

ORIGINAL ARTICLE

Early urinary biomarkers for renal tubular damage in spontaneously hypertensive rats on a high salt intake

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A high salt intake exacerbates hypertension and accelerates renal tubular damage in hypertensive patients. However, data concerning early biomarkers for renal tubular change induced by a high salt intake are limited. The objective of this study was to clarify the time course of new biomarkers for renal tubular damage during high salt intake in spontaneously hypertensive rats (SHR). Male SHR received a regular or high-salt diet from 9 to 17 weeks of age. At 10 weeks of age, a high salt intake caused renal tubular damage, which was further exacerbated at 17 weeks of age. Although albuminuria was detected in salt-loaded SHR at 14 weeks of age, urinary excretion of vanin-1 and neutrophil gelatinase-associated lipocalin (NGAL) was elevated in these animals from 10–17 weeks of age. However, kidney injury molecule-1 (Kim-1) was elevated at 15 weeks of age in salt-loaded SHR. These results suggest that urinary vanin-1 and NGAL are potentially early biomarkers for renal tubular damage in SHR under a high salt intake.

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INTRODUCTION

A high salt intake can markedly elevate blood pressure (BP) in hypertensive patients,¹ which is supported by clinical data showing that a reduction in salt intake causes a significant fall in BP in patients with essential hypertension.^{2,3} Elevated BP increases the risk for cardiovascular and cerebrovascular morbidity and mortality.⁴ In addition to its influence on BP, salt promotes organ damage, leading to cardiovascular^{5,6} and cerebrovascular⁷ diseases.

Renal injury is also caused by a high salt intake. Clinical studies have demonstrated that a high salt intake promotes albuminuria⁶ and a decline in glomerular filtration rate⁸ in hypertensive patients. However, data regarding the profiles of renal tubular damage during the onset of renal injury caused by high salt intake are limited in these patients.⁹

Recently, several new biomarkers for renal tubular damage have been identified. For example, increased urinary excretion of kidney injury molecule-1 (Kim-1)^{10–12} and neutrophil gelatinase-associated lipocalin (NGAL),^{13,14} have been shown to reflect proximal tubular damage. We also found that urinary excretion of vanin-1, which is an epithelial glycosylphosphatidylinositol (GPI)-anchored pantetheinase,^{15,16} is elevated before urinary increases of Kim-1 and NGAL in rats with nephrotoxicant- and drug-induced renal tubular injury.^{17,18} These data led us to speculate that, compared with urinary Kim-1 and NGAL, urinary vanin-1 is an early biomarker for renal tubular damage in hypertensive patients. This study is the first step to demonstrate this hypothesis. Time courses of urinary vanin-1, Kim-1

and NGAL were determined in spontaneously hypertensive rats (SHR), a model of human essential hypertension, under high salt intake.

METHODS

Experimental protocol

All animal procedures were approved by the Animal Care and Use Committee of Jichi Medical University (Shimotsuke, Japan) and were performed in accordance with the Guidelines for Animal Research. Male SHR (Charles River Japan, Yokohama, Japan) were obtained at 8 weeks of age and maintained under a specific pathogen-free condition and controlled temperature and humidity, with a 12-h light/12-h dark cycle (0700–1900 h/1900–0700 h). The rats were fed a regular diet (CE-2; CLEA Japan, Tokyo, Japan) and water *ad libitum* for 1 week before experimental use. Nine-week-old rats either were given a high-salt diet or continued to receive the regular diet. We chose an 8% NaCl diet, because many previous studies that examined the profiles of salt-induced renal damage used 8% NaCl in food.^{19,20} This enabled us to compare the present results with the previous data.

The following experiments were performed:

Experiment 1: at 9 weeks of age, SHR ($n=12$) were randomized to regular (0.8% NaCl) or high-salt (8% NaCl) diet groups. Once a week, a urine sample was collected in a metabolic cage for 8 weeks and stored at -80°C until use. Under the prewarmed conscious state, systolic blood pressure (SBP) was measured by the tail-cuff method (KN-210; Natsume Seisakusho, Tokyo, Japan). At 17 weeks of age, animals were anesthetized with pentobarbital sodium (50 mg kg^{-1} i.p.) to obtain blood and kidney samples for assessing the renal morphology and mRNA expression of renal tubular endocytic receptors.

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Experiment 2: at 9 and 10 weeks of age, which were apparently at an early stage of renal damage after salt loading, rats ($n=4$ at each time point in each group) were killed to determine an early change in renal tubular morphology and/or mRNA expression of renal tubular endocytic receptors.

Histopathology

Kidney tissues at 9 (experiment 2), 10 (experiment 2) and 17 (experiment 1) weeks of age were fixed in 4% paraformaldehyde overnight, dehydrated by passing through an ascending ethanol series, and embedded in paraffin wax. The sections (4 μ m) were stained with hematoxylin and eosin for the assessment of inflammation, with periodic acid-Schiff for the determination of tubular damage, and with Masson's trichrome (LSI Medience Corporation, Tokyo, Japan) for the assessment of interstitial fibrosis. The sections were examined with a light microscope and analyzed using semiquantitative scoring, ranging from grades 0 to 4 (grade 0, normal; grade 1, mild; grade 2, moderate; grade 3, severe; and grade 4, very severe). The grading was applied for typical hypertension-induced kidney lesions such as tubular degeneration and tubular dilatation. For each group, an average score was calculated.

