Increased renin excretion is associated with augmented urinary angiotensin II levels in chronic angiotensin II-infused hypertensive rats

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Increased renin excretion is associated with augmented urinary angiotensin II levels in chronic angiotensin II-infused hypertensive rats. Am J Physiol Renal Physiol 301: F1195–F1201, 2011. First published August 24, 2011; doi:10.1152/ajprenal.00339.2011. —Renin expression in principal cells of collecting ducts (CD) is upregulated in angiotensin II (ANG II)-dependent hypertensive rats; however, it remains unclear whether increased CD-derived renin undergoes tubular secretion. Accordingly, urinary levels of renin (uRen), angiotensinogen (uAGT), and ANG II (uANG II) were measured in chronic ANG II-infused Sprague-Dawley rats (80 ng/min for 14 days, n = 10) and sham-operated rats (n = 10). Systolic blood pressure increased in the ANG II rats by day 5 and continued to increase throughout the study (day 13; ANG II: 175 ± 10 vs. sham: 116 ± 2 mmHg; P < 0.05). ANG II infusion increased renal cortical and medullary ANG II levels (cortical ANG II: 606 ± 72 vs. 247 ± 43 femtomoles/g; P < 0.05; medullary ANG II: 2,066 ± 116 vs. 646 ± 36 femtomoles/g; P < 0.05). Although plasma renin activity (PRA) was suppressed in the ANG II-infused rats (0.3 ± 0.2 vs. 5.5 ± 1.8 ng ANG I·ml⁻¹·h⁻¹; P < 0.05), renin content in renal medulla was increased (12,605 ± 1,343 vs. 7,956 ± 765 ng ANG I·h⁻¹·mg⁻¹; P < 0.05). Excretion of uAGT and uANG II increased in the ANG II rats [uAGT: 1.107 ± 0.106 vs. 60 ± 26 ng/day; P < 0.0001; uANG II: 3,813 ± 431 vs. 2,080 ± 361 fmol/day; P < 0.05]. By day 13, despite suppression of PRA, urinary prorenin content increased in ANG II rats [15.7 ± 3 vs. 2.6 ± 1 × 10⁻³ enzyme units excreted (EUE)/day; P < 0.01] as was the excretion rate of renin (8.6 ± 2 × 10⁻⁶ EUE/day) compared with sham (2.8 ± 1 × 10⁻⁶ EUE/day; P < 0.05). Urinary renin and prorenin protein levels examined by Western blot were augmented ~10-fold in the ANG II-infused rats. Concomitant AT₁ receptor blockade with candesartan prevented the increase. Thus, in ANG II-dependent hypertensive rats with marked PRA suppression, increased urinary levels of renin and prorenin reflect their augmented secretion by CD cells into the luminal fluid. The greater availability of renin and AGT in the urine reflects the capability for intratubular ANG II formation which stimulates sodium reabsorption in distal nephron segments.

An intratubular formation of angiotensin II (II) in angiotensin II (II) secreted by the CD from AGT which is secreted by the kidney. This increased renin formation, has been associated with increased intratubular ANG II levels (14–16). The presence of AGT in the urine indicates that AGT traverses the distal nephron segments where it may then be cleaved to ANG I, to the extent that an adequate source of renin is available (36).

Although renin is synthesized primarily by the juxtaglomerular apparatus (JGA) cells (8), renin is also expressed in the proximal tubules (5, 11, 21, 39), connecting tubules, and cortical and medullary collecting duct (CD) cells from rat, mouse, and human (12, 30, 32, 33, 36). Renin in the CD is upregulated by chronic ANG II infusions, via an AT₁R-mediated mechanism (33), and is independent of blood pressure (34). The presence of apical renin immunostaining in CD cells of human and mouse tissues and renin secretion from isolated CD cells in vitro suggest that these cells may secrete renin (36). Kang et al. (12) showed renin mRNA and protein stimulation in M1 cells, a cortical CD cell line from mouse origin, when these cells were treated with ANG II. However, there is limited in vivo evidence that renin is secreted by the CD and whether it is increased in ANG II-dependent hypertension.

