Differential actions of renal ischemic injury on the intrarenal angiotensin system

ALICIA J. ALLRED, MARK C. CHAPPELL, CARLOS M. FERRARIO, AND DEBRA I. DIZ
Hypertension and Vascular Disease Center, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, North Carolina 27157-1032

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Allred, Alicia J., Mark C. Chappell, Carlos M. Ferrario, and Debra I. Diz. Differential actions of renal ischemic injury on the intrarenal angiotensin system. Am J Physiol Renal Physiol 279: F636–F645, 2000.—The present study determined the effect of either occlusion of the left renal artery for 60 min (ischemia) or sham operation on angiotensin (ANG) receptors and tissue and urinary levels of ANG peptides between 24 and 72 h recovery in male Sprague-Dawley rats. At 24 h postischemia, urinary concentrations of ANG I and ANG-(1–7) rose by an average of 83 and 64%, respectively (P < 0.05) but had declined to control levels by 72 h. Tissue ANG II rose at 24 h in postischemic kidneys by an average of 63% compared with the contralateral nonischemic kidney (P < 0.05). Whereas the enzymatic activity of angiotensin-converting enzyme and neprolysin was reduced after ischemia, renal renin activity in ischemic kidneys rose by 74% compared with sham-operated kidneys. Receptor autoradiography using 125I-labeled [Sar1, Thr8]ANG II (125I-Sarthran) (0.8 nM) revealed a decreased apparent density of ANG receptors (>80% AT1) in ischemic kidneys with a trend for a decrease in the contralateral nonischemic kidneys compared with the kidneys from sham-operated rats. Twenty-four hours after ischemia, ANG II receptors decreased by 68% in glomeruli (P < 0.05), 49% in the outer cortical tubulointerstitial area (P < 0.05), and 48% in the inner cortical-outer medullary area of the vasa recta (P < 0.05). Medullary binding decreased ~50% in both the ischemic kidney and the contralateral nonischemic kidney compared with sham. In all regions of the ischemic kidney, receptors recovered by 72 h to levels not different from sham control rats. The marked change in urinary ANG I and ANG-(1–7) at 24 h following occlusion indicates these peptides may be potential urinary markers for acute renal ischemia. The reduction of receptors in vascular and tubular regions of the ischemic kidney provides a mechanism for the loss of vasoconstrictor responses to ANG II following ischemia previously reported by others.

angiotensin II; angiotensin-(1–7), angiotensin receptors; AT1 receptor; AT2 receptor; renal ischemia; urinary angiotensins

THE ACUTE RESPONSE TO RENAL ISCHEMIA-REPERFUSION INJURY involves attenuation of both renal blood flow and glomerular filtration rate, as well as reduced tubular function. The possible mediators involved in ischemia-reperfusion injury include purine metabolites, reactive oxygen molecules, and vasoactive agents, including angiotensin II (ANG II), endothelin, thromboxane, and prostaglandins (12, 44). Clinically, ischemic renal disease may be causative in up to 15% of end-stage renal disease cases; its diagnosis is based on a clinically important reduction in glomerular filtration rate, which may arise from bilateral renal artery stenosis or unilateral renal artery stenosis in a solitary functioning kidney (35).

Normal renal function is modulated largely through the influence of the renal renin-ANG system (RAS). Receptors for ANG II are found on the renal vasculature, glomeruli, and tubules; binding to these receptors influences renal blood flow and sodium and water excretion. Regionally, binding density of ANG receptors varies across renal structures, with higher concentrations of ANG II receptors in glomeruli and low to moderate binding throughout the proximal convoluted tubules of the outer cortex. The outer medullary-inner cortical stripe has a high density of ANG binding compared with the inner medulla (48). In keeping with these findings, in situ hybridization for AT1 mRNA, showed that AT1 subtype receptors constitute a majority, ~95%, of ANG II binding in rat kidney (1, 18).

Ozono et al. (34) recently reported immunolocalization of low levels of the AT2 receptor to glomeruli of adult female rats, with increased expression in glomeruli and interstitium in response to sodium depletion. However, only in humans and other primates is there significant evidence for AT2 receptors in the vascular and tubular elements of the adult kidney (16, 48).

AT1 receptors mediate intense renal vascular constriction in response to ANG II. Although in acute renal failure the response to ANG II may be variable, immediately following a renal ischemic event, there is reduced responsiveness to the constrictor actions of intra-arterially administered ANG II (12, 31). Increased endogenous levels of ANG II (39) resulting in a reduction in AT1 receptors may explain this reduced response to exogenous ANG II in the renal circulation. Although there have been numerous studies of the intrarenal RAS after acute or chronic ischemic injury, few have investigated the cause of the reduction in
vasoconstrictor action of ANG II immediately following renal artery stenosis reported by McGiff and Itskovitz (31). In addition, there is no information on the regulation of expression of different ANG peptides within the kidney associated with acute renal ischemia following reperfusion. Thus we assessed the effects of renal ischemia on multiple components of the intrarenal RAS, including ANG II receptors, tissue and urinary peptide content, and enzymes involved in the processing of angiotensins.

