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Sacubitril/valsartan ameliorates renal tubulointerstitial injury through increasing renal plasma flow in a mouse model of type 2 diabetes with aldosterone excess

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ABSTRACT

Background. Aldosterone has been assumed to be one of aggravating factors in diabetic kidney disease (DKD). Natriuretic peptides/guanylyl cyclase-A/cGMP signalling has been shown to ameliorate aldosterone-induced renal injury in mice. Sacubitril/valsartan (SAC/VAL) is used clinically for chronic heart failure and hypertension, in part by augmenting natriuretic peptide bioavailability. The effects of SAC/VAL on renal pathophysiology including in DKD, however, have remained unclarified.

Methods. Eight-week-old male *db/db* mice fed on a high-salt diet (HSD) were treated with vehicle or aldosterone (0.2 μg/kg/min), and divided into four groups: HSD control, ALDO (aldosterone), ALDO + VAL (valsartan), and ALDO + SAC/VAL group. After 4 weeks, they were analysed for plasma atrial natriuretic peptide (ANP) levels, renal histology, and haemodynamic parameters including glomerular filtration rate (GFR) by FITC-inulin and renal plasma flow (RPF) by para-amino hippuric acid.

Results. The ALDO + SAC/VAL group showed significantly increased plasma ANP concentration and creatinine clearance, and decreased tubulointerstitial fibrosis and neutrophil gelatinase-associated lipocalin expression compared to ALDO and ALDO + VAL groups. SAC/VAL treatment increased GFR and RPF, and suppressed expression of *Tgfb1*, *Il1b*, *Ccl2*, and *Lcn2* genes compared to the ALDO group. The percentage of tubulointerstitial fibrotic areas negatively correlated with the RPF and GFR.

Conclusion. In a mouse model of type 2 diabetes with aldosterone excess, SAC/VAL increased RPF and GFR, and ameliorated tubulointerstitial fibrosis. Furthermore, RPF negatively correlated well with tubulointerstitial injury, suggesting that the beneficial effects of SAC/VAL could be through increased renal plasma flow with enhanced natriuretic peptide bioavailability.

Keywords: aldosterone, ARNI, diabetic kidney disease, GFR, RPF

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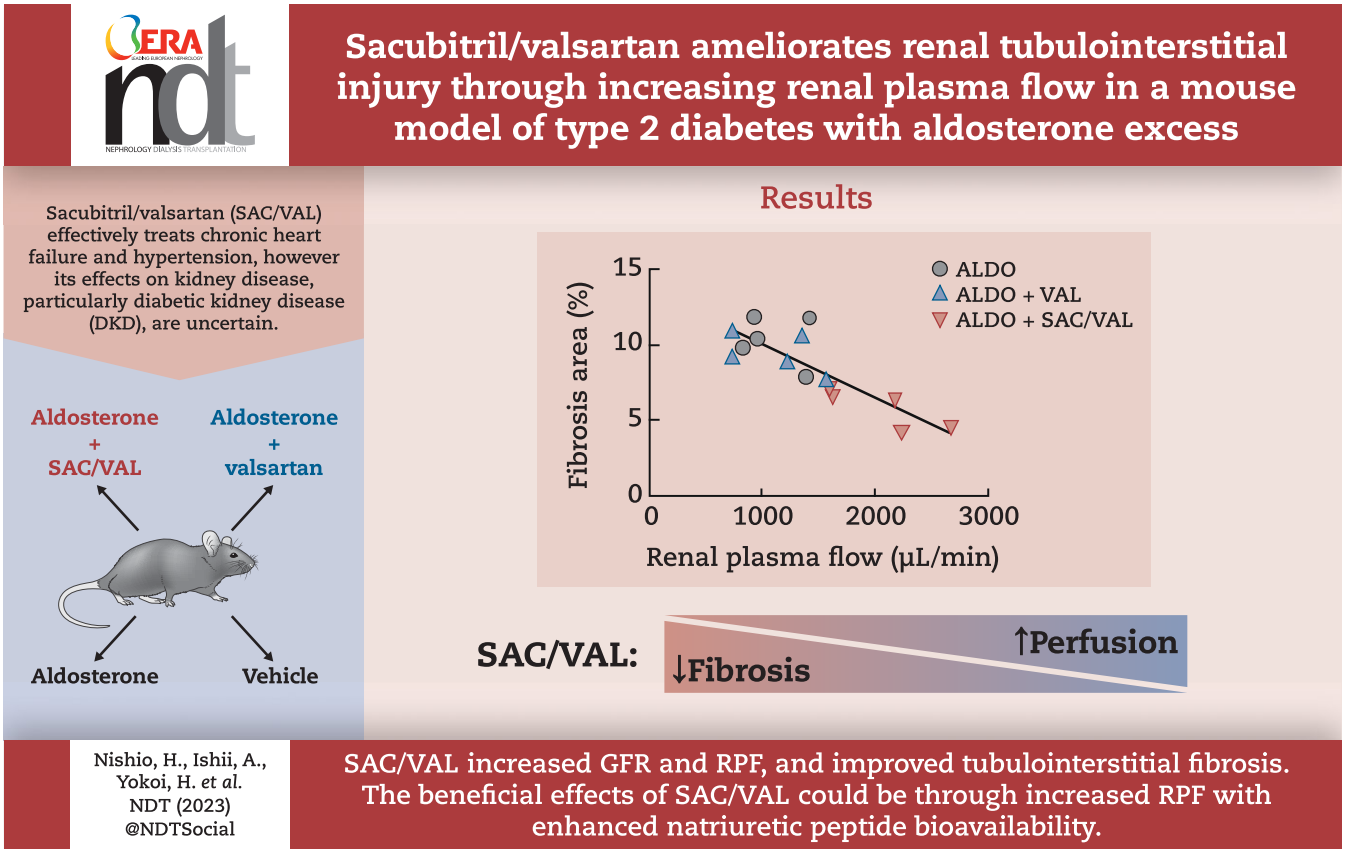
GRAPHICAL ABSTRACT



KEY LEARNING POINTS

What was known:

- The importance of aldosterone-mineralocorticoid receptor signalling as an aggravating factor in DKD has attracted attention.
- Clinical trials to date have demonstrated that sacubitril/valsartan has been proven effective for chronic heart failure and hypertension, but its effects on renal pathophysiology have not been fully clarified so far.

This study adds:

- In type 2 diabetic mice with aldosterone excess, sacubitril/valsartan improved RPF and GFR and ameliorated tubulointerstitial injury.
- We newly found that RPF and GFR negatively correlated well with tubulointerstitial fibrosis in this model, suggesting that sacubitril/valsartan can ameliorate renal tubulointerstitial damages probably by increased renal plasma flow.

