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Intestinal *Cckbr*-specific knockout mouse as a novel model of salt-sensitive hypertension via sodium over-absorption

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ABSTRACT

OBJECTIVES To investigate the value of CCKBR$^{fl/fl}$ villin-Cre mice as a mouse model of salt-sensitive hypertension (SSH).

METHODS In the first part, 2-month-old CCKBR$^{fl/fl}$ villin-Cre mice (CKO) and control CCKBR$^{fl/fl}$ mice (WT) were fed with normal diet (0.4% NaCl) or high salt diet (4% NaCl), separately for 6 weeks. In the rescue study, one week of hydrochlorothiazide or saline injection were treated with the CKO mice fed high salt diet. The blood pressure, biochemical indexes, and the expression of small intestinal sodium transporters (NHE3, NKCC1, eNaC) was detected. The organ injury markers (MMP2/MMP9) and the histopathological changes of kidneys were observed, whereas the changes of duodenal sodium absorption were detected by small intestinal perfusion in vivo.

RESULTS The CCKBR$^{fl/fl}$ villin-Cre mice with high salt intake exhibited high blood pressure, increased duodenal sodium absorption and urinary sodium excretion, and with renal injury. The protein expression of NHE3, NKCC1 and eNaC were also significant increase in the intestine of CKO-HS mice. Treatment with hydrochlorothiazide remarkably attenuated the elevated blood pressure by high salt absorption in the CCKBR$^{fl/fl}$ villin-Cre mice, but no significant histopathological changes were observed.

CONCLUSIONS These results support a crucial role of intestinal *Cckbr* deficiency on SSH development and the diuretic antihypertension effect in CCKBR$^{fl/fl}$ villin-Cre mice. The CCKBR$^{fl/fl}$ villin-Cre mice with the high salt intake may serve as a stable model of salt-sensitive hypertensive induced by sodium overloading.

Hypertension is a multifactorial cardiovascular disease that depends on the interaction of environmental, genetic and behavioral factors.[1] High sodium (Na^+) intake is one of the most significant environmental factors that cause high blood pressure (BP). Salt-sensitive hypertension (SSH), the majority of the hypertensive population, is characterized by an elevated blood pressure (at least 5 mmHg) in response to the sodium intake[2] and is related to an elevated risk of various diseases, such as the stroke and heart failure.[3]

Experimental animal models are a vital tool for disease pathogenesis research and drug development. Salt-sensitive rats were first derived from Sprague-Dawley rats selected with the highest BP response to a high salt diet by Dahl and became the most classic small animal models for examining the kidney, vasculature, and genetic abnormality in SSH.[4] In addition to the rats, mice were more widely used as experimental animal model because of their small size, ease of feeding, clear quality control standards and ease in genetic engineering. Nevertheless, there was no proper mouse model for SSH. Although C57BL/6J has been found to be salt-sensitive, the elevation of blood pressures after high salt diet was unstable or insignificant. Several transgenic mice showed salt-sensitive (SS) phenotypes in response to the regulation of renal sodium reabsorption,[5] which not be

able to more comprehensively imitate SSH diseases and not widely used. It is of necessity to build a stable and heritable model of salt-sensitive hypertension in mice.

In mammals, circulating sodium is filtered and reabsorbed by the kidney,[6] but most of the sodium ingested orally is absorbed by the gut, mainly by the small intestines.[7] The sodium anomalous absorption in the gastrointestinal system induced animal model is rarely mentioned. Na^+/H^+ exchanger 3 (NHE3) at the intestinal brush border occupies the majority of sodium absorbed both in the basal state and in the later stage.[8] Evidence suggests that selective deletion of *Cckbr* increases BP and sodium excretion in male and female mice fed with a high salt diet by stimulating NHE3 activity and transport.[9] According to the Genome-wide association study (GWAS), it was reported that the chromosomal loci of *Cckbr* (11P15.5) are in connection with human essential hypertension[10] and global knockout of *Cckbr* in mice showed salt-sensitive hypertension.[11] Thus, we hypothesized that the intestinal *Cckbr* may be a novel genetic modification target in mice to generate stable salt-sensitive hypertension mouse models.

In this study, we demonstrated that intestinal *Cckbr*-specific knockout mice (CCKBR$^{fl/fl}$villin-Cre) with high salt intake exhibited high blood pressure, increased sodium absorption and excretion, and kidney injury. Hydrochlorothiazide treatment significantly rescued the elevated blood pressure induced by high salt intake in CCKBR$^{fl/fl}$villin-Cre mice, confirming that CCKBR$^{fl/fl}$villin-Cre mice could be a novel salt-sensitive hypertension animal model and would be of great value in drug research and preclinical evaluations related to intestinal sodium absorption.

