Renal Uptake of Circulating Angiotensin II in Val^5-Angiotensin II Infused Rats Is Mediated by AT_1 Receptor

Li-Xian Zou, John D. Imig, Anka Hymel, and L. Gabriel Navar

Previous studies have demonstrated that augmentation of intrarenal angiotensin II (ANG II) levels during ANG II induced hypertension involves both endogenous formation and accumulation of circulating ANG II. The present work extends these findings and determines whether accumulation of infused ANG II in the kidney requires AT_1 receptor activation by using Val^5-ANG II as the infused peptide. Male Sprague-Dawley rats were uninephrectomized and divided into three groups: control (n = 6), Val^5-ANG II (exogenous form) infused (n = 8), and Val^5-ANG II infused rats treated with losartan (n = 8). Val^5-ANG II, which has the same biological and immunoreactive properties as endogenous ANG II, was infused at 40 ng/min via an osmotic minipump implanted subcutaneously. By day 12, systolic blood pressure (SBP) increased significantly in Val^5-ANG II infused rats (197 ± 7 mm Hg). As previously shown, the development of hypertension in ANG II infused rats was prevented by losartan treatment. Blood and kidney samples were harvested, subjected to HPLC to separate Val^5-ANG II (exogenous) from Ile^5-ANG II (endogenous) and the fractions were measured by radioimmunoassay. In the Val^5-ANG II infused rats treated with losartan, total plasma ANG II levels were elevated to a greater extent than in rats not treated with losartan (289 ± 20 *v* 119 ± 14 fmol/mL). However, losartan markedly decreased by 88% the enhancement of intrarenal Val^5-ANG II content that occurred in the rats infused with Val^5-ANG II alone. These results demonstrate that AT_1 receptor blockade markedly reduces the intrarenal uptake of circulating ANG II that occurs in ANG II induced hypertension. Am J Hypertens 1998; 11:570–578 © 1998 American Journal of Hypertension, Ltd.

KEY WORDS: **Val^5-angiotensin II, IIe^5-angiotensin II, angiotensin II induced hypertension, renin-angiotensin system.**

Previous studies have indicated that increased angiotensin II (ANG II) levels in the contralateral, nonclipped kidney may contribute to the pathogenesis of two-kidney, one-clip (2K1C) hypertension.[1–3] However, the mechanisms of intrarenal ANG II augmentation remain unclear. Although intrarenal ANG II is augmented in the nonclipped kidney of 2K1C rats, there is marked depletion of renal renin content and reduction of renal renin mRNA levels.[2,4–8] These findings are consistent with the possibility that there is a non–renin-dependent mechanism responsible for augmenting intrarenal

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ANG II levels in the nonclipped kidney of 2K1C rats. This possibility has received support from studies indicating that chronic infusion of subpressor doses of ANG II into uninephrectomized rats mimics 2K1C hypertension, increases intrarenal ANG II levels, and depletes renal renin content and renin mRNA levels in the remaining kidney, as has been observed in the nonclipped kidney of 2K1C rats.[2,5,9]

A recent study using Val^5-ANG II as the infused peptide demonstrated that a substantial fraction of the elevated intrarenal ANG II found in the kidneys consisted of exogenously infused Val^5-ANG II.[10] These data suggest that the kidney has the capability to take up circulating ANG II into intrarenal sites that protect against degradation and metabolism. Quantitatively, the tissue Val^5-ANG II contents were much greater than could be explained simply by nonspecific trapping of circulating ANG II. The observation that the AT_1 receptor antagonist, losartan, prevents intrarenal ANG II augmentation during chronic low dose ANG II infusion[11] also provides evidence that the renal uptake of ANG II is an active process and does not represent nonspecific intrarenal sequestration of circulating ANG II. In this previous study, heart and adrenal ANG II levels increased in parallel with the plasma ANG II levels during losartan treatment, suggesting that receptor mediated augmentation of renal ANG II is tissue specific. However, the available data did not allow us to determine whether the losartan was preventing uptake of circulating ANG II or reducing intrarenal ANG II formation. The present study was therefore designed to determine the involvement of AT_1 receptors in the renal uptake of circulating ANG II. Rats were infused with Val^5-ANG II, which is not endogenously produced in the rat but has the same biological and immunoreactive properties as endogenous Ile^5-ANG II and can be separated by high performance liquid chromatography (HPLC).[10] As previously described,[10] this approach allowed us to distinguish renal uptake of exogenously infused Val^5-ANG II from that of endogenously formed ANG II. In the present study, the extent to which this process involves AT_1 receptor activation was tested by treating a separate group of Val^5-ANG II infused rats with the AT_1 receptor antagonist, losartan.

