Angiotensin II- and Salt-Induced Kidney Injury through Rac1-Mediated Mineralocorticoid Receptor Activation

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ABSTRACT

Experiments with hyperaldosteronemic animals suggest that, despite lowering plasma aldosterone, salt worsens renal injury by paradoxical activation of the mineralocorticoid receptor (MR). Salt and aldosterone synergistically contribute to renal impairment through Rac1-mediated activation of the MR, but whether angiotensin II also promotes renal injury through this mechanism is unknown. Here, we placed angiotensin II-overproducing double transgenic Tsukuba hypertensive mice on a low- or high-salt intake for 6 weeks and treated some animals with adrenalectomy, the MR antagonist eplerenone, the Rac inhibitor EHT1864, or hydralazine. High-salt intake, but not low-salt intake, led to hypertension and prominent kidney injury. Adrenalectomy prevented angiotensin II/salt-induced nephropathy in mice receiving high-salt intake, which was recapitulated by aldosterone supplementation, suggesting the involvement of aldosterone/MR signaling. Plasma aldosterone levels, however, were lower in high- than low-salt conditions. Instead, angiotensin II/salt-evoked MR activation associated with Rac1 activation and was not dependent on plasma aldosterone level. Both EHT1864 and eplerenone repressed the augmented MR signaling and mitigated kidney injury with partial but significant reduction in BP with high-salt intake. Hydralazine similarly reduced BP, but it neither suppressed the Rac1-MR pathway nor ameliorated the nephropathy. Taken together, these results show that angiotensin II and salt accelerate kidney injury through Rac1-mediated MR activation. Rac inhibition may be a promising strategy for the treatment of CKD.

Activation of the renin–angiotensin–aldosterone system (RAAS) plays a major role in the progression of kidney disease. Randomized clinical trials proved the efficacy of angiotensin-converting enzyme inhibitors and angiotensin-receptor blockers to reduce proteinuria and improve long-term renal prognosis. Angiotensin (Ang) II has been regarded as the central player in the harmful effects of RAAS. Recent evidence revealed that aldosterone is another contributory factor. RAAS blockers not only inhibit the production or action of Ang II but also suppress the synthesis of aldosterone. Indeed, mineralocorticoid receptor (MR) blockers were shown to protect against various kidney diseases. Aldosterone is proposed to cause albuminuria, glomerulosclerosis, renal inflammation, and fibrosis.

There is an intricate crosstalk between RAAS and salt. When sodium intake is increased, the injurious actions of RAAS are augmented, although circulating RAAS is suppressed by a negative feedback mechanism. With regard to aldosterone, we previously reported that salt lowered plasma aldosterone but nevertheless, accelerated renal injury through paradoxical MR activation using hyperaldosteronemic animals. Our subsequent studies identified small GTPase Rac1 as the ligand-independent modulator of MR activity and showed that this alternative MR activation by Rac1 contributes to aldosterone/salt-induced renal impairment.
To date, the mechanisms of how salt enhances the deleterious effects of Ang II (e.g., whether salt augments Ang II receptor or MR signaling) remain to be elucidated. Several studies suggested the involvement of intrarenal Ang II and oxidative stress. In the present study, we hypothesized that Ang II, in concert with salt, promotes kidney injury through Rac1-MR activation and not through Ang II signal augmentation. Tsukuba hypertensive mice (THM) are Ang II-overproducing transgenic strains carrying both human renin and human angiotensinogen genes. Each transgene is conjugated to its own promoter, allowing normal tissue-specific expression pattern. Although single transgene carriers did not increase Ang II synthesis because of species specificity of renin for its substrate, dual transgenic mice overproduce Ang II by enhanced cleavage of human angiotensinogen by human renin, leading to the development of hypertension and vascular and kidney damage. Salt loading worsens aortic lesions in THM, although the effects on renal injury have not been explored. In the present study, we first examined the relationship between salt intake and Ang II-mediated nephropathy using the THM model. We also clarified the underlying mechanisms by which Ang II/salt promotes kidney injury, focusing on the dependence of the adrenal-derived hormone and Rac1-MR pathway.

