CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

The Effect of Angiotensin-(1-7) in Mouse Unilateral Ureteral Obstruction

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Angiotensin-(1-7) is a ligand for the Mas receptor and may protect against tissue injury associated with renin-angiotensin system activation. We determined the effects of endogenous or exogenous angiotensin-(1-7) in mice with unilateral ureteral obstruction (UUO). Mice with UUO were treated with or without the angiotensin-(1-7) antagonist A779 or with 6, 24, or 62 $\mu\text{g/kg}$ per hour exogenous angiotensin-(1-7). After 10 days, kidneys were harvested for histology, immunoblots, and measurement of NADPH oxidase. Compared with controls, A779 treatment significantly increased fibronectin, transforming growth factor- β , and α -smooth muscle actin expression in obstructed kidneys and enhanced tubulointerstitial injury, apoptosis, and NADPH oxidase. Unexpectedly, administration of angiotensin-(1-7) to mice with UUO caused injury in obstructed kidneys compared with controls and increased macrophage infiltration. In obstructed kidneys from mice with gene deletion of Mas (*Mas*^{-/-}), apoptosis and macrophage infiltration were increased compared with wild-type mice. Angiotensin-(1-7) (but not A779) further increased apoptosis and macrophage influx in obstructed kidneys from *Mas*^{-/-} mice, compared with untreated *Mas*^{-/-} mice. These data indicate that endogenous angiotensin-(1-7) protects against kidney injury in UUO. In mice with or without the Mas receptor, however, delivery of exogenous angiotensin-(1-7) worsens kidney damage. The results suggest dose-dependent effects of angiotensin-(1-7) in the kidney in UUO, with endogenous angiotensin-(1-7) promoting repair pathways via interaction with Mas and higher amounts exacerbating injury. (*Am J Pathol* 2015; 185: 729–740; <http://dx.doi.org/10.1016/j.ajpath.2014.11.013>)

The renin-angiotensin system (RAS) is critically involved in the regulation of extracellular fluid volume and blood pressure. This function is primarily mediated by the octapeptide angiotensin (Ang) II, which is formed by angiotensin-converting enzyme (ACE)-dependent cleavage of the inactive decapeptide Ang I. In the kidney, RAS activation occurs in disease states, and enhanced signaling of Ang II through G protein-coupled AT₁ receptors contributes to progressive nephron injury and tubulointerstitial fibrosis.¹ Thus, inhibition of Ang II production or pharmacologic blockade of AT₁ receptors significantly protects against kidney injury in experimental models and humans with kidney disease, independent of blood pressure lowering alone.^{1,2}

Ang-(1-7) is a heptapeptide member of the RAS, formed by the hydrolysis of Ang II by ACE2 and other peptidases.^{3,4} Ang-(1-7) interacts with its own G protein-coupled receptor, the Mas protein,⁵ exhibits vasodilatory properties,

and inhibits Ang II-mediated signaling in several tissues.⁴ The Ang-(1-7)/Mas axis is proposed to counterregulate the RAS/AT₁ receptor axis and thereby could serve as a therapeutic target in cardiovascular and kidney diseases. In this regard, Ang-(1-7) exerts beneficial effects on cardiovascular function in models of hypertension or cardiac disease and also protects against kidney injury in experimental diabetic nephropathy.⁴

Notwithstanding these protective effects of Ang-(1-7) considerable controversy surrounds the actions of Ang-(1-7) in the kidney, and cell- and nephron segment-specific actions were described. For example, Ang-(1-7) dilates pre-constricted afferent arterioles,⁶ inhibits Ang II-stimulated mitogen-activated protein kinase signaling in proximal

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tubular cells,⁷ and yet stimulates mitogen-activated protein kinase phosphorylation and production of fibronectin, collagen IV, and transforming growth factor- β 1 (TGF- β 1) in glomerular mesangial cells.⁸ To add to this complexity, Esteban et al⁹ reported that mice with gene deletion of the Mas receptor (*Mas*^{-/-}) that were subjected to unilateral ureteral obstruction (UUO) were protected against kidney injury, with reductions in extracellular matrix proteins, apoptosis, and inflammatory cell infiltrates in obstructed kidneys. Furthermore, wild-type mice with UUO infused with Ang-(1-7) exhibited more severe kidney fibrotic changes than did untreated mice.⁹ These data suggest that Ang-(1-7) may exert Mas-mediated adverse effects in UUO, exacerbating rather than protecting against kidney damage.

The potential role of Ang-(1-7) as a protector or mediator of kidney tubulointerstitial fibrosis therefore requires further investigation. Our aim was to examine the effects of endogenous Ang-(1-7) in UUO, by treating wild-type C57BL/6 mice with the Ang-(1-7) antagonist A779. We also studied the dose-dependent effects of exogenous Ang-(1-7) in this model and determined effects in the *Mas*^{-/-} mouse. Our results reveal that, although endogenous Ang-(1-7) mediates protection against kidney injury in UUO, administration of exogenous Ang-(1-7) exacerbates kidney damage, at least partly via non-Mas-dependent pathways.

Materials and Methods

UUO Model

Male C57BL/6 mice were obtained from Charles River Laboratories (Montreal, QC, Canada). At 12 weeks of age, mice were subjected to UUO surgery, essentially as described.^{9,10} Briefly, under halothane anesthesia a flank incision was made, and the left ureter was isolated and divided between two silk ligatures. The right, unobstructed kidney served as a control. Muscle incisions were closed with 6-0 silk ligatures, and skin incisions were closed with metal clips, which were removed after 7 days. In some experiments, C57BL/6 mice with deletion of the Mas proto-oncogene (*Mas*^{-/-}) [kindly provided by Dr. Michael Bader (Max-Delbruck Center for Molecular Medicine, Berlin, Germany) and Dr. Robson A. Santos (Federal University of Minas Gerais, Belo Horizonte, Brazil)] were subjected to UUO surgery. These mice were genotyped by PCR to confirm deletion of the *Mas* gene before use. At the time of surgery, mice were implanted with s.c. osmotic minipumps (Alzet model 1002; Alza Corp., Palo Alto, CA) for delivery of A779 at 31 μ g/kg per hour; Ang-(1-7) at 6, 24, or 62 μ g/kg per hour; or vehicle (sterile saline).^{9,11,12} Mice had free access to food and water at all time points and were sacrificed 10 days after UUO surgery, at which time tissue and blood were harvested for further analyses. All experimental protocols were approved by the University of Ottawa Animal Care and Use Committee and performed according to recommendations of the Canadian Council for Animal Care.

