High salt-induced weakness of anti-oxidative function of natriuretic peptide receptor-C and podocyte damage in the kidneys of Dahl rats

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Abstract

Background: Atrial natriuretic peptide (ANP) and its natriuretic peptide receptors A (NPR-A) and C (NPR-C) are involved in the regulation of physiological and pathophysiological process of blood pressure. The present study aimed to determine the role of NPR-C in the development of salt-sensitive hypertension.

Methods: The Dahl salt-sensitive (DS) and salt-resistant (DR) rats were used in this study. Animals were matched according to their age and weight, and then placed on either a high-salt (HS, 8%) or a normal-salt (NS, 0.4%) diet for 6 weeks randomly using random number table. The systolic blood pressure (SBP), plasmatic sodium concentration (PL_{Na}), urinary sodium excretion (UV_{Na}), and serum creatinine concentration (Scr) were measured. The concentration of ANP in blood and tissues (heart and kidney) was detected by enzyme-linked immunosorbent assay. The expression of ANP, NPR-A, and NPR-C in kidney was evaluated with western blot analysis. Regarding renal redox state, the concentration changes in malondialdehyde (MDA), lipofuscin, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox), and nitric oxide synthase (NOS) in kidney were detected by a spectrophotometric method. The kidney damage was evaluated using pathological techniques and the succinodehydrogenase (SDHase) examination. Furthermore, after an intra-peritoneal injection of C-atrial natriuretic peptide $(ANP)_{4–23}$ (C-$ANP_{4–23}$), an NPR-C receptor agonist, the SBP, biochemical values in blood and urine, and renal redox state were evaluated. The paired Student's t test and analysis of variance followed by the Bonferroni test were performed for statistical analyses of the comparisons between two groups and multiple groups, respectively.

Results: The baseline SBP in all groups was within the normal range. At the end of the 6-week experiment, HS diet significantly increased the SBP in DS rats from 116.63 ± 2.90 mmHg to 162.25 ± 2.15 mmHg ($t = -10.213$, $P < 0.001$). The changes of SBP were not significant in DS rats on an NS diet and DR rats on an NS diet or on an HS diet (all $P > 0.05$). The significant increase of PL_{Na}, UV_{Na}, and Scr related to an HS diet was found in both DS and DR rats (all $P < 0.05$). However, significant changes in the concentration ($t = -21.915$, $P < 0.001$) and expression of renal ANP ($t = -3.566$, $P = 0.016$) and the expression of renal NPR-C ($t = 5.864$, $P = 0.002$) were only observed in DS hypertensive rats. The significantly higher desmin immunochemical staining score ($t = -5.715$, $P = 0.005$) and mitochondrial injury score ($t = -6.325$, $P = 0.003$) accompanied by the lower SDHase concentration ($t = 3.972$, $P = 0.017$) revealed mitochondrial pathologic abnormalities in podocytes in DS rats with an HS diet. The distinct increases of MDA ($t = -4.685$, $P = 0.009$), lipofuscin ($t = -8.195$, $P = 0.001$), and Nox ($t = -12.733$, $P < 0.001$) but not NOS ($t = -0.328$, $P = 0.764$) in kidneys were also found in DS hypertensive rats. C-$ANP_{4–23}$ treatment significantly decreased the SBP induced by HS in DS rats ($P < 0.05$), which was still higher than NS groups with the vehicle or C-$ANP_{4–23}$ treatment ($P < 0.05$). Moreover, the HS-induced increase of MDA, lipofuscin, Nox concentrations, and Nox4 expression in DS rats was significantly attenuated by C-$ANP_{4–23}$ treatment as compared with those with HS diet and vehicle injection (all $P < 0.05$).

Conclusions: The results indicated that the renal NPR-C might be involved in the salt-sensitive hypertension through the damage of mitochondria in podocytes and the reduction of the anti-oxidative function. Hence, C-$ANP_{4–23}$ might serve as a therapeutic agent in treating salt-sensitive hypertension.

Keywords: Natriuretic peptide receptor-C; Nicotinamide adenine dinucleotide phosphate oxidase 4; Oxidative stress; Podocyte; Salt-sensitive hypertension

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Introduction

Hypertension is a chronic disease characterized by increased arterial blood pressure (BP) and damages to target organs.[1,2] Patients with hypertension tend to be more salt sensitive,[3] leading to significant morbidity and mortality.[4] In China, the detection rate of salt-sensitive hypertension in the adult population is 27.1% on average, but 58.7% in hypertensive patients.[1] Thus, exploring the pathogenesis of salt-sensitive hypertension is important to improve its prevention and treatment.[5-8]

Atrial natriuretic peptide (ANP) is a hormone that regulates BP by promoting natriuresis and vasodilation. It was originally identified in heart atria. It has two types of receptors: natriuretic peptide receptors A (NPR-A) and C (NPR-C).[9,10] NPR-C is usually known as the clearance receptor. It acts by internalizing and eliminating the hormone from the circulation. However, accumulating evidence indicated the involvement of NPR-C in the pathogenesis of hypertension. The precise role is still unclear and controversial. A study reported that the distribution of the expression of NPR-C was higher in spontaneously hypertensive rats (SHRs) than in normotensive rats. In addition, hypoxia-induced pulmonary hypertension was suppressed by binding to NPR-C with its selective agonist in rats.[11] However, another study showed that the number of NPR-C receptors significantly reduced in salt-loaded Dahl hypertensive rats.[12] These findings suggested that the discrepancies in the expression of NPR-C reflected its complex role in BP regulation.

