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ORIGINAL ARTICLE

Effects of renal Na^+/Ca^{2+} exchanger 1 inhibitor (SEA0400) treatment on electrolytes, renal function and hemodynamics in rats

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Abstract
Background Na^+/Ca^{2+} exchanger 1 (NCX1) controls intracellular Ca^{2+} concentration in various cell types. In the kidney, NCX1 is expressed mainly in the distal tubular basolateral membrane as well as in vascular smooth muscle. Tubular NCX1 is involved in Ca^{2+} reabsorption, and NCX1 in renal arterioles may control intraglomerular pressure. However, the functions of renal NCX1 have not been studied in vivo. Therefore, this study examined the effects of renal NCX1 blockade on water and solute metabolism, renal function and blood pressure in rats.
Methods Wistar-Kyoto rats were uninephrectomized, and an osmotic mini pump was implanted to infuse the remnant kidney cortex with a specific NCX1 inhibitor, SEA0400 (SEA), or vehicle for 7 days.
Results Serum Ca^{2+} concentration and urinary Ca^{2+} excretion were similar between the vehicle- and SEA-treated groups. However, serum phosphate was significantly decreased by 8 % in the SEA group, with similar urinary phosphate excretion between the two groups. Systolic blood pressure was higher in the SEA group (117 ± 3 vs. 126 ± 1 mmHg, $n = 9$–11), with a 1.6-fold increase in plasma aldosterone concentration. However, SEA significantly reduced urinary protein excretion and the glomerular sectional area by 16 and 8 %, respectively. Similar experiment in spontaneously hypertensive rats produced different results.
Conclusion Renal SEA treatment reduced serum phosphate concentration, urinary protein and glomerular size with higher systemic blood pressure compared to control Wistar-Kyoto rats. Further study on renal NCX1 may be beneficial in delineating the pathophysiology of glomerular pressure control and calcium/phosphate regulations.

Keywords Na^+/Ca^{2+} exchanger 1 (NCX1) · Blood pressure · Calcium · Phosphate · Aldosterone · Renal vasculature · Urinary protein

Introduction

Na^+/Ca^{2+} exchanger (NCX) maintains intracellular Ca^{2+} in many cell systems [1]. In the kidney, NCX type 1 (NCX1) is the predominant type of NCX, and NCX1.3 and NCX1.7 splice variants of NCX1 are abundantly expressed in the kidney [2].

There are two main sites of renal NCX1 expression. One is in the tubular epithelial cells. NCX1 is expressed in the basolateral membrane of the distal convoluted tubule and the connecting tubule, which is the renal segment where hormonally regulated, transcellular urinary Ca^{2+} reabsorption occurs. From in vitro experiments, NCX1 is postulated to be responsible for 15–70 % of urinary Ca^{2+} reabsorption in this segment [3].

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Na^+/Ca^{2+} exchanger 1 is also expressed in the vascular smooth muscle cells. Smooth muscle NCX1 of systemic vasculature may be involved in the pathogenesis of salt-sensitive hypertension [4]. In this hypothesis, a dietary NaCl-induced increase in endogenous Na^+/K^+ pump inhibitors raises intracellular Na^+, causing Ca^{2+} influx via NCX1 and smooth muscle contraction [5]. Renal arterioles also show significant NCX activity, which is higher in the afferent compared to efferent arterioles [6, 7]. Afferent arteriole NCX1 activity is reduced in some hypertensive animal models, such as salt-sensitive Dahl rats [8] and spontaneously hypertensive rats (SHR) [9]. In renal arteries, however, NCX1 inhibitor KBR-7943 increases the renal vascular resistance in isolated rat kidney perfusion [10].

It can be hypothesized that a renal NCX1 blockade may have significant effects on Ca^{2+} homeostasis and renal hemodynamics. However, the function of renal NCX1 has not been studied in vivo. We, therefore, performed chronic renal infusion of a specific NCX1 blocker, SEA0400, and examined its effects on water and solute metabolism, renal function and blood pressure in rats.