Blood Pressure Measurement

Systolic blood pressure (SBP) was monitored by tail cuff plethysmography (BP-2000; Visitech Systems, Apex, NC) as described.¹³ Values for SBP are expressed as the average of 8 to 10 measures per mouse.

Plasma Analytes and Hematocrit Measurements

At the time of sacrifice, blood samples were collected by cardiac puncture with the use of a heparinized syringe. Blood was transferred to 1.5-mL tubes that contained an ice-cold solution of protease inhibitor cocktail (0.01 mmol/L *p*-hydroxy-mercury benzoate, 1.5 mmol/L *o*-phenanthroline, 0.01 mmol/L phenylmethylsulfonyl fluoride, 0.05 mmol/L pepstatin A, and 10 mmol/L EDTA), centrifuged at 3000 $\times g$ for 10 minutes at 4°C, and then stored at -80°C. Plasma analytes were measured by Idexx Laboratories (Markham, ON, Canada). Hematocrit measurements were performed with a microhematocrit capillary tube reader (Critocaps; Oxford Lab, Baxter, Deerfield, IL).

Assay of Ang II and Ang-(1-7)

Kidney amounts of Ang II were measured with a commercial peptide radioimmunoassay kit that contained an Ang II-selective polyclonal antibody and ¹²⁵I-Ang II (Peninsula Laboratories, San Carlos, CA), and kidney Ang-(1-7) amounts were measured with a commercial peptide enzyme immunoassay kit that contained an Ang-(1-7)-selective polyclonal antibody (Peninsula Laboratories), as described.¹⁴ The manufacturer's instructions were followed for both assays. Briefly, kidney samples were homogenized in 1 mL of ice-cold methanol in the presence of protease inhibitors (50 mmol/L EDTA, 0.5 mmol/L *o*-phenanthroline, 1 mmol/L *N*-ethylmaleimide, and 0.1 mmol/L pepstatin A). Samples were centrifuged at 12,000 $\times g$ for 10 minutes at 4°C. The supernatant fluids were dried in a vacuum centrifuge, and the dry residue was reconstituted in 4 mL of 50 mmol/L sodium phosphate buffer (pH 7.4) that contained 0.1% bovine serum albumin and was divided into two equal fractions, one for Ang II assay and the other for Ang-(1-7) assay. C₁₈ Sep-Pak columns (Waters, Milford, MA) were equilibrated with 1 mL of a buffer that consisted of 60% acetonitrile, 1% trifluoroacetic acid, and 39% distilled water, followed by three 3-mL washes with 1% trifluoroacetic acid. After sample application, each C₁₈ Sep-Pak column was washed twice with 3 mL of 1% trifluoroacetic acid. The angiotensin peptides were eluted in polypropylene tubes with 2 $\times$ 1 mL of the same buffer used to equilibrate the columns. Eluants were concentrated to dryness by using a vacuum centrifuge and were stored at -80°C until the radioimmunoassay or enzyme immunoassay was performed. In preliminary experiments, the Ang II radioimmunoassay demonstrated 0% cross-reactivity to Ang-(1-7), and the Ang-(1-7) enzyme immunoassay showed 0% cross-reactivity with Ang II.

Measurement of NADPH Oxidase Activity

The lucigenin chemiluminescence assay was used to determine NADPH oxidase activity in tissue homogenates.¹⁵ Briefly, tissues were homogenized in lysis buffer (20 mmol/L KH_2PO_4 , 1 mmol/L EGTA, 1 $\mu\text{g}/\text{mL}$ aprotinin, 1 $\mu\text{g}/\text{mL}$ leupeptin, 1 $\mu\text{g}/\text{mL}$ pepstatin, and 1 mmol/L phenylmethylsulfonyl fluoride) and kept on ice. The reaction was initiated by adding 0.1 mmol/L NADPH to the suspension that contained 20 μg of prepared sample, 5 $\mu\text{mol}/\text{L}$ lucigenin, and assay phosphate buffer. Luminescence was measured with a luminometer (Lumistar Galaxy; BMG Technologies, Allmendgruen, Germany) every 1.8 seconds for 3 minutes. Buffer blank was subtracted from each reading.

Immunohistochemistry

At sacrifice, kidneys were fixed in 4% formalin, dehydrated, and embedded in paraffin. Tissue was cut into 5- μm sections, stained with periodic acid-Schiff, and analyzed in a blinded fashion (A.G.) for tubulointerstitial injury/fibrosis, apoptosis, and macrophage infiltration, using an Axioscope 2: Imager A1 microscope (Carl Zeiss Canada Ltd, Toronto, ON, Canada), with image analysis by ImagePro-Plus software version 7 (Media Cybernetics, Bethesda, MD). Tubulointerstitial injury/fibrosis was scored on five images per kidney section (at $\times 600$ magnification) by using a semi-quantitative scale, as described.¹⁶ For macrophage infiltration, sections were deparaffinized in xylene, rehydrated with 100% ethanol, and treated with proteinase K for 25 minutes at 37°C, followed by washing with 1 $\times$ phosphate-buffered saline. Sections were blocked with 10% donkey serum in 1% bovine serum albumin in phosphate-buffered saline for 1 hour before incubation with dilution 1:200 anti-F4/80 antigen rat monoclonal anti-mouse antibody (eBioscience, San Diego, CA) for 12 to 18 hours at 4°C. Endogenous peroxidase activity was blocked by incubating the slides with 0.3% H_2O_2 for 30 minutes at room temperature. Secondary biotinylated donkey anti-rat IgG (dilution 1:200) was applied for 1 hour at room temperature, followed by streptavidin-conjugated horseradish peroxidase (Vector Laboratories, Burlingame, CA) for 30 minutes and consequent diaminobenzidine substrate development. Five images were analyzed per section for positive staining area at $\times 600$ magnification. Results are expressed as percentage of positive F4/80 staining.

Apoptosis was assessed with the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) Apoptosis Detection Kit (Genscript, Piscataway, NJ) according to the manufacturer's instructions. Kidney sections (two per kidney, 10 fields/section) were visualized at $\times 600$ magnification. Results are expressed as the number of TUNEL⁺ nuclei per field. DNase-treated sections and intestinal sections were used as positive controls.