METHODS

Animals

Conditional intestine-specific *Cckbr* knockout mice (CCKBR$^{fl/fl}$villin-Cre) were generated and provided from Beijing View Solid Bio technology Company (China). All experimental procedures were carried out following the guidelines of our Institutional Animal Care and Use and Committee (ILAS-YZW19002).

In the first part, the CCKBR$^{fl/fl}$villin-Cre mice and wild-type mice (CCKBR$^{fl/fl}$) in week 8 were fed with normal salt diet (0.4% NaCl) or high salt diet (4% NaCl) for six weeks separately, and then divided into four groups (WT-NS, WT-HS, CKO-NS, and CKO-HS). In the second part, the CCKBR$^{fl/fl}$villin-Cre mice fed with high salt diet (4% NaCl) for six weeks were randomly split into two groups: the hydrochlorothiazide (25 mg/kg per day, one week) injection group (CKO-HS+HCTZ) and the saline (0.9%) injection group (CKO-HS).

Materials

Antibodies against NHE-3 (sc-136368), ENaC (sc-25354) and Villin antibody (sc-58897) were obtained from Santa Cruz Biotechnology Inc. Besides, antibodies against NKCC1 (13884-1-AP), Fibronectin 1 (66042-1-Ig), GAPDH (60004-1-Ig) were purchased from Proteintech Group, Inc. Protein concentration was quantified with the use of a modified BCA Protein Assay Kit (P0010) purchased from Beyotime Biotech Inc (China). Sodium Assay Kit (MAK247) was from Sigma Aldrich (USA).

Radio Telemetry Recording Blood Pressure Measurement

BP was measured in conscious mice based on the radio telemetry recording method following our previously published procedure.[12] The mice were exposed to the abdominal aortae via a midline incision under 2% isoflurane (inhaled) anesthesia. A BP sensor (model PTA-M) was inserted into the aorta to femoral bifurcation, extending up to the renal artery alongside the femoral artery. Then, a transmitter was stitched to the inside of the abdominal muscle wall. In addition, all incisions were sutured and carprofen (5 mg/kg) was performed for analgesia. After the surgery, the mice were housed separately. A Stellar Telemetry System (TSE Systems) was applied to acquire and document the continuous arterial pressure waveform. Moreover, the acknowledge software program was adopted for configuring the Stellar experiments and analyze BP.

At the end of the treatments, BP was also measured directly from a catheter inserted into the carotid artery, throughout the left carotid artery, under isoflurane (2%, inhaled) anesthesia. Blood, urine, kidneys, hearts and intestines were obtained after the mice were euthanized with 2% sodium pentobarbital.

Enzyme-linked Immunosorbent Assay (ELISA)

Electrolytes (sodium, potassium, chlorine), creatinine and hypertensive hormones (renin, angiotensin II, and aldosterone) in the serum and urine from WT and CKO mice were tested by commercial ELISA kits from Xinfan biotechnology Co., Ltd (Shanghai, China) in line with the manufacturer's instructions.

Histological and Immunohistochemical Staining

The organs in each mouse were harvested, formalin-fixed, and paraffin-embedded, which were cut into 4-μm sections, incorporating a 4-chamber view of the heart. The paraffin sections were exposed to hematoxylin-eosin (HE) staining and Masson trichrome staining (Huige biotechnology Co., Ltd, Beijing, China) to observe the pathological variations and the collagen deposition, respectively. The immunohistochemistry staining of fibronectin 1 was performed to examine the extent of fibrosis. The paraffin sections were deparaffinized with a series of xylene and alcohol. In addition, antigen unmasking was conducted by pressure-cooker treatment in a sodium citrate buffer. Sections were blocked using goat serum for 1 h at room temperature and then stained with primary antibodies at 4 °C overnight. The sections were rinsed with PBS and then subject to incubation at room temperature for 1h with secondary antibodies. After washing with PBS, the sections were next stained with chromogen DAB staining and haematoxylin counters. As for immunofluorescence, 4'6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen, CA) was applied to indicate nuclei and the secondary antibody (NHE3) was conjugated with Alexa Fluor 488 phalloidin. All of the staining results were visualized under an Olympus IX70 microscope equipped with an Olympus DP70 digital camera.

Western blot

Brush borders of mouse small intestine and mouse kidney or heart homogenates were prepared for western blot following our previously published procedure.[11] The samples were adjusted to the identical protein concentration. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and later probed with primary antibodies and appropriate horseradish peroxidase conjugated secondary antibodies. As for images, they were visualized by means of chemiluminescence (Tanon 5500).