Methods

Animals and SEA0400 infusion

All experimental procedures were approved by the Fukushima Medical University School of Medicine Animal Committee. On male Wistar-Kyoto rats (WKY, Japan SLC Inc., Sendai, Japan) at 8 weeks of age, uninephrectomy and osmotic mini pump implantation were performed, using a similar method as described previously [11]. SEA0400 (SEA, a gift from Taisho Pharmaceutical, Tokyo, Japan), a specific NCX1 inhibitor [12, 13], was used for renal NCX1 blockade. In brief, under intraperitoneal pentobarbital anesthesia (50 mg/kg), the right kidney was removed, and a catheter connected to an osmotic mini pump (1 μl/h, Alzet Corporation, Palo Alto, Calif., USA) containing vehicle or SEA in lipid emulsion diluted tenfold in phosphate-buffered saline was inserted into the cortical interstitium of the remaining left kidney. The mini pump infused SEA (100 pmol/h) for 7 days. The IC50 of SEA0400 is 5–92 nM, and SEA0400 is reported not to affect other transporters and channels, etc. up to 3 μM [12]. With the dose we used, theoretically maximal intrarenal SEA0400 concentration would be about 11 μM, assuming all of the SEA0400 administered during 7 days remained in the kidney. In reality, with half-life of SEA0400 being approximately 10 h with oral administration and kidney being an organ with high blood flow, the actual SEA0400 concentration would presumably be lower than 3 μM in most of the kidney.

Blood pressure and biochemical analysis

Unanesthetized systolic blood pressure and heart rate were measured by the tail-cuff method (Blood Pressure Analyzer model BP-98A; Softron, Tokyo, Japan). Urine was collected for 24 h using metabolic cages. Sodium, potassium, creatinine, plasma renin activity, plasma aldosterone concentration, and urinary protein concentrations were determined as described previously [14, 15, 16]. FGF23 concentration in serum or plasma was determined using FGF-23 ELISA kit (KAINOS Laboratories, Tokyo, Japan), and serum 1,25 $(OH)_2$ vitamin D concentration was determined by SRL, Inc. (Tokyo, Japan) using radioimmunoassay. Transtubular potassium gradient (TTKG) was calculated using potassium concentration and osmolarity in plasma and urine.

Quantitative real-time PCR

Rats were given pentobarbital anesthesia after 7 days of intrarenal infusion, and the kidneys were collected after drawing blood from the abdominal aorta. Blocks of kidney cortex were preserved in RNAlater Solution (Ambion, Inc., Austin, TX), and total RNA was extracted using RNeasy Plus Mini Kit (QIAGEN, Inc., Valencia, Calif., USA). Total RNA (0.25 μg) was reverse-transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, Calif., USA) in a 20 μl reaction volume. One μl of the resultant mixture was used for quantitative PCR for NCX1 using iQ SYBR Green Supermix and iQ5 real-time PCR detection system (Bio-Rad). The primers used were as follows; rat NCX1 forward: 5′-CAGTTGTGTTTGTC GCTCTTGG-3′, reverse: 5′-GTTGGCCGCATGGTAGA TGG-3′, rat GAPDH forward: 5′-GCAAGTTCAACGG-CACAGTCAAG-3′, reverse: 5′-ACATACTCAGCAC CAGCATCACC-3′. Quantitative PCR for NaPi2b was performed using TaqMan system and StepOnePlus according to the manufacturer's protocol. Beta actin was used as internal control.

The excised kidneys were fixed with HistoChoice Tissue Fixative (AMRESCO Inc., Solon, Ohio, USA), embedded in paraffin, sectioned into 2-μm slices, and stained with hematoxylin and eosin. Digitized photographs of kidney sections were taken with a light microscope and DP70 Digital Microscope Camera (Olympus, Corp. Tokyo, Japan). Sectional areas of renal corpuscle were measured using WINROOF software (Mitani, Tokyo, Japan) in a blind manner, and 15–40 renal corpuscles per rat were measured and averaged.

