Angiotensin II Type 1 Receptor–Mediated Augmentation of Urinary Excretion of Endogenous Angiotensin II in Val5-Angiotensin II–Infused Rats

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Abstract—Rats infused chronically with Val5-Angiotensin (Ang) II exhibit increased urinary excretion of endogenous Ile5-Ang II by the 12th day of infusion, suggesting the stimulation of endogenous Ang II formation by Val5-Ang II infusion. The present study determined the time course of increased urinary Ang II excretion and the effects of Ang II type 1 receptor blockade (candesartan, 2 mg/kg per day) on the urinary excretion rates of Ile5-Ang II in Val5-Ang II–infused (80 ng/min) rats. Ile5-Ang II was separated from Val5-Ang II by high-performance liquid chromatography and measured by radioimmunoassay. Systolic blood pressure increased progressively (215±2 mm Hg) in Val5-Ang II–infused rats (n=5), whereas the candesartan-treated group (n=6) remained normotensive (124±3 mm Hg). Candesartan treatment significantly increased the level of plasma Ile5-Ang II (24.0±7.6 versus 156.9±24.6 fmol/mL; P<0.01); in contrast, there was a markedly lower intrarenal Ile5-Ang II content (357.9±76.6 versus 21.1±2.8 fmol/g; P<0.01). Urinary Ile5-Ang II excretion rates were elevated by day 9 (2185.7±283.2 fmol/24 hours) in Val5-Ang II–infused rats but not in candesartan-treated rats (740.6±110.3 fmol/24 hours). Thus, Ang II type 1 receptor blockade prevents the increase in urinary excretion of endogenous Ang II in rats subjected to chronic Ang II infusion. These data indicate that the increased urinary excretion of endogenous Ang II in Val5-Ang II–infused rats is primarily attributed to Ang II type 1 receptor–dependent secretion into and/or de novo formation of Ang II within the tubular lumen. (Hypertension. 2010;56:378-383.)

Key Words: angiotensin II–induced hypertension ■ renin-angiotensin system ■ high-performance liquid chromatography ■ intrarenal angiotensin II ■ urinary angiotensin II

Previous studies have demonstrated that intrarenal angiotensin (Ang) II content is significantly elevated in chronic Ang II–infused rats.1–3 The increases in circulating Ang II lead to progressive augmentation of intrarenal Ang II. The plasma renin activity (PRA) is markedly reduced in the Ang II–infused rats,1,2,4–6 whereas the kidney renin content and renin mRNA are not reduced to the same extent, which indicates that the sustained intrarenal renin activity may contribute to the augmented or sustained intrarenal Ang II levels.1–7,9 Because Ang II type 1 (AT1) receptor blockers markedly diminish the increase in intrarenal Ang II content in Ang II–infused rats, the augmentation of intrarenal Ang II is partially attributed to AT1 receptor–mediated binding and internalization of circulating Ang II.2,3,7,10,11 In addition, renal angiotensinogen (AGT) activity, AGT mRNA, protein, and renal Ang I contents, as well as intrarenal angiotensin-converting enzyme activity, are either increased or not reduced from the control levels, suggesting that there is also enhanced de novo formation of Ang II in the kidney.2,7,12–15

Our recent study demonstrated that rats infused chronically with Val5-Ang II exhibit increased urinary excretion of endogenous Ile5-Ang II by the 12th day of infusion, indicating the stimulation of endogenous Ang II formation.16 Several other studies have reported that the augmentation of intrarenal AGT in chronic Ang II infusion depends on activation of AT1 receptors. Chronic Ang II infusion leads to increases in urinary excretion of AGT, and enhanced urinary excretion rates of AGT are the consequence of the stimulation of AGT production by the kidney.7,12,17,18 These results provide further evidence that there is an enhanced intrarenal endogenous production of Ang II with subsequent augmentation of endogenous Ang II excretion in the urine in Val5-Ang II–infused rats. However, the relative quantitative contribution of enhanced endogenous Ang II production versus accumulation of circulating Ang II has not been established. Also, it has not been determined whether the increased endogenous Ang II in the urine is attributed to increased filtered Ang II or increased intratubular formation and/or secretion. Finally, the specific role of AT1 receptors in mediating the increased intrarenal and urinary endogenous Ang II has not been established. The objective of this study was to determine the time course of increased endogenous

