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Genetic knockout and pharmacologic inhibition of NCX2 cause natriuresis and hypercalciuria

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ABSTRACT

The Na^+/Ca^{2+} exchanger (NCX) is a bidirectional transporter that is controlled by membrane potential and transmembrane gradients of Na^+ and Ca^{2+}. Although two isoforms of NCX1 and NCX2 are coexpressed on the basolateral membrane of the distal nephron, the functional significance of these isoforms is not entirely clear. Therefore, we used NCX1- and NCX2-heterozygote knockout mice (KO) and their double KO, as well as isoform-selective NCX inhibitors, to determine the roles of NCX isoforms in urine formation and electrolyte excretion in mice. NCX inhibitors, particularly NCX2-sensitive inhibitors, caused a dose-dependent natriuresis and in a higher dose, moreover, hypercalciuria. Consistently, NCX1-KO possessed normal renal function similar to wild-type mice (WT), whereas NCX2-KO and double KO exhibited moderate natriuresis and hypercalciuria. Notably, renal responses to YM-244769 were equivalently observed in NCX1-KO and WT, but disappeared in NCX2-KO and double KO. Thus, functional inhibition of NCX2 initially causes natriuresis, and further inhibition of NCX2 produces hypercalciuria, suggesting that the functional significance of NCX2 lies in Na^+ and Ca^{2+} reabsorption of the kidney.

1. Introduction

The kidney regulates the maintenance of body fluid and electrolyte balance. The blood is filtrated by the glomeruli and the electrolytes are reabsorbed from the pro-urine in several tubular segments, comprising the proximal, Henle's, and distal parts of the nephron. The distal part of the nephron, primarily in the distal convoluted tubule (DCT) and connecting segment (CNT), plays a pivotal role in the final tuning of electrolyte excretion by various ion transporters [1,2]. Several endogenous factors and hormones tightly control these ion transport processes.

The Na^+/Ca^{2+} exchanger (NCX) is expressed in the distal part of the nephron [3,4]. NCX is a bidirectional transporter controlled by membrane potential and transmembrane gradients of Na^+ and Ca^{2+} [5,6]. The mammalian NCX forms a multigene family encompassing three isoforms: NCX1, NCX2 and NCX3. Several splicing variants were identified for NCX1 and NCX3, whereas no alternate splicing variants were detected for NCX2. NCX1 is ubiquitously expressed in several tissues, including the heart, brain and kidney, whereas the expression of NCX2 and NCX3 is abundant in the brain and skeletal muscle [7]. In this study, we initially found that NCX2, as well as NCX1, is expressed in the basolateral membrane of the distal nephron. Although NCX is thought to contribute to the reabsorption of Ca^{2+} [1–4], the functional significance of the existence of different NCX isoforms in the kidney is not entirely understood. Therefore, the aim of the present study is to determine the physiological roles of NCX1 and NCX2 on renal function.

Specific NCX inhibitors are very useful for analyzing the physiological roles of NCX isoforms [8–10]. Pharmacologic actions of NCX inhibitors have been studied extensively since the development of KB-R7943, a prototype of benzyloxyphenyl NCX inhibitors, in 1996 [11]. Furthermore, recent experiments are actively progressing with more selective inhibitors, i.e., SEA0400 and YM-244769. YM-244769, as well as KB-R7943, inhibits NCX3 three fold more potently than NCX1 and NCX2, but at a high dose, these inhibitors almost completely suppress all three NCX isoforms [10,12]. On the other hand, SEA0400 specifically inhibits NCX1; SEA0400 only slightly suppresses NCX2 and has no effect on NCX3 [13]. In this study, we used these three NCX inhibitors to

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determine the functional roles of NCX isoforms in urine formation and electrolyte excretion in mice.

In addition, genetic knockout of each NCX isoform in mice is also a very useful strategy for studying the function of targeted NCX isoform. We created NCX1- and NCX2-heterozygote knockout mice (KO), in which the targeted NCX isoform is specifically reduced by about half, although both of these homozygote knockout mice die before birth [14,15]. NCX1-KO exhibit unique features, such as resistance to myocardial and renal ischemia [16,17] and salt-resistance to hypertension [18], suggesting that mild inhibition (~50%) of NCX1 may possess beneficial effects on cardiovascular and renal diseases [9,10]. On the other hand, NCX2-KO display altered colonic motility with decreasing acetylcholine release onto the myenteric neurons, suggesting the physiological role of NCX2 in intestinal motility [19]. In this study, to verify the functional significance of the existence of different NCX isoforms in the kidney, we compared urine formation, electrolyte excretion, and pharmacologic responses in wild-type mice (WT), NCX1-KO, NCX2-KO and their double KO.