Intestinal Perfusion Experiment

A fresh perfusate consisting of normal salt (133 mmol/L) or high salt (177 mmol/L) in PBS was prepared at the beginning of the experiment. The small intestinal sac of mice was applied as an in vivo model to investigate the effect of high salt levels on intestinal sodium absorption. Mice were anesthetized by the intraperitoneal injection of 2.5% tribromoethanol (0.2 mL/10 g) and positioned prone on the surgical table, with the intestine exposed through a mid-line incision. The perfusion pipe, filled with 2 mL perfusate beforehand, was inserted into both ends of the same section of jejunum (the length of the jejunum section was about 6 cm), and immobilized with suture. Hence, an extracorporeal circulation was established with an infusion pump at a pump flow rate of 23.6 mL/min. Sodium concentrations were detected in both the intestinal perfusion fluid and the intestinal epithelial cells scraped and lysed from the villi after 15-30 min of circling in each mouse jejunum.

Statistics

All quantitative data are shown to be mean ± SD and analyzed by adopting SPSS software, version 20.0. For comparisons among > 2 groups, one-way ANOVA followed by Turkey test was performed. *P*-value < 0.05 was perceived as statistically significant.

RESULTS

CCKBR$^{fl/fl}$ Villin-Cre Mice Showed Salt-Sensitive Hypertension During High Salt Diet

Compared with the WT group, CCKBR$^{fl/fl}$villin-Cre mice showed increased blood pressure (103 ± 5 *vs.* 88 ± 3 mmHg) during normal salt diet ($P < 0.05$). High blood pressure was induced in both CKO and WT mice induced after six weeks of high salt diet, suggesting that the CCKBR$^{fl/fl}$villin-Cre background is salt-sensitive. As shown in the two high salt groups, intestine specific *Cckbr* knockout significantly enlarged the elevation in BP caused by HS

diet compared with WT-HS group (Figure 1A). BP from the carotid artery was also measured constantly in conscious mice by radiotelemetry. Systolic BP was initially increased by the third week (123 ± 10 mmHg) and was elevated until the seventh week (137 ± 12 mmHg) of high salt (4% NaCl) diet, which was remarkably higher than in that in WT mice (from 94 ± 2 to 112 ± 11 mmHg) (Figure 1B).

The renin-angiotensin-aldosterone system makes a central role in the pathogenesis of SSH, and therefore the concentration of RAAS in the serum of each mouse was tested. The increased pattern in renin, angiotensin II, and aldosterone were matched with elevated blood pressure in four disparate groups (Figure 1C-E), which turned into another proof for the generation of the salt-sensitive hypertension animal model.

Intestine-specific *Cckbr* Deficiency Increased Intestinal Sodium Absorption

To verify the role of intestinal sodium absorption in CCKBR$^{fl/fl}$villin-Cre mice, luminal sodium concentration in CKO and WT mice with or without high salt intestinal perfusion *in vivo* was measured. The result shows that a high salt perfusion decreases sodium concentration in the duodenal cavity (Figure 2A), which increases in duodenal epithelial cells (Figure 2B), with intestinal *Cckbr* deficiency significantly intensifying those results. High sodium absorption induced by high salt intake in duodenal epithelial cells in CKO-HS is notably enlarged than that in WT mice (Figure 2B). Sodium absorption and excretion should be balanced in body. In order to further certify intestinal *Cckbr* deficiency promotes sodium absorption, we measured the urinary sodium concentration. We found that urinary sodium, and chloride (Figure 2C and 2D), instead of potassium (Figure 2E), were higher in CCKBR$^{fl/fl}$ villin-Cre mice with high salt diet, which indicated that more sodium was absorbed by intestinal *Cckbr* deficiency. These results confirmed that the elevated intestinal sodium absorption plays an essential function in salt-sensitive hypertension in CKO mice.

Intestine-specific *Cckbr* Deficiency Increased Sodium Transporters Expression

To probe the mechanism of sodium absorption in the intestine of CKO mice, the expression of several