For this study, we hypothesized that during conditions of stimulated CD renin, renin synthesized by principal cells of the CD is secreted into the lumen, and is available to act on luminal AGT delivered from the proximal tubules, thus contributing to increased intratubular ANG II formation. Increased urinary renin excretion in chronic ANG II-infused rats, which is a model that exhibits suppression of plasma renin activity (PRA), may reflect augmented renin secretion by CD cells and increased CD luminal renin activity. Our objective was to evaluate this hypothesis in male Sprague-Dawley rats chronically infused with ANG II for 2 wk, by examining the presence and nature of renin protein levels and its activity in the urine. Additional experiments were performed in rats with maximum PRA suppression due to addition of a high-salt diet, plus similarly treated rats subjected to AT₁ receptor blockade with candesartan.

METHODS

Experimental animals, sample collections, and tissue preparation.

The experimental protocols were approved by the Tulane Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (170 to 220 g, n = 30; Charles River Laboratories, Wilmington, MA) were cage-housed and maintained in a temperature-controlled room with a 12:12-h light-dark cycle, with free access to tap water and standard rat chow (Ralston Purina, St. Louis, MO) for 14 days. Rats were randomly selected and subjected to either sham operation (n = 10) or ANG II infusion (Human ANG II, Sigma, St. Louis, MO; n = 10) via a subcutaneous osmotic minipump (Alzet model 2002, Alza, Palo Alto, CA) at a rate of 80 ng/min for 14 days. Additional studies were performed in ANG II-infused rats maintained on a very high-salt diet.
(n = 5) to maximize the suppression of PRA and rats treated with candesartan to block AT1 receptors (n = 5). Systolic blood pressure (SBP) was monitored by tail-cuff plethysmography (ITC, Woodland Hills, CA) on 1 day before and 5, 8, and 12 days after ANG II infusion. Twenty-four-hour urine samples were collected on days −1, 2, 6, and 13 following sham operation or minipump implantation. At day 14, rats were subjected to conscious decapitation and blood and kidneys were harvested. Trunk blood was collected for measuring PRA and plasma ANG II levels. After decapsulation, kidneys were weighed, cross-sectioned, and renal cortices were dissected from inner medullas under stereomicroscopy for regional determination of kidney renin content (KRC).

**PRA and plasma ANG II levels.** The blood samples for PRA were collected into 5.0 mmol/l EDTA and were assayed as previously described (40). PRA was expressed as nanograms per milliliter per hour of generated ANG I. For plasma ANG II levels, blood samples were collected into tubes containing a mixed inhibitor solution (5 mmol/l of EDTA, 20 μmol/l of pepstatin, 20 μmol/l of enalapril, 1.25 mmol/l of 1,10-phenanthroline, and 10 μmol/l of PMSF) to avoid in vitro formation and degradation of angiotensin peptides and were assayed as described previously (38). Urinary ANG II concentrations were determined by radioimmunoassay (RIA) (Peninsula Laboratories, Torrance, CA) at 1:1,000 dilution (Sigma). Trypsinized urine samples were also subjected to the above protocol, 24-h urine volumes were similar between both groups (sham: 185 ± 5, ANG II: 189 ± 2 g), but on day 13, body weights were significantly lower in ANG II-infused rats compared with sham (286 ± 9 vs. 336 ± 8 g; P < 0.05). At the beginning of the study, SBP values were similar between both groups of rats (sham: 132 ± 4, ANG II: 127 ± 2 mmHg), but by day 5, SBP was significantly elevated in ANG II-infused rats compared with sham rats (163 ± 11 vs. 115 ± 2 mmHg; P < 0.05) and continued to increase in ANG II-infused rats throughout the study (day 12; 175 ± 10 vs. 116 ± 2 mmHg; P < 0.05).

