学　位　論　文

Effects of diuretics on SGLT2 inhibitor-induced changes in blood pressure in obese rats suffering from the metabolic syndrome

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Running title: SGLT2 inhibitor and diuretics in metabolic syndrome

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ABSTRACT

Objective: Experiments were carried out to investigate whether diuretics (hydrochlorothiazide + furosemide) impact on the effects of a sodium-dependent glucose co-transporter 2 (SGLT2) inhibitor on glucose metabolism and blood pressure in metabolic syndrome SHR/NDmcr-cp(+/+) rats (SHRcp).

Methods: Male 13-week-old SHRcp were treated with: (i) vehicle; (ii) the SGLT2 inhibitor luseogliflozin (10 mg/kg/day); (iii) diuretics (hydrochlorothiazide; 10 mg/kg/day + furosemide; 5 mg/kg/day); or (iv) luseogliflozin + diuretics (n = 5–8 for each group) daily by oral gavage for 5 weeks. Blood pressure and glucose metabolism were evaluated by a telemetry system and oral glucose tolerance test, respectively.

Results: Vehicle-treated SHRcp developed non-dipper type hypertension (dark- *vs.* light-period mean arterial pressure (MAP): 148.6±0.7 and 148.0±0.7 mmHg, respectively, *P=0.2*) and insulin resistance. Compared with vehicle-treated animals, luseogliflozin-treated rats showed an approximately 4000-fold increase in urinary excretion of glucose and improved glucose metabolism. Luseogliflozin also significantly decreased blood pressure and turned the circadian rhythm of blood pressure from a non-dipper to dipper pattern (dark- *vs.* light-period MAP: 138.0±1.6 and 132.0±1.3 mmHg, respectively, *P<0.01*), which were associated with a significant increase in urinary excretion of sodium. Addition of diuretics did not influence luseogliflozin-induced improvement of glucose metabolism and circadian rhythm of blood pressure in SHRcp.

Conclusion: These data suggest that a SGLT2 inhibitor elicits its beneficial effects on glucose metabolism and hypertension in subjects with metabolic syndrome undergoing treatment with diuretics.

INTRODUCTION

The metabolic syndrome is a constellation of impaired glucose metabolism and insulin resistance, overweight and obesity, dyslipidemia, and hypertension. It is associated with development of type-2 diabetes mellitus and cardiovascular disease [1]. Among several comorbidities, hypertension affects ≤85% of patients with the metabolic syndrome [2]. A multi-targeted and integrated approach is required to simultaneously treat hypertension, obesity and insulin resistance owing to the complex etiology of metabolic syndrome [3]. The thiazide diuretic hydrochlorothiazide (HCTZ) is recommended as first-line treatment for hypertensive patients suffering from the metabolic syndrome [4]. Furthermore, the effects of thiazides are potentiated in combination therapy with the loop diuretic furosemide [5].

Renal proximal tubular sodium-dependent glucose co-transporter (SGLT)2 mediates the transport of glucose and sodium in 1:1 stoichiometry [6]. SGLT2 is thought to be responsible for ≈90% of glucose reabsorption in the S1 segment of proximal tubules in the kidney [7]. Therefore, increasing excretion of urinary glucose by inhibition of SGLT2 is a new insight into treatment of type-2 diabetes mellitus [8]. Of interest, animal experiments and clinical trials have consistently indicated that treatment with SGLT2 inhibitors reduces blood pressure in type-2 diabetes mellitus [9-11]. The underlying mechanism of action of SGLT2 inhibitors is not clear, but the potential roles of weight loss [12], osmotic diuresis [13] or natriuresis [14] have been suggested.

A phase-I, single-center, open-label, fixed-sequence, two-period study [15] demonstrated the good tolerability of the SGLT2 inhibitor canagliflozin on HCTZ-treated healthy participants. In that study, co-administration of canagliflozin and HCTZ had no notable pharmacokinetic or pharmacodynamic interactions, and adverse

METHODS

Animals

All experimental procedures were carried out according to the guidelines for care and use of animals established by Kagawa University (Kagawa, Japan). Wistar–Kyoto (WKY) rats, spontaneously hypertensive rats (SHR) and SHRcp were purchased from Japan SLC Inc. (Shizuoka, Japan). Rats were housed in specific-pathogen-free animal facilities under a controlled temperature (24±2°C) and humidity (55±5%) with a 12-h light–dark cycle. Rats were given standard chow and had access to water *ad libitum*.

Drugs

The selective SGLT2 inhibitor luseogliflozin was provided by Taisho Pharmaceuticals Co., Ltd. (Omiya, Saitama, Japan) [17]. HCTZ was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Furosemide was purchased from Sigma–Aldrich, Inc. (Saint Louis, MO, USA). Doses of luseogliflozin, HCTZ and furosemide were determined on the basis of previous studies in rats [17-19].

Experimental protocols

Male 13-week-old SHRcp were divided into four groups (n=5–8 for each group) based on basal parameters: systolic blood pressure (SBP), mean arterial pressure (MAP) and body weight. Rats were treated with (i) vehicle (0.5% carboxymethyl cellulose), (ii) luseogliflozin (10 mg/kg/day), (iii) diuretics (HCTZ; 10 mg/kg/day + furosemide; 5 mg/kg/day), or (iv) luseogliflozin + diuretics. Vehicle, luseogliflozin and diuretics were administered daily by oral gavage during the experimental period. Age-matched WKY rats (n=6) and SHR (n=6) were used as normotensive and hypertensive controls,

Implantation of a telemetry system and measurement of blood pressure

A telemetry system (Data Science International, Saint Paul, MN, USA) was used for measurement of blood pressure in conscious animals, as described previously [21]. At 11 weeks of age, a telemetry catheter was inserted into the abdominal aorta, and animals were maintained under stress-free conditions for 2 weeks for recovery. All animals had a 24-h acclimatization period on a telemetry receiver panel, and drugs were administered 3 h before starting measurement of blood pressure for 24 h. Every week, 24-h blood pressure measurement was undertaken during the experimental period. Moreover, we calculated the 12-h dark-period (6 pm to 5 am) and 12-h light-period (6 am to 5 pm) MAP to evaluate the circadian rhythm of blood pressure after 5 weeks of treatment.

Oral glucose tolerance test (OGTT)

After 5 weeks of treatment, an OGTT was carried out, as reported previously [20]. Briefly, rats were allowed to fast overnight and glucose (2 g/kg body weight) administered by oral gavage. Blood samples were collected from the tail by needle puncture for measurement of plasma concentrations of glucose and insulin before as well as 30, 60, 90 and 120 min after glucose administration.

Immunohistochemistry of SGLT2

Immunostaining of SGLT2 was done in formalin-fixed paraffin-embedded kidney tissues. First, paraffin sections were deparaffinized and autoclaved with 10 mmol/L sodium citrate buffer (pH 6.0) for 20 min for antigen retrieval. After cooling and

[22]. All data showed the relative differences between vehicle-treated SHRcp and other groups after normalization to expression of the β-actin gene.

Western blotting

For determination of the protein abundance of different transporters in the kidney, we prepared membrane fractions from cortical and inner medullary tissues in accordance with a previous study [23]. Briefly, tissues were homogenized in homogenization buffer (250 mmol/L sucrose, 50 mmol/L Tris-HCl, 5 mmol/L EDTA (pH 7.6) with leupeptin, aprotinin, phenylmethylsulfonyl fluoride and protease inhibitor). Then, the homogenate was centrifuged at 300 (×g) for 10 min at 4°C to remove whole cells, nuclei and mitochondria. The supernatant was collected and ultracentrifuged at 17,000 (×g) for 20 min at 4°C. The pellet was resuspended in homogenization buffer to obtain the membrane fraction. The protein concentration of the membrane fraction was determined using the Bradford assay. Protein samples (30 μg) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel, transferred to a nitrocellulose membrane, and immunoblotted with specific antibody against sodium–hydrogen exchanger 3 (anti-NHE3; 1:1000 dilution; StressMarq Biosciences Inc., Victoria BC, Canada) [24], phosphorylated NHE3 (p-NHE3 antibody; 1:500 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) [25], sodium chloride co-transporter (NCC; 1:250 dilution), phosphorylated NCC (p-NCC; 1:250 dilution) [26], epithelial sodium channel-α (anti-ENaC-α; 1:5000 dilution; StressMarq), ENaC-β (anti-ENaC-β; 1:1000 dilution; StressMarq), ENaC-γ (anti-ENaC-γ; 1:1000 dilution; StressMarq) [27], aquaporin 2 (AQP2, C-17; 1:5000 dilution; Santa Cruz Biotechnology, Inc.) [28], and phosphorylated AQP2 (p-AQP2, anti-phospho-ser^{269} aquaporin 2; 1:1000 dilution; Phosphosolutions, Aurora, CO, USA) [29]. Equal loading was confirmed by re-probing

