**ORIGINAL ARTICLE**

# Renoprotective effect of GLP-1 receptor agonist, liraglutide, in early-phase diabetic kidney disease in spontaneously diabetic Torii fatty rats

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## Abstract

**Background** The aim of this study is to investigate the renoprotective effect of the GLP-1 receptor agonist, liraglutide, in early-phase diabetic kidney disease (DKD) using an animal model of type 2 diabetes with several metabolic disorders.

**Methods** Male 8-week-old spontaneously diabetic Torii (SDT) fatty rats ($n = 19$) were randomly assigned to three groups. The liraglutide group ($n = 6$) was injected subcutaneously with liraglutide. Another treatment group ($n = 6$) received subcutaneous insulin against hyperglycemia and hydralazine against hypertension for matching blood glucose levels and blood pressure with the liraglutide group. The control groups of SDT fatty ($n = 7$) and non-diabetic Sprague–Dawley rats ($n = 7$) were injected only with a vehicle.

**Results** The control group of SDT fatty rats exhibited hyperglycemia, obesity, hypertension, hyperlipidemia, glomerular sclerosis, and tubulointerstitial injury with high urinary albumin and L-FABP levels. Liraglutide treatment reduced body weight, food intake, blood glucose and blood pressure levels, as well as ameliorated renal pathologic findings with lower urinary albumin and L-FABP levels. Liraglutide increased expressions of phosphorylated (p)-eNOS and p-AMPK in glomeruli, downregulated renal expression of p-mTOR, and increased renal expressions of LC3B-II, suggesting activation of autophagy. However, these effects were not caused by the treatments with insulin and hydralazine, despite comparable levels of hyperglycemia and hypertension to those achieved with liraglutide treatment.

**Conclusions** Liraglutide may exert a renoprotective effect via prevention of glomerular endothelial abnormality and preservation of autophagy in early-phase DKD, independent of blood glucose, and blood pressure levels.

**Keywords** Diabetes · Diabetic kidney disease (DKD) · GLP-1 · GLP-1 receptor · Liraglutide

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## Introduction

Glucagon-like peptide-1 (GLP-1), which is an incretin hormone to be secreted mainly from enteroendocrine L cells existing in the intestine after eating, lowers blood glucose levels due to stimulation of insulin secretion via binding to the GLP-1 receptor in the pancreas. Therefore, GLP-1 receptor agonists (GLP-1RA) have been developed for enhancing actions of GLP-1 and are used as antidiabetic agents [1].

GLP-1RA reportedly possesses renoprotective effects apart from a lower glucose-dependent manner, because GLP-1 receptor is expressed in the kidney [2, 3]. Major clinical studies reported that GLP-1RA reduced albuminuria [4, 5]. Although it is unclear whether GLP-1RA prevents progression of renal dysfunction in type 2 diabetes patients with moderate-to-severe chronic kidney disease (CKD) [6, 7], administration of GLP-1RA from early-phase diabetic kidney disease (DKD) without renal dysfunction may be useful to diminish the progression to end-stage renal disease (ESRD).

A variety of mechanisms for renoprotection of GLP-1RA, which have antioxidant, anti-inflammatory, natriuretic, and renal vasodilation effects and promote autophagy, were reported in in vitro and in vivo studies [3, 8–10]. However, the role of GLP-1RA against early-phase DKD in type 2 diabetes has not been sufficiently investigated. We investigated the renoprotective effect of GLP-1RA, liraglutide, in early-phase DKD without renal dysfunction and determined the mechanisms underlying its effects in a rat model of type 2 diabetes using spontaneously diabetic Torii (SDT) fatty (SDT.Cg-Lepr$^{fa}$/JttJcl) rats, a novel model imitating the human pathophysiology of type 2 diabetes with obesity, hypertension, and hyperlipidemia [11, 12].

## Materials and methods

### Animals and drug administration

All procedures performed in studies involving animals were in accordance with the ethical standards of the St. Marianna University School of Medicine institution or practice at which the studies were conducted (Approval number:1908010). Male SDT fatty rats derived from a Sprague–Dawley (SD) colony were used as a type 2 diabetes model. The 5-week-old male SDT fatty ($n = 19$) and age-matched control SD rats ($n = 7$) were obtained from CLEA Japan (Tokyo, Japan) and allowed free access to usual laboratory chow and water. The SDT fatty rats at age

8 weeks ($n = 19$) were randomly assigned to three groups. The liraglutide group ($n = 6$) was injected subcutaneously with liraglutide. Another treatment group ($n = 6$) received subcutaneous insulin against hyperglycemia and hydralazine against hypertension for matching blood glucose levels and blood pressure with the liraglutide group. The control groups of SDT fatty ($n = 7$) and SD rats ($n = 7$) were injected only with a vehicle. Body weight, blood glucose level, and SBP in all rats and food intake in all SDT fatty rats were measured every 4 weeks from ages 8 to 16 weeks (Online Resource materials and methods 1).

### Blood pressure measurement

Systolic blood pressure (SBP) was measured using a tail-cuff apparatus (Softron BP-98A; Softron, Tokyo, Japan) every 4 weeks from ages 8 to 16 weeks. The averages of three SBP measurements per animal per time point were recorded [12].

### Serum and urinary biochemistry

The levels of serum cystatin C and insulin, urinary rat L-FABP, rat albumin and creatinine were measured using each commercially available kit. The levels of serum total cholesterol and triglyceride were measured in the SRL clinical laboratory testing services (Tokyo, Japan). Their urinary levels were reported as ratios relative to the urinary creatinine levels (Online Resource materials and methods 2).

