Infusion of angiotensin-(1–7) reduces glomerulosclerosis through counteracting angiotensin II in experimental glomerulonephritis

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Zhang J, Noble NA, Border WA, Huang Y. Infusion of angiotensin-(1–7) reduces glomerulosclerosis through counteracting angiotensin II in experimental glomerulonephritis, Am J Physiol Renal Physiol 298: F579–F588, 2010. First published December 23, 2009; doi:10.1152/ajprenal.00548.2009.—Recent identification of a counter-regulatory axis of the renin-angiotensin system, called angiotensin-converting enzyme 2-angiotensin-(1–7) [ANG-(1–7)]-Mas receptor, may offer new targets for the treatment of renal fibrosis. We hypothesized that therapy with ANG-(1–7) would improve glomerulosclerosis through counteracting ANG II in experimental glomerulonephritis. Disease was induced in rats with the monoclonal anti-Thy-1 antibody, OK-T3. Based on a three-dose pilot study, 576 μg kg–1·day–1 ANG-(1–7) was continuously infused from day 1 using osmotic pumps. Measures of glomerulosclerosis include semiquantitative scoring of matrix proteins stained for periodic acid Schiff, collagen I, and fibronectin EDA+ (FN). ANG-(1–7) treatment reduced disease-induced increases in proteinuria by 75%, glomerular periodic acid Schiff staining by 48%, collagen I by 24%, and FN by 25%. The dramatic increases in transforming growth factor-β1, plasminogen activator inhibitor-1, FN, and collagen I mRNAs seen in disease control animals compared with normal rats were all significantly reduced by ANG-(1–7) administration (P < 0.05). These observations support our hypothesis that ANG-(1–7) has therapeutic potential for reversing glomerulosclerosis. Several results suggest ANG-(1–7) acts by counteracting ANG II effects: 1) renin expression in ANG-(1–7)-treated rats was dramatically increased as it is with ANG II blockade therapy; and 2) in vitro data indicate that ANG II-induced increases in mesangial cell proliferation and plasminogen activator inhibitor-1 overexpression are inhibited by ANG-(1–7) via its binding to a specific receptor known as Mas.

ACTIVATION OF THE RENIN-ANGIOTENSIN system (RAS) and generation of angiotensin (ANG) II have long been known to play a crucial role in the pathogenesis of renal fibrosis (8, 23). Formation of ANG II involves two main steps: the aspartyl protease renin cleaves angiotensinogen to form the decapetide ANG I, and ANG I is then converted by ANG-converting enzyme (ACE) to ANG II. ANG II interacts with its type 1 (AT1) and type 2 (AT2) receptors (AT1R and AT2R) on kidney cells to induce responses that affect the structure and function of the kidney. Numerous experimental studies, using pharmacological or genetic genetic to manipulate the action of the classic axis of RAS, the ACE-ANG II-AT1R axis, as well as clinical studies, have shown that blockade of this axis reduces renal injury and fibrosis (19, 35, 46). Therapy with an ACE inhibitor (ACEi) or an AT1R blocker (ARB) is now the first-line strategy to reduce the progression of renal disease.

Recently, a new axis of RAS, called ACE2-ANG-(1–7)-Mas receptor axis, has been established (as summarized in Fig. 9) (4, 31, 32). In this axis, ANG I is converted to ANG-(1–7) by the catalytic activity of ACE2. ACE2 is a homolog of ACE that can directly form ANG-(1–7) through hydrolysis of ANG II or indirectly through hydrolysis of ANG I to ANG-(1–9), with subsequent conversion to ANG-(1–7) by ACE. ANG-(1–7) is then further metabolized by ACE to ANG-(1–5) (31). It has been shown that ANG-(1–7) has its own unique receptor, Mas, a G-protein-coupled receptor identified originally as a protooncogene (33). It is likely that actions of ACE and ACE2 in balancing ANG II and ANG-(1–7) expression influence the RAS in the kidney. Kidney diseases have been associated with a reduction in renal ACE2 expression. Low ACE2 levels have been reported in established renal disease, in the 5/6ths nephrectomy model of renal insufficiency, and in diabetic animal models and patients where ACE is significantly increased (15, 20, 29, 43, 47). The resulting high ACE-to-ACE2 ratio in diseased kidney may result in accelerated formation and accumulation of ANG II and increased catabolism of ANG-(1–7), thereby facilitating the damaging effects of ANG II in the progression of renal disease. Conversely, higher ACE2 relative to ACE may decrease ANG II and increase ANG-(1–7), facilitating ANG-(1–7) generation, which, in turn, may provide protection against the development of renal disease.