METHODS

Experimental Design Male Sprague-Dawley rats (Charles River, Wilmington, MA) were housed in wire cages and maintained in a temperature and light controlled room. Throughout the experiments, animals had free access to standard rat chow (Ralston-Purina, St. Louis, MO). All experiments were approved by the Tulane University Animal Care and Use Committee. Rats (180 to 200 g body weight) were anesthetized with pentobarbital anesthesia (50 mg/kg intraperitoneally) and the right kidney was removed. An osmotic minipump (Model 2002, Alza Corp, Palo Alto, CA) containing Val^5-ANG II was implanted subcutaneously in each rat. Val^5-ANG II (Novabiochem, San Diego, CA) was delivered continuously at a rate of 40 ng/min. Losartan (Du Pont-Merck Pharmaceutical Co, Wilmington, DE) was administered in the drinking water at a dose of 30 mg/kg/day to allow chronic treatment throughout the period of ANG II infusion. The results obtained in this group were compared to results obtained in other rats prepared in a similar manner including rats infused with vehicle or Val^5-ANG II but not treated with losartan.[10]

Systolic blood pressure (SBP) was measured in conscious rats by tail-cuff plethysmography (Harvard Apparatus, South Natick, MA) to monitor the progression of hypertension. For measurement of plasma renin activity (PRA), plasma and renal ANG I, Ile^5-ANG II, and Val^5-ANG II levels, the conscious rats were decapitated on day 13. Approximately 4 mL of trunk blood were collected in about 15 to 20 sec and the kidneys were immediately removed, quickly weighed, and homogenized in methanol. The time delay between decapitation and homogenization of the kidney did not exceed 60 sec.

Measurement of Val^5-ANG II and Ile^5-ANG II Levels in Plasma and Kidney *Collection and Extraction of Blood and Kidney* Trunk blood was collected in chilled tubes containing a mixed inhibitor solution (5 mmol/L EDTA, 10 μmol/L pepstatin, 20 μmol/L enalaprilat, and 1.25 mmol/L 1,10-phenanthroline) and kept at 4°C. To minimize in vitro generation of the peptides, blood samples were immediately centrifuged at 4°C for 10 min at 1000 *g*. After centrifugation, plasma was separated and immediately extracted by adsorption to and elution from a phenyl bonded, solid phase extraction column (Bond-Elut; Varian, Harbor City, CA). 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. One-half of each kidney was immersed in cold methanol (100%) and homogenized with a glass homogenizer immediately upon harvest. The supernatants from the kidney homogenates were dried overnight in a vacuum centrifuge. The dried residue was reconstituted in 4 mL assay diluent (50 mmol/L sodium phosphate buffer, pH 7.4, containing 0.1 mg human serum albumin/mL). These samples 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 methodology for the measurement of angiotensin peptides has been reported.[10,12] Briefly, the extract residue from each plasma and kidney sample was redissolved in 150-μL HPLC column equilibration solvent (35%