2. Materials and methods

2.1. Drugs

YM-244769 (N-(3-aminobenzyl)-6-{4-[(3-fluorobenzyl)oxy] phenoxy} nicotinamide) and SEA0400 (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline) were synthesized by procedures described previously [20,21], and were finally identified with ^{1}H NMR spectra and Mass spectra. KB-R7943 (2-[2-[4-(4-nitrobenzyloxyl)phenyl]ethyl] isothiourea methanesulfonate) was provided by Nippon Organon (Osaka, Japan). Trichlormethiazide was purchased from Wako Pure Chemical (Osaka, Japan).

2.2. Animals

The experimental designs and all procedures were conducted in accordance with the Animal Care Guidelines of the Animal Experimental Committees of Fukuoka University. Male C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan). NCX1-KO and NCX2-KO were generated as reported previously [14,15]. All mice used were males and they were housed under diurnal lighting conditions (light period 8:00 a.m. to 8:00 p.m.) and allowed access to normal raw chow and plain drinking water *ad libitum*. Systolic blood pressure was measured using a programmable tail-cuff sphygmomanometer MK-2000 (Muromachi, Tokyo, Japan).

2.3. Blood and urine biochemical analysis

Blood was drawn from the aorta under light anesthesia and centrifuged to measure plasma parameters. Mice were kept in metabolic cages for collection of 24-h urine. Plasma and urine Na^+, K^+, and Cl^- concentrations were measured using automated electrolyte analyzer (Jokoh, Tokyo, Japan). Plasma and urine Ca^{2+} and creatinine (Cr) concentrations were measured by commercial kits (Wako Pure Chemical Industries, Osaka, Japan).

2.4. Real-time PCR analysis

Total RNA from kidney cortex of each mouse strain was isolated using on RNeasy Mini kit (Qiagen, Hilden, Germany), and it was reverse-transcribed with a QuantiTect Reverse Transcription kit (Qiagen). Quantitative real-time PCR was performed using a QuantiTect SYBR green PCR kit (Qiagen), and analyzed on StepOne or 7500 Real-time PCR System (Applied Biosystems, CA, USA) according to manufacturer's protocol. Primers targeting the genes of interest and the housekeeping gene, namely glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as an endogenous control, were used (see Supplemental Table 1).

2.5. Immunohistochemistry

Frozen kidney cortex sections (16-μm) were fixed with acetone and incubated with primary antibody [polyclonal anti-NCX1 (1:50), monoclonal anti-NCX2 (1:100), polyclonal anti-TRPV5 (1:50) (Santa Cruz Biotechnology, CA, USA), or polyclonal anti-NCC (1:200) (Chemicon, CA, USA)] for 30 min and then with Alexa Fluor 488-labeled donkey anti-rabbit IgG (1:200) or Alexa Fluor 546-labeled goat anti-mouse IgG (1:200) (Molecular Probes, OR, USA) for 30 min. Rabbit polyclonal and mouse monoclonal antibodies against NCX1 and NCX2 were produced as described previously [22]. The immunofluorescence pictures were then taken with a confocal laser-scanning microscope FV1000-D (Olympus, Tokyo, Japan).

2.6. Statistical analysis

Data are presented as the means ± S.E.M. Statistical comparisons were made using a one- or two-way ANOVA followed by a Student's *t*-test, Dunnett's test, or Tukey's test. $P < 0.05$ was considered statistically significant. All analyses were performed using the StatFlex software ver.6 (Artek, Osaka, Japan).

3. Results

3.1. Renal expression of NCX isoforms in WT and NCX-KO mice

We initially examined the expression and localization of NCX1 and NCX2 isoforms in the kidneys of wild-type C57BL/6J mice using double immunostaining. As shown in Fig. 1A, immunofluorescence of NCX1 was observed in the basolateral membrane of the distal nephron as reported previously [3,4]. We found that NCX2 was colocalized with NCX1 within the similar portion of the distal nephron. Immunofluorescence of NCX2 was observed within the nephron segments in which TRPV5 (transient receptor potential channel subfamily V member 5) and NCC (Na^+–Cl^- cotransporter) were expressed, although they were detectable in the apical membrane of the distal nephron (Fig. 1A). It has been reported that TRPV5 and NCC were distributed in both the late DCT and CNT and in the early and late DCTs, respectively, of mouse distal nephron [4,23]. The NCX2-positive segment almost completely overlapped with the TRPV5-positive segment, whereas it partially overlapped with the NCC-positive segment, implying that NCX1 and NCX2 isoforms are distributed primarily in the late DCT and CNT segments.