**RESULTS**

**Body weight and SBP.** Body weights were similar at the initiation of the study (sham: 185 ± 5, ANG II: 189 ± 2 g), but on day 13, body weights were significantly lower in ANG II-infused rats compared with sham (286 ± 9 vs. 336 ± 8 g; P < 0.05; Fig. 1A). ANG II infusion significantly increased renal cortical and medullary ANG II contents compared with sham-operated rats (cortical ANG II: 606 ± 72 vs. sham: 247 ± 43 fmol/ml; P < 0.05; Fig. 1A). ANG II infusion significantly increased renal cortical and medullary ANG II contents compared with sham-operated rats (cortical ANG II: 606 ± 72 vs. sham: 247 ± 43 fmol/ml; P < 0.05; Fig. 1B). Urinary ANG II excretion was measured in sham-operated rats compared with values obtained in all the rats before minipump implantation. After 12 days of ANG II infusion, urinary ANG II was significantly higher compared with sham-operated rats (3,813 ± 431 vs. 2,080 ± 361 fmol/day; Fig. 2A).

**Urinary angiotensinogen excretion.** At the beginning of the protocol, 24-h urine volumes were similar between both groups (sham: 10 ± 1, ANG II: 13 ± 3 ml/day). However, due to pressure diuresis and the diuregenic effect of ANG II, the chronic ANG II-infused rats had increased urine volume compared with sham rats (52 ± 8 vs. 13 ± 1 ml/day; P < 0.05). At 13 days, urinary excretion rates of creatinine were similar between sham and ANG II rats (10,201 ± 388 vs. 9,246 ± 1,109 μg/day). The urinary angiotensinogen (uAGT) excretion rates (Fig. 2B) remained at control levels in sham rats (day −1: 58 ± 15 vs. day 12: 60 ± 26 ng/day), but they were markedly increased by ~20-fold in ANG II-infused rats (1,107 ± 106 vs. 60 ± 26 ng/day; P = 0.0001).

**PRA and KRC.** Chronic ANG II infusion alone or in combination with high-salt diet suppressed PRA (0.3 ± 0.2 and 0.2 ± 0.1 ng ANG I·ml⁻¹·h⁻¹) compared with sham-operated rats (5.5 ± 2 ng ANG I·ml⁻¹·h⁻¹; P < 0.05). Concomitant treatment with candesartan to chronic ANG II-infused rats fed a high-salt diet significantly increased PRA levels (41.3 ± 12.3 ng ANG I·ml⁻¹·h⁻¹; P < 0.0001). In contrast to PRA, renin content in renal medullary tissues, determined by measuring the amount of ANG I generated per hour from added substrate
corrected by wet tissue weights, was increased in chronic ANG II-infused rats compared with sham-operated rats (12,605 ± 1,343 vs. 7,956 ± 765 ng ANG I·ml⁻¹·h⁻¹; P < 0.05; Fig. 3A).

Urinary renin/prorenin excretion in chronic ANG II-infused rats: effects of concomitant administration of a high-salt diet and AT1R blockade. As depicted in the Fig. 3B by day 13 of the study, the urinary renin/prorenin (uRen) excretion was increased in ANG II-infused rats compared with sham-operated rats (8.6 ± 2 vs. 2.8 ± 1 × 10⁻⁶ EUE/day; P < 0.05). To maximize the suppression of PRA and avoid a substantial contribution of renin from the circulation into the urine, we further examined the uRen excretion in urine samples from chronic ANG II-infused rats fed a high-salt diet (8% NaCl). The urine of these rats showed values of renin content significantly higher than sham rats (9.6 ± 4 × 10⁻⁶ EUE/day; P < 0.05 vs. sham). The increases in uRen were prevented in rats receiving concomitant administration of 25 mg/l of candesartan in drinking water (4.7 ± 2.5 × 10⁻⁶ EUE/day) even though PRA levels were increased thus showing a dissociation between uRen and PRA. Importantly, the measurement of active renin content in the urine (Fig. 4A) and after trypsinization demonstrated that urinary prorenin excretion (Fig. 4B) was further augmented in ANG II-infused rats compared with sham-operated rats (prorenin: 15.7 ± 3 vs. 2.6 ± 1 × 10⁻³ EUE/day; P < 0.001), indicating that most of the renin in the urine is in the prorenin form.