RESULTS
Salt-Dependent BP Elevation and Renal Damage in THM
We used THM as the Ang II-overproducing transgenic model. Baseline plasma renin activity, plasma Ang II, and plasma aldosterone concentrations were significantly higher in THM compared with control C57BL/6J (C57) (Supplemental Figure 1, A–C). After 6 weeks of treatment period, systolic BP (SBP) was not elevated in THM on a low-salt (0.05% NaCl) diet (THM-LS) compared with C57. By contrast, SBP was significantly elevated in 1% NaCl-loaded THM (THM-HS; C57=113.9±3.6, THM-LS=121.8±4.1, and THM-HS=157.8±2.7 mmHg, P<0.01 versus C57 and THM-LS) (Figure 1A). Similarly, urinary albumin excretion did not differ between C57 and THM-LS, whereas high-salt intake in THM developed massive albuminuria (C57=6.3±1.2, THM-LS=22.5±4.8, and THM-HS 3022.5±540.9 μg/d, P<0.01 versus C57 and THM-LS) (Figure 1B). Periodic acid-Schiff (PAS) -stained kidney sections of THM-LS showed grossly normal morphology. However, THM-HS exhibited severe histologic abnormalities: glomerular lesions with enlargement of the glomerular tufts, increased mesangial cellularity, mesangial matrix expansion, and sclerotic changes that were accompanied by tubular cast formation, tubulointerstitial damage, and arteriolar hyalinosis (Figure 1C). Scoring of glomerular sclerosis and tubulointerstitial injury revealed only mild increment in THM-LS compared with
C57, whereas THM-HS showed marked elevation (Figure 1, D and E). Next, we examined the salt sensitivity of BP and kidney injury in C57. In contrast to THM, SBP and urinary albumin excretion showed no significant difference between C57 on a 0.05% NaCl diet (C57-LS) and C57 on a 1% NaCl water (C57-HS) (Figure 1, F and G). These findings indicate that salt sensitivity of BP and renal damage differs between THM and C57 and that Ang II excess plus high salt, but not high salt alone, is responsible for the increased salt sensitivity of BP and kidney injury in THM-HS.

Ang II/Salt May Enhance MR-Related Cascade in the Kidney of THM

Serum- and glucocorticoid-inducible kinase 1 (Sgk1), plasminogen activator inhibitor (PAI) -1, and connective tissue growth factor (CTGF) are postulated as direct or associated effectors of MR signaling in the kidney. Compared with C57, renal mRNA levels of Sgk1, PAI-1, and CTGF were not different in THM-LS but significantly upregulated in THM-HS (Figure 2A). These data suggest that MR signaling is activated in the kidney of THM in response to salt loading.

We next carried out MR immunostaining in the kidneys of THM-LS and THM-HS (Figure 2B). MR staining was localized predominantly in the nuclei of certain cells. Intense signals were detected in the distal nephron. Weaker but distinct stainings were also present in the glomeruli. The distribution pattern of positive signals within the kidney was essentially similar between THM-LS and THM-HS. The intensity of positive signals was stronger in THM-HS than THM-LS both in the distal nephron and glomeruli.

Adrenalectomy and Aldosterone Supplementation in THM-HS

To verify the involvement of the aldosterone/MR system in the pathogenesis of Ang II/salt-induced nephropathy in THM-HS, we next examined the effects of adrenalectomy and aldosterone supplementation in THM-HS. Adrenalectomy reduced plasma aldosterone to undetectable levels, and accordingly, it reduced BP and prevented albuminuria, renal histologic abnormalities, and podocyte injury (Figure 3, A–G). On the contrary, aldosterone supplementation, which reversed plasma aldosterone levels comparable with THM-HS, recapitulated BP elevation and kidney injury. Associated with these changes, the enhanced mRNA expressions of Sgk1, PAI-1, and CTGF in the kidneys of THM-HS were effectively attenuated by adrenalectomy and reversed by aldosterone supplementation (Figure 3H). Plasma corticosterone was hardly detectable in adrenalectomized, aldosterone-infused mice, ensuring successful adrenalectomy (data not shown).

