Unilateral ureteral obstruction attenuates intrarenal angiotensin II generation induced by podocyte injury

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1Division of Nephrology and Hypertension, Department of Internal Medicine, The Jikei University School of Medicine, Tokyo, Japan; 2Department of Internal Medicine, Tokai University School of Medicine, Kanagawa, Japan; 3Department of Pediatrics, Tokai University School of Medicine, Kanagawa, Japan; and 4Institute of Medical Science, Tokai University School of Medicine, Kanagawa, Japan; 5Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland; 6Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee; and 7Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee

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Okabe M, Miyazaki Y, Niimura F, Pastan I, Nishiyama A, Yokoo T, Ichikawa I, Matsusaka T. Unilateral ureteral obstruction attenuates intrarenal angiotensin II generation induced by podocyte injury. Am J Physiol Renal Physiol 308: F932–F937, 2015. First published February 11, 2015; doi:10.1152/ajprenal.00444.2014.—The renal tissue renin-angiotensin system is activated in chronic kidney diseases. We previously demonstrated that intrarenal ANG II is synthesized primarily from liver-derived angiotensinogen filtered through the glomerulus and that podocyte injury increases the passage of angiotensinogen into the tubular lumen and generation of ANG II. In the present study, we tested the effect of cessation of glomerular filtration by ureteral obstruction on renal ANG II generation in kidneys with podocyte injury under two experimental conditions. Ureteral obstruction is known to activate the renin-angiotensin system in nonproteinuric kidneys. Transgenic mice expressing hCD25 in the proximal tubular cell. On the other hand, we have recently demonstrated that kidney (proximal straight tubule)-specific Agt knockout (KO) mice showed no decrease in intrarenal Agt and ANG II contents, whereas liver-specific Agt KO mice had remarkably suppressed renal Agt and ANG II (16). These findings clearly indicate that the major source of renal ANG II is liver-derived Agt, with almost no contribution of renal Agt mRNA. Analysis in megalin KO mice revealed that a small fraction of liver-originated circulating Agt is filtered through the glomerulus and reabsorbed by proximal tubular cells via megalin (16, 26).

We also investigated the effect of podocyte injury on intrarenal ANG II (16) using the NEP25 mouse model (17), in which podocyte injury can be induced by a recombinant immunotoxin, LMB2. After induction of podocyte injury, a massive amount of plasma Agt was leaked into the tubular lumen and reabsorbed by proximal tubular cells via megalin. In parallel with the increase in intrarenal Agt, renal ANG II was also increased after podocyte injury. Again, in this condition, liver-specific, but not kidney-specific, Agt KO near completely abolished intrarenal ANG II (15), clearly indicating that the liver is the major source of renal ANG II both in the basal condition and in that with podocyte injury.

Since Agt content within the interstitial space of the kidney did not change after podocyte injury (15), it appeared likely that the filtered Agt is converted to ANG II within the kidney. In the present study, to further investigate the role of glomerular filtration of plasma Agt in renal ANG II generation, we tested the effect of ureteral obstruction on renal ANG II generation after the induction of podocyte injury. Unilateral ureteral obstruction (UUO) is commonly used to induce tubulointerstitial injury (2, 4, 5, 8), but this procedure also virtually nullifies glomerular ultrafiltration by increasing Bowman’s capillary pressure (28). Although bilateral kidneys were exposed to LMB2, the obstructed kidney was devoid of Agt staining and contained less ANG II. In the present study, we found that ureteral obstruction, which causes activation of the renin-angiotensin system (RAS) in kidneys without podocyte...
injury, suppresses renal ANG II generation in kidneys with podocyte injury.

MATERIALS AND METHODS

Animal experiments. The Animal Experimentation Committee of Tokai University School of Medicine approved all protocols in accordance with the Guide for the Care and Use of Laboratory Animals published by National Institutes of Health.

In experiment 1, six adult female NEP25 transgenic mice (16 wk of age) were used, backcrossed with the C57BL/6N strain >10 times. These mice were intravenously injected with LMB2 (1.25 ng/g body wt), and, 24 h later, the UUO operation was performed. The operation was done by ligating the right ureter at the ureteropelvic junction under anesthesia with 60 µg/g body wt pentobarbital sodium. These mice were euthanized 8 days after LMB2 injection. Each kidney was immediately harvested and cut in three. The center thin slice was fixed in 4% paraformaldehyde for histological analysis. The upper and lower half portions were frozen in liquid nitrogen and stored at −80°C: one for RNA and protein analyses and the other for ANG II analysis. The same number of age- and sex-matched wild-type C57BL/6N mice were left untreated and used as controls. In experiment 2, 10 adult male NEP25 mice (18–25 wk of age) were injected with 10 ng/g body wt LMB2, and their kidneys were harvested 4 days after the UUO operation.