Immunofluorescence staining

Immunofluorescence staining of the kidney was performed on frozen sections as previously described.¹⁷ Briefly, sections were incubated with goat anti-vanin-1 antibody (1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat anti-Kim-1 antibody (1:1000; R&D Systems, Minneapolis, MN, USA), and then with donkey Cy3-labeled anti-goat IgG, (1:200; Abcam, Cambridge, UK) and Alexa Fluor 488-labeled phalloidin (Life Technologies, Carlsbad, CA, USA). Images were acquired with an AX80 (Olympus, Tokyo, Japan).

Laboratory measurements

Urinary, serum and renal concentrations of vanin-1, Kim-1 and NGAL protein were measured using an ELISA kit from Uscn Life Science (Cloud-Clone, Houston, TX, USA), R&D Systems and BioPorto Diagnostics (Gentofte, Denmark), respectively. In brief, urine and blood samples were centrifuged at 1000 g for 10 min, and their supernatants were used. Tissue lysate was prepared from renal cortices of rats at 17 weeks of age, and 25 μ g of total protein measured with the BCA protein assay kit (Thermo scientific, Rockford, IL, USA) was loaded onto an ELISA microplate. Urinary albumin was measured with an autoanalyzer by immunonephelometry (MP Biomedicals Japan, Tokyo, Japan). Serum and urinary concentrations of creatinine were determined by the Jaffe method with a commercial kit (Wako Pure Chemical Industries, Osaka, Japan).

RNA extraction and real-time quantitative PCR

Total RNA isolated from the kidney tissues using an RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) was reverse-transcribed with a PrimeScript RT reagent Kit (Takara Bio, Otsu, Japan). Gene expression was analyzed by real-time PCR, performed using the Applied Biosystems StepOnePlus Real-Time PCR system (Life Technologies). Specific sets of primers and TaqMan probes were obtained from Life Technologies. To control for variation in the

amount of cDNA available for PCR in the different samples, expression of target sequences was normalized to that of an endogenous control, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The GenBank accession number, assay ID, and target exon were NM_030827.1, Rn00578067_m1 and 77-78 (*megalin*); NM_053332.2, Rn00584200_m1, and 39-40 (*cubilin*); NM_177421.3, Rn00598583_m1, and 4-5 (*24p3R*); NM_173149.1, Rn00597703_m1 and 5-6 (*Havcr1*); NM_017008.4, Rn99999916_s1 and 3-3 (*GAPDH*). Data were analyzed using the comparative threshold cycle method.

Statistical analysis

Data are expressed as the means \pm s.e.m. Comparisons were made by an unpaired *t*-test. For time course studies, data were compared using two-way ANOVA followed by Bonferroni's *post hoc* analysis. A value of $P < 0.05$ was considered statistically significant. All statistical analyses were conducted with GraphPad Prism, version 4.03 (GraphPad Software, San Diego, CA, USA).

RESULTS

Effects of high salt intake on SBP

At 9 weeks of age (before the initiation of salt loading), SBP was not significantly different between the groups (Figure 1). SBP in the group on a high-salt diet was significantly higher than that in the group on a regular diet at 10 and 17 weeks of age (Figure 1).

Effects of high salt intake on urinary albumin, creatinine clearance and renal histopathology

As shown in Figure 2a, urinary albumin excretion in the salt-loaded SHR was significantly higher at 14 weeks of age and further increased thereafter. However, a high salt intake had no significant influence on creatinine clearance at 17 weeks of age in these animals. Renal tubular damage such as tubular degeneration and dilatation was slightly induced at 10 and 17 weeks of age in SHR on a regular diet (Figures 2b and c). These changes were exaggerated in SHR on a high-salt diet. Interstitial inflammation (Figure 2d) and fibrosis (Figure 2e) were marked in SHR on a high-salt diet at 17 weeks of age, whereas these changes were mild in SHR on a high-salt diet at 10 weeks of age and on a regular diet at 17 weeks of age.

Effects of high salt intake on newly developed renal tubular biomarkers

Salt loading significantly elevated urinary excretions of vanin-1 and NGAL in SHR at 10 weeks of age, which persisted up to the end of the study (Figure 3). Urinary Kim-1 after salt loading was significantly elevated at 15 and 17 weeks of age.