We previously generated NCX1-KO and NCX2-KO (heterozygotes) as an animal model for a loss-of-function of NCX isoforms [14,15], although both of these homozygotes die before birth. NCX1-KO and NCX2-KO were apparently normal in survival, gross physical appearance, and organ morphology for 1 year. In the kidneys from NCX1-KO, NCX2-KO, and their double KO, the expression of targeted NCX isoform was specifically reduced by ~50% (Fig. 1B). On the other hand, the mRNA levels of other ion transporters were not significantly altered in the kidneys of NCX1-KO and NCX2-KO (see Supplemental Fig. 1).

3.2. Renal effects of NCX inhibitors in WT mice

To determine the roles of NCX1 and NCX2 isoforms on renal function, we orally administered specific NCX inhibitors (YM-244769, SEA0400 and KB-R7943) to wild-type C57BL/6J mice. As

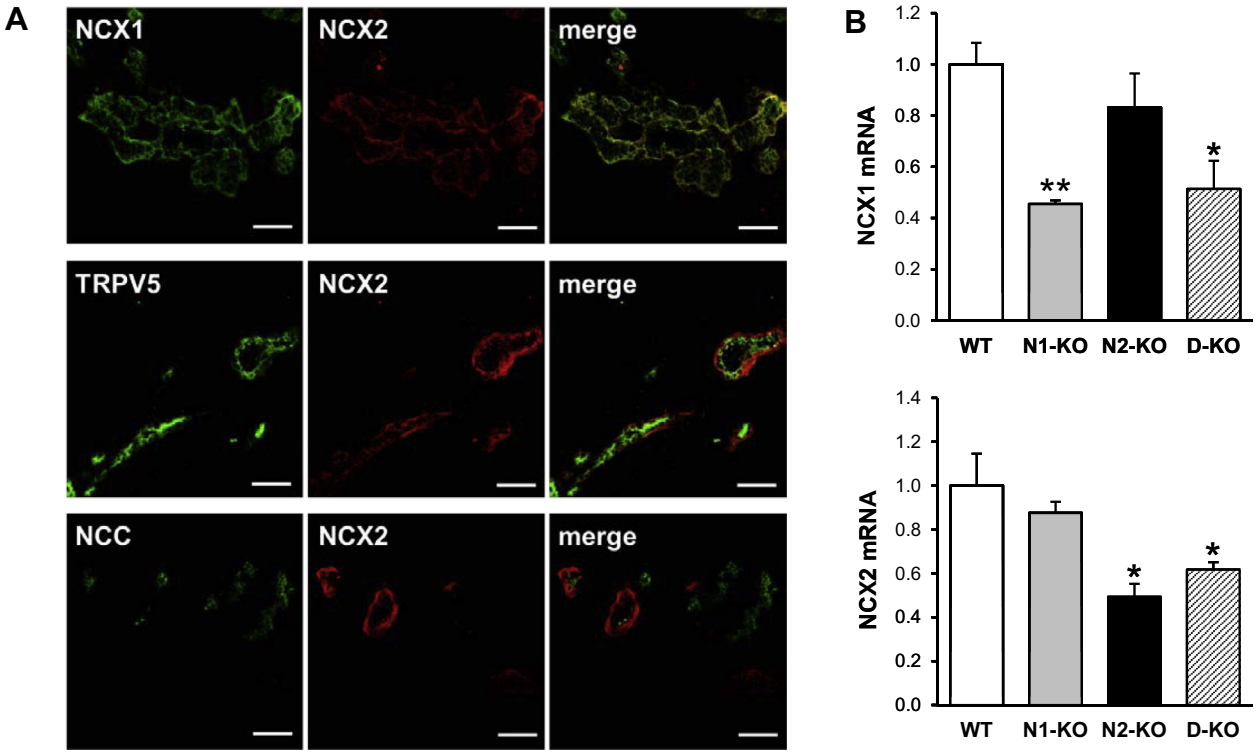

Fig. 1. Expression and localization of NCX isoforms in kidneys of WT and NCX-KO mice. (A) Double immunostaining of NCX1, NCX2, TRPV5, and NCC in kidney cortexes form WT mice. Frozen sections of kidney cortex were double-immunostained with polyclonal antibody (anti-NCX1, anti-TRPV5, or anti-NCC antibody) (green) and monoclonal anti-NCX2 antibody (red). Double-immunostaining appears yellow in the merged images. Scale bars = 50 μm. (B) Expression levels of NCX1 and NCX2 mRNA in kidney cortexes of WT, NCX1-KO (N1-KO), NCX2-KO (N2-KO), and double KO (D-KO). Quantitative real-time PCR was performed as described under Section 2. Each mRNA level is shown as a ratio to the targeted mRNA of WT. $^{*}P < 0.05$, $^{**}P < 0.01$ versus WT ($n = 3$).

shown in Fig. 2, administration of YM-244769 (0.1–1 mg/kg) caused a dose-dependent increase (up to approximately 200%) in urine volume and urinary excretion of electrolytes (Na^+, K^+ and Cl^-). Similar effects were observed by administration of KB-R7943 (1–10 mg/kg) or SEA0400 (1–10 mg/kg), although YM-244769 was most potent among these inhibitors. The natriuretic action of YM-244769 at a dose of 1 mg/kg was almost comparable to that of trichlormethiazide, an NCC inhibitor, at a dose of 10 or 30 mg/kg. Notably, a higher dose of YM-244769 or KB-R7943, but not of SEA0400, significantly increased urinary excretion of Ca^{2+} as well as Ca^{2+}/Cr ratio (Fig. 2). In contrast, trichlormethiazide (30 mg/kg) tended to decrease them.