Statistical analysis

All P values were two sided and P values of 0.05 or less were considered statistically significant. Statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University), which is a graphical user interface for R (The R Foundation for Statistical Computing). More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics. Statistical differences between groups were analyzed using t test or multi-factorial ANOVA where appropriate. Data are given as mean ± standard error unless otherwise noted.

Results

Body weight, intake, and urine and serum osmolalities were similar between the control and SEA-treated rats (Table 1). Neither urinary Ca^{2+} excretion (Table 2) nor serum Ca^{2+} concentration (Table 3) was altered by chronic renal SEA infusion. However, there was an 8 % reduction in serum phosphate concentration in the SEA-treated group (Table 3), without a significant difference in daily urinary phosphate excretion (Table 2). It has been reported that serum phosphate concentration exhibits diurnal variation and is also altered by feeding [17]. When serum phosphate was measured in fasting condition in the morning, there was a similar tendency for a reduction in serum phosphate in the SEA-treated group (control WKY 2.82 ± 0.05 mmol/l vs. SEA-treated WKY 2.66 ± 0.18 mmol/l, $n = 3$/group, ns), albeit with no significant difference possibly due to a small sample size. Vitamin D and FGF23 are important factors of phosphate metabolism, but there was no significant difference in FGF23 (control WKY 489 ± 100 vs. SEA-treated WKY 438 ± 172 pg/ml, $n = 4$–5/group, ns) or 1,25 $(OH)_2$ vitamin D (control WKY 92.0 ± 26.9 vs. SEA-treated WKY 110.4 ± 18.0 pg/ml, $n = 3$/group, ns) concentrations in blood between the groups. NCX1 transports 3 Na^+ for 1 Ca^{2+}, but SEA did not affect serum or urinary Na^+ concentrations. Measurements for K^+ and Mg^{2+} were also similar between the groups (Tables 2 and 3).

The lack of SEA effect on systemic Ca^{2+} homeostasis may have been due to compensatory upregulation of renal NCX1. However, the renal cortical expression of NCX1 mRNA was not significantly altered by SEA infusion (control vs. SEA: 100 ± 11 vs. 86 ± 8 % of control, $n = 4$/group).

Table 1 Weight and intake/output data in WKY

	Control ($n = 13$)	SEA ($n = 13$)	P
Body weight (g)	227 ± 3.8	226 ± 3.1	ns
Food intake (g/day)	17.7 ± 0.6	17.2 ± 0.4	ns
Water intake (ml/day)	36.0 ± 1.5	33.6 ± 1.0	ns
Urine volume (ml/day)	13.9 ± 0.8	12.9 ± 0.5	ns
u-osmolarity (mOsm/kgH_2O)	1406 ± 50	1443 ± 34	ns
s-osmolarity (mOsm/kgH_2O)	302.1 ± 3.1	303.7 ± 3.4	ns

u urinary, *s* serum, *ns* not significant

Table 2 Serum biochemical measurements and creatinine clearance in WKY

	Control ($n = 10$)	SEA ($n = 12$)	P
Na (mEq/l)	138 ± 0.5	139 ± 0.3	ns
K (mEq/l)	4.5 ± 0.2	4.0 ± 0.1	ns
Cl (mEq/l)	101 ± 0.6	102 ± 0.3	ns
Ca (mmol/l)	2.37 ± 0.02	2.40 ± 0.02	ns
P (mmol/l)	2.52 ± 0.03	2.32 ± 0.06	<0.05
Mg (mmol/l)	0.99 ± 0.01	1.03 ± 0.01	ns
BUN (mmol/l)	9.25 ± 0.5	9.57 ± 0.6	ns
Crea (μmol/l)	26.7 ± 0.8	25.9 ± 0.8	ns
UA (μmol/l)	51.7 ± 4.2	53.5 ± 3.0	ns
CCr (ml/day)	1975 ± 171	2112 ± 58	ns

