Salt-Induced Nephropathy in Obese Spontaneously Hypertensive Rats Via Paradoxical Activation of the Mineralocorticoid Receptor
Role of Oxidative Stress

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Abstract—Aldosterone is implicated in the pathogenesis of proteinuria and chronic kidney disease. We previously demonstrated the contribution of elevated serum aldosterone in the early nephropathy of SHR/NDmcr-cp (SHR/cp), a rat model of metabolic syndrome. In the present study, we investigated the effect of salt loading on renal damage in SHR/cps and explored the underlying mechanisms. SHR/cps fed a high-sodium diet for 4 weeks developed severe hypertension, massive proteinuria, and advanced renal lesions. High salt also worsened glomerular podocyte impairment. Surprisingly, selective mineralocorticoid receptor (MR) antagonist eplerenone dramatically ameliorated the salt-induced proteinuria and renal injury in SHR/cps. Although salt loading reduced circulating aldosterone, it increased nuclear MR and expression of aldosterone effector kinase Sgk1 in the kidney. Gene expressions of transforming growth factor-β1 and plasminogen activator inhibitor-1 were also enhanced in the kidneys of salt-loaded SHR/cps, and eplerenone completely inhibited these injury markers. To clarify the discrepancy between decreased aldosterone and enhanced MR signaling by salt, we further investigated the role of oxidative stress, a putative key factor mediating salt-induced tissue damage. Interestingly, antioxidant Tempol attenuated the salt-evoked MR upregulation and Sgk1 induction and alleviated proteinuria and renal histological abnormalities, suggesting the involvement of oxidative stress in salt-induced MR activation. MR activation by salt was not attributed to increased serum corticosterone or reduced 11β-hydroxysteroid dehydrogenase type 2 activity. In conclusion, sodium loading exacerbated proteinuria and renal injury in metabolic syndrome rats. Salt reduced circulating aldosterone but caused renal MR activation at least partially via induction of oxidative stress, and eplerenone effectively improved the nephropathy. (Hypertension. 2007;50:877-883.)

Key Words: aldosterone □ mineralocorticoids □ metabolic syndrome □ sodium □ dietary □ oxidative stress (kidney) □ chronic kidney disease □ proteinuria □ eplerenone

Salt plays an important role in the progression of target organ injury, as well as in the pathogenesis of hypertension. Epidemiologic studies revealed that higher sodium intake increases the risk of cardiovascular disease, independent of other risk factors, and causes poor prognosis of renal disease. Experimental animal studies indicated that increased dietary salt promotes renal fibrosis and progression of kidney disease. The deleterious effects of salt are, at least partially, independent of blood pressure (BP). Susceptibility to salt is reported to be augmented in certain disease conditions. For example, high salt exacerbates proteinuria in overweight but not nonoverweight subjects. However, the underlying mechanisms have not been clearly elucidated.

Accumulating evidence suggests that aldosterone is an important pathogenic factor in the progression of renal disease and heart failure. In the kidney, aldosterone was demonstrated to cause proteinuria, glomerulosclerosis, arteriopathy, and renal fibrosis. The postulated mechanisms include stimulation of transforming growth factor (TGF)-β1, plasminogen activator inhibitor (PAI)-1, and inflammatory responses. Indeed, mineralocorticoid receptor (MR) antagonists confer beneficial effects against proteinuria and renal injury. We previously reported the renoprotective effects of the selective MR antagonist eplerenone in Dahl salt-sensitive rats, one of the salt-induced renal injury models.

Metabolic syndrome, in which multiple risk factors, such as hypertension, visceral obesity, glucose intolerance, and dyslipidemia, coexist, is a highly predisposing condition for cardiovascular and renal diseases. We recently demonstrated the contribution of elevated serum aldosterone in the early nephropathy of a metabolic syndrome model SHR/NDmcr-cp (SHR/cp), a derivative of the spontaneously hy-
pertensive rat (SHR) with leptin receptor mutation.\textsuperscript{13} SHR/cp at this age did not exhibit renal arteriolar lesions, glomerulonephrosclerosis, or tubulointerstitial injury yet, but manifested glomerular podocyte injury and increased proteinuria, which were ameliorated by eplerenone. Considering the damaging effects of salt on renal functions as stated above, we investigated whether SHR/cps exert salt-sensitive deterioration of renal injury and explored the underlying mechanisms.