METHODS

Animal model and surgery. Studies were conducted in male Sprague-Dawley rats 10–12 wk of age (HARLAN, Indianapolis, IN). Rats were housed in plastic shoebox cages with free access to ordinary chow and tap water at an ambient room temperature of 25°C and a 12:12-h light-dark cycle (lights on 7:00 AM to 7:00 PM). One day prior to surgery rats were housed in metabolic cages to monitor food and water consumption. Urine was collected into tubes kept on dry ice to inhibit degradation of urinary peptides during collection. Rats were anesthetized with pentobarbital sodium (50 mg/kg ip; Abbott Laboratories, North Chicago, IL), and the left kidney was surgically isolated. The renal arteries and veins were exposed, and 500 U of heparin (Upjohn, Kalamazoo, MI) were injected into the artery 5 min prior to occlusion of the artery. The left renal artery, in the animals noted as ischemic, was occluded for 1 h and verified by the blanching of the renal surface after ligation. The clamp was removed 1 h after, and the incision was closed by sequential suturing with 3-0 coated Vicryl (polyglactin 910, braided suture; Ethicon, Somerville, NJ) of the muscular layer and the skin layer with MikRon Autoclip 9-mm wound clips (Clay-Adams, Becton-Dickinson, Sparks, MD). Rats belonging to the sham group were treated similarly but lacked vascular occlusion. All rats recovered from surgery on a warmed surface, then were returned to individual metabolic cages with water and pelleted chow ad libitum for 24- and 72-h reperfusion periods. Urine was collected on dry ice at 24 and 72 h.

Receptor autoradiography. Rats were killed by decapitation at the 24- and 72-h time points, and kidneys were harvested and frozen on dry ice immediately in an Optimal Cutting Temperature cryostat cutting compound (OCT; Miles, Elkhart, IN) and stored at −80°C until sectioned. Sections were cut on a cryostat (model OTC; Bright Instrument, Huntingdon, Cambridge, UK) with chamber temperatures between −15 and −17°C, at a thickness of 14 μm. These sections were then treated to Superfrost Plus glass slides (Fisher, Pittsburgh, PA) and stored at −80°C.

Autoradiography of ANG binding sites was determined in sections of sham, ischemic, and contralateral kidneys using 125I-labeled [Sar1,Thr9]ANG II (125I-Sarthran). This ligand was iodinated using the chloramine T method and purified by HPLC to a specific activity of 2,200 Ci/mmol, as described previously (9). Tissue sections were preincubated in the following buffer: 5 mM MgCl2, 1 mM EGTA, 10 mM HEPES, 5 mM NaCl, 0.5% bovine serum albumin, pH 7.4, with or without dithiothreitol (DTT, 1.25 mM) for 30 min at room temperature. Tissue was then incubated for 60 min in buffer containing 0.2 or 0.8 nM 125I-Sarthran and the following competitors: 10 μM lisinopril, an ANG-converting enzyme (ACE) inhibitor; 200 μM phenylmethylsulfonyl fluoride (PMF), a serine protease inhibitor; 10 μM Sch-33970, a nephrilysin inhibitor; 1 μM amastatin/bestatin, an aminopeptidase inhibitor; and 10 μM z-prolyl prolinal, a prolyl endopeptidase and prolyl carboxypeptidase inhibitor. Data from the 0.8 nM 125I-Sarthran study, a near-saturating concentration, were used as an indication of apparent maximal density of binding (42).

Urine, plasma, and renal tissue angiotensins. Urine collected on dry ice before and after surgery was thawed and extracted on Sep-Pak Vac 3-ml (200 mg) C18 cartridges (Waters, Milford, MA). Sep-Pak C18 cartridges were activated with 5 ml 90% ethanol (90%), 4% acetic acid, 3 ml methanol, and 5 ml 4% acetic acid; peptides were eluted with 10 ml 90% ethanol/4% acetic acid. Upon decapitation at 24 h postsurgery, trunk blood was collected into chilled Vacutainer blood collection tubes (Becton-Dickinson, Franklin Lakes, NJ) containing a mixture of peptidase inhibitors, as follows: 25 mM EDTA, 0.44 mM α-phenanthroline, 1 mM 4-chloromercuribenzoic acid (PCMB), 0.12 mM pepstatin A, and 3 μM acetyl-His-Pro-Phε-Val-statine-Leu-Phe, a specific rat renin inhibitor (25). After 20 min on ice, blood samples were centrifuged at 3,000 rpm for 20 min, and plasma was stored without disturbing the packed cells. Aliquots of plasma were stored at −80°C until radioimmunoassay (RIA) of angiotensin peptides. To ensure prompt inactivation of ANG metabolic enzymes, kidneys were quickly removed at 24 h postsurgery and immediately homogenized on ice in an acid ethanol (80% vol/vol 0.1 N HCl) solution containing the following peptidase inhibitors: EDTA, phenanthroline, PMSF, and PCMB and the renin inhibitor (7). A sample of homogenate was taken to determine total protein content, using the Bradford protein assay with bovine serum albumin as a standard (Bio-Rad Protein Assay Reagent; Bio-Rad Laboratories, Hercules, CA). The homogenate was centrifuged at 30,000 g for 20 min, and the supernatant was decanted and then further acidified with 1% (vol/vol) heptfluorobutyric acid (HFBA; Pierce, Rockford, IL). The solution was allowed to precipitate at 4°C overnight and then centrifuged at 30,000 g for 20 min. The supernatant was decanted into glass tubes and concentrated to 5 ml on a Savant vacuum centrifuge (model AS160 automatic SpeedVac with VaporNet outfitted with radiant cover model RC160; Savant, Farmingdale, NY). The concentrated extracts were applied to activated Sep-Paks, washed with 0.1% HFBA, and eluted with 5 ml 80% methanol, 0.1% HFBA. Percent recovery of ANG pep-
tides was determined by addition of \(^{125}\text{I}-\text{ANG-(1–7)}\) to homogenates with a comparison of total counts applied to the Sep-Pak to the counts recovered in the 5-ml eluate. Recovery of the ligand averaged \(76 \pm 3.1\%\) \((n = 4)\) with this procedure. Tissue content was not corrected for the recovery.

