〈Regular Article〉

Dipeptidyl peptidase-4 inhibitor linagliptin reduces urinary albumin excretion through the protection of glomerular endothelial function

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ABSTRACT **Background**: In most developed countries, diabetic kidney disease is the most common cause of chronic kidney disease, leading to end-stage renal disease, and it is also associated with cardiovascular diseases, including heart failure, and a higher risk of other microvascular complications. A recent clinical trial indicated that the dipeptidyl peptidase-4 inhibitor linagliptin prevents the occurrence and progression of albuminuria in patients with type 2 diabetes. Thus, this study aimed to elucidate the molecular mechanism underlying the inhibitory effect of linagliptin on albuminuria in diabetic kidney disease. **Methods**: Control C57BL/6 mice and diabetic *Ins2*$^{+/Akita}$ mice were orally administered linagliptin (5 mg/kg/day) every day for 8 weeks. **Results**: Compared to control mice, *Ins2*$^{+/Akita}$ mice had markedly elevated blood glucose and HbA1c levels, but there were no significant changes after linagliptin treatment. Furthermore, albuminuria and urinary 8-OHdG levels were significantly increased and glomerular mesangial area was significantly expanded in *Ins2*$^{+/Akita}$ mice compared to those in control mice; these changes were ameliorated by linagliptin treatment, which also improved the degradation of glomerular endothelial glycocalyx and enhancement of glomerular permeability of macromolecules. The activity of AMP-activated protein kinase and the expression of guanosine 5'-triphosphate cyclohydrolase I in human glomerular endothelial cells were significantly lower in high glucose conditions and were improved by linagliptin or GLP-1 administration. **Discussion**: These results together suggest that linagliptin reduced albuminuria in a blood glucose-independent manner via the reduction of oxidative stress and maintenance of the glycocalyx in endothelial cells. Thus, earlier treatment with linagliptin may slow the progression of diabetic kidney disease.

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Key words：Diabetic kidney disease, Glucagon-like peptide-1, Endothelial dysfunction, Oxidative stress, Glomerular permeability, Glycocalyx

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INTRODUCTION

Type 2 diabetes is a global public-health concern, and its incidence continues to increase; current treatment strategies are directed toward the control of hyperglycemia to prevent the development of microvascular and macrovascular complications. Furthermore, albuminuria is an important therapeutic target to prevent the progression of diabetic kidney disease (DKD) and subsequent complications. Recent clinical trials have shown that the progression of chronic kidney diseases, including DKD, can be suppressed by sodium glucose cotransporter 2 inhibitors and mineralocorticoid receptor blockers[1-3].

In the CARMELINA Randomized Clinical Trial, the dipeptidyl peptidase-4 (DPP-4) inhibitor, linagliptin, prevented the progression of albuminuria compared with placebo in patients with type 2 diabetes[4]. DPP-4 is a glycoprotein peptidase with multiple functions that is widely expressed in various cells; DPP-4 inhibitors comprise a new class of hypoglycemic agents for the treatment of type 2 diabetes, which have the advantage of a low risk of the consequent development of hypoglycemia. DPP-4 inhibitors prolong the half-life of incretins such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), thereby improving blood glucose levels via insulin secretion. Recent studies have suggested that DPP-4 inhibitors exert an organ-protective effect in animal models of DKD and that DPP-4 inhibitors are useful for reducing oxidative stress in vascular endothelial cells[5, 6].

Endothelial dysfunction is thought to play an important role in the development of vascular complications, which are a major cause of morbidity and mortality in patients with type 1 or type 2 diabetes[7, 8]. Nitric oxide (NO) produced by endothelial nitric oxide synthase (eNOS) in endothelial cells plays an important role in maintaining vascular homeostasis[9, 10]. We have previously reported that the imbalance between NO and reactive oxygen species (ROS) in glomeruli contributes to albuminuria in DKD, and uncoupled eNOS is one of the main sources of ROS generation in endothelial cells[11]. One of the mechanisms involved in eNOS uncoupling is the reduction in the level of tetrahydrobiopterin (BH4), an essential cofactor for eNOS[12, 13]. In diabetic patients, the levels of guanosine 5'-triphosphate cyclohydrolase I (GTPCH I), a rate-limiting enzyme for BH4 biosynthesis, are decreased because of accelerated proteasome-dependent degradation[14]. This accelerated degradation of GTPCH I reduces BH4 levels and causes eNOS uncoupling, resulting in endothelial dysfunction. Furthermore, AMP-activated protein kinase (AMPK) activation suppresses the proteasome-dependent GTPCH I degradation in vitro[15]. Additionally, we have reported that maintaining BH4 levels ameliorated albuminuria in DKD using endothelium-dominant GTPCH I transgenic mice[16].