Methods

Animals

All of the animal procedures were in accordance with the guidelines for the care and use of laboratory animals approved by University of Tokyo Graduate School of Medicine. Male SHR/cps (Disease Model Cooperative Research Association, Kyoto, Japan; n=74)\textsuperscript{13} at 13 weeks of age were fed a standard rodent chow (n=16), a high-salt diet containing 8% NaCl (n=16), or a high-salt diet supplemented with eplerenone (1.25 g/kg of chow; n=13) for 4 weeks. To evaluate the role of oxidative stress, SHR/cps were fed a high-salt diet with (n=13) or without (n=8) Tempol (1 mmol/L in drinking water) for 4 weeks. In an additional experiment, 13-week-old SHR/cps (n=8) and non-obese SHRs (n=8) were fed a high-salt diet for 4 weeks.

Systolic BP was measured by the tail-cuff method, and 24-hour urine was collected using a metabolic cage.\textsuperscript{13} Direct BP measurement was performed as described previously.\textsuperscript{14} After overnight fasting, rats were anesthetized with ether, and kidneys were harvested. Glomerular fraction was isolated by the graded sieving method.\textsuperscript{13} Plasma renin activity, serum aldosterone and corticosterone concentrations, and aldosterone content in the kidney were measured using radioimmunoassay.

Histological Analysis and Immunohistochemistry

For histological analysis and immunohistochemistry, please see the data supplement available online at http://hyper.ahajournals.org.

Real-Time RT-PCR

RNA extraction, reverse transcription, and real-time quantitative PCR were performed as described previously.\textsuperscript{11}

Electrophoretic Mobility Shift Assay

Nuclear proteins were extracted and subjected to electrophoretic mobility shift assay for the evaluation of nuclear factor κB and activator protein–1 activities, as reported previously.\textsuperscript{13}

Western Blotting

Western blotting was performed as described previously\textsuperscript{13} (please see the data supplement).

11β-Hydroxysteroid Dehydrogenase Type 2 Activity

For 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) activity in the kidney, please see the data supplement.

Urinary 8-Hydroxy-2′-Deoxyguanosine

The urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) concentration was determined as described previously.\textsuperscript{13}

Statistics

Data are expressed as mean±SE. Statistical analyses were performed by unpaired t test, ANOVA, and subsequent Tukey’s test or Kruskal–Wallis test followed by Mann–Whitney U test. P<0.05 was considered significant.

Results

Effects of Salt and Eplerenone on BP, Proteinuria, and Renal Histology in SHR/cps

We first examined whether high salt exacerbates nephropathy of SHR/cps, a metabolic syndrome model with elevated serum aldosterone. After 4 weeks, systolic BP was substantially elevated, and proteinuria was markedly increased in salt-loaded SHR/cps compared with the non–salt-loaded group (Figure 1A and 1B). Periodic acid-Schiff–stained micrographs revealed grossly normal appearance in control SHR/cps (Figure 1C, left). Salt-loaded SHR/cps exhibited advanced renal lesions, such as hypertrophic, sclerotic, and ischemic glomerular changes; arteriolar hyalinosis; and tubulointerstitial changes (Figure 1C, middle).

We next explored whether MR blocker exerts renoprotection in salt-loaded SHR/cps. Eplerenone partially reduced systolic BP and completely alleviated proteinuria in salt-loaded SHR/cps (Figure 1A and 1B). In addition, eplerenone dramatically mitigated renal histological abnormalities (Figure 1C, right). Semiquantitative analysis confirmed the reversal by eplerenone (Figure 1D). These findings suggest that SHR/cps are susceptible to salt-induced renal damage and that aldosterone plays a critical role in this process.