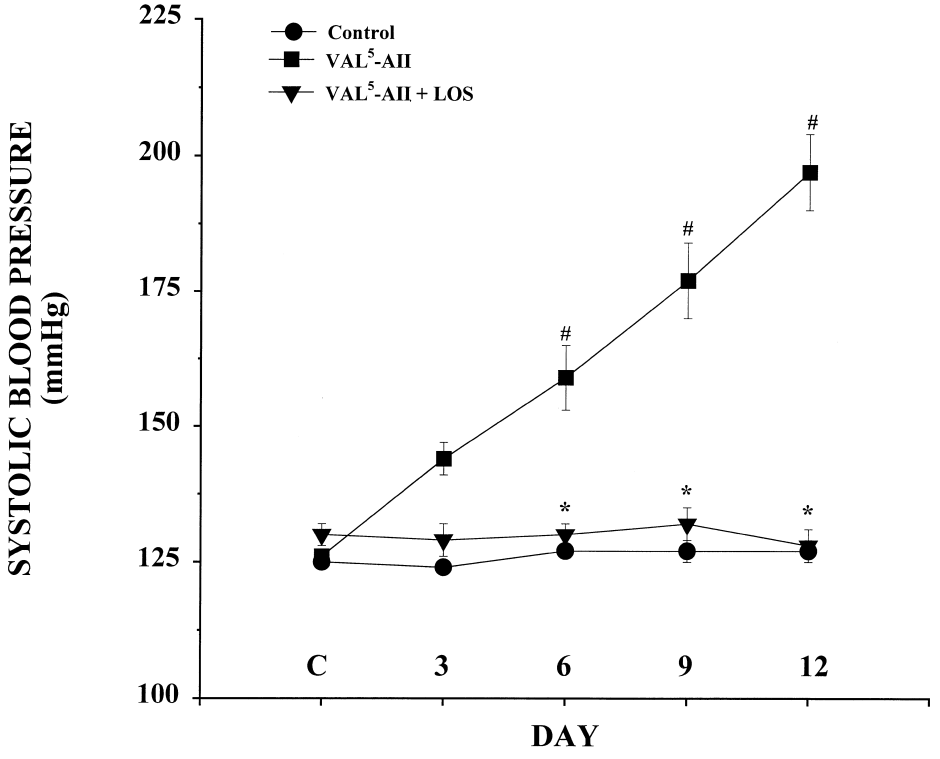

FIGURE 1. *Comparison of systolic blood pressure (SBP) in control (circles, n = 6), Val^5-ANG II infused (squares, n = 8), and Val^5-ANG II infused plus losartan treated (triangles, n = 8) groups before (control, C) and for 12 days after initiation of ANG II infusions. Values are mean ± SE.* #P < .05 v *control group;* *P < .05 v *Val^5-ANG II infused group.*

methanol, 65% water, 0.1% H_3PO_4) and chromatographed at 1 mL/min on a 25 × 0.46 cm, 5 μm Vydac C18 reversed-phase HPLC column (Separations Group, Hesperia, CA). To shorten the duration of the chromatography run, a combination of isocratic and step-gradient elution modes was used. After 12 min of isocratic elution with the equilibration solvent, the solvent was changed to 40% methanol, 60% water, and 0.1% H_3PO_4 and then the new solvent composition was continued for an additional 18 min. As previously described,[10] Val^5-ANG II eluted at 6 min (fractions 11 to 14) and Ile^5-ANG II had an elution peak of 9.5 min (fractions 18 to 22). Fractions were collected every 30 sec, evaporated to dryness, reconstituted in assay diluent, and measured directly by RIA.

Quantitation of Val^5-ANG II and Ile^5-ANG II by RIA The reconstituted plasma and kidney fractions were incubated with rabbit anti-ANG II antisera (Arnel, New York, NY), and ^{125}I-radiolabeled ANG II (Sigma Chemical Co., St Louis, MO) for 48 h at 4°C. Bound and free ANG peptides were separated by dextran coated charcoal and the supernatants were counted by a computer linked gamma counter for 3 min. As previously shown, the immunoreactivity of the antibodies for Val^5-ANG II and Ile^5-ANG II were virtually identical. Results are reported in femtomoles/gram kidney weight or femtomoles/milliliter plasma. The sensitivity of the ANG II assay was 1.46 ± 0.33 fmol. For the ANG II assays, the specific binding was 43.4% ± 2.3%, with a nonspecific binding of 1.3% ± 0.1%.

Plasma Renin Activity and ANG I Assays For renin determination, trunk blood was collected in chilled tubes containing EDTA (5 mmol/L). Plasma was separated and stored at −20°C until assayed using a commercially available ANG I RIA kit (Incstar, Stillwater, MN) as described previously.[2] For ANG I measurement, blood and kidney samples were collected, extracted, and quantitated by RIA as reported previously.[2]

Data Analysis All data are presented as mean ± SEM. The statistical analyses for plasma and kidney levels were performed using the one way analysis of variance (ANOVA) and Fisher's least significant difference (FLSD) post hoc test. Differences between and within groups for systolic blood pressure measurements were analyzed by two way ANOVA with repeated measures on one factor and the FLSD post hoc test. A value of $P < .05$ was considered statistically significant.

