Intrarenal RAS activity and urinary angiotensinogen excretion in anti-thymocyte serum nephritis rats

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Ohashi N, Yamamoto T, Huang Y, Misaki T, Fukušawa H, Suzuki H, Togawa A, Suzuki S, Fujigaki Y, Nakagawa T, Nakamura Y, Suzuki F, Kitagawa M, Hishida A. Intrarenal RAS activity and urinary angiotensinogen excretion in anti-thymocyte serum nephritis rats. Am J Physiol Renal Physiol 295:F1512–F1518, 2008. First published September 10, 2008; doi:10.1152/ajprenal.00058.2008.—The differential roles of circulating and intrarenal renin-angiotensin system (RAS) in glomerulonephritis have not been elucidated. In this study, we investigated the levels of circulating and intrarenal RAS activity and urinary angiotensinogen (AGT) excretion in anti-thymocyte serum (ATS) nephritis induced by an ATS injection (ATS group). The effect of olmesartan, an angiotensin II (ANG II) type 1 receptor blocker (ARB), on the development of nephritis was also examined (ATS+ARB group). In addition, the rats received a saline injection instead of ATS (control group). Mesangial proliferation with transient proteinuria, which peaked at day 7, was significantly increased in the ATS group compared with the control group. The levels of glomerular AGT mRNA, intrarenal ANG II, and urinary AGT excretion in the ATS group were increased significantly at day 7 compared with the control group. Administration of olmesartan (ATS+ARB group) significantly decreased the levels of renal lesions, proteinuria, and intrarenal RAS activity compared with the ATS group. In addition, the levels of urinary AGT excretion correlated with the levels of glomerular damage, urinary protein excretion, and immunoreactivity for AGT and ANG II in kidney. On the other hand, plasma renin activity was significantly lower in the ATS group compared with the control group and significantly higher in the ATS+ARB group than in the ATS group. These data suggest that an increase in kidney-specific RAS activity, which parallels urinary AGT excretion, plays an important role in the development of ATS nephritis.

In addition to the role of the circulating renin-angiotensin system (RAS) in blood pressure (BP) regulation and sodium homeostasis, intrarenal RAS recently has been shown to play an important role in the pathophysiology of hypertension in angiotensin II (ANG II)-infused, spontaneously hypertensive, and two-kidney, one-clip Goldblatt hypertensive rat models (6, 7, 11, 18). Angiotensinogen (AGT) is the only known substrate for renin, the rate-limiting enzyme of RAS. Kobori et al. (6, 7, 11) reported that intrarenal ANG II augments intrarenal AGT, and upregulation of intrarenal AGT leads to the elevation of ANG II levels in the kidney in hypertensive rats. They also demonstrated that the level of urinary excretion of AGT parallels intrarenal ANG II production and reflects intrarenal RAS activity in ANG II-infused and spontaneously hypertensive rats (8, 10, 11). However, intrarenal RAS activity in mesangial proliferative glomerulonephritis has not been elucidated. To investigate the role of circulating and intrarenal RAS in mesangial proliferative glomerulonephritis, we examined the levels of circulating and intrarenal RAS activity and urinary AGT excretion in anti-thymocyte serum (ATS) nephritis, which was induced by a single injection of ATS. The effects of olmesartan, an ANG II type 1 receptor blocker (ARB), on nephritis were also examined.

MATERIALS AND METHODS

Experimental Design

All animal procedures were conducted with the approval of the Animal Committee of the Hamamatsu University School of Medicine. ATS was raised in a nonpregnant adult female England Suffolk sheep by subcutaneous immunization with 1 × 10⁸ Wistar rat thymocytes in complete Freund’s adjuvant, followed by four injections of 1 × 10⁸ thymocytes in complete Freund’s adjuvant at 4-wk intervals. Sera obtained 1 wk after the last injection were heat inactivated (56°C for 30 min) and absorbed three times by Wistar rat erythrocytes.

