Role of AT₁ angiotensin II receptors in renal ischemic injury

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Kontogiannis, Jimmy, and Kevin D. Burns. Role of AT₁ angiotensin II receptors in renal ischemic injury. Am. J. Physiol. 274 (Renal Physiol. 43): F79–F90, 1998.—The present studies determined the effect of renal ischemia/reperfusion on components of the intrarenal renin-angiotensin system in rats and evaluated the effect of AT₁ angiotensin (ANG) II receptor blockade on functional recovery. After bilateral renal pedicle occlusion for 60 min, serum creatinine increased, peaking at 72 h, and returned to sham levels after 120 h. ANG II levels in ischemic kidneys were significantly increased 24 h after reperfusion but did not differ from levels in sham kidneys after 120 h. Both renal cortical angiotensinogen mRNA and proximal tubular AT₁ receptor mRNA were significantly reduced early after reperfusion, returning to sham levels by 120 and 72 h, respectively. AT₂ ANG II receptor mRNA was undetectable in proximal tubules from sham rats but was consistently present in ischemic rats at 120 h. By histoautoradiography, we found that binding of 125I-labeled ANG II was preserved in glomeruli but was decreased in whole cortex and outer medulla early after reperfusion and was completely blocked by the AT₁ antagonist losartan. Treatment of rats with losartan (25 mg/kg sc daily), starting at the time of reperfusion, had no effect on expression of proliferating cell nuclear antigen in cortical tubules but caused a significant decrease in serum creatinine at 72 h (ischemia: 334 ± 69 µM vs. ischemia + losartan: 135 ± 28 µM; P < 0.025, n = 6). These data indicate that renal ischemic injury causes an early increase in intrarenal ANG II levels, associated with reduction of mRNA for angiotensinogen and proximal tubular AT₁ receptors, and maintenance of glomerular ANG II binding. Losartan accelerates recovery of renal function, suggesting that activation of AT₁ receptors impairs glomerular filtration in the postischemic kidney. Angiotensin (ANG) II is a potent intrarenal vasconstrictor and also modulates glomerular filtration rate by exerting direct effects on afferent and efferent arteriolar tone and on mesangial cell function (8). In proximal tubule cells, ANG II stimulates hypertrophy by binding to apical or basolateral receptors (3, 36) and has been shown to augment epidermal growth factor (EGF)-stimulated proximal tubule cell mitogenesis (22). The proximal tubule contains mRNA for all components of the renin-angiotensin system, and high levels of ANG II are present in proximal tubular lumen (10⁻⁹ M) (27). This suggests that ANG II is synthesized locally and can act in an autocrine or paracrine fashion on tubular cells. The intrarenal effects of ANG II on hemodynamics and tubular growth responses are thought to be mediated by AT₁ ANG II receptors. In contrast, the localization and function of AT₂ ANG II receptors in the adult kidney remain unclear. AT₂ receptors are present in abundance in fetal kidney, disappear shortly after birth (28), and are linked to apoptosis in other tissues (38).

The role of the renin-angiotensin system in the recovery phase of renal ischemic injury remains incompletely understood. In the postischemic kidney, enhanced vascular sensitivity to sympathetic nervous stimulation has been reported to be due to intrarenal ANG II (24). In dogs, administration of angiotensin-converting enzyme inhibitors (ACEI) before renal artery clamping is associated with reduced severity of postischemic renal failure (17). In the present studies, we tested the hypotheses that renal ischemia alters expression of components of the intrarenal renin-angiotensin system in rats and that AT₁ receptor-mediated actions of ANG II modify the recovery of renal function. Our results reveal an increase in intrarenal levels of ANG II early after renal ischemia, accompanied by downregulation of cortical angiotensinogen and proximal tubular AT₁ receptor mRNAs, decreased renal cortical and medullary ANG II binding, and preservation of glomerular binding. New expression of AT₂ receptor mRNA occurs in proximal tubules and outer medulla 120 h after reperfusion. We demonstrate that blockade of AT₁ receptors with losartan, administered at the time of reperfusion, decreases serum creatinine levels, with no effect on proliferation of cortical tubular cells.