Origin of urinary vanin-1, NGAL and Kim-1

To clarify the localization of vanin-1 in the kidney, we carried out immunofluorescence analysis of vanin-1. As shown in Figure 4, vanin-

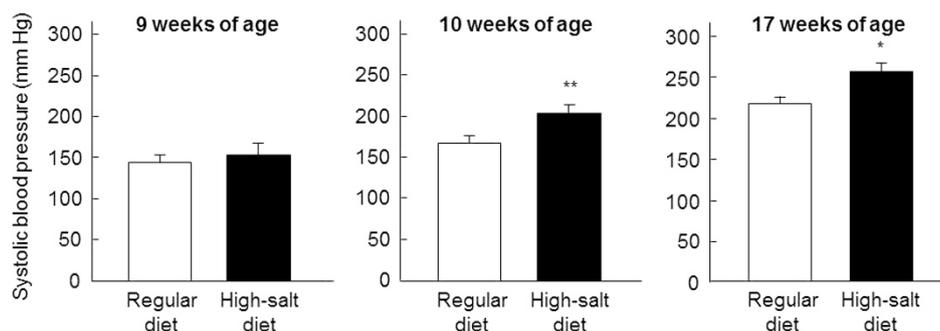


Figure 1 Effects of a high-salt diet on SBP in SHR. SBP was measured by the tail-cuff method in SHR on a regular or high-salt diet from 9 to 17 weeks of age. Data are the means \pm s.e.m. ($n=5-6$). * $P < 0.05$; ** $P < 0.01$ vs. SHR on a regular diet.

1 expression was detected in the tubules but not the glomeruli, which is consistent with our previous findings.^{17,18} These results indicate that urinary vanin-1 is derived from renal tubules. Of note, the cortex was positively stained for vanin-1 at 9, 10 and 17 weeks of age, and the medulla was also stained at 17 weeks of age, which was accompanied by the progression of injury to the medulla, as shown in Figure 4.

We also investigated the origins of urinary NGAL and Kim-1 in this study. At 17 weeks of age, serum and renal concentrations of NGAL protein were detected in each group, and there were no significant differences between the groups (Figure 5a). Because NGAL is reabsorbed in the proximal tubules via endocytic receptors including megalin, cubilin²¹ and 24p3R,²² we further investigated the influence of a high-salt diet on these receptors. A high-salt diet significantly reduced the renal mRNA expression of megalin and cubilin at 10 and 17 weeks, respectively, in SHR (Figure 5b). Renal 24p3R mRNA expression was not influenced by high salt intake.

A high-salt diet significantly elevated Kim-1 both in the serum and in the kidney in SHR (Figure 6a). Therefore, it remained probable that urinary Kim-1 was derived from the blood as well as the kidneys. To determine whether urinary Kim-1 was derived from the kidneys, we conducted an immunofluorescence analysis. As shown in Figure 6b, Kim-1 was mainly expressed on the apical side of proximal tubule epithelia in the salt-loaded SHR at 17 weeks of age. These results indicate that urinary Kim-1 originated in the kidney. Consistently with these results, the mRNA expression of renal Kim-1 in the salt-loaded SHR was ~80-fold higher than that in the controls at 17 weeks of age, whereas there was no significant difference between the groups at 10 weeks of age (Figure 6c).

DISCUSSION

In this study, the following changes were detected in SHR: (1) a high salt intake caused histologically renal tubular damage, which was

