Enhancement of Intrarenal Angiotensinogen in Dahl Salt-Sensitive Rats on High Salt Diet

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Abstract—This study was performed to examine whether there is an inappropriate regulation of intrarenal angiotensinogen in Dahl-salt sensitive rats (DS) fed a high salt diet (HS). Dahl salt-resistant rats (DR) and DS were maintained on HS (8% NaCl) or low salt diet (LS, 0.3% NaCl) for 4 weeks. Systolic blood pressure (SBP), measured by tail-cuff plethysmography, was unaltered in DR (DR+HS, 127±3 mm Hg, n=5; DR+LS, 126±3, n=5); however, SBP was significantly increased in DS+HS (208±7, n=9) compared with DS+LS (134±2, n=5). HS suppressed plasma renin activity in both strains (0.7±0.2 ng of angiotensin I/mL per hour in DS+HS, 3.1±0.5 in DS+LS, 0.8±0.2 in DR+HS, 5.1±0.7 in DR+LS). Plasma angiotensinogen levels, measured by Western blot analysis, were also suppressed by HS in both strains (36 919±2170 integrated densitometric unit in DS+HS, 53 028±2752 in DS+LS, 44 722±1721 in DR+HS, 55 782±3785 in DR+LS). However, kidney angiotensinogen levels were significantly increased in DS+HS (75 850±4171, integrated densitometric unit) compared with DS+LS (47 232±3470), DR+HS (44 748±8236), and DR+LS (42 504±4052). Urinary excretion of angiotensinogen, measured by radioimmunooassay of angiotensin I after incubation with excess renin, had a similar profile. Urinary excretion of angiotensinogen was significantly increased in DS+HS (2958±531 pmol/d) compared with DS+LS (56±4), DR+HS (31±12), and DR+LS (21±7). These data indicate that intrarenal angiotensinogen is enhanced in DS+HS, which is reflected by the increased urinary excretion of angiotensinogen. The results suggest that DS on HS have an inappropriate augmentation of intrarenal angiotensinogen, which may contribute to impaired sodium excretion during a high salt diet and the development of hypertension in this strain. (Hypertension. 2003;41:592-597.)

Key Words: angiotensin II ■ angiotensinogen ■ rats, Dahl ■ kidney ■ urine ■ hypertension, sodium-dependent ■ Western blot

Various epidemiological studies have showed a correlation of dietary salt intake with the prevalence and progression of hypertension.1 Although the degree of salt sensitivity is variable, some individuals are particularly prone to have hypertension in response to an increased dietary salt intake. Subjects with essential hypertension have a higher frequency of salt sensitivity than is found in the normotensive population.2 There is some evidence that salt sensitivity is associated with low plasma renin activity (PRA) and impaired renal sodium excretion. However, the mechanisms underlying this phenomenon are poorly understood.3 Dahl salt-sensitive (DS) rats have been used as a model of human salt-sensitive hypertension because salt loading exaggerates the development of hypertension in strains that are genetically predisposed to hypertension.4 Mature DS rats are reported to have low PRA levels, which has been interpreted as being indicative of an overall suppression of the renin-angiotensin system (RAS); however, few studies of angiotensinogen (AGT) have been carried out in these rats. Although generally considered to be characterized by a low activity of circulating RAS, recent studies indicate that treatment with angiotensin (Ang) I-converting enzyme inhibitors or Ang II type I receptor antagonists reduces cardiac and/or renal dysfunction in DS rat fed a high salt diet (HS).5-10 These findings suggest that the local RAS may be inappropriately activated and contribute to the development of hypertension in this animal model.

Previous studies have demonstrated that Ang II infusions to normal rats result in paradoxical increases in renal expression of AGT mRNA11,12 and protein.13 Furthermore, urinary excretion of AGT was significantly increased in Ang II–infused rats, which was associated with an enhancement of intrarenal Ang II levels.14 These results indicate that intrarenal AGT levels are not necessarily associated with increased plasma or renal renin levels. However, the extent to which this may occur in DS rats has not been determined. Therefore, this study was performed to determine if there is an inappropriate regulation of intrarenal AGT in DS rats fed HS and if enhanced
intragranal AGT in DS rats with HS is reflected by increased urinary excretion of AGT.