1

2 **Statistical analysis**

3 Data are the mean ± SEM. We used one-way analysis of variance followed by
4 Bonferroni post-test for all cross-sectional one-factor data to compare values in
5 vehicle-treated SHRcp with those treated with luseogliflozin, diuretics or luseogliflozin
6 plus diuretics. Cross-sectional two-factor SHRcp data (12-h dark-period and 12-h
7 light-period MAP) were analyzed by two-way analysis of variance followed by
8 Bonferroni post-test. All longitudinal data (postprandial blood glucose; SBP; MAP;
9 electrolyte balances; urinary excretion of glucose and electrolytes; urinary osmolality;
10 body weight; food intake; water intake and urine volume) were analyzed by two-way
11 analysis of variance for repeated measures followed by the Bonferroni post-test unless
12 indicated otherwise. A value of $P<0.05$ was considered significant.

13

SHRcp. Addition of diuretics to luseogliflozin had similar effects on Hb_{A1c} levels in these animals (Table 1).

Effects of luseogliflozin and diuretics on blood pressure

In the present study, we measured 24-h blood pressure in conscious rats using a telemetry system [21]. Effects of luseogliflozin and diuretics on blood pressure are depicted in Fig. 2A–E. In vehicle-treated SHRcp, 24-h SBP increased gradually during the experimental period. Luseogliflozin significantly blunted the development of hypertension in SHRcp. Moreover, a significant interaction (P=0.03) was observed for the SBP between vehicle- and luseogliflozin-treated SHRcp. Treatment with diuretics also decreased SBP in these animals. Concomitant treatment of luseogliflozin plus diuretics showed the same trend of blood pressure reduction as that seen for diuretics alone. However, after 3 weeks of treatment, combination therapy decreased blood pressure further (Fig. 2A). Though diuretics alone significantly interacted (P=0.008) with vehicle-treated SHRcp for SBP, a significant interaction (P=0.009) was also observed between vehicle- and luseogliflozin plus diuretics-treated SHRcp. Furthermore, a significant interaction (P=0.015) was observed for SBP between luseogliflozin- and diuretics-treated SHRcp but was not significant (P=0.145) for time variation during the experimental period. Twenty-four-hour MAP followed the same trend as that for SBP (Fig. 2B). Normotensive WKY rats and SHR exhibited greater dark (active)-period MAP (108.2±1.0 and 168.0±2.1 mmHg at week 5, respectively) than that of light (inactive)-period MAP (102.0±0.5 and 162.2±1.6 mmHg, respectively), suggesting a dipper type phenomenon of blood pressure (Fig. 2C-E). Conversely, vehicle-treated SHRcp showed a non-dipper type of hypertension because no significant difference was

(Fig. 3A). Luseogliflozin addition to diuretics caused a significant interaction with vehicle treated-SHRcp (P=0.037). A significant interaction was also observed between luseogliflozin- and diuretics-treated SHRcp (P=0.027). Thus, data of sodium balance suggest that luseogliflozin or luseogliflozin plus diuretics caused higher urinary excretion of sodium compared with vehicle-treated SHRcp. Urinary excretion of potassium or chloride was also increased significantly by treatment with luseogliflozin or luseogliflozin plus diuretics (Supplemental Fig. 3C and D). The interaction of potassium balance was also significant in vehicle- *vs.* luseogliflozin- (P=0.017), vehicle- *vs.* luseogliflozin plus diuretics- (P=0.002), and luseogliflozin- *vs.* diuretics- (P=0.047)-treated SHRcp (Fig. 3B). Chloride balance also interacted significantly between vehicle- and luseogliflozin- (P=0.002), vehicle- and diuretics- (P=0.009), vehicle- and luseogliflozin plus diuretics- (P<0.0001), or luseogliflozin- and diuretics (P<0.0001)-treated SHRcp (Fig. 3C). Significant differences in plasma levels of sodium, potassium or chloride were not observed among treatment groups (data not shown).

Effects of luseogliflozin and diuretics on other renal parameters

Administration of luseogliflozin or luseogliflozin plus diuretics tended to increase creatinine clearance in SHRcp, but these changes were not significant (Fig. 4A). However, urine flow increased remarkably (P<0.001) 1 day after administration of luseogliflozin or luseogliflozin plus diuretics (Supplemental Fig. 1D). Plasma levels of angiotensin II were not changed by luseogliflozin or diuretics, but were increased significantly (P<0.05) by concomitant treatment with luseogliflozin plus diuretics. No significant change was observed in plasma levels of aldosterone or vasopressin (Table 2). Urine osmolality was decreased significantly by luseogliflozin, diuretics or

However, expression of p-NHE3 protein was not changed among treatment groups (Fig. 6A). Conversely, luseogliflozin, diuretics or luseogliflozin plus diuretics significantly increased levels of p-NCC in renal cortical membrane fractions. NCC expression tended to increase in luseogliflozin- or diuretics-treated SHRcp, but significantly increased expression was observed only in luseogliflozin plus diuretics-treated SHRcp (Fig. 6B). ENaC-α protein expression in renal cortical tissues tended to increase in luseogliflozin-, diuretics- or luseogliflozin plus diuretics-treated SHRcp (Fig. 6C), whereas levels of ENaC-β and -γ subunit proteins were not altered by any treatment. None of the treatments affected levels of AQP2 or p-AQP2 proteins in the inner medulla of the kidney (Supplemental Fig. 4).

in which the difference between dark-period and light-period MAP was 5.3 mmHg ($P<0.01$). There is a direct relationship between the abnormality of a dipping pattern of blood pressure and cardiovascular events [30]. Hence, our data are consistent with the hypothesis that the cardiovascular-protective effects of a SGLT2 inhibitor are mediated (at least in part) by normalization of the circadian rhythm of blood pressure. However, in the present study, excluding the possibility that blood-pressure reduction during the light-period is due merely to the short-acting action of luseogliflozin is difficult. In this regard, a study has shown that a single dose of luseogliflozin (3 mg/kg body weight, p.o.) results in a significant increase in urinary excretion of glucose even after 8 h of treatment in rats with type-2 diabetes mellitus, suggesting that oral administration of luseogliflozin elicits relatively long-acting effects in rats [32]. In accordance with those findings, it can be speculated that blood-pressure reduction in the light-period induced by luseogliflozin cannot be explained only by the duration of the half-life of luseogliflozin. In the present study, concomitant treatment of luseogliflozin plus diuretics also decreased blood pressure in the dark-period and light-period, and increased differences in blood pressure during these periods (change in the difference between dark-period and light-period MAP = 7.4±2.3 mmHg, $P<0.01$).

Excretion of electrolytes in urine

Sodium retention plays a predominant part in the development of hypertension in patients suffering from the metabolic syndrome [33], which contributes to the development of a disrupted circadian rhythm of blood pressure [34, 35]. In the present study, blood-pressure reductions were associated with 44% and 76% increases in the urinary excretion of sodium by treatment with luseogliflozin and luseogliflozin plus

explained by differences in the animal model (SHRcp vs. normal Wistar rats), experimental setup (chronic vs. acute administration) or specificity for the SGLT2 inhibitor (luseogliflozin vs. phlorizin). Next, we measured the protein abundance of NCC, which is sensitive to thiazide and responsible for reabsorption of 5–10% of filtered sodium in the distal convoluted tubules [38]. Several studies have suggested that treatment with HCTZ [39–41] or furosemide [38, 40] significantly increases the protein abundance of NCC in renal cortical tissues, an effect that might be induced by compensatory effects against increases in sodium and water load at distal nephrons. Interestingly, luseogliflozin alone also showed similar increases in expression of NCC protein. Na et al. [40] reported that chronic administration of furosemide causes a significant increase in protein abundance of all ENaC subunits, whereas HCTZ increases only β-ENaC in the cortex. We observed that expression of ENaC-α protein (but not ENaC-γ or -β proteins) tended to increase in the renal cortical tissues of luseogliflozin-, diuretics- or luseogliflozin plus diuretics-treated SHRcp, but that these changes were not significant. Studies have demonstrated that furosemide administration does not change the protein abundance of AQP1–3 in the cortex and outer medulla of the kidney, despite a significant increase in urine output and decrease in urinary osmolality [40]. We also observed that none of the treatments altered expression of AQP2 protein. To explore further the mechanism responsible for luseogliflozin-induced urinary excretion of sodium, future studies will be undertaken to examine the effects of acute systemic administration of SGLT2 inhibitors on regulation of renal sodium transporters.