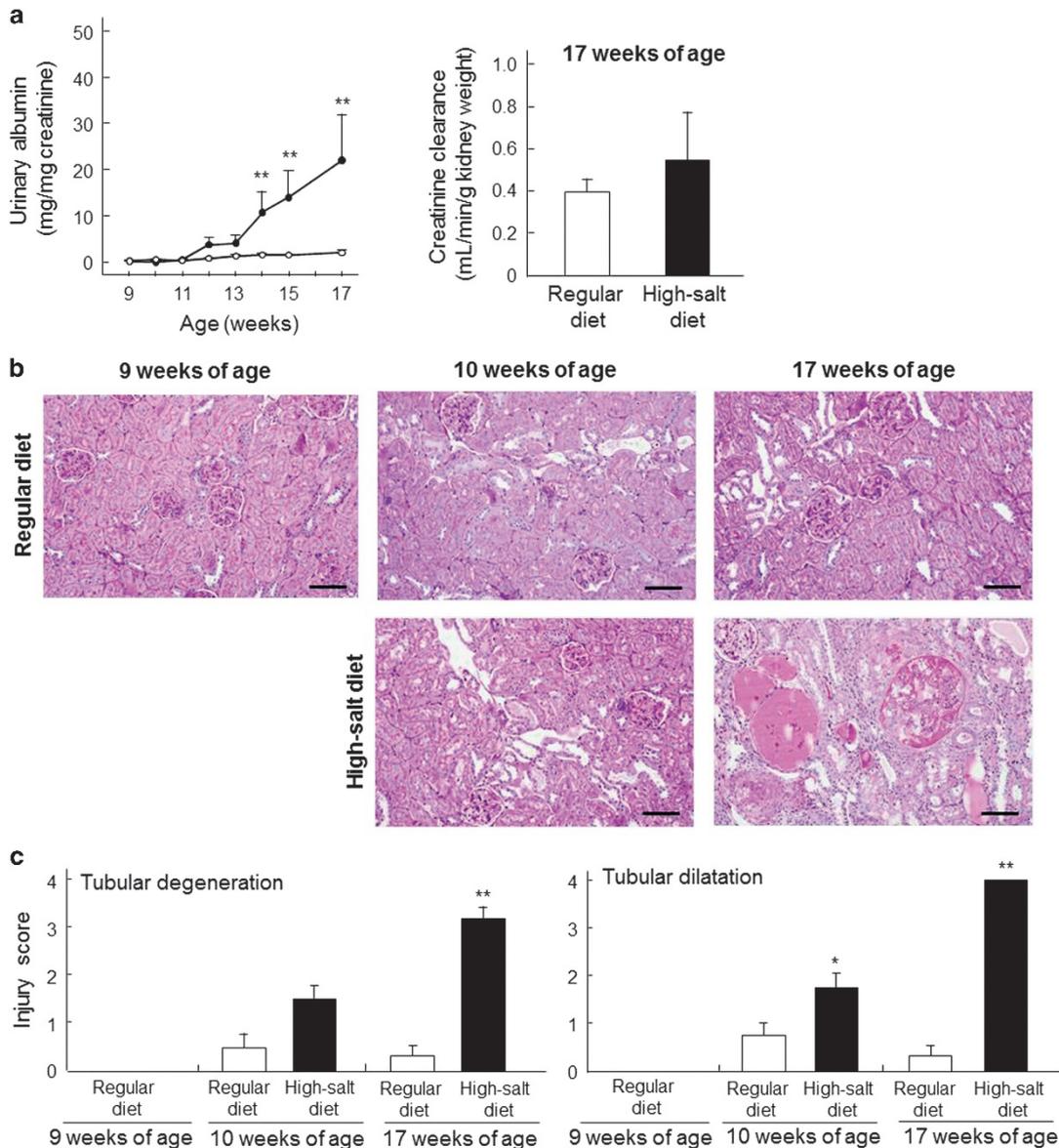


Figure 2 Continued.