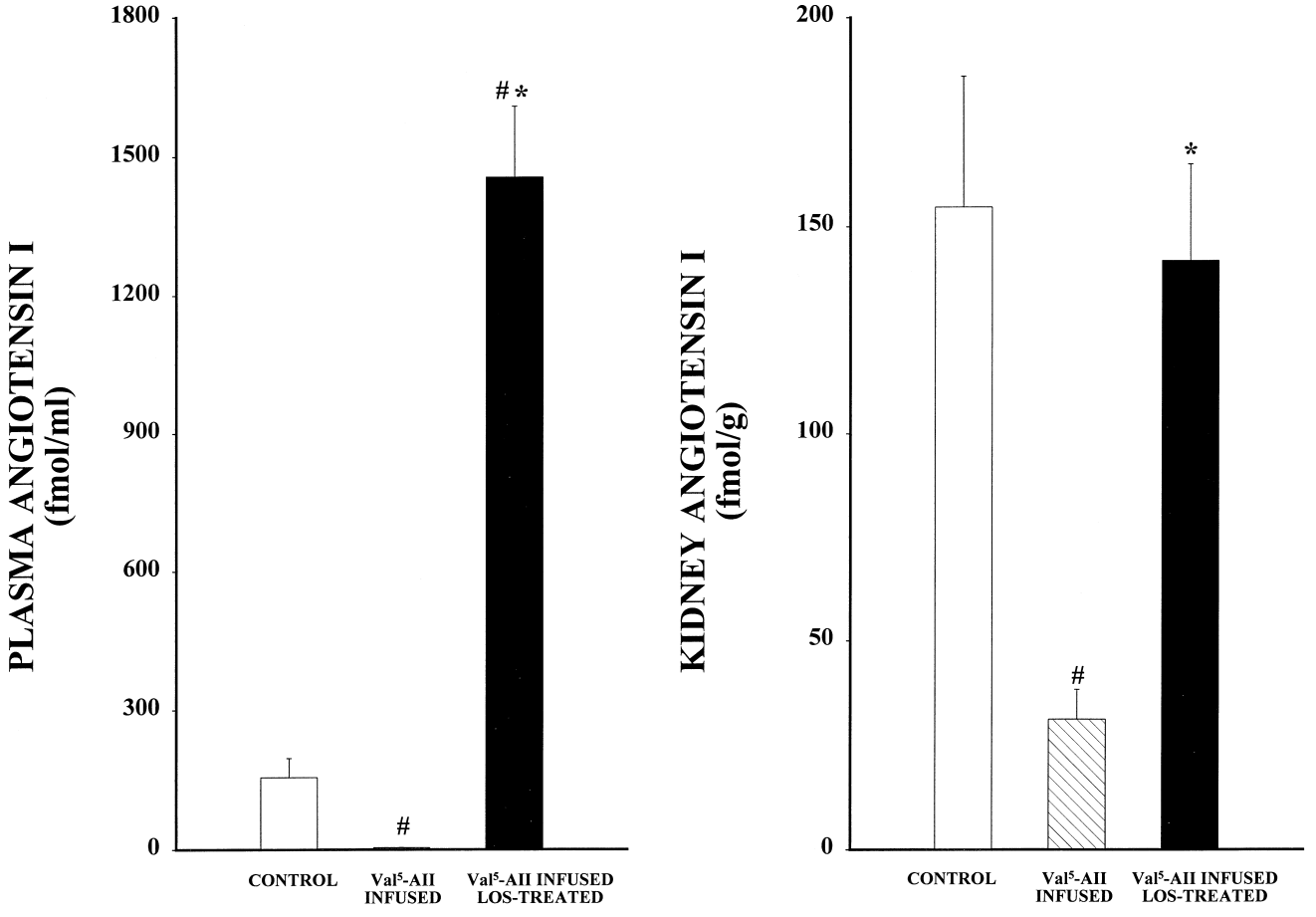

FIGURE 2. *Comparison of plasma and kidney angiotensin I levels in control (open bar, n = 6), Val^5-ANG II infused (striped bar, n = 8), and Val^5-ANG II infused plus losartan treated (solid bar, n = 8) groups. Values are mean ± SE.* #P < .05 v *control group;* *P < .05 v *Val^5-ANG II infused group.*

RESULTS

Influence of Losartan on Arterial Pressure During Val^5-ANG II Infusion (Figure 1) Before surgical manipulations, SBP values were normotensive with an average of 127 ± 1 mm Hg. As previously shown,[10] SBP in the Val^5-ANG II infused rats exhibited progressive increases over a 12 day period reaching a value of 197 ± 7 mm Hg at 12 days. This increase was similar to or slightly greater than that seen in rats infused with Ile^5-ANG II.[10] As shown for rats infused with Ile^5-ANG II,[11] losartan treatment prevented the development of hypertension in the Val^5-ANG II infused group and SBP remained at normal levels for the duration of the study.

Effects of Losartan on Plasma Renin and Plasma and Kidney ANG I During Val^5-ANG II Infusions As previously reported,[10] in vehicle infused controls, PRA averaged 4.96 ± 1.17 ng ANG I/mL/h, and the Val^5-ANG II infused rats had almost complete suppression of PRA with values of 0.21 ± 0.06 ng ANG I/mL/h. In contrast, losartan treatment in the Val^5-ANG II infused group exhibited a marked sevenfold increase in PRA at 13 days (36.35 ± 5.24 ng ANG I/mL/h).

As previously reported,[10] Plasma and kidney ANG I levels in the Val^5-ANG II infused rats were reduced markedly. The rats receiving losartan treatment, however, showed a ninefold increase in plasma ANG I. Nevertheless, intrarenal ANG I content was restored only to that found in control rats but was not significantly elevated beyond these values (Figure 2).