### Renal histologic and morphometric analyses

The midsection of each excised kidney was dissected in the minor axial direction. Both pieces were fixed in 10% buffered formalin or methyl Carnoy's solution (60% methanol, 30% chloroform, and 10% glacial acetic acid) and embedded in paraffin [12]. Serial Sects. (3 μm thick) were prepared for renal histologic assessment, including periodic acid–Schiff (PAS) staining and immunohistochemistry.

Glomerulosclerosis in PAS-stained sections was evaluated in 50 glomeruli for each animal by grading the extent of sclerosis in each glomerulus [12]. Tubular injury was measured based on the histopathological appearance, that is tubular dilatation, tubular epithelial attenuation, intra-luminal cast, and loss of brush border. For quantification, the tubular injury area was measured independently and expressed as rations relative to the areas of the entire cortical and outer medullary regions by image analyzer (WinRoof version 4.3; Mitani Corporation, Tokyo Japan) (Online Resource materials and methods 3).

## Renal immunohistochemical analysis

Target antigens in the preprocessed sections were stained using the indirect immunoperoxidase method, as previously described [12]. Briefly, the formalin-fixed, paraffin-embedded tissue specimens were used to stain for myofibroblasts and tubular cells exhibiting the epithelial–mesenchymal transition using a mouse monoclonal antibody specific for α-smooth muscle actin (α-SMA). The tissue specimens fixed in methyl Carnoy's solution were assessed immunohistochemically for macrophages using a mouse monoclonal antibody (ED-1) specific for CD68, for type I and III collagens using goat polyclonal antibodies specific for each type I and III collagen. For quantification, images from ten non-overlapping fields throughout the cortical and outer medullary regions were captured at ×200 magnification (Online Resource materials and methods 4).

## Real-time quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from the kidney tissues using a RNeasy midi kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions, and a 0.5-μg aliquot was reverse transcribed. TaqMan real-time polymerase chain reaction with StepOnePlus™ real-time polymerase chain reaction system (Applied Biosystems, Waltham, MA) was used to measure the mRNA levels of monocyte chemoattractant protein (*Mcp-1*), transforming growth factor-β (*Tgf-β*), and 18S ribosomal RNA (*18s*). The expression levels of all mRNAs were normalized to the levels of *18s* in all samples.

## Extraction of glomerular and renal protein, and Western Blot Analysis

The left kidney cortex was separated from the medulla and the cortical pieces were used for isolation of glomeruli [13]. Glomeruli were isolated by the differential sieving technique using stainless steel grids (80, 150, and 200 mesh size) and glomerular protein was extracted after homogenization in lysis buffer with protease and phosphatase inhibitors [13]. Next, renal proteins were extracted from a part of frozen right kidney tissues. Those protein concentrations were measured as previously described [12].

Extracts of glomerular protein (15 μg) or renal protein (30 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In the glomerular protein, primary antibodies against endothelial nitric oxide synthase (eNOS), phospho-eNOS, 5′AMP-activated protein kinase (AMPK), and phospho-AMPK were incubated overnight at 4 °C. Regarding the renal protein, primary antibodies against mammalian target of rapamycin (mTOR), phospho-mTOR,

microtubule-associated proteins-1 light chain 3B (LC3B), and autophagy related gene 5 (ATG5) were used. A rabbit monoclonal antibody to α-tubulin (1:4000, Abcam) was also used to detect α-tubulin on the same membranes. The expression levels of all proteins were quantified using ImageJ software (the National Institutes of Health, Bethesda, MD, USA). The ratios of phosphorylated to total proteins, of each protein expression level to α-tubulin were quantitated (Online Resource materials and methods 5).

## Statistical analysis

All values were expressed as means ± standard error of mean (SEM). $P < 0.05$ was considered statistically significant. Following the Kruskal–Wallis test, differences among each group were identified using the Steel test, and the differences among the four groups were compared using the Mann–Whitney *U* test. All statistical analyses were performed using JMP software version 13.0.0 (SAS Institute, Cary, NC, USA).

# Results

## Changes in body weight, blood glucose level, SBP, and food intake

Body weight of the SDT fatty rats was significantly greater than that of the SD rats at age 8 weeks (Table 1). Body weights of the SDT-Ins-Hyd group at ages 12 and 16 weeks were significantly greater than those of other groups at the same age.

Blood glucose levels were significantly higher in the SDT fatty rats than the SD rats throughout the experimental period (Table 1). Blood glucose levels in the SDT-Ins-Hyd group were matched with those in the SDT-Lira group as there were no significant differences between the SDT-Ins-Hyd and SDT-Lira groups.

SBP levels in SDT-Cont group at ages 12 and 16 weeks were significantly increased compared to the same group at age 8 weeks and the SD group at the same age (Table 1). In contrast, such an increase in SBP levels was not shown in the SDT-Ins-Hyd and SDT-Lira groups at ages 12 and 16 weeks. The SBP levels in the SDT-Ins-Hyd group were matched with those in the SDT-Lira group as there were no significant differences between the SDT-Ins-Hyd and SDT-Lira groups.