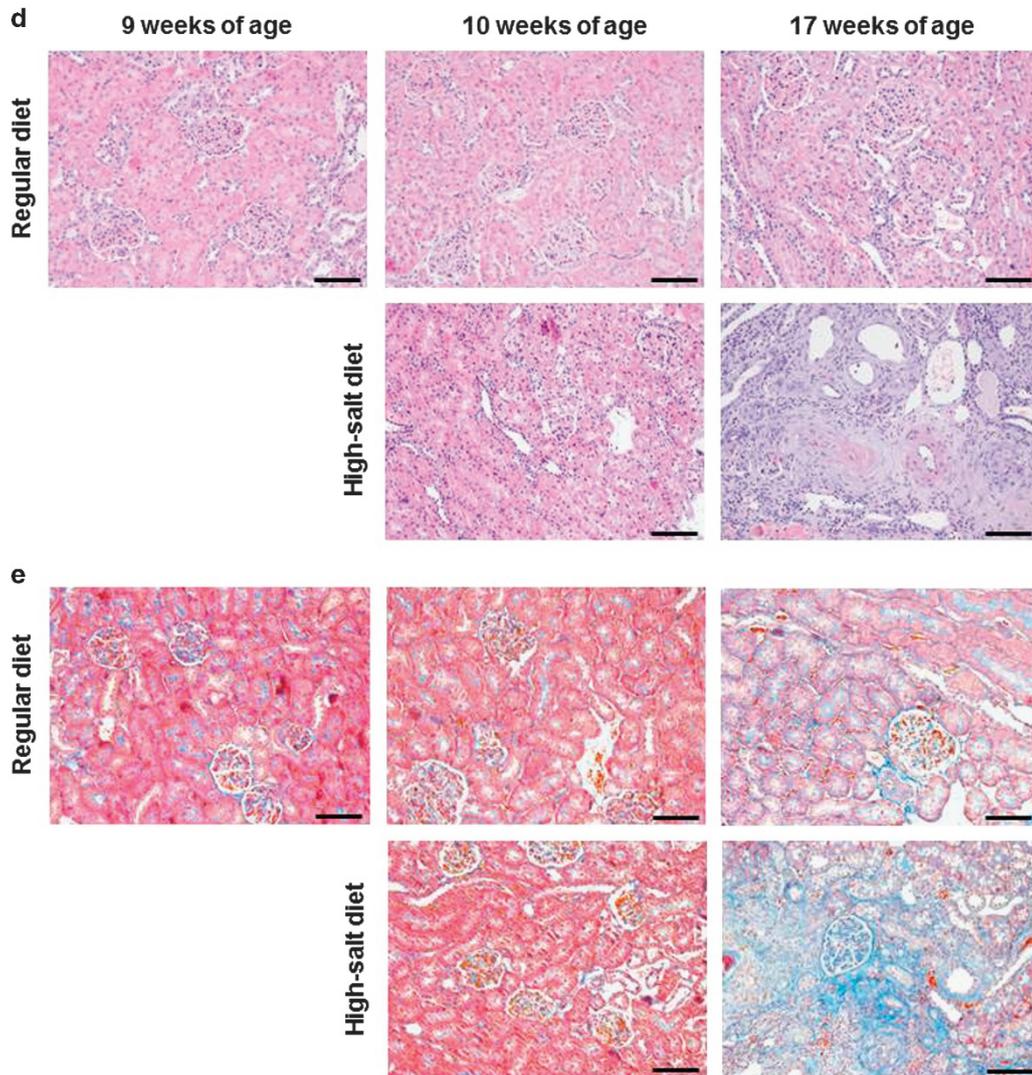


Figure 2 Effects of a high-salt diet on urinary albumin, creatinine clearance, and renal histopathology. (a) Twelve-hour urine samples of SHR on a regular diet (white circles) or a high-salt diet (black circles) were collected once a week from 9 to 17 weeks of age. Urinary albumin concentration was normalized to urinary creatinine concentration. Blood samples were taken from animals at 17 weeks of age. Data are the means \pm s.e.m. ($n=5-6$). $**P<0.01$ vs. SHR on a regular diet. (b) Representative photomicrographs of renal changes. Sections were stained by periodic acid-Schiff and hematoxylin. Scale bars, 100 μ m. (c) Renal tubular injury scores. Data are the means \pm s.e.m. ($n=5-6$). $*P<0.05$; $**P<0.01$ vs. SHR on a regular diet. (d, e) Representative photomicrographs of inflammation by hematoxylin and eosin stain (d) and fibrosis by Masson's trichrome stain (e). Scale bars, 50 μ m.

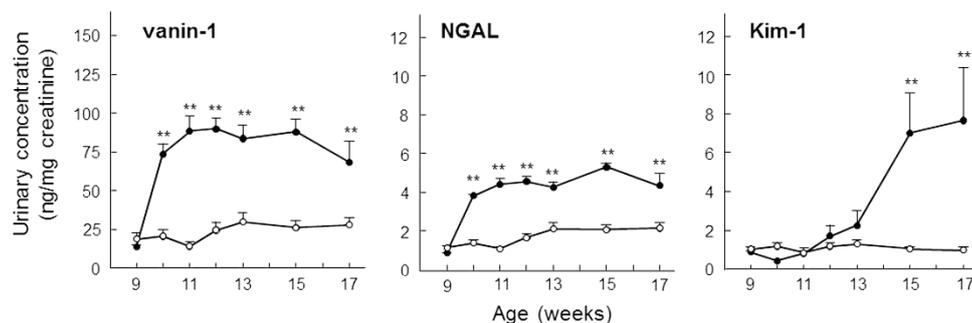


Figure 3 Effects of a high-salt diet on newly developed renal tubular biomarkers. Time course of urinary vanin-1, NGAL and Kim-1. Twelve-hour urine samples of SHR on a regular diet (white circles) or a high-salt diet (black circles) were collected once a week. Concentrations of these markers in urine were determined by ELISA and normalized to urinary creatinine concentration. Data are the means \pm s.e.m. ($n=5-6$). $**P<0.01$ vs. SHR on a regular diet.