Renal hemodynamics and renal parameters

Expression of glucose transporters in kidney

A growing body of evidence suggests that SGLT2 expression is increased in diabetic kidneys [43, 44]. Treatment with a SGLT2 inhibitor does not change expression of SGLT2 membrane protein in Akita mice with type-1 diabetes mellitus [43], whereas phlorizin causes an increase in SGLT2 expression in streptozotocin-injected Wistar rats [44]. We showed that vehicle-treated SHRcp exhibit higher expression of SGLT2 mRNA and more intense immunohistochemical staining compared with vehicle-treated WKY rats or SHR. Furthermore, luseogliflozin or luseogliflozin plus diuretics caused a significant reduction in expression of SGLT2 mRNA and protein in SHRcp. These data suggest that SGLT2 expression in the kidney is decreased by chronic treatment with a SGLT2 inhibitor in subjects with the metabolic syndrome. In agreement with another report [43], our data showed that expression of SGLT1 mRNA tended to decrease in luseogliflozin- or luseogliflozin plus diuretics-treated SHRcp. However, further studies are needed to examine the effects of a selective SGLT2 inhibitor on the activities of SGLT1 and SGLT2, as well as their relationship with sodium balance.

Clinical correlation and perspectives

Animal [17, 32] and clinical studies [45, 46] have suggested that luseogliflozin treatment shows beneficial effects on control of blood glucose in subjects with type-2 diabetes mellitus. In accordance with these findings, we showed that luseogliflozin treatment caused a significant improvement in glucose intolerance and insulin resistance in SHRcp. Insulin resistance has a crucial role in the development of hypertension in subjects with the metabolic syndrome [1]. Therefore, improvement of insulin resistance by luseogliflozin may contribute (at least in part) to its antihypertensive effect.

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systematic review and meta-analysis. *J Am Soc Hypertens* 2014; 8:262275.

10. Maliha G, and Townsend RR. SGLT2 inhibitors: their potential reduction in blood pressure. *J Am Soc Hypertens* 2015; 9:48-53.
11. Lin B, Koibuchi N, Hasegawa Y, Sueta D, Toyama K, Uekawa K, Ma M, Nakagawa T, Kusaka H, and Kim-Mitsuyama S. Glycemic control with empagliflozin, a novel selective SGLT2 inhibitor, ameliorates cardiovascular injury and cognitive dysfunction in obese and type 2 diabetic mice. *Cardiovasc Diabetol* 2014; 13:148.
12. Vasilakou D, Karagiannis T, Athanasiadou E, Mainou M, Liakos A, Bekiari E, Sarigianni M, Matthews DR, and Tsapas A. Sodium-glucose cotransporter 2 inhibitors for type 2 diabetes: a systematic review and meta-analysis. *Ann Intern Med* 2013; 159:262-274.
13. Haas B, Eckstein N, Pfeifer V, Mayer P, and Hass MD. Efficacy, safety and regulatory status of SGLT2 inhibitors: focus on canagliflozin. *Nutr diabetes* 2014; 4:e143.
14. Moses RG, Colagiuri S, and Pollock C. SGLT2 inhibitors: New medicines for addressing unmet needs in type 2 diabetes. *Australas Med J* 2014; 7:405-415.
15. Devineni D, Vaccaro N, Polidori D, Rusch S, and Wajs E. Effects of hydrochlorothiazide on the pharmacokinetics, pharmacodynamics, and tolerability of canagliflozin, a sodium glucose co-transporter 2 inhibitor, in healthy participants. *Clin Ther* 2014; 36:698-710.
16. Sueta D, Kataoka K, Koibuchi N, Toyama K, Uekawa K, Katayama T, Mingjie M, Nakagawa T, Waki H, Maeda M, *et al.* Novel mechanism for disrupted circadian blood pressure rhythm in a rat model of metabolic syndrome--the critical role of

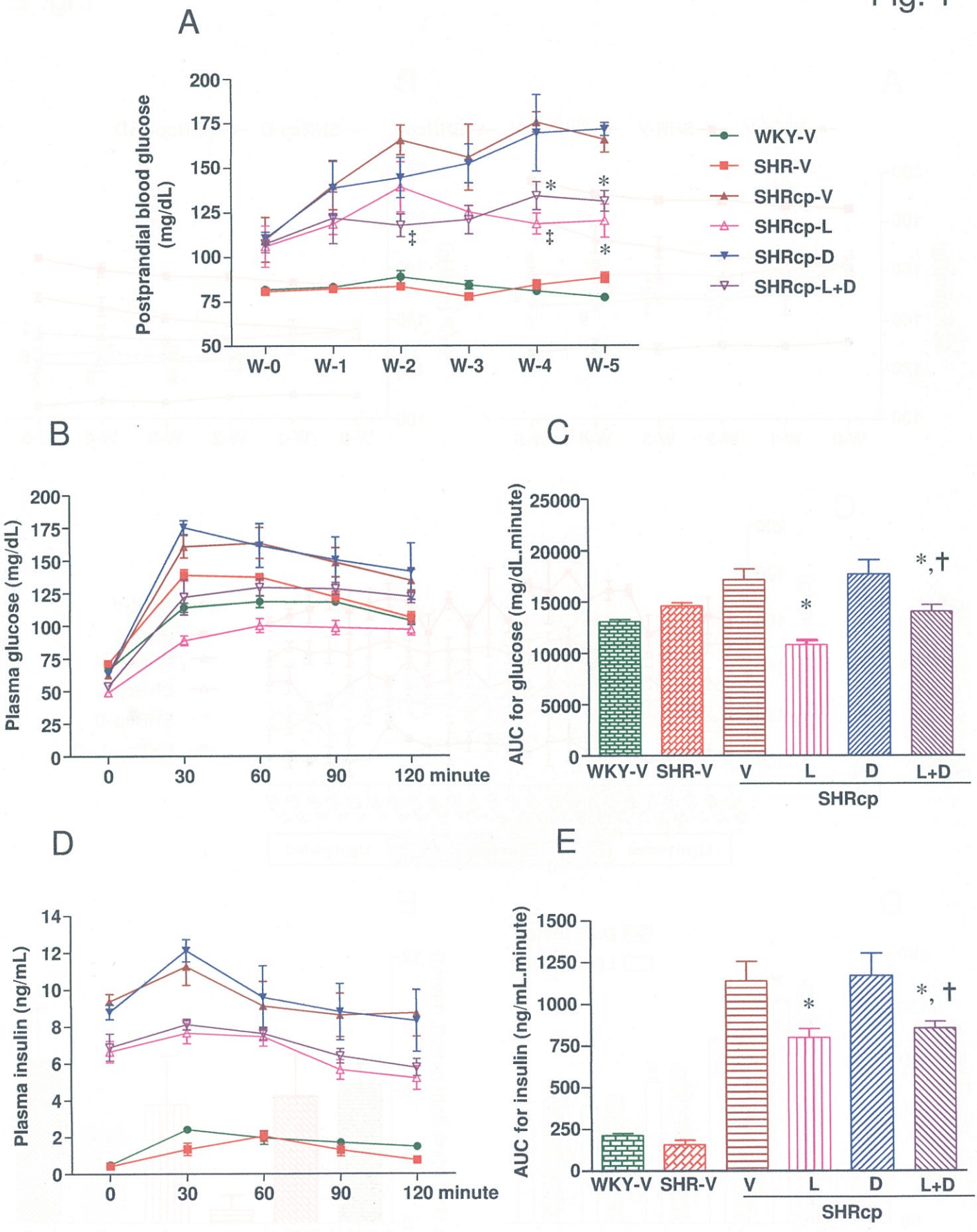
Nielsen S. Compensatory increase in AQP2, p-AQP2, and AQP3 expression in rats with diabetes mellitus. American journal of physiology. *Am J Physiol Renal Physiol* 2001; 280:F715-726.