Potential impact:

- In DKD with declining in GFR and RPF, sacubitril/valsartan may have beneficial effects through improving renal blood flow, in addition to suppressing aldosterone-induced renal interstitial damage.

INTRODUCTION

Diabetic kidney disease (DKD) is one of the most common underlying causes of end-stage renal failure globally, with a significant impact on patients' life expectancy, quality of life, and medical and social burden [1]. The advent of renin-angiotensin-aldosterone system (RAAS) inhibitors such as angiotensin converting enzyme inhibitor (ACEi) and angiotensin II receptor blocker (ARB), which are frequently prescribed as a standard therapy, are effective in retarding the progression of DKD, but their effects on renal prognosis, especially in advanced cases, are thought to be insufficient [2]. Recently, sodium-glucose cotransporter 2 (SGLT2) inhibitors have become a new treatment option for DKD, as they have been demonstrated to improve hyperfiltration, which is an important pathophysiological factor in DKD, along with significant protective actions in the heart and kidney [3]. In addition, the FIGARO-DKD and the FIDELIO-DKD clinical trials, large-scale interventional studies in patients with DKD, revealed that a novel mineralocorticoid receptor (MR) antagonist finerenone, when added to standard therapy, improved renal outcomes and reduced the risk of cardiovascular disease (CVD) [4, 5]. Aldosterone breakthrough event [6] in DKD, which occurs when RAAS inhibitors are used for a prolonged period in DKD, may worsen renal prognosis [7]. Additionally, it has been shown

that MR activity may be increased in diabetic circumstances [8], and aldosterone-MR signalling in DKD has received much attention [9].

Sacubitril/valsartan (SAC/VAL) is a fixed-dose combination of sacubitril, a prodrug of a neprilysin inhibitor (NEPi) LBQ657, and valsartan, an ARB, and is the first member of a drug category called angiotensin-receptor blocker/neprilysin inhibitor (ARNI) [10]. ARNI exerts organ-protective effects by inhibiting neprilysin, one of the main degradation enzymes of the natriuretic peptide (NP) family, consisting of atrial natriuretic peptide (ANP), brain or B-type natriuretic peptide, and C-type natriuretic peptide, thereby increasing blood NP levels and activating the NPs/guanylyl cyclase-A (GC-A)/cyclic guanosine monophosphate (cGMP) signalling. The PARADIGM-HF trial demonstrated that SAC/VAL improved the primary outcome in heart failure patients with lower left ventricular ejection fraction compared to the ACEi treatment group, which was a composite of death from cardiovascular causes or hospitalization for heart failure [11]. The UK Heart and Renal Protection (HARP)-III trial examined the short- to medium-term effects of SAC/VAL in patients with chronic kidney disease and found no difference in primary outcomes defined as a reduction in albuminuria or glomerular filtration rate (GFR) decline [12]. Meanwhile, a meta-analysis of randomized controlled trials that used NEPi and ARB, revealed a decrease in the frequency of acute renal events and estimated GFR decline over time [13]. Thus, the renoprotective effect of NEPi has been suggested. Clinical trials to date have shown that SAC/VAL is efficient for chronic heart failure and hypertension, but its effects on renal function and pathophysiology have not yet been fully clarified. Previously, our group has investigated the effects of the NPs/GC-A/cGMP signalling on the suppression of renal damage in animal models. We have previously reported that the NPs/GC-A/cGMP signalling could exert renoprotective effects on DKD [14], subtotal renal ablation [15], progressive glomerulonephritis [16], and renal injury induced by aldosterone [17, 18]. Therefore, we hypothesized that SAC/VAL could be beneficial in aldosterone-induced renal injury, and may be effective as a therapeutic agent for DKD [19]. In the present study, we examined the effects of SAC/VAL treatment using *db/db* mice treated with a high-salt diet (HSD) and aldosterone, as a progressive DKD model.

MATERIALS AND METHODS

Experimental animals and treatments

All animal experiments were performed in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions, and were approved by the Animal Experimentation Committee of Kyoto University Graduate School of Medicine (Approval number; MedKyo22537) and according to the ARRIVE guidelines [20]. BKS.Cg–*Dock7* ^{m} +/+ *Lepr*^{db}/Jcl mice (*db/m*) mice were purchased from Clea Japan Co. Ltd (Tokyo, Japan), and crossbred with each other. Eight-week-old male diabetic *db/db* mice were implanted with an osmotic minipump (ALZET 2004, Cupertino, CA, USA), and were administered with aldosterone (0.2 μg/kg body weight per minute, Sigma Aldrich, St. Louis, MO, USA) or vehicle (2% EtOH), as previously described [17]. All mice were fed with an HSD (5.79% NaCl, Clea Japan Co. Ltd, Tokyo, Japan). Vehicle (0.1% dimethyl sulphoxide (DMSO), Nacalai tesque, Kyoto, Japan), valsartan (VAL, 30 mg/kg/day, Tokyo chemical industry Co. Ltd, Tokyo, Japan), or SAC/VAL (30 mg/kg/day, Toronto Research, Toronto, Canada) was administered by dissolving solution in drinking water. For further details, please refer to the Supplementary Methods. Blood and kidney samples were harvested at 4 weeks. Mouse ANP levels were measured as previously described [21]. The sum of nitrate and nitrite in renal tissue was determined by using a Nitrite/Nitrate Assay kit (NK05, Dojindo, Kumamoto, Japan) as previously described [22]. In the experiment of renal haemodynamic parameters measurement described below, 8-week-old male *db/db* mice were similarly assigned to four groups, and blood pressure and renal haemodynamic parameters were measured at 0 and 4 weeks, and echocardiography was performed at 4 weeks using the Vevo 2100 Imaging system (FUJIFILM Visualsonics, Toronto, Canada).

RNA extraction and quantitative real-time PCR

RNA extraction and quantitative real-time PCR was performed as described previously [17, 23]. For further details, please refer to the Supplementary Methods. Primer and probe sets are described in Supplementary Table S1.

Histological, immunofluorescent and immunohistochemical studies

Histological and electron microscopic examinations were performed as described previously [17, 23]. For further details, please refer to the Supplementary Methods.