Immunoblot Analysis

Renal cortical tissue was homogenized with a COE CapMixer desktop homogenizer (GC America, Alsip, IL). Lysis buffer [1 $\times$ phosphate-buffered saline, 1% tert-Octylphenoxy poly(oxyethylene)ethanol, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris-HCl, 2.0 mmol/L Na_3PO_4 , 1 mmol/L phenylmethylsulfonyl fluoride, 1 $\mu\text{g}/\text{mL}$ leupeptin, 100 $\mu\text{g}/\text{mL}$ aprotinin, 100 $\mu\text{g}/\text{mL}$ pepstatin] was added to homogenized tissue, and samples were centrifuged at $13,000 \times g$ for 5 minutes. Supernatant fluid was collected and stored at -80°C for determination of protein concentration and immunoblotting. Protein concentration was measured by Bio-Rad DC Protein Assay (Bio-Rad, Mississauga, ON, Canada). Samples were prepared with equal amounts of protein lysate (20 μg) and 6 $\times$ loading buffer (7.0% 4 $\times$ Tris-HCl/SDS, 3.0% mL glycerol, 0.1 g/mL SDS, 6.0% β -mercaptoethanol, 0.12 mg/mL bromophenol blue) with distilled water to a total volume of 36 μL for loading. Samples were boiled for 5 minutes before loading on 7.5% to 15% SDS-polyacrylamide gels and were then transferred to nitrocellulose membranes (Bio-Rad). Membranes were washed in Tris-buffered saline (pH 7.6) that contained 0.1% Tween (TBS-T) before blocking with 5% skim milk in TBS-T for 1 hour at room temperature. Membranes were incubated with primary antibody for 12 to 18 hours at 4°C. After incubation, membranes were washed four times for 5 minutes with TBS-T before incubation with secondary antibodies for 1 hour at room temperature and washed four times for 5 minutes with TBS-T. Primary antibodies were used against TGF- β (dilution 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), fibronectin (dilution 1:5000; Sigma, St. Louis, MO), α -smooth muscle actin (α -SMA; dilution; 1:10,000; Sigma-Aldrich), ACE2 (dilution 1:1000; R&D Systems, Minneapolis, MN), ACE (dilution 1:1000; R&D Systems), and glyceraldehyde-3-phosphate dehydrogenase (dilution 1:5000; Millipore, Billerica, MA). Proteins were detected by enhanced chemiluminescence (GE Healthcare Bio-Sciences, Baie d'Urfe, QC, Canada). To control for protein loading, membranes were stripped and reprobed with antibody to glyceraldehyde-3-phosphate dehydrogenase. Immunoblot signals were quantified by densitometry and corrected for total protein amounts with the use of image-analysis software (Scion Image version 4.0.3.2; Scion Corporation, Houston, TX).

ACE2 and ACE Activity Assays

ACE2 activity in kidney homogenates was determined with a fluorogenic ACE2 synthetic substrate, with or without the ACE2 antagonist MLN-4760, as we have described.¹³ Kidney homogenate ACE activity was measured with a synthetic substrate in the presence or absence of captopril, as we have reported.¹⁴

Table 1 Effect of A779 on SBP in UUO

Blood pressure	Vehicle		A779	
	Baseline	UUO	Baseline	UUO
SBP (mm Hg)	102 ± 3	114 ± 5	109 ± 5	109 ± 7

Data are expressed as means ± SEM ($n = 12$).

SBP, systolic blood pressure; UUO, unilateral ureteral obstruction (10 days).

Statistical Analysis

Data were analyzed with SigmaStat software version 3.5 (Systat Software, San Jose, CA). All values are reported as means ± SEM. Statistical comparisons were made with two-way analysis of variance for multiple group comparisons or Student's *t*-test for comparison between two groups. $P < 0.05$ was considered significant.

Results

Effect of the Ang-(1-7) Antagonist A779 in UUO

Initial studies determined the effect of the Ang-(1-7) antagonist A779 (31 µg/kg per hour for 10 days) in the mouse UUO model. At baseline, SBP did not differ between mice that received vehicle or A779, and UUO did not significantly affect SBP in either group (Table 1). After 10 days of UUO, no significant differences were found in body weight, kidney weight, kidney/body weight, hematocrit, or plasma analyte concentrations (including urea and creatinine) between vehicle- or A779-treated mice (Table 2).

Table 2 Effect of A779 on Weights, Hematocrit, and Plasma Analytes in UUO

Variable	Vehicle	A779
BW (g)	(26.4 ± 0.3) 27.4 ± 0.3	(27.1 ± 0.1) 27.8 ± 0.1
UKW/BW (mg/g)	9.6 ± 0.6	9.8 ± 0.4
OKW/BW (mg/g)	8.1 ± 0.4	8.2 ± 0.4
HCT (%)	41.9 ± 1.2	41.7 ± 1.1
Na ⁺ (mmol/L)	146.2 ± 1.0	147.3 ± 0.4
K ⁺ (mmol/L)	4.9 ± 0.2	4.2 ± 0.1
HCO ₃ ⁻ (mmol/L)	23.5 ± 0.7	22.0 ± 1.5
Ca ²⁺ (mmol/L)	2.3 ± 0.1	2.4 ± 0.1
PO ₄ ²⁻ (mmol/L)	2.1 ± 0.2	2.3 ± 0.2
Albumin (g/L)	22.7 ± 3.7	27.7 ± 1.0
Cholesterol (mmol/L)	2.4 ± 0.2	2.6 ± 0.2
Triglycerides (mmol/L)	0.7 ± 0.2	0.9 ± 0.1
Urea (g/dL)	9.4 ± 1.1	8.7 ± 0.4
Creatinine (µmol/L)	12.2 ± 1.1	10.8 ± 0.9

Data are expressed as means ± SEM ($n = 6$), taken 10 days after UUO, except for results in parentheses (baseline).

BW, body weight; HCT, hematocrit; OKW, obstructed kidney weight; UKW, unobstructed kidney weight; UUO, unilateral ureteral obstruction.

Effect of A779 on Kidney Cortical Fibronectin, TGF-β, and α-SMA

UUO caused a significant increase in kidney cortical expression of the extracellular matrix protein fibronectin, the profibrotic cytokine TGF-β, and α-SMA in obstructed kidneys, determined by immunoblot analysis (Figure 1, A–C). Treatment with A779 caused a further significant increase in cortical expression of each of these proteins in obstructed kidneys. Furthermore, A779 increased expression of cortical fibronectin and α-SMA in unobstructed kidneys, compared with vehicle-treated controls.

Effect of A779 on Kidney NADPH Oxidase Activity

In mice treated with vehicle, UUO had no significant effect on renal cortical NADPH oxidase activity. By contrast, A779 induced a significant increase in cortical NADPH oxidase in obstructed kidneys (Figure 1D) ($n = 6$; $P < 0.01$ versus unobstructed kidneys).

