Rho kinase regulates renal blood flow by modulating eNOS activity in ischemia-reperfusion of the rat kidney

Amanda M. G. Versteilen,1 Iolente J. M. Korstjens,1 René J. P. Musters,1 A. B. Johan Groeneveld,2 and Pieter Sipkema1

1Laboratory for Physiology and 2Intensive Care Unit, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, The Netherlands

Submitted 1 November 2005; accepted in final form 17 December 2006

Versteilen, Amanda M. G., Iolente J. M. Korstjens, René J. P. Musters, A. B. Johan Groeneveld, and Pieter Sipkema. Rho kinase regulates renal blood flow by modulating eNOS activity in ischemia-reperfusion of the rat kidney. Am J Physiol Renal Physiol 291: F606–F611, 2006. First published March 8, 2006; doi:10.1152/ajprenal.00434.2005.—Renal ischemia-reperfusion (I/R) results in vascular dysfunction characterized by a reduced endothelium-dependent vasodilatation and subsequently impaired blood flow. In this study, we investigated the role of Rho kinase in endothelial nitric oxide synthase (eNOS)-derived regulation of renal blood flow and vasomotor tone in renal I/R. Male Wistar rats were subjected to 60-min bilateral clamping of the renal arteries or sham procedure. One hour before the clamping, the Rho kinase inhibitor Y27632 (1 mg/kg) was intravenously infused. After I/R, renal blood flow was measured using fluorescent microspheres. I/R resulted in a 62% decrease in renal blood flow. In contrast, the blood flow decrease in the group treated with the Rho kinase inhibitor (YI/R) was prevented. Endothelium-dependent vasodilatation of renal arcuate arteries to ACh was measured ex vivo in a pressure myograph. These experiments demonstrated that the in vivo treatment with the Rho kinase inhibitor prevented the decrease in the nitric oxide (NO)-mediated vasodilator response. In addition, after I/R renal interlobar arteries showed a decrease in phosphorylated eNOS and vasodilator-stimulated phosphoprotein, a marker for bioactive NO, which was attenuated by in vivo Rho kinase inhibition. These findings indicate that in vivo inhibition of Rho kinase in renal I/R preserves renal blood flow by improving eNOS function.

Acute renal failure; ROCK; vascular reactivity; vasodilator-stimulated phosphoprotein; nitric oxide synthase 3

In the critically ill, acute renal failure (ARF) requiring renal replacement therapy is associated with high morbidity and mortality. ARF is believed to frequently result from ischemia-reperfusion (I/R), as occurring in the course of shock, renal transplantation, or cardiovascular surgery. I/R leads to renal vascular dysfunction and subsequent impairment of blood flow, further increasing initial renal injury (4, 14). A variety of mechanisms may play a role, including increased constriction to neurohumoral agonists or failure to respond to physiological vasodilators. Vasomotor tone is strongly affected by endothelial nitric oxide synthase (eNOS)-derived nitric oxide (NO) (1). Decreased eNOS function is one of the hallmarks of endothelial dysfunction associated with ARF (6). Higher levels of eNOS expression after reperfusion are associated with enhanced recovery after renal transplantation (9), and increased eNOS activity induced by ischemic preconditioning has also been shown to protect kidneys from I/R (25), indicating an important role for eNOS in renal I/R. Downregulation of eNOS in cultured endothelial cells exposed to hypoxia seemed to be associated with increased activity of Rho kinase and could be reversed by inhibition of RhoA or Rho kinase (20). Rho kinase is one of the downstream mediators of the small G protein RhoA, which controls a wide variety of signal transduction pathways (22, 24). Gene transfer of constitutively active Rho/ Rho kinase mutants was shown to inhibit eNOS phosphorylation in cultured endothelial cells (13), whereas inhibition of Rho kinase demonstrated an increase in NO release in cultured endothelial cells (24). Renal I/R has been demonstrated to increase expression of RhoA mRNA and protein level during the ischemic phase (2) that decreases again on reperfusion. Interestingly, a recent study has shown that inhibition of Rho kinase protects against renal I/R injury in both pre- and postischemic treatment as demonstrated by improved renal function and histological scores (21). The mechanism of protection, however, is still not understood and may relate to effects of Rho kinase on the vasculature.