3.3. Renal functions and pharmacological properties in NCX-KO mice

To ascertain the roles of NCX1 and NCX2 isoforms in renal function, we examined urine formation and electrolyte excretion in NCX1-KO, NCX2-KO and their double KO, and compared with them in WT. As shown in Fig. 3, the basal urine volume and urinary excretion of Na^+ and K^+ were significantly higher in NCX2-KO, and moderately higher in double KO. In NCX1-KO, however, these urinary parameters were slightly, but not significantly, increased. Furthermore, the basal urinary excretion of Ca^{2+}, assessed by Ca^{2+}/Cr ratio, was significantly enhanced in double KO ($P < 0.01$), but not in NCX1-KO, and tended to increase in NCX2-KO. In addition, in NCX2-KO, plasma Ca^{2+} concentration was lower and creatinine clearance was higher ($P < 0.05$; Table 1). On the other hand, systolic blood pressure and plasma levels of Na^+, K^+ and Cl^- were no different in WT, NCX1-KO and NCX2-KO. Next, the renal responses to YM-244769 (1 mg/kg) were investigated in WT and NCX-KOs. Natriuretic actions of YM-244769 were equivalently observed in NCX1-KO and WT, but disappeared in NCX2-KO and double KO (Fig. 3).

4. Discussion

Previous studies based on *in situ* hybridization and immunohistochemistry have revealed that NCX1, a major isoform of NCX, is expressed in the distal part of the nephron [3,4]. Subsequently, using reverse transcriptase PCR, NCX2 transcripts have been also detected in the kidney [7]. Although NCX are generally thought to contribute to renal reabsorption of Ca^{2+} [1–4], the functional significance of the existence of different NCX isoforms in the kidney is not entirely understood. In this study, we first confirmed that NCX1 and NCX2 isoforms are colocalized to the basolateral membrane of the distal nephron, primarily in the late DCT and CNT segments (Fig. 1). On the other hand, TRPV5 and NCC are localized to the apical membrane in both the late DCT and CNT and in the early and late DCTs, respectively, as reported previously [4,23]. Thus, multiple Na^+- and Ca^{2+}-transporters including NCX1 and NCX2 are distributed in the late DCT, which plays a pivotal role in the final tuning of electrolyte excretion [1,2]. The aim of the present study is to investigate the physiological roles of NCX1 and NCX2 isoforms on renal function, using isoform-selective NCX inhibitors and NCX-heterozygote mouse lines.