SEA SEA0400 (NCX1 inhibitor), *Na* sodium, *K* potassium, *Cl* chloride, *Ca* calcium, *P* inorganic phosphate, *Mg* magnesium, *BUN* blood urea nitrogen, *Crea* creatinine, *UA* uric acid, *CCr* creatinine clearance, *ns* not significant

Table 3 Urinary electrolytes in WKY

	Control ($n = 10$)	SEA ($n = 12$)	P
u-Na (mEq/day)	1.61 ± 0.07	1.63 ± 0.06	ns
u-K (mEq/day)	2.89 ± 0.10	2.91 ± 0.07	ns
u-Cl (mEq/day)	1.87 ± 0.09	1.90 ± 0.07	ns
u-Ca (μmol/day)	60 ± 2	61 ± 4	ns
u-P (mmol/day)	0.68 ± 0.03	0.67 ± 0.03	ns
u-Mg (mmol/day)	0.33 ± 0.01	0.32 ± 0.02	ns

u- urinary, *ns* not significant

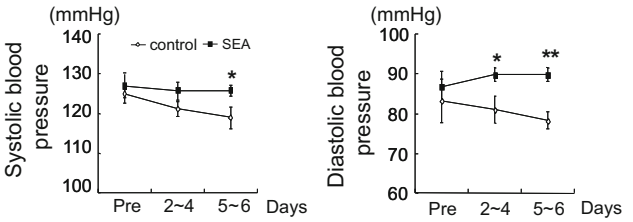

Fig. 1 Systolic and diastolic blood pressure of control and SEA-treated rats. Two-way ANOVA was performed to test for differences by time and treatment. No statistical significance was observed between blood pressure before and after SEA0400 treatment. $^{**}p < 0.01$ and $^{*}p < 0.05$, $n = 9$–12/group

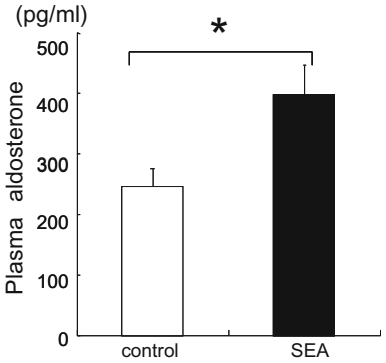

Fig. 2 Plasma aldosterone concentration of control and SEA-treated rats. *$p < 0.05$, $n = 12$–13/group

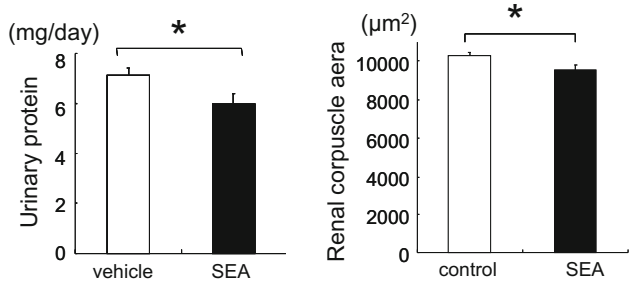

Fig. 3 **a** Urinary protein excretion of control and SEA-treated WKY. **b** Renal corpuscle sectional area of control and SEA-treated rats. Sectional area of 15–40 renal corpuscles were measured and averaged per rat. *$p < 0.05$, $n = 8$–9 rats/group

Table 4 Heart rate and urinary catecholamines in WKY

	Control	SEA	*P*
Heart rate (bpm)	366 ± 5	375 ± 4	ns
Adrenaline (μg/l)	4.3 ± 0.9	5.4 ± 0.9	ns
noradrenaline (μg/l)	39.4 ± 5.1	43.2 ± 3.8	ns
Dopamine (μg/l)	541 ± 65	447 ± 57	ns