Our hypothesis is that in vivo inhibition of Rho kinase improves renal blood flow by preserving eNOS activity in the renal vasculature in I/R. We therefore evaluated the effect of Rho kinase inhibition on renal blood flow and renal function. Also, we determined the amount of endothelium-dependent vasodilation and the phosphorylation level of eNOS in vasodilator-stimulated phosphoprotein (VASP), a marker for bioactive NO, in the renal vasculature after I/R.

MATERIALS AND METHODS

Animal model. The investigation conforms to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996), and the local ethics committee for animal experiments approved the procedures. Male Wistar rats (337 ± 35 g, total n = 66, Harlan, Horst, The Netherlands) were housed under standard conditions and were randomly divided into sham (n = 13), I/R (n = 17), and Rho kinase inhibitor-treated sham (Ysham, n = 10) and I/R (YI/R, n = 16) groups. Rats were anesthetized with pentobarbital sodium (60 mg/kg ip) and ketamine HCl (70 mg/kg im) with a pentobarbital sodium maintenance dose (30 min, 15 mg/kg pentobarbital ip). The rats were placed in a supine position on a heating pad maintaining body temperature at 37°C. An intraperitoneal (ip) catheter was placed to administer pentobarbital sodium. The trachea was intubated with polyethylene tubing to facilitate breathing. The animals...
received 75 IU/kg heparin intravenously (Leo Pharmaceutical Products, Weesp, The Netherlands) to prevent catheter clotting. The Rho kinase inhibitor Y27632 (Tocris Cookson, Bristol, UK) at a total dose of 1 mg/kg (8) or placebo (normal saline) was infused in a period of 15 min through a right jugular vein catheter 1 h before clamping of the renal arteries. N\textsuperscript{\textregistered}-nitro-L-arginine methyl ester (L-NAME) was given 30 min before the end of the experiment to YI/R (n = 5) and I/R rats (n = 5) (after 30 min of reperfusion) at a dose of 10 mg/kg in normal saline.

Systemic hemodynamic measurements. The right jugular vein, right carotid artery, and left femoral artery were cannulated with polyethylene tubing. The right jugular vein catheter and the left femoral artery catheter were connected to pressure transducers. Central venous pressure (CVP), mean arterial pressure (MAP), and heart rate were continuously monitored during the experiment. An acetone stripped pulmonary artery (PA) catheter leaving only the thermistor was placed in the thoracic aorta via the right femoral artery. Cardiac output (CO; Cardiac Output Computer 9520A, Edwards Lifesciences, Irvine, CA) was obtained every 30 min using the thermodilution method; 200 \( \mu \)l of saline were injected via the right jugular vein catheter as described previously (16). Blood flow was measured at the end of the experiment, after 1 h of reperfusion, using Fluospheres polystyrene microspheres (15 \( \mu \)m scarlet fluorescent (645/680, Molecular Probes Europe, Leiden, The Netherlands)). The renal blood flow in the left and right kidney was calculated using a reference blood sample as previously described in detail (17) and is expressed as the mean renal blood flow. The left and right triceps muscle blood flow was used to assess microsphere distribution.

Renal functional parameters. Blood was collected before the ischemic period and at the end of the experiment. Urine was collected until the ischemic period and from the ischemic period until the end of the experiment. The samples were analyzed for sodium, creatinine, and urea. Samples were analyzed using Modular Analytics (Roche Diagnostics, Mannheim, Germany). The samples taken before the ischemic period (data not shown) had comparable functional parameters. In rats with preserved urinary production, the creatinine clearance and the fractional sodium excretion were calculated.