Benzyloxyphenyl NCX inhibitors that have different isoform selectivities could be useful for discriminating among the functional characteristics of NCX isoforms [8–10]. YM-244769 and KB-R7943 are slightly selective to NCX3, but at a higher dose, both of these inhibitors almost completely suppress all three NCX isoforms [10,12]. SEA0400 is an NCX1-specific inhibitor; SEA0400 suppresses only weakly NCX2 and has no effect on NCX3 [13]. Intriguingly, YM-244769 (0.1–10 mg/kg) and KB-R7943 (1–10 mg/kg) exhibited dose-dependently natriuretic action in mice (Fig. 2). Although SEA0400 also tended to cause natriuresis,

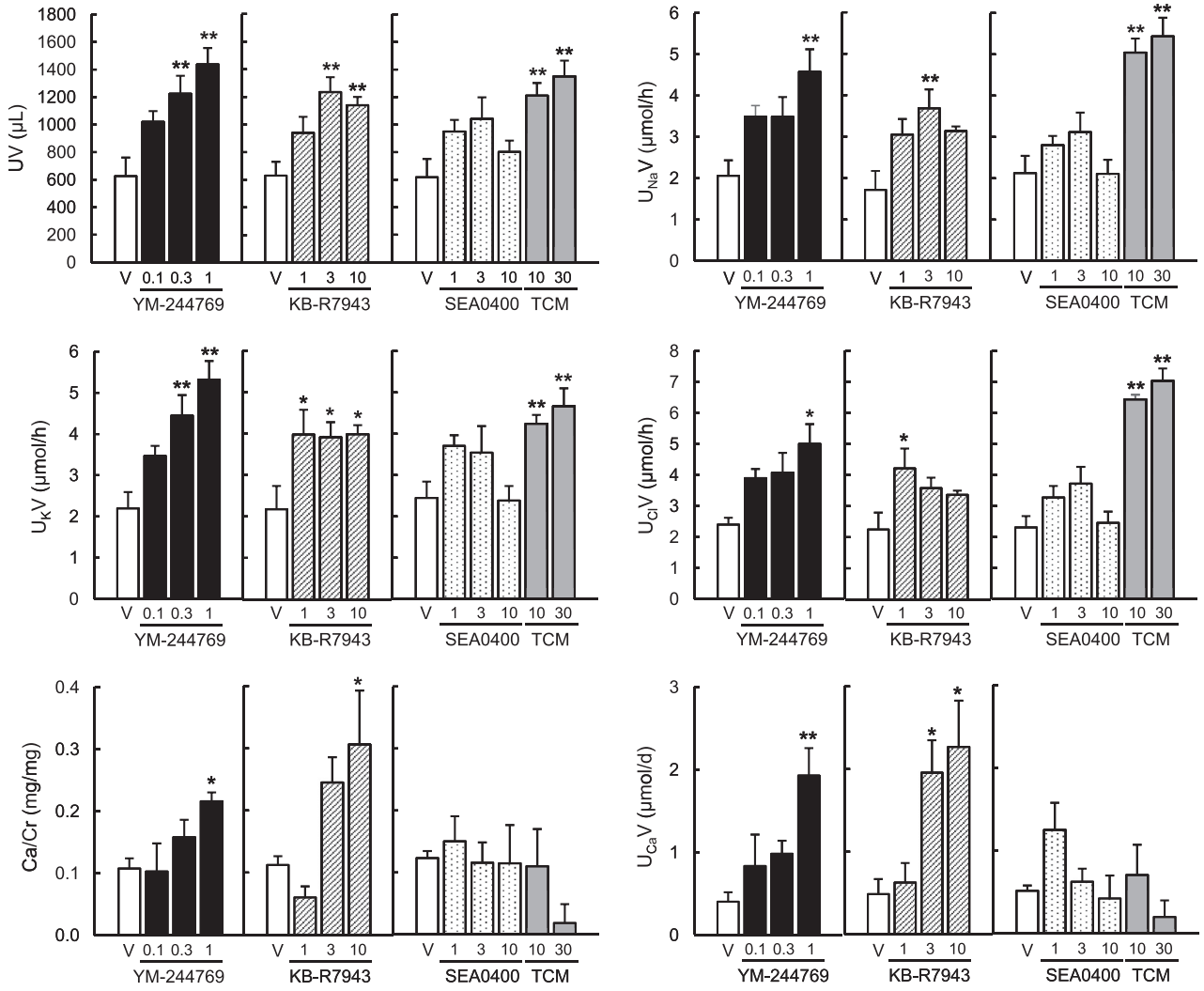

Fig. 2. Effects of NCX inhibitors on urine volume and electrolyte excretion in WT mice. After oral administration of NCX inhibitors (YM-244769, KB-R7943, or SEA0400) or trichlormethiazide (TCM) with 5% gum arabic (0.1 ml/10 g), WT mice (C57BL/6J) were kept in metabolic cages for collection of 24-h urine. Urine samples were used for measurements of renal parameters, such as urinary volume (UV), urinary excretion of Na^+, K^+, Cl^- and Ca^{2+} ($U_{Na}V$, U_KV, $U_{Cl}V$ and $U_{Ca}V$), as described under Section 2. $^*P < 0.05$, $^{**}P < 0.01$ versus each vehicle group (V) ($n = 5$ or 6).

YM-244769 had a more potent natriuretic action among these NCX inhibitors. Of note, a higher dose of YM-244769 or KB-R7943, but not of SEA0400, significantly increased urinary excretion of Ca^{2+} as well as Ca^{2+}/Cr ratio, whereas a high dose of trichlormethiazide tended to decrease it. These results suggest that pharmacologic inhibition of NCX2 as well as NCX1 may primarily cause natriuresis, and further inhibition of NCX2 induces hypercalciuria.

