Effects of Dietary Sodium and Genetic Background on Angiotensinogen and Renin in Mouse

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Abstract—Elements of a renin-angiotensin system expressed along the entire nephron, including angiotensinogen secreted by proximal tubule and renin expressed in connecting tubule, may participate in the regulation of sodium reabsorption at multiple sites of the nephron. The response of this tubular renin-angiotensin system to stepwise changes in dietary sodium was investigated in 2 mouse strains, the sodium-sensitive inbred C57BL/6 and the sodium-resistant CD1 outbred. Plasma angiotensinogen was not affected by sodium regimen, whereas plasma renin increased 2-fold under low sodium. In both strains, the variation in urinary parameters did not parallel the changes observed in plasma. Angiotensinogen and renin excretion were significantly higher under high sodium than under low sodium. Water deprivation, by contrast, induced significant activation in the tubular expression of angiotensinogen and renin. C57BL/6 exhibited significantly higher urinary excretion of angiotensinogen than did CD1 animals under both conditions of sodium intake. The extent to which these urinary parameters reflect systemic or tubular responses to challenges of sodium homeostasis may depend on the relative contribution of sodium restriction and volume depletion. (Hypertension. 2002;39:1007-1014.)

Key Words: angiotensinogen ▪ renin ▪ sodium ▪ mouse ▪ genetics ▪ urine

We have advanced the hypothesis that a paracrine tubular renin-angiotensin system operates along the entire nephron.1 Although angiotensinogen (AGT) is not filtered across the glomerular membrane, the protein2 and its mRNA3,4 have been detected in proximal tubule (PT), the protein is secreted to the apical side of PT cell monolayers,1 has been detected in final urine under normal physiological conditions,5 and was detected in luminal fluid of PT epithelium collected by micropuncture.6 Systemic renin is filtered and reabsorbed in the PT.7 Although not detected in situ, it may be expressed at low level in the PT.8,9 We have found that renin was also synthesized and secreted in connecting tubule (CNT).1 ACE and angiotensin (Ang) II receptors are expressed along the nephron.10,11 High luminal Ang II has been observed in the PT,12,13 where it stimulates sodium reabsorption.14 Some observations support a similar role in terminal segments of the nephron.15 The potential significance of this tubular renin-angiotensin system in blood pressure regulation is underlined by the observation that double transgenic animals overexpressing human renin systemically and human AGT in the PT develop hypertension.16

The impact of dietary sodium on the expression of renin and tubular AGT and the significance of their urinary excretion as indicators of the activity of this tissue system were tested in the mouse. Two strains were investigated, C57BL/6 and CD1. The C57BL/6 inbred differs from other inbred lines in its response to dietary sodium17; its sodium sensitivity has been demonstrated18,19 and exploited in an attempt to map genetic determinants of the arterial pressure response to dietary sodium.19 We have verified this sodium sensitivity under our experimental conditions. The outbred CD1 was selected as reference, as we have found that it was sodium resistant under such protocols (P. Lantelme, E. Hillas, A. Rohrwasser, J.-M. Lalouel, unpublished, 2001). We find that in both strains, the variation in urinary AGT and renin induced by dietary sodium did not parallel that observed in the general circulation. Furthermore, C57/BL6 animals exhibited significantly higher urinary AGT excretion than did CD1 animals under both high and low sodium intake.

Methods

Animal Protocols
All experiments were performed in 10- to 12-week-old male C57BL/6 and CD1 mice (Charles River, Wilmington, Mass), under...
controlled conditions of temperature and lighting following approved protocols (IACUC, University of Utah). Animals had unrestricted access to food (0.2% sodium, Purina) and water during the entire study. For in vivo studies, 8 C57BL/6 and 8 CD1 mice were housed singly in metabolic cages (Nalgene) for a 5-day habituation period, during which they were fed a high-sodium diet (3.15%, Purina). After this period, the observational part of the study consisted of a week of high sodium followed by a week of low sodium (0.03%, Purina). Body weight, food consumption, and water intake were measured daily. Twenty-four–hour urine was collected daily in the presence of 4–(2-aminoethyl)-benzenesulfonil fluoride (AEBSF, 0.5 mol/L, Fisher). Blood samples (50 μL) were obtained from tail snipping in half of the animals the day before and the day after dietary change (days 6 and 8), and in all animals at the end of the experiment.

For immunohistochemical studies, 9 C57BL/6 male mice were maintained under high sodium (3.15%) for 10 days, followed by a week of low sodium (0.03%, Purina). Three animals were euthanized at each of 3 time points: the end of the high-sodium period, after the first day of the low-sodium regimen, and on the last day of the study. Kidneys were removed, hemisected, and either snap-frozen or fixed in 10% formalin (Fisher).

