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\textsuperscript{+}\textendash Cl\textsuperscript{−} 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 (NHxs).13–18 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\textsuperscript{+}\textendash Cl\textsuperscript{−} 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 sodium excretion (UNaV) during distal blockade and UNaV 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.27–30

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.31 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.28,32,33 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.28 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 1.0% albumin, 4.5% polyfructosan (Inutest [inulin], Laevosan), and 1.5% para-aminohippurate (PAH; Merck Sharpe & Dohme) and was infused at a rate of 4 μL/min for a 60-minute equilibration period before urine collections.28,32,33

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 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.27–30

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.

### 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 infusions10 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.13,16–18,26,34,35 In particular, intraluminal Ang II can stimulate the NHE, Na+/Cl− cotransporter, ENaC, potassium channel, and H+–AT-Pase.16–18,36–38 Furthermore, luminal application of Ang II directly stimulates ENaC activity in the cortical collecting duct,13–15 even at a low concentration of 10−12 M.13 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,17,18,36–38 they do not provide quantitative estimates of the collective effect on distal nephron reabsorption rate. It is recognized that distal delivery and reabsorption...
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 augmentation of distal Ang II levels stimulates distal sodium reabsorption, 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.3,4,10,29,39,40 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 retention 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 emphasized 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 sodium nephron 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,36 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 infusions 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, UNaV 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 BPTZ, 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 reabsorbed 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 receptors 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 UNaV, 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 concentrations 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 augmentation 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.

Sources of Funding
This work was supported by National Heart, Lung, and Blood Institute grant HL-26371, by a Health Excellence Fund grant from the Louisiana Board of Reagents, and by the Centers of Biomedical Research Excellence grant P20RR0117659 from the Institutional Development Award Program of National Center for Research Resources.

Disclosures
None.

References
Enhanced Distal Nephron Sodium Reabsorption in Chronic Angiotensin II–Infused Mice
Di Zhao, Dale M. Seth and L. Gabriel Navar

Hypertension. 2009;54:120-126; originally published online June 1, 2009;
doi: 10.1161/HYPERTENSIONAHA.109.133785

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/54/1/120

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2009/05/26/HYPERTENSIONAHA.109.133785.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/
ONLINE SUPPLEMENT

Urine Ang II Radioimmunoassay Measurement
Di Zhao, Dale M. Seth, L. Gabriel Navar
Department of Physiology and Hypertension and Renal Center of Excellence
Tulane University School of Medicine
New Orleans, LA 70112

Corresponding author:
Di Zhao, MD, PhD
Tel: (504)-988-2611
Fax: (504)-988-2675
E-mail: dzhao@tulane.edu
The Ang II concentrations in the urine were measured by the previously described radioimmunoassay\textsuperscript{1-3}. 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.
References