Measurement of renal haemodynamic parameters

GFR and renal plasma flow (RPF) were determined in conscious mice by calculating the clearance of FITC-inulin (Sigma Aldrich, St. Louis, MO, USA) and para-amino hippuric acid (PAH, Sigma Aldrich, St. Louis, MO, USA) [24–26]. In brief, a cocktail of 1% PAH and 1% FITC-inulin (3.74 μl/g body weight; for further details, please refer to the Supplementary Methods) was injected via the tail vein in conscious mice and blood samples (25 μl) were collected each time through the tail tip using heparinized capillary tubes (CS-HMT-501; DWK Life Sciences, Melville, NJ, USA) at 1, 3, 7, 10, 15, 35, 55 and 75 min following injection. Samples were centrifuged at 10 000×*g* for 5 min at room temperature to separate the plasma. The concentration of FITC-inulin in the plasma samples was measured using a fluorescent plate reader (ARVO X5; PerkinElmer, Waltham, MA, USA), as described previously [24]. Then, the concentration of PAH in the samples was evaluated by a calorimetric assay using 15% trichloroacetic acid (TCA, Tokyo Chemical Industry Co. Ltd, Tokyo, Japan), 0.3% 4-dimethylaminocinnamaldehyde (DACA, Tokyo Chemical Industry Co. Ltd, Tokyo, Japan), and a microplate reader (Model 680, Bio-Rad, Hercules, CA, USA), as described previously [27]. On the basis of the measured FITC-inulin and PAH concentrations, clearances were calculated using two-compartmental pharmacokinetic data analysis, as previously described [24]. The value of RPF was estimated from PAH clearance using a renal extraction ratio of 0.7 [25]. Filtration fraction was calculated by dividing GFR by RPF.

Statistical analysis

Values are expressed as the mean ± standard error of the mean (SEM) and were analysed using Graph Pad Prism software (v.9.00, GraphPad, San Diego, CA, USA). Unpaired Student's *t* tests were used to analyse differences between the two groups, whereas comparisons of more than two groups were performed by one-way ANOVA with a Tukey *post hoc* test or a Dunnett *post hoc* test. Statistical significance was set at $P < 0.05$.

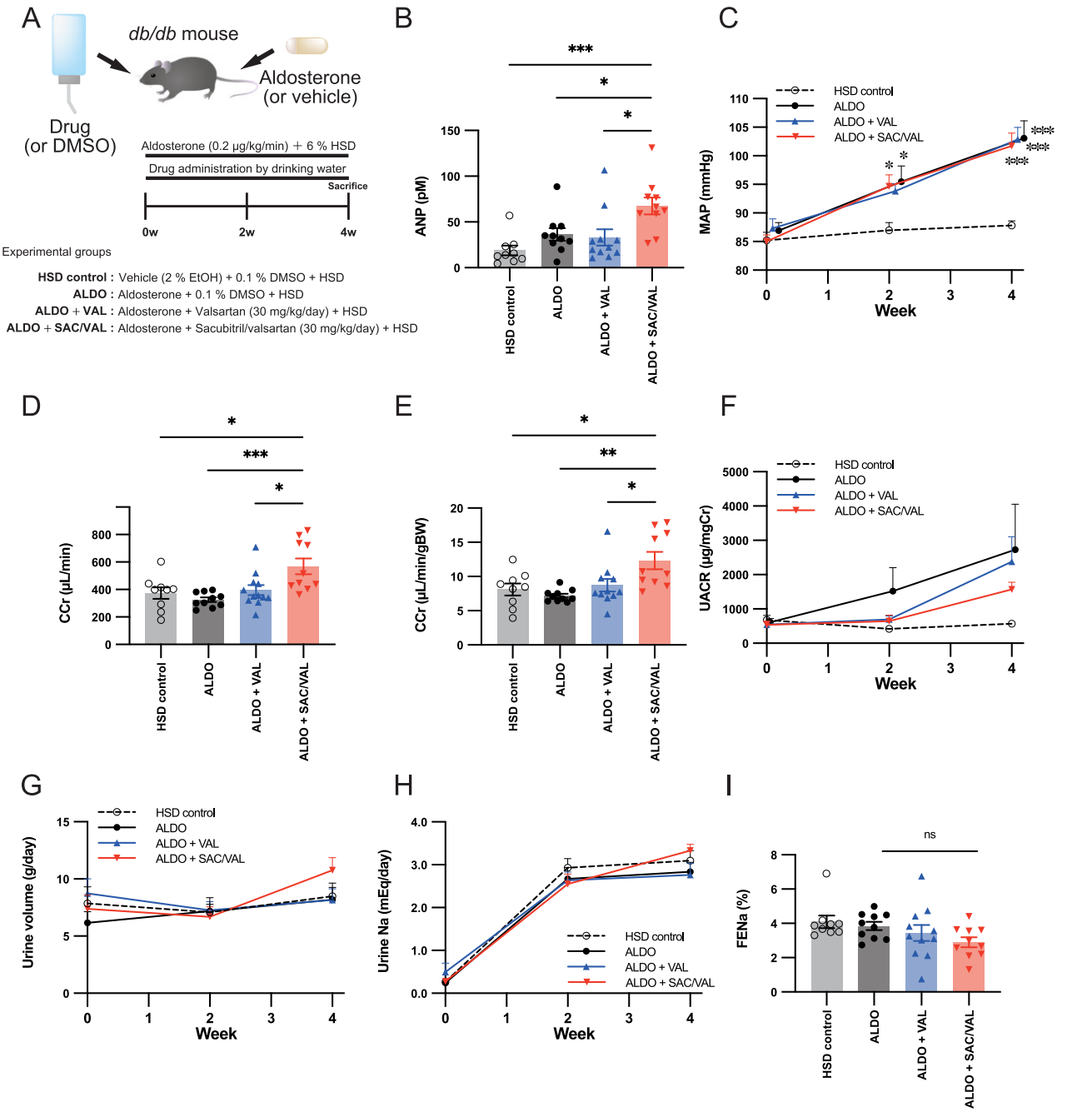


Figure 1: Sacubitril/valsartan (SAC/VAL) treatment increased serum ANP and creatinine clearance (CCr) in diabetic *db/db* mice treated with aldosterone and an HSD. (A) Schema of the experimental protocol. Eight-week-old male *db/db* mice fed an HSD were infused aldosterone by osmotic minipumps, and administered with 0.1% DMSO as ALDO ($n = 10$), valsartan (ALDO + VAL; $n = 11$), or SAC/VAL (ALDO + SAC/VAL; $n = 10$) by drinking water for 4 weeks. The vehicle-treated mice (HSD control) received operation by using osmotic minipumps filled with vehicle (2% EtOH) instead of aldosterone, and were also treated with an HSD and 0.1% DMSO ($n = 9$). (B) Plasma ANP was significantly increased by administration of SAC/VAL compared to other groups. (C) Mean arterial pressure (MAP) in experimental groups. Aldosterone administration increased MAP in mice compared to HSD control. (D, E) CCr (D) and CCr with body weight correction (E) were significantly increased by administration of SAC/VAL compared to other groups. (F–H) Time course of UACR (F), urine volume (G), and urinary sodium excretion (Urine Na; H). (I) The average of fractional excretion of sodium (FENa). Data are presented as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. HSD control or otherwise indicated, by one-way ANOVA analysis with a Tukey *post hoc* test.