Furthermore, the glycocalyx is a major component of the Endothelial surface layer (ESL) and covers most of the surface of endothelial cells, including glomerular endothelial cells (GECs). ESL possesses a highly negative charge, thereby regulating vascular permeability[17].

Previously, we demonstrated that the glomerular ESL is implicated in the regulation of glomerular wall permeability and that ROS-induced deterioration of the ESL exacerbates glomerular permeability in Zucker fatty rats[18].

Therefore, in this study, we hypothesized that DPP-4 inhibitors improve albuminuria in DKD by reducing oxidative stress in the GECs. To verify this hypothesis, we investigated the relationship between oxidative stress and glomerular permeability to albuminuria by using in vivo imaging and evaluated the suppressive effects of GLP-1 on ROS production in human GECs (hGECs).

MATERIAL AND METHODS

The experimental protocol (No.21-077) of this study was approved in advance by the Ethics Review Committee for Animal Experiments of the Kawasaki Medical School (Kurashiki, Japan), and this study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Kawasaki Medical School based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). C57BL/6 and *Ins2*$^{+/Akita}$ mice were purchased from Japan SLC (Shizuoka, Japan). Antibodies against AMPK (#2532) and phospho-AMPK (#2531) were purchased from Cell Signaling Technology (Danvers, MA, USA), antibodies against GTPCH I (sc-100749) and podocin (sc-21009) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and antibodies against β-actin (A1978) were purchased from Sigma-Aldrich Inc. (Saint Louis, MO).

Animal experiments

Male *C57BL/6* (control) and *Ins2*$^{+/Akita}$ mice (Akita, *C57BL/6* background) (n = 6 each) were housed in a temperature- and humidity-controlled room with a 12-hour light-dark cycle, were fed standard laboratory animal chow, and had free access to tap water. The mice were subdivided into four groups: (1) control, (2) control + linagliptin (Lina), (3) Akita, and (4) Akita + linagliptin (Akita + Lina). Linagliptin (5 mg/kg, by gavage) was administered daily for eight weeks to the corresponding groups. Mouse body weight was recorded, and blood pressure was measured using the tail-cuff method with an automatic sphygmomanometer (BP98A; Softron, Tokyo, Japan). Mice were then placed in metabolic cages for 24 h for urine collection and were then sacrificed under sevoflurane-inhalation anesthesia for the collection of kidney tissue and blood samples. Serum creatinine levels were measured using an enzyme assay (Nescoat VLII CRE Kit; Alfresa Pharma, Osaka, Japan). Urinary albumin levels were determined using a murine microalbuminuria ELISA kit (AlbuwellM; Exocell, Philadelphia, PA, USA). The amount of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in urine was measured using a competitive ELISA kit (Japan Institute for the Control of Aging) after protein exclusion with a 10 kDa molecular weight cut-off mesh to avoid interference by urinary protein. Furthermore, a two-photon laser microscopic in vivo imaging method was used to visualize glomerular filtration.

Immunohistochemical analysis

Cryostat sections (3-mm thickness) were used for immunohistochemical studies of podocin. Antibody binding was detected using the Histofine Simple Stain MAX-PO kit and diaminobenzidine (Sigma-Aldrich, Japan). A minimum of 100 glomeruli at 400× magnification were randomly selected from each mouse, and the mean score was calculated.