Glomerular Podocyte Damage

Immunostaining for desmin, an injured podocyte marker, was increased in the glomeruli of salt-loaded SHR/cps as compared with the non–salt-loaded group and reduced by eplerenone (Figure 2A). Conversely, immunofluorescence staining for nephrin, a slit diaphragm-associated protein, was attenuated in the high-salt group, which was prevented by eplerenone (Figure 2B). These effects were confirmed by quantitative analysis of nephrin mRNA and protein expressions (Figure 2C). Electron microscopic analysis revealed severe podocyte damage, such as foot process effacement, vacuolization, and accumulation of dense deposits in salt-loaded SHR/cps, which were alleviated by eplerenone (Figure 2D).

Renal Injury Markers

TGF-β1, PAI-1, and inflammatory cytokines are suggested to be involved in aldosterone-induced target organ damage.\textsuperscript{6} Renal expressions of TGF-β1, type 1 collagen, PAI-1, and macrophage chemotactic protein-1 were all significantly augmented in salt-loaded SHR/cps (Figure 3A). In parallel with the changes in macrophage chemotactic protein-1, infiltration of ED-1–positive macrophages was markedly increased by salt loading (Figure 3B). Electrophoretic mobility shift assay revealed that the DNA-binding activities of nuclear factor κB and activator protein–1 were significantly enhanced in salt-loaded SHR/cps (Figure 3C). Notably, salt-induced stimulations of these parameters were completely inhibited by eplerenone.

High Salt Reduces Serum Aldosterone But Causes MR Activation in the Kidney

We next explored how circulating aldosterone and renal MR signaling are affected by salt loading in SHR/cps. Plasma renin activity and serum aldosterone concentration were significantly lower in salt-loaded SHR/cps than in control
SHR/cps, possibly because of volume overload (Figure 4A and 4B). On the other hand, salt loading increased nuclear MR content and Sgk1 expression in the kidney (Figure 4C and 4E). Total (cytosolic plus nuclear) MR was also increased (Figure 4D). Both tissue aldosterone content and aldosterone synthase transcripts in the kidney were under the detection limit, excluding the possibility of enhanced aldosterone production within the kidney.

**Oxidative Stress Contributes to Salt-Induced MR Activation**

What are the mechanisms underlying the discrepancy between reduced aldosterone level and renal MR activation in salt-loaded SHR/cps? MR can be activated by endogenous glucocorticoids or reduced 11β-HSD2 activity. However, serum corticosterone levels were not elevated, and 11β-HSD2 activity was not reduced by salt loading (Figure 5A and 5B). We next tested the role of oxidative stress. Urinary 8-OHdG excretion was elevated in salt-loaded SHR/cps, which was reduced by antioxidant Tempol (Figure 5C and 5D). To our interest, Tempol significantly inhibited the increased nuclear MR contents in the kidney of salt-loaded SHR/cps and attenuated renal Sgk1 expression (Figure 5E and 5F).

**Tempol Alleviates Renal Injury**

Associated with MR inactivation, Tempol ameliorated proteinuria, histological abnormalities, and nephrin in salt-loaded SHR/cps (Figure 6). Tempol did not affect BP (227±5 mm Hg in the Tempol group versus 230±6 mm Hg in the nontreated group; P value not significant). These results support the notion that salt-induced oxidative stress contributes, at least in part, to the enhanced MR signaling and renal injury in salt-loaded SHR/cps.

**Direct BP**

We performed direct BP measurement to evaluate BP more accurately. Mean BP was 137±13 mm Hg in control SHR/cps (n=4); 155±9 mm Hg in salt-loaded SHR/cps (n=4); 139±6 mm Hg in salt-loaded SHR/cps treated with eplerenone (n=5); and 154±8 mm Hg in salt-loaded SHR/cps treated with Tempol (n=5).

**Salt-Induced Nephropathy in Nonobese SHRs and Obese SHR/cps**

Finally, we compared the salt-induced nephropathy in SHRs and SHR/cps (Figure S1). Although the BP level was similar (mean BP was 162±9 mm Hg in salt-loaded SHRs), proteinuria and renal injury were marked in salt-loaded SHR/cps. In addition, salt-loaded SHR/cps showed higher serum aldosterone and increased renal expressions of MR and Sgk1.