RESULTS

SAC/VAL increased plasma ANP concentration and creatinine clearance (CCr) in diabetic mice with aldosterone excess

We investigated the effect of treatment with VAL or SAC/VAL for 4 weeks on renal injury induced by aldosterone and an HSD in an 8-week-old male diabetic (*db/db*) mouse model (Fig. 1A). HSD control group mice were fed an HSD and 0.1% DMSO and treated with vehicle, and ALDO mice were fed an HSD and 0.1% DMSO, and received aldosterone. There was no significant difference in the body weight, pulse rate, or fasting blood glucose among four groups (Supplementary Fig. S1A–C), and in the actual dose of drugs

Table 1: Effect: of SAC/VAL on physical and biochemical characteristics in diabetic (*db/db*) mice treated with aldosterone and HSD.

	HSD control	ALDO	ALDO + VAL	ALDO + SAC/VAL
KW/BW, mg/g	8.94 ± 0.26	11.42 ± 0.37[c]	11.49 ± 0.27[c]	11.56 ± 0.25[c]
Blood urea nitrogen, mg/dl	23.1 ± 1.4	17.0 ± 1.3[b]	16.0 ± 1.3[b]	14.4 ± 0.7[c]
Creatinine, mg/dl	0.09 ± 0.01	0.12 ± 0.00	0.10 ± 0.01	0.08 ± 0.01[d]
Triglycerides, mg/dl	122.2 ± 18.0	127.1 ± 15.1	123.4 ± 16.4	137.5 ± 13.9
Cholesterol, mg/dl	144.2 ± 4.5	119.2 ± 5.6[a]	120.1 ± 6.2[a]	122.2 ± 3.9[a]
Na, mEq/l	148.2 ± 1.2	157.7 + 1.5[a]	153.0 ± 3.5	152.7 + 1.7
K, mEq/l	4.73 ± 0.21	3.48 ± 0.11[c]	3.53 ± 0.25[c]	3.26 ± 0.13[c]
HbA1c, %	6.6 ± 0.3	5.9 ± 0.6	6.0 ± 0.3	5.9 ± 0.4

KW, kidney weight; BW, body weight. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ vs. HSD control, [d]$P < 0.05$ vs. ALDO, by one-way ANOVA analysis with a Tukey *post hoc* test. HSD, high-salt diet; ALDO, aldosterone; VAL, valsartan; SAC/VAL, sacubitril/valsartan.

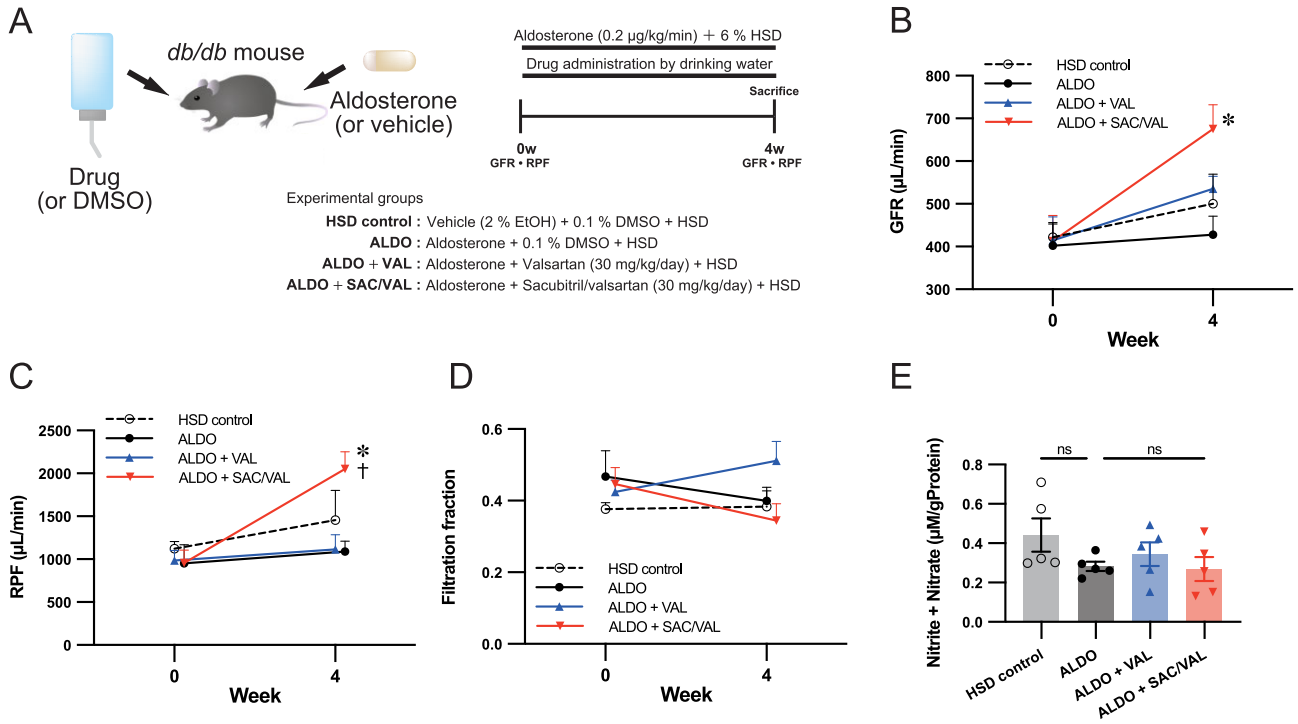


Figure 2: SAC/VAL treatment increased GFR and RPF significantly, while FF was not changed. (A) At 0 and 4 weeks, GFR and RPF were measured using a similar load performed on different mice ($n = 5$ per group). (B–D) Time course of GFR (B), RPF (C), and FF (D) in experimental groups. (E) The sum of nitrite and nitrate in renal tissue was measured. Data are presented as means ± SEM. *$P < 0.05$ vs. ALDO, †$P < 0.05$ vs. ALDO + VAL, by one-way ANOVA analysis with a Tukey *post hoc* test. HSD, high-salt diet; ALDO, aldosterone; VAL, valsartan; SAC/VAL, sacubitril/valsartan.

between VAL and SAC/VAL treatment groups (Supplementary Fig. S1D). Physical and biochemical characteristics were shown in Table 1. Plasma ANP concentrations in the SAC/VAL treatment group were significantly increased compared to all other groups (Fig. 1B). CCr was significantly increased in the SAC/VAL treatment group compared with the other three groups (Fig. 1D), even after body weight correction (Fig. 1E). Mean arterial pressure, urine volume, urinary sodium excretion, or fractional excretion of sodium (FENa) were not changed at 4 weeks among the ALDO, VAL, and SAC/VAL treatment groups (Fig. 1C and G-I). The mean urine albumin/creatinine ratio (UACR) was highest in the ALDO group and tended to reduce in the VAL and SAC/VAL treatment groups, but not significantly (Fig. 1F). Thus, the treatment with SAC/VAL was demonstrated to increase ANP and CCr.