Effect of A779 on Kidney Histology

In the UUO model, unobstructed kidneys showed no evidence of tubulointerstitial fibrosis, as expected (scores equal to zero). By contrast, obstructed kidneys from vehicle-treated mice demonstrated histologic tubulointerstitial injury, and treatment with A779 caused a further significant increase in tubulointerstitial fibrosis scores (Figure 2A). Similarly, although apoptosis by TUNEL staining was not observed in unobstructed kidneys, UUO was associated with a significant increase in apoptosis, and this was further exacerbated by A779 (Figure 2B). UUO caused a significant increase in kidney macrophage infiltration in vehicle-treated mice, although A779 had no effect on this response (Figure 2C).

Effect of Exogenous Ang-(1-7) in UUO

The effects of A779 suggested that endogenous Ang-(1-7) is protective in UUO, by preventing matrix protein production and tubulointerstitial fibrosis and apoptosis. Accordingly, further experiments determined the effects of exogenous delivery of Ang-(1-7) in the UUO model. Mice were treated with vehicle or Ang-(1-7) at three different doses (6, 24, or 62 µg/kg per hour) for 10 days after UUO.

At baseline, SBPs and body weights were not different between groups (data not shown). Treatment with Ang-(1-7) at any dose did not affect SBP, body weight, kidney weight, kidney/body weight ratios, hematocrit, or any plasma analytes, compared with mice that received vehicle alone (not shown).

Effect of Exogenous Ang-(1-7) on Kidney Cortical Fibronectin, TGF-β, and α-SMA

In obstructed kidneys, Ang-(1-7) at each of the three doses administered caused a significant increase in kidney

Figure 1 Effect of A779 on kidney cortical expression of fibronectin (A), TGF- β (B), α -SMA (C), and NADPH oxidase (D) activity in UUO. **A–C:** Expression of each protein in unobstructed (gray bars) and obstructed (black bars) kidney cortices is shown, from male C57BL/6 mice that underwent UUO for 10 days and received either vehicle or 31 μ g/kg per hour A779. Representative immunoblots are depicted above each graph. **B:** The representative blots are from two separate membranes derived from gels run and exposed at the same time (lanes 1–4, and lanes 5–8, respectively). Lanes 1 and 2, unobstructed, vehicle; lanes 3 and 4, obstructed, vehicle; lanes 5 and 6, unobstructed, A779; lanes 7 and 8, obstructed, A779. Data are expressed as means $\pm$ SEM. $n = 12$ for immunoblots; $n = 6$ for NADPH oxidase. $**P < 0.01$, $***P < 0.001$ versus unobstructed in same group (A–C); $^{\dagger}P < 0.05$ versus unobstructed, vehicle (A and C); $^{\ddagger}P < 0.05$, $^{\text{††}}P < 0.01$, and $^{\text{†††}}P < 0.001$ versus obstructed, vehicle (A–D); $^{\text{§§}}P < 0.01$ versus unobstructed, A779 group (D). α -SMA, α -smooth muscle actin; TGF- β , transforming growth factor- β ; UUO, unilateral ureteral obstruction.

cortical expression of fibronectin, compared with obstructed kidneys from vehicle-treated mice (Figure 3A). Renal cortical expression of TGF- β in obstructed kidneys was increased by Ang-(1-7) treatment at 6 and 24 μ g/kg per hour, compared with obstructed kidneys from vehicle-treated mice, whereas Ang-(1-7) at 62 μ g/kg per hour did not cause an additional increase (Figure 3B). Ang-(1-7)

increased α -SMA expression in obstructed kidneys, compared with obstructed kidneys, in vehicle-treated mice, although this only reached statistical significance at the dose of 62 μ g/kg per hour (Figure 3C). In unobstructed kidneys, 24 μ g/kg per hour Ang-(1-7) significantly increased expression of cortical fibronectin and TGF- β , compared with vehicle-treated controls.

Figure 2 Effect of A779 on tubulointerstitial fibrosis (A), apoptosis (B), and macrophage infiltration (C) in UUO. **A:** Periodic acid-Schiff staining of paraffin-embedded kidney sections from mice that underwent UUO and treatment with vehicle or 31 μ g/kg per hour A779. Shown are collapsed fibrotic glomerulus (arrow), dilated atrophic tubules (asterisks), and interstitial monocytes/macrophages (arrowheads). Graph below shows tubulointerstitial fibrosis scores after treatment with or without A779. Tubulointerstitial fibrosis scores were zero in unobstructed kidneys. **B:** TUNEL staining of kidney cortices from mice that underwent UUO for 10 days and treatment with vehicle or A779. Arrows indicate apoptotic cells. Graph shows quantification of apoptotic nuclei in obstructed kidneys. Unobstructed kidneys had only few apoptotic nuclei. **C:** Anti-F4/80 staining of paraffin-embedded kidney sections of mice that underwent UUO for 10 days and received vehicle or A779. Arrows show infiltrating macrophages. Graph shows quantification of F4/80 $^{+}$ area from obstructed (black bars) and unobstructed (gray bars) kidneys. Values are means $\pm$ SEM. $n = 6$. $***P < 0.001$. Original magnification, $\times 600$. O, obstructed kidney; PAS, periodic acid-Schiff; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; U, unobstructed kidney; UUO, unilateral ureteral obstruction.

Figure 3 Effect of exogenous angiotensin Ang-(1-7) on kidney cortical expression of fibronectin (A), TGF- β (B), α -SMA, (C) and NADPH oxidase (D) activity in UUO. Expression of each protein is shown in unobstructed (gray bars) and obstructed (black bars) kidney cortices from male C57BL/6 mice that underwent UUO for 10 days and received vehicle or 6, 24, or 62 μ g/kg per hour Ang-(1-7). Representative immunoblots are depicted above each graph. Lane 1, unobstructed, vehicle; lane 2, obstructed, vehicle; lane 3, unobstructed 6 μ g/kg per hour Ang-(1-7); lane 4, obstructed 6 μ g/kg per hour Ang-(1-7); lane 5, unobstructed 24 μ g/kg per hour Ang-(1-7); lane 6, obstructed 24 μ g/kg per hour Ang-(1-7); lane 7, unobstructed 62 μ g/kg per hour Ang-(1-7); lane 8, obstructed 62 μ g/kg per hour Ang-(1-7). Data are expressed as means $\pm$ SEM. $n = 6$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus unobstructed, vehicle group (A, B, and D); $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.001$ versus unobstructed in corresponding group (A and B); $^{\ddagger}P < 0.05$, $^{\ddagger\ddagger}P < 0.01$, $^{\ddagger\ddagger\ddagger}P < 0.001$ versus unobstructed, vehicle (A–D); $^{\S\S}P < 0.01$, $^{\S\S\S}P < 0.001$ versus unobstructed kidneys within same group (C); $^{\¶\¶\¶}P < 0.001$ versus unobstructed kidneys within corresponding group (D); $^{\¶}P < 0.05$ versus unobstructed, 6 μ g/kg per hour Ang-(1-7) (D). Ang, angiotensin; α -SMA, α -smooth muscle actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TGF- β , transforming growth factor- β ; UUO, unilateral ureteral obstruction.

