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Glucagon like peptide-1 modulates urinary sodium excretion in diabetic kidney disease via ENaC activation

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Diabetic kidney disease (DKD) is a leading cause of end stage kidney disease. Elevated salt sensitivity by epithelial sodium channel (ENaC) overexpression may be a residual risk factor for DKD. We found that combination therapy of linagliptin (LINA) to empagliflozin (EMPA), but not EMPA alone decreased phosphorylated Nedd4-2 (p-Nedd4-2) and ENaC levels in DKD rats in association with the increased urinary sodium excretion (USE). More extensive renoprotective effects were observed by the combination therapy of LINA and EMPA in deoxycorticosterone and high salt-treated mice. Acute injection experiments showed time-lagged administration of LINA to EMPA increased USE, and its effect sustained until 3 h. High salt and high glucose increased p-Nedd4-2 and ENaC levels in cultured distal tubules, which was inhibited by LINA or glucagon like peptide-1 (GLP-1), but there were no additive effects of LINA on GLP-1, the latter of which was blocked by GLP-1 receptor agonist. USE was higher and ENaC expression was lower in DKD patients received SGLT2is and DPP4is than those without. Our present findings suggest that addition of LINA to EMPA decreases p-Nedd4-2 and ENaC levels via the activation of GLP-1-receptor axis, which could ameliorate salt sensitivity and help prevent kidney injury in DKD.

Keywords GLP-1, GLP-1 receptor, DPP4 inhibitors, Diabetic kidney disease, SGLT2 inhibitors

According to the report of IDF Diabetes Atlas 2021, number of patients with diabetes is estimated to be 537 million and has been increasing worldwide[1]. Dietary salt intake and total body Na^+ concentration are known to be higher in patients with diabetes than non-diabetic individuals[2,3]. It has been demonstrated that activation of epithelial sodium channel (ENaC), a major channel for Na^+ reabsorption in distal nephron, increases salt sensitivity in patients with diabetes[4,5]; thereby, about 70% of patients with diabetes develop hypertension[6] that is strongly associated with an increased risk of progressive diabetic kidney disease (DKD)[7]. Furthermore, increased sodium retention enhances intraglomerular pressure that could lead to podocyte injury, mesangial expansion, and kidney fibrosis, histological characteristics of DKD. Thus, regulating ENaC to stimulate sodium excretion would be of importance as a therapeutic target to prevent the progression of DKD.

The increase in filtrated glucose is linked to upregulate sodium-glucose cotransporter 2 (SGLT2) expression in S1 segment of proximal tubules (PTC), thereby being involved in sodium retention in DKD[8]. Theoretically, the pharmacological inhibition of SGLT2 could promote sodium excretion into urine. However, although randomized clinical trials have revealed that SGLT2 inhibitors (SGLT2is) decreases blood pressure and reduces the risk of hospitalization for heart failure[9], urinary sodium excretion (USE) was not increased or only transiently increased by SGLT2is[10–12]. Thus, compensatory reabsorption of Na^+ in the distal nephron may play a role in failure to increase USE by SGLT2is. On the other hand, diuretics could be therapeutic candidates to maintain body fluids via promoting natriuresis in patients with DKD; but frequent use of diuretics is likely to exacerbate renal dysfunction due to diuretic-induced renal hypoxia[13]. Therefore, what pharmacotherapeutics in combination with SGLT2is could stimulate USE and improve salt sensitivity in diabetes remains unknown.

Besides normalizing sodium balance, controlling hyperglycemia is also essential to prevent the progression of DKD; but the glucose-lowering effect of SGLT2is is limited[14]. Therefore, other anti-diabetic agents may be

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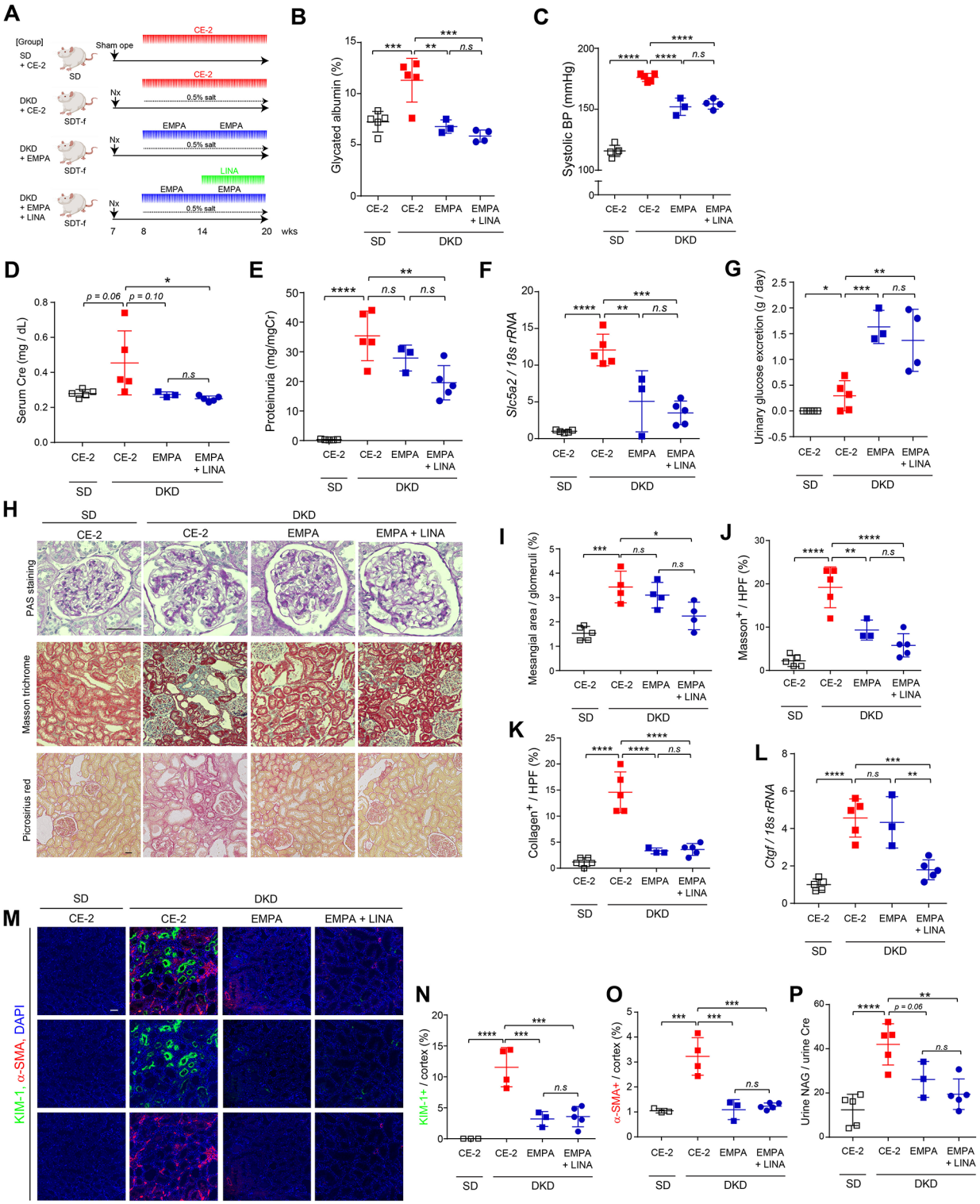


required to meet the therapeutic goals of glycated hemoglobin, which are recommended by several clinical guidelines[15,16]. DPP4 inhibitors (DPP4is) and GLP-1 receptor agonists (GLP-1RAs) have pleiotropic effects, including stimulation of natriuresis[17], regulating immune systems[18], and inhibiting senescence[19] as well as reducing blood glucose. A DPP4i was shown to downregulate Na^+/H^+ exchanger (NHE3) via GLP-1R activation or directly impairing NHE3 function in the proximal tubules[20]. Moreover, a couple of studies have suggested that DPP4is might induce natriuresis in the distal nephron as well[21,22]; however, it remains unknown whether DPP4is can regulate the distal nephron ENaC to stimulate the natriuresis, thus leading to improve salt sensitivity in patients with diabetes.

Therefore, in the present study, we investigated the synergistic effects of add-on therapy with DPP4is to SGLT2is on salt-sensitive hypertension, glomerular hyperfiltration, and kidney fibrosis in diabetic animals

◀**Fig. 1**. Combination of EMPA and LINA reduces kidney fibrosis and halts the progression of DKD. (**A**) Scheme of the experiment with SD rats with CE-2 and DKD rats with CE-2, EMPA, or EMPA + LINA. (**B**) Glycated albumin (%), (**C**) systolic BP, and (**D**) Serum Cre level (mg/dL) and (**E**) proteinuria (mg/gCr) in each group at 20 weeks. SD-CE-2 ($n = 5$), DKD-CE-2 ($n = 5$), DKD-EMPA ($n = 3$), DKD-EMPA + LINA ($n = 5$). (**F**) Real-time PCR for *Slc5a2* in kidneys of each group at 20 weeks. SD-CE-2 ($n = 5$), DKD-CE-2 ($n = 5$), DKD-EMPA ($n = 3$), DKD-EMPA + LINA ($n = 5$). (**G**) Urinary glucose excretion (g/day) in each group at 20 weeks. (**H**) Representative images of Masson-trichrome, Picrosirius red, and PAS staining in each group at 20 weeks. Scale bar: 50 µm. (**I**) Corresponding quantitation for mesangial area/glomeruli in PAS staining (%). (**J**) Corresponding quantitation for Masson$^+$ area/HPF (%) in Masson-trichrome staining. (**K**) Corresponding quantitation for collagen deposition area/HPF (%) in Picrosirius red staining. SD-CE-2 ($n = 5$), DKD-CE-2 ($n = 5$), DKD-EMPA ($n = 3$), DKD-EMPA + LINA ($n = 5$). (**L**) Real-time PCR for CTGF in kidneys of each group at 20 weeks. SD-CE-2 ($n = 5$), DKD-CE-2 ($n = 5$), DKD-EMPA ($n = 3$), DKD-EMPA + LINA ($n = 5$). (**M**) Representative images of KIM-1 and α-SMA-labelled kidneys. Scale bar: 50 µm. (**N**) The corresponding quantitation for KIM-1$^+$ area/cortex (%) and (**O**) α-SMA$^+$ area/cortex (%). SD-CE-2 ($n = 3$), DKD-CE-2 ($n = 5$), DKD-EMPA ($n = 3$), DKD-EMPA + LINA ($n = 5$). (**P**) Urine NAG/Cre in each group at 20 weeks. SD-CE-2 ($n = 5$), DKD-CE-2 ($n = 5$), DKD-EMPA ($n = 3$), DKD-EMPA + LINA ($n = 5$). Data are presented as means ± SD. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. CE-2, certified diet-experimental animal diet 2; DKD, diabetic kidney disease; EMPA, empagliflozin; LINA, linagliptin; BP, blood pressure; PAS, Periodic acid–Schiff; HPF, high power field; CTGF, connective tissue factor; KIM-1, kidney injury molecule-1; SMA, smooth muscle actin.

and explore the molecular mechanism by DPP4is increase the USE, especially focusing on ENaC. Also, we determined the therapeutic benefits of the combination of DPP4is and SGLT2is in patients with DKD.