Vasoreactivity experiments. Renal arcuate arteries were isolated (n = 6–8/group) and mounted in a pressure myograph. The mean arterial diameter was not different among groups (109.9 ± 3.9 \( \mu \)m). Diameters of arteries in response to various stimuli under 37°C were measured as previously described (10). MOPS buffer was used (in mM: 145 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgSO\(_4\), 1 NaH\(_2\)PO\(_4\), 3 MOPS, 2 pyruvate, 10 glucose, and 0.02 EDTA, pH 7.4) to fill the arteriole and pressure column. The organ chamber was filled with Krebs buffer (in mM: 110 NaCl, 5 KCl, 2.5 CaCl\(_2\), 1 MgSO\(_4\), 1 KH\(_2\)PO\(_4\), 10 glucose, 0.02 EDTA, and 24 NaHCO\(_3\), gassed with 95% air-5% CO\(_2\), pH 7.4). Vascular smooth muscle contractile function was studied by performing a cumulative concentration-response curve to determine norepinephrine (NE) sensitivity. As a measure of NE sensitivity, we determined a cumulative concentration-response curve to determine norepinephrine (NE) sensitivity. As a measure of NE sensitivity, we determined the log EC\(_{50}\) value; this is the NE concentration at which the artery is constricted 50%. This NE constriction level was used to test the endothelium-dependent vasodilatation response as determined previously (16). Blood flow was measured at the end of the experiment, after 1 h of reperfusion, using Fluospheres polystyrene microspheres (15 \( \mu \)m scarlet fluorescent (645/680, Molecular Probes Europe, Leiden, The Netherlands)). The renal blood flow in the left and right kidney was calculated using a reference blood sample as previously described in detail (17) and is expressed as the mean renal blood flow. The left and right triceps muscle blood flow was used to assess microsphere distribution.

Fig. 1. Hemodynamic variables measured during the experiment from sham, ischemia-reperfusion (I/R), and Rho kinase inhibitor (Y27632)-treated groups (YI/R, Ysham; n = 8). Values are means ± SE. A: mean arterial blood pressure (MAP). *P < 0.037 both I/R and YI/R vs. sham and Ysham. B: cardiac output (CO). *P < 0.023 vs. other groups. C: venous pressure (VP). *P < 0.032 vs. other groups.
phosphorylated and total protein levels, whereby the I/R and YI/R samples were expressed relative to the ratio of the control samples, which was set to 1. Four experiments in total were analyzed.

Statistical analysis. Data are expressed as means ± SE. Differences in ACh responses were tested with a repeated-measures ANOVA, and when differences were statistically significant, a post hoc Tukey test was performed. To compare differences in the arteriolar ACh response with and without L-NAME, a two-way repeated-measures ANOVA was done, and when differences were statistically significant, this was followed by a paired samples t-test. Comparisons among treatment groups were performed with one-way ANOVA, and when differences were statistically significant, a post hoc Tukey test was performed. Differences in relative density of Western blots were tested with Student’s t-test. Differences were considered statistically significant at P < 0.05.

RESULTS

Systemic hemodynamic variables during renal I/R. Hemodynamic variables were measured to study the acute effects of Rho kinase inhibition during renal I/R in vivo. There was a significant rise in MAP during clamping of the renal arteries in both the I/R group and the I/R group treated with the Rho kinase inhibitor (YI/R) (Fig. 1A). This increase in MAP was transient and restored during reperfusion. The CO of the I/R group decreased during reperfusion (Fig. 1B). In addition, a decrease in venous pressure (VP) was observed in the I/R group (Fig. 1C). This decrease in CO and VP was not observed in the YI/R group. There was no difference in heart frequency among groups (data not shown).