Methods

Preparation of Animals and Samples
The experimental protocol was approved by the Animal Care and Use Committees at Tulane University and Kagawa Medical University. Male Dahl salt-resistant (DR) and DS rats (200 to 220 g, Seac Yoshitomi, Fukuoka, Japan) were housed in metabolic cages and maintained in a temperature-controlled room regulated on a 12-hour light/dark cycle with free access to water. DR and DS rats were selected at random to receive commercially available rat chow containing HS (8% sodium chloride, Oriental Yeast) or low salt (LS, 0.6% sodium chloride, Oriental Yeast) for 4 weeks. Four groups of rats were studied: DR+HS (n=5), DR+LS (n=5), DS+HS (n=9), and DS+LS (n=5), respectively.

Systolic blood pressure (BP) was measured every week in conscious rats by tail-cuff plethysmography. Twenty-four-hour urine samples were collected 1 day before harvesting tissues in 0.6 mL distilled water containing 50 µg pepstatin A, 10 mg sodium azide, 300 nmol enalaprilat, and 125 µmol EDTA as previously reported.14 Urine samples were centrifuged and supernatant was separated and stored at −20°C until assayed for AGT concentrations. Blood and kidney samples were harvested at the end of the fourth week. After decapsulation, trunk blood was collected into chilled tubes with protease inhibitors. Plasma was separated and stored at −20°C until assayed for PRA, Ang II levels, and AGT as previously described.12,13 Immediately after removal, one of the kidneys was homogenized in cold methanol and processed for measurement of renal Ang II, as previously described.13 The contralateral kidneys were snap-frozen in liquid nitrogen and stored at −80°C until they were assayed.

Western Blot Analysis of Kidney Samples
Since renal AGT protein is located primarily in proximal tubular cells,12 the inner medulla was removed from kidney samples. Proteins were routinely extracted from kidney cortical samples after homogenization with protease inhibitors and quantified as previously described in detail.13 Kidney (10 µg protein) samples were electrophoretically separated by precast NuPAGE 4% to 12% Bis-Tris gel (Invitrogen). The proteins from the gel were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad) with the use of XCell II Mini-Cell (Invitrogen). The membrane was incubated with the primary antibody12–14 (sheep anti-rat AGT, 1:5000, provided by Dr Sernia, University of Queensland, Australia) for 2 hours, washed, incubated with the secondary antibody conjugated to horseradish peroxidase (donkey anti-sheep IgG, 1:5000, Amersham Pharmacia Biotech), and the blots were exposed to x-ray film (Hyperfilm-ECL; Amersham Pharmacia Biotech). Autoradiography films were scanned by means of Digital Imaging and Analysis Systems (Alpha Innotech) to obtain integrated densitometric values (IDV). To check for equal loading, membranes were reprobed with an antibody against β-actin. After first detection, membranes were incubated with Western Re-Probe solution (Geno Technology) for 1 hour. Membranes then were incubated with a monoclonal primary antibody (mouse against human/rat β-actin, 1:20000, Sigma) for 1 hour, washed, incubated with the secondary antibody conjugated to horseradish peroxidase (anti-mouse IgG, 1:5000, Amersham Pharmacia Biotech) for 1 hour, and washed. Detection and evaluation were done as described above.

To check the linearity between the IDV and the amount of kidney samples loaded, serial dilutions (20, 10, 5, 2.5, and 1.25 µg of kidney protein) of DS+LS and DS+HS samples were probed as described above. As depicted in Figure 1, the IDV and the amount of kidney samples preserve the linearity along the range of 1.25 to 10 µg. Therefore, we believe that our method for kidney angiotensinogen Western blot is reliable.