**Assays of Angiotensinogen**

Total AGT (t-AGT) in plasma or urine was measured by ELISA (A. Rohrwater, P. Lantelme, T. Cheng, J. Wu, J.-M. Lalouel, unpublished, 2000). Microtiter plates were coated with purified recombinant AGT. Samples or standards were added and incubated with polyclonal antiserum against AGT antibody.1 Bound antibody was detected after reaction of horseradish peroxidase conjugated swine anti-rabbit IgG with 3-amino-9-ethylcarbazole substrate (Sigma). The assay range was 5 ng/L to 10 ng. The assay was specific with no cross-reactivity to other proteins.

Uncleaved AGT (u-AGT) was measured in urine as the release of Ang I after complete digestion by highly purified renin.20 Urine samples (500 μL) were subjected to spin dialysis in a Microcon 30 (Millipore Co), diluted with 500 μL of a 10−4 M Tris-EDTA solution, and reconstituted. Sample aliquots (5 μL) were incubated for 2 hours at 37°C with purified mouse renin (2.5 nmol/L).1 and released Ang I was measured in duplicate by radioimmunoassay (NEN-DuPont).

**Measurement of Active Renin Concentration**

Renin activity was measured as the amount of Ang I generated after incubation with excess AGT. Two microliters of plasma were incubated for 20 minutes at 37°C with excess porcine AGT (4 μmol/L, Sigma) in a 10-μL reaction containing sodium-acetate (50 mmol/L, pH 6.5), AEBSF (2.5 mmol/L), 8-hydroxyquinoline (1 mmol/L), and EDTA (5 mmol/L). The reaction line was for at least 30 minutes in plasma using test samples from animals under high- or low-sodium diet. The reaction was stopped by boiling, and Ang I was measured by using an indirect radioimmunoassay (NEN-DuPont). Plasma renin concentration (PRC) was expressed as Ang I generated per hour per μL of plasma.

In contrast with plasma, Ang I release in urine was rapid initially (within seconds), with no further Ang I generation. This Ang I release is of renin origin because (1) basal Ang I, in the absence of substrate addition, was undetectable or very low; (2) Ang I release varied in direct proportion with the amount of sample used; (3) it increased in direct proportion to the amount of purified recombinant renin added to a urine sample; and (4) it was prevented in a dose-dependent manner by the renin inhibitor (H-77, Sigma). Consequently, active renin concentration was measured as the amount of Ang I released during a 15-minute incubation at 37°C. The dependence of the assay on physiological variation in pH, sodium, urea, protein, and des-Ang I–AGT was examined. Because sodium proved significant, the reaction buffer was adjusted to 500 mmol/L sodium chloride.

**Other Measurements**

Urine aldosterone was determined by radioimmunoassay (Coat-Count, Diagnostic Products). Urinary sodium was measured by atomic absorption (Perkin-Elmer, model 2380). Samples obtained from the high-sodium period were diluted 1:5000 in deionized water, whereas samples from the low-sodium period were diluted 1:250. All dilutions were performed in duplicate, and each sodium measurement consisted of the average of 3 consecutive readings. Creatinine was measured in duplicate using Jaffé’s reaction (Sigma). Assays were performed on undiluted plasma and 1:10 diluted urine. Protein concentrations were measured using the Micro bicinchoninic assay (BCA) method (Pierce).

**Immunohistochemistry**

Tissue slides, immunostaining of AGT and renin, and quantitation of CNT renin were performed as described.1 Angiotensinogen in the PT was expressed on a 0 to 3 scale, with 0 for no staining, 1 for staining of <20% of only the cortical PCTs, 2 for staining of 50% of all PCTs and 80% of the cortical PCTs, and 3 for strong and intense staining of >50% of all PCTs. PCTs were identified based on PAS counterstaining of brush borders. Scoring was performed by 2 investigators blinded to the experimental conditions and averaged.

**Quantitative Analysis of mRNA**

Total RNA was extracted from frozen tissues using Trizol reagent (Invitrogen). cDNA was prepared using reverse transcriptase (AMV reverse transcriptase, Promega) and the manufacturer’s standard protocol. Quantitative real-time polymerase chain reaction (PCR) analysis was performed by monitoring the fluorescence of SYBR Green (Molecular Probes) with the ABI PRISM 7700 detection system (Perkin Elmer Applied Biosystems). Oligonucleotide primers were designed to span at least 1 intron and to minimize primer-dimer formation. Samples were electrophoresed to verify specific amplification and the absence of primer-dimer formation. All PCR reactions were performed in triplicate with primers (1) specific for the corresponding mouse genes and (2) spanning at least 1 intron: 5’-GTG ACA GGA TTG TGG AAG ATG AAC T-3’ (mAGT Ex3), and 5’-TGG GAA GCT TGT CAT CAA CGA-3’ (mGAPDH). Amplification was performed during 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 68°C for 60 second. Water and genomic DNA samples were used as negative controls. Cloned mouse cDNAs for AGT and GAPDH were used to generate standard curves. AGT concentration was expressed relative to GAPDH.

