Augmentation of endogenous intrarenal angiotensin II levels in Val⁵-ANG II-infused rats

Weijian Shao, Dale M. Seth, and L. Gabriel Navar

Department of Physiology, Hypertension, and Renal Center of Excellence, Tulane University Health Sciences Center, New Orleans, Louisiana

Submitted 9 October 2008; accepted in final form 20 February 2009

Shao W, Seth DM, Navar LG. Augmentation of endogenous intrarenal angiotensin II levels in Val⁵-ANG II-infused rats. Am J Physiol Renal Physiol 296: F1067–F1071, 2009. First published February 25, 2009; doi:10.1152/ajprenal.90596.2008.—In angiotensin II (ANG II)-induced hypertension, intrarenal ANG II levels are increased by AT₁ receptor-mediated ANG II internalization and endogenous ANG II generation. The objective of the present study was to determine the relative contribution of de novo formation of endogenous ANG II. Male Sprague-Dawley rats were divided into three groups: sham operated (n = 6), Val⁵-ANG II-infused (n = 16), and Ile⁵-ANG II infused (n = 6). Val⁵-ANG II and Ile⁵-ANG II were infused at 80 ng/min via subcutaneous osmotic minipump for 13 days, followed by harvest of blood and kidney samples. In six Val⁵-ANG II-infused rats, urine was collected on the day before infusion and on day 12 of infusion. Extracted samples were subjected to HPLC to separate Val⁵-ANG II from Ile⁵-ANG II followed by RIA. Systolic blood pressure increased significantly from 121 ± 2 to 206 ± 4 mmHg in the Val⁵-ANG II-infused rats and from 124 ± 3 to 215 ± 5 mmHg in the Ile⁵-ANG II-infused rats. In the Val⁵-ANG II-infused rats, the plasma Ile⁵-ANG II levels increased 196.2 ± 70.1% compared with sham plasma Ile⁵-ANG II concentration. Val⁵-ANG II levels were 150.0 ± 28.2 fmol/ml which accounted for 53.5 ± 10.1% of the total ANG II in plasma. The kidney Ile⁵-ANG II levels in the Val⁵-ANG II-infused rats increased 69.9 ± 30.7% compared with sham kidney Ile⁵-ANG II concentrations. Intrarenal accumulation of Val⁵-ANG II accounted for 52.5 ± 5.3% of the total kidney ANG II during Val⁵-ANG II infusion while endogenous Ile⁵-ANG II accounted for 47.5 ± 8.6%. The urinary Ile⁵-ANG II excretion rate on day 12 increased 93.2 ± 32.1% compared with preinfusion level indicating increased formation of endogenous ANG II. Thus, the increases in intrarenal ANG II levels during chronic ANG II infusions involve substantial stimulation of endogenous ANG II formation which contributes to overall augmentation of intrarenal ANG II.

angiotensin II-induced hypertension; renin-angiotensin system; high-performance liquid chromatography