While food intake increased significantly at age 12 weeks compared to 8 weeks in the SDT-Cont group, it decreased significantly at ages 12 and 16 weeks compared to 8 weeks in the SDT-Lira group (Table 1). In the SDT-Lira group, food intake at age 12 weeks was significantly lower than that in the SDT-Cont and SDT-Ins-Hyd groups at the same age,

**Table 1** Time-related changes in body weight, blood glucose, systolic blood pressure, and food intake per weight

| Variable | Group | 8 weeks of age | 12 weeks of age | 16 weeks of age |
|---|---|---|---|---|
| Body weight (g) | SD | 264.63 (2.92) | 492.73$^{†}$ (6.42) | 531.57$^{†}$ (10.13) |
| | SDT-Cont | 390.76$^{*}$ (5.00) | 518.77$^{†}$ (11.77) | 535.89$^{†}$ (12.05) |
| | SDT-Ins-Hyd | 395.40$^{*}$ (4.67) | 586.62$^{*,#,†}$ (21.71) | 638.50$^{*,#,†}$ (23.41) |
| | SDT-Lira | 390.73$^{*}$ (3.86) | 523.15$^{§,†}$ (15.77) | 561.95$^{§,†}$ (41.81) |
| Blood glucose (mg/dL) | SD | 88.86 (4.94) | 95.00 (3.12) | 107.71$^{†}$ (5.53) |
| | SDT-Cont | 323.14$^{*}$ (29.11) | 453.14$^{*,†}$ (30.99) | 402.86$^{*}$ (23.39) |
| | SDT-Ins-Hyd | 350.00$^{*}$ (37.94) | 354.17$^{*}$ (15.84) | 326.83$^{*,#}$ (18.87) |
| | SDT-Lira | 309.00$^{*}$ (27.62) | 315.40$^{*,#}$ (14.01) | 319.40$^{*,#}$ (9.13) |
| Systolic blood pressure (mmHg) | SD | 118.86 (3.28) | 121.29 (3.26) | 122.43 (4.06) |
| | SDT-Cont | 123.29 (2.47) | 136.14$^{*,†}$ (1.87) | 138.86$^{*,†}$ (1.35) |
| | SDT-Ins-Hyd | 123.00 (3.90) | 129.00 (2.86) | 131.17 (3.66) |
| | SDT-Lira | 120.33 (1.54) | 119.67$^{#}$ (3.57) | 127.00$^{#,†}$ (2.00) |
| Food intake per weight (g/kg) | SD | – | – | – |
| | SDT-Cont | 52.90 (2.46) | 58.73$^{†}$ (2.78) | 56.21 (1.84) |
| | SDT-Ins-Hyd | 51.43 (0.67) | 56.63 (2.55) | 55.17 (2.36) |
| | SDT-Lira | 48.55 (1.30) | 40.02$^{#,§,†}$ (2.22) | 40.45$^{#,†}$ (3.62) |

All data were expressed as the mean (standard error of the mean (SEM))

$^{*}P < 0.05$ versus the SD group at the same age. $^{#}P < 0.05$ versus the SDT-Cont group among the SDT fatty rats at the same age; $^{§}P < 0.05$ versus the SDT-Ins-Hyd group between the SDT-Ins-Hyd and SDT-Lira groups at the same age; $^{†}P < 0.05$ versus the same group at age 8 weeks

and food intake at age 16 weeks was significantly lower than that in the SDT-Cont group at the same age.

## Comparison of serum parameters at age 16 weeks

Serum cystatin C levels were similar in all groups (Table 2). Serum total cholesterol and triglyceride levels were significantly higher in all SDT fatty rats than the SD group. The serum total cholesterol levels in the SDT-Lira group were significantly lower than those in the SDT-Cont and SDT-Ins-Hyd groups (Table 2). Serum triglyceride levels in the SDT-Ins-Hyd group were significantly higher than those in the SDT-Cont and SDT-Lira groups (Table 2). Serum insulin levels in the SDT-Ins-Hyd group were significantly higher than those in other groups, and levels in the SDT-Lira group were significantly higher than those in the SDT-Cont group (Table 2).

## Changes in urinary albumin and L-FABP levels

Throughout the experimental period, urinary albumin levels (Fig. 1a) in the SD group remained unchanged, while they were significantly higher in all SDT fatty rats than in the SD rats, and they remained high in the SDT-Cont and SDT-Ins-Hyd groups. Urinary albumin levels in the SDT-Lira group significantly decreased at age 16 weeks compared to 8 weeks, and the levels in the SDT-Lira group at ages 12 and 16 weeks were significantly lower than those in the SDT-Cont and SDT-Ins-Hyd groups at the same age.

In the SD group, urinary L-FABP levels significantly decreased at age 12 weeks and significantly increased at age 16 weeks compared to 8 weeks (Fig. 1b), but the levels were comparably low. Urinary L-FABP levels in all SDT fatty rats at age 8 weeks were significantly higher than those in the SD group at the same age, and urinary

**Table 2** Serum parameters and urinary sodium excretion at 16 weeks of age

| Variable | SD | SDT-Cont | SDT-Ins-Hyd | SDT-Lira |
|---|---|---|---|---|
| Cystatin C (ng/mL) | 3.94 (0.25) | 4.14 (0.14) | 4.34 (0.24) | 4.62 (0.19) |
| Total cholesterol (mg/dL) | 48.00 (1.94) | 141.14$^{**}$ (6.20) | 137.17$^{**}$ (3.81) | 115.83$^{**,#,§}$ (5.38) |
| Triglyceride (mg/dL) | 62.14 (10.52) | 458.00$^{**}$ (43.88) | 705.00$^{**,#}$ (81.79) | 418.83$^{**,§}$ (41.18) |
| Insulin (μIU/mL) | 34.74 (17.15) | 31.23 (2.4) | 163.84$^{**,##}$ (15.97) | 62.11$^{##,§§}$ (4.43) |

All date were expressed as the mean (SEM)