The glomerular ESL was also evaluated using fluorescein isothiocyanate (FITC)-conjugated tomato lectin (Sigma-Aldrich Japan G.K. Tokyo, JAPAN). For tomato lectin staining, kidney tissue was fixed in 4% paraformaldehyde and embedded in paraffin for histological analysis. The disarrangement scores for the amount of tomato lectin degradation were as follows: 0, none; 1, mild; 2, moderate; 3, severe; 4, global degradation. At least 50 glomeruli were randomly selected from each mice, and the mean score was calculated. To evaluate podocin-positive areas and disarrangement scores, the percentage was measured using a color image analyzer (WinLoof; Mitani Co., Fukui, Japan).

Histopathological examination

Kidney sections (4-μm thick) were prepared from paraffin-embedded tissues and stained with periodic acid-Schiff (PAS). Glomerular size and expansion of

the mesangial matrix were evaluated by examining 20 glomeruli from randomly selected tissue samples.

In vivo imaging of macromolecule hyperfiltration

Macromolecule hyperfiltration was imaged using a multiphoton excitation laser-scanning fluorescence microscopy confocal microscope system (TCS SP2AOBS MP; Leica Microsystems). In vivo imaging of glomerular microcirculation was performed as described previously[16]. Fluorescein-dextran (500 kDa; 2 mg/mL, anionic; excitation/emission maxima, 494/521 nm; Invitrogen, Tokyo, Japan) was infused through the jugular venous catheter to identify the glomerulus. To analyze glomerular permeability, 70 kDa fluorescein-dextran (2 mg/mL, anionic; excitation/emission maxima 494/518 nm; Invitrogen, Tokyo, Japan) was infused through the jugular venous catheter.

Cell Culture

Primary normal hGECs were purchased from Cell Systems (Kirkland, WA, USA) and cultured in endothelial cell basal medium-2 (Lonza, Walkersville, MD, USA) containing v/v 5% fetal bovine serum (FBS) under humidified conditions (95% air, 5% CO_2) at 37℃, according to the manufacturer's instructions. Confluent cells from passages 7 to 10 were used in the experiments. These cells were exposed to 5 mM D-glucose and 30 mM D-glucose in the presence or absence of 250 nM GLP-1 (Abnova Corporation, Taipei, Taiwan) and were incubated at 37℃ for 24 h.

Western blot analysis

Total cellular protein was extracted using an extraction reagent (T-PER Tissue Protein Extraction Reagent; Thermo Fisher Scientific, Rockford, IL, USA), according to the manufacturer's instructions, and SDS-PAGE was performed (30-50 μg protein/lane). Anti-β-actin, anti-GTPCH I, anti-AMPK, and anti-phospho-AMPK antibodies were used as primary antibodies. Signals were detected using an ECL system (Amersham Biosciences, Piscataway, NJ, USA). Relative optical densities of the bands were quantified using Image J software version 1.42.

Statistical analysis

Values are expressed as mean ± SEM. Statistical analyses were performed using the GraphPad Prism software (GraphPad Software). Parameters were evaluated using a two-tailed unpaired Student's *t*-test or one-way analysis of variance for comparison of multiple means. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Physiological and Biochemical Data

Physiological characteristics of the four groups of mice are summarized in Table 1. There were no significant differences in body weight, blood pressure, and serum creatinine levels among the groups. Urinary volume was significantly increased in Akita mice compared to control mice, but there was not significant change in urinary volume between Akita and Akita treated with linagliptin. In Akita mice, blood glucose and HbA1c levels were

Table 1. Physiological data and renal function

Group	Control	Lina	Akita	Akita+Lina
Body weight (g)	24.4 ± 2.8	23.4 ± 2.3	23.5 ± 1.6	21.8 ± 1.4
Urine volume (mL/day)	0.8 ± 0.4	0.9 ± 0.3	2.6 ± 1.5*	2.4 ± 0.5*
Blood pressure (mmHg)	95 ± 7.5	102 ± 8.0	107 ± 11.0	101 ± 10.0
Cre (mg/dL)	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.01

Cre, creatinine; Lina, linagliptin. Values are expressed as mean ± SEM. *$P < 0.05$ vs. control.