**Discussion**

We demonstrated that high-salt feeding caused severe hypertension and exacerbation of renal injury in the SHR/cp, a rat model of metabolic syndrome. What mechanisms are involved in the salt-induced renal damage in this model? First, elevated BP should be an important factor contributing to the salt-sensitive nephropathy in SHR/cps, because salt-loaded SHR/cps developed severe hypertension and renal lesions resembling malignant neph-
rosclerosis. It should be noted that malignant nephrosclerosis typically exhibits a BP threshold for injury, and even modest BP reductions below such a threshold are sufficient for dramatic renoprotection, which might be the case for eplerenone. In the present study, we evaluated BP by both the indirect tail-cuff method and the direct method via the arterial catheter. The limitation of our study, however, is that we only assessed BP over a short period in a few animals. Actually, we obtained somewhat discrepant results, showing a partial hypotensive effect of eplerenone according to the indirect method and complete BP reduction by the direct method. Recently, it was recommended that the quantitative relationships between BP and target organ damage should be evaluated by long-term BP monitoring through catheter implantation or radiotelemetry system. Although several reports indicated BP-independent renoprotection of hypotensive drugs, subsequent studies with more adequate BP measurements using telemetry proved a clear threshold relationship between BP and renal injury in a malignant nephrosclerosis model. Thus, we have to be very careful to judge whether the renal injury or renoprotection is BP dependent or not.

Alternatively, BP-independent factors might be involved in renal injury in obese SHR/cps. Accordingly, our data suggest that activation of MR signaling might underlie the salt-induced renal damage in SHR/cps, because nuclear MR content and Sgk1 expression were increased, and eplerenone completely prevented the injury. Recently, several studies suggested a synergistic interrelationship between salt-induced and MR-mediated tissue injury. First, salt loading in Dahl salt-sensitive rats caused augmented renal and cardiac MR signaling, as indicated by increased renal Sgk1 and cardiac MR mRNA induction, and MR antagonists corrected target organ injury in this model. MR antagonists are also protective against salt-evoked nephropathy in stroke-prone SHRs. Second, it is known that the deleterious effects of exogenously administered aldosterone are potentiated under inappropriately high-sodium intake. Indeed, the histological abnormalities and the pathogenetic factors involved in salt-induced renal damage of SHR/cps resembled those in aldosterone-infused rats together with high salt. Furthermore, several groups reported that knockout mice of Sgk1 and PAI-1 are protected from mineralocorticoid/salt-induced renal injury, although the role of PAI-1 is still controversial.

Salt loading decreased serum aldosterone concentration, whereas it caused MR activation in the target tissue. This apparently paradoxical response may be explained by several mechanisms. First, MR may be activated by endogenous glucocorticoids if salt loading increases circulating glucocorticoids or inhibits 11β-HSD2 activity in the target tissue. MR
might be activated by increased tissue aldosterone. However, our data did not support these possibilities.

We indicated that oxidant stress is one of the responsible factors. Salt loading is reported to enhance reactive oxygen species generation. For example, Kitiyakara et al.24 demonstrated that high salt intake augments oxidative stress, which is associated with activated renal reduced nicotinamide-adenine dinucleotide phosphate oxidase, and decreases intracellular superoxide dismutase. Oxidative stress was actually elevated in our salt-loaded SHR/cps. Reduction of oxidant stress by Tempol blocked the salt-evoked increment of nuclear MR and Sgk1 in the kidney. This inhibition was associated with improvement of proteinuria and renal injury. Compatible with our findings, Park et al.25 indicated that Tempol can mitigate the salt-induced injury in stroke-prone SHRs, in which MR signaling may be augmented.9 Tempol also alleviated salt-induced injury in Dahl salt-sensitive rats with elevated MR expression.18,19,26 Tempol may exert its protective effect at least partially via MR inactivation in these models. Funder27 proposed that oxidative stress may inhibit

Figure 3. A, Gene expressions of TGF-β1, type 1 collagen (Col I), PAI-1, and macrophage chemotactic protein-1 (MCP-1) determined by real-time PCR. n=8 per group. B, Immunostaining for ED-1 positive macrophages and semiquantitative analysis. n=4 per group. Bar, 100 μm. C, Representative autoradiographs and densitometric analysis of DNA-binding activity of nuclear factor (NF-κB; left) and AP-1 (right), determined by electrophoretic mobility shift assay. n=4 per group. High salt activated these mediators, which were completely inhibited by eplerenone. **P<0.01 vs SHR/cp; ###P<0.01 vs SHR/cp+HS.