Effects of Losartan on Plasma and Kidney ANG II During Val^5-ANG II Infusions As shown in Figure 3 and previously reported,[10] total plasma ANG II levels were elevated in the Val^5-ANG II infused group compared with controls. About half of the plasma ANG II was in the form of Val^5-ANG II, whereas Ile^5-ANG II levels were maintained at concentrations similar to those found in control rats in spite of marked renin depletion and reduction in ANG I con-

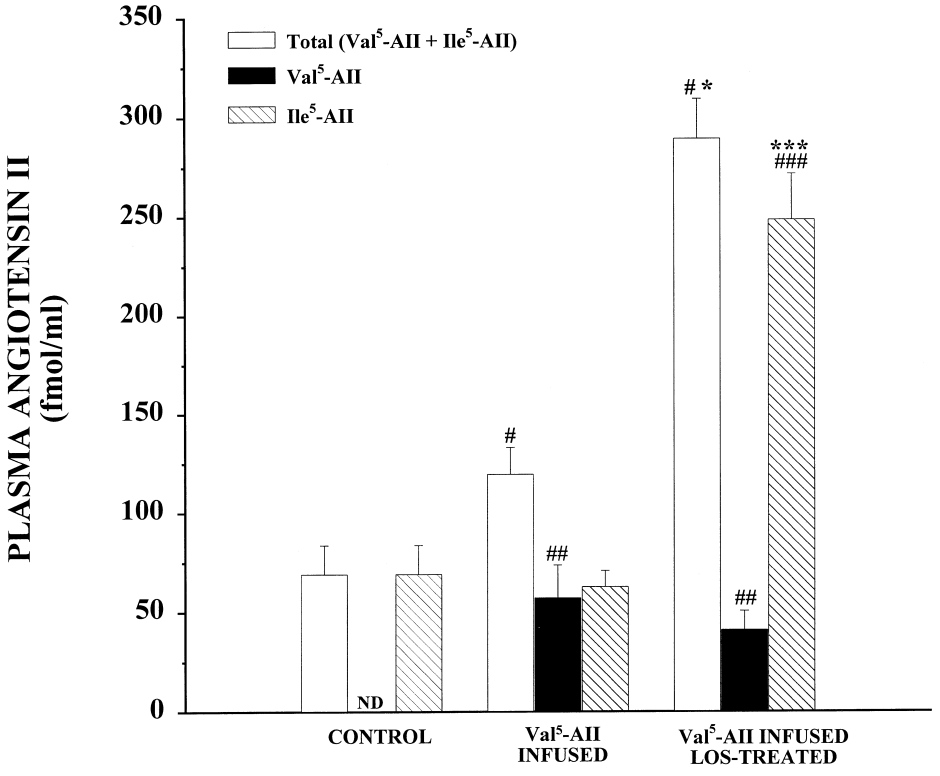

FIGURE 3. *Comparison of plasma Ile^5-ANG II (striped bar) and Val^5-ANG II (solid bar) levels in control (n = 6), Val^5-ANG II infused (n = 8), and Val^5-ANG II infused plus losartan treated (n = 8) groups. Values are mean ± SE.* #P < .05 v *total ANG II levels of controls;* ##P < .05 v *Val^5-ANG II levels of controls;* ###P < .05 v *Ile^5-ANG II levels of controls;* *P < .05 v *total ANG II levels of Val^5-ANG II infused group;* ***P < .05 v *Ile^5-ANG II levels of Val^5-ANG II infused group.*

centration. Losartan treatment caused substantial increases in total plasma ANG II levels in the Val^5-ANG II infused group. Total ANG II levels were 2.4-fold higher than in rats infused with Val^5-ANG II but not treated with losartan and fourfold higher than in the control group. A 3.6-fold elevation of Ile^5-ANG II was responsible for the elevated plasma ANG II levels, whereas the plasma Val^5-ANG II levels were not influenced by losartan treatment.

The intrarenal ANG II contents, however, showed a distinctly different pattern (Figure 4). As already reported,[10] Val^5-ANG II infused rats exhibited a marked increase in total intrarenal ANG II content (371 ± 57 *v* 116 ± 11 fmol/g controls). Interestingly, 70% of total intrarenal ANG II in the Val^5-ANG II infused rats was in the form of Val^5-ANG II and renal Val^5-ANG II content in the kidney was 256 ± 44 fmol/g, which greatly exceeded plasma concentrations of 57 ± 17 fmol/mL. The ratio of kidney to plasma Val^5-ANG II is 6.78 ± 1.73 (Figure 5). Additionally, intrarenal Ile^5-ANG II contents in the Val^5-ANG II infused rats were maintained compared with controls even though plasma renin activity and plasma and renal ANG I levels were markedly suppressed. In contrast to the changes observed for plasma ANG II levels, total intrarenal ANG II contents were decreased in the losartan treated rats. In particular, losartan treatment markedly reduced renal Val^5-ANG II content by 88% but did not significantly decrease renal Ile^5-ANG II contents in the Val^5-ANG II infused rats. The ratio of kidney to plasma Ile^5-ANG II was used as a reflection of the degree of intrarenal ANG II accumulation relative to the plasma levels. In the Val^5-ANG II infused rats this ratio was significantly reduced by losartan from 2.18 ± 0.49 to 0.37 ± 0.03 (Figure 5). Thus, the intrarenal levels of Ile^5-ANG II did not increase concomitantly with the plasma levels and were significantly reduced to ratios below 1 in the losartan treated rats, indicating that the intrarenal contents of Ile^5-ANG II could be attributed primarily to the extracellular fluid concentration.

