AT₁ receptor-mediated augmentation of angiotensinogen, oxidative stress, and inflammation in ANG II-salt hypertension

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The enhancement of intrarenal angiotensin II (ANG II) content contributing to hypertension and kidney damage is supported by the presence of all components of the renin-angiotensin system (RAS) in the kidney (27–29). During ANG II-dependent hypertension, intrarenal RAS activation is characterized by increased tissue levels of ANG II due to an augmented ANG II type 1 receptor (AT₁R) binding with concomitant internalization of circulating ANG II (5, 40) and by de novo formation of ANG II (30). In response to chronic ANG II infusions, there are 1) enhancement of angiotensinogen (AGT) synthesis and secretion by the proximal tubule cells (12), 2) increased urinary AGT (uAGT) excretion (13, 14), 3) stimulation of renin synthesis and secretion by the principal cells of the connecting tubules and collecting ducts (31–33), and 4) increased angiotensin-converting enzyme (ACE) activity along the nephron (1, 17, 33), which support further ANG II generation in distal nephron segments (31, 33).

The association of high salt (HS) consumption with cardiovascular and renal diseases including hypertension is well recognized. However, the mechanisms by which HS is translated into greater hypertension and renal injury remain unclear. While HS suppresses the RAS in normotensive individuals (2, 9), excessive salt intake exerts deleterious effects causing renal injury in hypertensive subjects through pressure-independent mechanisms (22, 24). It has been suggested that sustained RAS activity is involved in mediating the adverse effects of salt, since RAS blockade prevents or ameliorates salt-induced renal injury (44–46). Susic et al. (45) demonstrated that RAS activity in spontaneously hypertensive rats (SHR) was not suppressed or even augmented after 4 wk of salt loading, indicating that maintained intrarenal RAS combined with a HS diet contributes to greater renal damage in SHR (45). The increased RAS can act synergistically with reactive oxidative species (ROS), leading to salt-sensitive hypertension (2). In normal conditions, a low level of oxidative stress is maintained by the balance between the production and the degradation of ROS such as superoxide (O₂⁻). Reactive O₂⁻ is rapidly reduced by the enzyme superoxide dismutase or reacts with nitric oxide (NO); however, when there is enhanced O₂⁻ activity in the kidney, salt and water retention may occur (18, 23). In ANG II-infused rats and mice, augmented O₂⁻ is considered a key determinant in the development of salt-sensitive hypertension (19, 36). Conversely, in salt-sensitive hypertension ROS-dependent enhancement of AGT may be involved in the development and progression of renal injury (15, 26). Saeed et al. (37) showed that ANG II-infused rats on a HS diet (ANG II-salt hypertension) developed impairment of dynamic renal blood flow autoregulation, which was attenuated by tempol (37). However, the unique effect of HS on the pathogenesis of ANG II-salt hypertension has not been established.

The aim of the present study was to determine the consequences of HS intake alone, chronic ANG II infusion alone, and ANG II and HS on the stimulation of uAGT, renal oxidative stress, and kidney injury. Because the Sprague-Dawley rat is a salt-resistant model, we used an aggressive HS...
diet (8% NaCl) in the presence and absence of a chronic infusion of ANG II (80 ng/min). Since the HS diet did not alter systolic blood pressure (SBP) in normal rats, we were able to discriminate the pressor component mediated by ANG II and delineate a pressure-independent effect of HS diet on uAGT, ROS, and kidney tissue injury responses.

METHODS

Animals, experimental protocols, and samples handling. All animal procedures were approved by the Tulane University Animal Care and Use Committee. Twenty-five male Sprague-Dawley rats (175–200 g; Charles River Laboratories, Wilmington, MA) were randomly divided into five groups: 1) NS rats were fed a normal-salt diet (0.3% NaCl; diet TD 99414; Harlan-Teklad, Madison, WI) and sham operated; 2) ANG II rats were fed a NS diet and received ANG II (80 ng/min sc via minipumps for 14 days; Sigma, St Louis, MO); 3) HS rats were fed a high-salt diet (8% NaCl; diet TD 92012; Harlan-Teklad) and sham operation; 4) HS+ANG II rats were fed a HS diet and had ANG II infusion; and 5) HS+ANG II+ARB rats were fed a HS diet, infused with ANG II, and an AT1 receptor blocker [ARB; losartan (30 mg/kg) or candesartan (25 mg/kg)] was added to the drinking water for 14 days.