Seven-week-old male Wistar rats (150 g) used in these experiments were purchased from SLC (Hamamatsu, Japan) and were kept under a 12:12-h light-dark cycle. We previously reported that injection of ATS induces mesangial proliferative glomerulonephritis (29, 31). In the present study, rats were randomly assigned to three groups of rats: ATS+ARB, ATS, and control groups, as outlined below.

ATS+ARB group (n = 9). Rats received an intravenous injection of 0.35 ml of ATS at day 0 and were treated daily with CS-866, a prodrug type of olmesartan (ARB, Daiichi Sankyo, Tokyo, Japan). Olmesartan (10 mg·kg⁻¹·day⁻¹) dissolved in 0.5% methylcellulose solution was administered orally using gastric tubes. This amount of olmesartan was shown previously to normalize BP, urinary protein excretion, renal function and glomerulosclerosis effectively in Imai rats (22).

ATS group (n = 10). Rats received an ATS injection at day 0 and a daily oral administration of equal volumes of methylcellulose solution, the solvent of the olmesartan solution.

Control group (n = 9). Rats were injected with 0.35 ml of 0.9% saline solution instead of ATS at day 0, and then a methylcellulose solution was administered as described above.
BP was measured at day 5 after ATS injection by the tail-cuff method for animals that are conscious and restrained. To measure urinary AGT excretion, urine samples were collected using metabolic cages for 24 h at day 7 in tubes containing 50 µg pepstatin, 10 mg sodium azide, 300 nmol enalapril maleate (Sigma), and 125 µmol EDTA in distilled water (total volume, 0.6 ml). Urinary protein excretion was measured using a pyrogallol red-molybdate protein assay kit (Wako, Osaka, Japan) at day 7. Thereafter, blood samples were collected from the abdominal aorta, and the kidneys were perfused with ice-cold sterile 0.1 M PBS at pH 7.4 and dissected out as described previously (26).

Evaluation of Glomerular Lesions

Kidney tissues were fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Tissue sections (3 µm thick) were stained with periodic acid-Schiff (PAS) for histopathological evaluation. The percentage of the intraglomerular area occupied by mesangial matrix was estimated in 20 glomeruli from each rat and assigned values as follows: 0 (<1%), 1 (10–30%), 2 (30–50%), and 3 (>50%); the means of the values were calculated. All quantitative analyses were performed in a blinded manner to avoid bias.

Immunohistochemistry for AGT and ANG II

Immunostaining for AGT and ANG II in kidney sections was determined using a Histofine kit (Nichirei-Bioscience, Tokyo, Japan) according to the manufacturer’s instructions. The primary antibodies used were mouse monoclonal anti-AGT antibody (Swiss Swant Laboratories, Bellinzona, Switzerland) and rabbit polyclonal anti-ANG II antibody (Bachem, Torrance, CA). Nuclei were counterstained lightly with hematoxylin. Sections incubated without primary antibodies were used as controls. Specificity of the anti-AGT antibody was verified by preadsorption of the primary antibody with a synthetic oligopeptide corresponding to rat AGT epitopes (IBL, Takasaki, Gunma, Japan). For each rat, the cortical lesions in 20 consecutive microscopic fields were examined, and the intensities of AGT and ANG II immunoreactivity were calculated and averaged using Image Pro Plus software (Media Cybernetics, Bethesda, MD) as described previously (9, 11).

Intrarenal ANG II Contents

Intrarenal ANG II contents were measured by RIA using ANG II antibody (SRL, Tokyo, Japan) according to the manufacturer’s instructions. This ANG II antibody is certified by the manufacturer not to cross-react with angiotensin I (ANG I). The intrarenal ANG II concentration was normalized against the total protein concentration of homogenized kidney.