Furthermore, we applied NCX-heterozygote knockout mice to the verification of the functional difference of NCX1 and NCX2 isoforms in the kidney. In single and double NCX-KO lines, the targeted NCX isoform was specifically reduced by ~50% in the basolateral membrane of the distal nephron, whereas other multiple Na^+- and Ca^{2+}-transporters (see Supplemental Fig. 1), which are physiologically expressed in the distal nephron, were not altered. We found that NCX1-KO possessed similar renal function to WT, but NCX2-KO and double KO exhibited a moderate hyperfunction in urine formation and electrolyte excretion (Fig. 3). Notably, renal responses to YM-244769 were almost equivalently observed in NCX1-KO and WT, but disappeared in NCX2-KO and double KO. In addition, creatinine clearance, an indicator for the glomerular filtration rate, was significantly higher in NCX2-KO compared to WT (Table 1). These findings also support the interpretation that functional inhibition of NCX2 – rather than NCX1 – primarily causes natriuresis and hypercalciuria.

In the distal nephron comprising the early and late DCT and CNT, electrolytes are reabsorbed or secreted in the pro-urine via multiple ion transporters [1,2]. As for Ca^{2+} reabsorption, the late DCT and CNT primarily involve active Ca^{2+} transport. First, Ca^{2+} enters the renal epithelial cell through TRPV5. The entered Ca^{2+} is carried with calbindin 1 (Calb1), an intracellular Ca^{2+}-binding protein, from the apical side to the basolateral side. Finally, Ca^{2+} may be extruded across the basolateral membrane by NCX and/or plasma membrane Ca^{2+}-ATPase to the extracellular compartment [24]. The initial studies reported that TRPV5-homozygout knockout mice produces severe hypercalci uria [25,26], suggesting that TRPV5 serves as a gatekeeper in active Ca^{2+} transport. Calb1, which directly carries intracellular Ca^{2+}, can associate with TRPV5, resulting in the facilitation of Ca^{2+} transport by preventing the inactivation of TRPV5 [27]. In this study, we first experimentally demonstrated that NCX2-KO as well as mice administered with a NCX2-sensitive inhibitor (namely, YM-244769 or KB-R7943), caused hypercalciuria, providing evidence for a functional significance of NCX2 isoform as a