Oxidative stress is crucial for salt-sensitive hypertension. The production of reactive oxygen species (ROS) increased in salt-hypertensive Dahl rats,[13,14] which was detected in various organs of experimental animals including kidneys and cardiovascular system.[15-17] The activation of plasma membrane nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) is well known as the main source of excessive ROS generation.[18] Renal Nox has an important pathophysiological role in the development of hypertension. The O_2^- generation from Nox in renal tubules enhances arteriolar reactivity[19] and the tubuloglomerular feedback response to limit sodium chloride (NaCl) elimination, thereby contributing to salt sensitivity and hypertension.[20,21] Anti-oxidant interventions were reported to alleviate the severity of salt hypertension.[22,23] C-atrial natriuretic peptide $(ANP)_{4–23}$ (C-$ANP_{4–23}$), an NPR-C agonist, was also confirmed to decrease the enhanced oxidative stress in vascular smooth muscle cells of hypertensive rats. The probable mechanism is associated with the subsequent Nox-dependent superoxide anion generation.[24]

Interestingly, dietary salt supplementation increases plasma ANP concentrations in normotensive salt-resistant rats, whereas it does not show the same response in salt-sensitive SHRs.[11,25] These data suggested a key role of the renal ANP-receptor system in the development of salt-sensitive hypertension, but the related mechanism is not well known to date. The expression of NPR-C gene was influenced by salt loading in a tissue-specific manner and it markedly decreased in the kidneys of rats with salt-sensitive hypertension.[26] The present study using Dahl rats was performed to investigate (1) whether the renal ANP-receptor system was involved in the development of salt-sensitive hypertension and (2) whether the role of renal NPR-C was associated with anti-oxidation by the *in vivo* administration of C-$ANP_{4–23}$.

Methods

Ethical approval

This study was approved and supervised by the Animal Care and Use Committees of Shandong Provincial Hospital Affiliated to Shandong University (No. 2018-043) and performed in accordance with the international ethics for animal use. Significant efforts were made to minimize the number of animals and reduce their suffering.

Animal treatment

The male Dahl salt-sensitive (DS) rats and Dahl salt-resistant (DR) rats (aged 7–8 weeks, weighting 190–210 g; Beijing Vital River Laboratory Animal Technology Co., Ltd, Beijing, China) were used in this study. The animals were maintained in an environment with controlled humidity and temperature and had free access to tap water.

The present study comprised three series and the sample size was estimated according to the previous research.[27] In each experiment, rats were matched according to their age and weight, followed by being numbered and randomly assigned to the different treatment groups using a random number table. Protocol 1 was designed for detecting the effects of a high-salt (HS) diet on ANP function and its receptor expression in Dahl rats. Both DS and DR rats were divided into two groups ($n = 6$ for each group): (a) group fed with a normal-salt (NS) diet (0.4% NaCl) and (b) group fed with an HS diet (8% NaCl).[27] After 6 weeks, the rats were examined for systolic blood pressure (SBP), concentration of plasmatic sodium (PL_{Na}), urinary sodium excretion (UV_{Na}), and serum creatinine (Scr), ANP in plasma and organs, and the expression of ANP and NPR-A/C receptors in kidneys. Protocol 2 was designed for examining the effects of an HS diet on the factors associated with salt-sensitive hypertension, including podocyte damage and oxidative stress in DS rats. DS rats were given an NS or an HS diet as mentioned earlier ($n = 5$ for each group). After 6 weeks, the left kidney was removed and prepared for morphological observations, whereas the right kidney was prepared to examine the oxidative stress level. Protocol 3 was designed to verify the role of NPR-C in salt-sensitive hypertension in DS rats using its agonist, C-$ANP_{4–23}$. DS rats were raised in four different groups as follows ($n = 6$ for each group): (a) NS-C: fed an NS diet plus injected C-$ANP_{4–23}$ (10 nmol/kg body weight, purchased from GL Biochemistry, Shanghai, China) intra-peritoneally as described previously by Li *et al*,[28,29] twice per week; (b) NS-V: fed an NS diet plus injected vehicle intra-peritoneally; (c) HS-C: fed an HS diet plus injected C-$ANP_{4–23}$ intra-peritoneally; and (d) HS-V: fed an HS diet plus injected vehicle intra-peritoneally. The duration was 6 weeks. Then the rats were treated for projects designed for SBP, renal function parameters, and oxidative stress indexes.

SBP was measured in a conscious and non-invasive state using the tail-cuff method. After being anesthetized, the animals were infused with isotonic saline solution (0.15 mol/L NaCl) to guarantee the quantities of sampling. After stabilization, blood and urine were obtained. In the end, the rats were euthanized by decapitation. The hearts and kidneys were rapidly dissected out.

ANP enzyme-linked immunosorbent assay

ANP measurement was performed using an ANP-rat enzyme-linked immunosorbent assay (ELISA) commercial kit (SEA225Ra; Cloud-Clone Corp., Houston, TX, USA). Plasma and tissue homogenates were prepared and added to the appropriate microtiter plate wells with an ANP-specific biotin-conjugated antibody. After incubation with horseradish peroxidase-conjugated avidin and coloration, the color change was measured spectrophotometrically. The concentrations of ANP in the samples were then determined by comparing the optical density of the samples with the standard curve.

Western blot analysis

The tissue samples were homogenized and prepared for the following protocols. The expression of ANP, NPR-A, and NPR-C receptors, and Nox4 was examined using Western blot analysis. Pooled samples of renal tissue containing similar amounts of proteins were separated by electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA). They were transferred to a nitrocellulose membrane (Bio-Rad Laboratories) and incubated with goat polyclonal anti-ANP (1:800 dilution, sc-18811; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal-anti-NPR-A (1:3000 dilution, ab14356; Abcam, Cambridge, MA, USA), rabbit monoclonal anti-NPR-C (1:1000 dilution, ab177954; Abcam), or rabbit monoclonal anti-Nox4 (1:2000 dilution, ab109225; Abcam). After incubation with the secondary antibody (Proteintech, Wuhan, Hubei, China), the protein bands were visualized by enhanced chemiluminescence detection (W028; Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). The quantitative analysis of specific bands was performed by the densitometric scanning of autoradiographs (Tanon 5200, Shanghai, China) and quantified using Image J software (1.8 version; National Institutes of Health, Bethesda, MA, USA). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal standard (anti-GAPDH, 1: 2500 dilution, ab9485; Abcam) to avoid inaccuracies in protein loading. The target protein levels were expressed as the ratio between the optical densities of GAPDH bands corresponding to ANP, NPR-A, NPR-C, or Nox4.

Urine and plasma biochemical measurement

Urinary sodium, plasma sodium, and serum creatinine levels were measured by the spectrophotometric methods using commercial kits (C002, C011-1; Nanjing Jiancheng Bioengineering Institute). The concentration of creatinine and sodium was calculated using a standard formula. The urinary and plasma sodium concentrations were expressed as mmol/L and the creatinine level as μmol/L.

