Enhanced Distal Nephron Sodium Reabsorption in Chronic Angiotensin II–Infused Mice

Di Zhao, Dale M. Seth, L. Gabriel Navar

Abstract—Chronic angiotensin II (Ang II) infusions enhance urinary excretion of angiotensinogen, suggesting augmentation of distal nephron sodium reabsorption. To assess whether chronic Ang II infusions (15 ng/min for 2 weeks) enhance distal nephron sodium reabsorption, we compared sodium excretion before and after blockade of the 2 main distal nephron sodium transporters by IV amiloride (5 mg/kg of body weight) plus bendroflumethiazide (12 mg/kg of body weight) in male C57/BL6 anesthetized control mice (n=10) and in chronic Ang II–infused mice (n=8). Chronic Ang II infusions increased systolic blood pressure to 141±6 mm Hg compared with 106±4 mm Hg in control mice. After anesthesia, mean arterial pressure averaged 97±4 mm Hg in chronic Ang II–infused mice compared with 94±3 mm Hg in control mice, allowing comparison of renal function at similar arterial pressures. Ang II–infused mice had lower urinary sodium excretion (0.16±0.04 versus 0.30±0.05 μEq/min; P<0.05), higher distal sodium reabsorption (1.74±0.18 versus 1.12±0.18 μEq/min; P<0.05), and higher fractional reabsorption of distal sodium delivery (91.1±1.8% versus 77.9±4.3%; P<0.05) than control mice. Urinary Ang II concentrations, measured during distal blockade, were greater in Ang II–infused mice (1235.0±277.2 versus 468.9±146.9 fmol/mL; P<0.05). In chronic Ang II–infused mice treated with spironolactone (n=5), fractional reabsorption of distal sodium delivery was similarly augmented as in chronic Ang II–infused mice (94.6±1.7%; P<0.01). These data provide in vivo evidence that there is enhanced distal sodium reabsorption dependent on sodium channel and Na\(^+\)-Cl\(^-\) cotransporter activity and increased urinary Ang II concentrations in mice infused chronically with Ang II. (Hypertension. 2009;54:120-126.)

Key Words: tubular sodium reabsorption ■ filtered sodium ■ amiloride ■ bendroflumethiazide ■ renal plasma flow ■ glomerular filtration rate

Chronic angiotensin II (Ang II) infusions elicit hypertension through combined effects of increases in vascular resistance, decreases in sodium excretion, and suppression of the pressure-natriuresis relationship.\(^1\)–\(^4\) The latter effects are associated with enhanced formation and secretion of angiotensinogen by proximal tubular cells and increased angiotensinogen spillover into distal nephron segments reflected by increases in urinary excretion of angiotensinogen.\(^5\)–\(^10\) Because renin in collecting duct segments is also stimulated by chronic Ang II infusions,\(^11\)–\(^12\) increased spillover of angiotensinogen into distal nephron segments suggests increases in distal nephron Ang II levels, leading to stimulation of sodium reabsorption.\(^12\) Several studies have demonstrated that Ang II stimulates distal sodium transport processes, including the amiloride (AM)-sensitive epithelial sodium channels (ENaCs) and the sodium/hydrogen exchangers (NHEs).\(^13\)–\(^19\) However, there is no in vivo evidence that chronic Ang II infusions lead to increases in either absolute or fractional distal nephron sodium reabsorption that may play an important role in the progressive increase in arterial pressure during chronic Ang II infusions. In Ang II–infused rats, sodium excretion was lower for any given arterial pressure and there was marked suppression of pressure natriuresis,\(^3\)\(^,\)\(^19\) but the data did not allow segmental localization of the changes in sodium reabsorption. In chronic Ang II–infused dogs, the sodium excretion initially decreased and remained decreased if renal arterial pressure was prevented from increasing.\(^2\)\(^,\)\(^20\)\(^,\)\(^21\)