$^{*}P < 0.05$ and $^{**}P < 0.01$ versus the SD group; $^{#}P < 0.05$ and $^{##}P < 0.01$ versus the SDT-Cont group among the SDT fatty rats group; $^{§}P < 0.05$ and $^{§§}P < 0.01$ versus the SDT-Ins-Hyd group between the SDT-Ins-Hyd and SDT-Lira groups
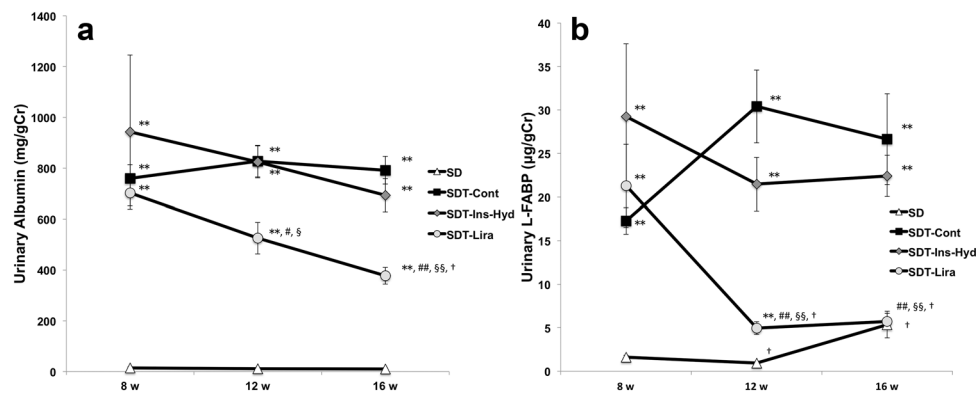
**Fig. 1** Evaluation of urinary parameters at ages 8, 12, and 16 weeks. **a** Urinary albumin levels. **b** Urinary liver-type fatty acid-binding protein (L-FABP) levels. All data were expressed as mean ± SEM. **P < 0.01 versus the SD group at the same age; #P < 0.05, ##P < 0.01

versus the SDT-Cont group among the SDT fatty rats at the same age; §P < 0.05, §§P < 0.01 versus SDT-Ins-Hyd group between the SDT-Ins-Hyd and SDT-Lira groups at the same age; †P < 0.05 versus the same group at age 8 weeks

L-FABP levels in the SDT-Cont and SDT-Ins-Hyd groups remained high throughout the experimental period. Urinary L-FABP levels in the SDT-Lira group at ages 12 and 16 weeks significantly decreased than those at age 8 weeks and were significantly lower than those in the SDT-Cont and SDT-Ins-Hyd groups at the same age.

## Evaluation of glomerular sclerosis

Focal glomerular sclerosis was observed in the PAS-stained kidney tissues of all SDT fatty rats (Fig. 2a), and significantly suppressed glomerular sclerosis was observed in the SDT-Lira group than in the SDT-Cont and SDT-Ins-Hyd groups (Fig. 2b). Type IV collagen immunolocalized to the mesangial areas in the glomeruli were significantly greater in all SDT fatty rats than those in the SD group (Fig. 2c,d).
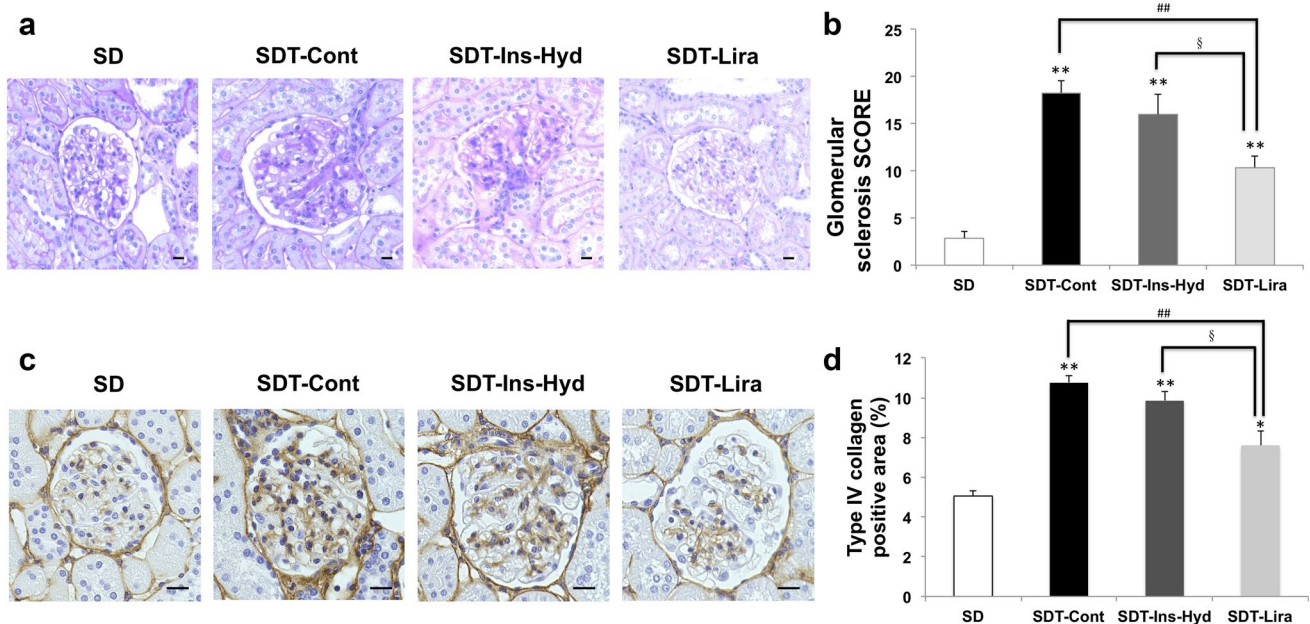


**Fig. 2** Histologic staining with PAS showing focal glomerular sclerosis (**a**) and semiquantitative assessment of focal glomerular sclerosis (**b**) (original magnification: ×100). Immunohistologic staining using an antibody against type IV collagen in the glomerulus (**c, d**) (original magnification: ×200). All data were expressed as mean ± SEM.