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urinary Ang II excretion and the effects of AT1 receptor blockade (candesartan) on the intrarenal endogenous Ang II and urinary excretion of endogenous Ang II in Val5-Ang II–infused rats. Rats were infused with Val5-Ang II, which is not formed by rats, but has the same biological and immunoreactive properties as endogenous Ile5-Ang II.3,16 The effect of Val5-Ang II is equivalent to that of Ile5-Ang II in raising arterial pressure and intrarenal Ang II levels.3,16 Because these 2 isoforms can be separated by high-performance liquid chromatography (HPLC), this substitution approach enabled determination of the relative contributions of uptake of exogenously infused Val5-Ang II versus endogenously formed Ile5-Ang II to the elevated intrarenal Ang II content and increased urinary Ang II excretion in rats subjected to chronic Ang II infusion.

Materials and Methods

Animal Preparation
Male Sprague-Dawley rats weighing 330 to 350 g (Charles River Laboratories) were housed in wire cages under controlled temperature and lighting conditions. Throughout the experiments, the animals had free access to tap water and standard rat chow (Ralston Purina). All of the experiments were approved by the Tulane University Animal Care and Use Committee. Rats were divided into 2 experimental groups: rats infused with Val5-Ang II (n = 5) and rats infused with Val5-Ang II and treated with AT1 receptor antagonist, candesartan (n = 6). Candesartan (AstraZeneca) was administered in the drinking water at a dose of 2 mg/kg per day to allow chronic blockade (candesartan) on the intrarenal endogenous Ang II and urinaryAng II excretion in rats subjected to chronic Ang II infusions. Urine was centrifuged, and representative 1.0 mL aliquots of the supernatants were extracted and evaporated as described above for plasma and subjected to HPLC.

Separation of Val5-Ang II From Ile5-Ang II by HPLC

The HPLC and radioimmunoassay methodologies for the measurement of Ang peptides were similar to those described previously, with some modification.3,11,19–21 Briefly, the dried extract residue from each sample was reconstituted with a 250-μL equilibration solvent, which included 65% mobile phase A (0.13% heptfluorobutyric acid) and 35% mobile phase B (80.00% acetonitrile/0.13% heptfluorobutyric acid). A 200-μL aliquot of supernatant was chromatographed by isocratic elution at 1 mL/min on a 250×0.46-cm, 5-μm Vyday C18 reverse-phase HPLC column (Separations Group). Val5-Ang II eluted at 9 minutes, and Ile5-Ang II had an elution peak at 12 minutes. Fractions were collected from 8 to 10 minutes for Val5-Ang II and from 11 to 13 minutes for Ile5-Ang II, evaporated to dryness, reconstituted in assay diluent, and measured directly by radioimmunoassay. For the present study, particular care was given to the prevention of any carryover of the peptides from the calibration procedures to the experimental samples.

Measurement of Val5-Ang II and Ile5-Ang II by Radioimmunoassay

The reconstituted fractions were incubated with rabbit anti–Ang II antisera (Peninsula Laboratories Inc) and [125I]–radiolabeled Ang II (Perkin Elmer Life and Analytic Sciences) for 48 hours at 4°C. Bound and free Ang peptides were separated by dextran-coated charcoal, and the supernatants were counted on a computer-linked gamma counter for 3 minutes. Immunoreactivities of the antibody for Val5-Ang II and Ile5-Ang II were virtually identical. The sensitivity of the Ang II assay was 0.97 fmol. For the Ang II assays, the specific binding was 76.8%, and nonspecific binding was 6.2%.