Quantitative Real-Time RT-PCR for AGT mRNA

Glomeruli were isolated using a graded sieving technique under sterile conditions (13), and total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan) as previously described (3, 28). Reverse transcription was performed with 5 µg of total RNA from glomeruli using a cDNA cycle kit (Invitrogen, Tokyo, Japan) using a LightCycler (Roche Diagnostics, Tokyo, Japan) was carried out to measure AGT mRNA levels. Data from quantitative real-time RT-PCR were normalized against GAPDH mRNA expression. The PCR-primer sequences were as follows: AGT: sense, 5'-TAGCTGTGCT- TGCTGGGCT-3' and antisense, 5'-GCTCTGCTGTAGTGAAGGA-3'; and GAPDH: sense, 5'-CAGAACATCATCCCTGCATC-3' and antisense, 5'-CTGCTTCACACACCTCTTGAGA-3'.

Measurement of Urinary AGT

Urinary excretion of AGT was measured as described elsewhere, except for using rat renin to convert rat AGT to ANG I (30). Briefly, the urine samples were collected for 24 h in tubes containing 50 µg pepstatin, 10 mg sodium azide, 300 nmol enalapril maleate, and 125 µmol EDTA and were incubated at 37°C for 27 h with 2.85 nM recombinant rat renin prepared as described previously (17). The levels of ANG I generated by renin were measured by ELISA for ANG I (Bachem). In a preliminary study with these samples, the amount of urinary AGT excreted daily was multiplied by the 24-h urinary volume to obtain the amount of urinary AGT excreted daily.

Plasma ANG II Concentration and Plasma Renin Activity

Plasma ANG II was measured using RIA for ANG II antibody (SRL) in a similar manner to intrarenal ANG II. Plasma renin activity (PRA) was measured by determining the generation rate of ANG I produced per milliliter per hour using an RIA kit (SRL).

Statistical Analysis

The differences among the groups with respect to various parameters were evaluated using ANOVA. To analyze correlations among

Fig. 1. Renal histology in anti-thymocyte serum (ATS) nephritis. A: representative photomicrographs of glomeruli in ATS nephritis rats at day 7. Increases in mesangial cell proliferation and matrix accumulation and marked glomerular enlargement were noted in the ATS group, and these lesions were ameliorated by administration of olmesartan (ATS+ARB group). Tissue sections were stained with periodic acid-Schiff (PAS). Original magnification ×400. B: glomerular damage scores in ATS nephritis at day 7. The percentage of intraglomerular area that was occupied by mesangial matrix was estimated in 20 glomeruli of each rat, and values were assigned as follows: 0 (<10%), 1 (10–30%), 2 (30–50%), and 3 (>50%). The means of the values were calculated. *P < 0.0001 vs. control group. #P < 0.0001 vs. ATS group.
the level of urinary AGT excretion, glomerular damage, urinary protein or intrarenal AGT and ANG II immunoreactivity, Pearson and Spearman single regression analysis was done. Statistical analysis was performed with StatView software (Abacus Concepts, Berkeley, CA). All data are presented as means ± SE. P < 0.05 was considered statistically significant.

RESULTS

BP

In ATS nephritis rats, no significant changes in both mean BP (MBP) and diastolic BP (DBP) were noted at day 5 among the three groups (MBP: control group: 110 ± 4 mmHg, ATS group: 112 ± 3 mmHg, ATS+ARB group: 104 ± 2 mmHg; DBP: control group: 94 ± 4 mmHg, ATS group: 97 ± 3 mmHg, ATS+ARB group: 90 ± 3 mmHg; means ± SE). On the other hand, a significant decrease in systolic BP (SBP) was noted in the ATS+ARB group compared with the ATS group (ATS group: 142 ± 2 mmHg; ATS+ARB group: 130 ± 2 mmHg, P < 0.01), although the level of SBP in the ATS group did not significantly differ from that in the control group (control group: 139 ± 4 mmHg).

Renal Histology and Proteinuria

In rats receiving the ATS injection, acute and transient increases in mesangial cell proliferation and matrix accumulation, which peaked at day 7, were noted (ATS group) (Fig. 1). A transient increase in proteinuria was also noted in the ATS group. The levels of glomerular lesions and proteinuria were significantly ameliorated by administration of olmesartan (ATS+ARB group) (Figs. 1 and 2).