Results

Combination of empagliflozin (EMPA) and linagliptin (LINA) reduces kidney fibrosis and halts the progression of DKD

EMPA is a potent SGLT2i used worldwide and short-term administration with EMPA showed natriuretic profile in patients with type2 diabetes and heart failure[23]. Meanwhile, LINA is a selective and long-acting DPP4i and shown to increase total fractional excretion of sodium in patients with type2 diabetes[22]. To determine whether the combination therapy of SGLT2is and DPP4is has a synergistic efficacy in slowing the progression of DKD, we performed chronic administration with a SGLT2i, EMPA or the combination of EMPA and a DPP4i, LINA into Spontaneously Diabetic Torii (SDT)-fatty rats, leptin receptor-mutated rats, that were given containing 0.5% NaCl (DKD rats). DKD rats were treated with or without EMPA for 12 weeks (DKD + EMPA) or EMPA for first six weeks and EMPA + LINA for last six weeks (DKD + EMPA + LINA). Experimental schematic is shown in Fig. 1A. Glycated albumin (GA) and systolic blood pressure (sBP) at 20 weeks were increased in DKD rats, which were inhibited by the treatment with EMPA or EMPA + LINA (Fig. 1B and C). Increased levels of serum creatinine (Cre) and proteinuria in DKD rats at 20 weeks were significantly prevented by EMPA + LINA, but not EMPA treatment alone (Fig. 1D and E). The levels of total ketone bodies, β-hydroxybutyrate and acetoacetate were increased by the treatment with EMPA, which was not affected by add-on therapy with LINA (Table 1). Clinical parameters of each group of the DKD rats were shown in Table 1. Gene expression of *Slc5a2* was increased in DKD rats, which was inhibited by EMPA or EMPA + LINA in association with further increase in urinary glucose excretion (Fig. 1F and G). Mesangial matrix expansion in PAS staining was significantly inhibited by EMPA + LINA, but not EMPA treatment (Fig. 1H and I). Kidney fibrosis with collagen deposition in masson-trichrome or picrosirius red staining was attenuated by EMPA or EMPA + LINA (Fig. 1H, J, and K). Increased gene expression of connective tissue growth factor (CTGF), a profibrotic cytokine in DKD rats was reduced by EMPA + LINA; but that was not affected by EMPA alone (Fig. 1L). The increase in kidney injury molecule-1 (KIM-1)- and α−smooth muscle actin (SMA)-positive tubules were ameliorated by EMPA or EMPA + LINA (Fig. 1M, N, and O). Furthermore, urine N-acetyl-β-D-glucosaminidase (NAG), a marker of tubular injury was significantly reduced by EMPA + LINA, but not EMPA alone, in 20-week-old DKD rats (Fig. 1P).

Addition of LINA to EMPA enhances USE via decreasing ENaCα expression in DKD rats

Compared with control SD rats fed with certified diet-experimental animal diet 2 (CE-2), a standard chow, USE assessed by 24 h urine sodium excretion (µmol/day) was significantly higher in DKD rats, which were further increased by EMPA + LINA, but not EMPA treatment alone (Fig. 2A). Likewise, urine Na/K, a clinical parameter of decreased ENaC activity, was further increased by EMPA + LINA, not EMPA treatment alone (Fig. 2B). Although compared with control rats, ENaCα expression in the whole kidneys was not increased in DKD rats, it was significantly increased in a marker of distal and collecting tubules, aquaporin 2 (AQP2)-positive cells, both of which were reduced by EMPA + LINA, but not EMPA alone (Fig. 2C, D, E, and F). EMPA or EMPA + LINA did not affect the expression levels of other sodium transporters, such as Na+-Cl − cotransporter (NCC) and Na–K–Cl cotransporter (NKCC2) (Supplementary Fig. 1A and 1B). Furthermore, the ratio of p-Nedd4-2 (phosphorylated Ser^{342} of Nedd4-2) to total Nedd4-2 as well as the ratio of p-Nedd4-2 to AQP2 were inhibited by EMPA + LINA, but not EMPA treatment alone (Fig. 2G, H, Supplementary Fig. 1C, and 1D). We found that GLP-1 concentration in the kidneys was decreased in DKD rats that were restored by EMPA or EMPA + LINA (Fig. 2I). However, the reduction in GLP-1R expression in AQP2-positive tubules in DKD rats was only restored by the combination therapy (Fig. 2J and K).

	SD+CE-2	DKD+CE-2	DKD+EMPA	DKD+EMPA/LINA
sBP(mmHg)	115.8 ± 4.17	176 ± 2.97***	152 ± 5.72***†††	154.3 ± 3.56***†††
dBP(mmHg)	75.8 ± 15.1	141.4 ± 11.2***	127.7 ± 9.7***	114.0 ± 6.4**†
GA (%)	7.2 ± 0.9	11.3 ± 1.9**	6.8 ± 0.5††	5.9 ± 0.5†††
BS (mg/dL)	133.8 ± 14.5	378.6 ± 79.2***	171.7 ± 12.4†††	128.8 ± 15.4†††
BUN (mg/dL)	17.6 ± 1.4	40.0 ± 4.6***	14.7 ± 0.5†††	14.0 ± 1.2†††
Cre (mg/dL)	0.28 ± 0.02	0.45 ± 0.16*	0.27 ± 0.01	0.25 ± 0.02†
Na (mmol/L)	144.6 ± 0.5	144.8 ± 1.9	147.0 ± 1.6	146.8 ± 2.5
K (mmol/L)	6.6 ± 0.2	6.2 ± 0.3	6.7 ± 0.2	5.3 ± 0.5***†###
Cl (mmol/L)	105.4 ± 1.0	101.6 ± 3.1	104.7 ± 1.2	100.8 ± 2.3
Total-C (mg/dL)	56.2 ± 3.8	435.2 ± 101.3***	260.0 ± 26.1**†	318.3 ± 59.0***
TG (mg/dL)	56.0 ± 25.1	854.2 ± 145.4***	676.7 ± 126.2**	620.8 ± 235.9**
Total ketone body (mmol/l)	962.6 ± 191.9	498.6 ± 292.7*	3304.7 ± 1733.1***†††	4916.2 ± 2851.9**††
β-hydroxybutyrate (mmol/l)	878.6 ± 187.2	410.2 ± 238.9**	2502.3 ± 1250.0***†††	4018.3 ± 2241.6*††
Acetoacetate (mmol/l)	84.0 ± 28.0	88.4 ± 66.7	802.3 ± 548.6**††	898.5 ± 777.3**†

Table 1. Clinical parameters of DKD rats treated with CE-2, EMPA, or EMPA/LINA at 20 weeks. Values are shown as mean ± SD or counts. SD=SD rats, CE-2=certified diet-experimental animal diet 2, DKD=SDT-fatty rats fed with high salt diet, EMPA=empagliflozin, LINA=linagliptin, sBP=systolic blood pressure, dBP=diastolic blood pressure, GA=glycated albumin, BS=blood sugar, BUN=blood urea nitrogen, Cre=creatinine, Na=sodium, K=potassium, Cl=chloride, Total-C=total cholesterol, TG=triglycerides. *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$ vs. SD; †: $P<0.05$, ††: $P<0.01$, †††: $P<0.001$ vs. DKD+CE-2, ###: $P<0.001$ vs. DKD+EMPA.

Addition of LINA to EMPA enhances USE via reduction of ENaC expression, reduces UAE, and inhibits kidney injury in deoxycorticosterone acetate/salt (DS)-administered, uninephrectomized mice

DS-administered, uninephrectomized mice (DS mice) have been known to exhibit glomerular hyperfiltration and severe kidney injury with ENaC activation[24]. Thus, to investigate whether the add-on therapy with LINA to EMPA could also protect against more severe kidney damage via the inhibition of ENaC, DS mice were treated with EMPA alone or EMPA + LINA for three weeks (Fig. 3A). sBP was increased and sustained until the end of the experiments in DS mice, which was inhibited by EMPA + LINA, but not EMPA (Fig. 3B). Kidney weight/body weight (KW/BW) was increased in DS mice, which was reduced by EMPA + LINA, not EMPA alone (Fig. 3C). USE was increased by EMPA + LINA, but not EMPA treatment alone (Fig. 3D). Expression levels of ENaCα in AQP2-positive tubules were higher in DS mice than sham-operated control mice, which was reduced by EMPA + LINA, but not EMPA treatment alone (Fig. 3E and F). While p-Nedd4-2/Nedd4-2 ratio was increased by EMPA alone compared with carboxymethyl cellulose (CMC)-treated DS mice, it was significantly reduced by EMPA + LINA (Fig. 3G and H). Although EMPA treatment alone did not affect UAE, mesangial expansion, or podocyte injury assessed by wilms tumor-1 (WT-1) expression in DS mice, combination therapy of EMPA and LINA significantly ameliorated these parameters (Fig. 3I, J, K, and L). Collagen IV deposition in the kidneys of DS mice was significantly reduced by EMPA alone, which effects were further potentiated by the co-treatment with LINA (Fig. 3J and M).