PREVIOUS STUDIES showed augmentation of intrarenal ANG II levels in two-kidney, one-clip (2K1C) hypertensive rats, chronic ANG II-infused rats, and TGR(Ren2) rats (8, 15, 25, 28, 31). The augmentation in the intrarenal ANG II levels in the ANG II-infused rats is prevented by AT₁ receptor antagonists, which markedly reduce the intrarenal ANG II content of kidneys harvested after 2 wk of ANG II infusion, indicating that the enhanced intrarenal ANG II is mediated by an active receptor-mediated process (12, 15, 30, 31). While the plasma renin activity (PRA) is markedly suppressed in the ANG II-infused rats (5, 17, 27, 28, 31), the reductions in kidney renin content (KRC) and renin mRNA are not as marked suggesting the possibility that continued intrarenal renin activity may contribute to the augmented or sustained intrarenal ANG II levels (7, 12, 22, 28). Previous studies showed that some of the intrarenal ANG II is due to accumulation of circulating ANG II (30, 32); however, there is also evidence for enhanced de novo formation of ANG II because both the renal angiotensinogen activity and kidney ANG I contents are either increased or not significantly reduced from control levels (10, 12, 29, 31). While both increased sequestration of circulating ANG II and increased intrarenal generation of ANG II could contribute to the increased intrarenal ANG II levels, the quantitative roles of these two processes to the overall increases in intrarenal ANG II levels remain uncertain. In particular, it has not been demonstrated that the increased circulating concentrations resulting from the ANG II infusions actually lead to increased intrarenal levels of ANG II from endogenous origin. In a previous study, Zou et al. (32) demonstrated that an important component of the intrarenal ANG II was derived from accumulation of circulating Val⁵-ANG II. Other studies, however, demonstrated that chronic ANG II infusions lead to substantial stimulation of intrarenal angiotensinogen which leads to increased angiotensinogen secretion into the tubular fluid and excretion in the urine (10, 12, 19, 20). These studies support a greater role for enhanced endogenous formation of ANG II during chronic ANG II infusions and prompted us to reexamine this issue. Accordingly, the objective of this study was to delineate the relative origins of the intrarenal ANG II to determine whether there is an increased endogenous formation of ANG II during chronic ANG II infusions. Rats were infused with Val⁵-ANG II, which is not endogenously produced in rats but has the same biological and immunoreactive properties as endogenous Ile⁵-ANG II (32). The effect of Val⁵-ANG II is equivalent to that of Ile⁵-ANG II in raising arterial pressure and intrarenal ANG II levels (32). Since these two isoforms can be separated by HPLC, this substitution approach enabled determination of the relative contributions of uptake of exogenously infused Val⁵-ANG II vs. endogenously formed Ile⁵-ANG II to the elevated intrarenal ANG II content.

MATERIALS AND METHODS

Animal preparation. Male Sprague-Dawley rats (240 to 270 g body wt; Charles River Laboratories) were housed in wire cages under controlled temperature and lighting conditions. Throughout the experiments, animals had free access to tap water and standard rat chow (Ralston Purina). All experiments were approved by the Tulane University Animal Care and Use Committee. Rats were divided into three experimental groups: sham operated rats (n = 6), rats infused with Val⁵-ANG II (n = 16), and rats infused with Ile⁵-ANG II (n = 6). The sham-operated rats underwent the same minipump implant surgery as the ANG II-infused rats except without the minipump. Our previous studies showed that implantation of vehicle-containing min-
impumps does not influence the results. Ang II was delivered continuously at a rate of 80 ng/min via osmotic minipump (model 2002, Duract, Cupertino, CA) that was implanted subcutaneously at the dorsum of the neck. Systolic blood pressure (SBP) was measured in conscious rats by tail-cuff plethysmography (Visitech Systems, Apex, NC) to monitor the progression of hypertension. For measurements of PRA, KRC, plasma and renal Ile-5-ANG II and Val-5-ANG II levels, the conscious rats were decapitated on day 13. Trunk blood was collected and the kidneys were immediately removed, quickly weighed, and homogenized in methanol. The time delay between decapitation and homogenization of the kidneys did not exceed 60 s.

**Collection and extraction of blood, kidney, and urine samples.**

Blood was collected in chilled tubes containing a mixed inhibitor solution (final concentration: 5 mMol/l EDTA, 20 μmol/l pepstatin, 10 μmol/l PMSF, 20 μmol/l enalaprilat, and 1.25 mmol/l 1,10-phenanthroline). After centrifugation at 4°C for 10 min at 1,000 g, plasma was separated and applied to phenyl-bonded solid-phase extraction columns (Bond-Elut, Varian) that had been prewashed with 90% methanol followed by water. After sample application, each solid-phase extraction column was washed sequentially with water, hexane, and chloroform. The water removed salts and other polar substances from the column. The hexane and chloroform eluted contaminating lipid and hydrophobic material from the column but did not affect the recovery of angiotensin peptides (6). Angiotensin peptides were eluted from the solid-phase extraction column with 90% methanol. The eluants were collected, evaporated to dryness under vacuum, and then subjected to HPLC to separate Val-5-ANG II from Ile-5-ANG II (6, 32).