Evaluation of oxidative stress

The renal samples were homogenized and centrifuged at 12,000× *g* for 20 min. The supernatants were subjected to detection reagents using a commercial kit (malondialdehyde, MDA: A014-1; Nox: A127; nitric oxide synthase, NOS: A014-1; Nanjing Jiancheng Bioengineering Institute; lipofuscin: ELISA kit, MBS7202025, San Diego, CA, USA). Briefly, the samples were mixed with the standard liquid, incubated, and colored. Finally, they were examined by a spectrophotometric method and expressed as U/mg protein. The activities of NOS and Nox were determined by detecting the concentrations of nitric oxide (NO) and nicotinamide adenine dinucleotide (NAD) and performing formula conversion according to the kit instruction.

Transmission electron microscopy

The kidney samples were first processed with fixation and dehydration, and then embedded in Araldite. Further, 70- to 80-nm sections were stained with 2% uranyl acetate and Reynolds lead citrate. A transmission electron microscope (410LS; Phillips, Amsterdam, the Netherlands) was used for examining glomeruli at 30,000× magnification.

The mitochondrial injury in podocytes was judged using the following scoring system, from 0 to 5[30]: 0, normal appearance; 1, minimal mitochondrial swelling; 2, mild mitochondrial swelling; 3, moderate mitochondrial swelling; 4, diffuse high-amplitude swelling and disruption of crystal membrane integrity; and 5, high-amplitude swelling with some mitochondrial flocculent densities and/or calcifications. Four photomicrographs of each kidney sample were depicted and five mitochondria per section were evaluated. This scoring was performed in a blinded manner.

Evaluation of mitochondrial injury

The renal tissues were prepared to isolate mitochondria. The samples were mixed with the isolation reagents (C3606; Nanjing Jiancheng Bioengineering Institute) and homogenized on ice. The mitochondria were isolated after centrifuging the samples two times, followed by the quantitative detection of protein. Succinodehydrogenase (SDHase) is an enzyme complex in the inner mitochondrial membrane, which participates in both the citric acid cycle and the electron transport chain. The SDHase activity in mitochondria samples was measured, since it is one of the key markers of mitochondrial function (SDHase kit: A022; Nanjing Jiancheng Bioengineering Institute). A spectrophotometric method was used and the SDHase concentration was expressed as U/mg protein.

Renal immunohistochemistry for the expression of desmin

The rats were sacrificed, and the renal tissues were fixed with 10% methanol and embedded in paraffin. The sections (5 μm thick) were used for immunohistochemical analysis. Briefly, after antigen retrieval, the sections were first incubated with 10% goat serum for preventing non-specific binding and then incubated with rabbit monoclo-

nal anti-desmin antibody (1: 2000 dilution, ab32362; Abcam) overnight at 4°C. Immunostaining was performed using a technique employing commercially modified avidin-biotin-peroxidase complex (PV-9000; ZSGB-BIO, Beijing, China), followed by counterstaining with hematoxylin. Histological sections were observed using a Nikon E400 light microscope (Nikon Instrument Group, New York, NY, USA).

Glomerular desmin staining was graded as follows: signal area in the glomerular capillary tuft was 0, 0; 1, 1% to 25%; 2, 26% to 50%; 3, 51% to 75%; and 4, 76% to 100%.[31] This scoring was performed in a blinded manner (100 glomeruli per section).

Statistical analysis

Quantitative data were expressed as mean ± standard deviation (normal distribution of data was checked by Kolmogorov-Smirnov test). Statistical analyses were conducted using SPSS 19.0 statistical software (IBM, Armonk, NY, USA). Comparisons between two groups in protocol one and two were made with paired Student's t test. Comparisons of multiple groups in protocol 3 were evaluated by analysis of variance followed by the Bonferroni test. The results were considered statistically significant at a P value less than 0.05.

Results

Effects of an HS diet on ANP function and its receptor expression in Dahl rats

Blood pressure

The baseline SBP in all groups was within the normal range.[32] At the end of the 6-week experiment, HS diet significantly increased the SBP in DS rats from 116.63 ± 2.90 mmHg to 162.25 ± 2.15 mmHg ($t = -10.213$, $P < 0.001$). The changes of SBP were not significant in DS rats on an NS diet ($t = -2.079$, $P = 0.092$) and DR rats on an NS diet ($t = -1.507$, $P = 0.192$) or on an HS diet ($t = -1.828$, $P = 0.127$) [Table 1].

Plasmatic sodium, urinary sodium, and serum creatinine concentrations

Plasmatic sodium concentration (PL_{Na}), urinary sodium excretion (UV_{Na}), and serum creatinine concentration (Scr) were tested for renal excretive function. The HS diet significantly increased the PL_{Na} in both the DR ($t = -17.195$, $P < 0.001$) and DS groups ($t = -15.966$, $P < 0.001$). Compared with the NS diet, the UV_{Na} was also increased with HS administration in both the DR ($t = -8.248$, $P < 0.001$) and DS groups ($t = -6.771$, $P = 0.001$), but it was more remarkable in the DR group. However, the HS diet led to greater Scr in both the DR ($t = -3.884$, $P = 0.012$) and DS ($t = -19.460$, $P < 0.001$) groups, which was more remarkable in the DS group [Table 2].

Plasma, intra-cardiac, and intra-renal ANP

ANP concentration (pg/mL) was measured using ELISA. Compared with the NS diet, HS obviously increased the ANP concentration in plasma ($t = -11.959$, $P < 0.001$) and heart ($t = -5.876$, $P = 0.002$) in the DR group. These changes were not distinct in the DS group, although a slight increase in the ANP concentration in the heart was found with HS treatment (Plasma: $t = 0.590$, $P = 0.581$; Heart: $t = -1.007$, $P = 0.360$). Unlike the DR experimental group ($t = -0.763$, $P = 0.480$), the intra renal ANP concentration significantly increased in the DS group in response to an HS diet compared with an NS diet ($t = -21.915$, $P < 0.001$) [Table 3], which was confirmed using western blot analysis (DR: $t = 0.222$, $P = 0.833$; DS: $t = -3.566$, $P = 0.016$) [Figure 1A and 1B].

Table 1: Effect of the high-salt diet on SBP in Dahl rats of four groups.