24. Dynia DW, Steinmetz AG, and Kocinsky HS. NHE3 function and phosphorylation are regulated by a calyculin A-sensitive phosphatase. *Am J Physiol Renal Physiol* 2010; 298: F745-753.
25. Schultheis PJ, Clarke LL, Meneton P, Miller ML, Soleimani M, Gawenis LR, Riddle TM, Duffy JJ, Doetschman T, Wang T, *et al.* Renal and intestinal absorptive defects in mice lacking the NHE3 Na+/H+ exchanger. *Nat Genet* 1998; 19:282-285.
26. Nishida H, Sohara E, Nomura N, Chiga M, Alessi DR, Rai T, Sasaki S, and Uchida S. Phosphatidylinositol 3-kinase/Akt signaling pathway activates the WNK-OSR1/SPAK-NCC phosphorylation cascade in hyperinsulinemic db/db mice. *Hypertension 2012;* 60:981-990.
27. Kakizoe Y, Kitamura K, Ko T, Wakida N, Maekawa A, Miyoshi T, Shiraishi N, Adachi M, Zhang Z, Masilamani S, *et al.* Aberrant ENaC activation in Dahl salt-sensitive rats. *J Hypertens* 2009; 27:1679-1689.
28. Nedvetsky PI, Tabor V, Tamma G, Beulshausen S, Skroblin P, Kirschner A, Mutig K, Boltzen M, Petrucci O, Vossenkamper A, *et al.* Reciprocal regulation of aquaporin-2 abundance and degradation by protein kinase A and p38-MAP kinase. *J Am Soc Nephrol 2010;* 21:1645-1656.
29. Fenton RA, Moeller HB, Hoffert JD, Yu MJ, Nielsen S, and Knepper MA. Acute regulation of aquaporin-2 phosphorylation at Ser-264 by vasopressin. *Proc Natl Acad Sci USA* 2008; 105:3134-3139.
30. Karaagac K, Tenekecioglu E, Yontar OC, Kuzeytemiz M, Vatansever F, Tutuncu A,

role of glucose metabolism, osmotic stress, and sodium-glucose cotransporter isoform-mediated transport on Na+/H+ exchanger isoform 3 activity in the renal proximal tubule. *J Am Soc Nephrol* 2014; 25:2028-2039.

38. Abdallah JG, Schrier RW, Edelstein C, Jennings SD, Wyse B, and Ellison DH. Loop diuretic infusion increases thiazide-sensitive Na(+)/Cl(-)-cotransporter abundance: role of aldosterone. *J Am Soc Nephrol* 2001; 12:1335-1341
39. Jang HR, Kim S, Heo NJ, Lee JH, Kim HS, Nielsen S, Jeon US, Oh YK, Na KY, Joo KW, *et al.* Effects of thiazide on the expression of TRPV5, calbindin-D28K, and sodium transporters in hypercalciuric rats. *J Korean Med Sci* 2009; 24:S161-169.
40. Na KY, Oh YK, Han JS, Joo KW, Lee JS, Earm JH, Knepper MA, and Kim GH. Upregulation of Na+ transporter abundances in response to chronic thiazide or loop diuretic treatment in rats. American journal of physiology. *Am J Physiol Renal Physiol* 2003; 284:F133-143.
41. Kim GH, Lee JW, Oh YK, Chang HR, Joo KW, Na KY, Earm JH, Knepper MA, and Han JS. Antidiuretic effect of hydrochlorothiazide in lithium-induced nephrogenic diabetes insipidus is associated with upregulation of aquaporin-2, Na-Cl co-transporter, and epithelial sodium channel. *J Am Soc Nephrol* 2004; 15:2836-2843.
42. Kobori H, Nangaku M, Navar LG, Nishiyama A. The intrarenal renin-angiotensin system: from physiology to the pathobiology of hypertension and kidney disease. *Pharmacol Rev* 2007; 59(3): 251-87.
43. Vallon V, Gerasimova M, Rose MA, Masuda T, Satriano J, Mayoux E, Koepsell H, Thomson SC, and Rieg T. SGLT2 inhibitor empagliflozin reduces renal growth and

Fig. 1


A
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175
150
125
100
75
50
W-0
W-1
W-2
W-3
W-4
W-5
WKY-V
SHR-V
SHRcp-V
SHRcp-L
SHRcp-D
SHRcp-L+D
B
Plasma glucose (mg/dL)
0
30
60
90
120 minute
C
AUC for glucose (mg/dL.minute)
25000
20000
15000
10000
5000
0
WKY-V
SHR-V
V
L
D
L+D
SHRcp
D
Plasma insulin (ng/mL)
14
12
10
8
6
4
2
0
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AUC for insulin (ng/mL.minute)
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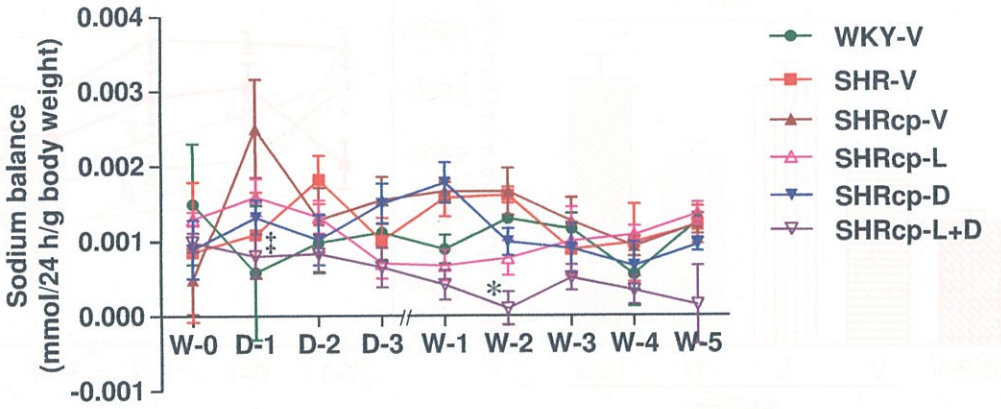
1250
1000
750
500
250
0

Fig. 3

A


Sodium balance
(mmol/24 h/g body weight)
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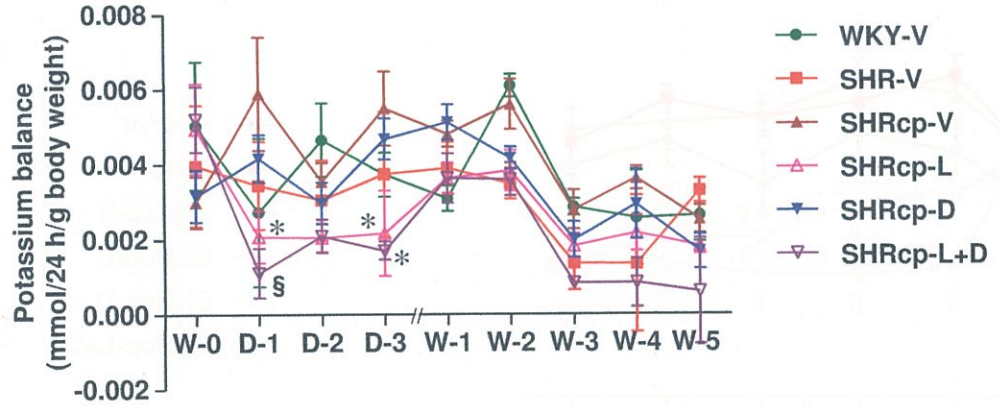
0.003
0.002
0.001
0.000
-0.001
W-0 D-1 D-2 D-3 W-1 W-2 W-3 W-4 W-5
WKY-V
SHR-V
SHRcp-V
SHRcp-L
SHRcp-D
SHRcp-L+D