Measurements of Urine Samples
Urine excretion of AGT was evaluated by radioimmunoassay (RIA) of generated Ang I as previously reported.14 RIA was performed to measure Ang I levels generated after incubation with an excess amount of exogenous porcine renin (Sigma). RIA of Ang I was performed with a commercially available kit (Incstar) by using the directions and reagents supplied by the manufacturer. Urinary concentration of protein was measured by a colorimetric assay with the use of a commercially available kit (Bio-Rad).

Statistical Analysis
Statistical analysis was performed by a 1-way ANOVA with a post hoc Scheffé F test. All data are presented as mean±SEM. A value of P<0.05 was considered significant.

Results

Temporal Profile of Systolic BP
Temporal profile of systolic BP was depicted in Figure 2A. Systolic BP levels were the same among the 4 groups at the beginning of the protocol (128±4 mm Hg in DS+HS, 124±3 in DS+LS, 124±3 in DR+HS, 121±4 in DR+LS). Systolic BP was unaltered during the protocol in DR+LS (126±3 at 4 weeks), DR+HS (127±3 at 4 weeks), and DS+LS (134±2 at 4 weeks) groups; however, the HS diet led to progressive increases in systolic BP in DS rats over the 4-week period to an average of 208±7 mm Hg.

Plasma Measurements
The HS diet significantly suppressed PRA in both strains (0.7±0.2 ng of Ang I/mL per hour in DS+HS, 3.1±0.5 in
DS+LS, 0.8±0.2 in DR+HS, and 5.1±0.7 in DR+LS). Plasma Ang II levels were suppressed by HS in both strains (21±3 fmol/mL in DS+HS, 37±6 in DS+LS, 34±3 in DR+HS, and 54±6 in DR+LS). Plasma AGT levels (Figure 2B), measured by Western blot analysis, were also suppressed by the HS diet in both strains (36 919±2170 IDV in DS+HS, 53 028±2752 in DS+LS, 44 722±1721 in DR+HS, and 55 782±3785 in DR+LS). When IDV were normalized by the average of the IDV of DR+LS rats, this suppression was preserved (Figure 2C 0.66±0.04 in DS+HS, 0.95±0.05 in DS+LS, 0.80±0.03 in DR+HS, and 1.00±0.07 in DR+LS).

It has previously been shown in Sprague-Dawley rats that plasma and liver samples have 2 forms of AGT; a 64-kDa, highly glycosylated form and a 52-kDa, slightly glycosylated form, whereas kidney and urine samples primarily have the 52-kDa AGT.12 In the present study, we observed that plasma samples of Dahl rats presented a very faint band at 64-kDa, which is unrecognizable in Figure 2B. We examined other plasma samples of Dahl rats, and the results consistently show that plasma AGT in Dahl rats is primarily of the slightly glycosylated form. We cannot address in the present study whether the difference of magnitude of glycosylated AGT is involved in the progress of hypertension in Dahl strain.

Kidney Measurements
Kidney Ang II levels were suppressed by HS in DR strain (180±14 fmol/g in DR+HS and 314±40 in DR+LS). However, kidney Ang II levels were not altered by HS in DS strain (222±24 in DS+HS and 180±14 in DS+LS).

HS diet did not alter the kidney AGT levels (Figures 3A and 3B) evaluated by Western blot analysis in DR rats; however, the HS diet significantly increased kidney AGT levels in DS+HS compared with DS+LS, DR+HS, and DR+LS groups (75 850±4171 IDV in DS+HS, 47 232±3470 in DS+LS, 44 748±8236 in DR+HS, and 42 504±4052 in DR+LS).

As a control to check for equal loading, membranes were reprobed with an antibody against β-actin (Figures 3C and 3D). IDV for β-actin were unaltered among the 4 groups (57 121±791 IDV in DS+HS, 56 058±2976 in DS+LS, 56 584±959 in DR+HS, and 56 107±486 in DR+LS).

The ratio of IDV for AGT to IDV for β-actin had a similar profile. The ratio was significantly increased in DS+HS (1.32±0.06, ratio to β-actin) compared with DS+LS (0.84±0.05), DR+HS (0.78±0.13), and DR+LS (0.76±0.07) groups. Only the DS+HS rats showed a distinct increase in kidney AGT level.