DISCUSSION

Previous studies demonstrating that intrarenal ANG II levels are augmented during ANG II-induced hypertension.[1–2] have led to the hypothesis that chronic increases in circulating ANG II lead to progressive augmentation of intrarenal ANG II content. Our recent observation that 70% of the elevated intrarenal ANG II found in the Val^5-ANG II infused rats was derived

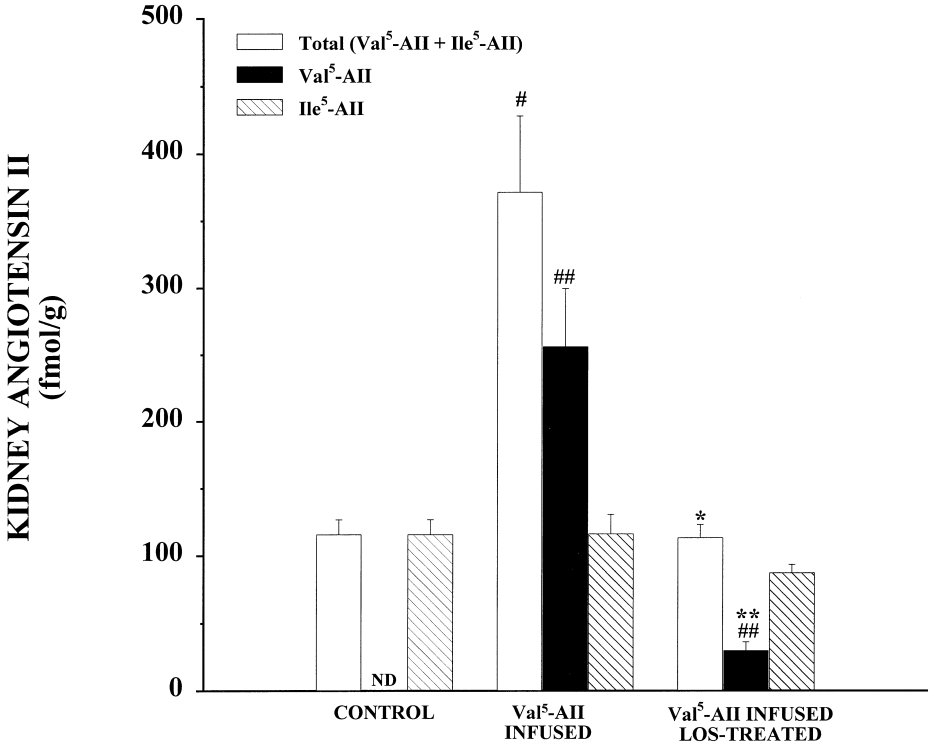

FIGURE 4. *Comparison of kidney Ile^5-ANG II (striped bar) and Val^5-ANG II (solid bar) levels in control (n = 6), Val^5-ANG II infused (n = 8), and Val^5-ANG II infused plus losartan treated (n = 8) groups. Values are mean ± SE.* #P < .05 v *total ANG II levels of controls;* ##P < .05 v *Val^5-ANG II levels of controls;* ###P < .05 v *Ile^5-ANG II levels of controls;* *P < .05 v *total ANG II levels of Val^5-ANG II infused group;* **P < .05 v *Val^5-ANG II levels of Val^5-ANG II infused group.*

from the exogenously infused Val^5-ANG II[10] indicates that augmentation of intrarenal ANG II is due, in large part, to uptake of circulating ANG II. In addition, the finding that endogenous Ile^5-ANG II is maintained in the kidneys of Val^5-ANG II infused rats in a setting where renin and ANG I levels are markedly reduced[10] suggests that endogenous production of ANG II is maintained under conditions of renin depletion. Additional recent results[11] demonstrate that intrarenal ANG II augmentation involves activation of AT$_1$ receptors, which may be responsible for renal uptake of ANG II or renal enhanced formation of ANG II. By using a different form of ANG II in the minipumps, the current study extends previous findings and specifically demonstrates the role of AT$_1$ receptor activation in mediating renal uptake of ANG II during ANG II infusion.