Renal function in renal I/R. Rats subjected to 1 h of renal I/R showed a marked deterioration of renal function, as demonstrated in Tables 1 and 2. The I/R group showed increased levels of blood urea nitrogen and plasma creatinine concentrations compared with the sham group. Urine analysis demonstrated in the I/R group a decrease in creatinine and urea clearance and an increase in fractional sodium excretion (FE\textsubscript{Na}). The Rho kinase inhibitor did not influence renal functional parameters at 1 h of reperfusion. The only observed difference between the two groups was the urine production, since in the I/R group fewer rats produced urine than in the YI/R group. The YI/R group produced urine more often, albeit at a slightly less amount than the I/R group, which gave some difficulty in analyzing all the parameters as indicated by the number of urinary analyses.

Renal blood flow following I/R. I/R induced a strong decrease in renal blood flow compared with the sham group (Fig. 2). Infusion of the Rho kinase inhibitor before the ischemic period maintained renal blood flow in the YI/R group at a level comparable to that in the sham group. The renal blood flow in the Ysham group was not different from that in the sham group. To determine the role of NO in the YI/R group, we used L-NAME, a NOS inhibitor. When NOS was inhibited by L-NAME in the YI/R group, the blood flow was comparable to the I/R group. NOS inhibition in the I/R group did not influence renal blood flow.

Vascular effects of NE and ACh after I/R. To determine the contractile function of the renal arcuate arteries after I/R, we made a cumulative concentration-response curve for NE (Fig. 3). The I/R group demonstrated, with an −log EC\textsubscript{50} value of 6.5 ± 0.1, a statistically significant lower sensitivity to NE-induced vasoconstriction than the YI/R (6.8 ± 0.1), sham (7.1 ± 0.1), or Ysham (7.0 ± 0.1) group. The −log EC\textsubscript{50} value was used to preconstrict the arteries, and the endothelium-dependent vasodilator response was tested with a cumulative concentration-response curve of ACh ex vivo in a pressure

---

**Table 1. Blood renal function parameters measured in I/R with Rho kinase inhibition**

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>Sham</th>
<th>I/R</th>
<th>Ysham</th>
<th>YI/R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium, mmol/l</td>
<td>139.6±0.9</td>
<td>139.9±1.2</td>
<td>138.5±0.7</td>
<td>139.0±0.7</td>
<td></td>
</tr>
<tr>
<td>Creatinine, µmol/l</td>
<td>44.5±2.0</td>
<td>100.1±3.2*</td>
<td>43.0±1.8</td>
<td>95.3±2.6*</td>
<td></td>
</tr>
<tr>
<td>Urea, mmol/l</td>
<td>7.2±0.4</td>
<td>11.2±0.3*</td>
<td>7.0±0.6</td>
<td>11.1±0.3*</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. I/R, ischemia-reperfusion. Blood analysis was measured 1 h after I/R. The groups treated with the Rho kinase inhibitor are indicated with Y (n = 8). *P < 0.001 both the I/R as the YI/R group vs. the sham and Ysham group.

**Table 2. Urinary renal function parameters measured in I/R with Rho kinase inhibition**

<table>
<thead>
<tr>
<th>Urine</th>
<th>Sham</th>
<th>n</th>
<th>I/R</th>
<th>n</th>
<th>Ysham</th>
<th>n</th>
<th>YI/R</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Production, %</td>
<td>100 (13/13)</td>
<td>35.3 (6/17)</td>
<td>100 (10/10)</td>
<td>68.8 (11/16)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Production, ml</td>
<td>0.82±0.09</td>
<td>0.58±0.20</td>
<td>0.72±0.09</td>
<td>0.43±0.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium, mmol/l</td>
<td>90.8±12.3</td>
<td>122.7±23.0</td>
<td>76.3±9.0</td>
<td>113.0±23.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine, µmol/l</td>
<td>8.3±0.6</td>
<td>1.0±0.3*</td>
<td>10.6±1.4</td>
<td>1.5±0.4*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea, mmol/l</td>
<td>698.5±65.5</td>
<td>71.8±17.0*</td>
<td>666.0±43.2</td>
<td>110.0±43.9*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl\textsubscript{Cr}, ml/min</td>
<td>1.26±0.14</td>
<td>0.03±0.01*</td>
<td>1.41±0.16</td>
<td>0.03±0.01*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FENa, %</td>
<td>0.37±0.07</td>
<td>14.69±4.05*</td>
<td>0.25±0.04</td>
<td>11.56±2.51*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of rats; Cl\textsubscript{Cr}, creatinine clearance; FENa, fractional excretion of sodium. Shown is analysis of urine collected during I/R. *P < 0.001 both the I/R as the YI/R group vs. the sham and Ysham group.
myograph. While the sham group demonstrated a dilation of >50%, the maximum ACh-dependent vasodilator response after I/R decreased to 10% (Fig. 4A). In vivo inhibition of Rho kinase before the ischemic period preserved the ACh response, because the arterioles of the YI/R group demonstrated 40% ACh-dependent vasodilation. Inhibition of NOS with L-NAME in the YI/R arterioles showed that the increase in ACh-induced endothelium-dependent vasodilation was mediated by NO (Fig. 4B).