METHODS

Animal model and surgery. Male Sprague-Dawley rats (250–350 g) were used for all studies and were housed in the University of Ottawa Animal Care Facility. The protocol used in these studies was approved by the University of Ottawa Animal Care Committee. Rats were anesthetized with som-
nated with a bolus of isotonic saline (20 ml/kg sc). Throughout surgery, all rats were kept at 37°C with a water-circulated heating apparatus (Micro-Temp pump; Seabrook Medical Systems, Cincinnati, OH). All rats underwent surgical exposure of the left and right renal pedicles via midline incision. To induce renal ischemia, both renal pedicles were occluded for 60 min with vascular clamps (no. RU-2955-10; Ryan Medical Distributor, Oakville, ON, Canada). Sham rats underwent the same procedure, without renal pedicle clamping. After 60 min, the clamps were removed, and kidneys were observed to undergo reperfusion. In some experiments, rats were treated with the AT₁ receptor antagonist losartan (25 mg·kg⁻¹·day⁻¹ sc) (32), starting at the time of reperfusion. The abdominal muscle layer was closed with an interrupted suture, and the skin layer was closed with a continuous subcutaneous suture. For analgesia, rats received topical lidocaine jelly (2%) to the wound for the first 24 h and one dose of acetaminophen (6.8 mg/kg pr) as deemed necessary by the Animal Care staff. All rats had free access to water and food. At various time points after kidney reperfusion (0, 1, 3, 6, 24, 72, or 120 h), rats were killed, and kidneys were rapidly removed for further analysis. Immediately before being killed, rats underwent intracardiac puncture for collection of blood for assay of blood urea nitrogen (BUN) and serum creatinine (measured by the Ottawa General Hospital Clinical Chemistry Laboratory). In each experiment, each sham rat was paired with a rat undergoing kidney ischemia/reperfusion. Northern analyses and reverse transcription-polymerase chain reaction (RT-PCR) were utilized to compare mRNA expression between sham and ischemic tissue, and histoautoradiography of kidney slices was used for comparison of ANG II binding.

Isolation of proximal tubule segments. Rat proximal tubule segments were isolated by the method of Vinay et al. (33). Briefly, renal cortices were dissected, gently minced, and suspended in a solution containing (in mM) 115 NaCl, 24 NaHCO₃, 5 KCl, 1.5 CaCl₂, 1.0 MgSO₄, 2.0 NaH₂PO₄, 5 glucose, 1.0 alanine, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.4), 0.03% collagenase (type IV; Sigma, St. Louis, MO), and 0.01% soybean trypsin inhibitor (Sigma). The suspension was gassed with 95% O₂:5% CO₂ for 30 min at 37°C. After digestion, the cortical suspension was strained through a 250-µm brass sieve and centrifuged for 1 min at 100 g. The pellet was resuspended in buffer A without collagenase or trypsin inhibitor and recentrifuged three times at 100 g. The pellet was then applied to a 40% Percoll solution of identical ionic composition as buffer A, which had been previously chilled to 4°C. The Percoll solution was centrifuged at 26,000 g for 30 min at 4°C, and the digested tissue was separated into four distinct bands, as described (33). The F₄ layer, enriched in proximal tubular segments, was removed and utilized immediately for RNA isolation. In preliminary studies, proximal tubule suspensions from sham or ischemic rat kidneys demonstrated a high degree of viability, determined by exclusion of trypan blue (>95% of cells).

Northern hybridization. Total RNA was isolated from rat kidney cortex, outer medulla, and freshly isolated proximal tubular segments by homogenization in 4 M guanidinium isothiocyanate solution (1.5% N-laurylsarcosine, and 1% β-mercaptoethanol, followed by ultracentrifugation on a 5.7 M CsCl gradient, as described (5)). In some experiments, RNA was isolated, using a commercial kit (RNeasy kit; Qiagen, Chatsworth, CA). RNA (5–15 µg) was run on 1% agarose-2.2 M formaldehyde gels and transferred overnight onto nylon membranes (Schleicher and Schuell, Keene, NH), followed by ultraviolet cross-linking (Bio-Rad UV GS Gene Linker; Bio-Rad, Montreal, PQ, Canada). Membranes were hybridized with probes for a 1-kb Acc I fragment of the rat angiotensinogen cDNA (kindly provided by Dr. K. R. Lynch, Univ. of Virginia (18)) or the 1.2-kb Sac I-Kpn I cDNA encoding the rat AT₁₃ ANG II receptor (kindly provided by Dr. T. Inagami, Vanderbilt Univ. (10)), labeled with [³²P]dCTP (3,000 Ci/mmol; Amersham, Mississauga, ON) by the random primer method (Multiprime DNA labeling system, Amersham). Hybridization was performed overnight at 42°C in a solution of 30% formamide, 5× SSC (1× SSC: 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 5× Denhardt’s, 50 mM sodium phosphate (pH 7.0), 0.1% sodium dodecyl sulfate (SDS), 100 µg/ml denatured salmon sperm DNA, 10% dextran sulfate, and [³²P]dCTP-labeled probe (sp act: 0.5–1.5 × 10⁶ counts-min⁻¹·µg⁻¹). The membranes were washed at low stringency (2× SSC, 0.1% SDS) for 40 min at 23°C and then at high stringency (0.2× SSC, 0.1% SDS) for 25 min at 65°C. Membranes were then exposed for 16 h at −70°C to Kodak X-OMAT film, with two Cronex intensifiers (Sigma).

In all experiments, quality and quantity of RNA were determined by measurement of optical density at 260 nm. Equality of RNA loading onto gels was determined by visualization of RNA samples on ethidium bromide-stained agarose-formaldehyde gels. RNA samples that were not of high quality or that showed appreciable degradation were excluded from further analysis. There was no difference in RNA quality or yield in proximal tubules isolated from sham vs. ischemic kidneys (not shown).