Increasing data indicate that ANG-(1–7) acts as a vasodilator, which antagonizes AT1R stimulation-mediated vasoconstriction (5, 18, 38). Infusion of exogenous ANG-(1–7) or oral administration of AVE-0991, a nonpeptide analog of ANG-(1–7), not only attenuated heart failure, but also reversed ANG II-induced cardiac myocyte hypertrophy and interstitial fibrosis (7, 9, 37). Furthermore, the inhibition of cellular signaling in the cardiovascular system by ANG-(1–7) was blocked by antisense oligonucleotides against Mas (37). Mas-deficient mice also showed an impairment of cardiac functions and increased collagen and fibronectin (FN) content in heart tissue (30), suggesting that these cardioprotective effects of ANG-(1–7) are at least partially mediated by stimulation of the Mas receptor, independent of blood pressure. Taken together, these results suggest that ANG-(1–7) may act as a counterregulator of ANG II, producing a blood-pressure-lowering effect and an organ-protective effect.

ANG-(1–7) has been measured in the kidney. It is present in concentrations comparable to ANG II (25). The role of ANG-(1–7) in the regulation of renal hemodynamics and pathogenesis of renal fibrosis is incompletely understood, and data are conflicting (5). However, ANG II blockade with ACEi or
ARBs increases endogenous ANG-(1–7) significantly (18), suggesting that ANG-(1–7) may be responsible for at least some of the benefit of ACEi and ARB treatment in renal disease. At this point, we hypothesized that therapy with ANG-(1–7) would ameliorate glomerulosclerosis in the anti-Thy-1 rat nephritis model. In vitro studies on renal mesangial cells (MCs) were then designed to test whether addition of ANG-(1–7) could directly limit the profibrotic effects of ANG II on MCs. Investigation of this hypothesis is of clinical relevance, because it should provide direction for further studies determining the efficacy of adding ANG-(1–7) therapy to ACEI or ARB therapy.

MATERIALS AND METHODS

Reagents

ANG-(1–7) and the inactive analog (D-Ala7)-ANG-(1–7) were purchased from Bachem Biosciences (Torrance, CA). The monoclonal anti-Thy-1 antibody, OX-7, was obtained from CCB, Biovest International (Minneapolis, MN). Unless specified, all other reagents were purchased from Sigma Chemical (St. Louis, MO).

Study 1. In vivo Studies of the Therapeutic Effect of ANG-(1–7) in Experimental Glomerulonephritis

Animals. Experiments in vivo were performed on male Sprague-Dawley rats (180–200 g) obtained from the SASCO colony of Charles River Laboratories (Wilmington, MA). Animal housing and care were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animal studies were approved by the Animal Care Committee of University of Utah. Glomerulonephritis was induced by tail vein injection of 1.75 mg/kg of the monoclonal anti-Thy-1 antibody, Ox-7. Ox-7 binds to a Thy-1-like epitope on the surface of MCs, causing immune-mediated, complement-dependent cell lysis, followed by exuberant matrix synthesis and deposition. Normal control animals were injected with the same volume of PBS (14).

Experimental design. Groups of six rats were assigned and treated as normal control, disease control, and nephritic rats treated with ANG-(1–7) at doses of 144, 288, and 576 μg·kg⁻¹·day⁻¹, respectively, based on previous reports (9, 17, 21). ANG-(1–7) was administered by continuous subcutaneous infusion via osmotic pumps. Treatment was started on day 1 (24 h after OX-7 injection) to day 5. Untreated normal control and nephritic rats served as control. The urinary protein excretion was measured by the Bradford method (Bio-Rad Protein Assay; Bio-Rad Laboratories, Hercules, CA) on urine collected from rats housed in metabolic cages for 24 h from day 5 to day 6.

On day 6, animals were anesthetized, 5–10 ml of blood were drawn from the lower abdominal aorta, and kidneys were perfused with 30 ml ice-cold PBS. For histological examination, cortical tissue was snap frozen for frozen sectioning or fixed in 10% neutral-buffered formalin, with periodic acid Schiff (PAS) staining. Glomeruli were isolated by graded sieving, as described previously (27). Five thousand glomeruli per milliliter per well were resuspended and cultured in serum-free RPMI 1640, supplemented with 0.1 U/ml insulin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 mmol/l HEPES buffer. After a 48-h incubation at 37°C/5% CO2, the supernatant was harvested and stored at −70°C until analysis of glomerular production of transforming growth factor-β1 (TGF-β1) by ELISA, as described previously (27).

Plasma renin activity. Plasma renin activity was determined as the ability of a sample to generate ANG I from nephrectomized rat serum substrate (containing angiotensinogen) as described (14). ANG I generated was quantitated using a commercially available RIA kit (Phoenix Pharmaceuticals, Belmont, CA).

Histological analyses. All microscopic examinations were performed in a blinded fashion. Three-micrometer sections of paraffin-embedded tissues were stained with PAS. The images (×400 magnification) of 20 random glomeruli per slide were captured using a Nikon D50 digital camera (Inkley’s-Ritz Camera, Salt Lake City, UT, www.ritzcamera.com; Nikon Capture 4, version 4.3, Nikon, Melville, NY), and the area of PAS-positive material in each glomerulus was quantified using a computer-assisted color image analysis system (ImageJ 1.38 for Windows; National Institutes of Health, http://rsb.info.nih.gov). The PAS-positive material area in the mesangium was divided by that in the total glomerular tuft area to obtain the rates of mesangial matrix, as indicated previously (11). Immunofluorescent (IF) staining for matrix proteins was performed on frozen sections and evaluated in 20 glomeruli from each rat. The percentage of positive area occupying each glomerulus was rated on a scale from 0 to 4, where 0 represents no positive staining; 1+ represents positive staining of <25% of the glomerulus; and 2+, 3+, and 4+ represent positive staining of 25–50, 50–75, and >75% of the glomerulus, respectively. A whole kidney average positive staining for matrix protein was obtained by averaging scores from all glomeruli (12).