Losartan treatment to rats infused with Val^5-ANG II for 13 days prevented the progressive increases in systolic blood pressure and markedly reduced the intrarenal augmentation of ANG II. As expected, the PRA levels, which were almost completely suppressed in the Val^5-ANG II infused rats, were markedly elevated during losartan treatment. These results are consistent with an important role of AT$_1$ receptor activation in the ANG II-induced negative feedback effect on renin release.[2,5,13–16] Chronic administration of losartan was also associated with elevated plasma ANG I and ANG II levels, which can be accounted for by the marked elevation of circulating renin activity leading to increased formation of ANG I from angiotensinogen and conversion to Ile^5-ANG II.

The significant finding of this study is that losartan treatment markedly diminished the increase in intrarenal Val^5-ANG II content in the Val^5-ANG II infused rats. These studies indicate that intrarenal ANG II augmentation is due, in large part, to AT$_1$ receptor mediated binding and internalization of circulating ANG II. These data are consistent with the findings by Anderson and Peach,[17] who demonstrated that ANG II is internalized via AT$_1$ receptors and that losartan can block ANG II internalization in cultured explant derived rat aortic vascular smooth muscle cells. Likewise, Wang et al[18] reported receptor mediated internalization of ANG II in primary cultures of bovine adrenal medullary chromaffin cells. Moreover, Ullian and Linas[19] showed the importance of receptor cycling in the regulation of angiotensin II surface receptor number and angiotensin II uptake in rat vascular smooth muscle cells. Our present observation provides complementary in vivo data that the ANG II receptor complex is internalized in the kidney. This

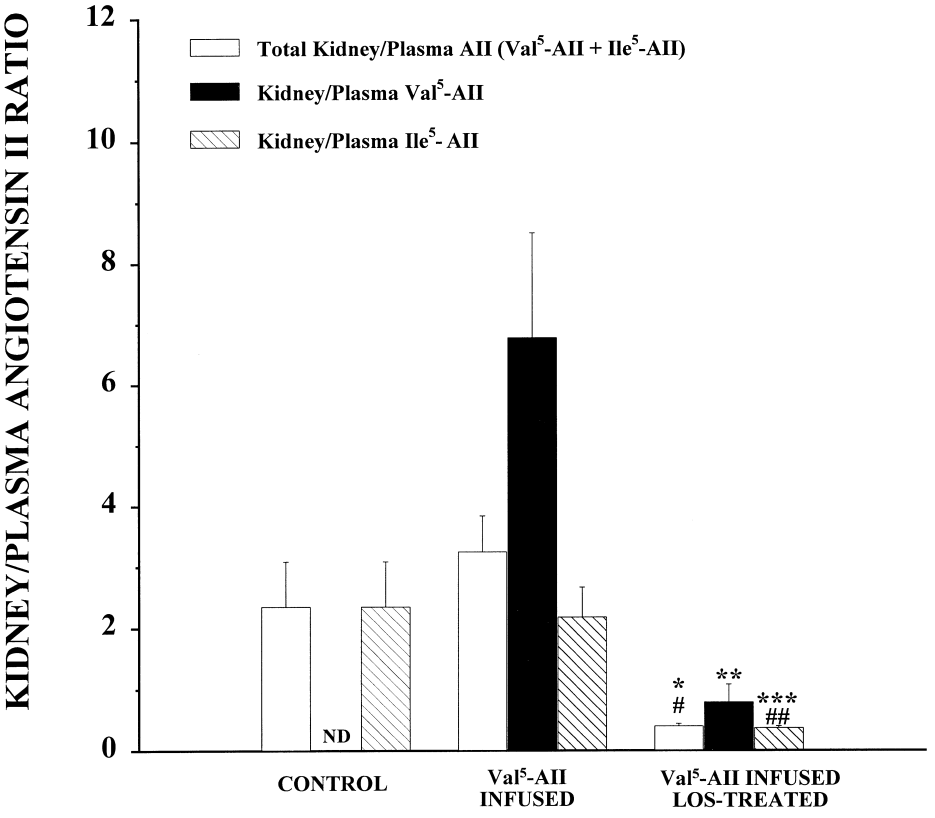

FIGURE 5. *Comparison of ratio of kidney ANG II to plasma ANG II levels in control (n = 6), Val^5-ANG II infused (n = 8), and Val^5-ANG II infused plus losartan treated (n = 8) groups. Values are mean ± SE.* #P < .05 v *total kidney/plasma ANG II ratio of controls;* ##P < .05 v *kidney/plasma Ile^5-ANG II ratio of controls;* *P < .05 v *total kidney/plasma ANG II ratio of Val^5-ANG II infused rats;* **P < .05 v *kidney/plasma Val^5-ANG II ratio of Val^5-ANG II infused rats;* ***P < .05 v *kidney/plasma Ile^5-ANG II ratio of Val^5-ANG II infused rats.*