PRA and Kidney Prorenin and Renin Content Assays

For PRA determinations, trunk blood was collected in chilled tubes containing EDTA (5 mmol/L). Plasma was separated and stored at −20°C until assayed with a commercially available GammaCoat Plasma Renin Activity 125I–Ang I Radioimmunoassay kit (DiaSorin Inc.).16 For kidney prorenin and renin content assessment, each kidney was immersed in cold kidney renin content homogenization buffer (2.6 mmol/L of EDTA, 3.4 mmol/L of hydroxyquinoline, 5.0 mmol/L of ammonium acetate, 200.0 μmol/L of PMSF, and 0.3 μmol/L of dimercaprol), minced, and homogenized.16 The homogenates were centrifuged and the supernatants were used to generate 1:1000 dilutions. Ten microliters of diluted kidney extract were incubated with trypsin (0.25 mg/mL) in the presence of BSA (10 mg/mL) and CaCl2 (5 mmol/L) for 1 hour at 4°C. The reaction was stopped by adding soybean trypsin inhibitor (0.5 mg/mL) for 10 minutes at room temperature. The diluted kidney extracts treated with trypsin and without trypsin were spiked with 1 μmol/L of synthetic renin tetradecapeptide substrate, and the generated Ang I was assayed with the Diasorin PRA radioimmunoassay kit for the prorenin and renin measurements.2,23

Statistical Analysis

Results are expressed as mean±SE. For Figures 1 and 6, the data were analyzed by repeated-measures ANOVA with post hoc Newman-Keuls multiple comparison test within each group, and the data were analyzed by 1-way ANOVA with post hoc Newman-Keuls multiple comparison test between 2 groups. We used student unpaired t test to analyze the data for Figures 2, 4, and 5. When compared with the third group from the previous publication in Figure 2, we applied a 1-way ANOVA. A value of P<0.05 was considered statistically significant.
Results

Effect of Candesartan on SBP During Val5-Ang II Infusion
SBP increased significantly from $113 \pm 10$ to $152 \pm 2$ mm Hg in the Val5-Ang II–infused rats. In the candesartan-treated rats, SBP was slightly lower initially and remained in the normotensive range throughout the duration of the experiment ($107 \pm 3$ to $124 \pm 3$ mm Hg; Figure 1).

Effect of Candesartan on PRA and Kidney Prorenin and Renin Content During Val5-Ang II Infusion
PRA was markedly suppressed in Val5-Ang II–infused rats compared with sham-operated rats in our previous report ($0.2 \pm 0.1$ versus $5.7 \pm 0.8$ ng of Ang I per milliliter per hour; $P<0.01$). In Val5-Ang II–infused rats treated with candesartan, PRA level was significantly elevated compared with that in Val5-Ang II–infused rats ($3.0 \pm 1.3$ versus $0.2 \pm 0.1$ ng of Ang I per milliliter per hour; $P<0.05$) but was not statistically different from the sham-operated rats (Figure 2). The kidney prorenin and renin contents were not statistically different between Val5-Ang II–infused rats and Val5-Ang II–infused rats treated with candesartan (Figure 3).

Effect of Candesartan on Plasma and Kidney Ang II Levels During Val5-Ang II Infusion
The total plasma Ang II concentration increased to $306.8 \pm 65.4$ fmol/mL in the Val5-Ang II–infused rats with most of it ($282.8 \pm 57.8$ fmol/mL) being attributed to Val5-Ang II, because the Ile5-Ang II concentrations were quite low ($24.0 \pm 7.6$ fmol/mL). In the Val5-Ang II–infused rats treated with candesartan, the total Ang II concentration was $373.6 \pm 58.7$ fmol/mL, but the Val5-Ang II concentration ($216.7 \pm 34.0$ fmol/mL) was not significantly lower compared with that in Val5-Ang II–infused rats not treated with candesartan; however, the Ile5-Ang II concentrations ($156.9 \pm 24.6$ fmol/mL) in the candesartan-treated group were significantly greater compared with the Ile5-Ang II levels ($24.0 \pm 7.6$ fmol/mL) in Val5-Ang II–infused rats ($P<0.01$; Figure 4). There were no differences in the plasma Ile5-Ang II levels between the sham-operated rats ($44.1 \pm 1.5$ fmol/mL) and Val5-Ang II–infused rats.