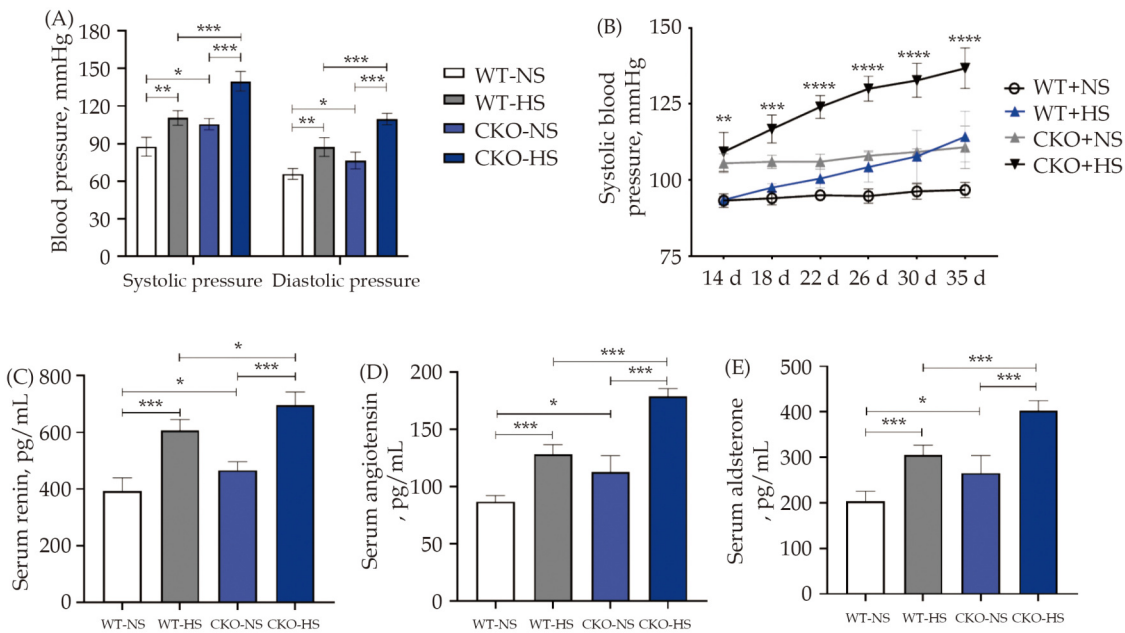

Figure 1 The blood pressure and serum levels of renin, angiotensin II and aldosterone were enhanced by high salt diet in CCKBR$^{fl/fl}$ villin-Cre mice. (A): The systolic blood pressure and diastolic blood pressure of CKO-HS group were notably higher when compared with those in the other three groups; (B): the systolic blood pressure gradually increased with elevated salt feeding; (C-E): The levels of renin, angiotensin II and aldosterone in the CKO-HS group were also evidently higher than those in the remaining three groups (*P < 0.05, **P < 0.01, ***P < 0.001, n = 6 per group, one-way ANOVA, Tukey test; values are expressed as mean ± SD). WT was wild-type mice (CCKBR $^{fl/fl}$); CKO was intestine-specific *Cckbr* knockout mice (CCKBR$^{fl/fl}$villin-Cre); NS represented the normal salt diet; HS denoted the high salt diet.

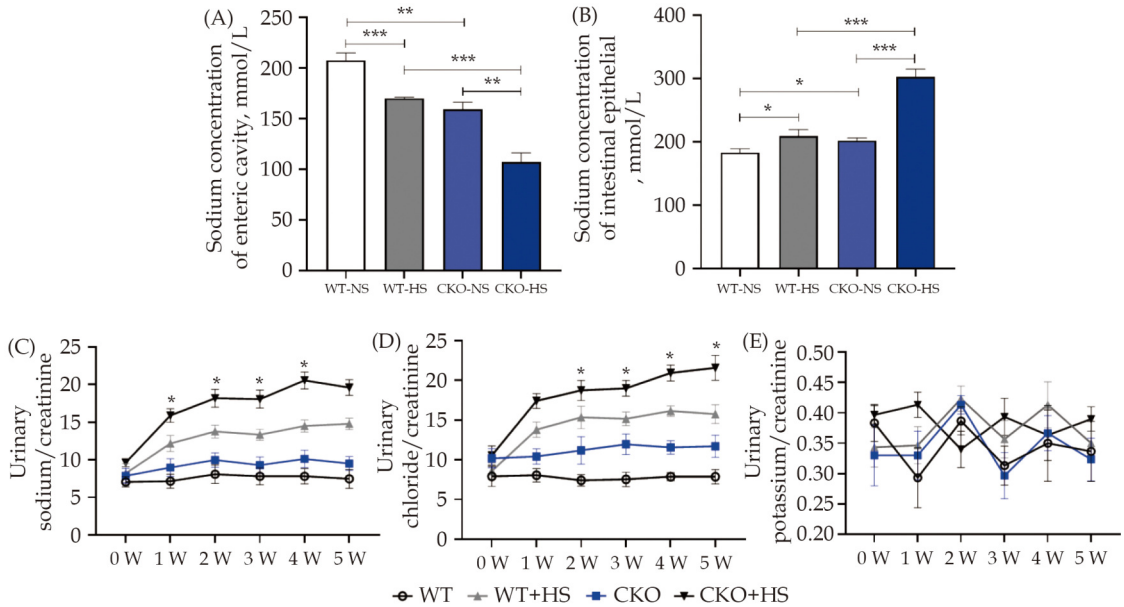

Figure 2 Intestinal sodium absorption elevated in CCKBR$^{fl/fl}$villin-Cre mice. (A): Concentration of sodium in the intestinal cavity; (B): concentration of sodium in the intestinal epithelial cells; (C): the ratio of urinary sodium to creatinine; (D): the ratio of urinary chloride to creatinine; and (E): the ratio of urinary potassium to creatinine (*P < 0.05, **P < 0.01, ***P < 0.001, n = 5 per group, one-way ANOVA, Tukey test; values are shown to be mean ± SD).