RNA preparation and Northern hybridization. Total RNA was extracted immediately from isolated glomeruli using Trizol Reagent (Gibco BRL, Gaithersburg, MD), according to the manufacturer’s instructions. RNA from each group was pooled, and Northern analysis of TGF-β1, plasminogen activator inhibitor-1 (PAI-1), FN, and type I collagen (Col I) mRNA expression was performed as previously described (12). Three blots per probe were performed. Autoradiographic films were scanned on a Bio-Rad GS-700 imaging densitometer (Bio-Rad Laboratories, Hercules, CA). For quantitative densitometric measurements of Northern blots, all of the signals were normalized compared with GAPDH levels used for equal loading. Glomerular renin, ACE, and ACE2 mRNA expression was determined by real-time RT/PCR, as described below.

Table 1. Primers used for real time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Location (Complementary to Nucleotides)</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rattus ACE</td>
<td>Forward</td>
<td>2428–2447</td>
<td>GAG GCA TCC</td>
</tr>
<tr>
<td>(NM_012544)</td>
<td></td>
<td></td>
<td>TCC CCT TTT</td>
</tr>
<tr>
<td>Rattus ACE2</td>
<td>Forward</td>
<td>2674–2693</td>
<td>CCA CAT GTT</td>
</tr>
<tr>
<td>(NM_001012006)</td>
<td></td>
<td></td>
<td>CCC TAG CAG</td>
</tr>
<tr>
<td>Reverse</td>
<td>168–187</td>
<td></td>
<td>GGA GAA TGC</td>
</tr>
<tr>
<td>Reverse</td>
<td>429–446</td>
<td></td>
<td>CCA AAA GAT</td>
</tr>
<tr>
<td>Rattus Renin</td>
<td>Forward</td>
<td>811–834</td>
<td>GAT CAT GAA</td>
</tr>
<tr>
<td>(NM_012642)</td>
<td></td>
<td></td>
<td>GGG GGT CGT</td>
</tr>
<tr>
<td>Reverse</td>
<td>1061–1084</td>
<td></td>
<td>TGT CCT GAA</td>
</tr>
<tr>
<td>Rattus PAI-1</td>
<td>Forward</td>
<td>681–700</td>
<td>TGG TGA AGC</td>
</tr>
<tr>
<td>(M24067)</td>
<td></td>
<td></td>
<td>CCC TCT ATT</td>
</tr>
<tr>
<td>Reverse</td>
<td>909–928</td>
<td></td>
<td>TGC GAG CAG</td>
</tr>
<tr>
<td>Rattus GAPDH</td>
<td>Forward</td>
<td>29–48</td>
<td>AGA CAG CCG</td>
</tr>
<tr>
<td>(NM_017008)</td>
<td></td>
<td></td>
<td>CAT CTT GCT</td>
</tr>
<tr>
<td>Reverse</td>
<td>151–170</td>
<td></td>
<td>TCC CCA TCC</td>
</tr>
</tbody>
</table>

ACE, angiotensin-converting enzyme; PAI-1, plasminogen activator inhibitor-1.
Table 2. *Body weight*

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight on Day of Injection</th>
<th>Body Weight on Day of Sacrifice</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>240 ± 5</td>
<td>271 ± 11</td>
</tr>
<tr>
<td>DC</td>
<td>239 ± 9</td>
<td>268 ± 15</td>
</tr>
<tr>
<td>Dose 1</td>
<td>241 ± 5</td>
<td>268 ± 6</td>
</tr>
<tr>
<td>Dose 2</td>
<td>240 ± 5</td>
<td>267 ± 9</td>
</tr>
<tr>
<td>Dose 3</td>
<td>243 ± 7</td>
<td>263 ± 6</td>
</tr>
</tbody>
</table>

Values are means ± SD in g; n = 6 in each group. NC, normal control rats; DC, untreated diseased rats; Dose 1, Dose 2, and Dose 3: diseased rats treated with angiotensin-(1–7) at doses of 144, 288, and 576 µg·kg⁻¹·day⁻¹ respectively.

Study 2. *In Vitro Studies of the Effect of ANG-(1–7) on ANG II-Induced Cell Proliferation and PAI-1 Overexpression by MCs*

**Cell culture.** Primary MCs derived from intact rat glomeruli of 4- to 6-wk-old male Sprague-Dawley rats were used between passages 5 and 8. Cells were maintained in RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS) (Hyclone Laboratory, Logan, UT), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 U/ml insulin, and 25 mM HEPES buffer at 37°C in a 5% CO₂ incubator. Subconfluent cells seeded on six-well plates were made quiescent in serum-free RPMI 1640 medium for 48 h before experimental studies.