Renal Expression of AGT

Focal and segmental immunoreactivity for AGT was noted mainly in proximal tubular cells, and several glomerular cells were weakly positive for AGT in control rats at day 7 (Fig. 3). No remarkable positive staining for AGT was noted in distal tubular cells in the control group. In the ATS group at day 7, diffuse and global immunoreactivity for AGT was noted in proximal tubular cells, and some glomerular cells and distal tubular cells were positive for AGT. Although there were no statistically significant differences between the control group and the ATS group, AGT immunoreactivity in the ATS group tended to be higher than that in the control group. Positive immunoreactivity for AGT was also noted in some glomerular cells and in proximal and distal tubular cells in the ATS+ARB group at day 7. However, its staining intensity tended to be less than that in the ATS group. Sections incubated without primary

![Fig. 2. Urinary protein excretion over 24 h in ATS nephritis rats at day 7. *P < 0.0001 vs. control group. #P < 0.05 vs. ATS group.](http://ajprenal.physiology.org/)

![Fig. 3. Immunohistochemical staining of angiotensinogen (AGT). A: representative photomicrographs of immunohistochemical staining for AGT at day 7 are shown. In the control group, focal and segmental immunoreactivity for AGT was noted mainly in some proximal tubular cells (indicated by arrows), with several glomerular cells weakly staining for AGT (indicated by arrowheads). No remarkable staining for AGT was noted in distal tubular cells (indicated by asterisks). In the ATS group, diffuse and global immunoreactivity for AGT was noted in proximal tubular cells (indicated by arrows). Some glomerular cells (indicated by arrowheads) and distal tubular cells (indicated by asterisks) were positive for AGT. In the ATS+ARB group, immunoreactivity for AGT in proximal (indicated by arrow) and distal (indicated by asterisks) tubular cells and glomerular cells (indicated by arrowhead) was less than in the ATS group. Original magnification ×400. B: semiquantitative analysis of the intensity of intrarenal AGT immunoreactivity at day 7. For each rat, 20 lesions in the cortex were examined, and the mean intensities of AGT immunoreactivity were obtained. The ratio was calculated relative to the control.](http://ajprenal.physiology.org/)
antibody did not stain, and preadsorption of the antibody with the synthetic oligopeptide corresponding to rat AGT epitopes caused a marked decrease in its immunoreactivity for AGT (data not shown). These data confirm the specificity of the anti-AGT antibody of these experiments.

Increased glomerular AGT mRNA expression was demonstrated at day 7 in isolated glomeruli from the ATS group, and administration of olmesartan decreased glomerular AGT mRNA expression in the ATS+ARB group (Fig. 4).

Renal Expression of ANG II

In control rats, ANG II immunoreactivity was noted at day 7 mainly in some distal tubular cells with weak staining in some proximal tubular cells (Fig. 5). In the ATS group, ANG II staining tended to increase in proximal and distal tubular cells at day 7 compared with that in the control group, and was significantly lower with administration of olmesartan (ATS+ARB group) compared with that in the ATS group. Intrarenal ANG II contents as measured by RIA, were increased significantly in the ATS group compared with the control. Intrarenal ANG II contents were significantly decreased in the ATS+ARB group compared with the ATS group (Fig. 6).

Urinary AGT Excretion

In the ATS nephritis rats at day 7, the levels of urinary AGT were significantly greater in the ATS group than in the control group, and significantly decreased by administration of olmesartan (ATS+ARB group) (Fig. 7).

Relationship Between Urinary AGT Excretion and Parameters of Renal Damage and Intrarenal RAS Components

The levels of urinary AGT excretion correlated positively with the level of glomerular damage, excretion of urinary

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**Fig. 4.** AGT mRNA expression in isolated glomeruli. Glomerular AGT mRNA expression was quantified in isolated glomeruli by real-time RT-PCR at day 7. Numbers indicate fold-induction relative to the control group. *P < 0.01 vs. control group. #P < 0.05 vs. ATS group.