bpm beats per minute, *ns* not significant

Table 5 Experimental parameters in SHR

	Control ($n = 8$)	SEA ($n = 8$)	*P*
Systolic BP (mmHg)	131 ± 4	126 ± 2	ns
Creatinine (μmol/l)	27.4 ± 0.9	26.5 ± 0.1	ns
Na (mmol/l)	141.5 ± 0.3	142.8 ± 0.3	<0.01
Cl (mmol/l)	105.8 ± 0.4	105.8 ± 0.3	ns
K (mmol/l)	4.09 ± 0.12	3.74 ± 0.10	≤0.05
Ca (mmol/l)	2.3 ± 0.0	2.3 ± 0.0	ns
P (mmol/l)	2.3 ± 0.1	2.2 ± 0.0	ns
Osmolarity (mOsm/kgH_2O)	306 ± 1	310 ± 1	<0.05
Renin activity (ng/ml/hr)	28.0 ± 2.6	27.9 ± 1.8	ns
Aldosterone (pg/ml)	200 ± 15	194 ± 21	ns
u-Na (mEq/day)	1.38 ± 0.08	1.29 ± 0.07	ns
u-K (mEq/day)	2.60 ± 0.12	2.52 ± 0.10	ns
u-protein (mg/day)	12.3 ± 0.4	11.7 ± 0.6	ns
TTKG	11.1 ± 0.3	12.3 ± 0.3	<0.05
Urine volume (ml/day)	1975 ± 171	2112 ± 58	ns
Corpuscle area (μm^2)	8927 ± 36	8728 ± 36	ns

BP blood pressure, *ns* not significant, *u* urinary, *TTKG* transtubular potassium gradient

In this study, chronic renal SEA infusion in rats on normal diet led to higher systolic and diastolic blood pressures by 9 and 10 mmHg, respectively, than vehicle-treated rats (Fig. 1). This is in contrast to a previous report of a systemic, acute administration of SEA in rats, where systemic blood pressure did not change during a normal diet, but rather decreased during a high-salt diet [18].

Interestingly, plasma aldosterone concentration significantly increased by 1.6-fold in the SEA group (Fig. 2). Plasma renin activity, serum creatinine, creatinine clearance, blood urea nitrogen and uric acid were similar between the groups (Table 2). Indirect measures of body fluid volume, such as serum albumin (3.2 ± 0.1 vs. 3.2 ± 0.1 g/dl, $n = 9$–10) and hematocrit (44.5 ± 0.8 vs. 44.8 ± 0.3 %, $n = 4$), were also not significantly different. Heart rate and urinary catecholamines, which are measures of sympathetic activity, were not significantly different between the groups (Table 4). Systemic administration of SEA0400 leads to liver dysfunction (personal communications from Taisho Pharmaceutical), but in this study no changes in aspartate aminotransferase or alanine transaminase were observed, which supports the local nature of intrarenal SEA administration.

There was some glomerular hyperfiltration in these rats due to unilateral nephrectomy. Generally, glomerular hyperfiltration leads to glomerular hypertrophy. In this study, chronic renal administration of SEA resulted in a significant reduction in urinary protein excretion (Fig. 3a) and decrease in glomerular size (Fig. 3b) despite higher blood pressure and increased plasma aldosterone concentration in WKY.

To determine whether the SEA effects may be different in other rat models, a similar experiment was performed using SHR. SHR showed significantly higher systolic blood pressure on normal diet compared to WKY at similar age. Also, it has been reported that uninephrectomy exacerbates proteinuria in SHR while it does not significantly affect urinary protein in WKY [19]. Results of the SHR study are shown in Table 5. Interestingly, intrarenal administration of SEA did not alter systemic blood pressure, plasma aldosterone, serum phosphate or urinary protein in SHR. However, there was a slight but significant increase in

serum sodium concentration and osmolarity with a decrease in serum potassium concentration in SEA-treated SHR. Although its mechanism is not clear, there may be an increased aldosterone effect in the tubules as TTKG was significantly higher in the SEA-treatment group.