For Ang II measurements, each kidney was immersed in cold methanol (100%), minced, and homogenized with polytron tissue tearor immediately after harvesting and weighing. The homogenates were centrifuged and the supernatants from the kidney homogenates were dried overnight in a vacuum centrifuge. The dried residue was reconstituted in 1 ml RIA buffer (19 mM monobasic sodium phosphate, 81 mM dibasic sodium phosphate, 0.05 M sodium chloride, 0.1% BSA, 0.1% Triton X-100, 0.01% sodium azide, pH 7.4). These samples were extracted and evaporated as described above for plasma and subjected to HPLC. Urine was collected in tubes containing the same mixed inhibitor solution as that for blood collecting for 24 h on the day before ANG II infusion and on day 12 after ANG II infusion. 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 Val-5-ANG II from Ile-5-ANG II by HPLC.** The HPLC and radioimmunoassay methodologies for the measurement of Ang II peptides were similar to those previously described (6, 30, 32). Briefly, the extract residue from each plasma, kidney, and urine sample was redivoluted in 250-μl HPLC column equilibration solvent (37% methanol, 63% water, 0.1% H₃PO₄) and chromatographed at 1 ml/min on a 25 × 0.46-cm, 5-μm Vydac C18 reverse-phase HPLC column (Separations Group). As shown in Fig. 1, Val-5-ANG II eluted at 6 min (fractions 11 to 14), and Ile-5-ANG II had an elution peak at 9 min (fractions 17 to 21; Fig. 1). Fractions were collected every 30 s, 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 Val-5-ANG II and Ile-5-ANG II by radioimmunoassay.** The reconstituted plasma, kidney, and urine fractions were incubated with rabbit anti-ANG II antiserum (Peninsula Laboratories) and 125I-labeled ANG II (Perkin Elmer Life and Analytical Sciences) for 48 h at 4°C. Bound and free ANG II peptides were separated by dextran-coated charcoal, and the supernatants were counted by a computer-linked gamma counter for 3 min. Immunoreactivities of the antibody for Val-5-ANG II and Ile-5-ANG II were virtually identical. Results are reported in femtomoles (fmol) per gram of kidney weight or fmol per milliliter of plasma and fmol per 24 h of urine. The sensitivity of the ANG II assay was 1.62 fmol. For the ANG II assays, the specific binding was 45.12%, and nonspecific binding was 3.26%.

**PRA and KRC 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 RIA kit (DiaSorin) (28). For KRC assessment, part of each kidney was immersed in cold KRC homogenation buffer (2.6 mM EDTA, 3.4 mM hydroxyquinoline, 5 mM ammonium acetate, 200 μM PMSF, 0.256 μM dimerco-prol), minced, and homogenized. The homogenates were frozen and thawed four times. The homogenates were centrifuged and the supernatants were used to generate 1:1000 dilutions that were spiked with 1 μM synthetic renin tetradecapeptide substrate and the generated ANG I was assayed with the Diasorin PRA RIA kit.

**Statistical analysis.** Data are expressed as means ± SE. The statistical analyses for plasma, kidney, and urine levels were performed using Student’s t-test between groups. A value of P < 0.05 was considered statistically significant.

**RESULTS**

**Effect of Val-5-ANG II and Ile-5-ANG II on SBP.** As shown in Fig. 2, SBP increased significantly from 121 ± 2 to 206 ± 4 mmHg in the Val-5-ANG II-infused rats and from 124 ± 3 to 215 ± 5 mmHg in the Ile-5-ANG II-infused rats by day 12 of infusion. In the sham-operated rats, SBP remained stable and normotensive throughout the duration of the experiment (123 ± 1 to 128 ± 4 mmHg; Fig. 2).