Blood pressure measurement and urinalysis. Conscious systolic blood pressure was measured by the tail-cuff method using MK-2000 (Muromachi Kikai, Tokyo, Japan) before the LMB2 injection and 1 day before death. Twenty-four-hour urine specimens were collected using metabolic cages on the same days. Concentrations of albumin and creatinine in urine were determined by the immunonephelometry method and enzymatic method, respectively, in an outside laboratory (SRL, Tokyo, Japan).

Histological analysis. Paraffin sections (2 μm) were incubated in methanol-H2O2 (100:1) solution at room temperature for 15 min, heated in citrate buffer at 100°C for 15 min, and blocked in normal goat serum (15 µl/ml in PBS). Sections were then incubated with rabbit polyclonal anti-mouse/rat Agt antibody (1:50, IBL, Fujioka, Gunma, Japan) at room temperature for 1 h followed by an incubation with a biotinylated goat anti-rabbit antibody (1:100, Vector Labs, Burlingame, CA) at room temperature for 1 h. The signal was visualized by the VECTASTAIN ABC KIT (Vector Labs) and diaminobenzidine. The specificity of this Agt antibody was confirmed using whole body Agt KO mouse tissues (16). This antibody perfectly detects Agt protein in the plasma, urine, liver, and kidney in Western blot analysis. However, it stains Agt protein only filtered through the glomerulus, but not Agt in vascular and interstitial spaces of the kidney, in heat-activated paraffin renal sections.

Western blot analysis. Western blot analysis for Agt protein was performed as previously described with some modifications (16). Kidney samples (5 μg total protein) were separated by electrophoresis in SDS-polyacrylamide gels under reduced condition. Proteins in the gels were electrophoretically transferred to polyvinylidene difluoride membranes. Agt protein was detected with rabbit anti-Agt antibody (IBL) diluted 1:500 with Can Get Signal immunoreaction enhancer solution (Toyobo, Tokyo, Japan). β-Actin was detected with rabbit monoclonal anti-β-actin antibody (Cell Signaling Technology, Tokyo, Japan) diluted 1:1,000. Densitometric analysis was performed with CS Analyzer 3.0 (Atto, Tokyo, Japan).

Assay for intrarenal ANG II content. Intrarenal ANG II content was determined by radioimmunoassay as previously described (16). Briefly, frozen kidney samples were homogenized in cold 10% methanol, and the supernatants were dried up. Residues were reconstituted in 50 mM sodium phosphate buffer (pH 7.4) containing 1 mM EDTA, 0.25 mM thimerosal, and 0.25% heat-inactivated BSA. These samples were applied to a phenyl-bonded solid phase extraction column (Bond-Elut, Agilent Technologies, Tokyo, Japan) and washed sequentially with water, hexane, and chloroform. ANG peptides were eluted with 90% methanol and evaporated under vacuum. They were reconstituted in the above buffer and added with 5,000 counts/min of 125I-labeled ANG II peptide and anti-ANG II antibody. After an overnight incubation at 4°C, free ANG II peptides were removed with charcoal, which was preincubated with dextran and human serum albumin, and radioactivity was then measured in a γ-counter for 3 min.

Real-time RT-PCR assay. Total RNA was extracted from frozen kidneys using ISOGEN II (Nippon Gene, Tokyo, Japan). Real-time RT-PCR was performed for Agt, renin, and Ace mRNAs as well as 18S rRNA using TaqMan primer probe sets with the StepOne Real Time PCR Systems (Thermo Fisher Scientific Japan, Yokohama, Japan). Relative amounts for Agt, renin, and Ace mRNAs were determined by ΔΔCt method (where Ct is threshold cycle).