B


Potassium balance
(mmol/24 h/g body weight)
0.008
0.006
0.004
0.002
0.000
-0.002
W-0 D-1 D-2 D-3 W-1 W-2 W-3 W-4 W-5
WKY-V
SHR-V
SHRcp-V
SHRcp-L
SHRcp-D
SHRcp-L+D

C

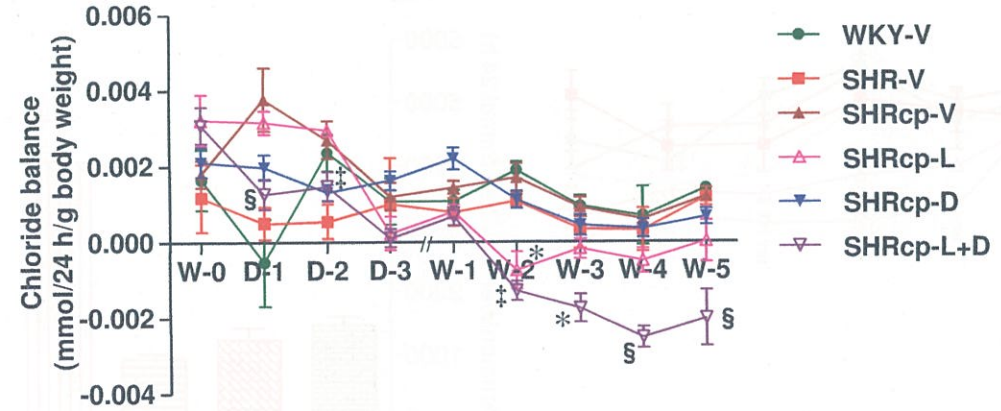
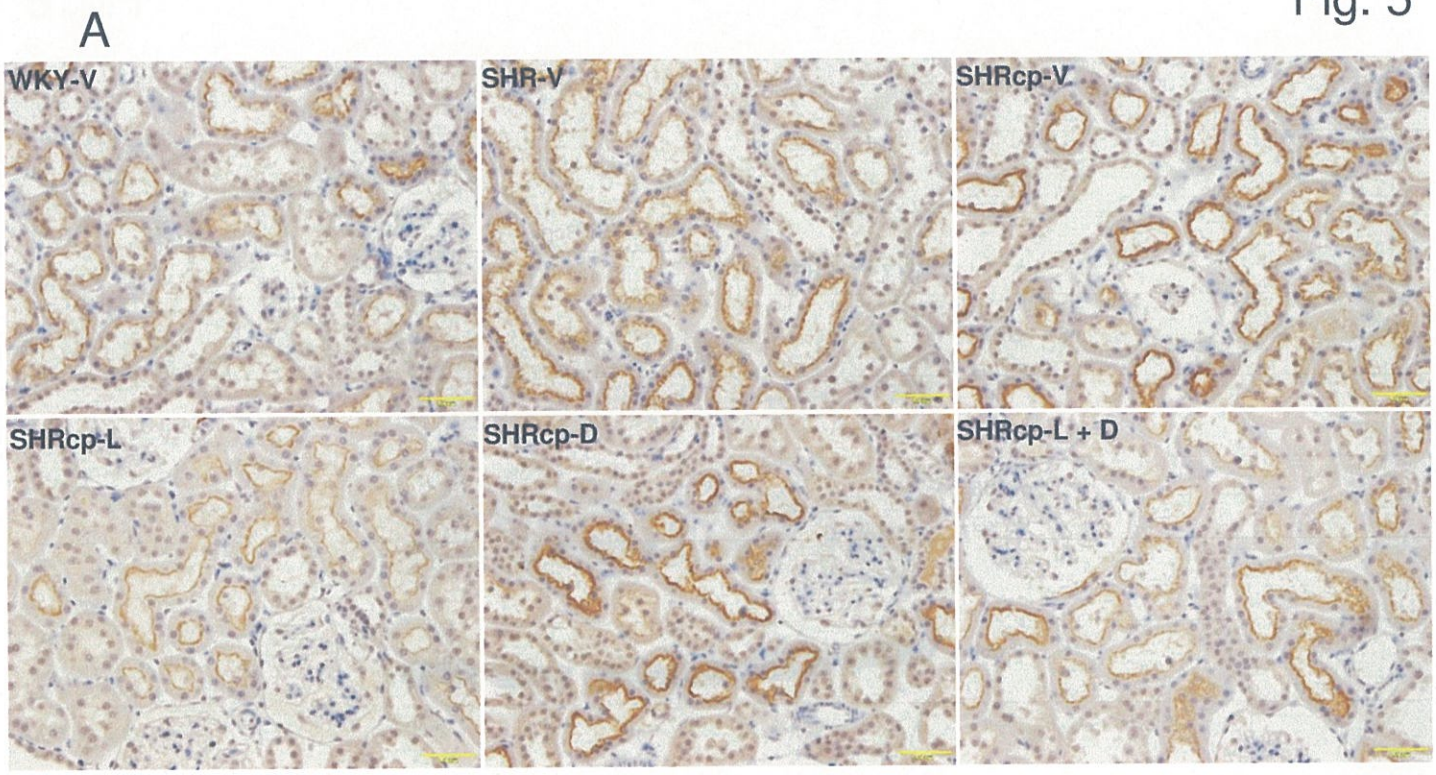
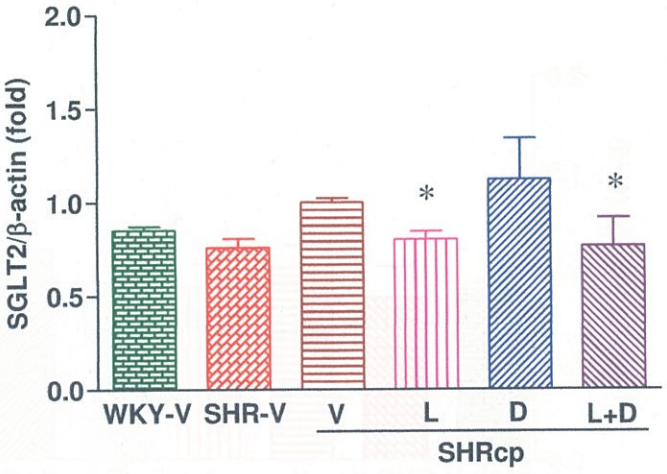
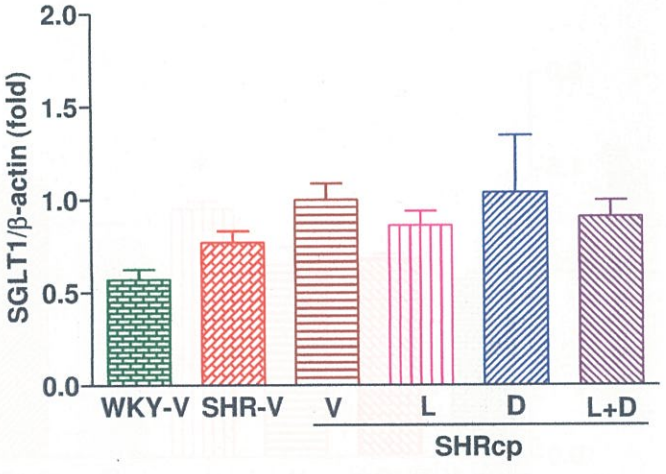
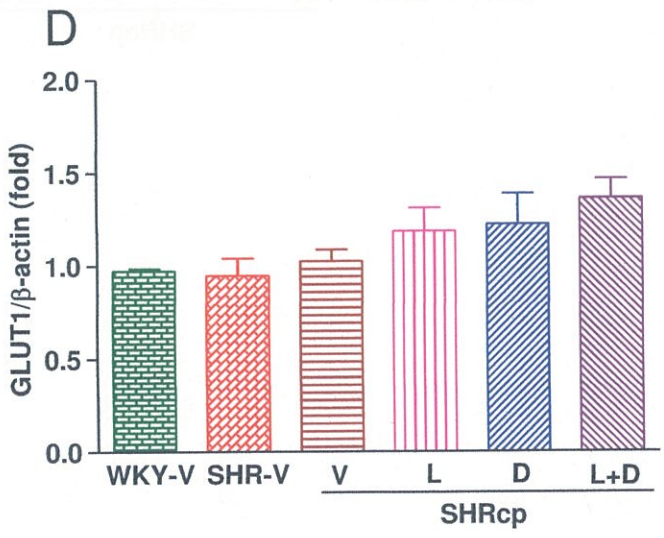