**ANG-(1–7) binding assay.** To determine specific binding of ANG-(1–7) to rat MCs, initial experiments were first performed to determine whether rat MCs express Mas gene by RT-PCR, identified as the receptor for ANG-(1–7). The binding studies were then performed on intact adherent rat MCs grown to confluence on 96-well culture plates, as described previously (16). Cells were incubated with a rhodamine-labeled ANG-(1–7) (Phoenix Pharmaceuticals, Belmont, CA) for 90 min at 4°C under serum-free medium. Saturation binding assay was performed with 0.5–10 nM rhodamine-labeled ANG-(1–7). Competi-

![Fig. 1. Effect of angiotensin (ANG)-(1–7) treatment on glomerular mRNA expression in anti-Thy-1 nephritis at day 6. Top: representative northern blot is shown. Bottom: the relative levels of glomerular mRNA expression of transforming growth factor (TGF)-β1, plasminogen activator inhibitor-1 (PAI-1), fibronectin (FN), and collagen type I (Col I) were standardized to GAPDH mRNA levels. Dose 1, Dose 2, and Dose 3: diseased rats treated with ANG-(1–7) [DC+ANG-(1–7)] at doses of 144, 288, and 576 µg·kg⁻¹·day⁻¹, respectively. *P < 0.05 vs. normal control (NC). #P < 0.05 vs. disease control (DC).](http://ajprenal.physiology.org/)

![Fig. 2. Effect of ANG-(1–7) treatment on urinary protein excretion (A) and glomerular matrix protein accumulation (B and C). A: 24-h urinary protein excretion from days 5 and 6. B: representative photomicrographs of glomeruli from NC, DC, and DC+ANG-(1–7)-treated nephritic rats at day 6. C: graphic representation of glomerular matrix score. *P < 0.05 vs. NC. #P < 0.05 vs. DC.](http://ajprenal.physiology.org/)
tion binding assay was performed by incubating 1.95 nM labeled ANG-(1–7) with increasing concentrations (10^{-10}-10^{-5} M) of unlabelled ANG-(1–7). After incubation, cells were washed with cold PBS at 4°C and then submitted to a fluorescence microplate reader (BioTek, Winooski, Vermont). The nonspecific binding was determined in the presence of an overdose of unlabelled ANG-(1–7) (10^{-5} M) and subtracted from each assay. The binding parameters were generated with GraphPad Prism software (version 3.00, GraphPad Prism Software, San Diego, CA).

**Cellular proliferation.** MC mitotic activity was evaluated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell proliferation assay, according to the manufacturer’s instructions, as described (13). The number of viable cells corresponds to the amount of MTT reduced by mitochondrial dehydrogenase in living cells. A preliminary study confirmed a significant correlation between counted cell numbers and optical density values at 520 nm. Each experiment was typically performed with n = 8 separate wells of MCs in 96-well plates under identical conditions. The administration of 10% FBS was used as the positive control.

**PAI-1 Western blot analysis.** After 36-h treatment, the cultured cell supernatant was harvested and centrifuged immediately at 2,000 rpm for 5 min to remove any floating cells or fragments. The equal volume of supernatant (40 µl) without concentration mixed with 13.3 µl of 4× loading buffer was then separated by 10% Tris-glycine gel electrophoresis (Novex Tris-Glycine Gels, Invitrogen Life Technologies, Carlsbad, CA) and transferred to a 0.45-mm immobilon-P transfer membrane (Millipore, Bedford, MA). The subsequent protein immunohybridization was performed as previously described (10). The rabbit-anti-rat PAI-1 IgG (stock solution: 250 µg/ml; American Diagnostica, Greenwich, CT, diluted 1:200 in 5% BSA in TBS/0.1% Tween-20 with 0.02% NaN3) was used as the primary antibody. The goat anti-rabbit horseradish peroxidase (stock solution: 400 µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA, diluted 1:2,000 in 5% nonfat milk powder in Tris-buffered saline) was used as the secondary antibody. Bound antibodies on the membrane were detected by developing the blots in ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 min. Quantitation of the bands on autoradiograms was performed using a Bio-Rad GS-700 imaging densitometer (Bio-Rad Laboratories, Hercules, CA).