The total intrarenal Ang II content averaged $743.3 \pm 45.8$ fmol/g with approximately equal amounts of Val5-Ang II...
ever, the total Ang II content (263.2 ± 35.5 fmol/g) was actually reduced the intrarenal Ile5-Ang II content (21.1 ± 8.7 fmol/g) in Val5-Ang II–infused rats. With candesartan treatment, however, the total Ang II content (263.2 ± 35.5 fmol/g) was significantly lower and similar to the content of only the Val5-Ang II (242.1 ± 32.7 fmol/g) in the group not treated with candesartan. Importantly, candesartan treatment markedly reduced the intrarenal Ile5-Ang II content (21.1 ± 2.8 fmol/g; \(P < 0.01\); Figure 5). Furthermore, the Val5-Ang II infusions caused a significant increase of intrarenal Ile5-Ang II in the kidney (357.9 ± 76.6 fmol/g) compared with that observed in sham-operated rats (206.1 ± 13.0 fmol/g) in our previous report.16

**Effect of Candesartan on Urine Volume, Urinary Osmolality, and Urinary Ang II Excretion Rates During Val5-Ang II Infusion**

Val5-Ang II infusion caused marked increases in urine flow and decreases in urinary osmolality presumably because of the well-recognized effect of Ang II to stimulate thirst.24 Candesartan treatment prevented the increase in urine volume, becoming statistically different by day 9 (37.6 ± 2.7 versus 10.9 ± 1.0 mL/24 hours; \(P < 0.01\)), and also prevented the decreases of urinary osmolality, becoming significantly different by day 3 (860.0 ± 119.9 versus 1571.6 ± 180.1 mmol/kg) relative to the Val5-Ang II–infused rats (\(P < 0.01\)). The urinary Val5-Ang II excretion rates in both groups of the Val5-Ang II–infused rats increased sharply at day 3, then returned to baseline at day 6 and remained stable until day 12. Candesartan treatment reduced the urinary Val5-Ang II excretion rates, with the difference achieving statistical significance at day 9 (3018.3 ± 391.1 versus 581.9 ± 86.7 fmol/24 hours; \(P < 0.01\)). The urinary endogenous Ile5-Ang II excretion rates in the Val5-Ang II–infused rats increased over the course of infusion, with perceptible increases observed by days 9 and 12 when they were significantly greater than measured before the start of Val5-Ang II infusion (1330.7 ± 299.0 versus 4620.5 ± 828.4 fmol/24 hours; \(P < 0.01\)). Candesartan treatment prevented the increases in urinary endogenous Ile5-Ang II excretion rates during Val5-Ang II infusion, and statistical significance was reached by day 9 compared with Val5-Ang II–infused rats (2185.7 ± 283.2 versus 740.6 ± 110.3 fmol/24 hours; \(P < 0.05\); Figure 6).

**Discussion**

Previous studies have shown that intrarenal Ang II levels are augmented during Ang II–induced hypertension1–3,13 because of AT1 receptor–mediated uptake of circulating Ang II, as well as increased formation of endogenous Ang II.2,3,11 In our recent study,16 we confirmed that there was an elevated Val5-Ang II in the kidneys of Val5-Ang II–infused rats. We further reported that there was a significant augmentation of endogenous Ile5-Ang II levels in the kidneys that was greater than could be explained from equilibration with the circulating Ile5-Ang II concentration, thus demonstrating increased de novo formation of endogenous Ile5-Ang II.16 The present study addressed the important question regarding the role of the AT1 receptor in mediating the augmentation of endogenous Ang II.