Time-lagged bolus injection of LINA after EMPA significantly increases USE in SD rats

To study acute effects of LINA on USE under the presence or absence of SGLT2is, we performed acute injection experiments with EMPA alone, LINA alone, concurrent administration with EMPA and LINA, and time-lagged bolus injection of LINA an hour after EMPA into the SD rats. All agents were injected via a tail vein under general anesthesia using isoflurane, and the experimental scheme is shown in Fig. 4A. USE evaluated as spot urine Na/Cre transiently increased an hour after injection with EMPA and then quickly returned to the baseline (Fig. 4B). While LINA treatment alone did not affect the USE, there was no significant difference of USE dynamics between EMPA alone group and concurrent administration with LINA and EMPA group (Fig. 4B and C). However, time-lagged injection of LINA to EMPA significantly increased the USE at 2 and 3 h when compared to those in other treatment groups (Fig. 4B and C). Area under the curve (AUC) values of USE from baseline to 3 h after treatments was significantly higher in time-lagged administration group than other treatment groups (Fig. 4D and E). Urine Na/K profiles were almost the same as USE; time-lagged administration of LINA to EMPA significantly increased urine Na/K at 2 and 3 h, while the other treatments did not affect the urine Na/K profile during the 3-hour experiments (Fig. 4F and G).

LINA decreases ENaCα expression in high sodium (HS) and high glucose (HG)-exposed tubular cells through the reduction of p-Nedd4-2 via GLP-1-receptor axis

To investigate whether ENaC activation occurs in response to exposure to HS and HG, we treated murine distal convoluted tubular cells (MDCTs) with HS, HG, and HS + HG. HS or HG treatment upregulated the expression of ENaCα, which levels were further enhanced by HS + HG (Fig. 5A and B). The increase in ENaCα expression of HS + HG-exposed cells was inhibited by LINA alone or EMPA + LINA, but not EMPA treatment alone (Fig. 5C

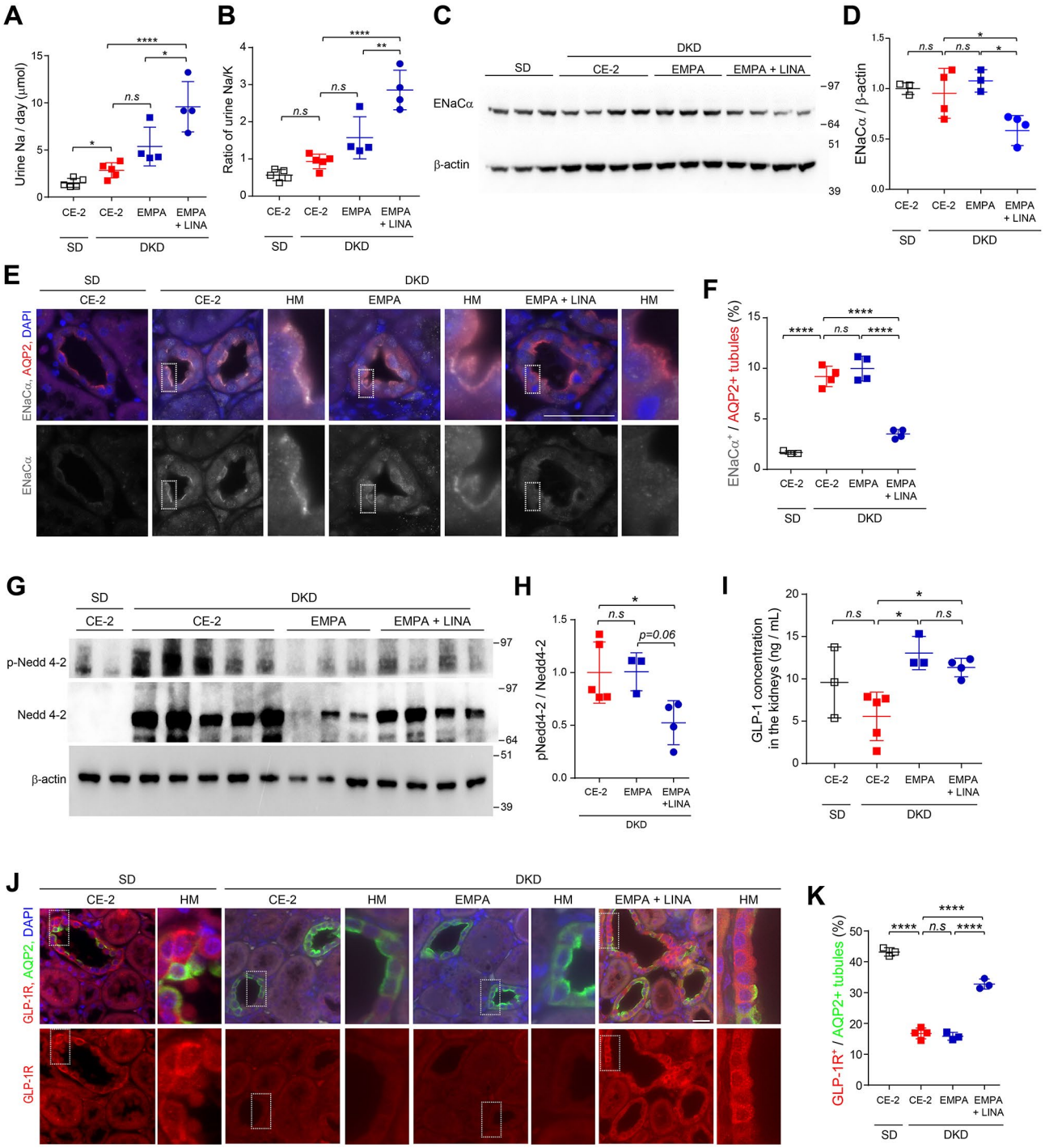
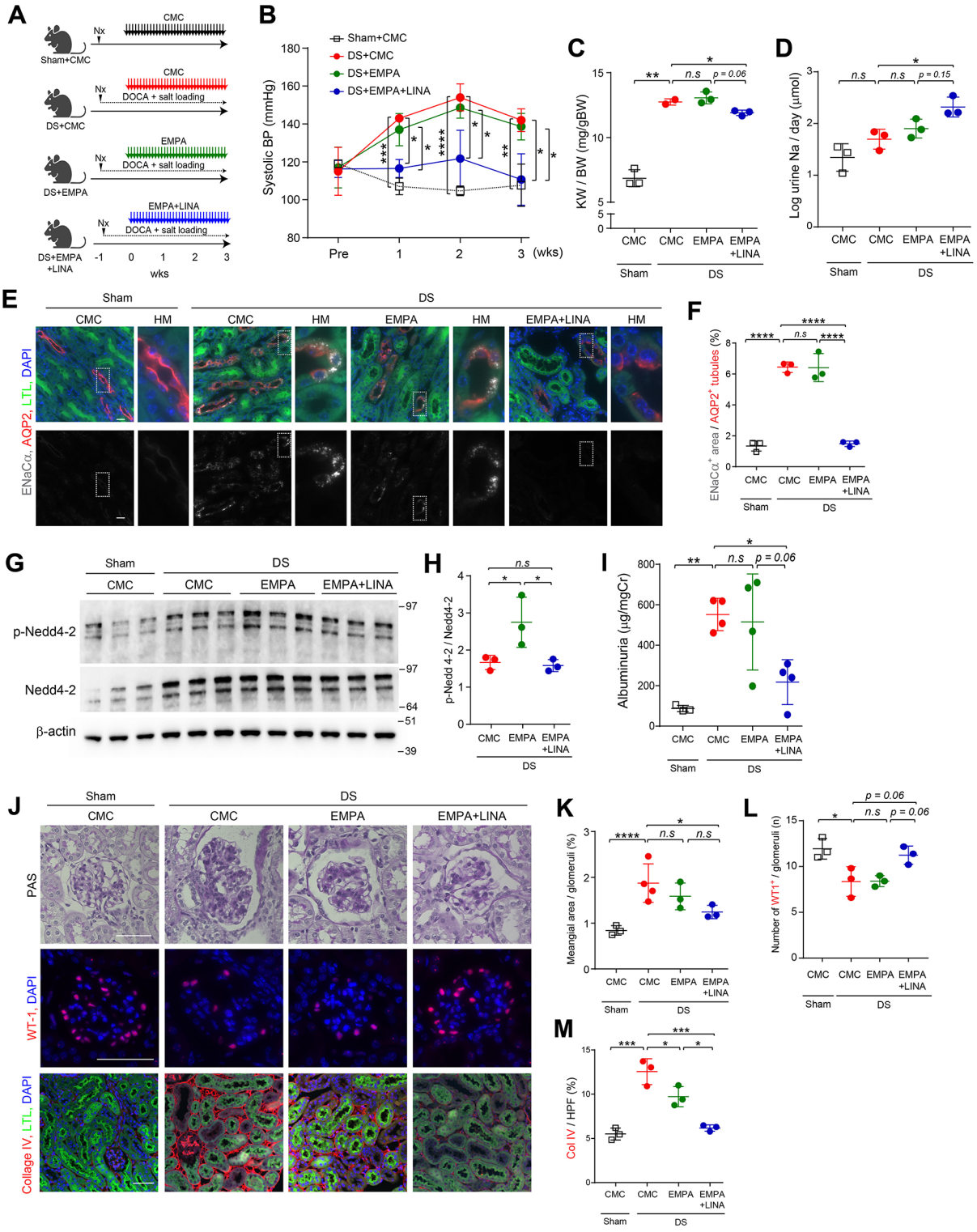


Fig. 2. Addition of LINA to EMPA enhances USE via decreasing ENaCα expression in DKD rats. (**A**) Urine Na/day (µmol) and (**B**) Urine Na/K at 20 weeks. SD-CE-2 ($n=5$), DKD-CE-2 ($n=5$), DKD-EMPA ($n=4$), DKD-EMPA + LINA ($n=4$). (**C**) Western blots for ENaCα and β-actin at 20 weeks and (**D**) quantitation for ENaCα/β-actin. SD-CE-2 ($n=3$), DKD-CE-2 ($n=4$), DKD-EMPA ($n=3$), DKD-EMPA + LINA ($n=4$). (**E**) Representative images of ENaCα- and AQP2-labelled kidneys at 20weeks. Scale bar: 50 µm. (**F**) The corresponding quantitation for ENaCα$^+$ area/AQP2$^+$ tubules (%). SD-CE-2 ($n=3$), DKD-CE-2 ($n=4$), DKD-EMPA ($n=4$), DKD-EMPA + LINA ($n=4$). (**G**) Western blots for p-Nedd4-2 and Nedd4-2 and (H) the ratio of p-Nedd4-2/Nedd4-2. DKD-CE-2 ($n=5$), DKD-EMPA ($n=3$), DKD-EMPA + LINA ($n=4$). (**I**) GLP-1 concentration in the kidneys of SD-CE-2 ($n=5$), DKD-CE-2 ($n=5$), DKD-EMPA ($n=2$), DKD-EMPA + LINA ($n=4$). (**J**) Representative images of kidneys labelled for GLP-1R and AQP2. Scale bar: 20 µm. (**K**) Corresponding quantitation for GLP-1R$^+$ area/AQP2$^+$ tubules in SD-CE-2 ($n=4$), DKD-CE-2 ($n=4$), DKD-EMPA ($n=4$), DKD-EMPA + LINA ($n=3$). Data are presented as means ± SD. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. CE-2, certified diet-experimental animal diet 2; EMPA, empagliflozin; LINA, linagliptin; SGLT2, sodium glucose transporter 2; ENaC, epithelial sodium channel; AQP2, aquapotin-2; Need4-2, neural precursor cell expressed developmentally down-regulated 4 – 2; GLP-1R, glucagon like peptide-1 receptor.



