+\). Because Y-27632 blocks vasoconstriction induced by high KCl and high perfusion pressure, both of which involve membrane depolarization, it was not possible to further test the hypothesis. Thus, consistent with the literature (5, 6, 36), one can conclude only that the Rho-Rho kinase pathway is very important in determining contractile behavior of the renal microcirculation.

The hydronephrotic kidney in vivo constricts in response to NOS inhibition (12) and in vitro responds normally to physiological levels of NO (46) but does not constrict or show enhanced autoregulation during NOS inhibition, indicating a lack of NO generation. The absence of constriction is likely due to reduced shear stress resulting from use of a cell-free perfusate. It is possible that this also accounts for the absence of effect of NOS inhibition on autoregulation, although it is also possible that the absence of modulation of autoregulation results from the loss of the relevant NO source, parenchymal iNOS or macula densa nNOS. Both isoforms are expressed constitutively in the kidney (34), and nNOS situated at or around the macula densa is a strong modulator of TGF (44, 45, 49, 53). Because of the known interaction between TGF and the myogenic mechanism (8, 55), it is quite conceivable that nNOS located in the macula densa could contribute to regulation of the myogenic mechanism.

We tested the involvement of these two NOS isoforms in regulation of myogenic autoregulation by means of i.a. infusion
of isoform-selective inhibitors. In each experiment, intrarenal infusion of L-NAME was included as a positive control and to assess by subtraction the contribution of eNOS. The inhibitors employed, the doses used, and the infusion protocols were based on inhibition kinetics for iNOS (13) and nNOS (2). No positive control is available for the experiment with 1400W, and it is difficult to ensure that nNOS blockade by L-VNIO was complete. However, 1400W and L-VNIO are inactivating (i.e., irreversible) inhibitors of iNOS and nNOS, respectively (2, 13), so that even if the selected doses were low, there would still be effective inhibition after a 30-min infusion. As expected, inhibition of iNOS had no effect on conductance or on RBF dynamics. Similar data (not shown) were acquired from a separate group of Brown-Norway rats. Thus the results of this exclusion experiment are consistent with the literature consensus that iNOS does not play a significant role in regulation of renal hemodynamics.

In contrast, the nNOS-selective inhibitor L-VNIO did affect RBF dynamics. This inhibitor is highly selective for nNOS vs. iNOS, which is of little relevance here, and is moderately selective for nNOS vs. eNOS. Selectivity can be increased by infusion of L-VNIO together with L-arginine (2), and this procedure was followed. In a variety of vascular beds and in several species, nonselective NOS inhibition results in increased incremental distensibility (10, 21). In a pressure-flow transfer function, with the vascular bed denervated, this is shown by an increase in gain at frequencies faster than the myogenic mechanism (10). Because L-VNIO did not increase gain in this region of the spectrum and caused only minor vasoconstriction, we can be confident that there was little inhibition of eNOS. Despite the absence of effect on eNOS, L-VNIO significantly augmented myogenic autoregulation. Interestingly and in contrast to L-NAME, this response did not appear to increase dynamic complexity, as shown by the lack of change of low-frequency coherence. Interaction between nNOS and TGF has been shown previously in several laboratories (17, 44, 45, 49, 53), and it has been demonstrated using the isolated blood-perfused juxtamedullary nephron preparation that this effect involves afferent arteriolar constriction (17, 18). There has been a strong presumption that TGF-mediated constriction is affected by inhibition of nNOS, because in most studies perfusion pressure was constant and response to a perturbation of load at the macula densa was assessed. The present results indicate, instead, that the effect is mediated at least partly by the myogenic mechanism. There are three conclusions to be drawn from these findings.

The first conclusion to be drawn is that the response to L-VNIO implicates macula densa nNOS, and thus TGF, in regulation of the myogenic mechanism. It thus provides a mechanism to the interaction that is known to occur between the two mechanisms (8, 55). Second, assuming effective blockade of nNOS by L-VNIO, this result indicates that both eNOS and nNOS contribute to the renal hemodynamic responses induced by nonselective NOS inhibition. Because inhibition of nNOS duplicates most or all of the autoregulatory response to nonselective NOS inhibition, it would appear that this isoform is the primary contributor to autoregulation. Vascular tone appears to be preferentially affected by eNOS, which may also contribute to regulation of RBF dynamics. Third, the significant augmentation of myogenic autoregulation in the presence of modest and nonsignificant vasoconstriction complements the results obtained in this study with phenylephrine and in other studies with vasopressin and ANG II concerning a potential interaction between vascular tone and RBF dynamics. In particular, the L-VNIO result indicates that vasoconstriction is not necessary for augmentation of myogenic autoregulation, whereas the phenylephrine, vasopressin (50, 52), and ANG II (24) results indicate that vasoconstriction is not sufficient for augmentation of myogenic autoregulation. These results imply that the effects of NOS inhibition on RBF dynamics and on vascular tone are at least conceptually separable.