Salt Reduced Plasma Aldosterone Concentration but Activated Renal Rac1-MR Cascade in THM

Although the results of adrenalectomized mice indicated the pivotal role for the aldosterone/MR pathway in the nephropathy of the Ang II/salt excess model, plasma aldosterone concentration was elevated in THM-LS compared with C57 and moderately suppressed in THM-HS (Figure 4A). This finding suggests that renal MR activation and kidney dysfunction in THM-HS were independent of plasma aldosterone status. We previously reported that the Rac1-MR system is an alternative pathway of MR activation and involved in renal dysfunction. Therefore, we examined whether the Rac1-MR cascade is enhanced in this model. Glutathione S-transferase (GST) pull-down assay revealed that the amount of guanosine triphosphate (GTP) -bound active Rac1 in the kidney was significantly higher in THM-HS than THM-LS (Figure 4B). Nuclear MR content and Sgk1 expression, markers for MR activation, were also increased in THM-HS (Figure 4, C and D). These results suggest that active Rac1 is increased by salt and causes MR activation and resultant kidney impairment in Ang II-overproducing THM.

We also analyzed Rac1 and MR activity in the kidneys of adrenalectomized mice.
Adrenalectomy suppressed both active Rac1 and MR cascade (nuclear MR and Sgk1) in the kidneys of THM-HS, which were reversed by aldosterone (Supplemental Figure 2). These results suggest that aldosterone is essential to both Rac1 and MR activity, which was previously reported.15

**Inhibition of Rac1-MR Cascade Ameliorated Hypertension, Albuminuria, and Renal Pathology in THM**

To substantiate the hypothesis that Ang II/salt exacerbates renal dysfunction through Rac1-MR activation, we treated THM-HS with a selective MR blocker eplerenone (Epl) or Rac inhibitor EHT1864 (EHT).20 SBP was modestly but significantly lowered in THM-HS+Epl and THM-HS+EHT compared with THM-HS (Figure 5A). Epl and EHT remarkably reduced prominent albuminuria to a similar extent (C57=6.3±1.2, THM-HS=3118.7±432.0, THM-HS+Epl=1018.5±318.2, and THM-HS+EHT=927.7±183.7 μg/d, P<0.01 versus THM-HS) (Figure 5B). In the PAS-stained kidney sections, glomerular and tubulointerstitial lesions in THM-HS were dramatically improved in THM-HS+Epl and THM-HS+EHT (Figure 5, C–E). Transmission electron microscopic analysis revealed severe degenerative changes of podocytes and foot process effacement in THM-HS. However, in parallel to albuminuria reduction, podocyte injury in THM-HS was markedly improved by Epl and EHT (Figure 5F). THM-LS showed almost intact podocyte ultrastructure (data not shown). Plasma aldosterone concentration in THM-HS+Epl was markedly elevated compared with THM-HS (Supplemental Figure 1D). It suggests that the secretion of aldosterone in THM-HS is under the control of normal physiologic regulation and that the amount of salt loading is not too much.

**Figure 3.** Adrenalectomy prevented salt-induced hypertension and renal damage in THM-HS. (A) SBP, (B) albuminuria, and (C) plasma aldosterone concentrations in THM-HS (n=7), adrenalectomized THM-HS (THM-HS+ADx; n=6), and adrenalectomized THM-HS with aldosterone infusion (THM-HS+ADx+Ald; n=5). (D) Representative micrographs of PAS-stained kidney sections of THM-HS+ADx and THM-HS+ADx+Ald. Histologic analysis of (E) glomerulosclerosis and (F) tubulointerstitial injury by semiquantitative morphometric evaluation. (G) Transmission electron micrographs of podocyte ultrastructure in the kidneys. Scale bar, 1 μm. (H) Gene expression was determined using quantitative real-time reverse transcription PCR in THM-HS, THM-HS+ADx, and THM-HS+ADx+Ald. Scale bar, 100 μm. The expression levels were normalized to the levels of β-actin and expressed relative to THM-HS. Data are expressed as mean ± SEM. *P<0.05 and **P<0.01 versus THM-HS; †P<0.05 and ‡P<0.01 versus THM-HS+ADx. n.d., not detected.
Rac Inhibitor and MR Blocker Suppressed Renal MR Activation in THM-HS

Rac inhibitor EHT significantly reduced the amount of GTP-bound Rac1 in the kidney of THM-HS (Figure 6A). Concomitantly, EHT suppressed renal MR activation, which was shown by nuclear MR content and Sgk1 expression, the efficacy of which was equivalent to Epl (Figure 6, B and C). These data indicated that Rac1 actually contributes to MR activation.