Although the proximal tubule is responsible for reabsorbing the bulk of the filtered load, the distal nephron segments are ultimately responsible for the fine regulation of sodium excretion.\(^13\)\(^,\)\(^22\)–\(^25\) Sodium reabsorption in connecting tubule and collecting duct segments is mainly mediated by AM-sensitive ENaCs and bendroflumethiazide (BFTZ) sensitive-Na\(^+\)-Cl\(^-\) cotransporters.\(^13\)\(^,\)\(^22\)\(^,\)\(^23\)\(^,\)\(^25\)\(^,\)\(^26\) Thus, treatment with AM plus BFTZ blocks most sodium transport in distal nephron segments, allowing sodium excretion under these conditions to provide a collective measure of sodium delivery to distal nephron segments.\(^27\)–\(^30\) Sodium reabsorption by distal nephron segments can, thus, be determined from the difference between urinary sodium excretion (U\(_Na\)V) during distal blockade and U\(_Na\)V during control conditions.

In this study, we tested the hypothesis that chronic Ang II–infused mice exhibit enhanced sodium reabsorption in distal nephron segments, which may contribute to sodium...
retention. Although technical limitations prevent direct assessment of the distal nephron and collecting duct Ang II concentrations, we measured concentrations in urine samples as an index of distal nephron Ang II levels. To minimize peptide degradation, we measured the urinary Ang II concentrations and excretion rates during the diuretic treatment, conditions where the urine would more closely reflect distal nephron tubular fluid. To obtain a collective assessment of distal nephron sodium reabsorption in the total nephron population, studies were performed during control conditions and after blockade of the 2 major distal nephron sodium transporters in mice infused chronically with Ang II. To investigate whether the observed changes in distal nephron sodium reabsorption were mediated by aldosterone during chronic Ang II infusions, we treated a group of chronic Ang II–infused mice with spironolactone.

Methods

Animals

Experiments were performed on 9- to 12-week–old male C57/BL6 mice (Jackson Laboratory, Bar Harbor, Maine) maintained on a 12:12-hour light-dark schedule (6 AM to 6 PM) at 25°C in the vivarium at Tulane University Health Sciences Center. Rodent chow containing normal salt content (0.5%) along with tap water was provided. The protocol was approved by the institutional animal care and use committee of Tulane University Health Sciences Center.

Experimental Protocol

Chronic Ang II–infused mice were prepared by administration of Ang II (Phoenix Pharmaceuticals) at 15 ng/min via osmotic minipump for 11 to 13 days to elicit a slow progressive pressor response. Systolic blood pressure in awake mice was measured by noninvasive computerized tail-cuff plethysmography (Visitech BP2000). Systolic blood pressure was monitored at 7 and 11 days after minipump implantation in chronic Ang II–infused mice. On the day of the experiment, mice were anesthetized with inactin (thiobutabarbital sodium) injected IP at 200 mg/kg of body weight. Mice were then administered tabarbital sodium (6 mg/kg of body weight per day), which was implanted at the same time as the minipump. Systolic blood pressure in awake mice was measured by noninvasive computerized tail-cuff plethysmography (Visitech BP2000). Systolic blood pressure was monitored at 7 and 11 days after minipump implantation in chronic Ang II–infused mice. One of the experiment, mice were anesthetized with inactin (thiobutabarbital sodium) injected IP at 200 mg/kg of body weight.

Once a stable level of anesthesia was obtained, judged by heart rate and lack of toe reflex, mice were placed on a surgical table (37°C) with servocontrol of temperature to maintain body temperature at 37°C and prepared for clearance experiments, as described previously. During surgery, an isotonic saline solution containing 6% albumin (bovine, Sigma Chemical Co) was infused at a rate of 4 μL/min. The bladder was catheterized with phycoerythrin 90 tubing via a suprapubic incision for urine collections. After surgery, the IV infusion solution was changed to isotonic saline containing 10% albumin, 4.5% polyfructosan (Inutest [inulin], Laevosan), and 1.5% para-aminohippurate (PAH; Merck Sharpe & Dohme) and was infused at 4 μL/min for a 60-minute equilibration period before urine collections.