RT-PCR. The mRNA for the rat AT₁ receptor was assayed by RT-PCR. After deoxyribonuclease digestion, samples of total RNA (1 µg) from outer medullas or proximal tubular segments were reverse transcribed, utilizing random hexamers and murine leukemia virus reverse transcriptase (Gene Amp kit; Perkin-Elmer, Roche Molecular Systems, Branchburg, NJ), in a total volume of 20 µl. After RT, the cDNA mixture was amplified by PCR, using AmpliTaq DNA polymerase (2.5 U) (all reagents from Perkin-Elmer) and 0.5 µM of oligonucleotide primers derived from the cDNA sequence of the rat AT₂ receptor (11) in a total volume of 100 µl. The upstream sense primer was 5'-TGAAGTCCCCATTTAAGTGC-3', and the downstream antisense primer was 5'-ACCACTGACATTTTCCAGG-3', generating a 536-base pair (bp) product, representing nucleotides 226–761 of the rat AT₂ receptor cDNA (11). PCR was carried out in a Perkin-Elmer Gene Amp 2400 PCR system by denaturing at 94°C for 30 s, annealing at 60°C for 30 s, and extending at 72°C for 45 s, for 40 cycles. RT-PCR products were run on 3% agarose gels and were directly visualized by ethidium bromide staining. All RT-PCR reactions included negative controls in which RNA samples were incubated in the absence of reverse transcriptase to rule out amplification of genomic DNA. The 536-bp AT₂ receptor RT-PCR product from rat kidney was subcloned into the plasmid pCRII-Script KS (+) (Strategene, La Jolla, CA) and partially sequenced by the dideoxy termination method (Sequenase version 2.0 DNA sequencing kit, Amersham). The AT₂ receptor cDNA product had 100% homology to the reported sequence of the rat AT₂ receptor in the region of interest (11).

RT-PCR was also performed to determine the presence of mRNA encoding AT₁₃ and AT₁₉ ANG II receptors in rat kidney. Primers specific for the AT₁₉ cDNA were upstream sense 5'-GCACACTGGCGAGATGTAAGC-3' and downstream antisense 5'-GTTGAAACAGAACAATGGAC-3', generating a 385-bp product, as described (19). Primers specific for the AT₁₃ receptor cDNA were upstream sense 5'-GCCCTGAAGTTGAATGATTTC-3' and downstream antisense 5'-TTTTAA-CAGTGCGTTTGCTTCC-3', generating a 204-bp product (19). PCR conditions were as described above for the AT₂ receptor,
except cycle number was 30 for the AT1a receptor mRNA and
33 for the AT1b receptor.

Equality of loading of samples of total RNA in RT-PCR
reactions was verified by visualization of RNA samples on
ethidium bromide-stained agarose-formaldehyde gels and by
RT-PCR amplification of mRNA encoding the cytoskeletal
protein β-actin, utilizing primers derived from the human
sequence, upstream sense 5′-AACGGAGAAGATGACCCCA-
GATCATGTTT 3′ and downstream antisense 5′-AGCAAGCC-
GTGGCGCATCTCTGGAGTGC 3′, generating a 352-bp
product (6). Semiquantitative RT-PCR of AT1a and AT1b
receptor mRNA was performed under noncompetitive condi-
tions to determine relative abundance of AT1a and AT1b
mRNAs (30). In preliminary experiments, image analysis of
PCR products demonstrated linearity of RT-PCR for serial
dilutions of total RNA between 0.08 and 2.00 µg. Accordingly,
semiquantitation was performed on total RNA samples be-
tween 0.60 and 0.76 µg. Densitometry was performed on PCR
products run on ethidium bromide-stained gels and corrected
for corresponding β-actin PCR products. Results are ex-
pressed as the ratio of PCR product signal intensities (sham/
ischemic).