RIA analysis of ANG peptide content in urine, plasma, and renal tissue was performed as recently published \((9)\). The angiotensin-(1–7) RIA fully recognizes ANG-(1–7) and ANG-(2–7), but cross-reacts less than 0.01% with ANG-(3–7), ANG II, ANG I, and their fragments. The ANG II RIA equally recognizes ANG III, ANG-(3–8), and ANG-(4–8), but cross-reacts less than 0.01% with ANG I and ANG-(1–7). The ANG I RIA fully recognizes ANG-(2–10) and ANG-(3–10), but cross-reacts with ANG II and ANG-(1–7) less than 0.01%. The limits of detection for each RIA were as follows: ANG-(1–7), 4 fmol/tube; ANG II, 0.5 fmol/tube; and ANG I, 5 fmol/tube.

**Tissue renin, nepri lysin, and ACE activity.** Upon removal of the kidney, the cortex was isolated, homogenized on ice, and centrifuged at 30,000 \(g\) for 20 min. The supernatant was collected for analysis of renin activity, and the pellet was resuspended and then centrifuged at 30,000 \(g\) for 20 min. The pellet from the second centrifugation was resuspended in PBS, and activities of neprilysin and ACE were measured. ACE activity was determined by incubation of cortical membranes \((50 \mu g)\) with the synthetic substrate Hip-His-Leu in the presence and absence of the ACE inhibitor lisinopril \((10 \mu M)\) as described \((10)\). ACE activity is expressed in nanomoles His-Leu produced per milligram protein per minute. Neprilysin was measured with the fluorescent substrate N-succinyl-Ala-Ala-Phe-amido-4-methyl coumarin incubated with 0.2 \(\mu\)g cortical membranes, with or without the neprilysin inhibitor Sch-39370 \((10 \mu M)\) \((50)\). Neprilysin activity was expressed in nanomoles amidomethylcoumarin produced per milligram protein per minute. Tissue renin activity is expressed in picomoles ANG I produced per milligram protein per hour.

**Statistics.** All data are expressed as means ± SE. To quantify differences in renal ANG II receptors or tissue ANG peptides, one-way ANOVA with Dunnett’s post hoc comparisons to sham-operated kidneys was used. Changes in urine volume and urinary peptides were analyzed using ANOVA and Dunnett’s post hoc test compared with before-surgery values in each group. Plasma ANG peptide concentrations were analyzed using the Student’s t-test for unpaired variables, with comparisons made between sham-operated rats and ischemic-operated rats at 24 h after surgery. Analysis of tissue enzyme activities (ACE, neprilysin, and renin) was determined by one-way ANOVA with Dunnett’s post hoc test comparisons with the control sham-operated kidneys at 24 h postsurgery. Differences were considered significant at \(P < 0.05\). Statistical analysis was carried out using Instat 2.1 (GraphPad Software, San Diego, CA).

**RESULTS**

**Effect of renal ischemia-reperfusion on urine volume.** Urine was collected on dry ice 24 h presurgery and again at 24 and 72 h post-renal ischemia or sham surgery. In the sham-operated group, there was a non-significant tendency for a decrease in urine volume compared with presurgical values \((11.2 ± 1.8\) before vs. \(6.0 ± 1.8\) ml/24 h after), with a return to presurgery levels by 72 h postsurgery \((10.0 ± 2.0\) ml/24 h). In rats subjected to unilateral renal ischemia, urine volume did not appreciably change at either 24 or 72 h postsurgery compared with presurgery levels \(9.6 ± 1.7\); 24 h postsurgery, \(9.2 ± 1.5\); and 72 h postsurgery, \(11.7 ± 0.33\) ml/24 h). There was no significant difference between the ischemic and sham-operated groups at any time point.