Urinary Excretions of AGT and Protein
Urinary excretion rates of AGT evaluated by RIA of Ang I production with excess renin are shown in Figure 4A. Urinary excretion of AGT was significantly increased in DS+HS (2958±531 pmol/d) compared with DS+LS (56±4), DR+HS (31±12), and DR+LS (21±7) groups. Urinary excretion of protein was also significantly increased in DS+HS (179±18 mg/d) compared with DS+LS (31±1), DR+HS (22±2), and DR+LS (11±1) groups. The ratio of urinary excretion of AGT to urinary excretion of protein was also significantly increased in DS+HS (18.1±2.0 pmol/mg) compared with DS+LS (1.8±0.1), DR+HS (1.7±0.6), and DR+LS (1.6±0.6) groups.

Correlation of Urinary Excretion Rates of AGT
Correlation studies were done to determine if there was an association between the urinary excretion rates of AGT and the systolic BP (Figure 4B) or kidney AGT levels (Figure 4C). Urinary AGT excretion rates showed a high degree of correlation with systolic BP (r=0.92) and kidney AGT levels (r=0.77).

Discussion
Previous studies have demonstrated that chronic Ang II infusion results in significant increase in renal expression of AGT protein as well as AGT mRNA. Furthermore, urinary excretion of AGT was significantly increased in
association with enhancement of intrarenal AGT and Ang II levels. These results prompted us to perform further experiments to evaluate the correlation between urinary excretion rate of AGT and intrarenal AGT levels in other animal models of hypertension.

It is well known that adult DS rats have low PRA levels, but studies have not addressed intrarenal Ang II and AGT regulation in DS rats because it has been assumed that the RAS is suppressed in DS rats fed a HS diet. However, recent studies suggest that treatment with Ang I–converting enzyme inhibitors or Ang II type I receptor antagonists reduces cardiac and/or renal dysfunction in DS rats made hypertensive by a HS diet. Furthermore, Nakaya et al reported that prepubertal treatment with an Ang II type I receptor antagonist causes partial attenuation of hypertension and ameliorates the renal damage in adult DS rats fed HS. These data suggest that the local RAS may contribute to development of hypertension and renal dysfunction in this model. As expected, systolic BP was increased by HS in DS rats but was unchanged in DR rats. The HS diet suppressed PRA in both strains, and PRA was significantly lower in the DS/H11001 LS group compared with the DR/H11001 LS group, as previously shown. The HS diet has been shown to suppress plasma and intrarenal expression of AGT in Sprague-Dawley and Wistar-Kyoto rats. Similarly, in the present study, plasma Ang II and AGT levels were also suppressed by HS diet in both DR and DS strains. However, in association with the HS-induced increase in systolic BP in DS rats, we observed a paradoxical enhancement of kidney AGT levels in DS rats but not in DR rats. Furthermore, urinary AGT was significantly increased in DS+HS compared with DS+LS, DR+HS, and DR+LS rats. Interestingly, urinary AGT excretion rates showed a high degree of correlation with systolic BP and kidney AGT levels. Thus, the present study provides evidence that intrarenal AGT levels are inappropriately enhanced by HS in DS rats and are associated with increases in urinary AGT excretion rates. This paradoxical enhancement may be an important factor that helps to explain the salt-sensitive characteristic of DS rats.

As previously shown, the HS diet induces progressive renal damage in DS rats, and it is possible that renal damage is involved in the enhanced urinary excretion of AGT of DS+HS rats in the present study. This is supported by the increased urinary excretion rates for protein observed in the DS+HS group. However, the ratio of urinary AGT to urinary protein is still significantly increased in DS+HS group, indicating a much greater increase in AGT than protein in the urine. Therefore, the enhancement of urinary AGT cannot be explained just by the increased protein excretion occurring in DS+HS animals.