Chloride balance
(mmol/24 h/g body weight)
0.006
0.004
0.002
0.000
-0.002
-0.004
W-0 D-1 D-2 D-3 W-1 W-2 W-3 W-4 W-5
WKY-V
SHR-V
SHRcp-V
SHRcp-L
SHRcp-D
SHRcp-L+D

Fig. 5


A
WKY-V
SHR-V
SHRcp-V
SHRcp-L
SHRcp-D
SHRcp-L + D


B
SGLT2/β-actin (fold)
2.0
1.5
1.0
0.5
0.0
*
*
WKY-V
SHR-V
V
L
D
L+D
SHRcp


C
SGLT1/β-actin (fold)
2.0
1.5
1.0
0.5
0.0
WKY-V
SHR-V
V
L
D
L+D
SHRcp


D
GLUT1/β-actin (fold)
2.0
1.5
1.0
0.5
0.0
WKY-V
SHR-V
V
L
D
L+D
SHRcp

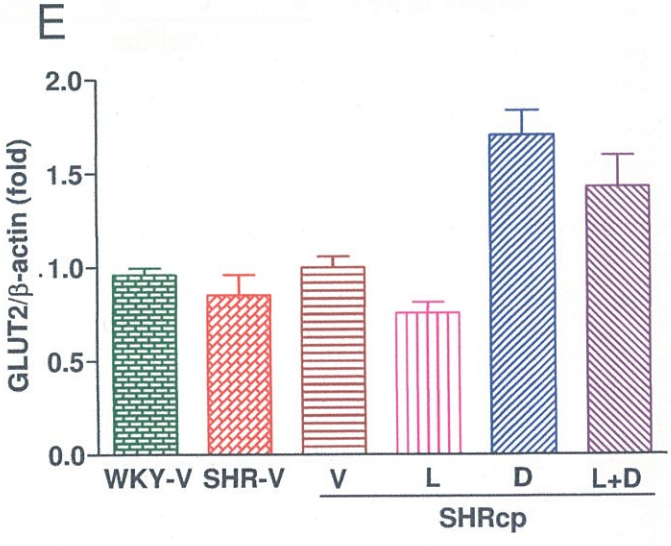
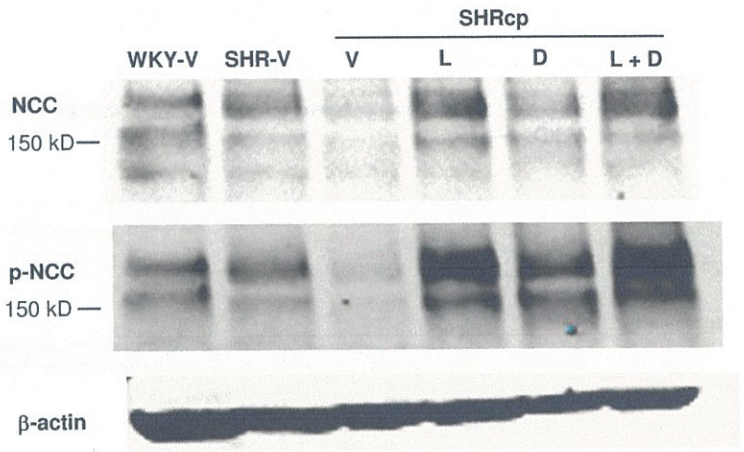
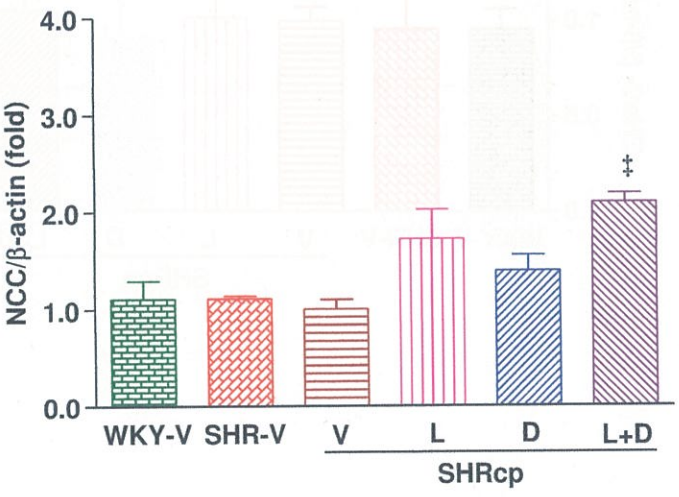

E
GLUT2/β-actin (fold)
2.0
1.5
1.0
0.5
0.0
WKY-V
SHR-V
V
L
D
L+D
SHRcp

Fig. 6 (Contd.)

B


SHRcp
WKY-V
SHR-V
V
L
D
L + D
NCC
150 kD
p-NCC
150 kD
β-actin


NCC/β-actin (fold)
4.0
3.0
2.0
1.0
0.0
‡
WKY-V
SHR-V
V
L
D
L+D
SHRcp

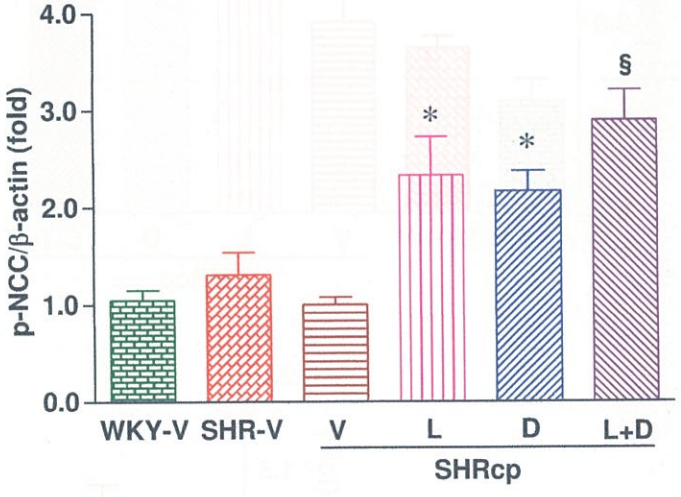

p-NCC/β-actin (fold)
4.0
3.0
2.0
1.0
0.0
*
*
§
WKY-V
SHR-V
V
L
D
L+D
SHRcp

FIGURE LEGENDS

Fig. 1. Glucose homeostasis and insulin resistance

Postprandial blood glucose levels during the 5-week treatment period (A). Luseogliflozin or luseogliflozin plus diuretics cause a significant decrease in post-prandial blood glucose levels. Responses of blood glucose and plasma insulin to an oral glucose load during oral glucose tolerance test, and calculated total area under curves (AUC) for blood glucose and plasma insulin are shown (B–E). Compared with WKY rats and SHR, vehicle- and diuretics-treated SHRcp show higher levels of glucose and insulin. Luseogliflozin or luseogliflozin plus diuretics blunt increases in blood levels of glucose and plasma levels of insulin. WKY-V, vehicle-treated Wister–Kyoto rats; SHR-V, vehicle-treated spontaneously hypertensive rats; SHRcp, SHR/NDmcr-cp rats; V, vehicle; L, luseogliflozin; D, diuretics (hydrochlorothiazide + furosemide); L+D, luseogliflozin + diuretics; W-0 to W-5, week of treatment. Values are the mean ± S.E.M. $^{*}P<0.05$; $^{\ddagger}P<0.01$, vehicle-treated SHRcp *vs.* luseogliflozin- or luseogliflozin plus diuretics-treated SHRcp; $^{\dagger}P<0.05$, diuretics-treated SHRcp *vs.* luseogliflozin plus diuretics-treated SHRcp.

Fig. 2. Blood pressure and its circadian rhythm

Twenty four-h systolic blood pressure (SBP) (A) and mean arterial pressure (MAP) (B). Vehicle-treated SHRcp develop hypertension during the observation period, whereas luseogliflozin blunts the increase in blood pressure. Diuretics treatment also decreases blood pressure. Adding luseogliflozin to diuretics further decreases blood pressure in SHRcp. Twenty four-h MAP after 5 weeks treatment (C). Twelve-hour dark- and 12-h

diuretics-treated SHRcp. A significant increase in urinary excretion of ammonia/ammonium is observed in luseogliflozin- and luseogliflozin plus diuretics-treated SHRcp (E). N.S., non significant $^{*}P<0.05$; $^{\ddagger}P<0.01$; $^{\S}P<0.001$, vehicle-treated SHRcp *vs.* luseogliflozin-, diuretics- or luseogliflozin plus diuretics-treated SHRcp.