The enhanced mRNA expressions of PAI-1 and CTGF in the kidneys of THM-HS were strikingly suppressed in THM-HS+Epl and THM-HS+EHT (Figure 7). MR stimulation is known to potentiate inflammatory cascades.\(^6,21\) Gene expressions of MCP-1 and Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES) were significantly increased in the kidneys of THM-HS and effectively attenuated by Epl and EHT. Consistently, adrenalectomy suppressed mRNA expression of proinflammatory MCP-1 and RANTES, which was reversed by aldosterone supplementation (Figure 3H).

**Inhibition of Rac1-MR Pathway Suppressed Inflammatory Cell Infiltration in THM-HS**

MCP-1 and RANTES are chemokines that play an important role in renal inflammation by attracting macrophages and T lymphocytes, respectively. Immunohistochemical staining illustrated that both CD68-positive macrophages and CD3-positive T lymphocytes were infiltrated into the kidney of THM-HS. Epl as well as EHT effectively suppressed the recruitment of inflammatory cells (Figure 8). These findings suggest that Rac1-MR activation is accompanied by enhanced renal inflammation, which may underlie kidney deterioration in THM-HS. However, we did not show direct evidence for the causative link between MR activation and inflammation. Alleviation of inflammation may have been secondary to alleviation of cellular damage.

**Hydralazine Did Not Suppress the Rac1-MR Pathway or Salt-Induced Kidney Injury**

Finally, we administered hydralazine (Hyd) to THM-HS, because Epl and EHT slightly but significantly reduced BP. Although Hyd lowered SBP to a level comparable with the levels in THM-HS+Epl and THM-HS+EHT, it did not ameliorate the salt-induced prominent albuminuria, renal histologic abnormalities, podocyte injury, and enhanced mRNA expression of Sgk1, MCP-1, and RANTES in THM-HS (Supplemental Figure 3). Hyd also failed to suppress Rac1 activation and increased nuclear MR and Sgk1 expression in THM-HS (Supplemental Figure 4). These data support our hypothesis that the Rac1-MR pathway plays a critical role in the pathogenesis of Ang II/salt-evoked kidney injury in THM-HS.

**DISCUSSION**

In our previous studies, we examined how salt augments aldosterone-mediated renal injury using a high-aldosterone model, and we found that salt reduces plasma aldosterone levels but causes paradoxical MR activation. Subsequently, we identified an alternative pathway of MR activation by Rac1 and showed that Rac1 is actually responsible for the above-mentioned salt-induced paradoxical MR activation. In the current study, however, we focused on the mechanisms of how
Ang II, in concert with salt, promotes kidney injury using Ang II-overproducing double transgenic THM. Conventionally, it is considered that Ang II injures kidney by acting on AT1R-expressing renal cells. We newly showed that Ang II/salt accelerates kidney injury through Rac1-mediated MR activation rather than an Ang II receptor-mediated pathway using Ang II-overproducing double transgenic THM, a model of enhanced RAAS. We also showed that inhibition of the Rac1-MR cascade by Rac inhibitor EHT 1864 or MR blocker eplerenone effectively ameliorates Ang II/salt-induced kidney injury in THM-HS.

We showed that salt is indispensable for the Ang II-mediated kidney damage in THM. Despite the elevated RAAS levels caused by transgenes, renal injury was minimal in THM under low-salt condition. The result is compatible with the epidemiologic finding that some tribes living in no-salt culture do not develop vascular disease, despite increased RAAS. However, Ang II overproduction, in concert with high-salt intake, resulted in severe podocyte injury, albuminuria, glomerulosclerosis, and tubulointerstitial injury. Such close relationship with salt reminds us of aldosterone/MR-mediated injury.