SBP was monitored by tail-cuff plethysmography (ITC Instruments, Woodland Hills, CA) 1 day before and 3, 7, and 11 days following sham operation or minipump implantation, as previously described (14, 33). One day after the SBP measurement, the body weight was taken and the rats were housed in individual metabolic cages in a temperature-controlled room regulated on a 12:12-h light-dark cycle with free access to chow and water. Twenty-four-hour urine samples were collected and centrifuged at 3,000 g for 5 min, and the supernatants were separated and stored at −20°C until assayed for total protein and urinary AGT (uAGT), as previously described (13). Rats were euthanized by conscious decapitation on day 14. Trunk blood samples were collected in chilled tubes containing 5 mmol/l EDTA, which were centrifuged at 2,000 g for 30 min at 4°C for plasma fraction separation to measure plasma renin activity (PRA) (31). Immediately following kidney harvesting, the poles of the right kidney were sectioned and immediately immersed in optimum cutting temperature compound (OCT, Tissue-Tek, Sakura Finetek, Torrance, CA) and used for O2 determination. Right kidney samples were snap frozen in liquid nitrogen and used for Western blotting and NADPH oxidase activity determinations. The left kidneys were perfused with cold PBS followed by 4% paraformaldehyde formaldehyde and fixation with formalin to be used for histological purposes.

ROS in the kidney cortex. The oxidative fluorescent dye dihydroethidium (DHE; Invitrogen, Carlsbad, CA) was used to detect ROS in kidney poles from NS, ANG II, HS, ANG II+HS, and ANG II+HS+ARB rats, as previously described (39, 41). Ten-micrometer cryosections from kidney poles, were stained with the superoxide-anion-selective fluorescent dye dihydroethidium (DHE; Invitrogen, Carlsbad, CA) and used for O2 determination. Right kidney samples were snap frozen in liquid nitrogen and used for Western blotting and NADPH oxidase activity determinations. The left kidneys were perfused with cold PBS followed by 4% paraformaldehyde formaldehyde and fixation with formalin to be used for histological purposes.

Histology and immunohistochemistry of kidney injury markers. Kidney sections (3 μm), paraffin embedded, were stained with Masson’s trichrome (Mass Histology, Worcester, MA) for assessment of glomerular and interstitial fibrosis and 2) periodic acid-Schiff (PAS) for the determination of glomerular lesions. Morphometry of glomeruli and the extent of the interstitial fibrotic-positive area were evaluated quantitatively using computerized image analysis, as described (7, 26). Mesangial expansion was quantified in 20 glomeruli per kidney per rat by dividing the PAS-positive stained area by the total area of the glomerulus and expressed as means ± SE of the 20 measurements in percentage values, as previously described (7, 26). For the immunoexpression of proliferating cell nuclear antigen (PCNA) and CD68, markers of cell proliferation and macrophage infiltration, respectively, monoclonal antibodies for PCNA and CD68 were used at 1:1,000 dilutions (7, 26), respectively. Data were expressed as fold-changes in the number of nuclei in proliferation (for PCNA) or number of macrophages (for CD68). All the stains were performed using Molecular Imager VersaDoc Imaging Systems (Bio-Rad, Hercules, CA). Densitometric determinations, using Image J Software (National Institutes of Health), were calculated as the ratio between the n-Tyr residues bands ranging from 120 to 12 kDa and GAPDH expression.

Statistical analysis. Results are expressed as means ± SE. The data were compared by one-way analysis of variance taking into account the treatment in the experimental groups. The significance of the difference was evaluated using the multiple comparative Bonferroni test. The values of all parameters were considered significantly different at P < 0.05.