**Fig. 5.** Immunohistochemical staining of ANG II. A: representative photomicrographs of immunohistochemical staining of ANG II at day 7. In the control group, immunoreactivity for ANG II was noted mainly in some distal tubular cells (indicated by arrows). Some proximal tubular cells (indicated by arrowheads) were weakly positive for ANG II. In the ATS group, ANG II staining was increased in proximal (indicated by arrowhead) and distal (indicated by arrows) tubular cells. In the ATS+ARB group, ANG II staining in proximal (indicated by arrowheads) and distal (indicated by arrows) tubular cells was decreased by administration of olmesartan. Original magnification ×400. B: semiquantitative analysis of intensity of intrarenal ANG II immunoreactivity at day 7. For each rat, 20 lesions in the cortex were examined, and the mean intensities of ANG II immunoreactivity were obtained. The ratio was calculated relative to the control. #P < 0.01 vs. ATS group.

**Fig. 6.** Intrarenal ANG II contents at day 7. The intrarenal ANG II contents were normalized against the total protein concentration in homogenized kidney. *P < 0.05 vs. control group. #P < 0.05 vs. ATS group.
protein, and immunoreactivity for intrarenal AGT and ANG II (Table 1).

**Plasma ANG II and PRA**

In the ATS nephritis rats, the levels of plasma ANG II and PRA changed in the opposite direction from those in kidney. In the ATS nephritis rats, the level of plasma ANG II tended to decrease in the ATS group compared with the control and were significantly increased by administration of olmesartan (ATS+ARB group) (Fig. 8). The levels of PRA were also significantly lower in the ATS group than the control and were significantly higher in the ATS+ARB group than the ATS group.

**DISCUSSION**

As with our previous reports (29, 31), a single injection of ATS caused mesangial proliferative glomerulonephritis. In ATS nephritis rats, the levels of PRA were significantly less than in control rats and increased markedly following administration of olmesartan. Similarly, the levels of plasma ANG II were relatively low in ATS nephritis rats compared with control rats, and increased significantly in ATS nephritis rats that were administered olmesartan daily. In contrast, the levels of intraglomerular AGT mRNA and intrarenal ANG II contents were increased significantly in ATS nephritis rats. Immunoreactivity for AGT and ANG II also tended to increase in ATS nephritis concurs with the hypothesis that AGT Elevated intrarenal RAS activity and the suppression of urinary AGT by expressing increased urinary AGT and ANG II expression in glomerular endothelial cells (1, 2, 6, 21, 27). In ATS nephritis, glomerular AGT mRNA was significantly elevated, and immunoreactivity for AGT tended to increase. Increased glomerular expression of AGT has also been reported in IgA nephropathy (9). Immunoreactivity for ANG II was mainly observed in distal tubules in control rats, and it tended to increase in proximal and distal tubular cells in ATS nephritis rats. Moreover, intrarenal AGT contents were increased significantly in ATS nephritis rats. In addition to circulating renin originating from the juxtaglomerular apparatus, it has been shown that renin is expressed in proximal tubules and it exists in proximal tubular fluid (4, 14, 16). It has been shown that angiotensin-converting enzyme (ACE) is expressed in the brush border of proximal tubules (24, 25).

Therefore, the observed increase in AGT and ANG II expression in ATS nephritis concurs with the hypothesis that AGT originating from either glomeruli or proximal tubules can be converted to ANG II in the tubular lumen and carried to distal tubules and that increased intrarenal ANG II can induce intrarenal AGT expression (5, 7, 11, 12). Administration of olmesartan decreased renal AGT and ANG II expression in ATS nephritis and ameliorated both renal lesions and proteinuria. These data suggest that enhanced intrarenal RAS activity is involved in the development of ATS nephritis.