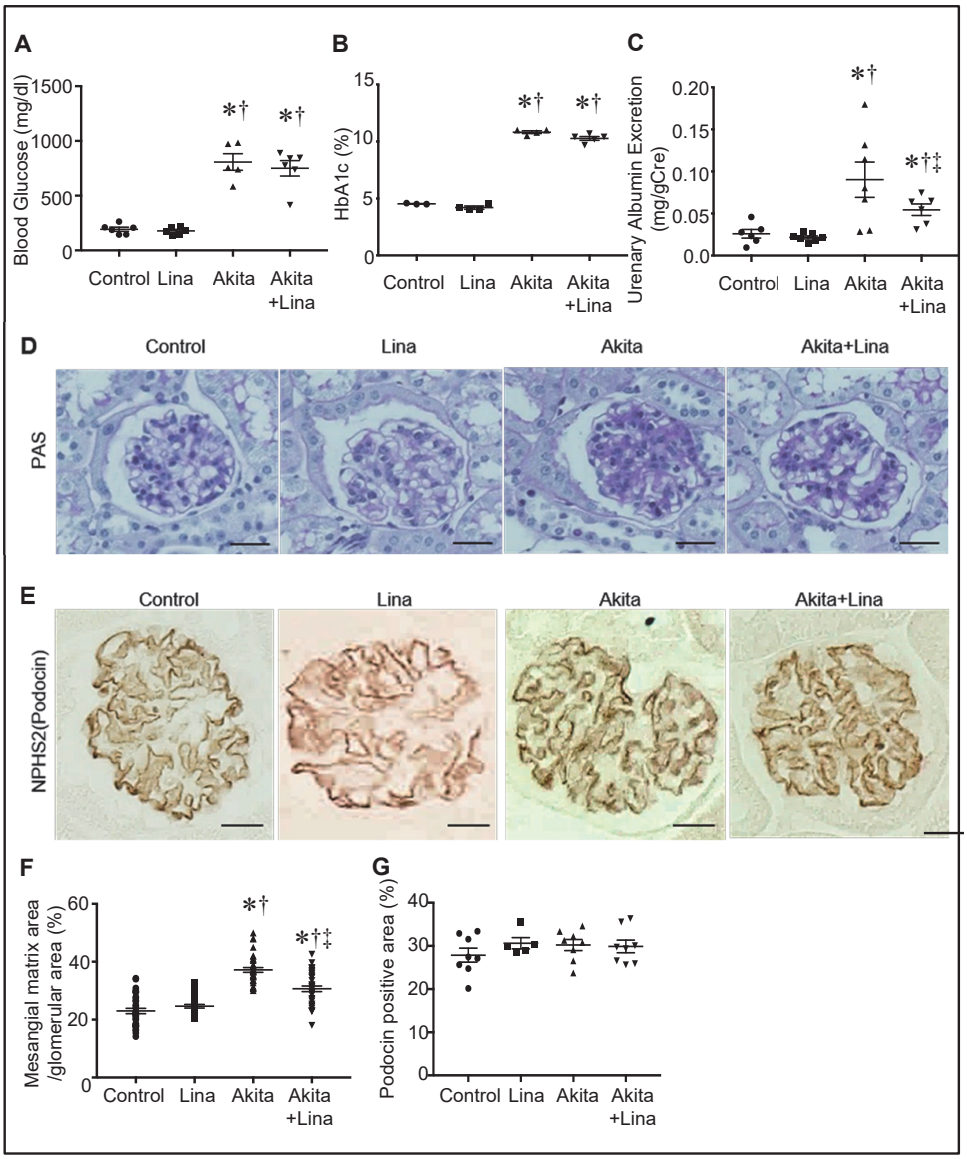

Fig. 1. Biochemical and histological findings for mice in all four groups.
(A-B) Blood glucose and HbA1c levels in the diabetic condition. (C) Urinary albumin excretion in the diabetic condition. (D) Glomerular morphological changes revealed by periodic acid-Schiff staining. (E) Immunohistochemical staining of podocin. (F) Evaluation of the mesangial matrix area. (G) Evaluation of the glomerular podocin-positive area. Data are expressed as mean ± SEM. * $P < 0.05$ versus control; † $P < 0.05$ versus Lina; ‡ $P < 0.05$ versus Akita. Bar = 40 μm. Lina, Linagliptin.

significantly higher than those in control mice, and these parameters were not reduced by linagliptin treatment (Figs. 1A and B).