RESULTS

Functional responses in ANG II-salt hypertensive rats. The combination of ANG II+HS slowed the normal body weight gain to the same extent as that observed in chronic ANG II-infused rats, while a HS diet alone did not (NS: 336 ± 8; HS: 323 ± 7; ANG II 286 ± 9 and ANG II+HS 242 ± 12 g; P < 0.05). In addition, ANG II+HS rats had increased Na+ excretion compared with the HS alone group (HS: 28 ± 2 and ANG II+HS: 49 ± 5 meq·24 h−1·100 g−1·24 h−1; P < 0.05); in both, Na+ excretion was much higher than in NS rats (0.82 ± 0.092
meq·24 h⁻¹·100 g⁻¹; P < 0.05). No differences in food intake were detected. The ANG II+HS rats demonstrated severe lethargy, assumption of a hunched posture, and piloerection, which along with a salt-losing condition are manifestations of malignant hypertension in the rat (25, 48).

Table 1. Functional responses in ANG II-salt hypertensive rats

<table>
<thead>
<tr>
<th>Rat Group</th>
<th>SBP, mmHg</th>
<th>PRA, ng ANG I·ml⁻¹·h⁻¹</th>
<th>Proteinuria, mg/day</th>
<th>uAGT, ng/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS</td>
<td>115 ± 2</td>
<td>6 ± 2</td>
<td>23 ± 3</td>
<td>40 ± 7</td>
</tr>
<tr>
<td>ANG II</td>
<td>175 ± 10*</td>
<td>0.3 ± 0.2*</td>
<td>46 ± 7*</td>
<td>1,109 ± 70*</td>
</tr>
<tr>
<td>HS</td>
<td>124 ± 4</td>
<td>0.1 ± 0.01*</td>
<td>21 ± 1</td>
<td>55 ± 9</td>
</tr>
<tr>
<td>ANG II+HS</td>
<td>221 ± 8†</td>
<td>0.2 ± 0.01*</td>
<td>127 ± 7†</td>
<td>7,200 ± 614†</td>
</tr>
<tr>
<td>ANG II+HS+ARB</td>
<td>116 ± 1</td>
<td>41 ± 12*</td>
<td>33 ± 3</td>
<td>55 ± 7</td>
</tr>
</tbody>
</table>

Values are means ± SE. SBP, systolic blood pressure; PRA, plasma renin activity; uAGT, urinary angiotensigen. Rat groups (n = 5) were divided as described in METHODS: normal salt intake (NS); chronic ANG II infusion (ANG II); high salt intake (HS); chronic ANG II infusion associated with a HS diet (ANG II+HS), and chronic ANG II infusion associated with a HS diet and treatment with an ANG II type 1 receptor blocker (ANG II+HS+ARB). *P < 0.05 vs. NS, †P < 0.05 vs. ANG II.

Systolic blood pressures were followed throughout the experimental period, and the data at the end of the study are shown in Table 1. Although a HS diet alone did not alter SBP, it caused a further increase compared with ANG II infusion alone. This effect was prevented by ARB treatment. Protein-
uria levels followed the same profile observed for SBP with a marked augmentation of proteinuria caused by combining HS with ANG II infusion (Table 1). Plasma renin activity was suppressed during ANG II and HS intake but was markedly increased by ARB treatment (Table 1).

Although HS alone did not change the uAGT levels, the combination of HS with chronic ANG II infusion markedly exacerbated the increases in uAGT by almost sevenfold compared with ANG II infusion alone (Table 1). This effect was also prevented by ARB treatment.