*P < 0.05, **P < 0.01 versus the SD group; #P < 0.05, ##P < 0.01 versus the SDT-Cont group among the SDT fatty rats; §P < 0.05 versus the SDT-Ins-Hyd group between the SDT-Ins-Hyd and SDT-Lira groups. The scale bars represent 20 μm

Significantly decreased positive areas were observed in the SDT-Lira group than in the SDT-Cont and SDT-Ins-Hyd groups.

## Evaluation of tubulointerstitial change

Tubular damage observed in the PAS-stained kidney tissues of the SDT fatty rats was significantly suppressed in the SDT-Lira group than in the SDT-Cont and SDT-Ins-Hyd groups (Fig. 3 a, b).

Infiltrated macrophages found in the interstitium was significantly greater in all SDT fatty rats than in the SD rats (Fig. 3c, d). The number of macrophages was significantly reduced in the SDT-Lira group than in the SDT-Cont and SDT-Ins-Hyd groups.

The positive areas of type I collagen observed in the interstitium were significantly greater in the SDT-Cont and SDT-Ins-Hyd groups than those in the SD group (Fig. 3e, f). Although the areas in the SDT-Lira group were not significantly different from those in the SD group, there were no significant differences between the groups of SDT fatty rats. The positive areas of type III collagen observed in the interstitium were significantly greater in all SDT fatty rats than in the SD rats (Fig. 3g, h), and there were no significant differences between the SDT fatty rats groups.

The extents of α-SMA positivity observed in the tubules and interstitium were significantly greater in the SDT-Cont group than those in the SD group (Fig. 3i, j), and the extents in the SDT-Ins-Hyd and SDT-Lira groups were not significantly different from those in the SD group. However, there were no significant differences between the SDT fatty rat groups.

## Inflammatory and profibrotic response

The renal gene expressions of MCP-1 were significantly upregulated in all the SDT fatty rats than in the SD rats ($p < 0.05$, Fig. 4a). However, the expression levels in the SDT-Lira group tended to be lower than in the SDT-Cont group ($p = 0.10$, Fig. 4a).

The gene expressions of TGF-β were significantly upregulated in the SDT-Cont and SDT-Ins-Hyd groups than in the SD group ($p < 0.05$, Fig. 4b). Although the expressions in the SDT-Lira group were not significantly upregulated compared to the SD group, there were no significant differences between the groups of SDT fatty rats.

## Evaluation of glomerular proteins

To evaluate endothelial abnormality in the glomerulus, phosphorylation levels of AMPK (Fig. 5a) and eNOS (Fig. 5b) were measured. These levels were significantly increased

in the SDT-Lira group than in the SDT-Cont and SDT-Ins-Hyd groups.

## Evaluation of renal protein related to autophagy

The phosphorylation levels of mTOR in the SDT-Cont and SDT-Ins-Hyd groups were significantly increased than those in the SD group; however, in the SDT-Lira group, such an increase was significantly suppressed (Fig. 6a). Furthermore, the expressions of LC3B-II in all SDT fatty rats were significantly lower than those in the SD group. However, the expressions of LC3B-II in the SDT-Lira group were significantly increased than those in the SDT-Cont and SDT-Ins-Hyd groups (Fig. 6b). The expressions of ATG5 were similar in all groups (Fig. 6c).

## Discussion

Our study showed that liraglutide prevented glomerular sclerosis, tubular damage, and interstitial inflammation, and an increase in urinary albumin and L-FABP levels in SDT fatty rats without renal dysfunction. In contrast, intervention of insulin and hydralazine did not reproduce protective effects comparable to liraglutide despite comparable levels of hyperglycemia and hypertension to those of liraglutide. The underlying molecular mechanism of the renoprotective effect by liraglutide was considered to be prevention of glomerular endothelial abnormality due to activation of eNOS via phosphorylation of AMPK in the glomerulus and to be preservation of autophagy due to an increase in LC3B-II via a decrease in phosphorylation of mTOR in kidneys of SDT fatty rats, subsequently leading to both suppression of glomerular type IV collagen expression and prevention of interstitial inflammation. Liraglutide may possess renoprotective action in early-phase type 2 diabetes with obesity, hypertension, and hyperlipidemia, independent of blood glucose, and blood pressure levels.