**Statistical Analyses**

Urinary parameters were expressed relative to creatinine to guard against incomplete urine recovery. Individual values are summarized as mean±SEM. All measurements were in the range of reference values21 or previously published observations.17,22 To examine the effects of the 2 factors, dietary sodium and genetic background, mean values were calculated for each animal over each period of sodium regimen and were subjected to 2-way ANOVA. Whenever significant heterogeneity of residual variance was indicated by Levene’s test, analysis was repeated after logarithmic transformation.

**Results**

Eight male mice of each of 2 strains, C57BL/6 and CD1, were housed singly in metabolic cages and observed during 2 consecutive weekly periods of high and low dietary sodium. The variables examined were expressed either as average concentration (for plasma) or average daily excretion (for urine) over each week of sodium regimen. Urinary parameters were examined without or with correction for body weight or for creatinine excretion. We verified that plasma creatinine was not significantly affected by either sodium
In marked contrast with plasma, urinary excretion of t-AGT, as measured by an indirect radioimmunoassay of Ang I released after incubation with excess renin, represented <6% of t-AGT excretion and was only marginally dependent on sodium intake and strain (Figure 1D).

By contrast with PRC, urinary excretion of active renin was higher under high than under low sodium intake (Figure 1E, $P<0.0001$), with no significant strain difference. Urinary aldosterone excretion measured in a subset of animals was below detection limits under high sodium, but exceeded detection threshold by 2 orders of magnitude under low sodium (Figure 1F).

Expression of PT AGT and CNT Renin
When animals were transferred from high to low dietary sodium, no significant difference in AGT expression could be detected by either semiquantitative immunohistochemistry of the protein (Figure 2A through 2D) or quantitation of mRNA (Figure 2E). CNT renin, evaluated only by semiquantitative histology, did not exhibit significant variation with dietary sodium (Figure 2F through 2I).

Discussion
The impact of dietary sodium on components of systemic and tubular renin-angiotensin system was examined in 2 mouse strains, C57BL/6 and CD1. The first was selected for its reputation as a sodium-sensitive strain\textsuperscript{17} and its ubiquitous use for transgenic models in circulation research. The CD1 outbred was also investigated, as our unpublished observations suggest that it is sodium resistant. There are both advantages and potential pitfalls in the use on either inbred or outbred strains. Inbred lines, quasi-homozygous at all loci, are genetically more stable, but dominance and epistatic effects contribute to the genetic determination of continuous phenotypes. Outbred lines such as CD1, derived from a small number of founders and maintained without deliberate inbreeding, are susceptible to genetic drift over time. Significant heterozygosity at most loci, however, affords greater additive contribution of individual loci to the genetic variance.

Plasma t-AGT concentration was not affected by either dietary sodium or genetic background. The size of circulating AGT ($61$ to $65$ kDa) precludes glomerular filtration under normal physiological conditions.\textsuperscript{23} Although degradation fragments of AGT could escape this exclusion, a direct
A. Total plasma angiotensinogen
Diet n.s.
Strain n.s.
Interaction n.s.