**Effect of ANG II infusion on PRA and KRC.** Infusion with either of the ANG II peptides resulted in marked suppression of PRA as previously shown (28). In the sham-operated rats, the PRA average level was 5.7 ± 0.8 ng ANG I·ml⁻¹·h⁻¹, whereas in Val-5-ANG II- and Ile-5-ANG II-infused rats, the PRA levels were 0.6 ± 0.3 and 0.3 ± 0.1 ng ANG I·ml⁻¹·h⁻¹, respectively (Fig. 3A). However, the KRC (470.6 ± 44.8 ng
ANG I·h⁻¹·mg⁻¹) was not different from the sham KRC (448.0 ± 28.7 ng ANG I·h⁻¹·mg⁻¹; Fig. 3B).

Effect of ANG II infusion on plasma, kidney, and urine ANG II levels. As shown in Fig. 4, the plasma Ile⁵-ANG II levels (130.5 ± 30.9, 253.5 ± 60.5 fmol/ml) increased 196.2 ± 70.1 and 475.3 ± 137.3% in both Val⁵-ANG II- and Ile⁵-ANG II-infused rats compared with sham-operated rats (44.1 ± 1.5 fmol/ml), P < 0.05. In the Val⁵-ANG II-infused rats, plasma Val⁵-ANG II levels were 150.0 ± 28.2 fmol/ml which accounted for 53.5 ± 10.0% of total ANG II in plasma. As depicted in Fig. 5, the kidney Ile⁵-ANG II levels (350.1 ± 63.2 fmol/g) in the Val⁵-ANG II-infused rats increased 69.9 ± 30.7% compared with the sham kidney Ile⁵-ANG II concentration (206.1 ± 13.0 fmol/g), P < 0.05, and kidney Val⁵-ANG II levels were 387.2 ± 39.4 fmol/g. Relative to the total intrarenal ANG II 52.5 ± 5.3% was due to accumulation of Val⁵-ANG II from the circulation during Val⁵-ANG II infusion, while endogenous Ile⁵-ANG II accounted for 47.5 ± 8.6%. In the Ile⁵-ANG II-infused rats, kidney Ile⁵-ANG II levels (519.3 ± 57.4 fmol/g) increased 152.0 ± 27.8% compared with the sham kidney Ile⁵-ANG II concentration, P < 0.01 (Fig. 5). In the Val⁵-ANG II-infused rats, the urinary Val⁵-ANG II excretion rate was 1,831.7 ± 251.6 fmol/24 h and the urinary Ile⁵-ANG II excretion rate was 2,183.7 ± 363.4 fmol/24 h which increased 93.2 ± 32.1% compared with the ANG II excretion rate on the day before infusion (1,130.5 ± 208.3 fmol/24 h; P < 0.05; Fig. 6).

**DISCUSSION**

Several animal models of hypertension, including 2K1C hypertensive rats, the Ren-2 gene transgenic rats, and ANG II-infused rats, have elevated intrarenal ANG II levels to extents greater than can be explained from equilibration with the circulating concentrations (8, 16, 27, 28, 31, 32). In our Val⁵-ANG II- and Ile⁵-ANG II-infused rats, the SBP began to increase at day 3 and progressively increased over the course of the 2 wk with both groups reaching similar arterial pressure levels by day 12. Intrarenal ANG II levels increased substantially in both groups of ANG II-infused rats. Importantly, the Val⁵-ANG II experiments demonstrate that these increases were due to increased formation of endogenous ANG II, as well as accumulation of the infused ANG II. The substantial increases in the intrarenal Val⁵-ANG II are not likely to be due only to nonspecific trapping but also reflect active accumulation via AT₁ receptors (30). As previously described (1, 13, 29, 30), ANG II is internalized via AT₁ receptors with accumulation in endosomes. In vivo studies also showed that losartan markedly reduced the intrarenal ANG II content of kidneys harvested after 2 wk of ANG II infusion (31). Cervenka et al. (3) reported that while plasma ANG II levels in AT₁A knockout mice are much higher than in the wild-type control, the kidney ANG II contents are lower in the AT₁A knockout mice. These findings indicate that ANG II binds to AT₁ receptors and leads to internalization of ANG II contributing to the overall increases in intrarenal ANG II levels and are in accord with an extensive recent study in AT₁A knockout mice (4). These increases are associated with endosomal ANG II accumulation and enhanced expression of AT₁ receptors in these intracellular compartments which can be prevented by concurrent administration of AT₁ receptor blockers (29). This uptake has also been shown to occur in proximal tubule cells through AT₁ receptors.
receptor-mediated endocytosis (13). Further studies in proximal tubule cells from AT1A null mice showed that AT1A receptors exert the predominant role in mediating the intrarenal ANG II accumulation (14).