and D). We next studied whether the inhibitory effects of LINA on ENaCα was dependent on GLP-1. When HS + HG-exposed MDCTs were treated with human GLP-1 recombinant protein in the presence or absence of LINA, LINA or GLP-1 significantly reduced p-Nedd4-2 as well as ratio of p-Nedd4-2 to total Nedd4-2, and there was no significant difference in p-Nedd4-2/Nedd4-2 levels between LINA and GLP-1 treatment groups (Fig. 5E and F). Indeed, 14-3-3 protein was increased by the exposure to HS + HG in MDCTs, which was prevented by the treatment with LINA or GLP-1. Furthermore, LINA did not potentiate the GLP-1-induced reductions in ENaCα, p-Nedd4-2/Nedd4-2 levels, and 14-3-3 protein of HS + HG-exposed MDCTs (Fig. 5G, H, Supplementary Fig. 2A and B). To determine whether GLP-1 regulates ENaC through the engagement of GLP-1R, we treated MDCTs with GLP-1 in the presence or absence of the indicated concentrations of GLP-1R antagonist. GLP-1R antagonist dose-dependently inhibited the GLP-1-induced decrease in ENaCα of MDCTs exposed to HS + HG (Fig. 5I and

◀**Fig. 3**. Add-on therapy with LINA to EMPA increases USE and prevents podocyte injury and kidney fibrosis in DS mice. (**A**) Scheme of the experiment employing DS mice treated with CMC, EMPA, or EMPA + LINA. (**B**) Time course of systolic BP (mmHg) at each week. (**C**) KW/BW (mg/g) and (**D**) Urine Na excretion (μmol/day) in each group at 3 weeks. (**E**) Representative images of IF for ENaCα, AQP2, and LTL and (**F**) the quantitation for ENaCα$^+$ area/AQP2$^+$ tubules. 15 tubules are analyzed in each group. Scale bar: 20 μm. (**G**) Western blots for p-Nedd4-2 and Nedd4-2 and (**H**) the ratio of p-Nedd4-2/Nedd4-2 in sham ($n = 3$), DS-CMC ($n = 3$), DS-EMPA ($n = 3$), and DS-EMPA + LINA ($n = 3$). (**I**) Urinary albumin excretion (μg/mgCr) in each group at 3 weeks. Sham-CMC ($n = 3$), DS-CMC ($n = 4$), DS-EMPA ($n = 4$), and DS-EMPA + LINA ($n = 4$). (**J**) Representative images of glomerulus in PAS and IF staining for WT-1 and Col IV at 3 weeks. Scale bar: 50 μm. (**K**) The quantitation for mesangial area/glomeruli (%), (**L**) number of WT-1$^+$ cells/glomeruli, and (**M**) Col IV$^+$ area/HPF in sham ($n = 3$), DS-CMC ($n = 3$), DS-EMPA ($n = 3$), and DS-EMPA + LINA ($n = 3$). Data are presented as means ± SD. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. DS, deoxycorticosterone acetate with high salt; CMC, carboxymethyl cellulose; EMPA, empagliflozin; LTL, Lotus Tetragonolobus Lectin; KW, kidney weight; BW, body weight; HW, heart weight; WT-1, wilms tumor-1; PAS, periodic acid–Schiff; IF, immunofluorescence; Nedd4-2, neural precursor cell expressed developmentally down-regulated 4 – 2; HPF, high power field; Col IV, collagen IV.

J). Meanwhile, GLP-1R was downregulated by EMPA alone when MDCTs were exposed to HG (Supplementary Fig. 2C and D).

Combination therapy of DPP4is and SGLT2is reduces ENaC activity in patients with DKD

To investigate whether add-on therapy of DPP4is to SGLT2is reduces USE in patients with DKD, we next conducted a retrospective clinical study of hospitalized patients with diabetes, who had been treated with or without SGLT2is alone or SGLT2is + DPP4is in Kurume University Hospital since 2010 to 2022. Renin-angiotensin system inhibitors (RASi) were administrated in all enrolled patients, and those taking diuretics, mineralocorticoid receptor antagonists (MRA), and immunosuppressants were excluded from this study. At 6 months after the initiation treatment, USE, urine Na/K, estimated GFR (eGFR) and the change in serum sodium concentration from baseline (Δsodium concentration) were evaluated (Fig. 6A). There were no significant differences in eGFR, glycemic or lipid parameters, liver function profiles, and hemoglobin levels among each group (Table 2). Compared with DKD patients without SGLT2is or DPP4is, urine Na/Cre, urinary Na concentrations, and urine Na/K were significantly higher in combination therapy, but not patients with SGLT2is treatment alone (Fig. 6B, C, and D). ΔSodium concentration was higher in patients receiving SGLT2is treatment alone than those without or with SGLT2is + DPP4is (Fig. 6E). To further examine the effects of SGLT2is alone or SGLT2is + DPP4is on ENaCα expression levels in the kidneys, we analyzed renal biopsy specimens obtained from DKD patients. Compared with normal kidney (NK) samples obtained from patients without diabetes who received a renal biopsy due to mild proteinuria or microhematuria, ENaCα expression levels in AQP2-positive tubules were significantly higher in DKD patients with neither agents and those with SGLT2is alone, but not those in the combination (Fig. 6F and G). Line profile analysis showed that apical membrane ENaCα expression was increased in DKD patients with neither agents and those with SGLT2is alone, but that was inhibited by the combination therapy of SGLT2is and DPP4is (Fig. 6H).

Discussion

Diabetes is a global public health issue, imposing a huge burden on social economy[25]. DKD is a leading cause of end stage kidney disease (ESKD) and associated with the increased risk of cardiovascular disease in diabetes[26]. Recent randomized clinical trials have shown that SGLT2is or GLP-1RAs reduce the risk of composite kidney outcomes in patients with diabetes[27]. However, despite the standard therapies for DKD, a substantial number of diabetic patients still continue to progress to ESKD, therefore there is some residual risk of the progression of DKD[28]. Salt-sensitive hypertension is a major risk factor to promote the progression of DKD, which may be the residual risk of DKD because SGLT2is have little effects on long-term USE[29]. Furthermore, although DPP4is are widely used oral hypoglycemic agents for type 2 diabetes[30], the synergistic effects of DPP4is and SGLT2is on USE and kidney protection were not fully understood. Therefore, in this study we studied whether add-on therapy with LINA to EMPA could improve renal damage in diabetic and/or hypertensive animal models by reducing ENaC. Novel findings of the present study are as follows; (1) add-on therapy with LINA to EMPA increased USE in DKD rats compared with EMPA alone in association with the decrease in p-Nedd4-2 and ENaCα expression in the kidneys, (2) EMPA + LINA therapy, but not EMPA treatment alone, significantly reduced serum Cre, proteinuria, urinary NAG excretion levels, mesangial matrix area, and *ctgf* gene expression in the kidneys of DKD rats, (3) combination therapy of EMPA and LINA increased USE and reduced sBP in DS mice, which were associated with the reduction in podocyte injury, mesangial matrix expansion, albuminuria, and ENaCα expression in DS mice, whereas all of the parameters were not affected by EMPA treatment alone, (4) time-lagged acute administration of LINA to EMPA, but not concurrent administration of the two, or either EMPA or LINA alone, increased USE, and its effect was sustained during 3 h after the treatment, (5) HS + HG treatment upregulated ENaCα expression in MDCTs, which was inhibited by LINA in association with the decreased p-Nedd4-2, whose effects were not enhanced by co-treatment of GLP-1, and (6) USE was higher and ENaCα expression was lower in DKD patients who received combination therapy of DPP4is and SGLT2is than those without DPP4is.

In the present study, we found that combination therapy of LINA with EMPA, but not EMPA alone significantly reduced ENaCα expression and the levels of p-Nedd4-2 in the kidneys of DKD rats, which were