In the Val5-Ang II–infused rats, the SBP begin to increase by day 3 and progressively increased over the course of the 2 weeks; candesartan treatment for rats infused with Val5-Ang II for 13 days prevented the progressive increase in SBP. The PRA levels were markedly suppressed in the Val5-Ang II–infused rats but not in the candesartan-treated rats, thereby indicating the importance of AT1 receptor activation in the Ang II–induced negative feedback effect on renin release.1,6,25–28 Interestingly, the kidney prorenin and renin contents were not suppressed in Val5-Ang II–infused rats. These results are consistent with previous reports that the kidney renin mRNA, renin protein, and kidney renin content are not suppressed to the same extent as PRA.1,8,26,27 Indeed, renin expression in collecting duct cells of the distal nephron actually increases in Ang II–infused rats.8,9 Thus, the KRC was not suppressed in the Val5-Ang II–infused rats, which would allow the possibility of increased endogenous intrarenal Ang II formation. In the Val5-Ang II–infused rats treated...
with candesartan, the plasma endogenous Ile\(^5\)-Ang II levels were elevated, which can be explained by the increased circulating renin activity.\(^2\)\(^-\)\(^3\) In contrast, the candesartan-treated Val\(^5\)-Ang II–infused rats exhibited reductions in kidney endogenous Ile\(^5\)-Ang II content by 94%, as well as in Val\(^5\)-Ang II content by 37%. The 37% reduction of Val\(^5\)-Ang II in the kidney reflects the blockade of AT\(_1\) receptor–mediated uptake of circulating Val\(^5\)-Ang II.\(^2\)\(^-\)\(^3\) The marked reduction in endogenous Ile\(^5\)-Ang II was because of both reduction of endogenous Ile\(^5\)-Ang II uptake and also a substantial reduction in intrarenal de novo formation of Ile\(^5\)-Ang II in the Val\(^5\)-Ang II–infused rats treated with candesartan. Zhuo et al\(^{13}\) showed that Ang II levels in renal cortical endosomes and intermicrovillar clefts are markedly increased in Ang II–infused hypertensive rats, and concurrent administration of candesartan prevented the increase in endosomes and intermicrovillar cleft Ang II levels. This study demonstrated that there is increased AT\(_1\) receptor–mediated intracellular trafficking/accumulation of circulating and/or intrarenal-formed Ang II into cortical tubular endosomes during Ang II–dependent hypertension, which is blocked by an Ang receptor blocker. The internalized Ang II or Ang II–AT\(_1\) receptor complex may migrate to the nucleus to exert genomic effects,\(^{20}\)\(^-\)\(^{21}\) because there are nuclear binding sites for Ang II in renal cells.\(^{32}\) In addition, chronic Ang II infusions increased intrarenal AGT mRNA levels causing enhanced intrarenal production of AGT, suggesting additional de novo Ang II generation, which contributes further to the increased intrarenal Ang II levels.\(^{7,12,14,28,33}\) In addition, AGT and Ang II are present in very high concentrations in proximal tubular fluid.\(^{17,18,34,35}\) In our current study, candesartan had a more profound effect on Ile\(^5\)-Ang II production than uptake of Val\(^5\)-Ang II into renal tissue, which provides quantitative evidence supporting the idea that there is a positive amplification mechanism resulting in the increases in de novo intrarenal endogenous Ang II formation in Ang II–induced hypertension.