Groups	*n*	Baseline SBP (mmHg)	SBP after 6 weeks (mmHg)	*t*	*P*
DR + NS	6	119.88 ± 1.87	122.31 ± 1.57	−1.507	0.192
DR + HS	6	117.44 ± 2.52	123.38 ± 1.65	−1.828	0.127
DS + NS	6	117.63 ± 2.76	121.19 ± 1.90	−2.079	0.092
DS + HS	6	116.63 ± 2.90	162.25 ± 2.15	−10.213	<0.001

Data are presented as mean ± standard deviation. SBP: Systolic blood pressure; DR + NS: Dahl salt-resistant rats with normal-salt diet; DR + HS: Dahl salt-resistant rats with high-salt diet; DS + NS: Dahl salt-sensitive rats with normal-salt diet; DS + HS: Dahl salt-sensitive rats with high-salt diet.

Table 2: Effect of the high-salt diet on sodium and creatinine concentrations in blood and urine in Dahl rats.

Items	DR + NS ($n = 6$)	DR + HS ($n = 6$)	*t*	*P*	DS + NS ($n = 6$)	DS + HS ($n = 6$)	*t*	*P*
PL_{Na} (mmol/L)	75.49 ± 1.21	94.59 ± 1.11	−17.195	<0.001	78.28 ± 5.10	108.94 ± 7.49	−15.966	<0.001
UV_{Na} (mmol/L)	36.68 ± 0.52	61.41 ± 6.02	−8.248	<0.001	36.11 ± 1.90	51.28 ± 3.76	−6.771	0.001
Scr (μmol/L)	61.58 ± 3.67	69.65 ± 4.22	−3.884	0.012	56.84 ± 4.55	111.08 ± 7.71	−19.460	<0.001

Data are presented as mean ± standard deviation. DR + NS: Dahl salt-resistant rats with normal-salt diet; DR + HS: Dahl salt-resistant rats with high-salt diet; DS + NS: Dahl salt-sensitive rats with normal-salt diet; DS + HS: Dahl salt-sensitive rats with high-salt diet; PL_{Na}: Plasmatic sodium concentration; UV_{Na}: Urinary sodium excretion; Scr: Serum creatinine concentration.

Expression of intra-renal NPR-A and NPR-C

Western blot analysis results shown in Figure 1A and 1C indicated that the level of NPR-C significantly decreased in the DS group fed an HS diet compared with an NS diet ($t = 5.864$; $P = 0.002$); however, no significant difference was observed in the DR group ($t = 0.724$; $P = 0.502$). No dramatic differences were found in the expression of NPR-A (DR: $t = -2.527$, $P = 0.053$; DS: $t = -1.306$, $P = 0.248$) [Figure 1A and 1D].

Effects of an HS diet on podocyte damage and oxidative stress in DS rats

Podocyte damage in kidneys

The injury to podocytes was roughly evaluated in DS rats fed an HS diet. The podocyte damage was assessed by analyzing the expression of desmin, in addition to a conventional morphological study by electron microscopy. The desmin immunostaining was positive in glomeruli along the capillary tufts [Figure 2A–D]. Transmission electron microscopy was performed and revealed that the mitochondria of podocytes showed vacuolation, mitochondrial crest reduction, and structural disorder in DS rats fed an HS diet [Figure 2E–H]. According to the semi-quantitative analyses, the desmin staining score was significantly enhanced in podocytes of DS rats fed an HS diet ($t = -5.715$, $P = 0.005$) [Figure 2I]. The change in mitochondrial morphology was also analyzed. The score of mitochondrial injury was significantly higher in the HS treatment group compared with the NS treatment group ($t = -6.325$, $P = 0.003$) [Figure 2J]. The SDHase concentration, one of the key markers of mitochondrial function, was significantly lower in DS rats fed an HS diet (17.53 ± 3.44 U/mg protein) compared with the rats fed an NS diet (24.78 ± 5.94 U/mg protein) ($t = 3.972$, $P = 0.017$) [Figure 2K].

Table 3: Effect of the high-salt diet on atrial natriuretic peptide concentration in blood and organs of Dahl rats.

Items	DR + NS ($n = 6$)	DR + HS ($n = 6$)	t	P	DS + NS ($n = 6$)	DS + HS ($n = 6$)	t	P
Plasma ANP (pg/mL)	92.22 ± 6.59	126.36 ± 8.68	−11.959	<0.001	91.59 ± 11.15	89.99 ± 11.94	0.590	0.581
Heart ANP (pg/mL)	213.94 ± 11.37	270.97 ± 17.99	−5.876	0.002	215.20 ± 11.04	222.80 ± 11.18	−1.007	0.360
Intrarenal ANP (pg/mL)	103.55 ± 1.26	106.20 ± 5.60	−0.763	0.480	102.77 ± 4.54	192.14 ± 21.10	−21.915	<0.001

Data are presented as mean ± standard deviation. ANP: Atrial natriuretic peptide; DR + NS: Dahl salt-resistant rats with normal-salt diet; DR + HS: Dahl salt-resistant rats with high-salt diet; DS + NS: Dahl salt-sensitive rats with normal-salt diet; DS + HS: Dahl salt-sensitive rats with high-salt diet.