At present, there are no established markers for MR activation in the kidney. We assessed MR activation by combining several strategies, such as effects of adrenalectomy, aldosterone supplementation, treatment with MR blockers, and nuclear MR content. Aldosterone was shown to increase nuclear MR content in the rodent kidney. As surrogate markers, we analyzed Sgk1, PAI-1, CTGF, MCP-1, and RANTES. Sgk1 is postulated as a downstream mediator of aldosterone/MR signaling through the analyses of knockout mice and knockdown experiments. It should be noted that these genes are not

**Figure 5.** MR blocker and Rac inhibitor reduced BP and ameliorated renal injury of salt-loaded THM. (A) SBP in C57, THM-HS, THM-HS treated with Epl (THM-HS+Epl), and THM-HS treated with EHT (THM-HS+EHT). (B) Albuminuria. Histologic analysis of (C) glomerulosclerosis and (D) tubulointerstitial injury by semiquantitative morphometric evaluation. (E) Representative PAS-stained kidney sections. Scale bar, 100 μm. (F) Transmission electron micrographs of podocyte ultrastructure in the kidneys. Scale bar, 1 μm. Data are expressed as mean ± SEM; n=6 for each group. *P<0.05 and **P<0.01 versus C57; #P<0.05 and ##P<0.01 versus THM-HS.
necessarily MR-specific direct targets. In addition, target gene profile may be cell type-specific. To our great surprise, Ang II/salt-induced nephropathy in THM-HS was dramatically prevented by adrenalectomy and recapitulated by aldosterone supplementation, indicating the importance of aldosterone/MR signaling rather than intrarenal Ang II. MR antagonist eplerenone improved renal injury in THM-HS and concomitantly suppressed the above-mentioned surrogate markers. Consistent with our observations, the work by Chander et al.28 showed, using adrenalectomized, saline-loaded hypertensive rats, that aldosterone infusion produced renal injury, whereas Ang II had lesser effects in the absence of the adrenals. The work by Lea et al.29 reported that MR antagonism by spironolactone or aldosterone synthase inhibitor FAD286 significantly reduced kidney injury of Ang II/salt-treated rats. All of these findings support our notion that MR activation plays a critical role in Ang II/salt-mediated renal injury.

Although the significance of adrenal-derived aldosterone and renal MR activation was suggested, salt suppressed the circulating aldosterone level, implicating that molecules other than circulating aldosterone would activate renal MR. MR may be activated by locally generated aldosterone or other MR ligands such as corticosterone under suppressed 11β-hydroxysteroid dehydrogenase-2 activity. However, renal expression of aldosterone synthase mRNA was below the detection level. Neither corticosterone nor 11β-hydroxysteroid dehydrogenase-2 expression was altered by salt loading (data not shown). Alternatively, MR activity may be modulated by ligand-independent mechanisms, and one of such factors is Rac1. In the present study, Ang II/salt increased active GTP-bound Rac1 and markers for MR activity in the kidney of THM and Rac inhibitor; additionally, eplerenone substantially inhibited MR cascade, resolving renal injury. Then, what mechanisms would underlie the Rac1 activation in THM-HS? Rac1 was reported to be activated by NaCl, Ang II, and aldosterone.30–32 According to the present observations, Ang II activated Rac1 under conditions of high- but not low-salt intake. Thus, it can be speculated that

**Figure 6.** Rac inhibitor suppressed the enhanced MR signaling in the kidneys of salt-loaded THM. (A) GTP-bound active Rac1 and total Rac1 expressions in the kidneys of THM-HS and THM-HS+EHT. The bar graph represents densitometric analysis of active Rac1 (n=6 for each group). (B) MR expressions in the nuclear fraction of THM-HS, THM-HS+Epl, and THM-HS+EHT. CREB was used as a loading control. The bar graph represents densitometric analysis of nuclear MR (n=6 for each group). (C) Sgk1 expressions in the kidneys of THM-HS, THM-HS+Epl, and THM-HS+EHT. Actin was used as a loading reference. The bar graph represents densitometric analysis of Sgk1 expression (n=6 for each group). EHT significantly repressed renal Rac1 activity and concomitantly reduced the expressions of nuclear MR and Sgk1 comparable with Epl. Data are expressed as mean ± SEM. *P<0.05 and **P<0.01 versus THM-HS.