Urinary Albumin Excretion and Glomerular Morphologic Changes

Urinary albumin excretion was significantly higher in Akita mice than in control mice (Fig.

1C). Representative photographs of glomeruli from each group are shown in Fig. 1D. The PAS-positive glomerular mesangial area was significantly expanded in Akita mice compared to that in control mice, and this mesangial expansion in Akita mice was prevented by linagliptin treatment (Fig. 1F). Podocin, a slit membrane protein, was detected in the podocytes of the glomerular capillaries in immunohistochemically stained sections of mice from each group (Fig. 1E). Quantitative analysis showed no significant differences in the podocin-stained area among groups (Fig. 1G).

Glomerular Capillary Permeability of Macromolecules

In control and Lina mice, 70-kDa dextran-

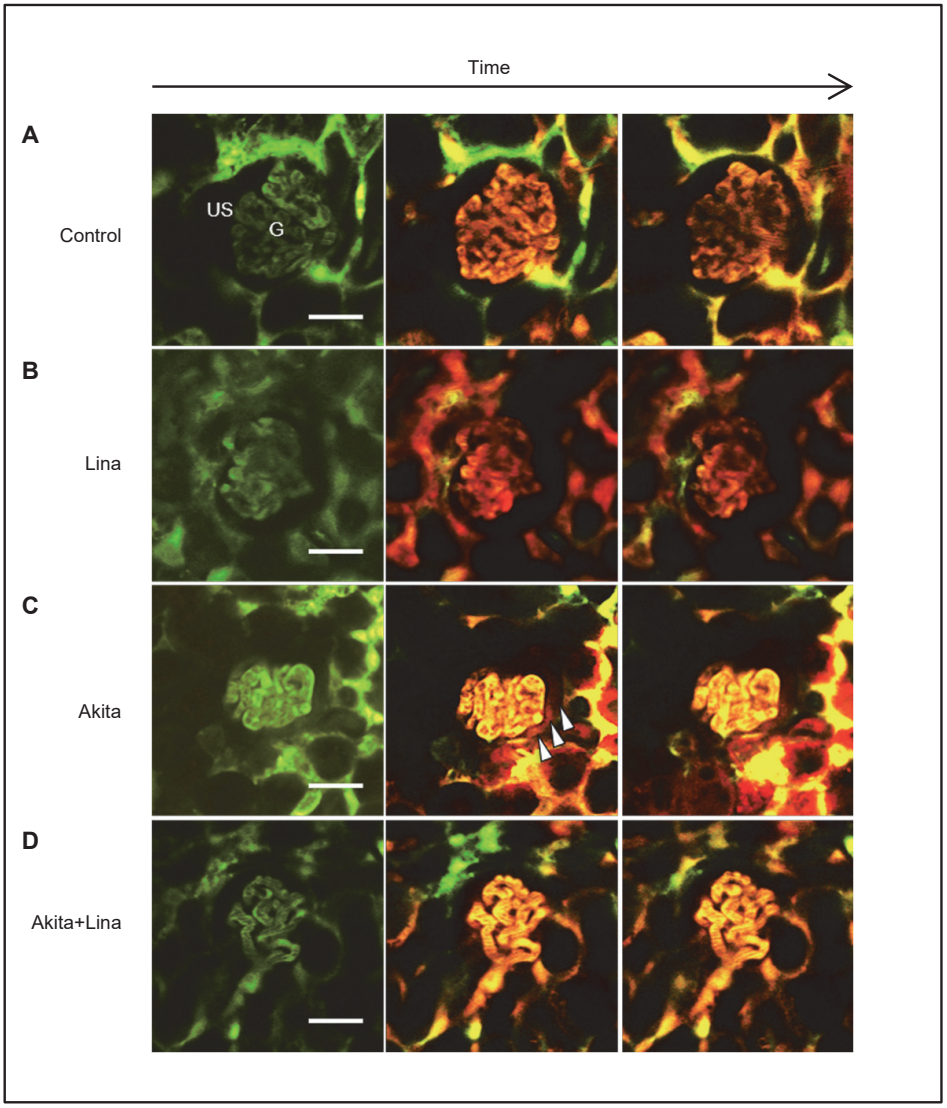

Fig. 2. Glomerular permeability assessed by in vivo imaging.