Renal plasma flow (RPF), glomerular filtration rate (GFR), urine flow, and sodium excretion were determined in control mice (n = 10), chronic Ang II–infused mice (n = 8), and chronic Ang II–infused mice treated with spironolactone pellet (n = 5; ~190 mg/kg of body weight per day), which was implanted at the same time as the minipump containing Ang II using a renal clearance protocol in mice, as described previously. A separate group of mice was used for the determination of urinary Ang II concentrations in Ang II–infused and control mice. Urine samples for urinary Ang II measurement were initially collected directly into an inhibitor mixture (5 mmol/L of EDTA, 20 μmol/L of pepstatin, 10 μmol/L of PMSF, 20 μmol/L of enalaprilat, and 1.25 mmol/L of 1-10-phenanthroline).

In each group, clearance periods 1 and 2 were performed for control measurements without diuretic treatment. An IV dose of AM (5 mg/kg of body weight) and BFTZ (12 mg/kg of body weight) was then administered followed by urine collections for assessment of distal sodium delivery. The peak sodium excretion during blockade was used as an estimate of sodium delivery to the distal nephron segments. By subtracting the average sodium excretion measured during periods 1 and 2, distal nephron sodium reabsorption was determined. At the end of the experiment, an arterial blood sample was collected from the arterial catheter for measurements of plasma PAH, inulin, and sodium concentrations. To determine whether the diuretic treatment altered RPF and GFR, we compared arterial pressure, RPF, and GFR between a group of mice treated with AM and BFTZ (n = 7) and a group of mice not treated with AM and BFTZ (n = 5). We did not observe any significant differences between untreated control mice and diuretic-treated mice in mean arterial pressure (MAP; 99 ± 2 versus 93 ± 2 mm Hg; P > 0.05), RPF (1.42 ± 0.32 versus 1.33 ± 0.30 mL/min; P > 0.05), and GFR (0.25 ± 0.05 versus 0.19 ± 0.02 mL/min; P > 0.05).

Urine and Plasma PAH, Inulin, and Sodium Concentrations

Urine and plasma PAH and inulin concentrations were measured using standard colorimetric techniques, as reported previously. RPF was estimated from the PAH clearance calculated as the ratio of urine:plasma PAH concentrations times urine flow. GFR was calculated as the ratio of urine:plasma inulin concentrations times urine flow. Urine output was determined gravimetrically, assuming a density of 1 g/mL. Urine and plasma sodium concentrations were measured using flame photometry (Flame Photometer IL 973, Instrumentation Laboratory).

Urine Ang II Radioimmunoassay Measurement

The detailed procedure is available in the online data supplement at http://hyper.ahajournals.org.

Calculations

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U_{\text{Na}}^v_{\text{c}} \text{ designates the control } U_{\text{Na}}^v \text{ (the average of periods 1 and 2). } U_{\text{Na}}^{V_{AM}+BFTZ} \text{ (distal sodium delivery) was based on the peak } U_{\text{Na}}^v \text{ after administration of AM plus BFTZ (period 3). Calculated distal sodium reabsorption was determined from } U_{\text{Na}}^{V_{AM}+BFTZ} \text{, } U_{\text{Na}}^v, \text{ and } U_{\text{Na}}^{V_{AM}+BFTZ}. \text{ Fractional reabsorption of distal sodium delivery was calculated as follows: } (U_{\text{Na}}^{V_{AM}+BFTZ} - U_{\text{Na}}^v) / U_{\text{Na}}^v.
\]

Statistical Analysis

The statistical analysis was performed by 1-way ANOVA with Bonferroni’s multiple-comparison posthoc tests using the GraphPad Prism program (GraphPad). The results are presented as mean ± SE. Significance was set at P < 0.05.