**Effects of renal ischemia-reperfusion on urinary concentration and excretion of ANG peptides.** The effects of ischemia on the renal RAS are shown in Table 1. In keeping with previous findings \((8, 46)\), basal levels of ANG I and ANG-(1–7) were significantly higher than those of ANG II. Urinary concentrations and excretion of ANG I showed a temporal and significant increase in rats subjected to renal ischemia at 24 h but not 72 h posts ischemia compared with control values. There were no significant changes in sham-operated animals in either urinary ANG I concentration or in ANG I excretion. Urinary ANG II concentrations did not significantly increase in the ischemic- or sham-operated rats at 24 h postsurgery compared with presurgery control levels. There was also no change in urinary ANG II excretion. Urinary ANG-(1–7) concentration significantly increased in ischemic rats at 24 h postsurgery compared with presurgery control levels. Urinary excretion of ANG-(1–7) followed a similar trend in ischemic animals. Sham-operated rats did not show any change in urinary ANG-(1–7) concentration or excretion. All values returned to presurgery levels by 72 h postsurgery.

**Effects of renal ischemia-reperfusion on plasma ANG peptides.** The ANG peptide concentrations from plasma collected at decapitation 24 h postsurgery are shown in Fig. 1. In sham-operated rats at 24 h postsurgery, the predominant ANG peptide measured was ANG I, with lower levels of ANG II and ANG-(1–7). A similar profile was found in plasma in the renal ischemia-reperfusion rats at 24 h postsurgery, and there were no significant differences compared with sham-operated rats.

**Effects of renal ischemia-reperfusion on tissue angiotensins.** As illustrated in Fig. 2, there was significantly higher tissue ANG II \((Fig. 2, middle)\) in the ischemic kidney compared with the sham-operated kidneys. The

**Table 1. Urinary angiotensin concentration and excretion**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Control ((n = 6–8))</th>
<th>24 h ((n = 6–8))</th>
<th>72 h ((n = 2–3))</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANG-(1–7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration, pM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>278 ± 58</td>
<td>370 ± 100</td>
<td>318 ± 8†</td>
</tr>
<tr>
<td>Ischemic</td>
<td>319 ± 48</td>
<td>575 ± 83*</td>
<td>229 ± 4</td>
</tr>
<tr>
<td>Excretion, ng/24 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>3.5 ± 1.0</td>
<td>3.0 ± 1.3</td>
<td>3.2 ± 0.7†</td>
</tr>
<tr>
<td>Ischemic</td>
<td>3.2 ± 0.8</td>
<td>5.3 ± 1.0</td>
<td>2.6 ± 0.03</td>
</tr>
<tr>
<td>ANG II</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Concentration, pM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>154 ± 39</td>
<td>229 ± 82</td>
<td>194 ± 72†</td>
</tr>
<tr>
<td>Ischemic</td>
<td>134 ± 18</td>
<td>296 ± 94</td>
<td>115 ± 17</td>
</tr>
<tr>
<td>Excretion, ng/24 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>1.9 ± 0.6</td>
<td>2.0 ± 0.9</td>
<td>2.1 ± 1.1†</td>
</tr>
<tr>
<td>Ischemic</td>
<td>1.3 ± 0.3</td>
<td>2.4 ± 0.6</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>ANG I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration, pM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>203 ± 67</td>
<td>396 ± 164</td>
<td>144 ± 26†</td>
</tr>
<tr>
<td>Ischemic</td>
<td>214 ± 41</td>
<td>1,098 ± 300*</td>
<td>146 ± 25</td>
</tr>
<tr>
<td>Excretion, ng/24 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>2.7 ± 1.0</td>
<td>2.7 ± 1.7</td>
<td>1.4 ± 0.02†</td>
</tr>
<tr>
<td>Ischemic</td>
<td>2.2 ± 0.5</td>
<td>10.4 ± 3.3*</td>
<td>1.7 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE, except as noted. †Mean ± SD, \(n = 2\). *\(P < 0.05\), compared with control values.
increase in ANG II levels in the ischemic kidney corroborates results of Boer et al. (5), who reported similar findings in kidneys with reduced perfusion pressure at 1–3 h following restriction of the abdominal aorta. In contrast, renal tissue ANG I (Fig. 2, top) and renal tissue ANG-(1–7) (Fig. 2, bottom) were not significantly altered in the ischemic or nonischemic kidneys compared with sham-operated kidneys. Moreover, there were no significant changes in ANG peptide content in the contralateral nonischemic kidneys at the 24 h time point.