Statistical analysis. A paired t-test was used to compare systolic blood pressure and body weight between before and after LMB2 injection. The urinary albumin-to-creatinine ratio was logarithmically transformed and compared by a paired t-test. For the Agt-to-β-actin ratio (determined by Western blot analysis), mRNAs for Agt, Ace, and renin, and intrarenal ANG II content, obstructed and contralateral kidneys were compared using a paired t-test, and other comparisons (wild-type vs. obstructed, wild-type vs. contralateral) were done with an unpaired t-test. P values were corrected by Holm’s method to minimize inflation of type I error due to multiple comparisons. Data in Figs. 2 and 5 are shown as means ± SE; other data are shown by means ± SD.

RESULTS

In experiment 1, podocyte injury was induced in NEP25 mice by injection of 1.25 ng/g body wt LMB2. To effectively hinder glomerular filtration, UUO surgery was performed 24 h after the LMB2 injection. Bilateral kidneys were exposed to the same dose of LMB2 since the half-life of circulating LMB2 is only 35 min (11). Seven days after the injection of LMB2, these mice developed massive proteinuria with an urinary albumin-to-creatinine ratio of >100 mg/mg (Table 1). Systolic blood pressure and body weight were not significantly changed during the experimental period.

The obstructed kidneys showed thinning of the renal parenchyma and diffuse dilation of renal tubular lumens and Bowman spaces, which are characteristic phenotypes of the UUO model. One of early signs of podocyte injury in the NEP25

| Table 1. Urinary albumin, blood pressure, and body weight (experiment 1) |
|------------------------|----------------------|-------------------------|
|                       | Before LMB2          | 7 Days After LMB2       |
| Urinary albumin-to-creatinine ratio, mg/mg | 0.0921 ± 0.0320 | 115.8 ± 16.1 |
| Systolic blood pressure, mmHg            | 112.7 ± 5.35       | 107.2 ± 12.8 |
| Body weight, g                           | 22.50 ± 1.19       | 23.25 ± 1.92 |

*Values represent means ± SD. Six NEP25 mice were injected with 1.25 ng/g body wt LMB2 on day 0, subjected to unilateral ureteral obstruction surgery on day 1, and euthanized on day 8. The urinary albumin-to-creatinine ratio was dramatically increased 7 days after LMB2 injection. Systolic blood pressure and body weight were not significantly changed during the experiment. *Log-transformed data were compared with a paired t-test.
model is downregulation of hCD25, the receptor for LMB2, which is driven by the nephrin promoter. Staining of hCD25 was markedly diminished in both obstructed and contralateral kidneys (data not shown), indicating that podocytes were severely injured in bilateral kidneys. However, as previously reported (14), glomerular morphology was relatively preserved in the obstructed kidney compared with the contralateral kidney. The contralateral kidney showed severe glomerular damage and massive proteinaceous casts. Agt was intensely stained in the contralateral kidney (Fig. 1). In contrast, Agt staining was dramatically suppressed in the obstructed kidney.

In parallel to the Agt immunostaining, intrarenal Agt protein as assessed by Western blot analysis was increased in the contralateral kidney of NEP25 mice with podocyte injury and suppressed in the obstructed kidney of the same mice (Fig. 2).

Intrarenal ANG II content was determined by radioimmunoassay. The contralateral unobstructed kidney of NEP25 mice with podocyte injury showed higher renal ANG II content than the wild-type kidney without podocyte injury. Intrarenal ANG II generation was significantly suppressed in the obstructed kidney compared with the contralateral kidney within the same NEP25 mice (Fig. 3).

Real-time RT-PCR assay revealed that the expression of renal Agt mRNA was upregulated not only in the unobstructed kidney but also in the obstructed kidney, to a lesser extent, compared with the untreated wild-type kidney (Fig. 4A). Renal renin mRNA was not significantly different among three groups (Fig. 4B). Transcription of Ace was downregulated in both obstructed and contralateral kidneys compared with the wild-type kidney without podocyte injury, and the decrease was larger in the obstructed kidney (Fig. 4C).

This larger decrease in Ace mRNA in the obstructed kidney was probably caused by tubular damage, as suggested by dilated tubules. We next evaluated the effect of UUO on renal ANG II in a condition with less severe tubular damage (experiment 2). NEP25 mice were injected with 10 ng/g body wt LMB2, subjected to UUO operation on the next day, and analyzed 4 days after UUO.