Results

Systolic Blood Pressure and AP

As shown in Figure 1A, systolic blood pressure measured in awake mice was increased after 2 weeks of chronic Ang II infusions as compared with control mice (141 ± 6 versus 106 ± 4 mm Hg; P < 0.001). In chronic Ang II–infused mice treated with spironolactone, systolic blood pressure was also higher as compared with control mice (143 ± 13 mm Hg; P < 0.01). As shown in Figure 1B, after anesthesia, mice infused chronically with Ang II had MAP averaging 97 ± 4 mm Hg. MAP in normal mice studied thus far are generally lower, averaging ~94 ± 3 mm Hg. In the group of chronic Ang II–infused mice treated with spironolactone, MAP averaged 93 ± 3 mm Hg.

RPF and GFR

As shown in Figure 2, RPF and GFR were not significantly different among the 3 groups and remained stable during the clearance periods. Although GFR tended to be slightly lower
in the Ang II–infused groups, the differences are not statistically significant.

**Urine Flow and UNaV**

As shown in Figure 3, urine flow was slightly lower in the chronic Ang II–infused mice, but this was not statistically different from urine flow in control mice. However, UNaV rate in the chronic Ang II–infused mice was lower compared with control (0.16±0.04 versus 0.30±0.05 µEq/min; P<0.05). After dual distal nephron blockade with AM plus BFTZ, the fractional UNaV rates increased markedly to 4.5±0.6% in control and 7.5±1.3% in Ang II–infused mice, thus reflecting the fractional sodium delivery to the terminal nephron segments. In the group of chronic Ang II–infused mice treated with spironolactone, urine flow was not statistically different as compared with control mice, whereas UNaV (0.08±0.02 µEq/min; P<0.01) was lower. However, urine flow and UNaV were not statistically different as compared with chronic Ang II–infused mice.
Distal Sodium Delivery and Sodium Reabsorption in Distal Nephron Segments

As shown in Figure 4, distal sodium delivery values were not significantly different between chronic Ang II–infused mice and control mice (1.90±0.25 versus 1.42±0.21 μEq/min; P>0.05). In contrast, distal sodium reabsorption in the chronic Ang II–infused mice was greater as compared with control mice (1.74±0.18 versus 1.12±0.18 μEq/min; P<0.05). Likewise, the fractional reabsorption of distal sodium delivery values was augmented in the chronic Ang II–infused mice (91.1±1.8% versus 77.9±4.3%; P<0.05). In chronic Ang II–infused mice treated with spironolactone, distal sodium reabsorption (1.82±0.28 μEq/min; P<0.05) and the fractional reabsorption of distal sodium delivery values (94.6±1.7%; P<0.01) were greater than in control mice, whereas distal sodium delivery values were not significantly different between chronic Ang II–infused mice treated with spironolactone and control mice (1.90±0.27 μEq/min; P>0.05). However, the distal sodium reabsorption and the fractional reabsorption of distal sodium delivery values were not statistically different as compared with chronic Ang II–infused mice.

Urinary Ang II Levels

As shown in Figure 5, urinary Ang II concentrations (1235.0±277.2 versus 468.9±146.9 fmol/mL; P<0.05) and urinary Ang II excretion rates (4.86±1.19 versus 2.06±0.36 fmol/min; P<0.05) were significantly higher in the chronic Ang II–infused mice during administration of AM plus BFTZ as compared with control.