Effect of renal ischemia-reperfusion on ANG processing enzymes in renal cortex. Additional experiments determined the effects of acute renal ischemia with 24-h reperfusion on the activity of cortical ACE, nepri-lysin, and renin, all of which are enzymes involved in either the production or degradation of ANG peptides within the kidney (14, 45). As shown in Fig. 3, top, tissue renin activity of the cortex of ischemic kidney increased approximately threefold compared with sham-operated and contralateral nonischemic kidneys. In contrast, renal ischemia with 24-h reperfusion decreased cortical ACE activity in both ischemic and contralateral nonischemic kidneys (Fig. 3, middle), and cortical neprilysin activity decreased in the contralateral nonischemic kidneys (Fig. 3, bottom), compared with cortical tissue from sham-operated kidneys.

Effects of renal ischemia-reperfusion on \(^{125}\text{T-Sartrhan binding.}\) The effect of renal ischemia-reperfusion on the density, distribution, and subtype of ANG receptors was determined by in vitro receptor autoradiography of rat kidney slices, using \(^{125}\text{T-Sartrhan as radioligand.}\) In Fig. 4, we show computer-generated, color images demonstrating localization of ANG receptors in sham-operated and ischemic kidneys at 24 h postsurgery. Binding of \(^{125}\text{T-Sartrhan}\) is found throughout the cortex and medulla, with the highest density of binding in the glomeruli and vasa recta (Total-24 h). In the ischemic-operated kidney at 24 h postsurgery, there is greatly diminished binding of \(^{125}\text{T-Sartrhan.}\) The juxtaposition of sham-operated and ischemic kidneys shows that the overall decrease in \(^{125}\text{T-Sartrhan binding in the ischemic kidney after 24 h reperfusion recovered to levels similar to sham-operated kidney by 72 h. In both ischemic kidneys and those from sham-operated animals at 24 h, the majority of the binding was displaced by an AT\(_1\) antagonist (losartan) and not by an AT\(_2\) antagonist (PD-123319).

Quantification of the decrease in binding in four major areas of the kidney is shown in Fig. 5. At 24 h, \(^{125}\text{T-Sartrhan binding was significantly decreased (P < 0.05) in cortical tubulointerstitium, glomeruli, and the region of the vasa recta in the ischemic kidney compared with sham-operated kidneys. Reductions of 30–50% occurred relative to values in sham-operated rats. Significant decreases in \(^{125}\text{T-Sartrhan binding also oc-
curred in the medullary region of both the ischemic (57%) and the nonischemic contralateral kidney (51%) at 24 h postsurgery. There were no differences in binding between the ischemic and nonischemic kidneys at 24 h.

Total binding recovered to levels not different from sham-operated kidneys in all areas of the ischemic and nonischemic kidneys by 72 h.

To investigate the relative contribution of AT₁ or AT₂ receptors to the binding, we determined competition for ¹²⁵I-Sarthran binding with the selective AT₂ antagonist PD-123319 and the selective AT₁ antagonist losartan for each of the four individual areas of the kidney analyzed. As shown in Table 2, competition for binding with the AT₁ antagonist losartan accounted for ~80–90% of the binding in sham, ischemic, and nonischemic kidneys. There was no significant competition by the AT₂ antagonist PD-123319 in sham kidneys and no significant alteration in the percentage of AT₂ receptors in the kidneys exposed to ischemia-reperfusion injury or the contralateral nonischemic kidneys. Since previous studies have shown that the reducing agent DTT augments binding to the AT₂ receptor (9), we further investigated the ability of PD-123319 to compete for ¹²⁵I-Sarthran binding in the presence of 1.25 mM DTT. No significant competition for binding was seen with PD-123319 in the ischemic, nonischemic, or sham-operated kidneys in the presence of DTT (Table 2). These results are consistent with few, if any, AT₂ receptors in the normal adult rat kidney, as reported by other investigators (1, 6, 49). The competition studies with the AT₁ and AT₂ antagonists revealed that there were no detectable differences in the relative proportion of AT₁ and AT₂ subtype receptors among sham, ischemic, and nonischemic kidneys, consistent with the majority of sites being AT₁ in all areas studied.

Previous studies by Ernsberger et al. (15) showed that ANG-(1–7) competed with relative high affinity (~20–40 nM) at 15–20% of the ANG II receptors in mesangial cells. To determine whether renal ischemia-reperfusion injury affects this high-affinity component of ANG-(1–7) binding, we studied competition for binding of ¹²⁵I-Sarthran with ANG-(1–7) at a concentration of 0.2 mM, illustrated in Table 2. ANG-(1–7) competed for ~25% of total binding in the sham-operated kidneys, and there was no significant difference in competition by ANG-(1–7) in the ischemic vs. nonischemic right kidneys. Only the cortical area of the contralateral nonischemic kidney showed significantly greater competition with ANG-(1–7) compared with sham.