In previous studies (30, 32), emphasis was placed on the uptake of the infused Val5-ANG II mediated via an AT1 receptor mechanism. In our current study, the increased plasma levels of Ile5-ANG II in Val5-ANG II-infused rats indicate continued formation of endogenous ANG I and ANG II even though PRA levels are markedly suppressed. The low PRA levels suggest that the endogenous ANG II peptides are formed in organs and tissues where renin may not be suppressed to the same extent. In this regard, several previous studies showed that the kidney renin mRNA, renin protein, and KRC are not suppressed to the same extent as PRA. The extent of suppression is dependent on the rate of ANG II infusion. Indeed, renin expression in collecting duct cells of the distal nephron actually increases in ANG II-infused rats (7, 22, 23, 28). In the current study, where we used an ANG II infusion rate that caused a slow progressive hypertension, the KRC was not suppressed in Val5-ANG II-infused rats. These data suggest that the increased endogenous ANG II formation is occurring in the kidneys with some of it spilling into the circulation.

Previous studies demonstrated that angiotensinogen and ANG II are also present in very high concentrations in proximal tubule fluid (2, 19, 20, 26). The ability of the chronic ANG II infusions to stimulate renal angiotensinogen mRNA and protein levels has been postulated to be responsible for additional de novo ANG II generation which contributes further to the increased intrarenal ANG II levels (10–12, 25). Other studies (7, 24) also demonstrated localization of angiotensinogen mRNA and protein in proximal tubules of mice and augmentation in response to ANG II infusions (7). The augmentation of intrarenal angiotensinogen in ANG II-infused rats is blocked by treatment with AT1 receptor blockers (12). It has also been shown that the renal tubular AT1 receptor mRNA and protein levels are maintained in ANG II-induced hypertension and do not show signs of downregulation (9). These data support the concept that there is enhanced intrarenal ANG II accumulation mediated by chronic ANG II infusion, but it has been difficult to provide in vivo evidence that there is actually an increased formation of endogenous Ile5-ANG II formed de novo. Also, we recognize that it is difficult to dissociate the effects of increases in arterial pressure from the direct effects of AT1 receptor stimulation particularly because there may be synergistic interactions. In the present study, however, emphasis was placed on the question of whether the endogenous intrarenal ANG II content is greater in the Val5-ANG II-infused rats than in sham-operated animals. In the Val5-ANG II-infused rats, the intrarenal Ile5-ANG II content was markedly increased above that measured in sham-operated rats. Furthermore, the urine Ile5-ANG II excretion rates were significantly greater than those measured in the same rats on the day before the start of Val5-ANG II infusions. Importantly, the overall increases in urinary ANG II levels also support the concept that intraluminal distal nephron ANG II concentrations are increased in ANG II-infused rats which may
The data from the Val5-ANG II-infused rats demonstrate that increased levels of intratubular ANG II concentrations contribute to overall augmentation of intrarenal ANG II leading to increased levels of intratubular ANG II concentrations.

ACKNOWLEDGMENTS

The authors thank D. Olovarrieta for assistance in the preparation of the manuscript.

GRANTS

This work was supported by grants from National Heart, Lung, and Blood Institute (HL-26371), from the Health Excellence Fund of the Louisiana Board of Regents, and by a COBRE grant (P20RR017659) from the Institutional Developmental Award program of the National Center for Research Resources.

REFERENCES