SAC/VAL improved GFR and RPF compared to the ALDO group

Because SAC/VAL treatment improved CCr compared to the other groups, we measured GFR and RPF to further assess renal haemodynamics in detail (Fig. 2A). In the HSD control group, both GFR and RPF increased over 4 weeks, but there was essentially no increase in either at 4 weeks in the ALDO group (Fig. 2B and C). In the SAC/VAL treatment group, both GFR and RPF were significantly higher than in the ALDO group, and RPF was higher than in the VAL treatment group (Fig. 2B and C, Supplementary Table S2). On the other hand, filtration fraction (FF) in the SAC/VAL treatment group remained constant and was comparable to that in the HSD control and ALDO groups (Fig. 2D). There was no difference in cardiac function at 4 weeks among the four groups

(Supplementary Table S2). To investigate the nitric oxide (NO) pathway, which is involved in renal haemodynamics along with the NPs/GC-A/cGMP signalling, the sum of nitrite and nitrate in renal tissue was measured and no differences were found among the four groups (Fig. 2E). These results indicate that SAC/VAL therapy increased RFP through a mechanism distinct from the NO pathway.

SAC/VAL improved tubulointerstitial injury and tubulointerstitial fibrosis

We evaluated the histological findings. Periodic acid–Schiff (PAS) staining showed no evidence of gross tubulointerstitial damage such as tubular cell necrosis or cast formation (Fig. 3A). Immunofluorescent NGAL staining revealed a 2-fold increase in NGAL-positive areas in the ALDO group compared to HSD control, and a predominant improvement in the SAC/VAL treatment group, indicating that SAC/VAL treatment reduced tubulointerstitial injury (Fig. 3B and E). Sirius red staining showed mild fibrosis of the interstitium in the HSD control group, but increased positive areas in the ALDO and the VAL treatment groups (Fig. 3C and D). On the other hand, the SAC/VAL treatment group showed significantly fewer positive areas than the ALDO and VAL treatment groups, suggesting that treatment with SAC/VAL improves tubulointerstitial fibrosis (Fig. 3C, D and F). Next, we evaluated glomerular damage (Supplementary Fig. S2A–E). PAS staining of glomeruli showed no difference in glomerular diameter or mesangial area among the four groups. (Supplementary Fig. S2F and G). The number of WT1-positive cells decreased in the ALDO group compared with the HSD control group, but there was no improvement after drug administration (Supplementary Fig. S2H). There was no difference in the staining of nephrin among the four groups (Supplementary Fig. S2C). Electron microscopy revealed that the podocyte damage, which is represented by increased foot process width, was ameliorated in the SAC/VAL and VAL treatment groups, compared to the ALDO group (Supplementary Fig. S2E and J), and there was no difference in the GBM thickness among the four groups (Supplementary Fig. S2I). In this study, the therapeutic effects of the drugs on glomerular injury were limited to podocyte protection. As a result, the impact of SAC/VAL on histological findings was more pronounced in amelioration of the tubulointerstitial injury.

SAC/VAL suppressed gene expressions of inflammation, tubulointerstitial damage, and fibrosis

We then analysed gene expression in renal tissue using whole kidney samples (Fig. 4). Treatment with SAC/VAL significantly suppressed *Tgfb1, Il1b, Ccl2*, *Lcn2* compared to the ALDO group (Fig. 4A and C–E). Gene expression of *Col1a1* and *Kim1* tended to reduce in the VAL and SAC/VAL treatment groups, but not significantly (Fig. 4B and F). No difference in *Nos3* gene expression was observed among the four groups (Fig. 4G). These findings suggest that SAC/VAL reduced inflammation, tubulointerstitial damage, and fibrosis. Furthermore, we evaluated gene expression in glomerular samples and found no change among the four groups (Supplementary Fig. S3A–G). Gene expression analysis demonstrated that the therapeutic effect of SAC/VAL was pronounced in improvement of the tubulointerstitial damages.

Tubulointerstitial fibrosis was negatively correlated with RPF and GFR in diabetic mice with aldosterone excess

As SAC/VAL upregulates NP concentration and NPs increase renal blood flow [28], we speculated that renal blood flow may correlate with renal pathological findings. The percentage of NGAL-positive areas in the histological findings was positively correlated with the percentage of tubulointerstitial fibrosis areas. (Fig. 5A). Dot plot analysis of GFR, RPF and tubulointerstitial fibrosis areas showed that GFR and RPF were negatively correlated with tubulointerstitial fibrosis areas in the three groups treated with aldosterone (Fig. 5B and C), indicating that increase of GFR could be due to increase of RPF and that increase of RPF can improve renal tubulointerstitial fibrosis.

DISCUSSION

Recently, the importance of aldosterone-MR signalling as an exacerbating factor in DKD has attracted much attention. As demonstrated by the FIGARO-DKD and the FIDELIO-DKD studies, in which finerenone, a non-steroidal selective MR antagonist, has shown to inhibit the progression of renal damage in diabetic nephropathy [4, 5, 9]. In our present study, we demonstrate that SAC/VAL could improve renal haemodynamics and tubulointerstitial damage in diabetic mice treated with an HSD and aldosterone. In particular, SAC/VAL significantly improved RPF and alleviated tubulointerstitial injury compared to the VAL treatment alone. Valsartan, which blocks upstream pathway of aldosterone-MR receptor system, showed a certain effect including improvement of podocyte foot process effacement, possibly because valsartan blocked the positive feedback loop of the RAAS induced by aldosterone. Although aldosterone is located downstream of renin–angiotensin system, it is suggested to activate upstream pathway by increasing ACE and angiotensinogen (AGT) to form a positive feedback loop [29, 30]. Therefore, valsartan is expected to exert renoprotective effects in this model. In addition, SAC/VAL showed an improvement over valsartan on the increase of GFR and RPF and the improvement of tubulointerstitial fibrosis, indicating SAC/VAL has additional effects on the kidney.