B. Plasma renin concentration
Diet p<0.0001
Strain n.s.
Interaction n.s.

C. Total urinary angiotensinogen
Diet p<0.0001
Strain p<0.0001
Interaction p<0.01

D. Uncleaved urinary angiotensinogen
Diet p<0.001
Strain n.s.
Interaction n.s.

E. Urinary renin
Diet p<0.0001
Strain n.s.
Interaction n.s.

F. Urinary aldosterone
Diet p<0.0001
Strain n.s.
Interaction n.s.

Figure 1. Measured responses to changes in dietary sodium as a function of genetic background. Results are expressed as means of daily average excretions within strain under each sodium regimen. Solid bars and empty bars refer to C57BL/6 (n=8) and CD1 (n=8) strains, respectively.
Figure 2. Assessment of AGT and renin expression in kidney of C57BL/6 animals as a function of dietary sodium: immunostaining for AGT (A through C) for each observation point (days 9, 10, and 16), mean semiquantitative scores±SEM for AGT immunostaining (D), quantitative real-time reverse transcription–PCR for AGT expression (E), and immunostaining for renin (F through H) with mean semiquanti-
tative scores±SEM for CNT renin immunostaining (I).
examination of immunoreactive AGT in plasma after electrophoresis and Western blotting confirms that smaller cross-reacting species constitute at best a very minor fraction of total circulating AGT (Rohrwasser, unpublished observations). It follows that urinary AGT is of PT origin. Urinary excretion of t-AGT was consistently higher in C57BL/6 than in CD1 under both conditions of sodium intake. This difference was observed whether t-AGT was expressed relative to body weight or relative to creatinine excretion. Several elements indicate that increased glomerular leakage of systemic AGT induced by increased dietary sodium is unlikely to account for this observation: (1) the modest rise in mean arterial pressure (10 to 15 mm Hg) induced by short-term (10 to 15 days) increase in dietary sodium is not likely to induce significant glomerular damage; (2) the difference observed between strains is present under either high or low sodium intake.
intake; and (3) urinary protein excretion (Table) is not significantly affected by either sodium regimen or strain. This observation may reflect a genetic difference in renal sodium handling between the 2 species. Uncleaved AGT, as measured by an indirect radioimmunoassay, accounted for ≈6% of t-AGT, suggesting that most of the renin substrate released in luminal fluid may be consumed in the nephron or in the lower urinary tract. We cannot exclude, however, the possibility that some hydrolysis occurred during timed collection of voided urine, as measurement of urinary renin precluded aspartyl proteinase inhibition.

Although PRC was inversely related to dietary sodium, urinary excretion of active renin paralleled dietary sodium. The origin of urinary renin remains unclear. Micropuncture studies of rat PTs have provided direct evidence that this small protein (36 to 40 kDa) can be found in glomerular ultrafiltrate at a fifth of its plasma concentration and that it is rapidly reabsorbed along the PT.7 The escape of even a small fraction from such fate could contribute substantially to renin in final urine, however. Although renin may also be synthesized in PTs,3 this contribution is likely to be small at best, as it is not detected by immunohistochemistry of renal tissue. We have reported renin synthesis and secretion by principal cells of connecting tubule or by CNT cells.1 Evidence that renin may be added to the urine in a distal segment of the nephron was provided by stop-flow studies in dogs.24 De novo renin synthesis in distal nephron segments has also been reported in rats subjected to subtotal nephrectomy.25

The decrease in urinary AGT excretion observed under low sodium appeared at odds with the previous observation that AGT mRNA in PT was increased by a combination of dietary sodium restriction and furosemide treatment.4 No quantitative difference could be noted in AGT mRNA under the present experimental conditions (Figure 2). In an ancillary experiment, animals were subjected to 24-hour water deprivation. A significant increase in immunoreactive AGT was observed in proximal tubules after water deprivation (Figure 3A through 3C, P=0.003). Using expression analysis, we observed a similar trend that did not reach statistical significance (Figure 3D). We speculate that the lack of statistical significance might be the result of our small sample size (n=3). These data suggest that sodium restriction and volume depletion exert different effects on AGT expression in PTs. In conditions in which only dietary sodium is manipulated, changes in urinary excretion of AGT that do not correlate with variation in synthesis may reflect the dependence of reabsorption of the protein in PT on net tubular flow in this nephron segment. Our previous observation of an acute increase in urinary u-AGT concentration when animals are switched to a low sodium diet may have resulted from dehydration concurrent with the 24-hour food deprivation that preceded this dietary change.1

Urinary renin excretion has been examined under conditions of either dietary sodium restriction or sodium and volume depletion induced by loop diuretics. The observation that urinary renin parallels26,27 or is dissociated from a rise in plasma renin28 may depend on the relative contributions, in the experimental maneuver applied, of sodium restriction on one hand and sodium or volume depletion on the other. In our experiments, manipulations of dietary sodium in solid food while maintaining free access to water led only to moderate increases in PRC that did not impact on urinary excretion. Volume depletion, by contrast, may lead to enhanced urinary renin through either one or both of 2 mechanisms: (1) the marked rise in systemic renin, leading to increased renin in the ultrafiltrate, may saturate the reabsorbing capacity of PT24,29; and (2) activation of CNT renin may also contribute to enhanced urinary renin excretion. In dehydrated animals, we have observed significant elevation of CNT renin (Figure 3E through 3G, P=0.02). As in the case of AGT, decreased tubular flow under conditions of dietary sodium restriction may lead to increased fractional reabsorption of filtered renin. The modest rise in systemic renin induced by euvelomic sodium restriction may not have been sufficient to compensate for or to saturate this transport.

In conclusion, urinary excretion of AGT and renin reflect the interplay of systemic and tubular changes that affect both secretion and reabsorption of the proteins. Although sodium restriction and volume depletion exert different effects on synthesis of the 2 proteins, excretion in final urine may predominantly reflect changes in net tubular flow. Recent work and literature reviewed therein30 supports the significance of sodium reabsorption in PTs as an independent determinant of blood pressure response to sodium in human hypertension. Our observations on AGT excretion as a function of dietary sodium suggests that this parameter may correlate with net sodium and water reabsorption in this segment and may serve as indicator of PT function.

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