possibility has received support from several studies showing that binding of ANG II to its receptor initiates internalization of the complex in hepatoma cells,[20] adrenocortical cells,[21] and vascular smooth muscle cells.[17,22] However, these previous studies did not determine whether the internalized ANG II was immediately degraded or accumulated. The approach used in the current study allowed us to detect increased amounts of intrarenal ANG II suggesting that some of the internalized ANG II is protected from degradation and is possibly used for some intracellular process. This hypothesis is supported by results from experiments in isolated or cultured cells showing intracellular localization of ANG II and ANG II specific binding sites.[17,23–28]

Although the present studies demonstrate that receptor mediated internalization of ANG II occurs in the kidney, the specific cells responsible for renal internalization of ANG II remain undetermined. It is now clear that AT_1 receptors are abundant on rat proximal tubules.[29] Poggioli et al[25] provided further evidence by showing that AT_1 receptors are predominant in intact rat proximal tubule cells and are coupled to both IP_3-Ca^{2+} and cAMP signaling pathways. Douglas and Hopfer[28] also found that AT_1 receptors of proximal tubular epithelial cells are linked to a complex combination of signal transduction pathways. In addition, it has been found that ANG II leads to increases in proximal tubule AT_1 receptor mRNA and receptor binding.[30] In regard to the functional properties of receptor mediated ANG II internalization, Schelling and Linas[31] reported that ANG II dependent proximal tubule sodium transport involves receptor mediated endocytosis. Becker and Harris[32] using LLCPK epithelial cells demonstrated that ANG II binding to the apical AT_1 receptor and subsequent endocytosis is required for PLA_2 activation. In preliminary studies ANG II stimulated Na^+ flux was inhibited by the calcium independent PLA_2 inhibitor HELSS, raising the possibility that internalization of the ANG II AT_1 receptor complex is required for full expression of biological activity.[32] Moreover, a large body of studies in vivo have indicated that the blockade of AT_1 receptors by losartan is associated with decreases in fractional proximal reabsorption and proximal tubular transport.[33–34] Collectively, these findings suggest that

renal proximal tubular cells may be involved in receptor mediated ANG II internalization and receptor mediated cellular signal transduction.

Because losartan also prevented the development of hypertension during the 2 weeks of ANG II infusion, it is also possible that the renal ANG II uptake in the ANG II infused rats may be secondary to renal injury occurring as a consequence of hypertension.[35] The ANG II infused rats clearly develop extensive renal injury to the vasculature, glomeruli, tubules, and interstitium.[11,35] Thus, renal injury could serve as a stimulus for both ANG II uptake and intrarenal ANG II formation. Because losartan prevented hypertension, it is also possible that the reduction in intrarenal ANG II content in the losartan treated rats was due, in part, to the prevention of hypertension. At present, the relative contributions of the hypertension associated renal injury versus the increased circulating ANG II concentrations to the increased intrarenal uptake of ANG II cannot be established. However, it was recently shown that the increased intrarenal ANG II levels still occurred to the same extent in ANG II infused rats subjected to renal denervation, which markedly attenuated the magnitude of the hypertension.[36]

The current data also showed that losartan treatment did not alter intrarenal Ile^5-ANG II contents in the Val^5-ANG II infused rats. Although this would seem to suggest that intrarenal handling of Ile^5-ANG II was not influenced by losartan, it should be noted that losartan stimulated renin release and markedly increased the circulating ANG II concentrations. Thus, when considered as the ratio of intrarenal content to circulating ANG II concentration, it can be noted that the relative intrarenal content of Ile^5-ANG II was reduced because it was a much smaller fraction of circulating Ile^5-ANG II during losartan treatment than before. In all likelihood, losartan also prevented receptor mediated internalization of endogenous ANG II but it is difficult to determine how much of the intrarenal Ile^5-ANG II was formed de novo as opposed to being derived from circulating ANG II.

In summary, the results of the present study indicate that losartan markedly reduced renal Val^5-ANG II contents in the Val^5-ANG II infused rats. These results indicate that circulating ANG II is bound and taken up into kidney cells primarily through an AT_1 receptor mediated process, which may play a major role in augmenting intrarenal ANG II levels in conditions of ANG II dependent hypertension caused by elevated circulating ANG II concentrations.

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