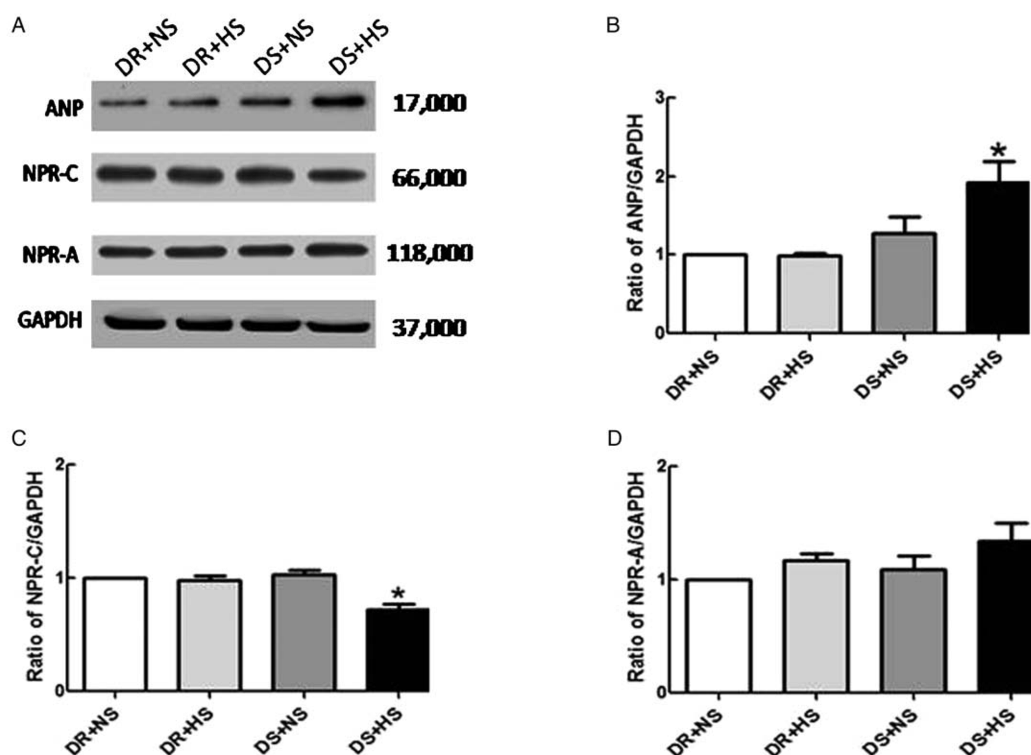

Figure 1: Effects of the high-salt diet on expressions of ANP and its receptors in Dahl rats. (A) Western blot analysis for the expressions of ANP and its receptors (NPR-C and NPR-A) in kidneys. GAPDH was used as an internal control. The relative expression levels of ANP (B), NPR-C (C), and NPR-A (D) in kidneys detected by Western blot. $^*P < 0.05$ compared with DS + NS group. ANP: Atrial natriuretic peptide; NPR-C: Natriuretic peptide receptors C; NPR-A: Natriuretic peptide receptors A; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; DR + NS: Dahl salt-resistant rats with normal-salt diet; DR + HS: Dahl salt-resistant rats with high-salt diet; DS + NS: Dahl salt-sensitive rats with normal-salt diet; DS + HS: Dahl salt-sensitive rats with high-salt diet.