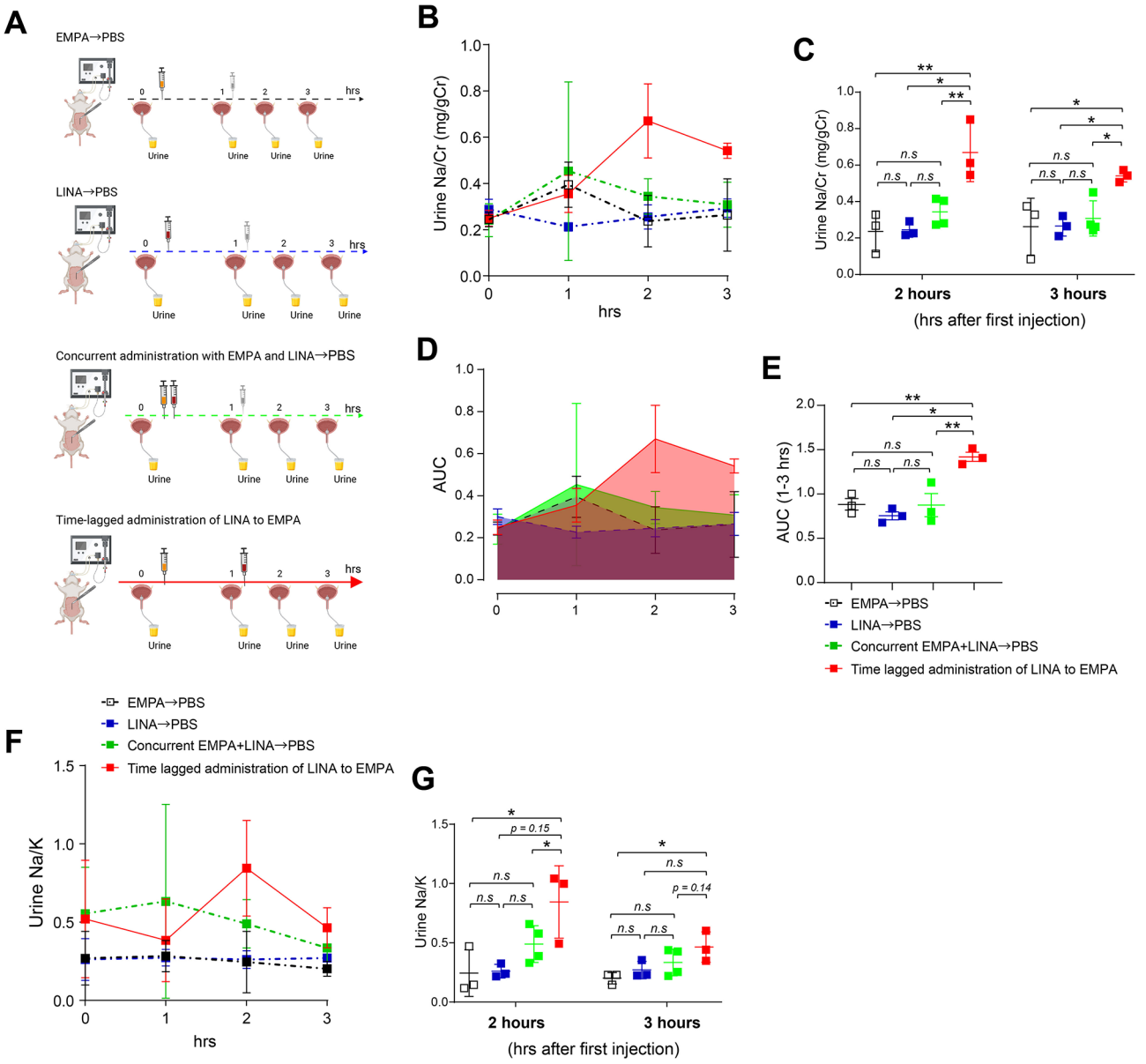


Fig. 4. Time-lagged bolus injection of LINA after EMPA significantly increases USE in SD rats. (**A**) Scheme of the experiment, (**B**) Urine Na/Cre (mEq/mgCr) over time after bolus injections with EMPA, LINA, concurrent administration with EMPA and LINA, and time-lagged administration of LINA to EMPA. The horizontal axis indicates time (hours) after the injections. (**C**) Urine Na/Cre (mEq/mgCr) at 2 h and 3 h after first bolus injections in EMPA, LINA, concurrent administration with EMPA and LINA, and time-lagged administration of LINA to EMPA group. $n = 3–4$ in each group. (**D**) Representative image of AUC in urine Na/Cr from 0 through 3 h after the first injection. (**E**) The quantitation for AUC value from 1 to 3 h in each group. (**F**) Urine Na/K value after bolus injections with EMPA, LINA, concurrent administration with EMPA and LINA, and time-lagged administration of LINA to EMPA. (**G**) Urine Na/K at 2 h and 3 h after first bolus injections in EMPA, LINA, concurrent administration with EMPA and LINA, and time-lagged administration of LINA to EMPA group. $n = 3–4$ in each group. Open squares, blue squares, green squares, and red squares indicate EMPA→PBS, LINA→PBS, concurrent administration with EMPA and LINA→PBS, and time-lagged administration of LINA to EMPA, respectively in Fig. 4. Data are presented as means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. EMPA, empagliflozin; LINA, linagliptin, AUC, area under the curve.

associated with the increase in USE. ENaC, comprised of three different but homologous subunits (α, β, and γ), is responsible for Na^+ reabsorption in distal nephrons[31] and its α subunit is critical in channel functionality[32]. Nedd4-2, an E3 ubiquitin-protein ligase is responsible for degradation of ENaC, and its function is inhibited when serine residue at position 342 is phosphorylated (p-Nedd4-2)[33,34]. These findings suggest that addition of LINA to EMPA could promote the degradation of ENaC by reducing the p-Nedd4-2 levels, which led to the increase in USE; mRNA levels of ENaCα were not affected by either treatment or combination therapy

(data not shown), further supporting that post-transcriptional modification of ENaC by the addition of LINA to EMPA may contribute to the increase in USE. In this study, HS + HG increased ENaCα expression levels in MDCTs, which was inhibited by LINA irrespective of the presence or absence of EMPA. Furthermore, LINA or GLP-1 prevented the increase in ENaCα and p-Nedd4-2 levels in HS + HG-exposed MDCTs, but there were no additive effects of GLP-1 to LINA on these protein expressions. GLP-1R antagonist dose-dependently inhibited the effects of GLP-1 on ENaCα levels in HS + HG-exposed MDCTs. In addition, GLP-1 levels in whole kidneys and GLP-1R expression in AQP2-positive distal tubules of DKD rats were restored by the addition of LINA to EMPA (Fig. 2I, J, and K). EMPA-induced increase in serum ketone bodies may be associated with the increased GLP-1 concentration in the DKD rats[35,36]; meanwhile, EMPA alone decreased GLP-1R in HG-exposed MDCTs (Supplementary Fig. 2C and D), indicating an insufficient activation of GLP-1-receptor axis by EMPA alone. Therefore, our observations suggest that activation of GLP-1-receptor axis by LINA could increase the USE via reducing ENaCα and p-Nedd4-2 levels in the kidneys of DKD rats.

GLP-1R agonist, liraglutide has been reported to decrease 14-3-3 protein, an inhibitory binding partner of Nedd4-2 in mouse and human islets through the interaction with GLP-1R[37]. Compatible with that, we identified that 14-3-3 protein expression was increased in HS + HG-exposed MDCTs, which was inhibited by LINA or GLP-1. LINA did not potentiate the GLP-1-induced reductions in 14-3-3 protein; thus, GLP-1-regulated 14-3-3 protein may promote Nedd4-2-dependent ENaC ubiquitination. Taken together, LINA may increase USE by stimulating Nedd4-2-dependent ENaC ubiquitination via GLP-1-receptor axis-mediated reduction of 14-3-3 protein.

Secondary hypertension is caused by a genetic mutation of ENaC in Liddle syndrome, whose high BP was ameliorated by kidney transplantation[38]. Therefore, the increase in ENaC expression and activity could be associated with hypertension and may cause renal damage in DKD rats. However, in this study, there were no significant differences of sBP and glycated albumin levels between EMPA + LINA-treated DKD rats and EMPA alone group. Therefore, renoprotective effects of LINA addition to EMPA in DKD rats, such as decreases in serum Cre levels, mesangial area expansion, and renal *Ctgf* gene expression, were totally independent of BP and blood glucose-lowering effects. Inhibition of ENaC by serin protease inhibitors has been shown to reduce profibrotic markers, while high salt diet activates fibroblasts[31]. Thus, LINA may protect against renal damage in DKD rats partly through anti-fibrotic effects via reduction of ENaC expression.

In the present study, we also found that the combination of EMPA and LINA, but not EMPA alone significantly decreased p-Nedd4-2 and ENaCα levels in kidneys of more severe kidney injury model, DS mice, which was associated with the decrease in USE. Moreover, the combination therapy, but not EMPA alone, restored number of podocytes, inhibited mesangial matrix expansion, and reduced UAE in DS mice. In addition, add-on therapy of LINA to EMPA reduced renal interstitial collagen IV deposition. In DS mice, combination therapy, but not EMPA alone, completely inhibited the increase in sBP. This may partly explain the reason why drastic kidney protection was acquired by the addition of LINA to EMPA in this model.

In acute injection experiments, we further showed that USE was increased by time-lagged administration of LINA to EMPA. Since EMPA-induced increase in USE was transient, a continuous increase in USE by time-lagged addition of LINA to EMPA may improve salt sensitivity and reduce sBP in a long term via increasing USE, which might lead to kidney protection. Combination therapy of DPP4is with SGLT2is has been shown to have the potential to reduce sBP in a previous clinical trial[39], thus supporting our hypothesis. Moreover, we found in our retrospective study that combination of SGLT2is and DPP4is reduced the apical membrane ENaCα expression in distal tubules in DKD patients, which was associated with the increase in USE. These observations suggest that addition of DPP4is to SGLT2is may play a protective role against renal injury beyond the glucose-lowering effects, that is, through the reduction of ENaCα.

There are some limitations in the present study. Although we demonstrated that time-lagged injection with DPP4is after SGLT2is promoted USE in acute injection experiments, it remains unknown whether oral administration of time-lagged regimen with DPP4is after SGLT2is could maintain high level of USE and help prevent kidney injury in longitudinal and prospective clinical trial. Further, we conducted a retrospective clinical observation regarding the effects of the combination therapy of DPP4is and SGLT2is in DKD patients with stage 3b-5 of CKD category. It remains unclear whether the combination therapy could prevent the progression of renal injury in patients with early stages of DKD.