**DISCUSSION**

In the present studies, we investigated the effects of renal ischemia-reperfusion on the enzymatic, receptor, and peptide components that comprise the intrarenal RAS. Specifically, we characterized the density and subtype of ANG receptors, urinary ANG peptide excretion and concentration, renal ANG content, and the activities of renal cortical enzymes involved in processing and degradation of ANG peptides. The model of unilateral renal ischemic injury involved clamping the left renal artery for 60 min. In the ischemic kidney, we found a significant increase in renal tissue levels of ANG II and a tendency for elevation of ANG I, but no change in ANG-(1–7). The increased levels of ANG II may be a direct result of the observed increase in cortical renin activity, especially given that renal cortical ACE activity decreased following ischemia. Kontogiannis and Burns (28) also observed an increase in tissue levels of renin and ANG II at 24 h following bilateral renal ischemia, with a return to sham levels by 120 h. Renal cortical angiotensinogen was de-
Fig. 4. In vitro receptor autoradiography. In vitro receptor autoradiography with $^{125}\text{I}-[\text{Sar}^1,\text{Thr}^8]\text{ANG II}$ (0.8 nM) was used to determine ANG receptor density in different regions of the kidney, with the highest intensity of radioligand binding indicated by red and orange, and lesser intensities of binding indicated by blue. Glomeruli can be seen as small punctate areas of binding in the outer cortical region. Total binding at 24 h (Total-24 h) postsurgery was higher in sham-operated (SHAM) than ischemic kidneys (ISCH). In adjacent sections from those same animals at 24 h postsurgery, losartan (+Losartan), the AT$_1$ antagonist, competes for almost all the binding. In contrast, the AT$_2$ antagonist did not compete to any significant degree in any area (+PD-123319) in kidneys from either the ischemic or sham-operated animals at 24 h. At 72 h postsurgery (Total-72 h), total binding was similar in sham-operated and ischemic kidneys.

Fig. 5. Quantitative analysis of receptor density. ANG receptor density is shown in four areas of the ischemic (Isch, $n = 3$), nonischemic kidneys (N-Isch, $n = 3$) at both 24 h (solid bars) and 72 h (cross-hatched bars) postsurgery, and the sham kidneys (24 and 72 h postsurgery data combined; open bars, $n = 4$). In A, cortical-interstitial receptor density is significantly decreased in the ischemic kidney at 24 h postsurgery compared with sham-operated kidneys, with a return to sham-operated levels of binding by 72 h postsurgery. A similar decline in receptor binding was observed in glomeruli (B) and area of the vasa recta (D) of the ischemic kidneys at 24 h postsurgery compared with sham-operated rats, with returns to sham levels by 72 h postsurgery. C: medullary binding was decreased in both the ischemic and the nonischemic kidney compared with sham at 24 h postsurgery, with returns to sham levels by 72 h postsurgery. There were no statistical differences between the level of binding in the ischemic kidneys and nonischemic kidneys at 24 or 72 h postsurgery. * $P < 0.05$. 

![Figure 4: In vitro receptor autoradiography.](image1)

![Figure 5: Quantitative analysis of receptor density.](image2)

![Figure 6:](image3)
creased in their study (28), again suggesting that increased production of ANG peptides within compartments of the kidney may be due to the increased availability of renin.

In contrast to the elevated tissue levels of ANG II, ANG-(1–7) and ANG I excretion increased in the urine during the first 24 h posts ischemia. While ANG I conversion to ANG II occurs in the vasculature of the kidney, consistent with the presence of ACE in the renal endothelium, uptake of circulating ANG II also contributes to tissue levels of the peptide in the kidney (33). ANG I and ANG II are found in the juxtaglomerular cells, and angiotensinogen is found in the proximal tubule epithelium, facing the lumen of the proximal tubule (21, 24). Also, there is renin mRNA expression and production of active renin in the proximal tubule (32). This evidence would suggest that angiotensin peptides, such as ANG I and II, may be produced intracellularly and secreted into the tubular lumen, resulting in increased urinary values of ANG peptides. There is also release of ANG II from isolated glomeruli (3). Although our results show no difference in plasma ANG peptides 24 h following renal ischemia-reperfusion, we observed significant increases in urinary levels of ANG peptides (37, 9). The increased tissue and urinary ANG peptides following ischemia implies that intrarenal production does not rely on delivery of ANG peptides from the plasma but that the intrarenal RAS may be stimulated by renal ischemia-reperfusion.

Our data further suggest an intrarenal, nonvascular site of production of ANG-(1–7). In studies of isolated renal brush border membranes from both rat and pig, ANG-(1–7) was the primary product from ANG I metabolism (26, 38). Moreover, we and others found that there was little contribution of ACE in this preparation, consistent with previous reports of low ACE activity in renal brush border (23). Due to the high content of nephrilysin in cortical tubules (26), it is likely that ANG-(1–7) may be one of the major intraluminal ANG peptides. Significant, although transient, increases in urinary levels of ANG I and ANG-(1–7) at 24 h post-ischemia-reperfusion injury, were observed in the present study. Decreased cortical membrane nephrilysin activity was observed, however, signifying that the increases in urinary ANG peptides could be due to increased renin activity, supplying increased substrate (ANG I) to both renal tissue and tubular sites. In addition, the increase in ANG-(1–7) may have been influenced by decreased ACE activity, resulting in reduced metabolism of the peptide (10). ANG-(1–7) exhibits high affinity for ACE, comparable to that of only kinins (10). Changes in urinary levels of ANG I and ANG-(1–7) following unilateral renal ischemia-reperfusion may reflect altered secretion of peptides from the tubule into luminal fluid. In contrast, changes in tissue levels of ANG II may be due to regulation within the vasculature and interstitial tissue of the kidney.