Although distribution of GLP-1R had been reported in various kidney cells [2, 10], recent in situ hybridization results revealed that the gene of GLP-1R was expressed in glomerular capillary walls and renal arteries, but not in tubules [3]. Other reports showed that its protein expression was induced in preglomerular vascular walls including afferent arterioles in hypertensive rats using a monoclonal antibody directed against the extracellular domain of GLP-1R [2], which our evaluation was supporting (Online Resource Fig. 1). Furthermore, we did not show the effect on effacement of the foot processes by liraglutide treatment (Online Resource Fig. 2). Liraglutide might prevent abnormality of glomerular endothelial cells [14] and improve glomerular hemodynamics via relaxation of the artery and afferent arterioles mediated by nitric oxide [8, 15]. Our results, which
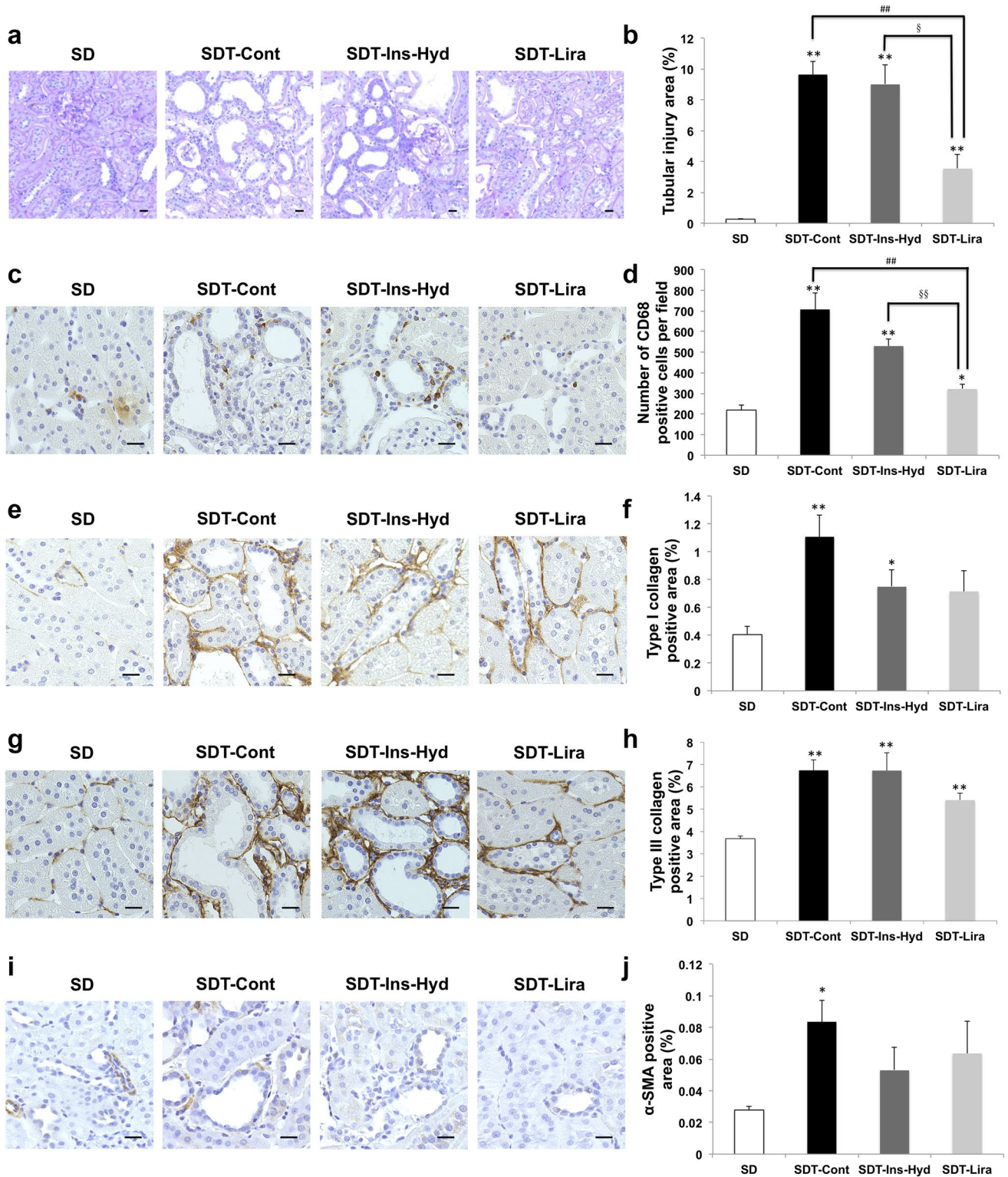
**Fig. 3** Histological staining with periodic acid–Schiff showing tubular injury (**a**) and semiquantitative assessment of tubular injury area (**b**) (original magnification: ×100). Immunohistological staining using antibodies against ED-1(**c**, **d**), type I collagen (**e**, **f**), type III collagen (**g**, **h**), α-smooth muscle actin (α-SMA) (**i**, **j**) (original magnification: ×200). All data were expressed as the mean±standard error of the mean *$p<0.05$, **$p<0.01$ versus the SD group. #$p<0.05$, ##$p<0.01$ versus the SDT-Cont group among the SDT rats; §$p<0.05$ between the SDT-Ins-Hyd group and the SDT-Lira group. The scale bars represent 20 μm

**Fig. 4** Gene expression analysis for renal inflammatory and profibrotic cytokines. Gene expression of *MCP-1* (**a**) and *TGF-β* (**b**). All data were expressed as the mean ± standard error of the mean. *$p < 0.05$, **$p < 0.01$ versus the SD group
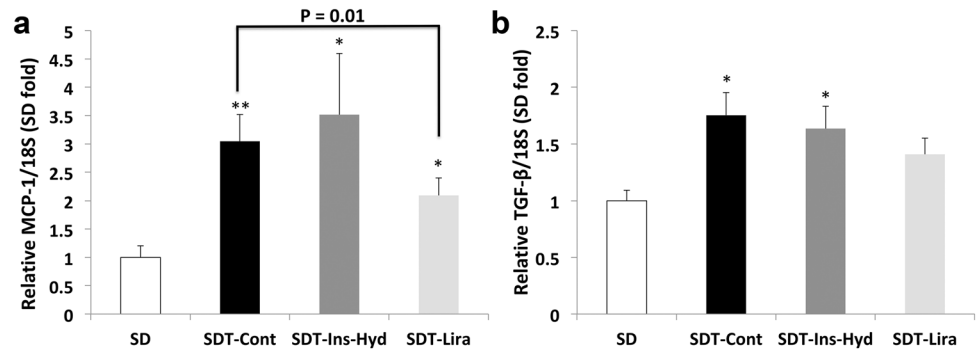