Histoautoradiography. Histoautoradiography of ANG II
binding was determined on sham and ischemic rat kidneys,
using [125I]-labeled [Sar1, Ile8]ANG II (sp act 2,000 Ci/mmol,
Amersham), essentially as described (20). Immediately after
rats were killed, kidneys were perfused with phosphate-
buffered saline (PBS; 0.9% NaCl in 10 mM sodium phosphate
buffer, pH 7.4) via intracardiac puncture, removed, and
rapidly preserved on dry ice. Kidneys were cut into 20-µm
sagittal sections and thaw mounted on glass microscope
slides. After drying overnight in a dessicator under reduced
pressure at 4°C, tissue sections were preincubated in an
isotonic solution consisting of (in mM) 150 NaCl, 5 disodium
EDTA, 0.3 bacitracin, and 0.2% bovine serum albumin (BSA;
fraction V; Sigma) for 15 min at 23°C, followed by a 60-min
incubation in an identical solution with addition of 100 pM
[125I]-labeled [Sar1, Ile8]ANG II. Tissue sections were then
washed four times for 1 min each in ice-cold 50 mM tris(hy-
droxymethyl)aminomethane buffer (pH 7.4) via intracardiac puncture, removed, and
rapidly preserved on dry ice. Kidneys were cut into 20-µm
galgal sections and thaw mounted on glass microscope
slides. After drying overnight in a dessicator under reduced
pressure at 4°C, tissue sections were preincubated in an
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fraction V; Sigma) for 15 min at 23°C, followed by a 60-min
incubation in an identical solution with addition of 100 pM
125I-labeled [Sar1, Ile8]ANG II. Tissue sections were then
washed four times for 1 min each in ice-cold 50 mM tris(hy-
droxymethyl)aminomethane buffer (pH 7.4), dried under
warm air, and then exposed to Hyperfilm enhanced chemilu-
mescence (Amersham) at −70°C for 24-48 h. The film was
developed with Kodak developer D-19 and fixed with Kodak
rapid fixer (Sigma). In preliminary experiments, preincuba-
tion of kidney slices with an excess of unlabeled ANG II (10−6
M) completely displaced radiolabeled ANG II binding, confirm-
ing specificity of receptor binding.

Immunocytochemistry. Localization of cortical tubular nu-
clei staining for proliferating cell nuclear antigen (PCNA)
was performed on postischemic kidney sections from rats
treated with or without losartan. Briefly, 5- to 6-mm kidney
sections were fixed in 4% paraformaldehyde in 0.1 M sodium
phosphate buffer (pH 7.4) for 4 h. Tissue sections were then
rinsed in PBS and stored in 0.6 M sucrose in 0.1 M sodium
phosphate buffer. Tissue was frozen on dry ice and cut into 8-
µm sections with a cryotome, and sections were thaw mounted
on slides. For PCNA staining, slides were incubated in PBS
with 1% SDS for 5 min, followed by a 10-min wash in PBS and
a 20-min incubation in PBS with 1% BSA. Slides were then
incubated with mouse monoclonal antibody to PCNA (Dako
done PC10, lot 016; Cedarlane Laboratories, Hornby, ON) at
1:10 dilution in PBS with 1% BSA for 60 min at room
temperature. This was followed by three washes in PBS.
Slides were then incubated with secondary antibody, goat
anti-mouse immunoglobulin G2a gamma (Caltag Laborato-
ries, San Francisco, CA) coupled to fluorescein isothiocyanate
(1:20 dilution), in PBS with 1% BSA for 60 min at room
temperature, followed by three washes in PBS. To stain all

Fig. 1. Effect of renal ischemia/reperfusion on serum creatinine (A)
and blood urea nitrogen (BUN; B) in rats. Values of serum creatinine
and BUN from rats with renal ischemia were significantly elevated
above sham values, starting at 6 h after reperfusion and continuing
through 72 h. At 120 h, serum creatinine and BUN did not signifi-
cantly differ between the 2 groups. Values represent means ± SE. ●,
Shams; ○, ischemia/reperfusion.
nuclei, slides were also incubated for 1 min with 5 µg/ml of the dye Hoechst 33258 (Aldrich, Milwaukee, WI) before mounting. Sections were examined with a Zeiss Axioplan Universal microscope equipped for epifluorescence. To quantify PCNA staining, the number of Hoechst-positive nuclei that stained positively for PCNA was counted in 30 cortical tubules/section by an observer blinded to the experimental protocols. Results are expressed as percentage of PCNA-positive nuclei per kidney.

Assay of intrarenal ANG II. Intrarenal levels of ANG II were measured in ischemic and sham rat kidneys at 24 and 120 h after reperfusion by high-performance liquid chromatography (HPLC) and radioimmunoassay, using the method of Ruzicka et al. (26). Briefly, kidneys were perfused in situ with PBS to remove blood, via intracardiac injection, and then removed and snap frozen in liquid nitrogen. Snap-frozen kidneys were weighed, minced, and then boiled for 20 min in 1 M acetic acid. Tissue was then homogenized for 1 min, using a hand-held homogenizer (Tissue Tearor; Biospec Products, Racine, WI), and centrifuged at 10,000 g for 10 min at 4°C. The aqueous phase was removed and passed through a C18 Sep-Pak cartridge (Waters, Milford, MA) that had been preconditioned with 10 ml of deionized distilled water and 10 ml 100% methanol, followed by 10 ml deionized distilled water, at a rate of 5 ml/min. The sample was applied to the cartridge at a rate of 1 ml/min, followed by rinsing with 5 ml of 0.1% acetic acid, and eluted in 6 ml of 4% acetic acid in ethanol at a rate of 1 ml/min. Eluants were dried in a Speed Vac concentrator for further analysis. HPLC separation of ANG II was kindly performed by the laboratory of Dr. F. Leenen (Univ. of Ottawa), according to published protocol (26). After HPLC, ANG II levels were measured in the eluants by radioimmunoassay, utilizing a commercial kit (Amersham) that employs 125I-[Sar1,Ile8]ANG II and rabbit antiserum to ANG II. Standard curves were prepared, using [Asn1,Val5]ANG II (Sigma) as substrate. Results are expressed as femtomoles ANG II per gram kidney.