The present study demonstrates that the urinary Ile\(^5\)-Ang II excretion rates by day 12 were significantly greater than those measured in the same rats on the day before the start of Val\(^5\)-Ang II infusion. Furthermore, the urinary Ile\(^5\)-Ang II excretion rates increased progressively and were significantly elevated by day 9 in the Val\(^5\)-Ang II–infused rats and significantly greater than in the candesartan-treated rats. In candesartan-treated rats, the Ile\(^5\)-Ang II excretion rate was reduced by 82%, and Val\(^5\)-Ang II excretion rate was reduced by 30%. These percentage reductions are similar to the reductions of intrarenal Ang II content, being 94% for Ile\(^5\)-Ang II and 37% for Val\(^5\)-Ang II. These results indicate that the increased urinary Ang II excretion rate is positively related to the elevation of intrarenal Ang II during Ang II–induced hypertension and that changes in the urinary Ang II excretion rates are a reflection of changes in intrarenal Ang II levels. Zou et al\(^{2}\) reported that, in Ang II–infused rats, plasma Ang II levels were elevated by day 3 of Ang II infusion; however, the intrarenal Ang II, measured at days 3, 7, 10, and 13, increased slightly by day 7 and were significantly elevated by day 10. The differences in the time-related changes in circulating and intrarenal Ang II levels suggested a slowly developing effect of circulating Ang II to enhance intrarenal Ang II. The time pattern of the augmentation of intrarenal Ang II observed by Zou et al\(^2\) is closely matched by the enhanced urinary Ile\(^5\)-Ang II excretion rates that we observed. Also, urinary excretion rates of AGT in Ang II–infused rats become significantly increased at day 8 compared with sham rats,\(^{33}\) and basal renal plasma flow and glomerular filtration rate levels in chronic Ang II–infused rats and those treated with a Ang receptor blocker are similar.\(^{36}\) These results support the hypothesis that the augmented urinary endogenous Ang II originates from the kidney and not from the filtration of circulating endogenous Ang II, which was actually decreased in the Val\(^5\)-Ang II–infused rats.

Administration of Ang II increases mRNA expression of renal and hepatic AGT production\(^{28}\) and directly increases AGT production and AGT mRNA levels in cultured proximal tubular cells,\(^{37}\) supporting the hypothesis that intrarenal AGT protein is predominately localized to proximal tubular cells.\(^{14,37,44}\) Furthermore, the increased AGT in the urine in Ang II–dependent hypertension indicates that AGT traverses the entire nephron segments.\(^{7}\) Komlosi et al\(^{45}\) reported that Ang II was formed intraluminally in the collecting duct in the presence of angiotensin-converting enzyme. Furthermore, the increases in renal AGT and urinary AGT excretion rate are mediated by AT\(_1\) receptor activation\(^{7}\) and lead to increased AGT secretion into the tubular lumen, which would lead to increased spillover of AGT into distal nephron segments, where it could then be acted on by renin and angiotensin-converting enzyme in collecting duct cells, leading to enhanced urinary excretion rates of Ang II.\(^9\) Accordingly, the present data support the hypothesis that the increased urinary endogenous Ile\(^5\)-Ang II excretion in Val\(^5\)-Ang II–infused rats is not derived from the circulating Ile\(^5\)-Ang II but is attributed to enhanced secretion into and/or de novo formation of intrarenal endogenous Ile\(^5\)-Ang II mediated by AT\(_1\) receptors.

**Perspectives**

This study demonstrates that chronic Ang II infusions to normal rats significantly increase urinary excretion of endogenous Ile\(^5\)-Ang II in a time-dependent manner that was associated with elevated intrarenal endogenous Ile\(^5\)-Ang II levels, and they are both mediated by AT\(_1\) receptors. The urinary excretion of endogenous Ang II may be an indicator of increased distal nephron Ang II production in Ang II–dependent hypertension, which could augment distal sodium reabsorption. It is possible that, in human subjects, the measurement of urinary excretion of Ang II can be used as one of the biomarkers in hypertensive patients to monitor the efficacy of drugs that block the actions of the renin-angiotensin system and, thus, contribute to an optimized treatment.

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