ROS generation in kidney cortex of chronically ANG II-infused SD rats subjected to a HS diet. Figure 1 shows DHE fluorescence as an indication of ROS production [superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), or hydroxyl radical (OH$^-$)] in the rat kidney sections. ANG II and ANG II+HS rats exhibited increased DHE fluorescence from 17 ± 2.8 (NS) to 45 ± 2.0 and 46 ± 1.8 arbitrary units ($P < 0.001$), respectively; while ARB treatment prevented ROS formation. In contrast, HS rats showed a decrease in DHE fluorescence compared with the NS rats (7 ± 1.3 arbitrary units; $P < 0.01$). In contrast, NADPH oxidase activity (Fig. 2A) was increased in HS rats and ANG II+HS rats with the activity increasing more than twofold in ANG II+HS rats [NS: 7.841 ± 1.101; ANG II: 12.738 ± 3.537; HS: 14.310 ± 2.720; ANG II+HS: 17.747 ± 1.519 relative light units (RLU)-mg$^{-1}$-min$^{-1}$; $P < 0.05$]. Since it has been established that HS intake increases NO formation (8, 20, 49), we postulated that NO contributes to the consumption of the O$_2^-$ by generating peroxynitrite (ONOO$^-$). Thus we used the detection of nitrotyrosine residues in the proteins of the kidney as a marker of local peroxynitrite levels (6, 20, 47). Interestingly, rats fed a HS diet alone exhibited the highest levels of peroxynitrite activity (Fig. 2B), thus explaining the reduced DHE fluorescence in the HS rats. Importantly, the combination of ANG II+HS did not manifest the increases in nitrotyrosine residues observed with HS alone. ARB treatment prevented increases in DHE fluorescence, NADPH oxidase activity, and nitrotyrosine residues.

A HS diet exacerbates renal interstitial and glomerular fibrosis, glomerular expansion, cell proliferation, and interstitial macrophage infiltration in ANG II-salt hypertensive rats. The renal fibrosis examined by Masson’s trichrome staining in glomeruli and in the tubulointerstitial areas (Fig. 3A) showed that in both cases, the combination of chronic ANG II infusion and HS intake exacerbated collagen deposition compared with the ANG II and HS rats (Fig. 3, B and C). However, HS alone caused significant increases in glomerular and interstitial fibrosis even though these rats were not hypertensive. ARB treatment prevented peroxynitrite in the glomeruli (Fig. 3B) but not in the interstitium (Fig. 3C).

Using PAS staining (Fig. 4), ANG II, HS, and ANG II+HS rats exhibited a pattern of diffuse mesangial expansion. Indeed, the quantification of positive PAS-stained areas showed that the two groups of rats subjected to a HS diet presented the highest degree of glomerular expansion and that this effect was partially prevented by ARB treatment (NS: 11 ± 0.5; ANG II: 32 ± 1.3; HS: 37 ± 0.9; ANG II+HS: 36 ± 0.9; ANG II+HS+ARB: 19 ± 0.8%; $P < 0.01$; Fig. 4F).

The changes in PCNA immunostaining indicated that a HS diet exacerbated the proliferation of the tubular epithelial cells stimulated by ANG II (Fig. 5); however, HS alone did not elicit an increase in PCNA immunostaining. ARB treatment prevented the increase caused by HS+ANG II (ANG II: 1.7 ± 0.09-fold; ANG II+HS: 2.7 ± 0.1-fold increase in nuclei in proliferation).

The extent of glomerular and tubulointerstitial macrophage infiltration was quantified by the presence of CD68-positive cells in glomeruli and in the tubulointerstitium (Fig. 6). Similar increases in the accumulation of CD68-positive cells in glomeruli in ANG II, HS, and ANG II+HS rats were observed. The infiltration of CD68-positive cells in the renal interstitium was observed in rats fed a HS diet and was exacerbated in the ANG II-salt hypertensive rats (Fig. 6B). Treatment with ARB completely prevented this effect (Fig. 6B).

**Discussion**

The present study demonstrates that a HS diet given to chronic ANG II-infused Sprague-Dawley rats for 2 wk exacerbates ANG II-dependent hypertension and leads to malignant hypertension, as reflected by the reduced body weight gain, severe lethargy, piloerection, and assumption of a hunched posture, along with increased Na$^+$ excretion and proteinuria.
ANG II has been shown to cause skeletal muscle wasting (cachexia) via an increase in muscle catabolism, associated with an increase in proteasome activity mediated, at least in part, by NADPH oxidase-derived ROS (39). Furthermore, ANG II infusion leads to decreases in body mass with decreases in retroperitoneal and epididymal fat but with no alterations in food consumption (34). The physical characteristics of the rats are similar to the ones described in the Cyp1a1-Ren2 rats, a model of ANG II-dependent malignant hypertension associated with increased PRA and high circulating and intrarenal ANG II levels (25, 48).