critical sodium transporters in intestine was measured. The results showed that NHE3 was up-regulated in CKO mice and continuously elevated by high salt diet (CKO-HS), which was consistent with our former discovery(Figure 3A and C).[9] Furthermore, it was found that extra sodium intake also contributed to the increase of the expression of eNaC and NKCC1 in small intestine, which was markedly accentuated in high salt fed CCKBR$^{fl/fl}$villin-Cre mice (Figure 3A, 3B and 3D). The immunofluorescence image showed that CCKBR$^{fl/fl}$villin-Cre mice with high salt diet had increased NHE3 expression in the surface of intestine epithelial cells (Figure 3E).

Intestine-specific *Cckbr* Deficiency Aggravated Renal Injury

Chronic excessive BP causes organ damage in renal and cardiovascular systems.[13] Compared with the wild-type mice fed with a high salt diet (WT-HS), interstitial fibrosis and collagen deposition were particularly evident in the kidney of CCKBR$^{fl/fl}$villin-Cre mice fed with high salt diet (CKO-HS) (Figure 4A). The organ injury markers, MMP (matrix metalloproteinase)2 and MMP9 were significantly increased in the kidney (Figure 4B), but no difference was found in the heart (Figure 4C) in the CKO-HS group.

Intestine-specific *Cckbr* Deficiency Responds to Hydrochlorothiazide Treatment

To test the applicability of the CCKBR$^{fl/fl}$villin-Cre mice as a SSH mouse model, hydrochlorothiazide (HCTZ) was utilized to treat the hypertensive CCKBR$^{fl/fl}$villin-Cre mice after six weeks during high salt diet. It turned out that HCTZ could significantly prevent the high salt induced BP in CCKBR$^{fl/fl}$villin-Cre mice. Systolic blood pressure starts to decrease on the third day of injection (131 ± 5 mmHg) and continued to decline until the end of the week, which were distinctively lower than that of the CKO-HS mice (123 ± 4 *vs.* 141 ± 3 mmHg) (Figure 5A). HCTZ excluded excess sodium accumulation to lower blood pressure, again demonstrating that the intestine sodium excessive absorption is a crucial mechanism in salt-sensitive CCKBR$^{fl/fl}$villin-Cre mice.

Along with the anti-hypertensive treatment, the activation of the renin-angiotensin-aldosterone system was terminated. Though there were no differences in serum renin and angiotensin II between the CKO-HS and the CKO-HS+HCTZ (Figure 5C & 5D), the serum aldosterone has already begun to decrease after the HCTZ treatment (Figure 5B). Also, the natriuresis increased (Figure 5F) because of the