Fig. 5. Expression of glucose transporters

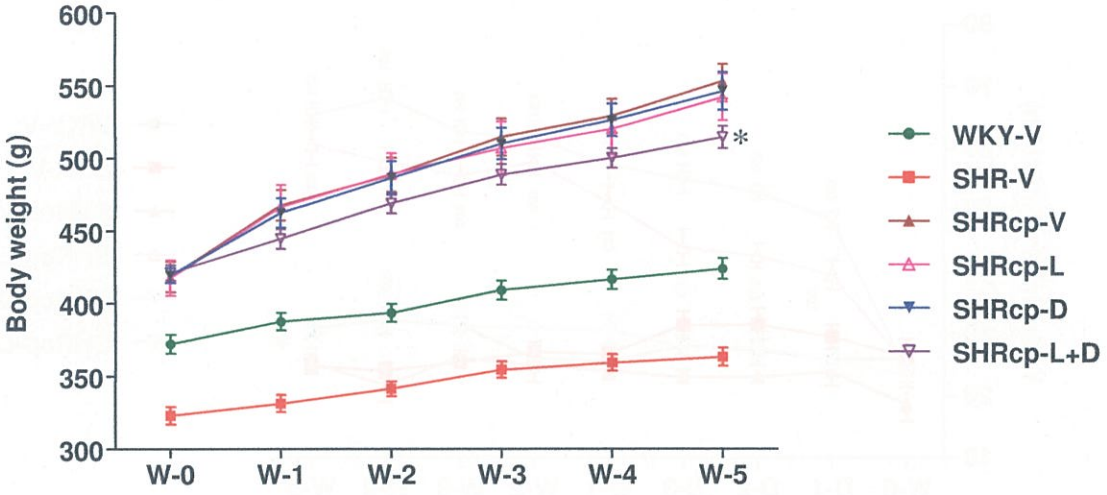
Representative microphotograph of kidney sections of immunostaining for sodium-dependent glucose co-transporter (SGLT)2 (A). Scale bar, 50 μm. SGLT2 (B), SGLT1 (C), glucose transporter (GLUT)1 (D) and GLUT2 (E) mRNA levels in renal cortical tissues are shown. SGLT2 immunostaining and mRNA expression are decreased significantly in luseogliflozin- or luseogliflozin plus diuretics-treated SHRcp. $^{*}P<0.05$; vehicle-treated SHRcp *vs.* luseogliflozin- or luseogliflozin plus diuretics-treated SHRcp.

Fig. 6. Protein levels of several sodium transporters in the kidney

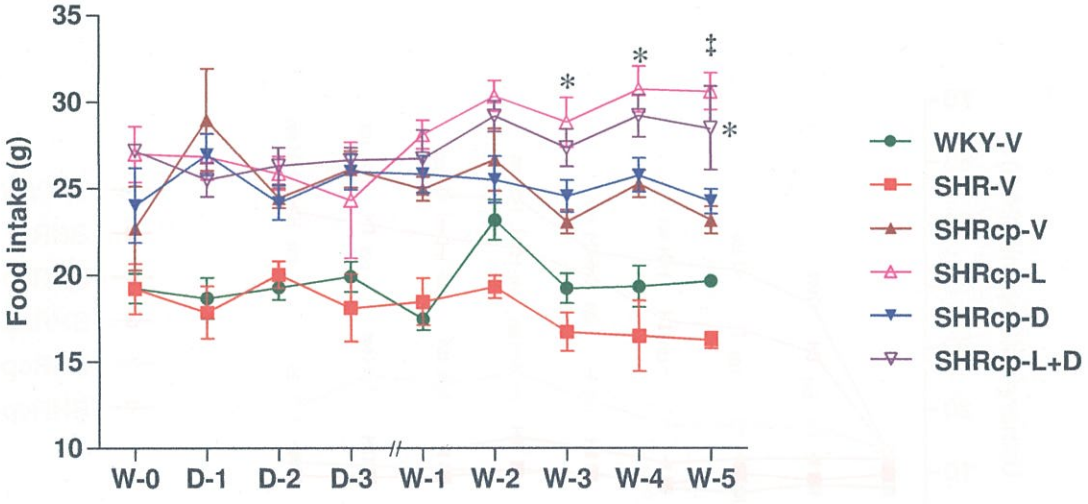
Protein expression of sodium–hydrogen exchanger 3 (NHE3) and phosphorylated NHE3 (A) in renal cortices. Expression of sodium chloride co-transporter (NCC) and phosphorylated NCC protein in renal cortical tissues (B). Expression of epithelial sodium channel-α (ENaC-α), ENaC-β and ENaC-γ proteins in renal cortical tissues (C). A significant increase in levels of NHE3 and NCC proteins are observed in luseogliflozin- or luseogliflozin plus diuretics-treated SHRcp. $^{*}P<0.05$; $^{\ddagger}P<0.01$; $^{\S}P<0.001$, vehicle-treated SHRcp *vs.* luseogliflozin-, diuretics- or luseogliflozin plus diuretics-treated SHRcp.