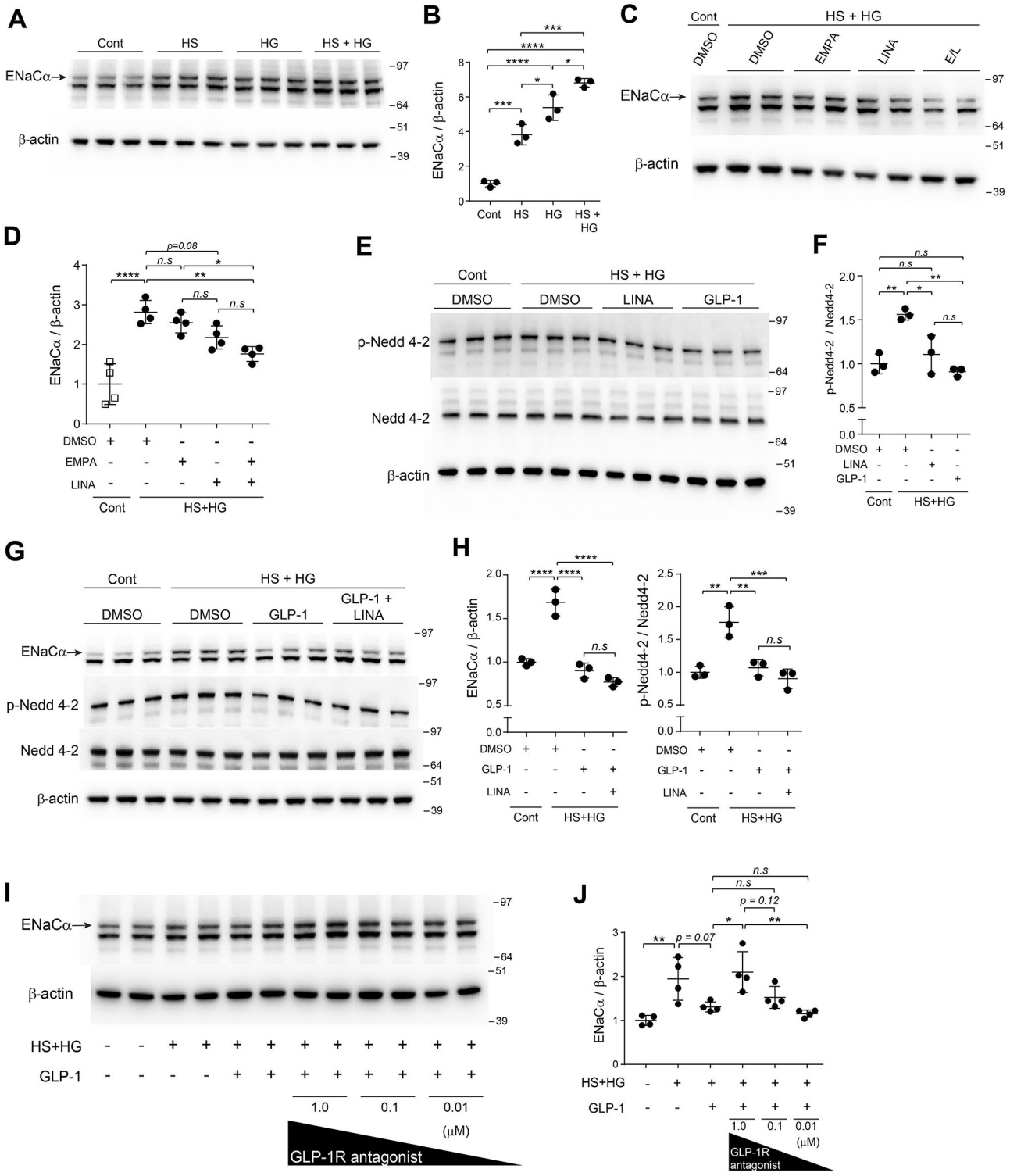
Conclusion

Our present findings suggest that addition of LINA to EMPA decreases 14-3-3 protein, p-Nedd4-2 and ENaCα levels in the kidneys of DKD rats and DS mice probably via the activation of GLP-1-receptor axis (Fig. 7). The combination therapy decreases ENaCα expression in association with the increase in USE in patients with DKD, and therefore add-on therapy with DPP4is to SGLT2is might become a potent therapeutic combination to attenuate salt sensitive hypertension and ameliorate kidney damage.

Methods

Animal preparation

Sprague Dawley rats (SD rats) and Spontaneously Diabetic Torii fatty rats (SDT-fatty rats), which are known as an obese type 2 diabetic model, were purchased from Japan CLEA (Tokyo, Japan). Also, C57BL/6J mice were purchased from Japan CLEA. Eight-week-old SDT-fatty rats were given water supplemented with 0.5% salt to induce hypertension and divided into 3 groups as followings; (1) SDT fatty rats given certified diet-experimental animal diet 2 (CE-2), a standard chow provided from CLEA (CLEA, Tokyo, Japan) (DKD-vehicle), (2) SDT fatty rats given CE-2 containing empagliflozin (EMPA)(30 mg/kg, Boehringer-Ingelheim, Germany)for 12 weeks (DKD-EMPA), and (3) SDT fatty rats given empagliflozin diet for first 6 weeks and switched to CE-2 containing



EMPA and linagliptin (LINA) (3 mg/kg, Boehringer-Ingelheim, Germany) (DKD-EMPA + LINA). SD rats given CE-2 and normal water were given as control. Hypertension was induced in C57BL/6J by uninephrectomy with the administration of DOCA (50 mg, a 21-day continuous-release, Innovation Research, USA) and 1% NaCl-containing drinking water was given. EMPA and LINA were dissolved with CMC and administrated by oral gavage. C57BL/6J mice performed sham operation fed with normal diet were used as control group. CMC was administrated into the control group. Twelve to fourteen-week-old SD rats were anesthetized with 2% isoflurane (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) delivered via an inhalation mask. Following anesthesia, a polyethylene catheter (Eastsidemed, Tokyo, Japan) was surgically inserted into the bladder. The catheter was secured in place to ensure proper urine collection. Rats were divided into four groups as following categories; (1) intravenous administration with EMPA (10 mg/kg, Adipogen life sciences, Liestal, Switzerland) followed by administration with PBS an hour after the administration with EMPA, (2) intravenous administration with LINA (10 mg/kg, Adipogen life sciences, Liestal, Switzerland) followed by administration

◀**Fig. 5**. GLP-1 regulates p-Nedd4-2 and ENaCα expression through the engagement with GLP-1 receptor. (**A**) Western blots for ENaCα in MDCTs treated with HS, HG, and HS + HG and (**B**) the quantitation for ENaCα/β-actin. $n = 3$ in each group. (**C**) Western blots for ENaCα and β-actin in MDCTs treated with HS + HG in the presence of EMPA, LINA, or EMPA + LINA and (**D**) the quantitation for ENaCα/β-actin. $n = 2–4$ in each group. (**E**) Western blots for p-Nedd4-2 and Nedd4-2 in MDCTs treated with HS + HG in the presence of LINA or GLP-1 and (**F**) the quantitation for the ratio of p-Nedd4-2/Nedd4-2. (**G**) Western blots for ENaCα, p-Nedd4-2, and Nedd4-2 in MDCTs treated with HS + HG in the presence or absence of GLP-1 or GLP-1 + LINA and (**H**) the quantitation for ENaCα/β-actin and p-Nedd4-2/Nedd4-2. $n = 3$ in each group. (**I**) Western blots for ENaCα in HS + HG-treated MDCTs in the presence of GLP-1 with or without GLP-1R antagonist and (**J**) the quantitation for ENaCα/β-actin. $n = 4$ in each group. Data are presented as means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. MDCTs, murine distal convoluted tubular cells; HS, high salt; HG, high glucose; GLP-1, glucagon-like peptide-1, Nedd4-2, neural precursor cell expressed developmentally down-regulated 4 – 2; EMPA, empagliflozin; LINA, linagliptin.

with PBS at an hour, (3) concurrent administration with EMPA (10 mg/kg) and LINA (10 mg/kg) followed by administration with PBS at an hour, and (4) intravenous administration with EMPA followed by administration with LINA at an hour (10 mg/kg). Urine samples were collected every single hour up to 4 h after anesthesia. Rodents were housed in metabolic cages for 24 h before sacrifice to collect urine samples. All animals were housed in a temperature- and humidity-controlled room with a 12-hour light–dark cycle. SDT-fatty rats and DS mice were euthanized by isoflurane inhalation at an overdose for prolonged time periods. All experiments were performed in accordance with relevant guidelines and regulations, including ARRIVE guideline (https://arriveguidelines.org/) and the experimental protocol approved by the Ethics Review Committee for Animal Experimentation at Kurume Medical School, Kurume, Japan.

Measurement of blood pressure and heart rate

Systolic blood pressure(sBP) of rodents were measured by a tail-cuff sphygmomanometer using an automated system with a photoelectric sensor (BP-98 A; Softron, Tokyo, Japan). Average sBP and heart rate values following three measurements at each session were used for analysis.

Measurement of clinical parameters

Blood was collected and centrifuged, then plasma and serum were obtained and stored at -80 °C. The urine samples were placed on ice during collection and then stored at -80℃.The plasma levels of total cholesterol, Triglyceride, Glycated albumin, urinary albumin excretion, urinary glucose excretion and urinary sodium, potassium and chloride were measured using an auto-analyzer (Nihondenshi Co., Tokyo, Japan). Glucose concentrations were measured using Ascencia Contour Blood GlucoSDeter (Bayer Healthcare, Newbury, UK). Plasma concentration of insulin, glucagon, GLP-1 and urinary NAG levels were measured using commercially available ELISA kit for rats (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan; Mercodia, Uppsala, Sweden; CMIC Holdings Co., Tokyo, Japan). Insulin resistance was calculated by means of the homeostatic model assessment index (HOMA-IR) using the relationship between the fasting blood glucose, according to the following formula: HOMA-IR = Insulin(μU/mL) × Blood glucose(mM)/ 22.5.

Cells Preparation and treatment

Mouse distal convoluted tubule cells (MDCTs) were purchased from ATCC (ATCC Cat# CRL-3250, RRID: CVCL_CZ90) and maintained in DMEM supplemented with 5% fetal bovine serum (FBS), 1% penicillin-streptomycin in a humidified incubator at 37 °C with 5% CO2. For high salt (HS) and high glucose (HG) stimulation, MDCTs were seeded in 12-well plates and the cells reached 80% confluency, they were subjected to high salt (20 mM) and high glucose (30 mM) conditions for 48 h. The treatment with EMPA (10μM), LINA (10μM), GLP-1 (500nM), or GLP-1 receptor antagonist 1 (100nM, MedChemExpress, MCE, USA) started at the same time when MDCTs were exposed to HS, HG, or HS + HG. EMPA, LINA, and GLP-1 antagonist 1 were dissolved with DMSO and GLP-1 was dissolved with PBS.