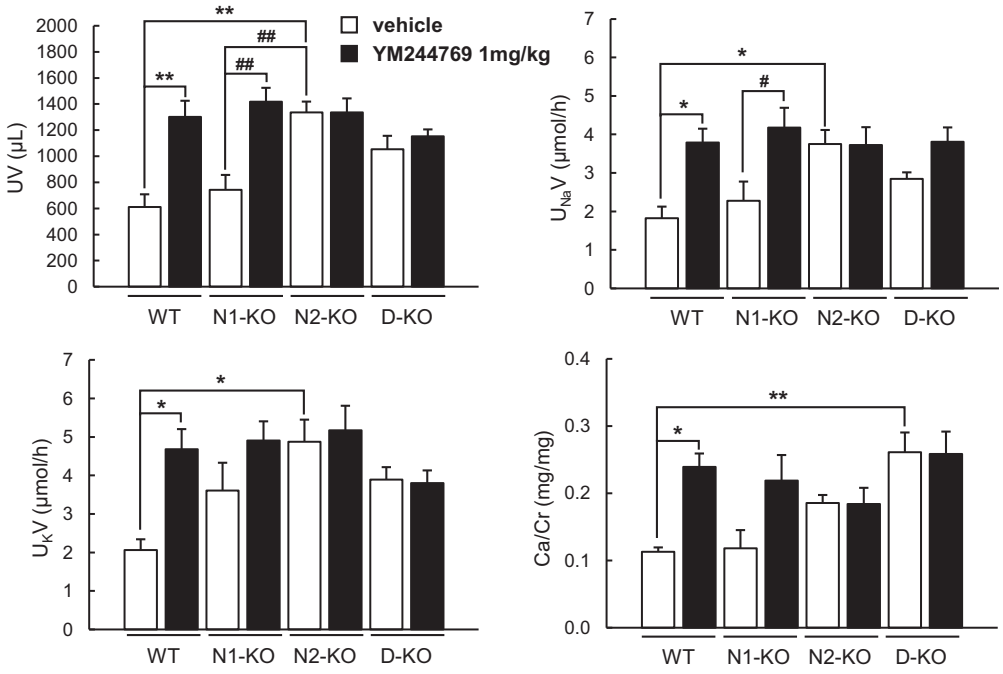

Fig. 3. Urine formation, electrolyte excretion, and pharmacologic effects in WT and NCX-KO mice. YM-244769 (1 mg/kg) was orally administered into WT, NCX1-KO (N1-KO), NCX2-KO (N2-KO) and double KO (D-KO). After drug administration, mice were kept in metabolic cages for collection of 24-h urine. Urine parameters were measured as described under Section 2. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#\#}P < 0.01$ by ANOVA ($n = 5$ or 6).

Table 1
Plasma and urine parameters of WT and NCX-KO mice.

	WT	N1-KO	N2-KO
Plasma concentration			
Na^+ (mmol/L)	149 ± 0.6	147 ± 0.8	148 ± 1.1
K^+ (mmol/L)	5.0 ± 0.3	5.1 ± 0.3	5.4 ± 0.4
Cl^- (mmol/L)	114 ± 0.9	113 ± 0.7	113 ± 0.6
Ca^{2+} (mmol/L)	2.2 ± 0.08	2.1 ± 0.05	1.9 ± 0.08*
Ccr (μL/min)	250 ± 23	263 ± 28	412 ± 59*
SBP (mm Hg)	92.2 ± 0.7	91.6 ± 0.7	91.8 ± 0.9

Ccr; creatinine clearance, SBP; systolic blood pressure.
* $P < 0.05$ vs WT ($n = 5$).

basolateral Ca^{2+} extrusion system. Hypercalciuria may be induced by either facilitated glomerular Ca^{2+} filtration and/or suppressed Ca^{2+} reabsorption.

As for Na^+ reabsorption, the distal nephron also participates significantly in active Na^+ transport; apical Na^+ uptake by NCC as well as ENaC (epithelial Na^+ channel) and basolateral Na^+ extrusion by Na^+,K^+-ATPase. Previous studies reported that $WNK4^{D561A/+}$ knockin mice, in which serine/threonine-protein kinase WNK4 mutation causes a gain-of-function of NCC, produce hyperkalemia, hypercalciuria, and acidosis [28,29]. In $WNK4^{D561A/+}$ knockin mice, Na^+ reabsorption is accelerated by overexpression of apical NCC in the DCT, which causes low luminal Na^+ concentration in the cortical collecting duct [28]. The low luminal Na^+ concentration may also stimulate ENaC. In our recent experiments, we observed that natriuresis, but not hypercalciuria, induced by YM-244769 are abolished in $WNK4^{D561A/+}$ knockin mice (unpublished observation). From this finding, we speculate that functional inhibition of NCX2 may indirectly suppress Na^+ reabsorption via NCC or ENaC, resulting in natriuresis.

In summary, this comprehensive study using isoform-selective NCX inhibitors and NCX-heterozygote mouse lines reveals that functional inhibition of NCX2 initially causes natriuresis, and further inhibition of NCX2 produces hypercalciuria, suggesting that the functional significance of NCX2 in Na^+ and Ca^{2+} reabsorption of the kidney. Genetically altered mice and specific inhibitors for NCX2 are useful tools for elucidating the pathological mechanism underlying abnormal calcium metabolism.

Conflict of interest

The authors have declared no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.12.016.

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