Effect of Exogenous Ang-(1-7) on Kidney NADPH Oxidase Activity

Although UUO was not associated with increases in kidney cortical NADPH oxidase activity in vehicle-treated mice, exogenous Ang-(1-7) caused a significant increase in NADPH oxidase activity in obstructed kidneys, at each of the three delivered doses (Figure 3D). Ang-(1-7) at 24 and 62 μ g/kg per hour also induced a significant increase in NADPH oxidase activity in unobstructed kidneys, compared with vehicle-treated mice.

Effect of Exogenous Ang-(1-7) on Kidney Histology

In obstructed kidneys, exogenous Ang-(1-7) increased tubulointerstitial fibrosis scores compared with vehicle-treated mice, although this only reached statistical significance at a dose of 62 μ g/kg per hour (Figure 4A). Ang-(1-7) increased apoptosis in UUO, compared with vehicle-treated mice, but only at doses of 6 and 62 μ g/kg per hour (Figure 4B). At each of the three administered doses, Ang-(1-7) caused a significant increase in macrophage infiltration in obstructed kidneys, compared with obstructed kidneys from vehicle-treated mice (Figure 4C). Macrophage infiltration was unaffected in unobstructed kidneys.

Effect of A779 and Ang-(1-7) in *Mas*^{-/-} Mice

To determine the role of the Mas receptor in responses to A779 and exogenous Ang-(1-7) in UUO, studies were conducted in C57BL/6 mice with *Mas*^{-/-}, treated with or without A779 at 31 μ g/kg per hour or Ang-(1-7) at 62 μ g/kg per hour for 10 days. At baseline, SBP, body weight, kidney weight, kidney/body weight ratio, and hematocrit did not differ between wild-type mice and *Mas*^{-/-} mice, and

treatment with Ang-(1-7) or A779 had no significant effect on any of these variables (data not shown).

In obstructed kidneys from *Mas*^{-/-} mice, cortical expression of fibronectin, TGF- β , and α -SMA was significantly increased compared with unobstructed kidneys (Figure 5). Although expression of fibronectin and α -SMA did not differ between obstructed kidneys from wild-type mice and *Mas*^{-/-} mice, expression of TGF- β was significantly enhanced in *Mas*^{-/-} mice (Figure 5). Treatment with A779 or Ang-(1-7) had no significant effect on cortical expression of fibronectin, TGF- β , or α -SMA in obstructed kidneys from *Mas*^{-/-} mice, compared with vehicle-treated *Mas*^{-/-} mice. No significant differences were found in expression of these proteins in unobstructed kidneys, across the groups.

Histologic analysis revealed significant tubulointerstitial injury in obstructed kidneys from *Mas*^{-/-} mice, although scores did not differ from those in obstructed kidneys from wild-type mice (Figure 6A). In *Mas*^{-/-} mice, A779 or exogenous Ang-(1-7) did not significantly increase injury scores in obstructed kidneys. Obstructed kidneys from *Mas*^{-/-} mice exhibited significant increases in apoptosis and macrophage infiltration compared with wild-type mice with UUO (Figure 6, B and C). In *Mas*^{-/-} mice, exogenous Ang-(1-7) exacerbated apoptosis and macrophage infiltration ($P < 0.05$ and $P < 0.01$ versus *Mas*^{-/-} alone, respectively), whereas A779 had no significant effect (Figure 6, B and C).

Effect of UUO on Kidney Ang II and Ang-(1-7) Amounts

In vehicle-treated mice with UUO, obstructed kidneys demonstrated increased amounts of Ang II, compared with unobstructed kidneys (Figure 7A). Treatment with A779 caused a further significant increase in Ang II amounts in obstructed kidneys ($P < 0.01$ versus vehicle), with no effect

Figure 4 Effect of exogenous Ang-(1-7) on tubulointerstitial fibrosis (A), apoptosis (B), and macrophage infiltration (C) in UUO. **A:** Periodic acid-Schiff staining of paraffin-embedded kidney sections from male C57BL/6 mice that underwent UUO and treatment with vehicle or 6, 24, or 62 μg/kg per hour Ang-(1-7). Shown are collapsed fibrotic glomeruli (arrows), dilated atrophic tubules (asterisks), and interstitial monocytes/macrophages (arrowheads). Graph at right shows tubulointerstitial fibrosis scores. Scores were zero in unobstructed kidneys. **B:** TUNEL staining of kidney cortices. Arrows indicate apoptotic cells. Graph shows quantification of apoptotic nuclei, as assessed by TUNEL assay. Unobstructed kidneys had few apoptotic nuclei. **C:** Anti-F4/80 staining of paraffin-embedded kidney sections. Arrows indicate infiltrating macrophages. Graph shows quantification of F4/80⁺ area from obstructed and unobstructed kidneys. Values are means ± SEM. $n = 6$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus vehicle (A and B); †† $P < 0.01$ versus unobstructed, vehicle (C); ††† $P < 0.001$ versus unobstructed kidneys within corresponding group (C); §§§ $P < 0.001$ versus obstructed, vehicle (C). Original magnification, ×600. Ang, angiotensin; O, obstructed kidney; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; U, unobstructed kidney; UUO, unilateral ureteral obstruction.

on Ang II amounts in unobstructed kidneys. Administration of exogenous Ang-(1-7) was also associated with a dose-dependent increase in Ang II in obstructed kidneys, which significantly exceeded amounts in vehicle-treated mice at the dose of 62 μg/kg per hour ($P < 0.001$). In obstructed kidneys from *Mas*^{-/-} mice, Ang II amounts were significantly elevated compared with unobstructed kidneys but did not differ from those in wild-type mice. In *Mas*^{-/-} mice, neither A779 nor Ang-(1-7) at 62 μg/kg per hour significantly affected kidney Ang II (Figure 7B).