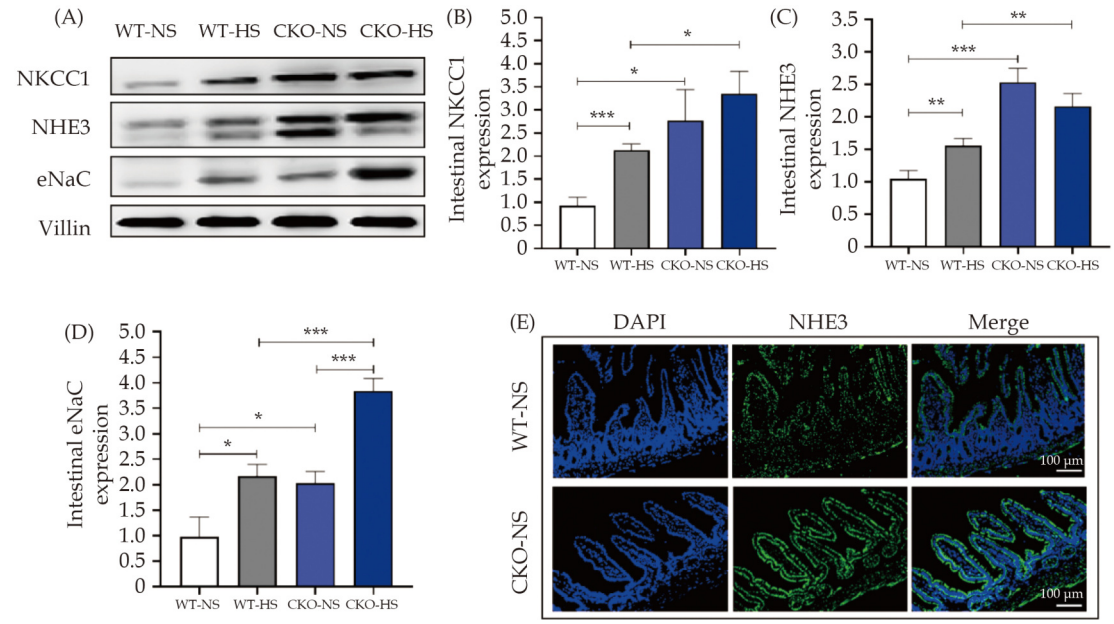

Figure 3 Expression of sodium transporters in CCKBR$^{fl/fl}$villin-Cre mice was fed with high salt diet. (A): Expression of NKCC1, NHE3 and eNaC in the intestine; (B): quantification of NKCC1 expression in intestine; (C): quantification of NHE3 expression in intestine; (D): quantification of eNaC expression in intestine; and (E): expression and location of NHE3 (green) in the mouse duodenum (*P < 0.05, **P < 0.01, ***P < 0.001, n = 5 per group, one-way ANOVA, Tukey test; values are denoted to be mean ± SD).

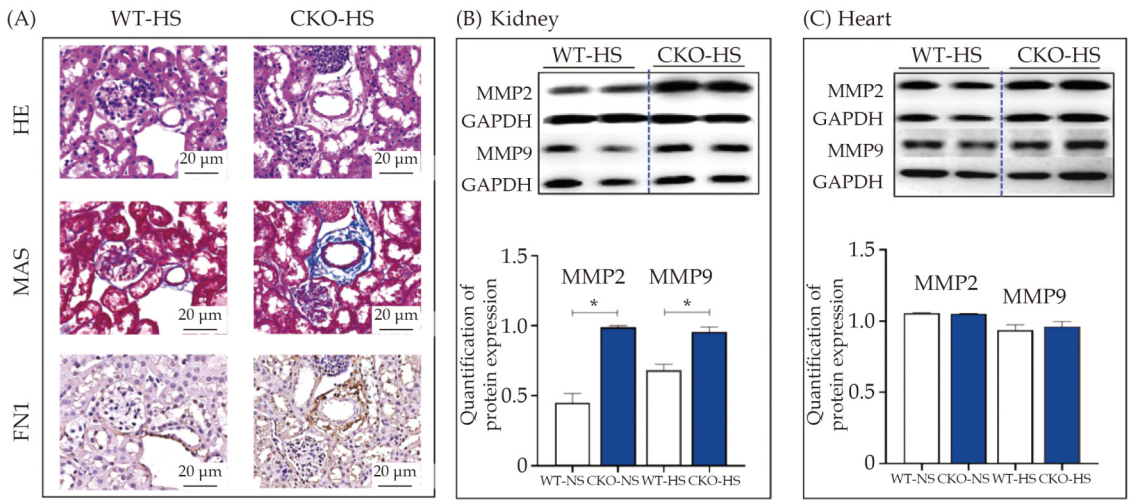

Figure 4 Renal fibrosis aggravated in CCKBR$^{fl/fl}$villin-Cre mice fed with high salt diet. (A): HE, Masson's trichrome stain, and fibronectin 1 stain in the kidney; (B): MMP2 and MMP9 protein expressions were quantified by the western blot in the kidney (*P < 0.05 vs WT-HS, n = 5 per group, one-way ANOVA, Tukey test; values are expressed as mean ± SD); (C): MMP2 and MMP9 protein expressions were quantified by the western blot in heart. FN1: fibronectin 1; MAS: Masson's trichrome stain; MMP2: matrix metalloproteinase-2; MMP9: matrix metalloproteinase-9.

diuretic effect, while the chloride and potassium have not varied compared to those in the placebo group (CKO-HS) (Figure 5G & 5H).

In both groups, organ damage was detected. The ratio of blood urea nitrogen to creatinine showed a slight decrease, but no statistical significance was detected in the CKO-HS+HCTZ group (Figure 5E). The morphology of the kidney and the renal fibrosis, indicated by histological and immunohistochemical staining, shared similarity in both CKO-HS and CKO-HS+HCTZ groups as well (Figure 5I).