In experiment 2, again, renal Agt staining was strongly suppressed (data not shown), and Agt protein was decreased in dilated tubules.
the obstructed kidney (Fig. 5). Intrarenal ANG II was also significantly suppressed in the obstructed kidney compared with the contralateral kidney within the same mice (Fig. 6). Renal Agt mRNA was increased in both obstructed and contralateral kidneys, and this increase was larger in the contralateral kidney (Fig. 7A). Renal renin mRNA was increased only in the obstructed kidney (Fig. 7B). The expression of renal Ace mRNA was similarly downregulated in obstructed and contralateral kidneys with podocyte injury, and no significant difference was observed between obstructed and contralateral kidneys (Fig. 7C).

DISCUSSION

As previously reported (15, 16), the present study showed that renal Agt protein and ANG II peptide were increased in contralateral unobstructed kidneys of NEP25 mice with podocyte injury. Our previous study (15) demonstrated that liver-specific but not kidney-specific Agt KO remarkably attenuated the contents of renal Agt and ANG II, indicating that the source of them is liver-originated Agt. In the present study, blockage of glomerular filtration by ureteral obstruction attenuated the amounts of renal Agt and ANG II in NEP25 mice with podocyte injury under two experimental conditions. In addition, in a separate study similar to experiment 1 using seven NEP25 mice, we observed that renal ANG II was suppressed in the obstructed kidney compared with the contralateral kidney (121 ± 81 vs. 210 ± 110 fmol/g kidney, P = 0.047; data not shown). These results indicate that Agt filtered through the glomerulus is converted to ANG II. This suggests that the increase in Agt protein in the tubular lumen and/or within the tubule cell, but not that in the capillary lumen or in the interstitial area, is responsible for the increase in renal ANG II content. This notion is consistent with our previous findings that the amount of Agt protein measured in the kidney perfusate did not change after podocyte injury (15).

The results of the present study may appear somewhat odd in view of the well-established notion that ureteral obstruction activates the renal RAS in a setting without podocyte injury. Many studies have shown that renin production is increased in the obstructed kidney and that inhibition of RAS attenuates...
tubulointerstitial injuries (2, 4, 8, 24, 25, 29, 30), and a few studies have shown increased renal ANG II in the obstructed kidney (6, 7), although not consistently (3, 25). Our previous study (15) showed that liver Agt KO mice have very low renal ANG II despite very high renin mRNA, indicating that the availability of Agt protein is more important than renin activity for renal ANG II generation in this condition. Since massive Agt protein is filtered through the glomerulus in the nephrotic condition, its blockage by ureteral obstruction has a greater impact on renal ANG II in proteinuric than nonproteinuric conditions and may surpass alterations in other factors affecting renal ANG II generation.

The level of ANG II in the obstructed kidney was not lower in experiment 1 and significantly higher in experiment 2 compared with the wild-type kidney without podocyte injury. Since the UUO operation was done 24 h after LMB2 injection, a significant amount of plasma Agt leaked through the glomeruli during the 24 h, which can be a source of ANG II. Moreover, the level of renin mRNA in the obstructed kidney was higher in experiment 2 or tended to be higher in experiment 1 compared with that in the wild-type kidney. The increased renin in the obstructed kidney may generate more ANG II than that in wild-type intact kidney but was not sufficient to generate more ANG II than that in the contralateral kidney, which was supplied with huge amount of Agt protein.

Renal Agt mRNA was correlated with renal ANG II content. However, our previous study ruled out the role of renal Agt mRNA in renal ANG II generation. Although Ace mRNA was more suppressed in the obstructed kidney than in the contralateral kidney in experiment 1, there was no difference between the two kidney groups in experiment 2. In both experiments, renal ANG II content were significantly suppressed in the obstructed kidney, indicating that renal ANG II in the obstructed kidney is determined independently by Ace mRNA level.

In addition to hindering glomerular filtration, ureteral obstruction potentially causes many drastic changes, including hypoxia, oxidative stress, inflammation, and fibrosis. It remains possible that other mechanisms may also underlie the attenuation of renal ANG II by UUO, although many of those cause activation, not deactivation, of the renal RAS.

In conclusion, we demonstrated that hindrance to glomerular filtration by UUO decreases intrarenal ANG II and Agt contents, which are markedly increased in proteinuric kidneys, indicating that the major source of increased renal ANG II in podocyte injury is filtered Agt.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.O., Y.M., F.N., T.Y., I.I., and T.M. conception and design of research; M.O. performed experiments; M.O. and T.M. analyzed data; M.O. and T.M. interpreted results of experiments; M.O. prepared figures;
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