**Fig. 5** Western blot analysis of glomerular proteins related to endothelial function. Glomerular protein expressions of phosphorylated AMPK (**a**) and phosphorylated eNOS (**b**). All data were expressed as the mean ± standard error of the mean. *$p < 0.05$ versus the SD group; #$p < 0.05$, ##$p < 0.01$ versus the SDT-Cont group among the SDT rats; §$p < 0.05$ among the SDT-Ins-Hyd group and the SDT-Lira group
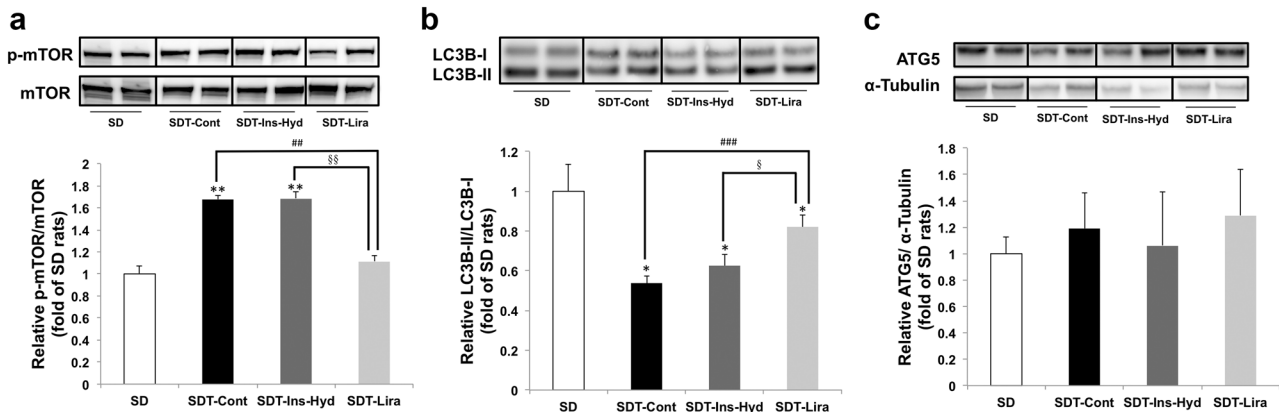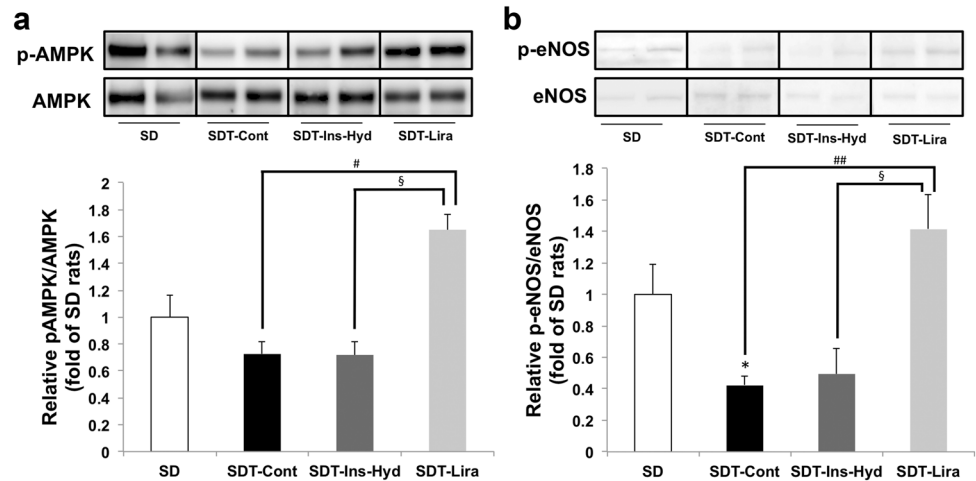




**Fig. 6** Western blot analysis of renal protein related to autophagy. Renal expressions of phosphorylated mTOR (**a**), LC3 (**b**) and ATG5 (**c**). All data were expressed as the mean ± standard error of the mean.

*$p < 0.05$, **$p < 0.01$ versus the SD group; ##$p < 0.01$, ###$p < 0.001$ versus the SDT-Cont group among the SDT rats; §$p < 0.05$, §§$p < 0.01$ among the SDT-Ins-Hyd group and the SDT-Lira group

showed that liraglutide treatment improved depressed activations of AMPK and eNOS in the glomerulus and attenuation of glomerular sclerosis in SDT fatty rats, also supported those of previous studies. Since eNOS, a major enzyme of NOS in renal vasculature, is a critical protective factor of glomerular endothelial cells against the progression of DKD [16, 17], liraglutide may possess vasoprotective and

vasodilator properties via the AMPK/eNOS axis and thereby prevented glomerular damage and decreased urinary albumin levels in early-phase DKD.

Autophagy has an important role to maintain cellular homeostasis, and the disorder of autophagy via activation of mTOR contributes to the pathogenesis of DKD [18]. Our study found that liraglutide suppressed activation of

mTOR in SDT fatty rats and increased expression of LC3B-II, which is related to the autophagosome membrane, by almost the same degrees as those in SD rats, although there were no differences in renal expression of ATG5 between the SDT fatty and SD rats. Since ATG5 is expressed to a certain degree and is predominantly diffusely distributed throughout the cytoplasm under physiologic conditions [19], upregulation of ATG5 expression might not be needed for revival of autophagy activation in early-phase DKD due to type 2 diabetes. Furthermore, promotion of autophagy by liraglutide was reported in non-diabetic CKD model [9], and liraglutide may work as a facilitator of autophagy in kidney disease.