DISCUSSION

In our study, we constructed a novel salt-sensitive hypertension model by conditionally knocking

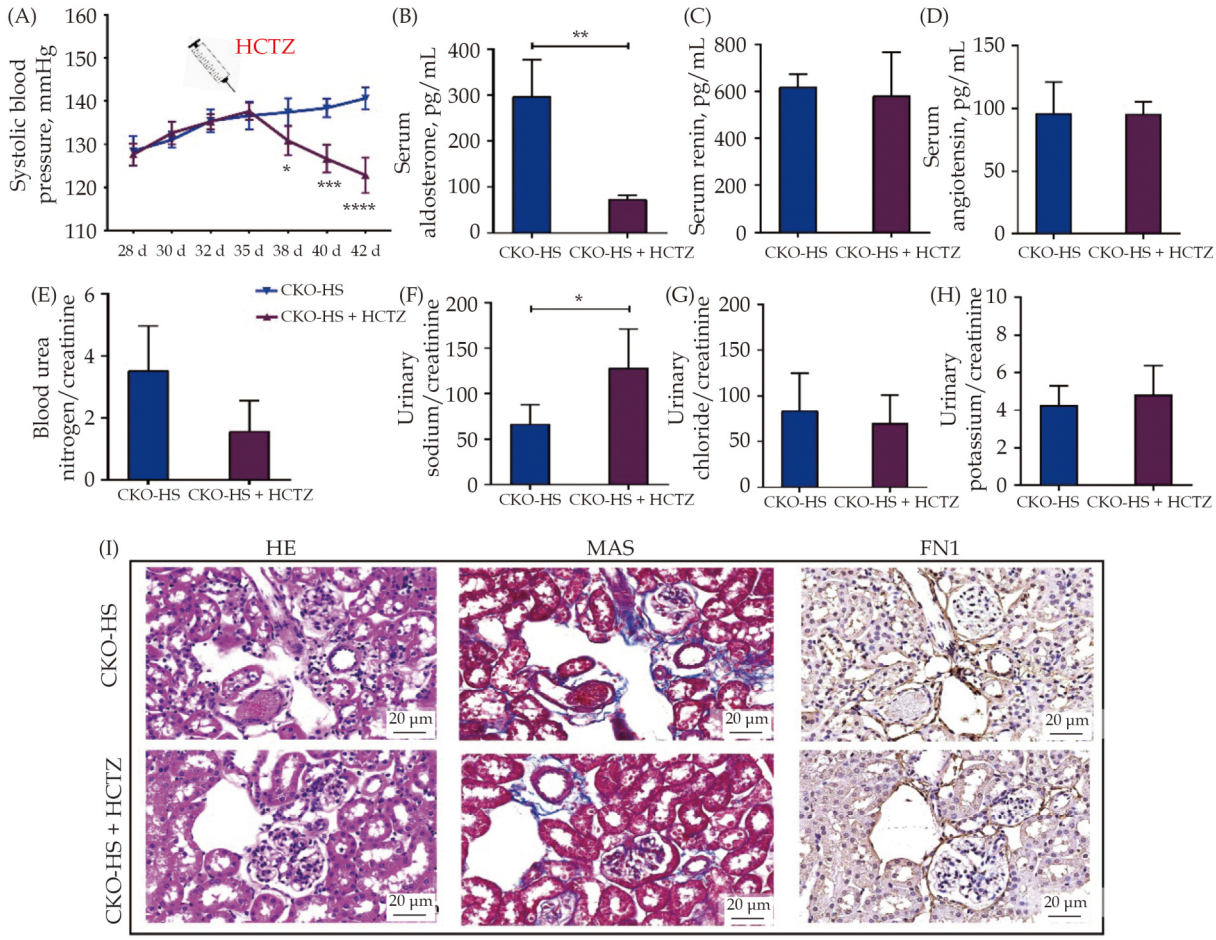

Figure 5 The hydrochlorothiazide (HCTZ) treatment decreased SBP, inhibited aldosterone, and increased natriuresis, but failed to improve the kidney damage. (A): The systolic blood pressure continued to decline after the HCTZ injection; (B-D): represented levels of serum renin, angiotensin II, and aldosterone in CKO-HS and CKO-HS+HCTZ mice; (E-H): represented ratios of sodium, potassium, chloride, and blood urea nitrogen to creatinine; (I): HE denoted Masson's trichrome stain, and fibronectin 1 stain in the kidney (*P < 0.05, **P < 0.01, ***P < 0.001 *vs.* CKO-HS, n = 5 per group, one-way ANOVA, Tukey test. Values are shown to be mean ± SD).

out the intestinal *Cckbr* gene (CCKBR$^{fl/fl}$ villin-Cre mice). Our study highlighted: (1) the significance of excessive sodium retention in the development of SSH via intestine *Cckbr* dysfunction; and (2) CCKBR$^{fl/fl}$ villin-Cre mice as stable model maximally mimics the clinical phenotype of human salt-sensitive hypertension, which filling a gap of mouse models in abnormal intestinal sodium absorption induced SSH.