Figure 4. A, Plasma renin activity. n=8 per group. B, Serum aldosterone concentration. n=8 per group. C, Nuclear MR contents in the kidney. n=4 per group. D, Total MR contents in the kidney. n=4 per group. E, Renal Sgk1 expression. n=4 per group. High salt suppressed circulating renin and aldosterone, whereas it increased expressions of renal MR and Sgk1. **P<0.01 vs SHR/cp.
11β-HSD2 activity through depletion of its substrate, nicotinamide-adenine dinucleotide, which was not the case in our salt-loaded SHR/cp model. It can be speculated that MR activation consequently stimulates reduced nicotinamide-adenine dinucleotide phosphate oxidase and generates reactive oxygen species, which further activate MR, culminating in the formation of a positive feed-forward loop. It should be taken into consideration that the inhibitory effects of Tempol on proteinuria and histological abnormalities were incomplete, whereas eplerenone entirely inhibited the nephropathy in the present study. It might be because of an insufficient dose of Tempol. Alternatively, other factors might also contribute to the salt-elicited MR activation, including insulin, local angiotensin II, renal sympathetic nerve activation, or still-identified MR modulators. Salt might affect transcriptional activity of MR by modulating coactivators or corepressors. Further studies are necessary to fully elucidate the mechanisms.

We showed that salt sensitivity of renal injury is exaggerated in obese SHR/cps compared with nonobese SHRs (Figure S1). Clinically, it is reported that urinary protein excretion is positively correlated with the amount of dietary sodium intake in obese but not in nonobese subjects. Likewise, the nephropathy was worsened by a high-sodium diet in Wistar fatty rats, a model of obese diabetes, but not in Wistar lean rats. These findings suggest that some unifying factor(s) related to obesity might be involved in the mechanisms and that salt restriction is critically important in the management of obesity-related disorders, including metabolic syndrome. Although a high-salt diet reduced circulating aldosterone in SHR/cps, this inhibition was blunted in this model as compared with salt-resistant animals (Figure S1). We reported previously that SHR/cps on a standard diet exhibit elevated serum aldosterone concentration as compared with nonobese SHRs and implicated that adipocyte-derived factors that stimulate aldosterone production in the adrenal glands might contribute to the aldosterone excess of this model. This aldosterone-releasing activity of adipocytes is angiotensin II independent and, thus, may not be suppressed by salt loading. As a result, suppression of circulating aldosterone is less than expected for the amount of salt intake, which might be another mechanism of salt-sensitive renal injury in this model.

![Figure 5](image_url) A, Serum corticosterone concentration. n=4 per group. B, 11β-HSD2 activity in the kidney. n=4 per group. C and D, Urinary 8-OHdG excretion was elevated in SHR/cps fed a high-salt diet (SHR/cp+HS) and reduced by tempol (SHR/cp+HS+TEMP), n=8 per group. E, Nuclear MR in the kidney. n=4 per group. F, Renal Sgk1 expression. n=4 per group. Tempol inhibited the salt-induced enhancement of nuclear MR and Sgk1 expressions. **P<0.01 vs SHR/cp; #P<0.01; #P<0.05 vs SHR/cp+HS.