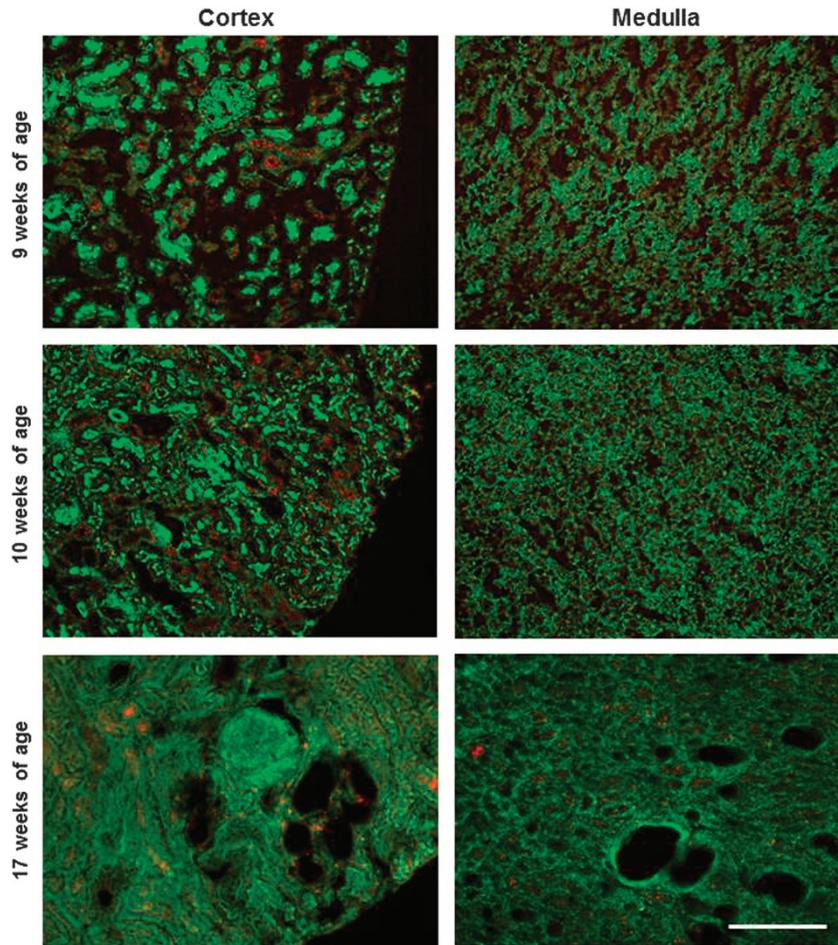


Figure 4 Origin of urinary vanin-1. A representative image of immunostaining of vanin-1 in the kidney of SHR on a high-salt diet. Cryosections (4 μ m) were stained with an antibody specific for vanin-1 (red) and phalloidin (green). Scale bars, 200 μ m.

detected before an appearance of albuminuria; (2) urinary excretion of vanin-1 and NGAL was elevated at 1 week after salt loading; and (3) urinary Kim-1 showed a significant increase at 6 weeks after salt loading. These data suggest that, in addition to urinary vanin-1, NGAL is potentially an earlier biomarker for salt-induced renal tubular damage than urinary Kim-1 in SHR, which differs from the previous findings that urinary vanin-1 is elevated before the increases in urinary Kim-1 and NGAL occur in rats with nephrotoxicant- and drug-induced renal tubular injury.^{17,18} The differences in the time course of urinary vanin-1, NGAL and Kim-1 might be partly explained by the following mechanisms. After salt loading, oxidative stress causes renal damage.²³ Lai *et al.*²⁴ have shown that increased reactive oxygen species has a role in salt-induced renal damage in mice. Vanin-1 has been shown to be induced by reactive oxygen species *in vivo*.²⁵ Because reactive oxygen species are involved in the onset of the inflammation process, vanin-1 seems to be induced in the early phase of injury in the salt-loaded SHR. In this study, urinary NGAL appeared early in salt-loaded SHR. Of note, the present results clearly demonstrated that a high-salt diet significantly reduces the renal mRNA expression of megalin in the SHR at 10 weeks of age. Because megalin is involved in the endocytic system, it is possible that urinary NGAL could be attributed to the defect in tubular reabsorption. However, urinary Kim-1 increased later in the course of the damage, partly because Kim-1 is upregulated during the regeneration of tubular epithelial cells

after injury.¹⁰ In addition, Kim-1 expression has been reported to be low in the kidneys of young SHR (12 weeks of age), probably because of a malfunction in the ongoing renal maturation process.²⁶ Consistently with these reports, this study showed that there was no detectable Kim-1 mRNA expression in salt-loaded SHR at 10 weeks of age, whereas Kim-1 was markedly upregulated at 17 weeks of age.

The origins of these urinary biomarkers might be renal tubules and/or blood.

(1) Urinary vanin-1. Previous studies have shown that vanin-1 is mainly expressed in the kidneys, and vanin-1 is expressed in the epithelial cells of renal cortical tubules but not glomeruli.¹⁶ Previously, we also demonstrated that urinary vanin-1 was derived from renal proximal tubules in rats with nephrotoxicant- and drug-induced renal injury.^{17,18} In this study, the immunofluorescence analysis of vanin-1 also showed that vanin-1 is expressed in renal tubular cells in the salt-loaded SHR. Therefore, urinary vanin-1 might be released from renal tubular epithelial cells in salt-loaded SHR. In hypertensive renal injury, hypertensive vascular damage occurs first in the juxtamedullary glomeruli, leading to impairment of renal medullary circulation and medullary tubulointerstitial damage.²⁷ The cortex is reported to be well protected from pressure-induced injury because renal cortical blood flow exhibits a high degree of autoregulation, and cortical renal damage is rarely found.²⁷ Therefore, the tubulointerstitial damage and glomerular sclerosis in the cortex have been considered to be induced