Supplemental Fig. 1

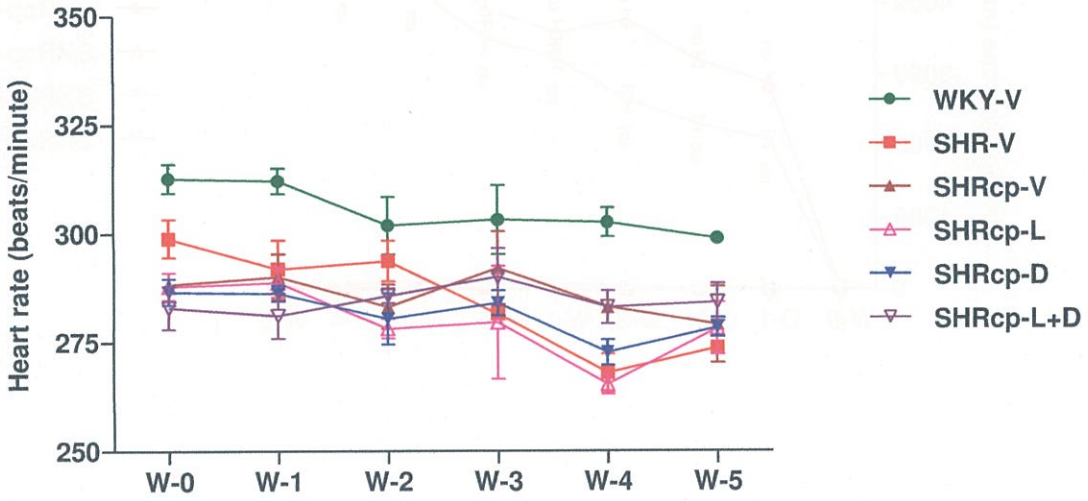
A



B

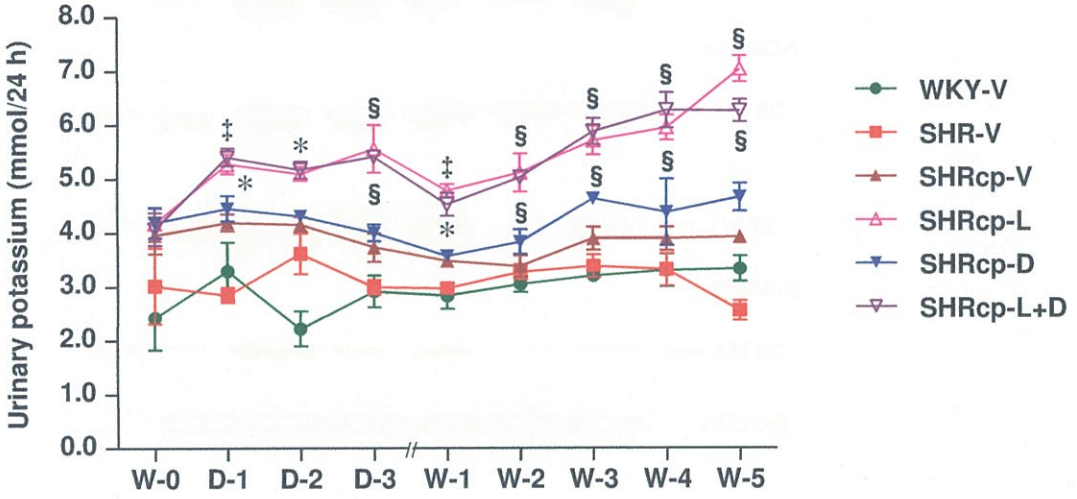


Supplemental Fig. 2

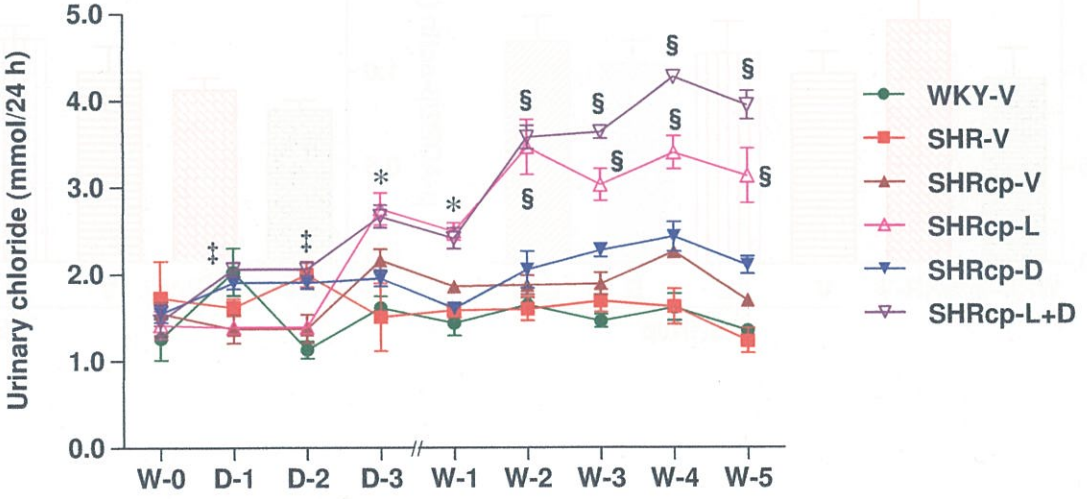


Supplemental Fig. 3 (Contd.)

C



D



SUPPLEMENTAL FIGURE LEGENDS

Supplemental Fig. 1. Body weight, food intake, water intake and urine volume

Body weight (A), food intake (B), water intake (C) and urine volume (D) are shown. Body weight is decreased significantly in luseogliflozin plus diuretics-treated SHRcp. Food intake is increased significantly in luseogliflozin- or luseogliflozin plus diuretics-treated SHRcp. Water intake and urine volume are also increased significantly within 1-day administration of luseogliflozin or luseogliflozin plus diuretics. WKY-V, vehicle-treated Wister–Kyoto rats; SHR-V, vehicle-treated spontaneously hypertensive rats; SHRcp, SHR/NDmcr-cp rats; V, vehicle; L, luseogliflozin; D, diuretics (hydrochlorothiazide + furosemide); L + D, luseogliflozin + diuretics; W-0 to W-5, weeks of treatment; D-1 to D-3, days of treatment. $^{*}P<0.05$; $^{\ddagger}P<0.01$; $^{\S}P<0.001$, vehicle-treated SHRcp *vs.* luseogliflozin-, diuretics- or luseogliflozin plus diuretics-treated SHRcp.

Supplemental Fig. 2. Heart rate

Twenty four-h heart rate during the experimental period. None of the treatment significantly alter the heart rate.

Supplemental Fig. 3. Urinary excretion of glucose and electrolytes

Time-dependent changes in 24 h urinary excretion of glucose (A) and sodium (B). Luseogliflozin or luseogliflozin plus diuretics significantly increase urinary excretion of glucose. However, urinary excretion of sodium reaches a significant difference after 3-day administration of luseogliflozin or luseogliflozin plus diuretics. Urinary excretion

Table 1. Parameters related to the metabolism of lipids and glucose

	WKY-V	SHR-V	SHRcp			
			V	L	D	L+D
Total cholesterol (mg/dL)	123±5	77±2	137±4	135±4	128±12	136±5
NEFA (mEq/L)	1.1±0.1	1.0±0.1	2.3±0.2	1.9±0.2	1.8±0.1	2.0±0.2
Triglyceride (mg/dL)	58±13	65±10	369±26	361±23	328±29	319±18
Hb_{A1c} (%, NGSP)	4.0±0.1	3.8±0.1	4.4±0.1	4.0±0.1*	4.4±0.1	3.9±0.1*†

WKY-V, vehicle-treated Wistar–Kyoto rats; SHR-V, vehicle-treated spontaneously hypertensive rats; SHRcp, SHR/NDmcr-cp rats; V, vehicle; L, luseogliflozin; D, diuretics (hydrochlorothiazide + furosemide); L+D, luseogliflozin + diuretics; NEFA, non-esterified fatty acid.

$^{*}P<0.05$, vehicle-treated SHRcp *vs.* luseogliflozin- or luseogliflozin plus diuretics-treated SHRcp; $^{\dagger}P<0.05$, diuretics-treated SHRcp *vs.* luseogliflozin plus diuretics-treated SHRcp.

Supplemental Table 1. Summary of mRNA and protein abundance of different transporters in kidney

	WKY-V	SHR-V	SHRcp			
			V	L	D	L+D
mRNA						
SGLT1 (relative ratio)	0.6±0.1	0.8±0.1	1.0±0.1	0.86±0.1	1.0±0.2	0.9±0.1
SGLT2 (relative ratio)	0.9±0.02	0.8±0.04	1.0±0.02	0.8±0.04*	1.1±0.2	0.7±0.2*
GLUT1 (relative ratio)	0.9±0.01	0.9±0.1	1.0±0.06	1.2±0.2	1.6±0.4	1.4±0.1
GLUT2 (relative ratio)	0.9±0.03	0.9±0.1	1.0±1.06	0.9±0.11.	1.7±0.1	1.4±0.2
Protein abundance						
NHE3 (relative ratio)	0.9±0.04	1.1±0.01	1.0±0.1	1.3±0.04*	1.1±0.08	1.4±0.05‡
p-NHE3 (relative ratio)	0.9±0.04	0.8±0.2	1.0±0.1	0.9±0.1	0.9±0.1	1.1±0.1
NCC (relative ratio)	1.1±0.2	1.1±0.03	1.0±0.1	1.7±0.3	1.4±0.2	2.1±0.1‡
p-NCC (relative ratio)	1.0±0.1	1.3±0.23	1.0±0.08	2.3±0.4*	2.2±0.2*	2.9±0.3§
ENaC-α (relative ratio)	0.6±0.1	1.2±0.1	1.0±0.1	1.3±0.2	1.3±0.2	1.3±0.03
ENaC-β (relative ratio)	0.9±0.1	0.9±0.2	1.0±0.1	1.0±0.1	0.9±0.2	1.0±0.2
ENaC-γ (relative ratio)	1.1±0.1	1.3±0.1	1.0±0.1	0.9±0.1	0.9±0.1	1.0±0.1
Aquaporin 2 (relative ratio)	0.9±0.2	1.3±0.2	1.0±0.1	1.1±0.2	1.0±0.1	1.2±0.1
p-Aquaporin 2 (relative ratio)	0.8±0.1	0.9±0.1	1.0±0.1	1.2±0.1	0.8±0.1	0.8±0.1

WKY-V, vehicle-treated Wistar–Kyoto rats; SHR-V, vehicle-treated spontaneously hypertensive rats; SHRcp, SHR/NDmcr-cp rats; V, vehicle; L, luseogliflozin; D, diuretics (hydrochlorothiazide + furosemide); L+D, luseogliflozin + diuretics.

*$P<0.05$; ‡$P<0.01$; §$P<0.001$, vehicle-treated SHRcp *vs.* luseogliflozin-, diuretics- or luseogliflozin plus diuretics-treated SHRcp.