Representative series of images showing the glomerular permeability of macromolecules Green indicates 500-kDa FITC-conjugated dextran, and red indicates 70-kDa rhodamine B-labeled dextran. Time per frame = 550 ms. Bar = 40 μm. The arrowhead shows the leakage of rhodamine B-labeled dextran. Lina, Linagliptin.

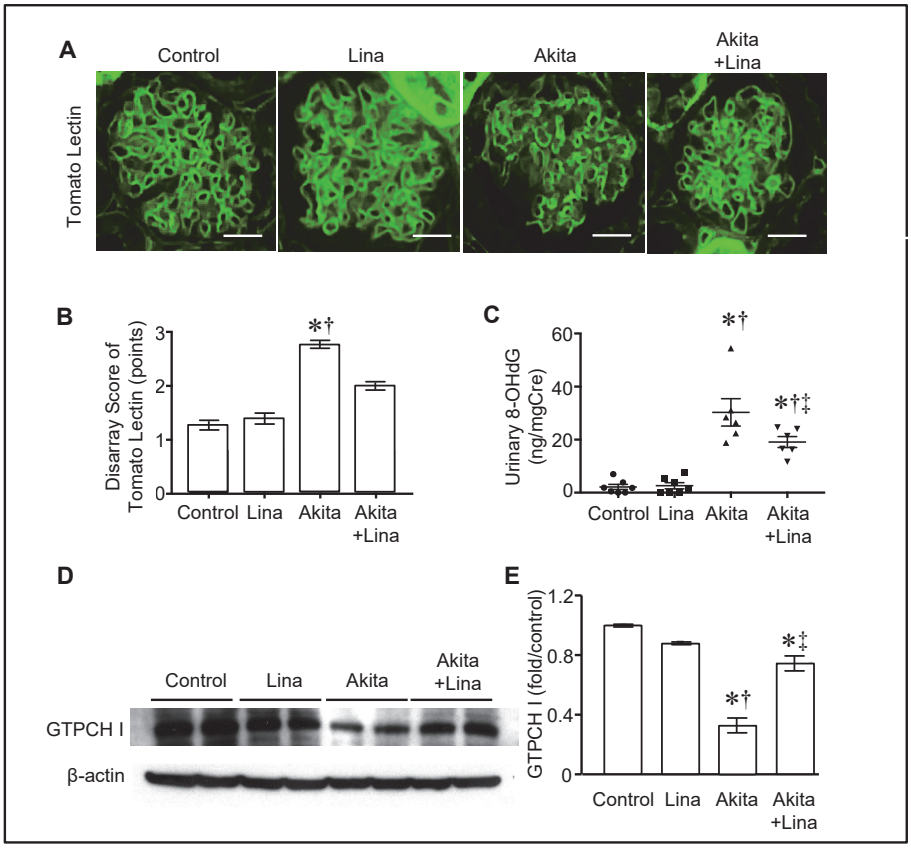

Fig. 3. Evaluation of glomerular epithelial surface layer (ESL), superoxide production, and GTPCH I expression.
(A) Glomerular ESL was detected by tomato lectin staining. (B) Disarray score of tomato lectin staining. (C) Quantification of urinary 8-OHdG excretion to evaluate glomerular oxidative stress in the diabetic condition. (D) Western blot analysis quantifying kidney expression of GTPCH I. (E) GTPCH I protein level expressed as fold change. Data are expressed as mean ± SEM. * $P < 0.05$ versus control; † $P < 0.05$ versus Lina; ‡ $P < 0.05$ versus Akita. Lina, Linagliptin.

rhodamine, which mimicked albumin, did not leak out to the Bowman's capsule from the glomerular capillaries (Figs. 2A and B). In Akita mice, dextran leaked into the Bowman's capsule and tubules (Fig. 2C), and this leakage was significantly reduced by linagliptin treatment (Fig. 2D).