To further explore the role of TGF in myogenic autoregulation, we assessed the effects of a high dose of furosemide and subsequent L-NAME, both administered systemically, on RBF dynamics. As expected, furosemide caused complete inhibition of the TGF signature in the transfer function (1, 22). The modest, though significant, vasoconstriction that was also induced is not unprecedented. Such vasoconstriction is a common, but by no means uniform, finding (eg., 1) and appears to be due to elevation of circulating catecholamines (20). It is relevant here, because it suggests that furosemide is not inhibiting myogenic autoregulation via a direct action on the vascular smooth muscle (35). The effect of furosemide to reduce the slope of gain to ~20 dB/decade and the phase peak to $-\pi/4$ rad suggests removal of positive, TGF-dependent modulation from the myogenic mechanism, leaving simpler, first-order dynamics. Presumably, the modulation exposed by furosemide is not mediated by NOS. Such modulation is consistent with the results of the L-VNIO experiment and the results of Schnermann and Briggs (38), who showed that autoregulation of glomerular capillary pressure was more effective with TGF clamped on than with it clamped off. It is also consistent with the known TGF-mediated vascular interaction among neighboring nephrons (16, 25).

In this experiment, the response of RBF dynamics to subsequent administration of L-NAME was highly significant, although numerically the response appeared to be less profound than in the other experiments in the present study. However, in this experiment the drugs were given systemically, whereas in the other experiments they were administered into the renal artery. Earlier results (see above) suggested that placement of the intrarenal cannula somewhat impaired autoregulation. We therefore chose to compare the effect of L-NAME after furosemide with that of L-NAME alone using data recalculated from a previously published study (51). In the previous study, there was remarkable consistency among normotensive strains (Wistar, Sprague-Dawley, and Long-Evans) in the effect of L-NAME on RBF dynamics. In the previous study and after administration of L-NAME, slopes of gain reduction ranged from 46 ± 7 to 58 ± 7 dB/decade, phase peaks ranged from 1.56 ± 0.11 to 1.85 ± 0.10 rad, and Coh05–08 ranged from 0.66 ± 0.04 to 0.50 ± 0.04 (recalculated from Ref. 51). Statistical analysis in the previous report (51) and subsequently reveals no differences in RBF dynamics among strains in the presence of L-NAME. Values from the previous study are numerically different from those shown in Table 2 when L-NAME was given after furosemide. Slope and phase are numerically higher and Coh05–08 values are numerically lower. Whether one compares the present results with the results from Wistar rats in the previous report or with the pooled results from the previous report, one reaches the same conclusion: the effect of nonselective NOS inhibition on the
myogenic response is blunted in the presence of furosemide. We thus conclude that this experiment plus the L-VNIO experiment indicate a role for TGF in regulating myogenic autoregulation and that this regulation involves NO produced by macula densa nNOS.

Just and Arendshorst (24) recently reported a similar, but much stronger, interaction between TGF and nonselective NOS inhibition in regulating the myogenic mechanism. In their study, furosemide blunted the constrictor response to l-NAME and abrogated the otherwise strong enhancement of myogenic autoregulation. The results of the study by Just and Arendshorst and of the present study are largely consistent: Both studies show strong modulation of myogenic autoregulation by NO and demonstrate involvement of TGF in this modulation. They differ in the extent to which the effects of NOS inhibition are attributed to TGF. We do not have an explanation for this discrepancy, but we do find their observation somewhat surprising given 1) the additional effect of l-NAME on RBF dynamics after inhibition of nNOS by L-VNIO and 2) the extensive diffusion of NO within the renal cortex (30).

In summary, the present results are consistent with a major role for the Rho-Rho kinase pathway in mediating the effects of NO on myogenic autoregulation. They demonstrate that vasoconstriction is neither necessary nor sufficient to explain the augmentation of myogenic autoregulation by NO inhibition. Because of the experimental separation of constrictor and dynamic responses, the results suggest the presence of another level of complexity in NO actions in the renal microcirculation. The results demonstrate interaction between TGF in the myogenic system, with the former influencing the latter, and they implicate macula densa nNOS in this interaction. Furthermore, involvement of nNOS and TGF in regulation of the myogenic mechanism suggests coupling of the myogenic mechanism to renal function.

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