**Fig. 3.** Concentration-response curve to norepinephrine (NE) of isolated renal arcuate arterioles in sham (n = 7), I/R (n = 8), Ysham (n = 6), and YI/R (n = 8) groups. Values are means ± SE. The log EC50 value of the I/R group was statistically different (*P < 0.05) from the other groups.

**Fig. 4.** Concentration-response curves to ACh of isolated renal arcuate arterioles. A: ACh responses were tested in a pressure myograph after 50% preconstriction with NE in sham (n = 7), I/R (n = 8), Ysham (n = 6), and YI/R (n = 8) groups. Values are means ± SE. *P < 0.029, I/R vs. YI/R. **P < 0.005, I/R vs. sham and Ysham. B: ACh response of renal arcuate arterioles from the YI/R group was tested in the presence of NOS inhibitor L-NAME (n = 5). *P < 0.03.

**Fig. 5.** Western blots (A and C) and corresponding densitometric analyses expressed as a ratio relative to the sham group (B (*P < 0.023; n = 4) and D (*P < 0.019; n = 3)) showing phosphorylation (p) levels of endothelial NOS (eNOS) and vasodilatory-stimulated phosphoprotein (VASP) in renal interlobar arteries of control, IR, and YI/R rats. Values are means ± SE.

**VASP and eNOS phosphorylation in renal arteries following I/R.** To determine whether the reduced NO-dependent vasodilator response corresponds to a reduction in eNOS activity, p-eNOS levels were measured as shown in Fig. 5, A and B. After I/R, p-eNOS levels decreased, which could be prevented by inhibition of Rho kinase in vivo. p-VASP was used to assess the amount of bioactive NO (15, 18). The level of p-VASP in renal interlobar arteries was decreased after I/R (Fig. 5, C and D). Inhibition of Rho kinase prevented this decrease in p-VASP levels. Tubulin detection was used to determine the amount of total protein, and there was no indication that there was a difference in total protein levels after 1 h of reperfusion (data not shown).

**DISCUSSION**

In the present study, we have shown that in vivo inhibition of Rho kinase in renal I/R is able to restore renal perfusion by preventing decreased eNOS function.
We demonstrated that the Rho kinase inhibitor Y27632 had a significant influence on the hemodynamic variables measured in renal I/R. During bilateral clamping of the renal arteries, MAP increased, which can be explained by the release of vasoactive substances by the injured kidneys. Rho kinase inhibition in vivo was not able to prevent an increase in MAP during bilateral clamping of the renal arteries, indicating that there was apparently no effect on the vasoconstrictor properties of the vascular bed. Most interestingly, inhibition of Rho kinase prevented a decrease in VP and CO during the reperfusion phase. A decrease in CO in renal I/R has been observed previously (16) and has been attributed to an increase in vascular resistance. The renal function parameters did not indicate an acute protective effect of the Rho kinase inhibitor.