**Cellular RNA isolation and real-time RT-PCR.** Total cellular RNA was isolated immediately from cultured MCs using Trizol Reagent (Gibco BRL, Gaithersburg, MD), according to the manufacturer’s instructions. Two micrograms of total RNA were reverse-transcribed using the superscript III first-stand synthesis system for RT-PCR kit (Invitrogen). Real-time RT-PCR was performed using a SYBR green dye I (Applied Biosystems, Foster City, CA) with the ABI 7900 Sequence Detection System (PE Applied Biosystems). cDNA was first denatured at 95°C for 15 min and then amplified through 40 amplification cycles, according to the manufacturer’s protocol as follows: denatured at 95°C for 15 s, and annealed/extended at 60°C for 30 s. Fluorescence signals were recorded in each cycle. Relative quantitation of gene expression was carried out using the standard curve method and analyzed with RQ-manager 1.2 (ABI 7900 Sequence Detection System, Applied Biosystems). Samples were run as triplicates in separate tubes to permit quantification of the target gene normalized to GAPDH used for equal loading. Sequences of primers used are listed in Table 1. The specificity of the PCR products was confirmed on a 1.5% agarose gel by showing a specific single band with the expected size.

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Fig. 3. Glomerular immunofluorescent staining score for extracellular matrix (ECM) proteins (A and B), number of monocytes/macrophages infiltrating glomeruli (C), and glomerular production of TGF-β1 (D) in anti-Thy-1 nephritis at day 6. IF, immunofluorescent. *P < 0.05 vs. NC. #P < 0.05 vs. DC.
Statistical Analysis

All data are expressed as means ± SD. Statistical analyses of differences between the groups were performed by ANOVA and subsequent Student-Newman-Keuls or Dunnett testing for multiple comparisons. Comparisons with a P value < 0.05 were considered significantly different. In study 1, the disease-induced increase in a variable was defined as the mean value for the disease control group minus the mean value of the normal control group (100%). The percent reduction in disease severity in an ANG-(1–7)-treated group was calculated as follows: \( \frac{100 \times [\text{Normal control group mean} - \text{Disease control group mean}]}{[\text{Normal control group mean} - \text{ANG-(1–7)-treated group mean}]} \). In study 2, duplicate wells were analyzed for each experiment, and each experiment was performed independently a minimum of three times.

RESULTS

Study 1: Therapeutic Efficacy of ANG-(1–7)

Rat body weight. There were no significant differences among all animal groups in body weight during the experiment (Table 2).

Dose effect of ANG-(1–7) on glomerular expression of mRNAs for TGF-β1, PAI-1, FN, and Col I. A pilot study was first carried out to determine an effective dose of ANG-(1–7) in nephritic rats by measuring the reduction in glomerular mRNA expression after treatment. As shown in Fig. 1, glomerular mRNA analysis revealed a robust increase in TGF-β1, PAI-1, FN, and Col I mRNA expression in disease control.
ANGIOTENSIN-(1–7) REDUCES GLOMERULOSCLEROSIS

Fig. 6. Effect of ANG-(1–7) (A) or Dla (B) on ANG II-induced rat MC proliferation. Quiescent rat MCs were treated with or without 10−7 M of ANG-(1–7), or 10−5 M Dla, or 10−5 M of ANG-(1–7) plus 10−5 M Dla, in addition to 10−8 M of ANG II for 24 h (n = 8). The administration of 10% FBS was used as the positive control. The proliferative response was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results were expressed as the mean optical density (OD) of MTT absorbance in control and treated wells. *P < 0.05 compared with the untreated control. #P < 0.05 compared with the ANG II alone treated cells.

Effects of ANG-(1–7) on glomerular production of TGF-β1. OX-7 administration resulted in 7.3-fold increases in TGF-β1 levels of PAI-1 mRNA expression by rat MCs. Quiescent rat MCs were incubated in the absence (open bars) or presence (solid bars) of 10−8 M ANG II (A) or 10−5 M ANG-(1–7) (B) for the indicated times, and real-time RT-PCR was performed. Relative values of PAI-1 mRNA expression by rat MCs are expressed relative to the no-additive, zero-time control, which was set at unity. *P < 0.05 compared with the untreated control.

Effects of ANG-(1–7) on monocyte/macrophages infiltration in anti-Thy-1 nephritis. The number of monocyte/macrophages was determined in kidney sections from all rats in each group (Fig. 3C). Nephritic glomeruli from disease control rats contained higher numbers of monocyte/macrophages than did glomeruli from normal control rats (13.6 ± 0.7 vs. 0.98 ± 0.1, P < 0.001). The average number of monocyte/macrophages per glomerular cross section in nephritic rats treated with ANG-(1–7) was 37% lower than that for disease controls (8.9 ± 0.5 vs. 13.6 ± 0.7, P < 0.05).

Effects of ANG-(1–7) on urinary volume and urinary protein excretion in anti-Thy-1 nephritis. Twenty-four-hour urine and urinary protein excretions were measured from day 5 to day 6. Nephritic rats exhibited a similar urinary volume for 24 h compared with normal rats (12.2 ± 2.68 vs. 12.3 ± 3.88 ml/rat, P > 0.05), but infusion of ANG-(1–7) resulted in significant increases in urinary volume compared with untreated disease rats (25.6 ± 12.58 vs. 12.2 ± 2.68 ml/rat, P < 0.02). As shown in Fig. 2A, disease induced a dramatic increase in urinary protein excretion, and this induction of urinary protein excretion was reduced 75.6% by ANG-(1–7) treatment (26 ± 16 vs. 57 ± 15 mg/24 h, P < 0.05).