We recently reported the increases in urinary AGT excretion in patients with chronic kidney disease having augmented intrarenal RAS activity and the suppression of urinary AGT by administration of losartan, another ARB (30). The present results demonstrate that urinary AGT excretion correlates with the level of glomerular damage, the excretion of urinary protein and immunoreactivity for intrarenal AGT and ANG II, thus confirming our previous findings. Several recent reports have demonstrated stimulation of AGT expression by the action of ANG II in the kidney (5, 7, 11, 12). Renin in collecting ducts has also been shown to be upregulated by the action of ANG II (20). Taken together, it is conceivable that intrarenal ANG II increases locally through the action of activated intrarenal RAS and that increased intrarenal ANG II can induce AGT expression and result in increased urinary AGT excretion and kidney damage. However, we cannot exclude the possible contribution of disrupted glomerular permeability to the increased urinary AGT excretion observed in ATS nephritis rats in the present study. Therefore, it is possible that the observed elevation in urinary AGT was just a nonspecific consequence of proteinuria. However, this possibility is unlikely because in our previous study we found no significant

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**Table 1. Relationship between urinary AGT and parameters of renal damage and intrarenal RAS components**

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<thead>
<tr>
<th></th>
<th>Correlation Coefficient</th>
<th>P Value</th>
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<tbody>
<tr>
<td>Glomerular damage score</td>
<td>0.745</td>
<td>0.0039</td>
</tr>
<tr>
<td>Urinary protein excretion</td>
<td>0.688</td>
<td>0.0113</td>
</tr>
<tr>
<td>Immunoreactivity for AGT</td>
<td>0.890</td>
<td>&lt;0.0001</td>
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<tr>
<td>Immunoreactivity for ANG II</td>
<td>0.836</td>
<td>0.0003</td>
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AGT, angiotensinogen; ANG II, angiotensin II.
negative correlation between circulating and urinary AGT levels in patients with chronic kidney diseases, in whom urinary excretion of AGT was increased in patients with augmented intrarenal ANG II activity (30). In addition, no significant increase in urinary AGT excretion was reported in deoxycorticosterone acetate salt-induced, volume-dependent but ANG II-independent, hypertensive rats despite concomitant increases in urinary protein excretion to the levels observed in ANG II-dependent hypertensive rats exhibiting increased urinary AGT excretion (10).

It is well known that hypertension plays an important role in the progression of renal lesions in a variety of kidney diseases. However, it would seem that BP made little contribution to the progression of renal damages in acute ATS nephritis, because no significant differences in MBP, DBP, and SBP were noted between control and ATS groups at day 5, when significant renal lesions had progressed in the ATS group. On the other hand, the data in the present study did not completely deny the role of BP in the progression of renal injury in acute ATS nephritis, because the significant decrease in SBP in the ATS+ARB group was associated with the amelioration of renal lesions. However, the amelioration of renal injury in the ATS+ARB group was also associated with the decreases in renal AGT expression, renal ANG II contents, and urinary AGT excretion, despite significant increases in PRA and plasma ANG II. Using transgenic mice overexpressing AGT in the kidneys, Sachetelli et al. (23) recently demonstrated that the increase in intrarenal RAS resulted in the development of hypertension and renal injury, and they were suppressed by RAS blockers. It has also been reported that whereas antihypertensive drugs non-RAS blockades prevent the development of hypertension, they fail to ameliorate kidney damage (11, 15). In the present study, it remained to be clarified whether the ameliorative effect of olmesartan on the renal lesions in acute ATS nephritis was attributed to the suppression of intrarenal RAS, BP, or both.

In summary, acute mesangial proliferation with transient proteinuria and the levels of intrarenal RAS activity, which is regulated independently of circulating RAS activity, were significantly increased in ATS nephritis rats, and administration of olmesartan significantly ameliorated these effects. In addition, the levels of urinary AGT excretion correlated with the levels of glomerular damage, urinary protein excretion, and intrarenal RAS activity. These data suggest that increased kidney-specific RAS activity, which parallels urinary AGT excretion, plays an important role in the development of nephritis.

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REFERENCES