In the present study, we observed a significant increase in plasma ANP in the SAC/VAL group compared to the other groups, while no difference was observed in evaluation related to the NO pathway, such as NO metabolites or *Nos3* gene expression in renal tissues, suggesting that the renal protective effect of SAC/VAL over ARB was mainly due to the NPs/GC-A/cGMP signalling. In previous studies, we found that the NPs/GC-A/cGMP signalling can suppresses *Tgfb1*, a master regulator of fibrosis, and other fibrosis-related genes, thereby ameliorating podocyte injury and tubulointerstitial damage in a renal injury model induced by an HSD and aldosterone [17, 18]. In the current study, we found that the expression of *Tgfb1* tended to be upregulated in the DKD model with aldosterone excess. Of note, SAC/VAL suppressed its expression to almost the same level as the vehicle. Furthermore, SAC/VAL alleviated the tubulointerstitial damage and fibrosis to a level comparable to a vehicle, even at a low dose with no appreciable antihypertensive effect. Thus, we revealed the therapeutic effect of SAC/VAL on aldosterone-induced injury in diabetic mice. Our findings suggest that SAC/VAL can directly antagonize hyperglycaemia-induced as well as aldosterone-induced tubulointerstitial damages (Fig. 6). Because SAC/VAL is shown to reduce the

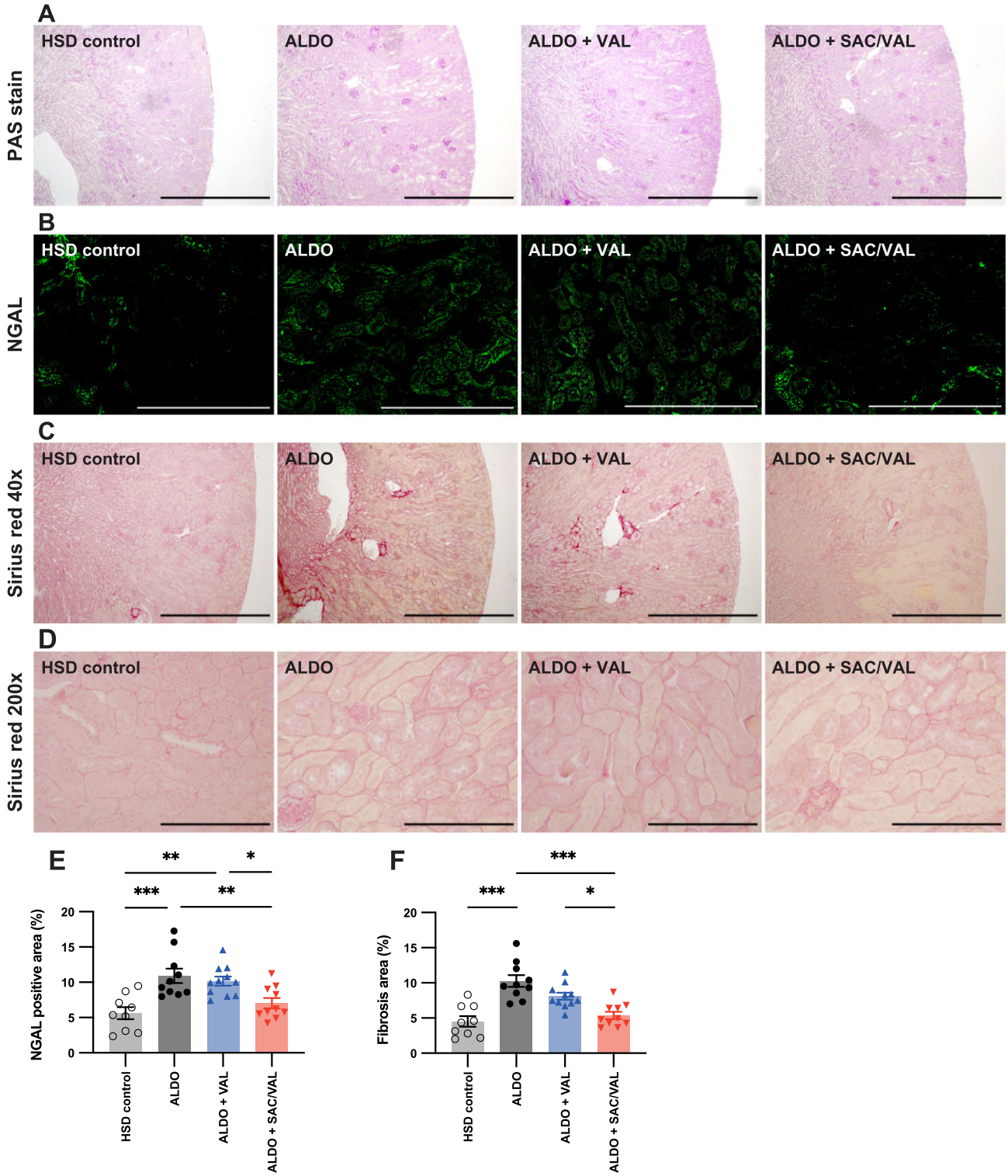


Figure 3: SAC/VAL treatment ameliorated tubulointerstitial injury in diabetic *db/db* mice treated with aldosterone and an HSD. (A) Representative images of periodic acid–Schiff (PAS) staining of kidney sections (magnification, ×40, Bars, 1 mm). (B) Immunofluorescence staining of kidney section for NGAL (magnification, ×200, Bars, 200 μm). (E) NGAL-positive areas were quantified (n = 9–11 per group). (C, D) Sirius red staining with lower magnification (C; magnification, ×40, bars, 1 mm), and higher magnification (D; magnification, ×200, bars, 200 μm). (F) The percentage of fibrosis was measured for each image of Sirius red staining in four groups. Data are presented as means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 by one-way ANOVA analysis with a Tukey *post hoc* test. ALDO, aldosterone; VAL, valsartan; SAC/VAL, sacubitril/valsartan.