**Figure 7.** MR blocker and Rac inhibitor attenuated the enhanced expressions of PAI-1, CTGF, MCP-1, and RANTES mRNA in the kidneys of salt-loaded THM. Gene expression was determined in C57, THM-HS, THM-HS+Epl, and THM-HS+EHT as described in Figure 2. Data are expressed as mean ± SEM; n=6 for each group. *P<0.05 versus EHT; **P<0.05 versus THM-HS.
Ang II and salt interdependently activate Rac1 in THM-HS, culminating in MR activation and renal injury. It should be noted that eplerenone did not completely alleviate kidney damage in THM-HS. It suggests that other mechanisms are involved in this Ang II/salt-induced nephropathy, such as direct renal actions of the Ang II/AT1R signaling and hemodynamic factors (hypertension, glomerular hypertension, and hyperfiltration). Ang II was reported to be generated locally in the kidney to induce vasoconstriction, glomerular hypertension, renal blood flow reduction, increased oxidative stress, and inflammation, and its production was increased by salt. However, recent studies have highlighted the importance of crosstalk between the Ang II/AT1R and aldosterone/MR systems. In vascular smooth muscle cells, for example, the work by Jaffe and Mendelsohn reported that Ang II transactivates MR through an Ang II receptor without the presence of aldosterone. Some of the signaling pathways activated by Ang II receptors are dependent on MR and vice versa. We speculate that the Ang II/AT1R and aldosterone/MR signaling pathways are synergistically involved in the pathophysiology of renal injury.

In summary, we newly showed that Ang II/salt promoted kidney injury through Rac1-mediated MR activation in a mouse model of enhanced RAAS. Several clinical conditions or animal models are reported to have increased plasma or tissue Ang II, including obesity, metabolic syndrome, CKD, and renovascular hypertension. Kidney injury in such patients may be deteriorated by high-salt intake through Rac1-mediated MR activation. Additional studies are necessary to identify clinically relevant conditions.

CONCISE METHODS

Animal Experimental Design

All animal procedures were conducted in accordance with the guidelines for the care and use of laboratory animals approved by the University of Tokyo Graduate School of Medicine. THM, double transgenic mice carrying both the human renin and human angiotensinogen genes were purchased from RIKEN Bio Resource Center (Ibaraki, Japan). In brief, single transgenic mice harboring either the human renin or angiotensinogen gene were produced by injecting the 15-kb human renin gene with 3 kb of 5'-flanking sequence and 1.2 kb of 3'-flanking sequence or the 14-kb human angiotensinogen gene with 1.3 kb of 5'- and 3'-flanking regions into the male pronuclei of mouse (C57BL/6J) fertilized eggs. THM were created by crossmating the above-mentioned single transgenic mice heterozygous for either the renin (R+/2) or angiotensinogen (2+/A) gene. It was noted that heterozygotes bearing the human renin gene expressed this transgene predominantly in the kidney, and the expression of the human angiotensinogen gene was found predominantly in the liver and kidney. C57BL/6J mice were used as controls. Male mice (12 weeks of age) were assigned to the following protocols. Urine was collected for 24 hours using metabolic cages (Natsume, Tokyo, Japan). The amounts of urinary albumin were determined using a mouse albumin ELISA kit (Shibayagi, Gunma, Japan). SBP was measured by the tail-cuff method (BP-98A; Softron, Tokyo, Japan). After 6 weeks of treatment period, the animals were anesthetized with ether, and blood and kidneys were harvested. Kidneys were dissected and frozen in liquid nitrogen, and they were fixed in 4% paraformaldehyde solution or 2% paraformaldehyde and 2.5% glutaraldehyde mixed solution.
For protocol 1, THM were fed a low-salt (0.05% NaCl) diet (THM-LS, n=5) or given drinking water containing 1% NaCl (THM-HS, n=8). C57 mice on a normal chow served as controls (C57, n=6). C57 mice were fed a 0.05% NaCl diet (C57-LS, n=6) or given 1% NaCl water (C57-HS, n=6).

For protocol 2, THM were divided into three groups: THM given 1% NaCl (THM-HS, n=7), adrenalectomized THM-HS (HS+Adx, n=6), and adrenalectomized THM-HS with continuous aldosterone infusion by osmotic minipump (HS+Adx+Ald, n=5).