Renin protein levels in the urine. The identity of the specific bands of renin (38 kDa) and prorenin (48 kDa) found in the urine samples of sham-operated and ANG II-infused rats was assessed by Western blot in the presence of positive controls using recombinant pure renin and prorenin proteins (Fig. 5A). The specificity of the antibody used to detect renin and prorenin in urine samples, as well as the identity of prorenin and renin bands were addressed by preadsorption of the renin antibody using 2-fold excess of purified recombinant human prorenin peptides (Lee Biosolutions, St. Louis, MI; Fig. 5B). After normalization for uCr, the amounts of renin (uRen-W/uCr) and prorenin (uProren-W/uCr) proteins measured by Western blot were significantly augmented in ANG II rats compared with sham rats (Table 1).

DISCUSSION

Several animal models of ANG II-dependent hypertension, including two-kidney, one-clip hypertensive rats, Cyp1a1-Ren2 gene transgenic rats, and chronic ANG II-infused rats, exhibit increased intrarenal ANG II levels to an extent greater than can be explained from equilibration with the circulating...
concentrations (22, 24, 25, 42). The demonstration that AGT and ANG II are present in high concentrations in the proximal tubule fluid and in the urine of ANG II-dependent hypertensive rats (15, 24) indicates continued formation of intratubular ANG I and ANG II despite marked suppression of PRA. Thus, for intratubular ANG I generation, a different source of renin other than from JGA cells is needed. Importantly, the models of ANG II-dependent hypertension described exhibit a marked enhancement of renin gene expression and protein in CD cells (30, 32, 34). Renin upregulation in principal cells of CD segments in ANG II-dependent hypertensive rats provides the means for enhanced intratubular ANG II formation in hypertension by the combination of CD renin acting on proximal tubule-derived AGT delivered to form ANG I (26, 31) and with subsequent conversion to ANG II by ACE present in the CDs (3, 4, 17). The critical finding of the present study is the demonstration of augmented prorenin and renin protein levels and increased renin activity in the urine of chronic ANG II-infused rats compared with control rats. Increased renin activity in the urine of chronic ANG II-infused rats reflects augmented renin activity in CD tubular fluid that may provide a final mechanism to increase intratubular ANG I formation. It is unlikely that increased prorenin and renin in the urine of ANG II-hypertensive rats are derived from filtered renin, since JGA renin and PRA are markedly suppressed in the chronic ANG II-infused model of hypertension. Furthermore, although basal secretion of renin by the principal cells into the peritubular capillaries and from these into the systemic circulation is possible, the contribution of filtered renin to the augmented prorenin and renin in the urine of ANG II-hypertensive rats seems unlikely since the chronic ANG II-infused rat model has marked suppression of JG renin and PRA levels. Campbell et al. (2) reported in Dahl salt-sensitive rats that renal anti-renin immunoreactivity and renin mRNA in JG cells changed in proportion to plasma prorenin not plasma renin between low- and high-salt diets. In the Dahl sensitive rats that also exhibit PRA suppression, plasma prorenin levels were significantly lower than Dahl resistant rats at 7 wk of age (2). Moreover, differences in urinary renin are not due to variations in the collection of the urine samples since the uCr rates were similar between ANG II and sham rats at day 13. Rohrwasser et al. (36) and Kang et al. (12), respectively, showed that renin can be secreted by CD cells using immunoblots of isolated CD from mouse kidneys (36) and cultures of immortalized mouse cortical CD cells (M-1 cells) treated with ANG II (12). The