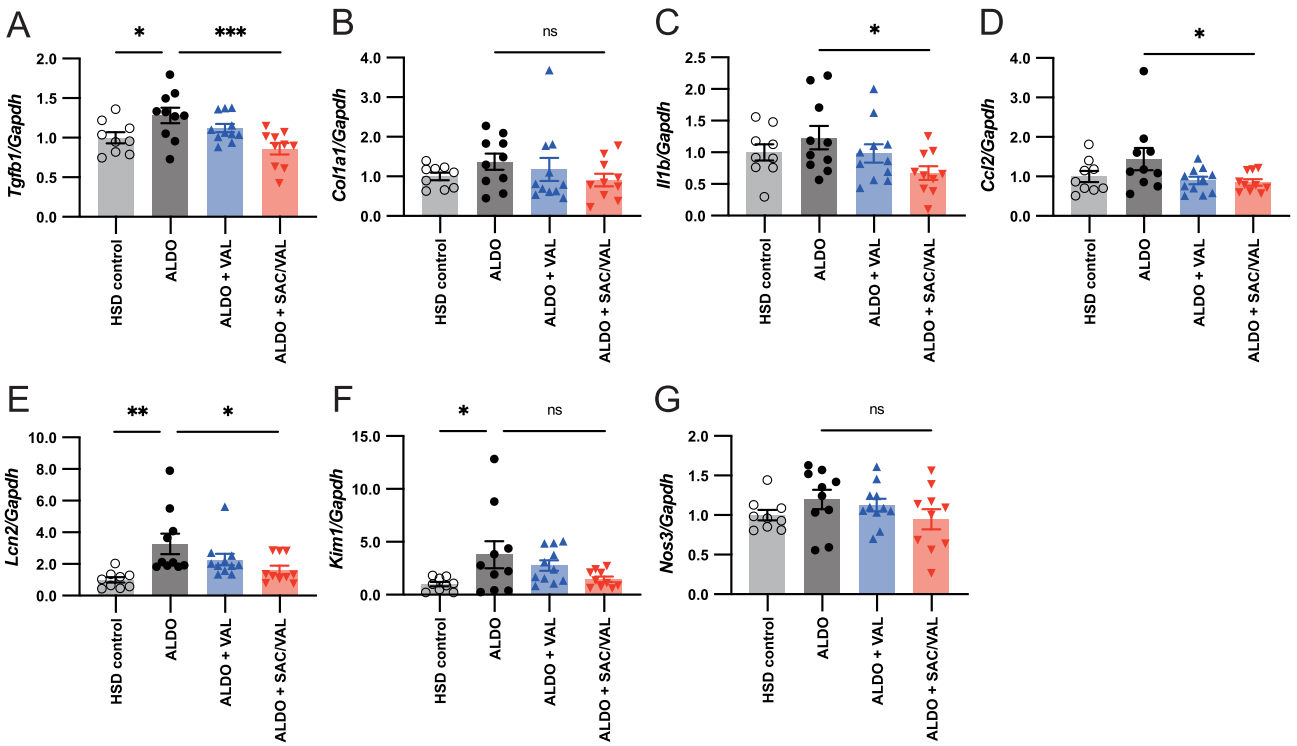


Figure 4: Quantitative PCR analysis of mRNA levels in the whole kidney. (A)*Tgfb1*, (B) *Col1a1*, (C) *Il1b*, (D) *Ccl2*, (E) *Lcn2*, (F) *Kim1*, and (G) *Nos3* mRNA expression in the whole kidney from each group. The level in HSD control mice was defined as 1.0. Data are presented as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. ALDO or otherwise indicated, by one-way ANOVA analysis with a Dunnett *post hoc* test. HSD, high-salt diet; ALDO, aldosterone; VAL, valsartan; SAC/VAL, sacubitril/valsartan. $n = 9$–11 per group.

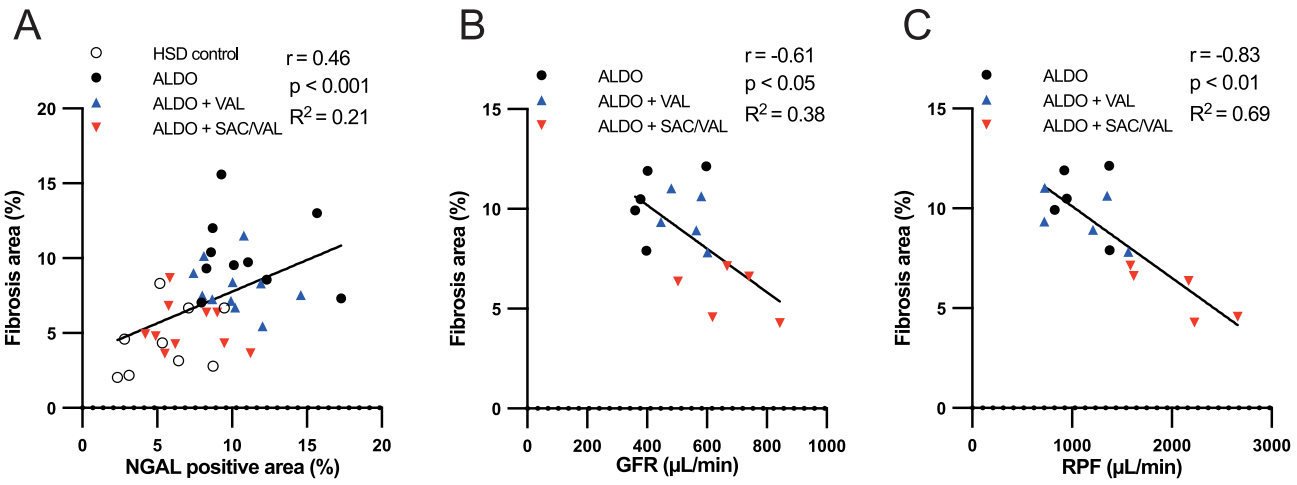


Figure 5: Correlation between histological findings and renal haemodynamics parameters. Relationship between fibrosis area and (A) NGAL-positive area ($n = 9$–11 per group), (B) GFR, and (C) RPF ($n = 5$ per group). r denotes Pearson's correlation coefficient. Each approximate curve was created based on the data of all groups (A) or aldosterone treatment groups (B–C). HSD, high-salt diet; ALDO, aldosterone; VAL, valsartan; SAC/VAL, sacubitril/valsartan.

frequency of aldosterone breakthrough phenomenon [31], and because the NPs/GC-A/cGMP signalling suppresses aldosterone secretion [32], it should be suggested that SAC/VAL has the potential to antagonize aldosterone pathway in multiple ways.