Densitometry of Northern blots and histoautoradiographs. Northern blots and histoautoradiographs were analyzed, using a computer-based image analysis software program (Image 1.47). In preliminary experiments, densitometry of autoradiographs of Northern blots revealed that signal intensity for the AT1 receptor was linear for RNA concentrations between 3 and 18 µg. For Northern blots, signal intensities were quantitated and are expressed as relative intensity of ischemic kidney to sham kidney signals. For histoautoradiographs, the images of kidney cortical and outer medullary regions were captured and outlined, and the mean signal density was recorded. Results are expressed as mean density of 125I-[Sar1,Ile8]ANG II binding (ischemic kidney/sham kidney).

Statistics. Results are expressed as means ± SE. The Z score test, which uses a standardized normal distribution,
was used to analyze data from Northern analyses and histoautoradiography. The Student’s t-test (unpaired) was used for single comparisons. Significance was assigned at \( P < 0.05 \).

**RESULTS**

Demonstration of acute renal failure with renal ischemia/reperfusion. In rats with renal ischemia/reperfusion injury, both serum creatinine concentration and BUN increased significantly, peaking 72 h after reperfusion and returning to levels not different from those in sham rats at 120 h (Fig. 1). In renal histological sections from rats with ischemia/reperfusion, extensive tubular necrosis was evident after 24 h, with preservation of glomerular morphology (not shown). At 120 h after reperfusion, kidney sections revealed intact tubules with flattened epithelium and papillary fronds.
extending into tubular lumens, representing proliferation of tubular epithelial cells, as previously described (1, 29). Apoptotic bodies were also present within tubules (Fig. 2). Together, these data indicate that our model induced extensive acute tubular necrosis, with recovery of filtration function by 120 h, consistent with other studies on rat renal ischemia/reperfusion injury (1).

Intrarenal ANG II levels. ANG II levels were measured in sham and postischemic kidneys 24 and 120 h after reperfusion. As shown in Fig. 3, intrarenal ANG II levels were significantly increased 24 h after reperfusion (sham: 102 ± 67 vs. ischemic: 529 ± 119 fmol/g; P < 0.025, n = 4) but did not differ from levels in sham kidneys after 120 h [sham: 143 ± 40 vs. ischemic: 90 ± 26 fmol/g; P = not significant (NS), n = 6]. Intrarenal ANG II levels in sham kidneys were comparable to those previously reported in adult rat kidney (39).

Expression of angiotensinogen and AT₁ receptor mRNAs in the postischemic kidney. To characterize further the effect of renal ischemia/reperfusion on the intrarenal renin-angiotensin system, renal angiotensinogen mRNA expression was studied by Northern blot analysis of renal cortical total RNA. As depicted in Fig. 4, in ischemic renal cortex at 6, 24, and 72 h after reperfusion, angiotensinogen mRNA was markedly decreased [angiotensinogen mRNA intensity (ischemic/sham) at 6 h: 0.42 ± 0.14, P < 0.04 vs. sham, n = 4; 24 h: 0.01 ± 0.01, P < 0.001 vs. sham, n = 5; 72 h: 0.02 ± 0.01, P < 0.001 vs. sham, n = 4]. Renal angiotensinogen gene expression recovered at 120 h after reperfusion, relative to sham rats [mRNA intensity (ischemic/sham): 0.82 ± 0.15, P = NS, n = 5].

Northern blot analysis was also utilized to study renal mRNA expression of AT₁ receptors after ischemia/reperfusion. In whole kidney cortex, AT₁ receptor mRNA did not significantly change after ischemia/reperfusion, although there was a tendency for increased expression at 1 and 3 h after reperfusion (Fig. 5). Proximal tubules are a major component of renal cortex and are targets of ischemia/reperfusion injury (2, 35). As shown in Fig. 6, there was a decrease in proximal tubular AT₁ mRNA expression immediately after 60 min of renal ischemia [AT₁ mRNA intensity (ischemic/sham) at 0 h: 0.45 ± 0.09; P < 0.001 vs. sham, n = 3], and mRNA continued to be significantly decreased at 1, 3, and 24 h after reperfusion, compared with sham rats (1 h: 0.60 ± 0.06, P < 0.001 vs. sham, n = 3; 3 h: 0.40 ± 0.07, P < 0.001 vs. sham, n = 3; 24 h: 0.30 ± 0.16, P < 0.03 vs. sham, n = 4). The decrease in AT₁ mRNA expression was not due to cell death, since the viability of Percoll gradient-isolated proximal tubule segments was >95%, determined by exclusion of the vital dye trypan blue. After 72 and 120 h of reperfusion, however, there were no significant differences between sham and ischemic rats in proximal tubule AT₁ receptor mRNA expression.