Histological analysis in vivo experiments

Kidneys were fixed in paraformaldehyde and embedded in paraffin which in turn were sectioned at five μm intervals. Kidney sections were stained for Masson-trichrome staining and Sirius-red staining for evaluating kidney fibrosis with collagen deposition. The stained sections were imaged, and collagen deposition was analyzed by BZ-X800 (Keyence, Osaka, Japan). We analyzed five $AQP2^{+}$ distal tubules for GLP-1R expression levels and calculated the average as an animal result in the DKD rats and DS mice. Meanwhile, six glomeruli were analyzed for mesangial area per glomeruli area and the number of WT1 positive cells per glomeruli and the average was calculated as an animal result. Immunofluorescence staining was performed to analyze tubular and podocyte injuries, localization of ENaCα in patients without diabetes who received kidney biopsy for mild proteinuria or microhematuria and those with diabetes mellitus. Briefly, 4 μm-sections were incubated in TE buffer and performed antigen retrieval using pressure cooker for 45 min. They were next incubated with 1% BSA / TBS-T containing 0.1% triton and 3% donkey serum for blocking. The sections were incubated with primary antibodies at the indicating concentration overnight. Secondary antibodies were added after washing sections with PBS twice. An hour later, mounting medium containing DAPI was added to prevent the fade of fluorescence. Primary antibodies against KIM-1(R and D Systems Cat# MAB1817, RRID: AB_2116445) and α-SMA (Novus Cat# NB600-531, RRID: AB_10000930) were used at a dilution of 1:200. Similarly, WT-1 (1:100,

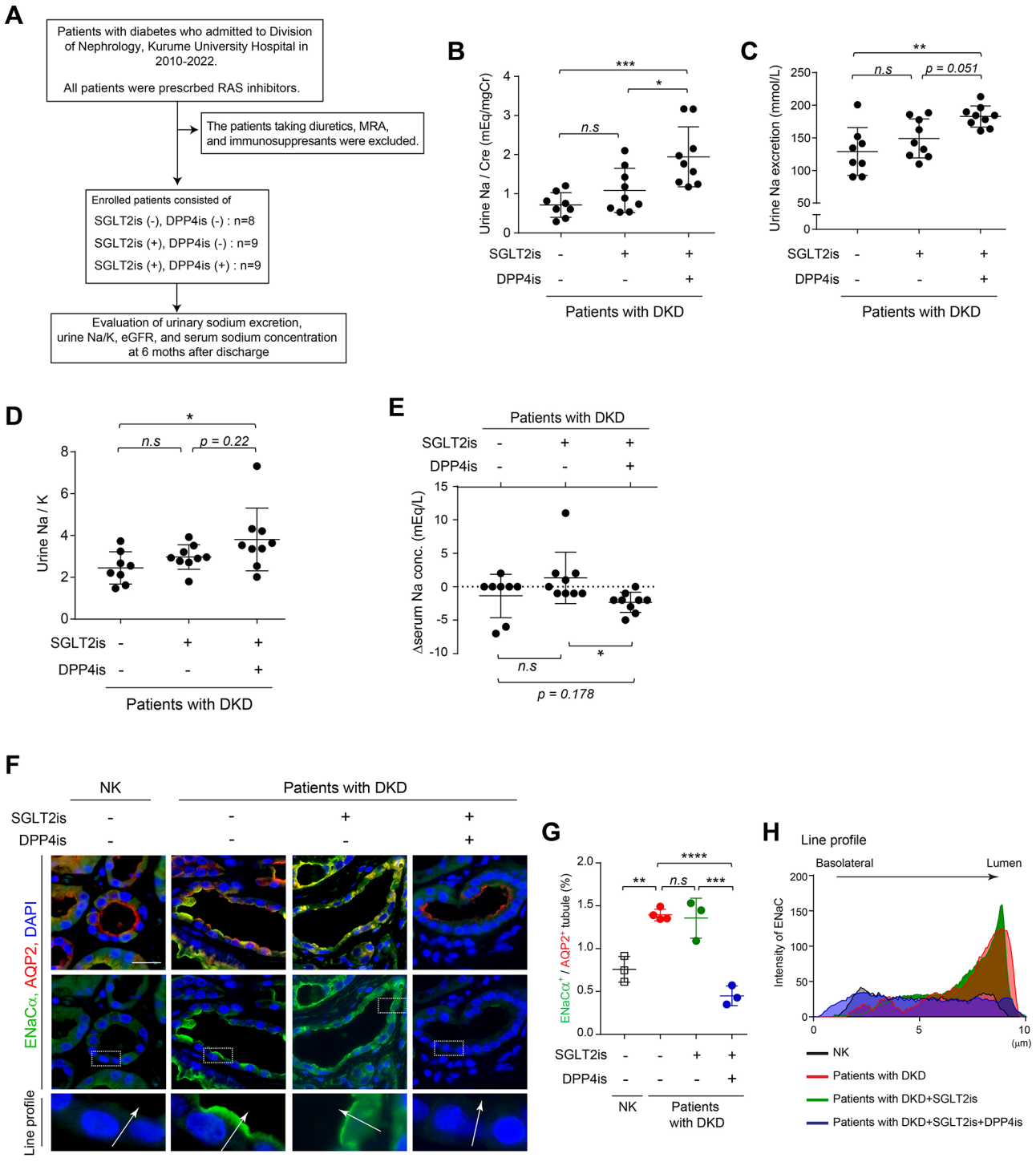


Fig. 6. Combination of SGLT2is and DPP4is reduces ENaCα and promotes USE in patients with DKD. (**A**) Scheme of the retrospective clinical study. (**B**) Urinary Na/Cre in patients with DKD in the presence of SGLT2is or SGLT2is and DPP4is, (**C**) Urinary Na excretion (mmol/L), (**D**) Urinary Na/K, and (**E**) Δserum Na concentration from baseline after 6 months. $n = 8–9$ in each group. (**F**) Representative images of ENaCα in AQP2$^+$ distal tubules in kidney biopsies from patients with NK and diabetic patients with SGLT2is or the combination with SGLT2is and DPP4is. Scale bar: 20 μm. (**G**) Corresponding quantitation for ENaCα$^+$ area/AQP2$^+$ tubules in NK($n = 3$), the kidneys of DKD patients without SGLT2is and DPP4is ($n = 4$), the kidneys of those with SGLT2is alone ($n = 3$), and the kidneys of those with SGLT2is and DPP4is ($n = 3$). (**H**) ENaCα expression intensity in the kidneys of NK ($n = 3$), the kidneys of DKD patients without SGLT2is and DPP4is ($n = 4$), the kidneys of those with SGLT2is alone ($n = 3$), and the kidneys of those with SGLT2is and DPP4is ($n = 3$). Data are presented as means ± SD. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. AQP2, aquaporin2; HM, high magnification; NK, normal kidneys; SGLT2is, sodium glucose transporter2 inhibitors; DPP4is, dipeptidyl peptidase-4 inhibitors, Na, sodium; K, potassium.

	DKD	DKD+SGLT2is	DKD+SGLT2is+DPP4is
No. of subjects	8	9	9
Men: Women	5:3	6:3	8:1
Age	66.5 ± 8.3	59.8 ± 13.0	66.6 ± 10.8
Body Mass Index (kg/m^2)	25.1 ± 5.1	25.2 ± 3.8	25.5 ± 4.5
Hemoglobin (g/dL)	11.9 ± 1.0	11.8 ± 1.0	12.1 ± 1.6
Albumin (g/dL)	3.7 ± 0.5	3.7 ± 0.5	3.9 ± 0.3
HbA1c (%)	7.7 ± 2.1	7.9 ± 1.4	6.4 ± 0.6
BS (mg/dl)	157.1 ± 105.9	131.6 ± 27.8	144.4 ± 36.7
BUN (mg/dL)	42.9 ± 9.5	47.4 ± 21.5	37.2 ± 14.5
Creatinine (mg/dL)	3.3 ± 1.4	3.9 ± 2.8	3.9 ± 2.8
eGFR (ml/min/1.73m^2)	15.2 ± 4.6	21.8 ± 18.5	26.4 ± 12.4
Na (mmol/L)	139.4 ± 4.2	138.3 ± 3.8	140.8 ± 2.1
K (mmol/L)	4.9 ± 0.4	4.7 ± 0.7	5.0 ± 0.6
Cl (mmol/L)	105.8 ± 5.4	103.6 ± 4.5	106.3 ± 1.8
AST (U/L)	27.1 ± 7.0	18.9 ± 6.7*	20.2 ± 5.5
ALT (U/L)	24.5 ± 10.1	16.9 ± 7.7	19.0 ± 8.4
Triglycerides (mg/dL)	185.6± 164.5	167.3 ± 69.1	133.8 ± 87.4
HDL-cholesterol (mg/dL)	52.7 ± 14.6	47.9 ± 8.7	56.5 ± 15.2
LDL-cholesterol (mg/dL)	83.7 ± 29.8	82.3 ± 20.5	106.7± 41.7
Urinary protein (g/gCr)	3.7 ± 1.5	4.3 ± 4.1	3.6 ± 2.3

Table 2. Clinical characteristics of patients with DKD enrolled in the retrospective clinical study. Values are shown as mean ± SD or counts. Abbreviations: DKD=Diabetic kidney disease, SGLT2is=SGLT2 inhibitors, DPP4is=DPP4 inhibitors, HbA1c=glycated hemoglobin, BS=blood sugar, BUN=blood urea nitrogen, eGFR=estimated glomerular filtration rate, AST=aspartate aminotransferase, ALT=alanine aminotransferase, Na=sodium, K=potassium, CL=chloride, HDL=high-density lipoprotein, LDL=low-density lipoprotein. *, P<0.05 vs. DKD group.

Abcam Cat# ab89901, RRID: AB_2043201) served as the primary antibody for evaluating podocyte injury in C57 BL/6J mice. Double staining was conducted for KIM-1 and α-SMA, AQP2 (1:200, Santa Cruz Cat# sc-515798) and GLP-1R (1:200, Bioss Cat# bs-1559R, RRID: AB_10857357) in rodent samples, as well as for ENaCα (SIGMA Cat# SAB5201770-100UG) and AQP2 (Abcam Cat# ab62628, RRID: AB_2227510) in patient samples. Secondary antibodies included Secondary donkey IgG directed against goat IgG conjugated with Cy5(Jackson ImmunoResearch Labs Cat# 705-175-147, RRID: AB_2340415), Secondary donkey IgG directed against rabbit IgG conjugated with Cy3(Jackson ImmunoResearch Labs Cat# 711-165-152, RRID: AB_2307443) and Secondary donkey IgG directed against mouse IgG conjugated with 488(Jackson ImmunoResearch Labs Cat# 715-545-150, RRID: AB_2340846), both at a dilution of 1:200 (Molecular Probes, Eugene, OR, USA). For all specimens, nuclear and renal structures were counterstained with DAPI (Thermo Fisher Scientific). The whole kidney was scanned, and the targeted protein-positive area was analyzed using BZ-X800 (Keyence, Osaka, Japan). Line profile was evaluated by Image J[40].