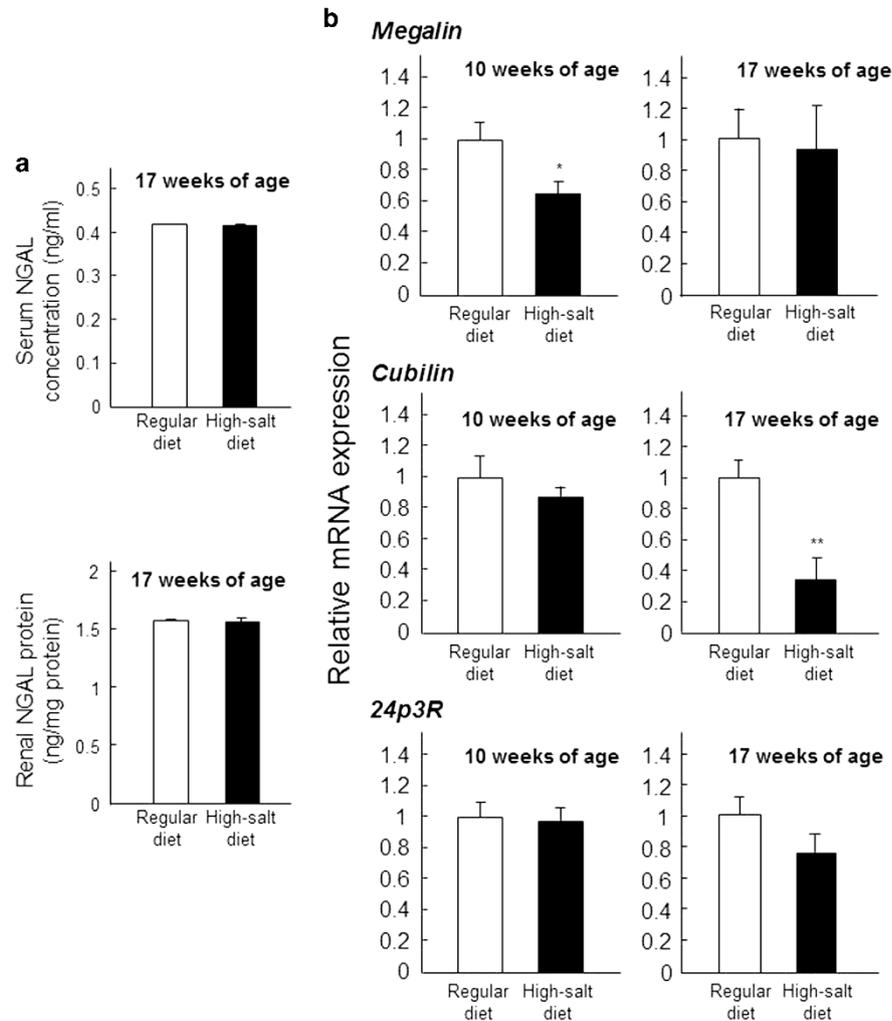


Figure 5 Origin of urinary NGAL. (a) Blood samples and kidney tissues were taken from animals at 17 weeks of age. Data are the means \pm s.e.m. ($n=5-6$). (b) Kidney tissues were taken from animals at 9, 10 and 17 weeks of age. The mRNA expression levels of *megalin*, *cubilin* and *24p3R* were normalized to those of *GAPDH*. Data are the means \pm s.e.m. ($n=4$ in each group). * $P<0.05$; ** $P<0.01$ vs. SHR on a regular diet.

by a direct effect of intrarenal angiotensin II rather than by elevation of BP. Angiotensin II has been reported to initiate and enhance inflammation and oxidative stress.^{28,29} Because vanin-1 reacts with oxidative stress, and our results revealed that the cortex was stained positively for vanin-1 (Figure 4), urinary vanin-1 might originate from the injured tubules mainly in renal cortex in the salt-loaded SHR.

(2) Urinary NGAL. NGAL was detected in blood and kidney in this study. NGAL is a small protein (21 kD) of the lipocalin superfamily and is filtered in urine. In renal proximal tubular epithelial cells, endocytosis receptors such as megalin, cubilin and 24p3R are responsible for the reabsorption of glomerular-filtered proteins including NGAL.^{21,22,30} Megalin binds to cubilin with a high affinity and contributes to the internalization of cubilin-ligand complexes.³¹ Therefore, the impairment of these endocytosis receptors could lead to decreased renal uptake of NGAL. In this study, serum NGAL did not differ between the groups, but urinary NGAL was elevated in salt-loaded SHR, which might have been caused by salt-induced decreases in the renal tubular megalin-cubilin complex.

(3) Urinary Kim-1. Blood and renal concentrations of Kim-1 and its urinary excretion were elevated in salt-loaded SHR at 17 weeks of age in this study. To evaluate the origin of urinary Kim-1, we conducted

immunofluorescence analysis. Our results revealed that Kim-1 is localized mainly in the apical sides of renal tubules, which supports the idea that urinary Kim-1 is derived from the apical side of renal tubules. In this study, interstitial inflammation and fibrosis were marked in SHR on a high-salt diet at 17 weeks of age, whereas these changes were mild in SHR on a high-salt diet at 10 weeks of age. Of note, urinary excretions of vanin-1 and NGAL were elevated from 10 weeks of age, but urinary Kim-1 excretion was elevated from 15 weeks of age in SHR on a high-salt diet. These data indicate that interstitial inflammation and fibrosis are involved in the mechanism of the enhanced urinary Kim-1 in salt-loaded SHR.

Urinary albumin is reabsorbed via the megalin-cubilin complex. Therefore, the reabsorption of urinary albumin is potentially reduced in the salt-loaded SHR at 10 and 17 weeks of age. However, albumin has negative charge and a large molecular radius, and it is normally difficult to filter through the pores.^{32,33} Indeed, glomerulopathy was mild at 10 weeks of age (Supplementary Figure); thus, transglomerular passage of albumin may be low at 10 weeks of age. Therefore, the decrease in megalin gene expression was not reflected in urinary albumin excretion at 10 weeks of age.