In each group of mice, obstructed kidneys demonstrated reduced cortical ACE2 expression and ACE2 activity

compared with unobstructed kidneys, although no significant differences were found between groups (Supplemental Figure S1). Kidney cortical ACE expression and ACE activity were also decreased in obstructed kidneys from all groups of mice, although no significance was found between group differences (Supplemental Figure S2).

The effect of exogenous Ang-(1-7) on kidney Ang-(1-7) amounts was determined by a peptide enzyme immunoassay. Obstructed kidneys from mice treated with each of the three doses of Ang-(1-7) exhibited increased amounts of Ang-(1-7), compared with vehicle-treated mice or unobstructed kidneys, although this only reached statistical

Figure 5 Effect of A779 and exogenous Ang-(1-7) on kidney cortical expression of fibronectin (A), TGF- β (B), and α -SMA (C) in *Mas*^{-/-} mice with UUO. Expression of each protein is shown in unobstructed (gray bars) and obstructed (black bars) kidney cortices from mice that underwent UUO for 10 days. At far left of each graph are data from vehicle-treated wild-type mice. Data to the right are from *Mas*^{-/-} mice that received vehicle, 31 μ g/kg per hour A779, or 62 μ g/kg per hour Ang-(1-7) for 10 days. Representative immunoblots are depicted above each graph. Lane 1, unobstructed, wild-type, vehicle; lane 2, obstructed, wild-type, vehicle; lane 3, unobstructed, *Mas*^{-/-} + vehicle; lane 4, obstructed, *Mas*^{-/-} + vehicle; lane 5, unobstructed *Mas*^{-/-} + A779; lane 6, obstructed *Mas*^{-/-} + A779; lane 7, unobstructed *Mas*^{-/-} + 62 μ g/kg per hour Ang-(1-7); lane 8, obstructed *Mas*^{-/-} + 62 μ g/kg per hour Ang-(1-7). Data are expressed as means $\pm$ SEM. $n = 6$. ** $P < 0.01$, *** $P < 0.001$ versus unobstructed, within the corresponding group (A–C); † $P < 0.05$ versus obstructed, vehicle, wild-type (B). Ang, angiotensin; α -SMA, α -smooth muscle actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TGF- β , transforming growth factor- β ; UUO, unilateral ureteral obstruction.

significance at the doses of 6 and 62 μ g/kg per hour (Figure 7C). Treatment with A779 had no effect on Ang-(1-7) amounts in obstructed or unobstructed kidneys, compared with vehicle-treated mice. *Mas*^{-/-} mice had similar kidney amounts of Ang-(1-7) compared with wild-type mice (obstructed or unobstructed). Treatment of *Mas*^{-/-} mice with A779 did not affect kidney Ang-(1-7) amounts, whereas 62 μ g/kg per hour exogenous Ang-(1-7) caused an increase within obstructed kidneys and to a lesser extent in unobstructed kidneys (Figure 7D).

Discussion

Our study has several major findings that pertain to the role of Ang-(1-7) in the mouse UUO model of kidney fibrosis. First, inhibition of endogenous Ang-(1-7) with A779 significantly exacerbated injury in obstructed kidneys. Second, and unexpectedly, the administration of exogenous Ang-(1-7) at three separate doses in this model worsened kidney injury and stimulated kidney macrophage infiltration. Third, in mice with genetic deletion of the Mas protein [the putative receptor for Ang-(1-7)⁵], A779 had no significant effect in the UUO model. By contrast, although 62 μ g/kg per hour exogenous Ang-(1-7) did not affect expression of fibronectin, TGF- β , and α -SMA in obstructed kidneys from *Mas*^{-/-} mice, it increased apoptosis and macrophage infiltration, compared with untreated *Mas*^{-/-} mice. Finally, we observed increased Ang II amounts in obstructed kidneys from wild-type mice treated with A779 or Ang-(1-7). Taken together, these data suggest that endogenous Ang-(1-7) protects against the development of kidney fibrosis in UUO, via Mas-dependent pathways. However, exogenous Ang-(1-7) exacerbates kidney injury and promotes apoptosis and inflammation, at least partly via non-Mas receptor pathways.

Protective effects of Ang-(1-7) were reported in several experimental models of kidney disease. In hypertensive rats, 24 μ g/kg per hour exogenous Ang-(1-7) significantly reduced blood pressure and proteinuria, associated with diminished renal vascular injury.¹⁷ Endogenous Ang-(1-7) is also renoprotective in this model, because treatment with A779 exacerbated blood pressure and diminished proteinuria reduction by hydralazine or the ACE inhibitor captopril.¹⁸ Ang-(1-7) infusions reduced glomerulosclerosis in experimental glomerulonephritis,¹⁹ and the Mas agonist AVE0991 protected against tissue damage and leukocyte infiltration in experimental ischemic acute kidney injury.²⁰ In streptozotocin-diabetic rats, 24 μ g/kg per hour Ang-(1-7) reduced proteinuria,¹¹ and in mice with type 2 diabetes, Ang-(1-7) at doses of 15 or 21 μ g/kg per hour prevented mesangial expansion and kidney expression of TGF- β and fibronectin²¹ and decreased intrarenal oxidative stress, inflammation, and lipid accumulation.²² The protective effects of Ang-(1-7) are supported by studies in diabetic mice with gene deletion of ACE2 (*ACE2*^{-/-}), which demonstrate enhanced kidney injury compared with wild-type mice.²³ Indeed, *ACE2*^{-/-} mice subjected to UUO exhibit increased kidney tubulointerstitial fibrosis and inflammation compared with wild-type mice, attributed to enhanced Ang II signaling.²⁴

Similarly, our studies strongly support a protective role for endogenous kidney Ang-(1-7) in preventing tubulointerstitial injury in UUO. A779 treatment caused an increase in expression of profibrotic markers TGF- β , fibronectin, and α -SMA in obstructed kidneys, exacerbated histologic injury scores and apoptosis, and increased NADPH oxidase activity, compared with vehicle-treated controls. Because A779 did not increase kidney macrophage infiltration, compared with obstructed kidneys from controls, the enhanced kidney NADPH oxidase activity with A779 may be because of up-regulation of the subunit p22^{phox} in renal tubules, which was