Real-Time RT-PCR

Whole kidney total RNA was extracted with RNeasy mini kit (Qiagen), and 1 μg of total RNA was transcribed with Prime Script RT Master Mix kit (Takara Bio, Otsu, Japan). TaqMan probe for Slc5a2 (Rn00574917_m1), Ctgf (Rn01537279_g1) and 18s (Rn03928990_g1) ribosomal RNA were purchased from Applied Biosystems (Foster City, CA, USA). Real-time PCR was performed with StepOne (Applied Biosystems, Foster City, CA, USA). The expression of mRNAs for Slc5a2, Ctgf was calculated as a ratio to 18s mRNA expression. Statistical analysis of results was performed with the ΔCt (threshold cycle) value (Ct gene of interest – Ct 18s ribosomal RNA). Relative gene expression was obtained from the ΔΔCt method (Ct sample-Ct calibrator).

Western blot analysis

Whole kidney tissues were homogenized and lysed with 25 mmol/L Tris–HCl (pH 7.4) containing 1% Triton X-100, 0.1% SDS, 2 mmol/L EDTA, and 1% protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). The supernatant was then separated through SDS–PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). Protein concentration was determined using the BCA Protein Assay (Thermo Fisher Scientific). Membranes were incubated with the following primary antibodies, ENaCα (StressMarq Biosciences Cat# SPC-403, RRID: AB_10640131), Nedd4-2(Abcam Cat# ab245522), p-Nedd4-2(Cell Signaling Technology Cat# 12146, RRID: AB_2797829), GLP-1R (1:200, Bioss Cat# bs-1559R, RRID: AB_10857357), 14-3-3 protein (Cell Signaling Technology Cat# 7413, RRID: AB_10950820) and β-actin (Cell Signaling Technology Cat# 4967, RRID: AB_330288) during overnight. Horseradish peroxidase-conjugated anti-mouse (Cell Signaling Technology Cat# 7076, RRID: AB_330924) and anti-rabbit (Cell Signaling Technology Cat# 7074, RRID: AB_2099233) secondary antibodies (1:2000) were applied. Protein expressions were visualized by Signal Enhancer HIKARI (Nacalai

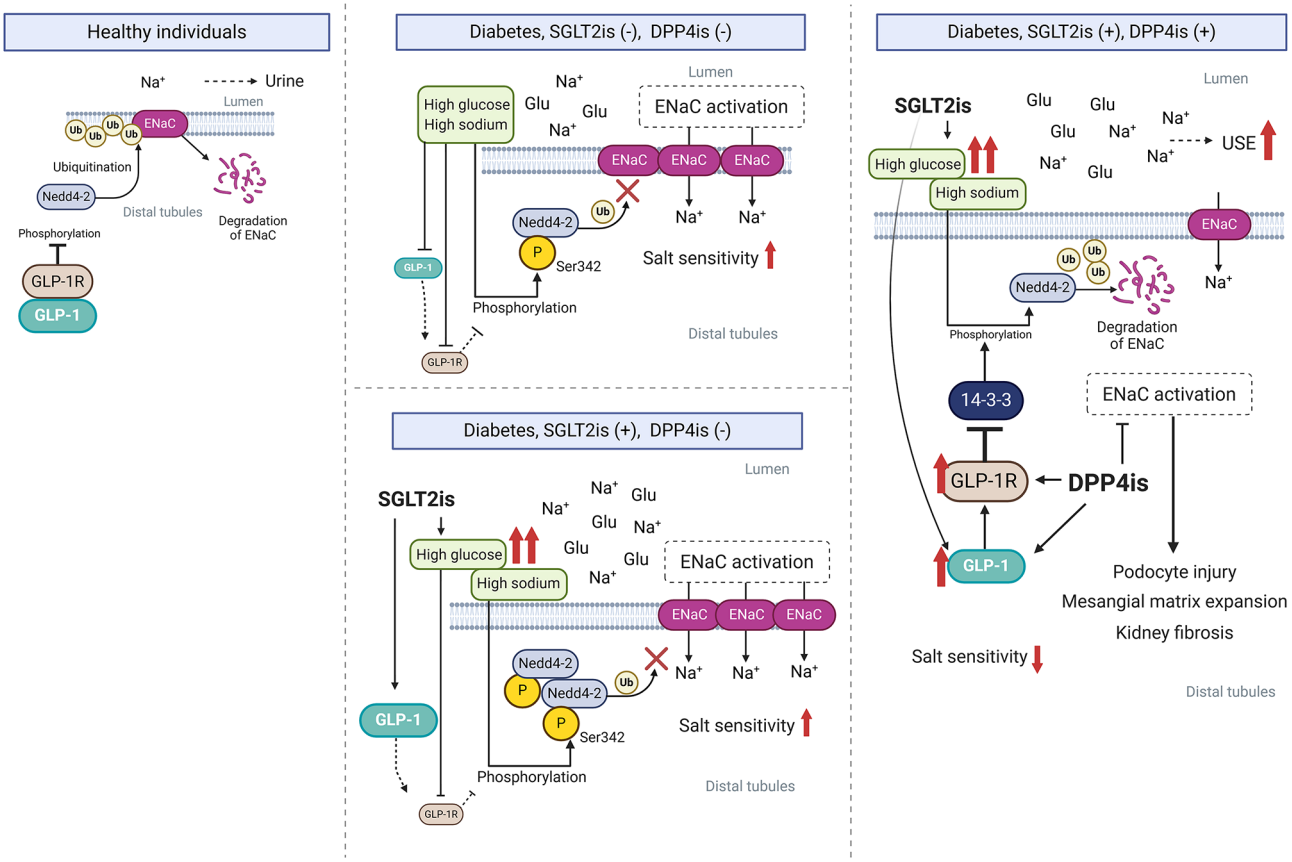


Fig. 7. Mechanism of how add-on therapy with DPP4is to SGLT2is improves salt sensitivity in DKD. Under normal condition, ENaC is continuously regulated by Nedd4-2-dependent degradation. In the context of DKD, increased sodium and glucose in urine reduces GLP-1 and GLP-1R expression in distal tubules, leading to the increase in p-Nedd4-2, 14-3-3 protein and ENaC, resulting in the increase in Na^+ reabsorption and high salt sensitivity. DPP4is, but not SGLT2is, increases the expression of GLP-1R in distal tubules of DKD, which reduces p-Nedd4-2, 14-3-3 protein, ENaC, and salt sensitivity. Thus, regulating ENaC by add-on therapy with DPP4is to SGLT2is via the activation of GLP-1-receptor axis prevents podocyte injury, mesangial matrix expansion, and kidney fibrosis. DKD, diabetic kidney disease; HS, high salt; HG, high glucose; SGLT2is, sodium glucose transporter2 inhibitors; DPP4is, dipeptidyl peptidase-4 inhibitors; Nedd4-2, neural precursor cell expressed developmentally down-regulated 4 – 2; p-Nedd4-2, phosphorylated Ser^{342} of Nedd4-2ENaC, Epithelial sodium channel.

Tesque, Inc., Kyoto, Japan). Western blots were visualized using Fusion Solo S (Vilber, Osaka, Japan), and protein expression was analyzed using ImageJ.

Study design of retrospective clinical study and patient selection

The single-center retrospective clinical study was conducted at Division of nephrology, Kurume University Hospital. The study protocol was approved by the institutional ethics committees of Kurume University School of Medicine, Japan (Ethical No.22162). All methods in the clinical study were performed in accordance with relevant guidelines and regulations. Due to the retrospective nature of the study, Kurume University Institutional Review Board waived the need of obtaining informed consent. The patients with DKD taking renin-angiotensin system inhibitors, such as angiotensin converting enzyme inhibitors and angiotensin receptor blocker, were enrolled and then those prescribed with diuretics, MRA, and immunosuppressants, including glucocorticoids were excluded from this clinical observation because the medications are likely to affect urinary sodium excretion. Urinary sodium and potassium concentrations other than clinical parameters measured in standard laboratory tests were measured in all patients on the day when they were admitted to the division of nephrology, Kurume University Hospital. Also, the patients were followed-up in outpatient clinic of the division of nephrology from April 2010 to August 2022 and the clinical parameters were investigated at least once 6 months after the admission to the hospital. The patients were classified as followings; (1) patients with DKD without SGLT2is and DPP4is, (2) those with DKD taking SGLT2is alone, and (3) those with DKD taking both of SGLT2is and DPP4is. Urinary sodium excretion was calculated according to the following formula: Sodium excretion = 21.98 × [urinary Na (mEq/L) ÷ urinary Cre (mg/dL) ÷ 10 × 24-hour urinary Cre excretion] ^ 0.392. In addition, to investigate the expression pattern and levels of ENaCα in the patients with diabetes taking SGLT2is, DPP4is, or the combination of the two agents, the paraffin samples were collected from the renal

biopsy database of the division of nephrology, Kurume university hospital. Normal kidney (NK) specimen was obtained from patients without diabetes who received kidney biopsy for mild proteinuria or microhematuria. No histological changes and immunoglobulin deposition were observed in PAS, periodic acid-methenamine silver, and immunofluorescence staining, so that the patient was diagnosed with minor glomerular abnormalities by pathologists. With those samples, we performed immunofluorescence staining for ENaC and AQP2.

Statistical analysis

All values are expressed as the means ± standard error of the mean (SD). Data were statistically analyzed using GraphPad Prism version 8.0 (San Diego, California USA, www.graphpad.com) or JMP Pro ver. 14 software (SAS Institute Inc., Cary, NC, USA). Comparison between two groups was performed by using an unpaired or paired 2-tailed Student's t-test. Comparison between multiple groups was performed by using one-way ANOVA followed by post-hoc Turkey test. P values < 0.05 were considered statistically significant.

Data availability

The datasets used and/or analyzed during the current study can be available from the corresponding author on reasonable request.

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Author contributions

GK, SI, YY, KM, ST, YM, and YY performed experiments, collected data, and analyzed the data. KT designed the experiments and created the manuscript. SY and KF helped with experimental design and manuscript preparation.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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