A decrease in vasoconstrictor responses to ANG II occurs following renal ischemia in dogs and rats (12, 31). Our results indicate a transient decrease in AT1 receptors throughout the ischemic kidney at 24 h, with a recovery to sham levels by 72 h, which may explain the reduced vasoconstrictor responses. These receptor changes corroborate recent studies by Kontogiannis and Burns (28), who found a decrease in cortical ANG II receptors by autoradiography and a decrease in proximal tubular AT1 mRNA expression at 24 h after bilateral renal artery occlusion; both recovered to sham levels at 72 and 120 h (28). The present study, however, differs in that only the left renal artery was occluded, providing an ischemic and nonischemic contralateral kidney for comparison. We saw similar trends for reductions in ANG II receptors in the nonischemic contralateral kidney. Thus our study allows dissection of the direct effects of ischemia vs. changes in circulating or tissue ANG II or altered hemodynamics in the contralateral kidney as the mechanism for observed changes. As previous studies have noted, increased levels of ANG II may promote internalization

### Table 2. Characterization of ANG II receptor subtypes in ischemic kidneys

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Region</th>
<th>Sham (n = 4)</th>
<th>Ischemic (n = 6)</th>
<th>Nonischemic (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1 (losartan)</td>
<td>Corticotubulointerstitium</td>
<td>78 ± 4</td>
<td>82 ± 5</td>
<td>87 ± 4</td>
</tr>
<tr>
<td></td>
<td>Glomeruli</td>
<td>90 ± 2</td>
<td>90 ± 1</td>
<td>90 ± 1</td>
</tr>
<tr>
<td></td>
<td>Medulla</td>
<td>83 ± 3</td>
<td>79 ± 6</td>
<td>89 ± 2</td>
</tr>
<tr>
<td></td>
<td>Vasa recta</td>
<td>78 ± 5</td>
<td>85 ± 3</td>
<td>89 ± 2</td>
</tr>
<tr>
<td>AT2 (PD-123319)</td>
<td>Corticotubulointerstitium</td>
<td>10 ± 4</td>
<td>4 ± 2</td>
<td>9 ± 4</td>
</tr>
<tr>
<td></td>
<td>Glomeruli</td>
<td>9 ± 4</td>
<td>9 ± 5</td>
<td>11 ± 5</td>
</tr>
<tr>
<td></td>
<td>Medulla</td>
<td>2 ± 1</td>
<td>4 ± 3</td>
<td>15 ± 8</td>
</tr>
<tr>
<td></td>
<td>Vasa recta</td>
<td>2 ± 1</td>
<td>4 ± 4</td>
<td>7 ± 5</td>
</tr>
<tr>
<td>ANG-(1–7)</td>
<td>Corticotubulointerstitium</td>
<td>25 ± 2</td>
<td>23 ± 5</td>
<td>41 ± 4*</td>
</tr>
<tr>
<td></td>
<td>Glomeruli</td>
<td>26 ± 3</td>
<td>33 ± 4</td>
<td>39 ± 5</td>
</tr>
<tr>
<td></td>
<td>Medulla</td>
<td>30 ± 2</td>
<td>23 ± 7</td>
<td>32 ± 9</td>
</tr>
<tr>
<td></td>
<td>Vasa recta</td>
<td>28 ± 4</td>
<td>37 ± 8</td>
<td>43 ± 4</td>
</tr>
</tbody>
</table>

Values are means ± SE, presented as competition in percent total binding for the 24- and 72-h time points combined. Numbers in parentheses represent competition with PD-123319 in the presence of DTT. *P < 0.05, compared with sham-operated kidneys.
of AT\textsubscript{1} receptors (40) and subsequent downregulation of AT\textsubscript{1} mRNA (28). It has been shown by Clark et al. (11) that ANG-(1–7) may downregulate AT\textsubscript{1} receptors in cultured vascular smooth muscle cells. In the ischemic kidney, increased renal tissue levels of ANG II at 24 h posts ischemia or increased urinary levels, reflective of increased tubular fluid content, of ANG-(1–7) may be a mechanism responsible for the downregulation of AT\textsubscript{1} receptors. However, homologous regulation of ANG II receptors in the kidney is quite variable with increased plasma ANG II associated with either a decrease (37) or no change (2) in receptor density. These responses may be due to differential regulation of glomerular and vascular components of the kidney compared with tubular elements. Expression of renal ANG receptor mRNA is altered with dietary restriction. A decrease in AT\textsubscript{1} receptor mRNA of the whole kidney is associated with the elevated ANG II compared with a high-salt diet (37), whereas glomerular ANG II receptor density is decreased with low-salt diet (36). In addition, recent studies suggest that nitric oxide can also decrease AT\textsubscript{1} mRNA at the transcriptional level (22). Addition of the nitric oxide donor S-nitroso-acetyl-DL-penicillamine dose dependently decreased expression of vascular smooth muscle cell AT\textsubscript{1} mRNA as quickly as 3 h, and by 24 h, only 7% of mRNA remained compared with control mRNA levels. Nitric oxide also plays a role in ischemic events in both the ischemic and nonischemic kidney (12). Thus the contribution of this factor to the heterologous regulation of ANG II receptors may account for the reductions of ANG receptors in both the ischemic and contralateral kidneys, with greater effects in the ischemic tissue.