Because our Northern analyses could not distinguish between AT₁A and AT₁B receptors, known to be present in rat kidney (9), RT-PCR was performed on proximal tubule RNA at 120 h after reperfusion, utilizing primers specific for AT₁A or AT₁B receptors. In both sham and ischemic proximal tubule segments, cDNA bands for both AT₁A and AT₁B receptor products were generated by RT-PCR. AT₁A or AT₁B receptor mRNA quantities, corrected for β-actin PCR product, did not differ between sham and ischemic proximal tubule segments at 120 h after reperfusion (Fig. 7), suggesting recovery of mRNA for both receptor subtypes at this time point [AT₁A mRNA (sham/ischemic): 1.03 ± 0.03, P = NS, n =
Kidney cortex generated faint cDNA bands for the AT2 receptors, RT-PCR of total RNA isolated from normal rat proximal tubular segments and not from ischemia/reperfusion injury affected expression of AT2 receptor mRNA expression. RT-PCR for the AT2 receptor was also performed on RNA isolated from freshly dissected outer medulla. As shown in Fig. 9, faint signals for AT2 receptor cDNA were detectable inconsistently (2 of 3 experiments) by RT-PCR from outer medullas of sham rats at 120 h. In contrast, AT2 receptor cDNA was readily and consistently amplified from RNA isolated from outer medullas of rats at 120 h after ischemia/reperfusion.

Histoautoradiographic studies. To determine the effect of renal ischemia/reperfusion on ANG II binding sites, histoautoradiography was performed on rat kidney slices, using $^{125}$I-$\text{[Sar}^1,\text{Ile}^8\text{]}\text{ANG II}$. Image analysis of histoautoradiograms revealed that ischemia/reperfusion caused a significant decrease in total renal cortical binding 24 h after reperfusion, with no significant difference in binding between sham and ischemic kidneys at 0, 3, or 120 h after reperfusion [Fig. 10A; cortical image density (ischemic/sham) at 24 h: 0.48 ± 0.06, P < 0.001 vs. sham, n = 4]. Examination of histoautoradiograms revealed preservation of glomerular ANG II binding at all reperfusion times, with reduction in cortical binding in periglomerular areas 24 h after reperfusion (Fig. 10B). In outer medulla, renal ischemia/reperfusion was associated with significant reduction in ANG II binding at 3 and 24 h after reperfusion but not at 0 or 120 h [Fig. 11; outer medulla image density (ischemic/sham) at 3 h: 0.49 ± 0.10, P < 0.02 vs. sham, n = 5; 24 h: 0.35 ± 0.07, P < 0.001 vs. sham, n = 4].

In the adult rat, histoautoradiography of kidney slices has previously demonstrated almost complete displacement of radiolabeled ANG II binding by the AT1 receptor antagonist losartan, with little effect of the AT2 receptor antagonist PD-123319 (10^{-5} M) largely abolished binding of $^{125}$I-$\text{[Sar}^1,\text{Ile}^8\text{]}\text{ANG II}$, with no effect of addition of the AT2 antagonist PD-123319 (10^{-5} M; not shown). This suggests a predominance of
might be involved in the pathophysiology of acute renal failure in this model. Accordingly, studies were performed to determine the effect of $\text{AT}_1$ receptor blockade on tubular cell proliferation and on recovery of renal function after ischemic injury. Treatment of rats with losartan (25 mg/kg sc daily), starting at the time of reperfusion, had no effect on the percentage of cortical tubular cells expressing PCNA 24 and 72 h after reperfusion (Fig. 12; 24 h ischemic: 15.5 ± 1.6% vs. ischemic + losartan: 14.9 ± 2.2%, $P = \text{NS, n} = 3$; 72 h ischemic: 16.5 ± 2.9% vs. ischemic + losartan: 21.3 ± 3.0%, $P = \text{NS, n} = 6$). In contrast, although treatment with losartan had no effect on serum creatinine values 24 h after reperfusion (Fig. 13; ischemic: 253 ± 33 µM vs. ischemic + losartan: 263 ± 27 µM, $P = \text{NS, n} = 8$), it caused a significant decrease in serum creatinine values at 72 h (ischemic: 334 ± 69 µM vs. ischemic + losartan: 135 ± 28 µM, $P < 0.025, n = 6$).

**DISCUSSION**

$\text{ANG II}$ regulates intrarenal blood flow and stimulates renal tubular cell growth. The purpose of the present studies was to examine the effect of renal ischemia/reperfusion on expression of the intrarenal renin-angiotensin system and to determine the role of $\text{AT}_1$ receptors in the early postischemic phase in this model.

**Fig. 10.** A: Bar graph showing cortical density of $^{125}\text{I}-\text{[Sar}^1\text{,Ile}^8\text{]}\text{ANG II}$ binding at various reperfusion times compared with cortical binding in sham rats. Results are means ± SE. Values in parentheses are numbers of separate experiments. *$P < 0.001$ vs. sham. B: Representative histoautoradiograph of sham and ischemic kidney slices 24 h after reperfusion. Cortical and outer medullary $\text{ANG II}$ binding densities are reduced after reperfusion, with preservation of glomerular binding (intense black dots in cortex).