For protocol 3, THM were divided into three groups: THM given 1% NaCl (THM-HS, n=6), THM-HS given a selective MR blocker eplerenone (1.67 mg kg\(^{-1}\) d\(^{-1}\) chow; THM-HS+Epl, n=6), and THM-HS administered a Rac inhibitor EHT1864 (40 mg kg\(^{-1}\) d\(^{-1}\)) by daily subcutaneous injection (THM-HS+EHT, n=6). Administration of eplerenone and EHT1864 was started 5 days before the beginning of the experiments. C57 mice on a normal chow served as controls (C57, n=6).

For protocol 4, THM were divided into two groups: THM given 1% NaCl (THM-HS, n=6) and THM-HS given an Hyd (4 mg kg\(^{-1}\) d\(^{-1}\) in 1% NaCl water; THM-HS+Hyd, n=5).

**Adrenalectomy and Aldosterone Infusion**

Bilateral adrenalectomy was performed with dexamethasone replacement (12 µg kg\(^{-1}\) d\(^{-1}\) subcutaneously, 3 d wk\(^{-1}\)) as described previously. Some adrenalectomized mice received aldosterone supplementation (1 µg d\(^{-1}\) subcutaneously) through osmotic minipumps (ALZET model 2006). The doses of dexamethasone and aldosterone were chosen to provide near-physiologic replacement based on published studies.

**Histomorphometric Analysis**

We fixed kidneys in 4% paraformaldehyde solution and embedded them in paraffin. For morphologic evaluations, we stained transverse sections (4 µm) with PAS reagents. A blinded observer semiquantitatively assessed the degrees of glomerulosclerosis using at least 50 randomly selected glomeruli in each specimen and tubulointerstitial injury using at least 20 randomly selected cortical fields in each specimen according to an established scoring system. Glomerular sclerosis was defined as synchiae formation by PAS staining with focal or global obliteration of capillary loops, and then, it was graded: 0, normal; 1, 0–25% of glomerular area was affected; 2, 25–50% was affected; 3, 50–75% was affected; 4, 75–100% was affected. Tubulointerstitial injury was graded on the basis of the percentage of tubular cellularity, basement membrane thickening, cell infiltration, dilation, atrophy, sloughing, or interstitial widening: 0; no change; 1, 0–10% tubulointerstitial injury; 2, 10–25% tubulointerstitial injury; 3, 25–50% tubulointerstitial injury; 4, 50–75% tubulointerstitial injury; 5, 75–100% tubulointerstitial injury.

**Transmission Electron Microscopy**

Ultrastructure of glomerular podocytes was analyzed using the Hitachi transmission electron microscope H-7000 (Tokyo, Japan) as described previously.

**Real-Time Reverse Transcription PCR**

Total RNA was extracted using an RNeasy Kit (Qiagen). Gene expression was determined by TaqMan real-time quantitative reverse transcription PCR using Step One Plus Real-Time PCR System (Applied Biosystems).

**Western Blot Analysis**

Western blotting was performed as described previously. We prepared nuclear extracts with a commercially available kit (BioVision). The membranes were incubated with rabbit anti-human Sgk1 (Abcam), mouse anti-rat MR (clone 1D5; a gift from Gomez–Sanchez, University of Mississippi), or mouse anti-human Rac1 (clone 23A8; Millipore) overnight followed by horseradish peroxidase-conjugated secondary antibodies. The signals were detected using enhanced chemiluminescence, and the images were analyzed using ImageQuant LAS 4000 mini (Fujifilm). Membranes were rehybridized with rabbit antiactin (Sigma) or rabbit anti-CREB (Millipore) to normalize protein loading.

**Rac1 Activation Assay**

We assessed the activities of Rac1 with a commercially available GST pull-down assay kit (Millipore). We homogenized samples in the magnesium lysis buffer and incubated at 4°C for 60 minutes with glutathione beads coupled with GST fusion protein corresponding to the p21-binding domain of human PAK1. We determined the Rac1 content in these samples by SDS-PAGE and immunoblotting.

**Immunohistochemistry**

Immunostaining was performed as described previously with some modifications. Briefly, 6-µm-thick cryosections or 4-µm-thick paraffin sections were incubated with rat anti-mouse CD68 (Serotec) or rabbit anti-human CD3 (Dako Cytomation), respectively, and antiactin (Sigma) or rabbit anti-CREB (Millipore) to normalize protein expression was determined by TaqMan real-time quantitative reverse transcription PCR using Step One Plus Real-Time PCR System (Applied Biosystems).