In the present study, ANG II-salt hypertensive rats also exhibited infiltration of CD68-positive cells in the kidney interstitium, sustained $\text{O}_2^-$ production, tissue injury, and exacerbation of urinary excretion of AGT. These responses are mediated by AT1 receptor activation since ARB treatment prevented or ameliorated the development of these changes. Although a HS diet alone caused increased NAPDH oxidase

Fig. 3. Representative photomicrographs of collagen deposition visualized by Masson’s trichrome stain in glomeruli ($\times$400) and tubulointerstitial areas ($\times$200; A). Twenty different microscopic fields per tissue section per animal ($n = 3$/group) were analyzed from paraffin kidney sections ($3 \mu m$). The extent of the interstitial fibrotic-positive area was evaluated quantitatively by automatic image analysis (B and C), which determined the area occupied by interstitial tissue staining positive in Masson’s trichrome-stained sections (blue). Values are means ± SE expressed as a percentage of the blue areas/field. $^*P < 0.01$ vs. NS. $^{**}P < 0.01$ vs. ANG II.
activity, \( \text{O}_2^- \) levels did not increase due to increased scavenging of the excess of \( \text{O}_2^- \), leading to increased peroxynitrite formation. The increased peroxynitrite formation was not apparent when HS was given to ANG II-infused rats, allowing accumulation of \( \text{O}_2^- \) levels and greater ROS activity. The increased \( \text{Na}^+ \) excretion likely reflects a pressure-natriuresis effort of the increased SBP. Since we did not observe an increase in SBP in the HS rats, we were able to discriminate the pressor component mediated by ANG II and delineate pressure-independent effects of HS on uAGT, ROS, and kidney tissue responses.

uAGT is a marker for intrarenal RAS activity, but not a primary consequence of the elevated arterial pressure or of hypertension-induced proteinuria (14, 16). The present data indicate that in the ANG II-salt hypertensive rats, uAGT is markedly elevated to much greater levels than those seen in ANG II-infused rats. In contrast, normal rats fed HS remained at basal uAGT excretion rates similar to those in NS rats. It has been demonstrated that HS alone suppresses systemic RAS and renal AGT mRNA expression (8, 10, 38, 43). However, during ANG II infusion, salt-sensitive hypertension develops and HS paradoxically stimulates intrarenal RAS activity, as reflected by exacerbation of the uAGT despite inhibition of PRA. Through augmentation of oxidative stress, HS synergizes with ANG II, culminating in much greater increases in uAGT and injury. Our data support the observations of Franco et al. (4) demonstrating that blood pressure is positively correlated with renal ANG II concentrations but not plasma ANG II. The combined effects of HS intake and the exacerbated intrarenal RAS activity are associated with increased SBP and proteinuria, which support previous findings (4, 38, 48).

Our data demonstrate that the HS either alone or in combination with ANG II increases \( \text{O}_2^- \) formation as demonstrated by increased NAPDH oxidase activity. However, with HS alone, there is also a marked increase in peroxynitrite formation that actually reduces the \( \text{O}_2^- \) levels due to increased NO formation, which may be critical in preventing increases in blood pressure and renal injury (8, 20, 49). When HS is