Fig. 3. Renal medulla renin content and urine renin content. Kidney medullary renin content was measured in inner renal medullary samples from chronic ANG II-infused rats and sham-operated rats (A). Urinary renin content (uRen; B) was measured in 24-h urine samples collected on day −1 of ANG II infusions (n = 20) and in collections from day 13 of sham-operated rats (n = 10). ANG II-infused rats (n = 10). ANG II-infused rats fed a high-salt (HS; 8% NaCl; n = 5) diet, and ANG II + HS + candesartan (26 mg/l in drinking water for 14 days; n = 5). Values are means ± SE. *P < 0.05 ANG II rats vs. sham rats.

Fig. 4. Active renin (A) and prorenin measured in the presence of trypsin (B) in the urine of chronic ANG II-infused rats and sham-operated rats. Notice that the amount of prorenin expressed in enzymatic units excreted (EUE) per day of urinary renin (uRen) after trypsinization (B) is 1,000 times higher than renin (A). Values are means ± SE. *P < 0.01 ANG II rats vs. sham rats.
The present study supports the hypothesis that the augmented renin enzymatic activity and content in the urine of chronic ANG II-infused rats reflect augmented renin secretion into the CD tubular fluid of ANG II-hypertensive rats. We also examined the content of renin and prorenin in the urine of the rats of this study and found that there was abundant renin and even greater prorenin content in the urine of ANG II-infused rats. In diabetic rats which is a model of high circulating prorenin, CD cells are the main source of prorenin (12). Nguyen and associates (27) cloned the prorenin receptor [(P)RR] that binds renin and prorenin. Receptor-bound prorenin exhibits full enzymatic activity comparable to that of active renin (27). Recent findings by Cousin et al. (7) demonstrated that the full-length form of (P)RR is processed intracellularly by cleavage leading to a soluble form [s(P)RR] that can be secreted and bind prorenin and renin. We recently demonstrated that the urinary levels of the soluble form of (P)RR were augmented in ANG II-infused rats (10). These findings, along with the increases in prorenin excretion rate and augmented renin activity in the urine of the ANG II rats, further support a functional role for the s(P)RR in the tubular fluid facilitating the generation of ANG I from AGT delivered to distal nephron segments from the proximal tubules during ANG II-dependent hypertension.

The molecular weight of renin by gel filtration or SDS-PAGE has been reported to be between 38 and 42 kDa (6, 9, 19). The present study characterized the prominent bands detected by immunoblotting with an estimated molecular weight of 33 and 40 kDa, respectively, as renin and prorenin because they have the expected molecular size of the bands reported for the used antibody; but more importantly, because the specificity and identity of the detected bands by Western blot were confirmed by preadsorption and by the use of human recombinant renin and prorenin, as positive controls in the blots.

Enhanced intrarenal ANG II content in ANG II-infused rats is partially due to stimulation of intrarenal ANG II uptake via an AT1R-mediated mechanism (22, 25, 42). Indeed, it has been shown that AT1R-mediated uptake of circulating [125I] Val5-ANG II in the kidney, particularly by the proximal tubule cells, contributes to the increase in ANG II content within the kidney and in the urine after it is secreted inchronic [125I] Val5-ANG II-infused wild-type mice (18). Although it is likely that part of the ANG II content in the kidney tissue and urine is a consequence of uptake of ANG II from the circulation, Shao et al. (38) demonstrated that over 50% of intrarenal and urine levels of ANG II are derived from de novo local formation. Importantly, the fact that the urinary excretion of AGT in ANG II-infused rats increased ~20-fold compared with that of sham rats, while urinary ANG II and renin activity both increased ~2-fold in ANG II-infused rats, indicates that urinary protein/renin availability rather than the substrate AGT is rate limiting for local ANG I generation. Although the increased AGT in the urine of ANG II rats is most likely due to augmented endogenous synthesis and secretion by the proximal tubules cells, the coexistence of proteinuria (ANG II: 45.8 ± 8.0 vs. 14.7 ± 1.0 μg/day; P < 0.05) raises the possibility that an increase in filtered AGT contributes to the increased uAGT (29). Regardless of the source, however, the increased AGT in the urine reflects increased substrate availability in the lumen of distal nephron segments, which by combined local action of intraluminal renin and ACE may lead to increased ANG I and ANG II generation. Demonstration of ACE in the CD fluid and urine (3, 4), as well as its secretion by isolated CD cells (3, 4, 17) indicate that CD ACE contributes to the increased ANG II formation, thus leading to enhanced distal nephron sodium reabsorption and the maintenance of high blood pressure (17, 28). Indeed, we found that the uANG II excretion rate was markedly increased in ANG II-infused rats. The present study does not allow us to delineate the percentage of ANG II in the urine of ANG II rats derived from