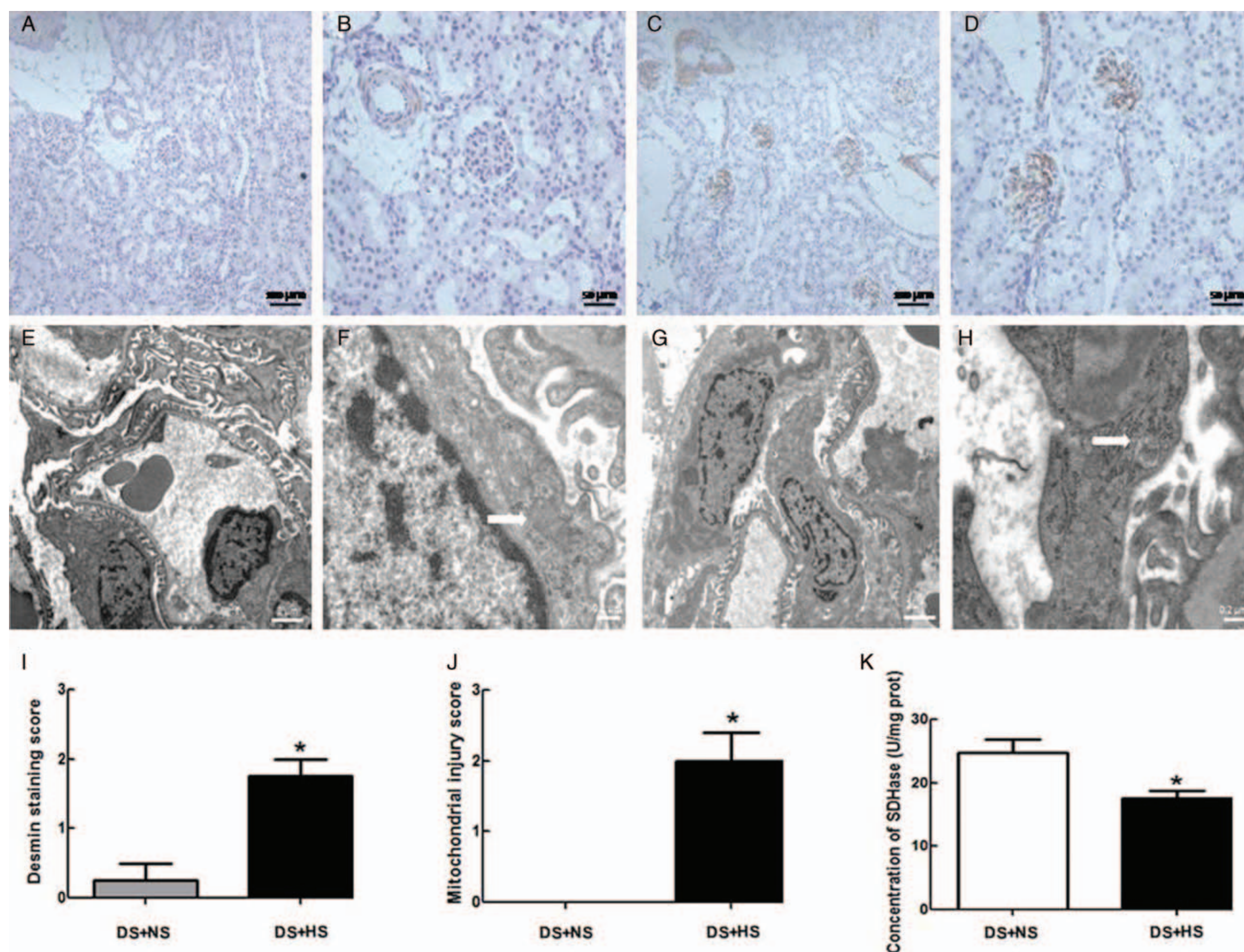

Figure 2: Effects of the high-salt diet on podocyte injury in Dahl salt-sensitive rats. Immunohistochemical examination for desmin in the glomeruli of Dahl salt-sensitive rats 6 weeks after a normal-salt (A, original magnification ×200; B, original magnification ×400) and a high-salt diet (C, original magnification ×200; D, original magnification ×400). Transmission electron micrographs of mitochondria in podocytes from Dahl salt-sensitive rats 6 weeks after a normal-salt (E, original magnification ×3000; F, original magnification ×30,000) and a high-salt diet (G, original magnification ×3000; H, original magnification ×30,000). Arrow, mitochondria. Analyses of desmin staining score in the glomeruli (I) and the morphological injury score for mitochondria in the podocytes (J) were showed respectively. (K) The SDHase concentrations in kidneys were compared between the Dahl salt-sensitive rats fed a normal-salt and a high-salt diet. $^{*}P < 0.05$ compared with DS + NS group. SDHase: Succinodehydrogenase; DS + NS: Dahl salt-sensitive rats with normal-salt diet; DS + HS: Dahl salt-sensitive rats with high-salt diet; prot: protein.

Oxidative stress indexes in kidneys

MDA and lipofuscin concentrations were measured as indirect markers to evaluate the degree of possible kidney damage caused by ROS. As shown in Figure 3A, the storage of MDA ($t = -4.685$, $P = 0.009$) and lipofuscin ($t = -8.195$, $P = 0.001$) in DS rats was significantly elevated with HS treatment for 6 weeks. Then the concentration of Nox was tested. The results showed that HS enhanced Nox concentration in the kidneys of DS rats ($t = -12.733$, $P < 0.001$) [Figure 3B]. HS was found to cause no significant difference in NOS concentration ($t = -0.328$, $P = 0.764$).

Role of NPR-C receptor agonist (C-ANP$_{4-23}$) on salt-sensitive hypertension in DS rats

Effect of C-ANP$_{4-23}$ treatment on the development of high BP, sodium concentration, and creatinine metabolism

C-ANP$_{4-23}$ treatment significantly decreased the SBP induced by HS in DS rats ($P < 0.05$), which was still higher than NS groups with the vehicle or C-ANP$_{4-23}$ treatment ($P < 0.05$) [Table 4]. Compared with Dahl rats on an NS diet, HS rats on an HS diet showed higher PL_{Na} and UV_{Na}. The treatment with C-ANP$_{4-23}$ increased sodium excretion in HS rats as compared with the vehicle injection group, followed by reduced sodium concentration in the blood, and these differences were significant (both $P < 0.05$). Serum creatinine is one of the markers for judging renal impairment. The HS diet significantly exacerbated the Scr level in the DS group, which was improved by C-ANP$_{4-23}$ treatment ($P < 0.05$) [Table 4].

Effect of C-ANP4-23 treatment on oxidative stress indexes in kidneys

Oxidative stress indexes were examined to investigate whether the attenuation of BP in DS hypertensive rats by the treatment with C-ANP$_{4-23}$ was associated with the anti-oxidative function. The results are shown in Figure 4. The HS-induced increase of MDA and lipofuscin concentration in DS rats was significantly attenuated by C-ANP$_{4-23}$ treatment as compared with those with HS diet and the

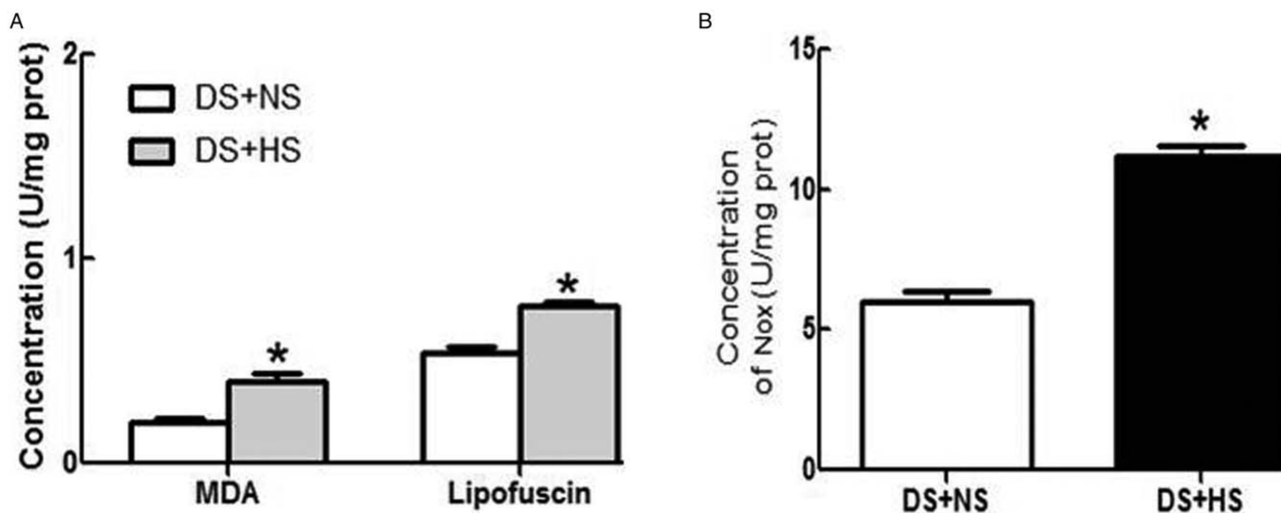

Figure 3: Effects of the high-salt diet on oxidative stress indexes in kidney from Dahl salt-sensitive rats. The concentrations of MDA, lipofuscin (A) and Nox (B) in the kidneys were compared between Dahl salt-sensitive rats with a normal-salt and a high-salt diet for 6 weeks. $^{*}P < 0.05$ compared with DS + NS group. MDA: Malondialdehyde; Nox: Nicotinamide adenine dinucleotide phosphate oxidase; DS + NS: Dahl salt-sensitive rats with the normal-salt diet; DS + HS: Dahl salt-sensitive rats with the high-salt diet; prot: protein.

Table 4: Effect of C-ANP$_{4–23}$ treatment on the SBP and renal function parameters in Dahl salt-sensitive rats.