**Fig. 11.** Histoautoradiography of $\text{ANG II}$ binding in outer medulla. Bar graph shows density of $^{125}\text{I}-\text{[Sar}^1\text{,Ile}^8\text{]}\text{ANG II}$ binding in outer medulla at various reperfusion times compared with outer medullary binding in sham rats. Results are means ± SE. Values in parentheses are numbers of separate experiments. *$P < 0.02$, **$P < 0.001$ vs. sham.
Effect of renal ischemia on angiotensinogen mRNA and intrarenal ANG II.

Effect of renal ischemia on angiotensinogen mRNA and intrarenal ANG II. In rats with 40 min of renal artery occlusion, Rosenberg and Paller (25) determined that renal renin mRNA was undetectable 1 h after reperfusion but returned to normal levels at 24 and 48 h. Preliminary studies by Kim et al. (13) utilizing this model demonstrated marked suppression of renal renin gene expression 1 h after reperfusion, with only 50% recovery at 72 h. Plasma renin activity, in contrast, increases early after ischemic renal injury in humans (16). In the present studies, angiotensinogen mRNA was profoundly decreased in renal cortices up to 120 h after reperfusion, yet intrarenal ANG II levels were elevated at 24 h. Together, these data are consistent with the hypothesis that preformed substrate components of the renin-angiotensin system may be released early after ischemia, causing enhanced local ANG II production. It is also possible that ischemic injury causes release of preformed ANG II from proximal tubular cells, thought to be sources of ANG II production (27), or that other peptidases may be activated that are capable of cleaving released substrates, generating ANG II locally. This might explain why ACEI have variable effects on recovery after renal ischemic injury in dog and rat models (15, 17), whereas we observed that direct blockade of AT1 receptors caused a significant reduction in serum creatinine levels at 72 h after reperfusion.

In the present studies, the decrease in cortical angiotensinogen mRNA was not simply due to tubular necrosis, since we determined that tubules isolated after Percoll gradient centrifugation excluded trypan blue, and, indeed, mRNA levels were only barely detectable at 24 and 72 h after reperfusion (Fig. 4). Clearly, inhibition of gene transcription and/or reduction in mRNA stability accounts for the reduction in angiotensinogen mRNA. In this regard, renal EGF mRNA levels are also markedly reduced after ischemic injury because of ischemia-mediated interruption of the function of an upstream promoter region of the preproEGF gene (23). It is also noteworthy that a 12-kDa protein that stabilizes angiotensinogen mRNA by binding to its 3'-untranslated end has recently been isolated from liver polysomes (14). Whether this mRNA stabilizing protein exists in kidney and is disrupted by ischemia requires further study.

Effect of renal ischemia on intrarenal AT1 receptors. Our data are consistent with the hypothesis that tubular responsiveness to ANG II is diminished in the early postreperfusion period. Both proximal tubular AT1 receptor mRNA and renal cortical and outer medullary ANG II binding were decreased early after reflow. Interestingly, Northern analysis of RNA from total cortex did not demonstrate differences in AT1 mRNA, although cortical binding was diminished by densitometry. In contrast, histoautoradiographs revealed complete preservation of glomerular ANG II binding at all reperfusion times. This suggests that regulatory mechanisms for AT1 receptor mRNA differ between glomeruli and tubular cells. Indeed, our previous studies demonstrated that, whereas glomerular ANG II receptors are

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**Fig. 12.** Effect of losartan on cortical tubular expression of proliferating cell nuclear antigen (PCNA) at 24 and 72 h after reperfusion. Rats were treated without (ischemia) or with losartan (25 mg/kg sc daily; ischemia + losartan), starting at time of reperfusion, and kidneys were harvested for immunocytochemistry as described in METHODS. Results are percentages of nuclei staining for PCNA, expressed as means ± SE; n = 3 for 24 h and n = 6 for 72 h.

**Fig. 13.** Losartan causes a reduction in serum creatinine levels 72 h after reperfusion. Rats were treated without (ischemia) or with losartan (25 mg/kg sc daily; ischemia + losartan), starting at time of reperfusion. At indicated times, rats were killed, and blood was collected for measurement of serum creatinine. Results are means ± SE; n = 8 for 24 h and n = 6 for 72 h. *P < 0.025 vs. ischemia at 72 h.
downregulated by elevated levels of ANG II, proximal tubular AT₁ receptors are upregulated by ANG II (4). Our present data, however, suggest that this mechanism is not responsible for the differences in AT₁ receptor mRNA and ANG II binding, since intrarenal ANG II levels were elevated 24 h after reperfusion, a time at which proximal tubular AT₁ mRNA is decreased and glomerular binding is preserved.