Discussion

Various studies have demonstrated that, in addition to its actions on earlier nephron segments, Ang II can stimulate transport activity in distal nephron segments. In particular, intraluminal Ang II can stimulate the NHE, Na\(^+\)/Cl\(^-\) cotransporter, ENaC, potassium channel, and H\(^+\)-ATPase. Furthermore, luminal application of Ang II directly stimulates ENaC activity in the cortical collecting duct, even at a low concentration of 10\(^{-12}\) M. These studies demonstrate that Ang II directly stimulates sodium reabsorption in distal nephron segments; nevertheless, in vivo quantitative data in support of enhanced distal sodium transport in chronic Ang II–infused models of hypertension have been difficult to obtain. Although micropuncture and isolated perfused tubule and cell studies provide direct data indicating the actions of Ang II on the transport mechanisms, they do not provide quantitative estimates of the collective effect on distal nephron reabsorption rate. It is recognized that...
the present approach using transport inhibitors that block the
distal nephron sodium transport systems provides only an
indirect estimate of distal sodium reabsorption; nevertheless,
it is an effective means of providing a collective quantitative
estimate of the net sodium reabsorption in the distal nephron
segments.28–30

The present study provides in vivo evidence that chronic
Ang II infusions elicit a chronic stimulatory influence on
sodium reabsorptive processes in distal nephron segments.
These results are consistent with the hypothesis that augmenta-
tion of distal Ang II levels stimulates distal sodium reab-
sorption, thus contributing to the progressive hypertension
that develops during chronic Ang II infusions. Previous
studies have shown that chronic Ang II–infused rats have a
rightward shift of the pressure-natriuresis relationship caused
primarily by a decrease in fractional excretion of sodium.3,4,19,20,39,40
In chronic Ang II–infused rats and mice, circulating Ang II levels increase within a few days and lead
to augmentation of intrarenal angiotensinogen and Ang II that
reduce sodium excretion, resulting in progressive increases in
arterial pressure.1,6–10,36,41 Hall et al.20 reported that Ang II
infusions caused sodium retention in dogs when renal arterial
pressure was prevented from rising with a servocontrolled
aortic occluder. Chronic Ang II infusions caused sodium reten-
tion for several days before sodium balance was achieved at an
elevated MAP in dogs.21

Although inappropriate stimulation of sodium reabsorption
in any segment of the nephron can lead to excess sodium
retention and hypertension,42 various studies have empha-
sized the important role of sodium transport in the terminal
distal nephron segments, in particular, the collecting duct
system.12,16,23,25 Several monogenetic mutations in human
subjects with hypertension involve overactivation of distal
sodium transport systems,24,43 including activation of ENaCs
and the Na\(^+\)Cl\(^-\) cotransporter, which are found in the distal
convoluted tubule and collecting duct segments.16,23,25,26 In
addition, chronic Ang II infusions have been shown to
upregulate ENaC expression in the collecting duct,16 and Ang
II has been shown to enhance trafficking of the distal tubule
Na\(^+\)Cl\(^-\) cotransporter29 and to activate ENaCs.13,15 These
and related findings discussed earlier support an enhanced
distal nephron sodium reabsorption in Ang II–dependent
hypertension.

In the present study, RPF and GFR were not significantly
different in chronic Ang II–infused mice and control mice, as
reported previously by Cervenka et al.44 These results suggest
that GFR is less responsive in mice than in rats, because
previous studies in rats showed that GFR is significantly
decreased by chronic Ang II infusions; however, RPF was not
different, consistent with the present results.3,45 Notably,
renal vascular Ang II type I receptor expression is maintained
in chronic Ang II–infused rats,26 and losartan prevents the
decreases of GFR in chronic Ang II–infused rats.3 Furthermore,
Ang II–mediated increases in oxidative stress may be
involved in the regulation of arterial pressure and renal
vascular resistance in chronic Ang II–infused mice.31