Items	DS + NS + V ($n = 6$)	DS + NS + C ($n = 6$)	DS + HS + V ($n = 6$)	DS + HS + C ($n = 6$)	F	P
SBP (mmHg)	117.67 ± 1.82	117.17 ± 1.99	166.17 ± 2.01*,†	128.50 ± 2.14*,†,‡	201.299	<0.001
PL_{Na} (mmol/L)	79.03 ± 3.62	76.29 ± 6.21	106.44 ± 4.69*,†	92.90 ± 3.09*,†,‡	27.346	<0.001
UV_{Na} (mmol/L)	37.36 ± 2.03	41.54 ± 5.66	48.78 ± 4.28*,†	59.47 ± 6.41*,†,‡	19.918	<0.001
Scr (μmol/L)	59.34 ± 5.70	55.68 ± 6.32	112.08 ± 4.66*,†	65.30 ± 3.53†,‡	136.119	<0.001

Data are presented as mean ± standard deviation. $^{*}P < 0.05$ compared with DS + NS + V group. $^{†}P < 0.05$ compared with DS + NS + C group. $^{‡}P < 0.05$ compared with DS + HS + V group. C-ANP$_{4–23}$: C-atrial natriuretic peptide (ANP)$_{4–23}$; SBP: Systolic blood pressure; DS + NS + V: Dahl salt-sensitive rats with normal-salt diet and vehicle injection; DS + NS + C: Dahl salt-sensitive rats with normal-salt diet and C-ANP$_{4–23}$ injection; DS + HS + V: Dahl salt-sensitive rats with high-salt diet and vehicle injection; DS + HS + C: Dahl salt-sensitive rats with high-salt diet and C-ANP$_{4–23}$ injection; PL_{Na}: Plasmatic sodium concentration; UV_{Na}: Urinary sodium excretion; Scr: Serum creatinine concentration.

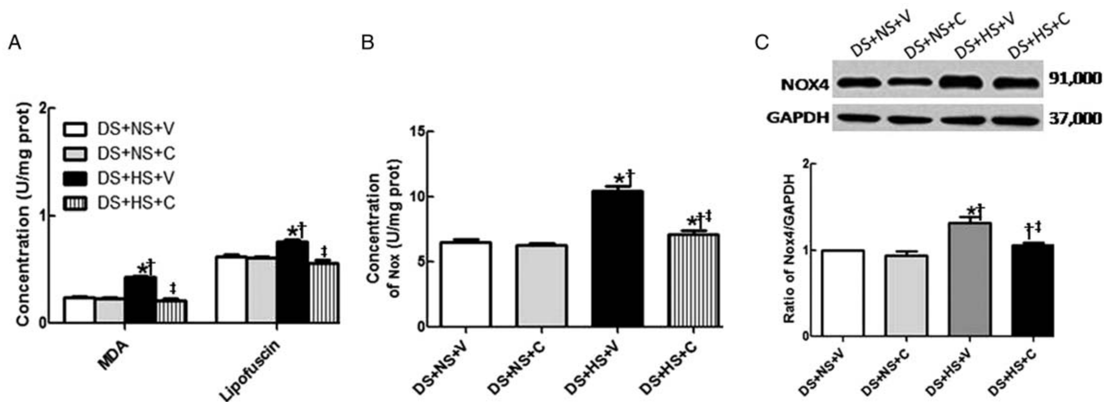

Figure 4: Effects of C-ANP$_{4–23}$ treatment on oxidative stress indexes in kidneys of Dahl salt-sensitive rats. Concentrations of MDA, lipofuscin (A) and Nox (B) in kidneys of Dahl salt-sensitive rats at 6 weeks after a normal-salt or a high-salt diet with vehicle or C-ANP$_{4–23}$ injection. (C) Dahl salt-sensitive rats with a normal-salt or a high-salt diet plus vehicle or C-ANP$_{4–23}$ injection for 6 weeks were subjected to Western blot analysis using antibodies against Nox4. The expression was normalized to the housekeeping protein GAPDH. $^{*}P < 0.05$ compared with DS + NS + V group. $^{†}P < 0.05$ compared with DS + NS + C group. $^{‡}P < 0.05$ compared with DS + HS + V group. C-ANP$_{4–23}$: C-atrial natriuretic peptide (ANP)$_{4–23}$; MDA: Malondialdehyde; Nox: Nicotinamide adenine dinucleotide phosphate oxidase; Nox4: Nicotinamide adenine dinucleotide phosphate oxidase 4; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; DS + NS + V: Dahl salt-sensitive rats with normal-salt diet and vehicle injection; DS + NS + C: Dahl salt-sensitive rats with normal-salt diet and C-ANP$_{4–23}$ injection; DS + HS + V: Dahl salt-sensitive rats with high-salt diet and vehicle injection; DS + HS + C: Dahl salt-sensitive rats with high-salt diet and C-ANP$_{4–23}$ injection; prot: protein.

vehicle ($P < 0.05$). Moreover, C-ANP_{4-23} treatment significantly attenuated the increase of Nox concentration and Nox4 expression in DS rats with HS diet ($P < 0.05$), which was still higher than those with NS diet and C-ANP_{4-23} treatment ($P < 0.05$).

Discussion

The present study reported that the renal ANP-receptor system was involved in the development of hypertension in Dahl rats by increasing ANP production and decreasing the expression of NPR-C receptor in response to high salt. Moreover, the findings revealed that salt-sensitive hypertension was involved in mitochondrial damage in podocytes and the weakness of anti-oxidative function of the renal NPR-C receptor.