Effect of losartan on tubular cell proliferation. ANG II has been shown to promote mitogenesis in proximal tubular cells in the presence of EGF (22) and to stimulate DNA synthesis in thick ascending limb cells (37). Our results, however, are consistent with the hypothesis that AT₁ receptor activation does not contribute to enhanced tubular cell proliferation in the early phase after ischemia/reperfusion injury. Proximal tubular AT₁ mRNA was downregulated up to 72 h after reperfusion. Expression of a nuclear marker for proliferation (PCNA) in cortical tubules was unaffected by treatment of rats with losartan at 24 and 72 h after reperfusion, times when tubular cell mitosis is increased after ischemic injury (7, 35). The percentage of PCNA-positive tubular cells in renal cortex in our study (14.9–21.3%) is consistent with values reported in rats with unilateral renal artery occlusion for 40 min (35). Our data do not exclude the possibility, however, that AT₁ receptors may affect proliferation in specific segments such as the S3 segment of proximal tubule in the cortex or the outer stripe of the outer medulla.

In the proximal tubule, ANG II stimulates synthesis of transforming growth factor (TGF)-β (36), a polypeptide considered to promote glomerulosclerosis and interstitial fibrosis. Basile et al. (1) demonstrated increased levels of TGF-β1 mRNA and protein early after acute renal ischemic injury in the rat, with persistent expression for 14 days (1). TGF-β was localized to regenerating tubular epithelial cells in the outer medulla and in proximal tubules, including within papillary proliferations (1). Our data suggest that increased intrarenal levels of ANG II early after reperfusion, associated with decreased tubular AT₁ receptors, might modulate local TGF-β production. Conceivably, TGF-β synthesis in the postischemic kidney could inhibit ANG II-stimulated proliferative responses.

Effect of losartan on serum creatinine. Our results reveal that daily losartan treatment caused a significant reduction in serum creatinine levels at 72 h after reperfusion. We selected both the 24- and 72-h time points for analysis, as our earlier studies revealed significant elevations of serum creatinine at these times (Fig. 1), signifying impaired glomerular filtration rate. As discussed above, this effect of losartan was unlikely to be due to modulation of tubular cell proliferation. Rather, we speculate that AT₁ receptor blockade has beneficial effects on intrarenal hemodynamics. Postischemic acute renal failure is associated with an increase in afferent arteriolar tone, at least partly due to impaired autoregulation (12), enhanced sensitivity to sympathetic nervous stimulation and local ANG II (24), and activation of tubuloglomerular feedback (31). Increased afferent arteriolar resistance results in reduction in glomerular perfusion pressure and contributes to filtration failure. Because we observed increased intrarenal levels of ANG II at 24 h after reperfusion, associated with preservation of glomerular ANG II binding by histoautoradiography, it is possible that the protective effect of losartan is due to reduction in afferent arteriolar tone and improved filtration.

AT₂ receptors in the postischemic kidney. A significant finding in the present studies is that new expression of AT₂ receptor mRNA occurs in proximal tubules and outer medulla in the postischemic kidney. AT₂ receptor mRNA was detected inconsistently in renal cortex from sham rats and at low levels in outer medulla. It is possible that S3 segments of proximal tubules are the source of AT₂ receptor mRNA in the postischemic outer medulla, since these segments are extensively injured in this model (35). Although we could not demonstrate AT₂ binding sites by histoautoradiography, this method may not be sensitive enough to detect AT₂ receptor protein on tubular cells. Previous studies revealed that AT₁ receptors are abundant in fetal kidney and that expression decreases rapidly after birth (28). The function of intrarenal AT₂ receptors remains unclear. Activation of AT₂ receptors has been linked to apoptosis in other cell types (38). The possible role of induction of AT₂ receptor mRNA in the postischemic kidney in mediating tubular cell apoptosis is unknown, although it is of interest that apoptosis is present in rat kidneys up to 4 mo after ischemic injury (29). Increased AT₂ receptor mRNA has been reported in the infarcted rat heart after 7 days (21) and in rat skin wounds after 3 days (34), suggesting involvement in remodeling. Because the intermediate filament protein vimentin, present in mesenchymal cells but not epithelial cells, is expressed in proximal tubule after ischemic injury (35), new expression of AT₂ receptor mRNA in proximal tubule may also signify dedifferentiation and recapitulation of developmental stages.

In summary, these studies demonstrate that renal ischemia/reperfusion causes an early increase in intrarenal ANG II levels, associated with reduction of mRNA for angiotensinogen and proximal tubular AT₁ receptors. Cortical ANG II binding is reduced, with maintenance of glomerular binding. AT₂ receptor mRNA is expressed at 120 h. Treatment of rats with losartan causes a reduction in serum creatinine at 72 h after reperfusion but has no effect on cortical tubular cell proliferation. This suggests that in the postischemic kidney, AT₁ receptor activation decreases glomerular filtration rate, perhaps by stimulation of intrarenal vasoconstriction.

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