Competition studies were used to determine whether the contribution of AT\textsubscript{1} and AT\textsubscript{2} angiotensin receptors to the overall binding of the kidney was altered following renal ischemia. The majority of receptors were characterized as AT\textsubscript{1} in sham-operated, ischemic, and contralateral nonischemic kidneys. Although the AT\textsubscript{2} subtype receptor does not appear to contribute to ANG II receptor binding in the adult rat kidney (49), it is widely expressed early in fetal development (17), has increased expression in adults associated with apoptosis (47), wound repair (41), and vascular injury (30), and is upregulated by chronic sodium depletion (34) and after global brain ischemia (29). We hypothesized that AT\textsubscript{2} receptors would increase, at least in the renal tubular areas, following ischemia-reperfusion injury in rat kidney, coincident with the degeneration and repair. However, we detected few if any AT\textsubscript{2} receptors in sham, ischemic, or contralateral nonischemic kidneys at 24 or 72 h postsurgery. We speculated that the lack of DTT might diminish the observed contribution of AT\textsubscript{2} receptors to ANG II binding in kidney, since the AT\textsubscript{2} receptor contains essential thiol groups (27). Although DTT is known to enhance binding at the AT\textsubscript{2} receptor (9, 49), most studies of ANG II receptors in the kidney do not include DTT in the binding buffer, because there is an inhibitory effect at high concentrations on binding to the AT\textsubscript{1} receptor (49). However, the results of our experiments with DTT present in the binding buffer were similar to those in the absence of DTT. Kontogiannis and Burns (28) found that renal ischemia-reperfusion stimulated AT\textsubscript{2} receptor mRNA in the outer medulla and in proximal tubules 120 h after reperfusion, but they were unable to detect these sites by receptor binding techniques. Thus it is not clear whether the increase in AT\textsubscript{2} mRNA at this later time point is accompanied by an increased expression of the AT\textsubscript{2} receptor protein or whether the level of expression remains below detection with the receptor binding methodology used.

ANG-(1–7) has natriuretic and diuretic effects on the whole kidney and isolated cortical tubules through release of prostaglandins (13, 19, 20). Previous reports indicate relative high affinity of 20–40 nM for ANG-(1–7) for a subpopulation (~20%) of ANG II receptors in mesangial cells in culture (15). To determine whether ischemia-induced an increased expression of the high-affinity component of ANG-(1–7) binding, we performed competition studies with ANG-(1–7) at 0.2 μM. This concentration of ANG-(1–7) is approximately 5-fold higher than the \(K_i\) at this site and yet still is 10-fold lower than its \(K_i\) at a typical AT\textsubscript{1} receptor (11). We observed ~20–25% competition by ANG-(1–7) in control kidneys. Although our results indicated no significant difference in competition with ANG-(1–7) in the ischemic kidney, competition by ANG-(1–7) in the corticotubulointerstitial area of the contralateral nonischemic kidney was significantly greater than in sham kidney. A similar trend was observed in the glomeruli and area of the vasa recta. Whether this indicates an upregulation of ANG-(1–7) binding sites in the contralateral nonischemic kidney, as a consequence of the humoral or hemodynamic factors that accompany the unilateral renal ischemia, remains to be fully explored.

To summarize, we found that unilateral renal ischemia-reperfusion injury resulted in a profound, albeit transient decrease in AT\textsubscript{1} receptor binding, primarily in the ischemic kidney associated with significant increases in renal tissue levels of ANG II, and no change in tissue ANG I or ANG-(1–7). The changes were short-lived, with a return to near-control levels by 72 h postsurgery, providing a mechanism for transient reduction in ANG II-induced vasoconstriction following ischemia. In contrast, renal ischemia-reperfusion injury caused an increase in the urinary ANG I and ANG-(1–7) levels compared with baseline, or presurgery levels. The different pattern of expression of ANG peptides in the urine compared with the tissue may be indicative of differential regulation of the metabolism of ANG I by specific processing enzymes present within compartments in the kidney. Previous studies indicate a poor correlation of ANG II with alterations associated with action of the intrarenal RAS (4, 5, 43). Thus our data suggest that urinary ANG I and ANG-(1–7) may be better markers of acute activation of the intrarenal RAS than urinary ANG II.

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