Evaluation of Oxidative Stress and the ESL

In Akita mice, urinary 8-OHdG excretion was significantly higher than that in control mice, but linagliptin treatment ameliorated this increase in urinary 8-OHdG excretion in Akita mice (Fig. 3C). The disarray score of glomerular ESL evaluated by tomato lectin staining was significantly higher in Akita mice treated with linagliptin than in Akita mice (Fig. 3B). Furthermore, the expression of GTPCH I was lower in Akita mice than in the control group, and this change was significantly improved by linagliptin treatment (Figs. 3D and E).

Evaluation of AMPK Activation, GTPCH I Expression, and ROS production in hGECs

We evaluated the effects of linagliptin on AMPK activation and GTPCH I expression (Figs. 4A-C).

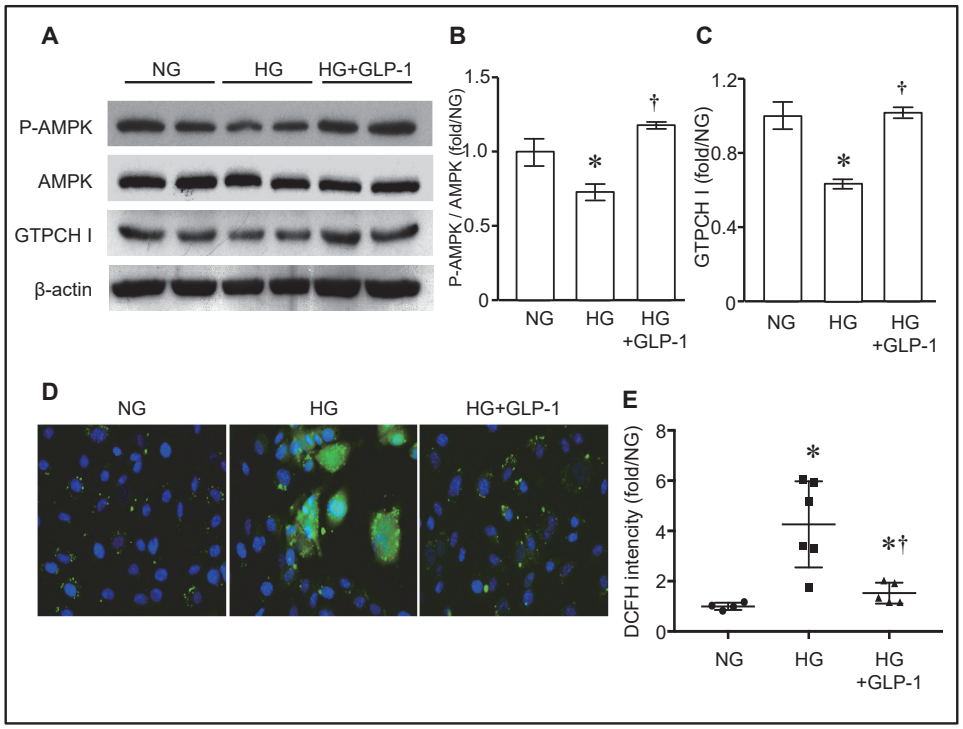

Fig. 4. Evaluation of GTPCH1 and p-AMPK/AMPK expression in hGECs.
(A) Western blot analysis for total AMPK, phospho-AMPK, and GTPCH1 in hGECs. (B) Results are presented as the phospho-AMPK/AMPK ratio, expressed as fold change. (C) Evaluation of GTPCH I expression in the high glucose condition. (D) DCFH-DA staining for detection of intracellular ROS production by fluorescence microscopy. Green indicates ROS production and blue indicates nucleus. (E) DCFH intencity expressed as fold change. Data are expressed as mean ± SEM. * $P < 0.05$ vs. NG; † $P < 0.05$ vs. HG. AMPK, AMP-activated protein kinase; DCFH-DA, dichlorofluorescein diacetate; GLP-1, Glucagon-like peptide-1; GTPCH Ⅰ, guanosine triphosphate cyclohydrolase I; hGEC, human glomerular endothelial cells; HG, high concentration of glucose; NG, normal concentration of glucose; p-AMPK, phospho-AMPK.