Discussion

In our study, renal administration of NCX1 inhibitor, SEA0400, did not significantly affect urinary Ca^{2+} excretion or serum Ca^{2+} concentration. This was rather surprising because distal tubular NCX1 is known as a major pathway of urinary Ca^{2+} reabsorption. Because Ca^{2+} level is tightly regulated in the body, effects on tubular NCX1 may be compensated by hormonal alterations on intestinal calcium absorption or bone metabolism. It could also be that a higher concentration of SEA may have been necessary to inhibit tubular Ca^{2+} reabsorption because SEA is reported to be more effective in blocking the Ca^{2+}-influx mode than Ca^{2+}-exit mode of NCX1. In contrast, renal SEA treatment significantly reduced serum phosphate concentration in WKY. Although its mechanism did not become clear in our study, it is possible that renal NCX1 inhibition may directly or indirectly and in combination affect endocrine factors that modulate calcium and phosphate handling, such as vitamin D, parathyroid hormone and klotho-FGF23.

Renal NCX1 inhibitor treatment lead to significantly higher systemic blood pressure and plasma aldosterone concentration compared to control rats in WKY. Intrarenally infused SEA0400 may have reached the adrenal gland because of its proximity to the kidney and directly increased aldosterone secretion. It is also possible that SEA0400 reduced glomerular perfusion pressure through the constriction of afferent arterioles. SEA0400 may act directly on renal arteriole smooth muscle cells. NCX1 inhibitor treatment on perfused kidney increases renal vascular resistance [20]. Moreover, as NCX1 activity is higher in the afferent compared to efferent arterioles [6, 7], relative contraction of the afferent arterioles may decrease intraglomerular pressure. It is also possible that SEA0400 may have increased afferent arteriolar tone indirectly through its action on connecting tubule NCX1 because the connecting tubule has direct contact with the afferent arteriole, and the connecting tubule can regulate afferent arteriolar tone by a mechanism called connecting tubule glomerular feedback [21, 22]. If so, subsequent activation of the renin-angiotensin aldosterone system could raise blood pressure, but reduced glomerular perfusion pressure may decrease urinary protein excretion and glomerular size in SEA-treated WKY.

It is also of interest that effects of renal SEA infusion were different in SHR. Although there was no significant difference in blood pressure, serum phosphate, plasma aldosterone or urinary protein excretion between control and SEA-treated SHR, SEA-treated SHR exhibited increased serum sodium and reduced serum potassium concentration. NCX1 is abundantly expressed in the distal tubular segments where much of sodium and potassium regulation occurs. NCX1 may alter the tubular transport of Na^+ and K^+ possibly by changing the cellular Na^+ and Ca^{2+} gradients.

One limitation of this study is that renal administration of SEA does not ensure the restriction of SEA effects to the kidney. Iwamoto et al. acutely infused 10 μg/kg/min SEA0400 into rat femoral artery and found no change in blood pressure [18]. In our study, the rate of infusion was much less, approximately 3 ng/kg/min to localize the drug effect in the kidney. Another limitation is the possibility of SEA actions other than NCX1 blockade. Studies using other NCX1 inhibitors or gene knockdown are necessary to elucidate this. However, other NCX1 inhibitors such as KBR-7943 are not specific enough for NCX1 [23, 24], and in vivo renal NCX1 siRNA administration has not achieved sufficient knockdown efficiency.

In summary, chronic renal treatment of WKY rats with NCX1 inhibitor, SEA0400, did not alter serum or urinary calcium but significantly reduced serum phosphate concentration. Higher systemic blood pressure and plasma aldosterone concentration with NCX1 inhibitor were accompanied by significantly reduced urinary protein and glomerular size. As SEA0400 decreased serum phosphate concentration and urinary protein excretion, further study on the role of NCX1 in the kidney may yield beneficial information on the control of phosphate metabolism and renal function.

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Conflict of interest Employment (endowed department): Junichi Yatabe and Tsuyoshi Watanabe (MSD, CHUGAI PHARMACEUTICAL, Dainippon Sumitomo Pharma, Takeda Pharmaceutical, TEIJIN PHARMA, AstraZeneca, Baxter International, and Daiichi Sankyo).

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