DS and DR rats were put on a 0.4% or 8% salt diet for 6 weeks. Salt loading elevated the BP only in DS rats. The expression of the NPR-C in kidneys reduced in DS hypertensive rats, whereas the expression of the NPR-A did not alter. This result was consistent with a previous study by Nagase *et al*.[26] They examined the transcript levels of natriuretic peptide receptors influenced by an HS diet in different tissues. The NPR-C messenger RNA (mRNA) level markedly decreased only in the kidneys of DS rats.[26] The NPR-C in vascular endothelial cells was more sensitive to changes in salt concentrations compared with the NPR-A.[33] This study demonstrated that high salt supplement attenuated the expression of renal NPR-C gene and increased the concentration of serum sodium, which was greater in DS rats than in DR rats. The results suggested a key role of NPR-C in regulating sodium balance and BP in salt-sensitive hypertension. The kidney is one of the major end-organ targets of hypertension. The renal damage increases in both human and experimental salt-sensitive hypertension.[34] The dysfunction of kidneys has been reported to occur even after 2 weeks of HS feeding and becomes worse after 5 weeks.[35,36] In agreement with this finding, the results of the present study indicated that 6 weeks of the HS diet led to the disorder of sodium excretion and mitochondrial damage in podocytes. However, the mechanisms underlying renal damage associated with salt-sensitive hypertension are not well known. Oxidative stress might be one of the most important reasons.[37] The production of ROS is enhanced in various organs of salt-hypertensive rats, including kidneys.[38,39] The mitochondrial electron transport chains and cellular Nox are primary sources of ROS in kidneys. The present study showed a more pronounced increase in the concentration of MDA, lipofuscin, and Nox in kidneys of salt-hypertensive Dahl rats, implying that oxidative stress was stimulated by HS loading. It was also confirmed that ANP served as an anti-oxidant by activating the NO system in SHRs.[40] However, the level of NOS did not differ between the DS strain and DR control strain fed an HS diet in the present study. Taylor and Cowley[13] also reported that NO might not participate in the development of salt-sensitive hypertension.

HS induced a major elevation in Nox-dependent ROS production in both medulla and cortex of kidneys in salt-hypertensive DS rats,[41,42] which might be involved in different pathogenic pathways. Among Nox subtypes, Nox4 is a key source of ROS responsible for kidney damage such as albuminuria and fibrotic glomeruli.[43,44] The glomerular injury was prevented by deleting Nox4.[45] In podocytes, Nox4 is predominately localized in mitochondria.[45] The accumulation of mitochondrial ROS results in mitochondrial dysfunction.[46] Consistent with these results, Das *et al*[47] suggested that mitochondrial Nox4 up-regulation by transforming growth factor-β1 was responsible for ROS production, mitochondrial dysfunction, and apoptosis, in part contributing to the development and progression of glomerular diseases such as diabetic nephropathy. A rich mitochondrial network was identified in podocytes.[48] This *in vivo* study showed that ROS production and expression of Nox4 increased in Dahl hypertensive rats. Also, mitochondrial damage was seen in podocytes of the same animal group. The findings of the present study were a further indication that, except for NPR-C down-regulation, the mitochondrial damage in podocytes might be involved in oxidative stress-induced kidney damage in DS hypertension. Multiple experimental studies have confirmed that renal NPR-C mRNA is predominantly localized in glomeruli, especially in podocytes.[49] Moreover, the marked reduction of NPR-C receptor in podocytes in DS rats may be involved in the development of salt-sensitive hypertension by resulting in the abnormal function of podocytes.[26] Some studies demonstrated that the treatment with C-ANP_{4-23}, an NPR-C receptor-specific agonist, attenuated the enhanced oxidant production, including O_2^-, Nox activity, and expression of Nox4, and then decreased the high BP in SHRs.[28] In this *in vivo* study, C-ANP_{4-23} was found to have an anti-oxidant effect by decreasing MDA, lipofuscin, and Nox-dependent superoxide production, as well as expression of Nox4, in the kidneys of salt-hypertensive Dahl rats. These results provided strong evidence that the NPR-C receptor might be a new therapeutic target for preventing hypertension by decreasing oxidative stress, at least partly through Nox pathway. This study confirmed the anti-oxidative role of a high dose of C-ANP_{4-23} in salt-sensitive hypertension and the related dose-dependent function needs to be further explored.

Chronic salt loading affects the ANP level in plasma and internal organs. However, the results are controversial. Some animal studies reported that high salt intake increased the concentration of plasma ANP and the expression of atrial ANP mRNAs.[11,26] Other studies contradicted that the plasma ANP concentration did not differ in response to an HS diet.[27,50] Relevant findings on human hypertension indicated that an HS diet increased the arterial pressure but decreased the plasma ANP concentration in salt-sensitive African Americans.[51] The conflicting results might be due to different procedures followed in HS experimental models. The present study demonstrated that the ANP concentration in blood and organs generally increased in response to an HS diet in two animal groups. Different from DR, the ANP concentration was elevated in kidneys but not in plasma and heart of DS rats, accompanied by a decrease in the expression of renal NPR-C receptor. Several possible explanations exist for interpreting this finding in salt-sensitive hypertension. First, it is well known that the NPR-C receptor has a major

role as a clearance receptor to remove ligands from the circulation. Gu *et al*[52] demonstrated that local ANP was potentiated by the down-regulation of the NPR-C receptor in preeclampsia. Therefore, a reduction of the NPR-C receptor in DS rats may cause a compensatory effect by reducing the elimination of ligands from the renal circulation. Second, the reduction of renal NPR-C in DS rats may be a primary factor in the mechanism of salt-sensitive hypertension. Accumulating pieces of evidence revealed that NPR-C has multiple effects on the cardiovascular system, overcoming its clearance effect.[12] For example, NPR-C is related to vascular smooth muscle cell hypertrophy through α-Gq protein/phospholipase C-β1 (Gqα/PLCβ1) proteins and ROS-mediated signaling in hypertensive rats.[53] Furthermore, Sun *et al*[11] demonstrated the involvement of potential factors such as growth factors and protein kinases in the HS-diet-induced down-regulation of the expression of NPR-C gene in kidneys. The results of the present study supported that NPR-C served as an anti-oxidant in the kidneys of Dahl hypertensive rats.

The previous observation suggests that ANP modulates BP and exhibits protection against the development of hypertension.[40] Chronic treatment with ANP not only lowered BP but also decreased the damage in the kidneys of male and female SHR rats.[40] Hence, it was speculated that the ANP production in this study might be stimulated in compensation for the NPR-C decrease in the kidney. In addition, renal ANP increase would be associated with a variety of factors such as the angiotensin system.[54] Hence, the mechanism underlying salt-sensitive hypertension needs further investigation.

In conclusion, the present study demonstrated that chronic salt loading induced high BP and renal function damage in Dahl rats, which might be partly related to the mitochondrial injury in podocytes and the weakness of the anti-oxidative function of the local NPR-C receptor. These findings suggested a pivotal role of NPR-C receptor in the development of salt-sensitive hypertension, and $C\text{-}ANP_{4\text{-}23}$ might be a potential drug in the future.

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Conflicts of interests

None.

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