Because the protective effect of Rho kinase inhibition with a larger time scale has been reported (21), we assumed that 1 h of reperfusion is not enough for the kidneys to recover from I/R, independent of Rho kinase inhibition. The only observed difference between the I/R and YI/R group is that the rats in the YI/R group produced urine more often. This observation could be the result of improved microvascular perfusion of the kidney.

I/R reduced renal blood flow to <50% on reperfusion compared with the sham group, in accordance with the literature (16). The Rho kinase inhibitor was able to maintain renal blood flow after I/R to a level comparable to the sham group. In vivo inhibition of NOS in the YI/R group was able to reduce renal perfusion to I/R levels, indicating that the increase in perfusion mediated by the Rho kinase inhibitor is probably NO dependent.

The vascular smooth muscle contractile function was determined ex vivo by examining the sensitivity to NE-induced contraction. The I/R group demonstrates a decreased sensitivity to NE that was absent in the group treated with the Rho kinase inhibitor. The decrease in sensitivity to NE has been described previously by Conger et al. (3) and has been attributed to a vascular smooth muscle defect. Activated Rho kinase has been shown to stimulate vascular smooth muscle cell contraction by inactivating myosin phosphatase and subsequently increasing myosin light chain phosphorylation (19). The decrease in sensitivity to NE was less pronounced than in vitro, in accordance with the results previously obtained by Conger et al. (3) and has been attributed to a vascular smooth muscle defect. Activated Rho kinase has been shown to stimulate vascular smooth muscle cell contraction by inactivating myosin phosphatase and subsequently increasing myosin light chain phosphorylation (19).

From these data, we concluded that activation of Rho kinase during renal I/R leads to an inactivation of eNOS, that less NO is produced, and that this may lead to a decrease in vasorelaxation and subsequent reduction in blood flow.

Rho protein is able to modulate the actin cytoskeleton (22), and a link between Rho protein, the actin cytoskeleton, and eNOS regulation has been described. Laufs et al. (11) prevented actin cytoskeletal changes by using a Rho protein inhibitor or a cytoskeleton depolymerizer, which leads to increased eNOS expression and activity mediated by an increase in eNOS mRNA half-life. These findings are supported by data published by other groups (5, 13, 20); however, these observations do not correspond with the data obtained in our study, because there was no indication of a decrease in eNOS protein. Wolfrum et al. (24) demonstrated in human endothelial cells an acute effect of Rho kinase on Akt and eNOS activity, which was dependent on phosphatidyl inositol 3-kinase activity. A study published by Luo et al. (12) demonstrated that Akt signal transduction has a role in ACh-induced eNOS phosphorylation, resulting in an increased arterial diameter and blood flow. In our experiments, we found a significant decrease in Akt phosphorylation (unpublished observations) in the renal arcuate arteries; however, the experiments of Luo et al. (12) demonstrated that a dominant negative mutant of Akt decreased ACh-dependent vasodilatation ~15%. Also, Luo et al. found no effect of a dominant negative mutant of Akt on basal blood flow in the femoral artery. In our experiments, we have found a difference of 40% ACh-dependent vasodilatation and a strong effect on blood flow, indicating that the acute effects of Rho kinase inhibition in our experiments are probably not mediated by Akt but could be the result of other vascular effects of Rho kinase (19).

In conclusion, Rho kinase inhibition in renal I/R resulted in an increase in renal blood flow and NO-mediated ACh response in renal arteries, associated with an increase in p-eNOS and p-VASP in renal arterioles after I/R. These findings indicate that in vivo inhibition of Rho kinase preserves renal blood flow by maintaining eNOS function. Because a Rho kinase inhibitor is available for human application in Japan, the possibility of targeting Rho kinase for therapeutic benefits in I/R and investigating its potential in clinical trials might be an important step in future research.

ACKNOWLEDGMENTS

The authors thank Dr. G. P. van Nieuw Amerongen of the Department of Physiology, VU University Medical Center, Amsterdam, for a critical reading of the manuscript.

REFERENCES