**Statistical Analyses**

Data are expressed as mean±SEM. For multiple comparisons, statistical analysis was performed by ANOVA and subsequent Tukey’s test. Nonparametric analysis with the Mann–Whitney U test was used for comparisons between two groups. Histologic data were analyzed using nonparametric analysis with the Kruskal–Wallis test followed by the Mann–Whitney U test. P values<0.05 were considered to be significant.

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DISCLOSURES

None.

REFERENCES


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Supplemental Figure 1. Basal renin-angiotensin-aldosterone system components of THM are highly enhanced compared with C57. (A) Plasma renin activity. (B) Plasma angiotensin II. (C) Plasma aldosterone concentration. THM on normal salt diet (n=6) and C57 on normal salt diet (n=6). (D) Plasma aldosterone concentration in C57 (n=12), THM-LS (n=12), THM-HS (n=12), THM-HS+Epl (n=6) and THM-HS+EHT (n=6). Data are expressed as mean ± SEM; * P < 0.05, ** P < 0.01 versus C57, ## P < 0.01 versus THM-HS.
Supplemental Figure 2. Renal Rac1 and MR activation were suppressed by adrenalectomy in THM-HS. (A) GTP-bound active Rac1 and total Rac1 expressions in the kidneys of THM-HS (n=7), THM-HS+ADx (n=6) and THM-HS+ADx+Ald (n=5). (B) Renal MR expressions in the nuclear fraction. CREB was used as a loading control. (C) Renal Sgk1 expressions. Actin was used as a loading reference. Bar graphs show the results of densitometric analysis. Data are expressed as mean ± SEM; * P < 0.05, ** P < 0.01 versus THM-HS; # P < 0.05, ## P < 0.01 versus THM-HS+ADx.
**Supplemental Figure 3**

(A) Systolic blood pressure measured by the tail-cuff method in THM-HS (n = 6), THM-HS on 1% NaCl drinking water with hydralazine (THM-HS+Hyd) (n = 5). (B) Urinary albumin excretion. (C) Representative micrographs of PAS–stained kidney sections. Scale bar: 100 µm. (D) Histological analysis of glomerulosclerosis and (E) tubulointerstitial injury by semiquantitative morphometric evaluation. (F) Transmission electron micrographs of podocyte ultrastructure in the kidneys. Scale bar: 1 µm. (G) Gene expression was determined using quantitative real-time RT-PCR. The expression levels were normalized to those of β-actin and expressed relative to THM-HS. Data are expressed as mean ± SEM; * P < 0.05 versus THM-HS.

**Supplemental Figure 3.** Hydralazine effectively lowered blood pressure but did not suppress salt-induced kidney injury and MR activation in THM-HS. (A) Systolic blood pressure measured by the tail-cuff method in THM-HS (n = 6), THM-HS on 1% NaCl drinking water with hydralazine (THM-HS+Hyd) (n = 5). (B) Urinary albumin excretion. (C) Representative micrographs of PAS–stained kidney sections. Scale bar: 100 µm. (D) Histological analysis of glomerulosclerosis and (E) tubulointerstitial injury by semiquantitative morphometric evaluation. (F) Transmission electron micrographs of podocyte ultrastructure in the kidneys. Scale bar: 1 µm. (G) Gene expression was determined using quantitative real-time RT-PCR. The expression levels were normalized to those of β-actin and expressed relative to THM-HS. Data are expressed as mean ± SEM; * P < 0.05 versus THM-HS.
Supplemental Figure 4. Hydralazine (Hyd) did not suppress the enhanced Rac1 and MR signaling in the kidneys of salt-loaded THM. (A) GTP-bound active Rac1 and total Rac1 expressions in the kidneys of THM-HS (n=6) and THM-HS+Hyd (n=5). The bar graph represents densitometric analysis of active Rac1. (B) MR expressions in the nuclear fraction of THM-HS and THM-HS+Hyd. CREB was used as a loading control. The bar graph represents densitometric analysis of nuclear MR. (C) Sgk1 expressions in the kidneys of THM-HS and THM-HS+Hyd. Actin was used as a loading reference. The bar graph represents densitometric analysis of Sgk1 expression. Data are expressed as mean ± SEM; *P < 0.05 versus THM-HS.