PAS staining. Representative glomeruli stained with PAS are shown in Fig. 3B. The glomeruli from the disease control rats showed marked accumulation of extracellular matrix (ECM) expressed as PAS-positive material at day 6 (Fig. 2B, DC) compared with normal glomeruli (Fig. 2B, NC). Treatment with ANG-(1–7) resulted in much less mesangial matrix accumulation in glomeruli at day 6 [Fig. 2B, DC+ANG-(1–7)]. Figure 2C shows a graphical representation of the mean ± SD of PAS score for each group. PAS score increased from 1.31 ± 0.2 in normal control rats to 2.28 ± 0.48 in disease control rats as a result of nephritis. ANG-(1–7) treatment decreased matrix score significantly (P < 0.05) from 2.28 ± 0.48 in the disease control group to 1.82 ± 0.44. This is a 47% reduction in the disease-induced increase in PAS staining score.

IF staining. The results of the semiquantitative analysis of IF staining for matrix proteins are shown in Fig. 3. Compared with the disease control group, the staining score was significantly lower in the ANG-(1–7)-treated group at day 6 for FN-EDA+ (1.34 ± 0.18 vs. 1.14 ± 0.08; P < 0.01; Fig. 3A) and Col I (0.90 ± 0.16 vs. 0.64 ± 0.16; P < 0.01; Fig. 3B). These represent decreases in disease-induced ECM accumulation of 35% for FN and 31% for Col I.

Fig. 7. Time course effects of ANG II and ANG-(1–7) on PAI-1 mRNA expression by rat MCs. Quiescent rat MCs were incubated in the absence (open bars) or presence (solid bars) of 10−8 M ANG II (A) or 10−5 M ANG-(1–7) (B) for the indicated times, and real-time RT-PCR was performed. Relative values of PAI-1 mRNA expression by rat MCs are expressed relative to the no-additive, zero-time control, which was set at unity. *P < 0.05 compared with the untreated control.
production of glomeruli isolated at day 6 (Fig. 3D). TGF-β1 production was reduced with ANG-(1–7) by 34% (P < 0.01).

Plasma renin activity and renal renin, ACE, and ACE2 mRNA expression in anti-Thy-1 nephritis. As shown in Fig. 4A, there were no significant differences in plasma renin activity between nephritic and normal rats. Administration of ANG-(1–7) resulted in a significant increase in plasma renin activity by 2.3-fold (P < 0.05). Similarly, glomerular renin mRNA expression did not differ between nephritic rats and normal rats. Administration of ANG-(1–7) resulted in a dramatic 16-fold increase in glomerular renin mRNA expression at day 6 (Fig. 4B). Disease significantly increased ACE expression by 2.5-fold (Fig. 4C), while ACE2 expression was dramatically decreased by 3.5-fold in nephritic glomeruli compared with normal glomeruli (Fig. 4D) (both, P < 0.01). The ratio of ACE to ACE2 was 8.8-fold higher in diseased compared with normal rats. Glomerular ACE2 mRNA expression was further reduced in ANG-(1–7)-treated nephritic rats, but the decrease did not reach statistical significance (P = 0.071). Administration of ANG-(1–7) had no effect on glomerular ACE mRNA expression compared with untreated disease group (P = 0.066).

Study 2: Effects of ANG-(1–7) on the Action of ANG II in Vitro in Cultured MCs

Characteristics of ANG-(1–7) binding to cultured MCs. Using RT-PCR analysis, we showed that that Mas mRNA was present in rat MCs (data not shown). Incubation of MCs with labeled ANG-(1–7) at 4°C revealed that ANG-(1–7) bound MCs in a concentration-dependent manner (Fig. 5A). This binding was significantly inhibited by the unlabeled ANG-(1–7) in a dose-dependent manner (Fig. 5C). A similar inhibition of ANG-(1–7)-specific binding was further observed with the inactive analog of ANG-(1–7) acting as the Mas receptor antagonist, (D-Ala7)-ANG-(1–7) (Dla) (44), but not with ANG II (Fig. 5D). Scatchard analysis from both specific binding data revealed the existence of one high-affinity binding site of ANG-(1–7) with an apparent Kd = 1.95 nM for MCs (around...
2 × 10³ cells per well, determined by dye counting) (Fig. 5B). These studies confirm the presence of a specific, Mas receptor-mediated binding site for ANG-(1–7) on these cells.

Effects of ANG-(1–7) on ANG II-induced MC proliferation. In a previous report (13), our laboratory observed that primary cultures of MCs in RPMI 1640 with 10% FBS grew quickly, reaching ~90% confluence by 24 h. Addition of ANG II in serum-free RPMI 1640 increased cell proliferation rates, seen in Fig. 6 (left 2 bars). In contrast, addition of ANG-(1–7) at 10⁻⁷ M had no effect on MC proliferation. When both ANG-(1–7) and ANG II were added together for 24 h, the cell proliferation seen with ANG II was partially inhibited by ANG-(1–7). This inhibition was significantly reversed by additional coadministration of 10⁻⁵ M Dla, while Dla alone or together with ANG-(1–7) had no effect on MC proliferation, or Dla alone had no effect on ANG II-induced cell proliferation (Fig. 6). These results indicate that ANG-(1–7) inhibits ANG II-mediated MC proliferation via the Mas receptor.