As regards attenuation of tubulointerstitial injury and prevention of interstitial inflammation by treatment of liraglutide, decreased urinary albumin levels associated with amelioration of glomerular changes may be related to attenuation of interstitial inflammation via suppressed expression of inflammatory cytokine, MCP-1, in renal tubular cells in addition to preservation of autophagy, leading to alleviation of tubular damage because the relationship between elevated urinary albumin and progression of tubulointerstitial damage has been widely recognized [20].

While hyperglycemia and hypertension are risk factors for DKD progression, treatments with insulin, and hydralazine aggravated obesity and hypertriglyceridemia and did not demonstrate the renoprotective effects comparable to liraglutide, despite similar blood glucose levels and SBP with liraglutide treatment. This result indicated that progression of DKD in the SDT fatty rats used in our study was influenced by various factors such as DKD in patients with type 2 diabetes. Moreover, liraglutide decreased food intake, serum total cholesterol and triglyceride levels, and urinary excretion of sodium (Online Resource Table 1) in our study. Decreased food intake may be attributable to caloric, protein, lipid, and sodium restriction, which are effective for protection of kidney disease. Therefore, the renoprotection of liraglutide may be due not only to intrarenal effects of the kidney via activation of renal GLP-1R but also to extrarenal

effects of liraglutide via dietary cure or improvement of various metabolic disorders [21].

Our results may be limited by several factors. First, we did not confirm the eNOS activated by GLP-1RA in glomerular endothelial cells using immunohistochemistry although the eNOS activation in glomerular protein was shown by western blot analysis. Second, we did not observe the effect of liraglutide for prevention of progression to ESRD. In our study focused on early-phase DKD with very mild renal fibrosis, liraglutide did not prevent renal expressions of type I and III collagens, and α-SMA, and the antifibrotic effect of liraglutide was not shown. At present, a placebo-controlled trial of other GLP-1RA with primary renal end-points (FLOW) is in progress and the usefulness of GLP-1RA against ESRD will be published in 2024. Third, while liraglutide administration induced reduced food intake, loss of body weight was not observed in our study. Muscle weight levels of hind leg in the SDT fatty rats with liraglutide were similar to those in the SDT fatty rats without treatment (data not shown). There is a possibility that, as utilization of glucose in various tissues including skeletal muscle was accelerated by increase in insulin due to injection of liraglutide, subsequently leading to suppression of body weight loss in spite of decrease in food intake. Although the previous study using obese Zucker rats of a metabolic syndrome model also did not report detectable changes in body weight with liraglutide [22], there is a possibility that increase in uptake of glucose in various tissues by further analysis for measurement of body composition is required. Finally, although restoration of autophagy by liraglutide was found in the kidneys, we did not specify the renal cells with autophagy activity. Activation of AMPK is related not only to activation of eNOS, but also to activation of autophagy. However, both suppression of autophagy in glomerulus of the SDT fatty rats and activation of autophagy by liraglutide were not found in the present study (data not shown). Therefore, such changes in autophagy activation may occur in renal tubular cells of the SDT fatty rats. In addition, the
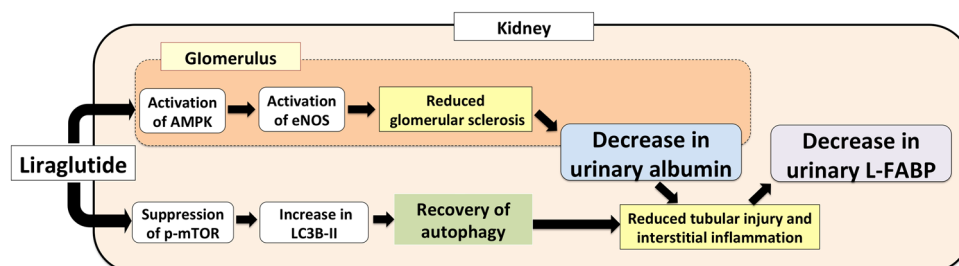


Fig. 7 Renoprotective mechanism of GLP-1 receptor agonist, liraglutide, in the early-phase DKD. Liraglutide induced the activation of AMPK and eNOS in the glomerulus, and it subsequently reduced glomerular sclerosis and decreased the urinary albumin level, leading to the prevention of tubulointerstitial damage progression and a decrease in the urinary L-FABP level. Furthermore, liraglutide contributed to autophagy recovery via the suppression of mTOR phosphorylation and increase in LC3B-II. The autophagy recovery may be related to the amelioration of renal tubular morphological changes

autophagic flux was not evaluated using a special method using chloroquine. However, as the retention of autophagic flux leads to cellular damage [23], liraglutide with renoprotective effect might promote both the autophagic flux in addition to the autophagy activation in renal tubular cells via the improvement of various metabolic disorders discussed above.

## Conclusion

To conclude, liraglutide may exert a renoprotective effect via prevention of glomerular endothelial abnormality and preservation of autophagy in early-phase DKD, independent of blood glucose, and blood pressure levels (Fig. 7).

## Compliance with ethical standards

**Conflict of interest** TS is the Director and a senior scientist at CMIC Holdings Co., Ltd., and KO is a scientist at CMIC Holdings Co., Ltd., which produced the kits for L-FABP analysis. All other authors declare no conflicts of interest.

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