Loss of the main sodium-hydrogen exchanger (NHE3) significantly reduces intestinal sodium transport,[14,15] while it brings with it significant side effects such as severe diarrhea.[16–18] Dominguez, *et al*[19]. also found that the survival rate of NHE3 intestine-conditional knockout mice was approximately 0. In addition to NHE3, eNaC-mediated electrogenic Na^+ absorption is important for fluid and electrolyte absorption in the distal colon.[20,21] Decreased eNaC activity is involved in the pathogenesis of diarrhea.[22] Thus, the use of NHE3/eNaC as the target to regulate intestinal sodium absorption and to construct SSH models is not appropriate and stable. Other studies by using genetically modified animal models apply aldosterone/salt treatment to induce hypertension, but either aldosterone or high salt loading alone is considered insufficient for significant BP elevation.[23,24] Therefore, indirectly inhibition of NHE3/eNaC by specific knockout intestinal *Cckbr* become a promising strategy to build an ideal SSH mouse animal model.

In our study, stable salt-sensitive hypertensive phenotypes developed in CCKBR$^{fl/fl}$ villin-Cre mice

after six weeks high salt intake, including more severely damaged kidney fibrosis in context of excessive salt compared to normal diet. Excessive sodium passes through the intestinal tract into the circulatory system leading to salt-sensitive hypertension and organ damage.[25] Disturbances in sodium balance will lead to clinical situations of volume depletion or overload, and will lead to arterial hypertension and heart failure.[26] Patients with hypertension, particularly those with salt-sensitive hypertension, shows renal arteriolar damage and lymphocyte accumulation in early histological studies and with an associated increase in renal end-organ damage.[27] In our study, high salt fed CCKBR$^{fl/fl}$ villin-Cre mice showed serious renal fibrosis and collagen deposition, which well mimics the clinical phenotype of organ damage.

Mechanism study validated the increased expression of NHE3 in the brush border membrane of the small intestine when CCKBR$^{fl/fl}$ villin-Cre mice were given a high salt diet. Other intestinal sodium transporters, eNaC and NKCC1, were also significantly elevated by the high salt diet. Evidence showed that activation of p38 mitogen-activated protein (MAP) kinase, followed by phosphatidylinositol 3-kinase (PI3-K) and protein kinase C (PKC), leads to brush border membrane eNaC and NKCC1 stimulation.[28,29] In human renal proximal tubule cells and brush border membrane of jejunum, *Cckbr* inhibits Na^+/K^+-ATPase and NHE3 activity via the PI3-K and PKC pathway.[9,30] Therefore, whether intestinal *Cckbr* regulate induced sodium transporter synergistic reaction through PI3-K/PKC-dependent pathway still need to further investigations.

Diuretic antihypertensive experiment shows that hydrochlorothiazide (HCTZ) treatment significantly reduced high salt induced blood pressure and aldosterone level. As the first hormone to respond to the stimulatory signal (HCTZ), decreased aldosterone indicate again that impaired sodium overload is the most important mechanism in CCKBR$^{fl/fl}$ villin-Cre mice and the separated mechanism between aldosterone and the other two elements in RAAS system.[31] Aldosterone also promotes sodium reabsorption as an electrogenic sodium transport through the amiloride-sensitive epithelial sodium channel (eNaC).[32] HCTZ treatment may further down regulate sodium absorption by aldosterone induced eNaC activation in CCKBR$^{fl/fl}$ villin-Cre mice. In additional, the histopathological staining showed no difference in renal impairment in either CKO-HS mice with or without HCTZ, which shows difference to few study in Dahl salt-sensitive rats as they have treated for over 6 weeks.[33] Thus, long-time treatment may be performed in further studies. Combined phenotype and antihypertensive effect of diuretic in CCKBR$^{fl/fl}$ villin-Cre mice, our CCKBR$^{fl/fl}$ villin-Cre mouse model was highly consistent with the clinical manifestations of salt-sensitive hypertension. This model provides a well-characterized tool for an in-depth study of the mechanism of sodium absorption in the gut and for the development of targeted drugs of salt-sensitive hypertension.

In summary, CCKBR$^{fl/fl}$ villin-Cre mice with high salt diet can steadily develop salt-sensitive hypertension. The CCKBR$^{fl/fl}$ villin-Cre mice with the high salt intake may serve as a stable model of salt-sensitive hypertensive induced by sodium overloading, and would be suitable for mechanistic studies and drug screening for the treatment of SSH, optimizing the defect of unstable SS in C57BL/6J mice.

DISCLOSURE

Conflicts of Interest

None.

Foundings

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