Effects of ANG-(1–7) on ANG II-induced PAI-1 mRNA expression and protein production by MCs. PAI-1 mRNA expression was elevated significantly by 10⁻⁸ M ANG II from 1 h to at least 12 h (with a peak of 5-fold at 8 h) (Fig. 7A). In contrast, 10⁻⁷ M ANG-(1–7) had no significant effect on PAI-1 mRNA expression compared with medium alone (Fig. 7B). The 8-h incubation was then chosen for further treatments with ANG II. Rat MCs cotreated with 10⁻⁸ M ANG II and 10⁻⁷–10⁻⁵ M ANG-(1–7) for 8 h showed significantly reduced induction of PAI-1 mRNA with ANG II treatment, and this effect is dependent on the dose of ANG-(1–7) added (Fig. 8A). The ability of ANG-(1–7) to reduce ANG II-induced PAI-1 mRNA overexpression was decreased by adding 10⁻⁵ M Dla, the Mas receptor antagonist, although Dla alone had no effect on PAI-1 expression (Fig. 8B), or Dla alone had no effect on ANG II-induced PAI-1 overexpression (Fig. 8C).

Similarly, ANG II at 10⁻⁸ M induced a more than twofold increase in PAI-1 protein production by MCs at 24-h incubation. This induction of PAI-1 production was also blocked by pretreatment of MCs with 10⁻⁷ M ANG-(1–7), an effect further reversed by 54.4% by coadministration of 10⁻⁵ M Dla (Fig. 8D). These results indicate that ANG-(1–7) inhibits ANG II-mediated PAI-1 overexpression via the Mas receptor.

**DISCUSSION**

Our in vivo data clearly indicate that exogenous infusion of ANG-(1–7) into nephritic rats at an effective dose has a diuretic effect and significantly reduces disease, as measured by proteinuria, pathological ECM expansion, matrix components (FN and collagen) deposition, glomerular macrophage infiltration, and mRNA expression and protein production of profibrotic molecules. These results indicate that increasing ANG-(1–7) levels produces antifibrotic effects in diseased glomeruli, and these effects may be independent of blood pressure, since the model of nephritis in rats is normotensive (22, 24). Similar protective effects of ANG-(1–7) are reported in renal wrap hypertensive rats, where ANG-(1–7) infusion prevented the aggravating effects of ovariectomy on glomerular and tubulointerstitial fibrosis, independent of blood pressure (17). In addition, infusion of ANG-(1–7) was also shown to prevent activation of NADPH oxidase and renal vascular dysfunction in diabetic hypertensive rats or spontaneously hypertensive rats treated with the nitric oxide synthesis inhibitor N⁵-nitro-l-arginine methyl ester (2, 3). Moreover, a new finding in the present study, revealed by reduced glomerular macrophage infiltration after treatment of ANG-(1–7), indicates that ANG-(1–7) may have anti-inflammatory effects in the kidney. These observations suggest that long-term treatment with ANG-(1–7) in patients with kidney disease could be effective in subsequent prevention of renal injury by regulating blood pressure, by limiting production of reactive oxygen species, renal inflammation, and ECM proteins, as observed in the heart (1, 38, 42). In contrast, chronic infusion of ANG-(1–7) into rats with

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**Figure 9.** Schematic of how ANG-(1–7) may protect against ANG II-induced organ damage. AT₁ receptor was stimulated by ANG II and the subsequent cellular signaling, including NADPH oxidase activation, reactive oxygen species (ROS) generation, protein kinase C (PKC) activation, calcium (Ca²⁺) loading, and phosphorylation of mitogen-activated protein kinase (MAPK). Many deleterious effects of ANG II in organ damage are implicated, which can be reduced or prevented by ANG-(1–7) through stimulating vasodilatation and decreasing proliferation, hypertrophy, and fibrosis. Endothelins (ETs), nuclear factor-κ-light-chain-enhancer of activated B cells (NF-κB), and activator protein-1 (AP-1) are nuclear transcription factors. NO, nitric oxide; eNOS, endothelial nitric oxide synthase; ET-1, endothelin 1; +, stimulates; −, inhibits.
strepotozotcin-induced diabetes was reported to accelerate renal injury (34). In addition, ANG-(1–7) infusion had no protective effect against renal injury in an adriamycin-induced nephrotic syndrome rat model (34), while the oral AVE 0991 ANG-(1–7) analog exerted renoprotective and anti-inflammatory effects in the same model in mice (36). While differences in dose and experimental design could contribute to these discrepancies, further study with a higher dose of ANG-(1–7) given for a longer term is needed.