Figure 6 Effect of A779 and exogenous angiotensin Ang-(1-7) on tubulointerstitial fibrosis (A), apoptosis (B), and macrophage infiltration (C) in *Mas*^{-/-} mice with UUO. **A:** Periodic acid-Schiff staining of paraffin-embedded kidney sections (obstructed kidneys) from wild-type mice or *Mas*^{-/-} mice that underwent UUO and treatment with vehicle, 31 μ g/kg per hour A779, or 62 μ g/kg per hour Ang-(1-7). Shown are collapsed fibrotic glomeruli (arrows), dilated atrophic tubules (asterisks), and interstitial monocytes/macrophages (arrowheads). Graph at right shows tubulointerstitial fibrosis scores. Scores were zero in unobstructed kidneys (not shown). **B:** TUNEL staining of kidney cortices (obstructed kidneys) from wild-type mice or *Mas*^{-/-} mice that underwent UUO and treatment with vehicle, 31 μ g/kg per hour A779, or 62 μ g/kg per hour Ang-(1-7). Arrows indicate apoptotic cells. Graph at right shows quantification of apoptotic nuclei. Unobstructed kidneys from all groups had only few TUNEL⁺ nuclei (not shown). **C:** Anti-F4/80 staining of paraffin-embedded kidney sections (obstructed kidneys) from wild-type mice or *Mas*^{-/-} mice that underwent UUO and treatment with vehicle, 31 μ g/kg per hour A779, or 62 μ g/kg per hour Ang-(1-7). Arrows show infiltrating macrophages. Graph at right shows quantification of F4/80⁺ area. Unobstructed kidneys had no significant macrophage infiltration. Values are means $\pm$ SEM. $n = 6$. *** $P < 0.001$ versus vehicle, wild-type (B and C); $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ versus vehicle-treated *Mas*^{-/-} (B and C); $^{\ddagger}P < 0.05$ versus *Mas*^{-/-} + A779 (B). Original magnification, $\times 600$. Ang, angiotensin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; UUO, unilateral ureteral obstruction.

described in mice with UUO.²⁵ A779 also increased expression of fibronectin and α -SMA in unobstructed kidneys, suggesting that endogenous Ang-(1-7) modulates basal amounts of these proteins in the uninjured kidney. However, in kidneys from *Mas*^{-/-} mice, expression of fibronectin, α -SMA, and tubulointerstitial scores were not significantly enhanced, although TGF- β expression, apoptosis, and macrophage infiltration were stimulated in obstructed kidneys, compared with kidneys from wild-type mice. These results differ from a previous report that found higher amounts of kidney cortical extracellular matrix proteins in *Mas*^{-/-} mice, by immunofluorescence.²⁶ Our results suggest that gene deletion of *Mas* may indeed lead to up-regulation of compensatory pathways that protect against kidney injury. In this regard, we observed that, although A779 increased obstructed kidney Ang II amounts (compared with vehicle treatment), kidney Ang II amounts were not further augmented in *Mas*^{-/-} mice, compared with wild-type mice. Importantly, we did not observe adverse effects of A779 treatment in *Mas*^{-/-} mice with UUO, suggesting that the protective effects

of endogenous Ang-(1-7) are mediated via its interaction with the Mas receptor. In this regard, in cultured proximal tubular cells Ang-(1-7) inhibits Ang II-mediated profibrotic signaling pathways via activation of a protein tyrosine phosphatase, effects that are blocked by A779.^{7,27} This endogenous mechanism may thereby limit tubulointerstitial fibrosis in UUO.

Adverse effects of Ang-(1-7) in kidney disease were also reported, which may depend on the experimental model, dose of Ang-(1-7), and cell type examined. In streptozotocin-diabetic rats, an infusion of Ang-(1-7) at 25 μ g/kg per hour for 6 weeks increased renal injury and mRNA and protein expression of TGF- β .²⁸ Rats with subtotal nephrectomy treated with Ang-(1-7) at 24 μ g/kg per hour for 10 days experienced increased blood pressure and accelerated cardiac hypertrophy and fibrosis, compared with untreated controls.²⁹ In healthy rats, infusion of Ang-(1-7) at 24 μ g/kg per hour for 10 days caused an induction of proximal tubular α -SMA expression and increased gene expression of matrix proteins.³⁰ Moreover, exposure of cultured rat tubular epithelial cells (NRK-52E) to Ang-(1-7) led to up-regulation of α -SMA and

Figure 7 Effect of A779 and Ang-(1-7) on kidney amounts of Ang II or Ang-(1-7) in mice with UUO. **A:** Graph depicts effect of 31 µg/kg per hour A779 and 6, 24, and 62 µg/kg per hour Ang-(1-7) on unobstructed (gray bars) and obstructed (black bars) kidney amounts of Ang II in wild-type mice with UUO. **B:** Graph depicts unobstructed and obstructed kidney amounts of Ang II in wild-type mice (far left, vehicle) or *Mas*^{-/-} mice with UUO treated for 10 days with vehicle, 31 µg/kg per hour A779, or 62 µg/kg per hour Ang-(1-7). By all groupwise comparison, unobstructed kidneys in the *Mas*^{-/-} group had significantly lower Ang II amounts than obstructed kidneys in *Mas*^{-/-} mice ($P < 0.01$). **C:** Graph depicts amounts of Ang-(1-7) in unobstructed and obstructed kidneys from wild-type mice with UUO treated with 31 µg/kg per hour A779 or 6, 24, or 62 µg/kg per hour Ang-(1-7) for 10 days. **D:** Graph depicts amounts of Ang-(1-7) in unobstructed and obstructed kidneys from wild-type mice or *Mas*^{-/-} mice with UUO treated for 10 days with vehicle, 31 µg/kg per hour A779, or 62 µg/kg per hour Ang-(1-7). Values are means $\pm$ SEM. $n = 6$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Ang, angiotensin; UUO, unilateral ureteral obstruction.

induction of a myofibroblast phenotype, effects blocked by A779.³⁰ Consistent with these reported adverse effects of Ang-(1-7) in the present studies increases in expression of fibronectin and α -SMA were observed in UUO kidneys from mice treated with Ang-(1-7) at all three doses, and TGF- β expression also increased, although only at doses of 6 and 24 µg/kg per hour. Ang-(1-7) did not affect blood pressure but did increase kidney NADPH oxidase activity, histologic injury scores, apoptosis, and macrophage infiltration. The lack of consistent dose-dependent effects of exogenous Ang-(1-7) in our UUO model (eg, tubulointerstitial fibrosis, apoptosis) is difficult to explain but could partly relate to sensitivity of some of the assays. Note, however, that no beneficial effects of Ang-(1-7) were observed at any of the exogenous doses in this study.