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**Fig. 4.** Representative photomicrographs of mesangial expansion by periodic acid-Schiff staining (PAS) of kidney sections (3 μm) from rats subjected to NS intake (A), chronic ANG II infusion (B), HS intake (C), chronic ANG II infusion associated with a HS diet (ANG II+HS; D), and chronic ANG II infusion associated with a HS diet and treatment with ARB (ANG II+HS+ARB; ×400; E). The extent of mesangial expansion was quantified in 20 different microscopic fields per tissue section per animal (n = 3/group) using an automatic image analyses of the area occupied by PAS-positive staining per glomerular area. Values are means ± SE expressed as a percentage of the blue areas/field. *P < 0.01 vs. NS. **P < 0.01 vs. ANG II.
combined with ANG II infusion, the increased $O_2^-$ formation is sustained or even slightly increased but the peroxynitrite generation is reduced, thus leading to the increase in $O_2^-$ levels, which contributes to the exacerbation of the uAGT and increased renal injury. A biphasic effect of peroxynitrite activity has been demonstrated where lower concentrations produce vasorelaxation and higher concentrations induce tissue injury (11, 35). The formation of peroxynitrite during HS intake may be a key molecular mechanism in preventing increases in SBP. However, the sustained formation of peroxynitrite in rats fed a HS diet may induce sulphhydryl oxidation, protein nitration, and lipid peroxidation, which can also contribute to kidney injury (3), as reflected by the glomerular expansion and kidney fibrosis even at SBP in the normal range. These findings further support the notion that pressure-independent factors stimulated by HS may predispose the kidneys to greater injury in response to ANG II infusions (49). HS independently stimulates transforming growth factor β1 (49) and other intracellular signaling pathways, leading to an enhanced response to ANG II. An enhancement in $O_2^-$ generation may also contribute to the early developmental phase of ANG II-induced salt sensitive hypertension (20). Therefore, the exacerbation in kidney damage exhibited by the ANG II infused rats fed a HS diet could be explained, at least in part, by the interaction between the RAS and ROS. In the present study, the ANG II+HS rats, had marked increases in uAGT excretion rats which paralleled the increases in $O_2^-$ in renal tissue. The critical involvement of AT1R in mediating this impairment is supported by the findings that ARB treatment prevented the increases in NAPDH oxidase activity, reducing the $O_2^-$ levels to a normal range. Since the ARB treatment was initiated in parallel with the ANG II infusion and HS diet, we cannot determine whether the increased formation of ROS is a direct effect or due to a reduction of the RAS. However, ARB treatment was able to block the vicious cycle between RAS and ROS. Although the HS diet did not alter SBP, it was able to cause mesangial expansion and kidney fibrosis, without eliciting cell proliferation. The combination of HS with chronic ANG II
infusions associated with higher levels of SBP clearly led to marked exacerbation of the ANG II effects on mesangial expansion, kidney fibrosis, and tubular epithelial cell proliferation. The fact that ARB treatment was able to prevent fibrosis in the glomeruli but not in the interstitium suggests that despite potential protection of glomeruli by ARB, the lesions in the interstitium may be sustained and progress to chronic kidney disease. Furthermore, an interesting finding was the observation of the infiltration of inflammatory CD68-positive cells into the renal interstitium. Growing evidence demonstrates that the immune system contributes to the mechanism of exacerbation of hypertension in ANG II-infused rats subjected to a HS diet (4). Franco et al. (4), using Sprague-Dawley rats infused with ANG II for 2 wk and then given HS, demonstrated an increase in inflammatory cells in the tubulointerstitial areas. We demonstrated that the combination of a HS diet and ANG II infusion leads to the infiltration of inflammatory cells, although no infiltration was observed when HS diet or ANG II infusion was administered alone. The detection of macrophages in the renal interstitium of the ANG II-salt hypertensive rats most likely contributed to greater activation of intrarenal RAS during HS intake, leading to further accumulation of O$_2^-$.

In summary, the results from this study demonstrate increased O$_2^-$ formation with HS alone, ANG II alone, or ANG
II in combination with HS. However, with HS alone there is also a marked increase in peroxynitrite formation. In contrast, ANG II infusion in combination with a HS diet reduced the generation of peroxynitrite but NADPH oxidase activity was maintained or further increased, shifting the balance to O$_2^-$ accumulation. This event explains the mechanism by which HS exacerbates the deleterious effects of ANG II. In essence, HS alone causes only modest changes but predisposes the kidney to greater injury when associated with chronic ANG II infusions. Thus in ANG II-salt hypertension the inappropriate intrarenal activation of the RAS, as demonstrated by the exacerbation of the uAGT, and increased ROS by the increases in O$_2^-$ in the renal tissue, synergize to cause greater glomerular and interstitial fibrosis and macrophage infiltration to the renal interstitium, leading to increased kidney injury.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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31. Prieto-Carrasquero MC, Botros FT, Kobori H, Navar LG.

30. F94 AT1R-MEDIATED RAS/ROS ACTIVITY IN ANG II-SALT HYPERTENSION


28. F94 AT1R-MEDIATED RAS/ROS ACTIVITY IN ANG II-SALT HYPERTENSION