The sodium excretion responses to chronic Ang II infu-
sions are complex and depend on the dose and duration of the
Ang II infusions, as well as the magnitude of the blood
pressure responses. In this study, U\(_{\text{NaV}}\) in the anesthetized
chronic Ang II–infused mice was significantly lower than in
control mice, thus suggesting that the higher arterial pressures
measured in vivo are required to maintain sodium balance in the
awake mice.2,20 These studies suggest initial sodium retention
during chronic Ang II infusions, with restoration of
sodium balance achieved at the elevated arterial pressures.21
After dual distal nephron blockade with AM plus BFTZ, the
fractional sodium excretion rates increased to 4.5% in control
and 7.5% in chronic Ang II–infused mice, thus reflecting the
fractional sodium delivery to the terminal nephron segments.
These data support our basic rationale that the dual distal
blockade effectively inhibits the bulk of the sodium reab-
sorbed by the distal nephron segments. Furthermore, absolute
distal sodium delivery was similar, suggesting that sodium
loads arriving at distal nephron segments were similar. These
results, thus, suggest that much of the augmented sodium
reabsorption responsible for the reduced sodium excretion in the
chronic Ang II–infused mice occurred in distal nephron
segments.

Chronic Ang II infusions stimulate production and release
of aldosterone, which activates the mineralocorticoid recep-
tors in principal cells of collecting ducts and, thus, augments
the abundance of Na/K ATPases, ENaCs, and potassium
channels.47–50 However, in the present study, there was a
trend toward a lower U\(_{\text{NaV}}\), which was not statistically
significant. Also, fractional reabsorption of distal sodium
delivery between chronic Ang II–infused mice treated with
spironolactone and chronic Ang II–infused mice was not
significantly different. These results suggest that increased
aldosterone is not responsible for the enhanced distal sodium
reabsorption in chronic Ang II–infused mice, and they are
consistent with the recent study by Ortiz et al.51

Although proximal tubule fluid Ang II concentrations have
been measured,35,36 it has not been possible to obtain an index
of the Ang II concentrations in distal nephron tubular and
collecting duct fluid. Because of major technological issues,
micropuncture34 and microcatheterization procedures are not
feasible and would require extensive surgical procedures and
unrealistic collection periods. We reasoned that the markedly
increased urine output occurring during dual distal blockade
would provide samples that more closely reflect distal tubular
fluid or collecting duct fluid and might have Ang II concen-
trations approaching those existing in the tubular fluid. We
found significant increases in Ang II concentrations, as well
as in Ang II excretion rates, in the chronic Ang II–infused
mice. These data provide in vivo evidence that tubular fluid
Ang II concentrations in distal nephron segments are indeed
higher in the chronic Ang II–infused mice.

**Perspectives**

Although elevated intrarenal Ang II levels may augment
sodium reabsorption in multiple nephron segments, the aug-
mentation of distal Ang II levels may play a particularly
important role in the progressive hypertension during chronic
Ang II infusions. These effects to stimulate the distal sodium
reabsorption leading to reabsorption of 90% of the distal
sodium delivery provide the final influence to achieve
marked sodium retention, which leads to progressive hyper-
tension. The efficacy of thiazide diuretics and AM in certain hypertensive cases may be attributed to their ability to counteract the enhanced distal sodium reabsorption. The noninvasive approach used in the present study is applicable to studies in transgenic mice and also to translational experiments that could test this hypothesis in human subjects.

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Disclosures

None.

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Urine Ang II Radioimmunoassay Measurement
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The Ang II concentrations in the urine were measured by the previously described radioimmunoassay. Briefly the urine that had been collected into inhibitor cocktail was measured to record the volume and then transferred into excess ice-cold methanol to denature the proteins. The samples were quickly vortexed and centrifuged at 4000 RPM at 4°C for 30 minutes and the supernatants were decanted and vacuum dried. The pellets were reconstituted in radioimmunoassay buffer (Peninsula) and two 100ul replicates were assayed. Each replicate received 100ul Anti-Ang II antibody (RIA antibody-Peninsula) and 100ul $^{125}$I-Ang II (15,000 CPM/100ul initial activity, Perkin-Elmer). The samples were incubated along with an Ang II standard curve for 48hr at 4°C. The bound and free radiolabel was separated from each other by the addition of dextran-charcoal and centrifugation. The bound activity was counted on a Wallac-Wizard 1470 Automatic Gamma Counter. The sample CPMs were plotted against the Ang II standard curve to yield fmole/ml.
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