Table 1. Urinary parameters after 14 days of ANG II infusions

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Units</th>
<th>Sham</th>
<th>ANG II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine volume</td>
<td>ml/day</td>
<td>13 ± 1</td>
<td>52 ± 8*</td>
</tr>
<tr>
<td>uCr</td>
<td>μg/day</td>
<td>10,201 ± 388</td>
<td>9,246 ± 1,109</td>
</tr>
<tr>
<td>uRen-R/uCr</td>
<td>μmol ANG I/mg</td>
<td>5 ± 1 × 10⁻⁷</td>
<td>13 ± 3 × 10⁻⁷*</td>
</tr>
<tr>
<td>uRen-W/uCr</td>
<td>du-mg⁻¹-day⁻¹</td>
<td>4.4 ± 0.2</td>
<td>52.9 ± 14.0*</td>
</tr>
<tr>
<td>uProren-W/uCr</td>
<td>du-mg⁻¹-day⁻¹</td>
<td>8.6 ± 2.2</td>
<td>45.7 ± 6.5*</td>
</tr>
</tbody>
</table>

Data are means ± SE of urine volume, urinary creatinine excretion rate (uCr), urine renin and prorenin protein levels in chronic ANG II-infused rats and sham-operated rats. The amounts of urinary renin determined by RIA (uRen-R/uCr) and renin and prorenin proteins determined by Western blot (uRen-W/uCr and uProren-W/uCr) were normalized with the urinary creatinine and expressed as ratios. uRen, urinary renin excretion rate; uProren, urinary prorenin excretion rate; -R, renin activity by RIA; -W, band intensity measured by Western blot; du, integrated densitometric unit. *P < 0.05 vs. sham.
local production; however, Shao et al. (38) documented that the kidney levels of endogenous Ile5-ANG II increased ~70% in Val5-ANG II-infused rats compared with that in sham kidneys. In rats infused with Val5-ANG II, the urinary Ile5-ANG II excretion rates at 2 wk infusion increased 93% compared with preinfusion levels, indicating augmented formation of endogenous ANG II. The results from Shao et al. (38) demonstrate that the increases in intrarenal and urinary ANG II levels during chronic ANG II infusions involve substantial stimulation of endogenous ANG II formation, which supports the conclusion that a major fraction of the ANG II content of the ANG II rats is newly formed in the kidney (38).

In summary, the results indicate that the increased renin content in medullary tissues and augmented renin enzymatic activity in the urine of ANG II-infused hypertensive rats contribute to the maintenance of intratubular ANG II levels observed in this model of experimental hypertension. The availability in distal nephron segments of ACE (3, 17, 28, 35) along with increased urinary levels of AGT (15), active renin, prorenin as well as sPRR collectively contribute to elevated and sustained intratubular ANG I and ANG II generation in chronic ANG II-hypertensive rats. The resultant ANG II-mediated effects on the apical transporters of CD cells could be acting synergistically and in concert with aldosterone to stimulate distal nephron sodium reabsorption and contribute to overall enhanced sodium reabsorption (1, 28, 41).

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GRANTS

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DISCLOSURES

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

REFERENCES