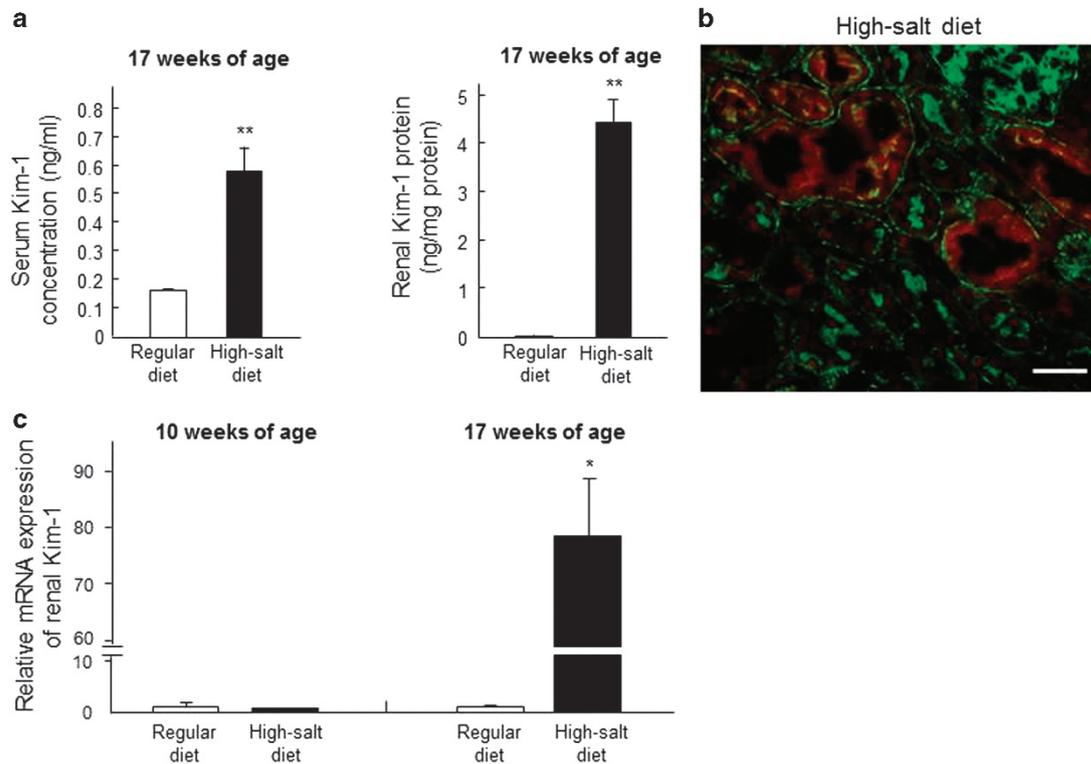


Figure 6 Origin of urinary Kim-1. (a) Blood samples and kidney tissues were taken from animals at 17 weeks of age. Data are the means \pm s.e.m. ($n=5-6$). ** $P<0.01$ vs. SHR on a regular diet. (b) A representative image of immunostaining of Kim-1 in the renal cortices of SHR on a high-salt diet. Cryosections ($4\mu\text{m}$) were stained with an antibody specific for Kim-1 (red) and phalloidin (green). Scale bars, $100\mu\text{m}$. (c) The mRNA expression levels of *Kim-1* normalized to those of *GAPDH*. Data are the means \pm s.e.m. ($n=4$ in each group).

Although BP was elevated (>200 mm Hg) in SHR on a regular diet at 17 weeks of age, renal tubular damage was relatively mild and was not reflected in the increases of urinary biomarkers in this study. These findings indicate that the role of salt might be greater than that of BP elevation in the development of renal tubular damage in SHR on a high-salt diet. The following mechanisms might be involved in the high-salt-induced renal tubular damage observed in this study. Salt loading is known to suppress renin release from the renal juxtaglomerular apparatus and cause a reduction in systemically generated angiotensin II. However, Nath *et al.*³⁴ have reported that, despite the suppression of circulating angiotensin II with salt loading, the locally generated intrarenal renin-angiotensin-aldosterone system (RAAS) is stimulated by salt, and the angiotensin II content of proximal tubular fluid increases. Therefore, it is probable that the stimulated intrarenal RAAS is involved in salt-induced renal tubular damage.

Damage in the tubular epithelial cells causes tubulointerstitial injury and fibrosis,³⁵ which is a common pathway for progression of many forms of renal disease.³⁴ Clinically, it is important for detecting renal tubular damage at an early stage and initiating appropriate therapeutic intervention. The present study provides the hypothesis that urinary vanin-1 and NGAL might potentially be early biomarkers of renal tubular damage in hypertensive patients under a high-salt diet. To address this hypothesis, prospective clinical studies are required.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Hypertension Research website (<http://www.nature.com/hr>)