Kidney Ang II levels were suppressed by HS in the DR strain, along with plasma Ang II and AGT levels. In contrast, kidney Ang II levels in DS strain tended to increase by HS, but this change is not statistically significant. However, it is important to emphasize that kidney Ang II levels were not suppressed by HS in the DS strain. Furthermore, it was recently reported that the expression of Ang II type I receptor is not suppressed by HS in the DS strain. The combination of maintained kidney Ang II levels and Ang II type I receptor expression and enhanced kidney AGT levels by HS in DS
may contribute to an enhanced activity of the renal RAS, thus leading to the development and maintenance of hypertension in this strain. Because the level of AGT is close to the $K_M$ value for renin,20,21 it is possible that AGT levels can control the activity of the RAS, and its upregulation may lead to elevated Ang II levels and increases in BP. Moreover, recent evidence has demonstrated the important role of AGT in the development of hypertension in human subjects22 and in gene-targeted animal models.23,24 Therefore, it is possible that elevated AGT levels serve a contributory factor mediating hypertension in DS rats fed a HS. These results are consistent with the observations that the pressure-natriuretic response is attenuated, as might be expected with reductions of intrarenal Ang II levels.35 The increases in intrarenal Ang II may be linked to the enhanced excretion of urinary AGT, which may contribute to the development and maintenance of hypertension.

In our study, kidney AGT levels in DS rats were significantly enhanced by HS; however, kidney Ang II levels in DS rats were not statistically increased by HS, although an upward trend was noted. One possible explanation for the apparent discrepancy between kidney AGT and Ang II levels may be linked to the enhanced excretion of urinary AGT, which is thought to reflect primarily AGT in the tubular fluid compartment. Most of the intrarenal AGT mRNA and protein are localized in proximal tubular cells.12,30,31 Once AGT is synthesized in and secreted from proximal tubular cells, AGT can be metabolized to Ang II by renin or renin-like enzymes32 and Ang I–converting enzyme33,34 present on proximal and/or distal tubular cells. However, there are abundant angiotensinases also present that may prevent substantial accumulation of Ang II.35 The increases in intrarenal Ang II may be restricted to the tubular compartment and therefore kidney Ang II levels may fail to show significant increases despite the enhanced kidney AGT and urinary AGT levels in this study.

It appears in the present study that the increase in renal AGT protein in DS + HS rats was relatively small compared with the huge increase in urinary excretion of AGT. As previously described,12,30,31 AGT mRNA and its protein are mainly synthesized in the proximal tubular cells predominantly on the apical side. Once AGT protein is synthesized, it is easily secreted into the lumen and secreted AGT traverses along nephrons and is finally excreted to urine. Under normal conditions, only a tiny fraction of the secreted AGT spills into the distal nephron and urine. Therefore, the measured urinary AGT is small. However, when the renal production of AGT is stimulated so that there is greater spillover into the distal nephron and urine, a small absolute increase in the amount that finally reaches the urine results in a large fractional increase because the basal levels are so low.

**Perspectives**

This study was performed to examine if there is an inappropriate regulation of intrarenal AGT in DS rats fed HS. DR and DS were maintained on HS or LS. SBP was unaltered in DR; however, SBP was significantly increased in DS + HS compared with DS + LS. HS suppressed PRA in both strains. Plasma AGT levels were also suppressed by HS in both strains. However, kidney AGT levels were significantly increased in DS + HS compared with DS + LS, DR + HS, and DR + LS. Urinary excretion of AGT was significantly increased in DS + HS compared with DS + LS, DR + HS, and DR + LS. These data indicate that the hypertension that resulted when DS rats were fed the HS diet was associated with increases in intrarenal AGT levels and urinary excretion rates of AGT. These paradoxical responses were not observed in DR rats fed the HS diet. The results suggest that DS rats on HS have an inappropriate augmentation of intrarenal AGT, which may contribute to the development of hypertension in this strain. It is possible that one hallmark of salt sensitivity in both animal
models and susceptible human subjects is an inability to appropriately suppress intrarenal RAS in response to a high salt diet. Together with our previous findings showing that Ang II infusions increased renal AGT mRNA and protein levels, the data support the hypothesis that urinary excretion of AGT provides a specific index of intrarenal Ang II production in Ang II–dependent hypertension.

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