![Figure 6](image_url) A, Proteinuria. n=8 per group. B, Representative micrographs of PAS-stained renal sections from SHR/cps fed a high-salt diet (SHR/cp+HS; left) and SHR/cp+HS treated with tempol (SHR/cp+HS+TEMP; right). Bars, 100 μm. C, Glomerulosclerosis (GS) index, vascular injury (VI) score, and tubulointerstitial injury (TII) score were semiquantitated. n=4 per group. D, Expression of nephrin in the glomeruli. n=4 per group. Tempol ameliorated proteinuria and histological abnormalities of SHR/cp+HS. **P<0.01, #P<0.05 vs SHR/cp+HS.
Perspectives

We demonstrate that a high-salt diet drastically aggravates the nephropathy of the SHR/cp, a rat model of metabolic syndrome, along with BP elevation. Salt-induced renal injury is accompanied by activated MR signaling in the kidney, without elevation of serum aldosterone. Notably, salt-evoked oxidant stress is suggested to be involved in this process. MR blockade by eplerenone perfectly ameliorates proteinuria and renal injury of salt-loaded SHR/cps. Recent studies have shown that MR antagonists confer renoprotection in high aldosterone states, such as primary aldosteronism, metabolic syndrome, renal insufficiency, and heavy proteinuria.5,10,13,31–33 Our results implicate that MR antagonist can also be renoprotective in patients with activated MR signaling in the target tissue, of which the circulating aldosterone level is not necessarily high, as is seen in metabolic syndrome–model rats fed a high-salt diet.

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Disclosures

None.

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Expanded Methods

Histological Analysis and Immunohistochemistry

Renal sections (3 μm thick) were stained with periodic acid-Schiff (PAS) and examined under light microscopy. Glomerulosclerosis index was semiquantitatively graded as 0 to 4+; vascular injury score as 0 to 3+; and tubulointerstitial injury score as 0 to 5+, according to the criteria reported previously.1 Ultrastructure of glomerular podocytes was analyzed using transmission electron microscope, as described previously.1

Immunohistochemistry of desmin, nephrin, and ED-1 was carried out as described previously.1 The number of ED-1-positive cells was counted in 20 randomly selected high-power fields (x200) and averaged.2 Glomerular desmin staining was semiquantitatively graded as 0 to 4+.1 All morphometric measurements were performed by a blinded observer (n=4 for each group).

Western Blotting

Western blotting for nephrin and Sgk1 was performed as described previously.3 For MR, homogenates of nuclear proteins or total proteins extracted with RIPA buffer (25 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and proteinase and phosphatase inhibitor cocktails) were subjected to SDS-PAGE, incubated with anti-MR antibody (1:1000, Perseus Proteomics, Tokyo, Japan), and visualized with ECL Advanced Western blotting detection system (GE
Measurement of 11β-Hydroxysteroid Dehydrogenase Type 2 (11β-HSD2) Activity

11β-HSD2 activity in the kidney was determined by measuring the rate of conversion of [3H]corticosterone (B) to [3H]11-dehydrocorticosterone (A) at 37°C for 10 minutes. The reaction mixture contained 250 μg of kidney homogenates, 14.5 nmol/L of [3H]corticosterone, 400 μmol/L of NAD⁺, and 50 nmol/L of corticosterone. The reaction products were subjected to C18 reverse-phase Sep-Pak columns, and extracted with methanol. After evaporation with nitrogen gas, [3H]B and [3H]A were separated by high-performance liquid chromatography (LC-10AT, Shimadzu, Kyoto, Japan).
References


Figure Legends

Figure S1. Non-obese SHR and obese SHR/cp at 13 weeks of age were fed a high salt diet for 4 weeks (SHR+HS, SHR/cp+HS). A, Systolic blood pressure measured by the tail-cuff method. n=8 per group. B, Urinary protein excretion per 24 hours. n=8 for each group. C, Glomerulosclerosis (GS) index, vascular injury (VI) score, and tubulointerstitial injury (TII) score were semiquantitated. n=4 per group. D, Expression of nephrin in the glomeruli. n=4 per group. E, Serum aldosterone concentration. n=8 per group. F, Mineralocorticoid receptor (MR) contents in the nuclear fraction of the kidney. n=4 per group. G, Expression of Sgk1 in the kidney. n=4 per group. Although elevation of BP was similar, proteinuria and renal injury were marked in salt-loaded SHR/cp. In addition, salt-loaded SHR/cp showed higher serum aldosterone levels, and increased expressions of MR and Sgk1 in the kidney. **P<0.01 versus SHR+HS; *P<0.05 versus SHR+HS.