The mechanisms by which endogenous Ang-(1-7) protects against renal injury, whereas exogenous Ang-(1-7) promotes damage in UUO, are likely complex but could at least partly involve direct activation of Mas receptors. Thus, biphasic, dose-dependent effects of Ang-(1-7) on proximal tubular fluid and bicarbonate reabsorption were reported, mediated by Mas receptors.³¹ Recent elegant studies indicate that AT₁ receptors on infiltrating macrophages protect against kidney fibrosis in a mouse model of UUO by inhibiting release of proinflammatory cytokines.³² It is possible that exogenous Ang-(1-7) potentially inhibits this process, via Mas activation, resulting in conversion from a renoprotective to a profibrotic, proapoptotic, and proinflammatory phenotype. However, mouse peritoneal macrophages were shown to express functional Mas receptors, which are up-regulated by lipopolysaccharide.³³ Ang-(1-7) has an anti-inflammatory effect in these cells, via inhibition of cytokine expression, which is blocked by A779.³³ Thus, the relative intensity of opposing effects of Mas activation on inflammatory responses may dictate the phenotype observed with exogenous Ang-(1-7) in UUO.

Our data in *Mas*^{-/-} mice with UUO nonetheless suggest that exogenous Ang-(1-7) induces injury at least partly via non-Mas receptor-mediated signaling. In *Mas*^{-/-} mice, exogenous Ang-(1-7) exacerbated apoptosis and macrophage infiltration, while not significantly affecting tubulointerstitial fibrosis or expression of fibronectin, TGF- β , or α -SMA, compared with obstructed kidneys from vehicle-treated *Mas*^{-/-} mice. Several mechanisms for non-Mas-mediated effects of Ang-(1-7) can be postulated. First, the existence of several *Mas*-related genes (*MRGPRs*) that encode G protein-coupled receptors³⁴ raises the possibility that Ang-(1-7) may interact with these receptors and stimulate adverse signaling pathways. Second, exogenous Ang-(1-7) may lead to cross-activation of angiotensin receptors in the kidney. Both AT₁ and AT₂ receptors are involved in activation of the proinflammatory NF- κ B pathway in mouse UUO, and it may be that Ang-(1-7) stimulates these receptors when at high amounts.³⁵ In the *Mas*^{-/-} mouse, kidney mRNA expression for the AT₁ receptor is increased by threefold compared with wild-type mice.²⁶ Angiotensin receptor expression was not measured in our studies, although enhanced AT₁ receptors in the *Mas*^{-/-} kidney could have affected responses to A779 with UUO or promoted receptor cross-activation by exogenous Ang-(1-7). Further studies that involve angiotensin receptor assays and use of receptor antagonists will be useful to address this hypothesis. Finally, Santos and colleagues^{36,37} have recently described the antifibrotic and antihypertensive properties of alamandine, a novel heptapeptide generated from Ang-(1-7) that binds to a Mas-related G protein-coupled receptor, member D. Studies directed at the effects of alamandine in models of kidney disease will clearly be of interest.

Our results have important differences compared with the findings reported by Esteban et al⁹ about Ang-(1-7) in the mouse UUO model. Consistent with our data, they reported that infusion of approximately 20 µg/kg per hour Ang-(1-7) to wild-type mice with UUO worsened kidney injury and

led to increased proapoptotic proteins and inflammatory cell infiltrate.⁹ They also showed that obstructed kidneys from *Mas*^{-/-} mice with UUO had significantly less kidney damage compared with wild-type mice, with decreased apoptosis and inflammatory response.⁹ By contrast, our data revealed that obstructed kidneys from *Mas*^{-/-} mice exhibited enhanced apoptosis and macrophage infiltration after 10 days, consistent with the hypothesis that endogenous Ang-(1-7) is renoprotective via interaction with Mas receptors. Furthermore, Esteban et al⁹ showed that infusions of approximately 60 µg/kg per hour Ang-(1-7) to healthy mice without UUO for 5 days caused significant increases in kidney interstitial cell inflammatory infiltrate, an effect we did not observe in unobstructed kidneys in our studies. The conflicting observations in these two studies are difficult to reconcile, but they might be because of differences in the models, including dose of Ang-(1-7) and assay approaches. Furthermore, the *Mas*^{-/-} mice used in the present studies were derived independently of the mice used in the study by Esteban et al,⁹ and substrain differences might account for variant results by using the gene knockout model.

UUO is characterized by early activation of components of the kidney RAS.³⁸ Although we observed elevated intrarenal amounts of Ang II in obstructed kidneys from mice treated with A779 or escalating doses of Ang-(1-7), whether Ang II is a mediator or a consequence of increased kidney injury requires further study. Elevated blood pressure was not observed in any of the groups of mice with UUO; therefore, we cannot account for enhanced kidney damage. The lack of blood pressure increase might be because of compensatory responses in sodium excretion by the unobstructed kidneys, which did not demonstrate increased Ang II amounts. With regard to the mechanism for enhanced Ang II amounts in UUO, expression and activity of kidney ACE2 was decreased in obstructed kidneys, although no differences were detected among the treatment groups. Moreover, reductions in ACE expression and activity were observed, which would be predicted to decrease Ang II generation. Thus, other components of the RAS or other peptidase pathways may account for elevated kidney Ang II in this model. Finally, although exogenous Ang-(1-7) increased Ang-(1-7) amounts in the obstructed kidneys in our study, it is of interest that unobstructed kidney amounts of Ang-(1-7) were unaffected [except for a small increase in *Mas*^{-/-} mice treated with Ang-(1-7)]. Because ACE mediates degradation of Ang-(1-7) to Ang-(1-5),³⁹ decreased ACE expression and activity in obstructed kidneys may have contributed to accumulation of the peptide.

Conclusion

These studies indicate that endogenous Ang-(1-7) exerts protective effects against kidney fibrosis, apoptosis, oxidative stress, and inflammation in UUO, via interaction with Mas receptors. Infusions of Ang-(1-7) at doses between 6 and 62 µg/kg per hour, however, exacerbate kidney injury in

this model, at least partly via non-Mas receptor-mediated pathways. Although Ang-(1-7) and its Mas receptor are promising targets to prevent progressive kidney fibrosis, caution must be exercised because of contrasting effects between endogenous (low dose) and exogenous (high dose) effects in the kidney. Indeed, our results support the need for further development of selective agonists that specifically target the Mas receptor and do not exert non-Mas actions.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2014.11.013>.

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