The effect of SAC/VAL on renal haemodynamics has been demonstrated in animal models of pathological conditions including cardiorenal syndrome and DKD [33–35]. Uijl *et al.* reported that SAC/VAL treatment predominantly increased RPF and GFR compared to the control by using TGR (mREN2)27 rats treated with streptozotocin (STZ) [34]. These changes in renal haemodynamic parameters were similar to those observed in our study. Novel findings in this study were that the improvement of RPF and tubulointerstitial fibrosis, and an increase in plasma ANP by SAC/VAL treatment. There were no differences in blood pressure and cardiac function in echocardiography, which were assessed simultaneously, suggesting that these effects on renal haemodynamics are mainly due to the direct effects of SAC/VAL on renal vessels. NPs have been shown to be protective against

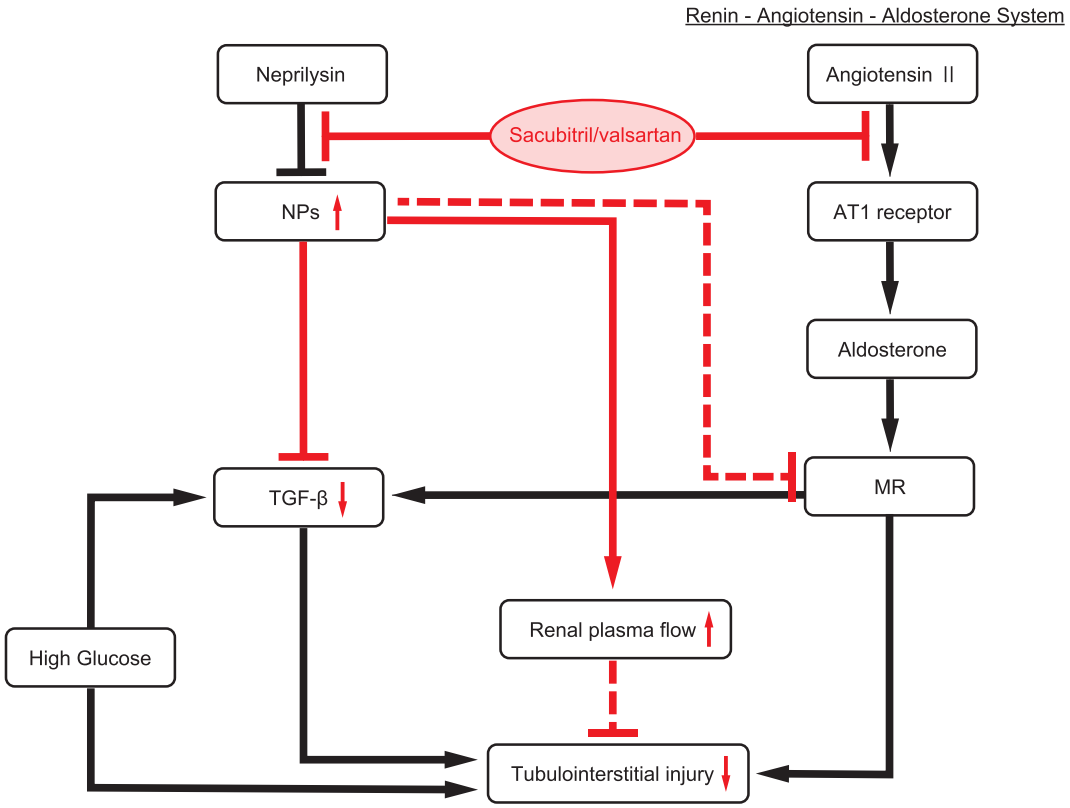


Figure 6: Effect of SAC/VAL on diabetic *db/db* mice treated with aldosterone and an HSD. Red solid lines indicate the observed effects of SAC/VAL. Red dashed lines indicate the inferred effects of SAC/VAL from previous reports. SAC/VAL increased the concentration of NPs by inhibiting neprilysin, appeared to increase RPF, suppress TGF-β, and improve tubulointerstitial injury. MR, mineralocorticoid receptor.

tubulointerstitial injury [36]. Therefore, the effect of SAC/VAL on renal haemodynamics and renal fibrosis is also likely to be mediated by NPs.

The relationship between impaired renal perfusion and disease progression in renal diseases, including DKD, is still unclear. Using arterial spin labelling (ASL) MRI, which can assess renal blood flow (RBF), Mora-Gutierrez *et al.* reported that renal perfusion is reduced in patients with early stage DKD compared to healthy subjects, despite no change in GFR [37], suggesting that this may be due to decreased NO concentration in the renal cortex and increased oxidative stress associated with chronic hyperglycaemia [38]. Other studies using ASL MRI have reported that RBF tends to decrease as DKD progresses, and assessment of renal perfusion by non-invasive methods such as ASL MRI is gaining attention as a prognostic factor for DKD [39]. In the present study, correlation analysis showed that tubulointerstitial injury and fibrosis tended to get worse as RPF decreased. Although these findings are not sufficient to explain the mechanism of tubulointerstitial injury, it suggests that decreased renal perfusion may contribute to tubular injury in DKD with aldosterone excess. Jenifer *et al.* reported that patients with dysregulation of aldosterone secretion, including aldosterone excess, tended to decrease RPF in the long term, further suggesting an increased risk of cardiovascular events in such patients [40]. Further research is required to determine the pathological relevance of reduced renal perfusion in patients with DKD and aldosterone excess.

There are some limitations in our study. First, the assessment of RPF is based on the measurement of PAH clearance, which may have variations in measurement. In the present study, PAH clearance in *db/db* mice of the baseline and vehicle groups was generally comparable to that of equine uric acid clearance reported by Gartner *et al.* [41]. However, renal injury with tubular damage may inhibit PAH uptake and secretion into the renal tubules, which may lead to underestimation when compared to true RPF evaluated by other modalities [42]. In this study, the possibility of underestimation of RPF cannot be denied in the ALDO and VAL treatment groups, in which tubular damage was observed prominently, but the GFR and FF measured at the same time were parallel to RPF, suggesting that the measured results are reasonable. Second, although we found a correlation between RPF and tubulointerstitial fibrosis, the pathogenetic mechanism by which tubulointerstitial fibrosis is exacerbated when RPF is decreased has not been elucidated. Further studies are needed to determine how and why fibrosis is exacerbated in the persistently reduced RBF in the DKD model with excess aldosterone.

In conclusion, we demonstrate that DKD with aldosterone excess induced tubulointerstitial fibrosis in association with RPF reduction. Furthermore, the treatment with SAC/VAL successfully improved renal tubulointerstitial damage probably through maintaining of RPF, suggesting that SAC/VAL could be a new treatment option for preventing the progression of DKD with RAAS activation.

SUPPLEMENTARY DATA

Supplementary data are available at *ndt* online.

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AUTHORS' CONTRIBUTIONS

H. Nishio, A. Ishii, and H. Yokoi conceived and designed the study. H. Nishio and N. Minamino conducted experiments. A. Ishii, H. Yamada, Y. K.P. Mori, K. Kato, S. Ohno, T. Handa, S. Sugioka, T. Ishimura, A. Ikushima, Y. Inoue, M. Minamino, and M. Mukoyama interpreted the results and contributed to discussion. H. Nishio, A. Ishii, and H. Yokoi wrote the manuscript. M. Yanagita supervised this study. H. Nishio, A. Ishii, and H. Yokoi are the guarantors of this work and had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All authors approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data underlying this article will be shared on reasonable request to the corresponding author.

CONFLICT OF INTEREST STATEMENT

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