In the anti-Thy-1 nephritis model, ACEi or ARB therapy consistently and repeatedly reduces glomerulosclerosis, suggesting that local renal RAS is activated and renal ANG II generation and action are increased (26, 27, 46). Here we show that glomerular ACE mRNA expression is increased, and ACE2 expression is decreased, resulting in a significant increase in the ACE-to-ACE2 ratio in diseased rats, consistent with previous observations in the kidneys or glomeruli and proximal tubular cells of both diabetic animals and patients (20, 29, 43). In nephritic glomeruli, this increased ACE and decreased ACE2 may result in accumulation of ANG II and less generation of ANG-(1–7). While increased ANG II is clearly a key factor in the progression of renal diseases, little is known about the effect of reduced renal ANG-(1–7) concentration in disease. Since we show here that directly increasing ANG-(1–7) has a protective role in renal fibrosis, the present study suggests that a reduction in renal expression of ANG-(1–7) may exacerbate renal injury. Increasing local ANG-(1–7) by exogenous infusion does not affect renal ANG II concentration, since it did not affect plasma ANG II levels (data not shown) and had no effect on increased ACE or decreased ACE2 expression observed in nephritic glomeruli. However, increased ANG-(1–7) significantly stimulated plasma and renal renin activation and generation, which is often known to happen when ANG II’s action is reduced in vivo through a RAS feedback mechanism. Thus the protective effects of ANG-(1–7) in renal fibrosis may be mediated by inhibiting ANG II’s action. Recently, it was found that ANG-(1–7) levels are significantly increased (5- to 25-fold) by chronic treatment with ACEi and ARBs (18). Our results further indicate that the increased ANG-(1–7) may contribute at least partially to the beneficial effects of these drugs by inhibiting ANG II’s action. However, the cellular and molecular mechanisms involved in the renal protective effects of ANG-(1–7) at this point are not fully understood.

The in vitro binding study, together with the finding that renal MCs express Mas receptor mRNA, revealed that ANG-(1–7) specifically binds renal MCs via the Mas receptor. That ANG II competes poorly with ANG-(1–7) for the binding sites on MCs suggests that the binding receptor of ANG-(1–7) on these cells has a low affinity for ANG II and is distinct from the ATαR and ATβR. ANG II-induced increases in cell proliferation and PAI-1 overexpression are inhibited by ANG-(1–7) via activation of the Mas receptor, suggesting that ANG-(1–7) may act by reversing the profibrotic effects of ANG II. This idea is supported by previous studies showing that genetic deletion of the Mas receptor in mice led to glomerular hyperfiltration, microalbuminuria, and increased expression of scarring proteins in both renal mesangium and interstitium (28). It has been reported that ANG-(1–7) also binds ATαR with a very low affinity compared with the Mas receptor (33). This binding requires micromolar plasma concentrations of ANG-(1–7). The concentration of ANG-(1–7) obtained by infusion in the present study should be <10 nM. Data from other groups also suggest that the plasma concentration of ANG-(1–7) in rats receiving a similar infusion dose of ANG-(1–7) is around 1 nM (39). In addition, a high concentration of the inactive analog of ANG-(1–7), Dla (10–5 M) had no effect on ANG II’s action in cultured MCs. It appears unlikely that binding of ANG-(1–7) to the ATαR is involved in the therapeutic effects of ANG-(1–7) observed in the present study. Clearly further work is necessary to understand the signaling and downstream effects of ANG-(1–7) in counteracting the ANG II-induced renal injury.

Of interest in the kidney is that MCs primarily generate ANG II when incubated with ANG I, since MCs have limited ACE2 (43). In contrast, glomerular podocytes predominantly leads to ANG-(1–9) and ANG-(1–7) formation and ANG II catabolism, since podocytes express ACE2 but limited ACE (40, 41). It has been found that injection of the monoclonal anti-Thy-1 antibody (OX-7) rapidly induced podocyte foot process swelling, podocyteuria, and MC proliferation (6, 45), which may be a response to the reduced ACE2 and increased ACE expression in nephritic glomeruli observed in this model of the present study. Our findings in vitro raise the possibility that exogenous administration of ANG-(1–7) to diseased glomeruli could be effective in reducing glomerulosclerosis through restoring the altered glomerular balance between ANG II and ANG-(1–7) and counteracting the actions of increased ANG II locally. Thus this study suggests that ANG-(1–7) may help regulate tissue repair in glomeruli.

In summary, the in vitro and in vivo data presented here extend previous work in the heart and kidney and support the notion that increasing ANG-(1–7) is therapeutic in reducing renal fibrosis. The mechanism may involve ANG-(1–7) binding to the Mas receptor and counteracting the profibrotic effects of ANG II, as summarized in the scheme in Fig. 9. However, further studies are needed to determine whether additional increases in ANG-(1–7) levels by exogenous administration are beneficial for reducing renal fibrosis when ACEi or ARB is being used and endogenous ANG-(1–7) is being increased significantly.

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DISCLOSURES

All of the authors declared no competing interests.

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