High glucose concentrations (HG) reduced levels of AMPK-Thr172 phosphorylation, the active form of AMPK, and GTPCH I in hGECs; however, linagliptin treatment attenuated these effects. In addition, linagliptin ameliorated HG-induced ROS production in hGECs (Figs. 4D and E).

DISCUSSION

This study elucidated the anti-albuminuric effect of linagliptin in mice with diabetes. Linagliptin treatment significantly decreased urinary albumin excretion in diabetic Akita mice. In addition, linagliptin improved glomerular tissue changes such as an enlargement of the mesangial region characteristic of DKD, and urinary oxidative stress markers. In the evaluation of glomerular permeability of maclomolecules using in vivo imaging, linagliptin reduced the glomerular permeability of maclomolecules in Akita mice. Glycocalyx, which is highly degraded under oxidative stress conditions, was maintained by linagliptin treatment. Glycocalyx is involved in the regulation of glomerular permeability of maclomolecules, and maintenance of Glycocalyx was considered to be the cause of reducing albumin leakage from glomeruli. These effects of linagliptin were independent of blood glucose levels. Furthermore, GTPCH I expression was preserved

and ROS production was reduced in hGECs treated with GLP-1. These results suggest that linagliptin prevents the progression of DKD by improving endothelial dysfunction via maintenance of plasma GLP-1 level.

The increase in urinary 8-OHDG level and decrease in renal GTPCH I expression in Akita mice were improved by linagliptin administration. The accumulation of oxidative stress in endothelial cells is implicated in the development of diabetes and diabetes-associated renal and cardiovascular disorders. In this study, high glucose concentrations induced lower AMPK activity and lower GTPCH I expression in hGECs compared to normal glucose concentrations, and these changes were ameliorated by the addition of GLP-1. Thus, linagliptin improved AMPK activity through the effect of GLP-1 and reduced oxidative stress in endothelial cells by maintaining GTPCH I expression. We have previously reported that NADPH oxidase and uncoupled NO synthase are major sources of glomerular superoxide in diabetic conditions, resulting in ROS/NO imbalance[11]. Furthermore, the degradation of GTPCH I via the ubiquitin-proteasome system due to decreased AMPK activity is a major factor in eNOS uncoupling[16]. Chao *et al.* have reported that DPP-4 inhibitors prevent high glucose-induced apoptosis via the activation of AMPK in endothelial cells[19]. In the present study, it was assumed that AMPK activation by linagliptin contributed to the maintenance of GTPCH I.

In this study, we showed that linagliptin improved glomerular vascular permeability by preserving the glycocalyx, a major component of ESL, which covers the cellular surface and is composed of large amounts of glycoproteins such as heparan sulfate proteoglycan[20]. Because of the presence of sulfated sugar chains in heparan sulfate proteoglycan, ESL possesses a highly negative charge, thereby regulating vascular permeability. We have previously demonstrated that glomerular ESL is implicated in the regulation of glomerular wall permeability and that ROS-induced deterioration of the ESL exacerbates glomerular permeability in Zucker fatty rats[18]. Thus, the alleviation of oxidative stress by linagliptin was considered to maintain the endothelial glycocalyx, leading to an improvement in glomerular permeability.

In this study, linagliptin did not decrease serum glucose level and HbA1c. Hence, linagliptin improved urinary albumin excretion and suppressed oxidative stress independent of blood glucose levels. Akita mice have a spontaneous mutation of the insulin 2 gene. Because this mutation causes misfolding of insulin in the endoplasmic reticulum leads to the development of hyperglycemia as a consequence of endoplasmic reticulum stress-induced β-cell apoptosis, Akita mice are recognized as a type 1 diabetic model mice. The effect of DPP-4 inhibitors on hyperglycemia is mediated by GLP-1 and GIP, which enhance the function of pancreatic β-cells and promote insulin secretion in a glucose-dependent manner[21]. The reason why linagliptin did not change the blood glucose level is considered to be that the decrease in β-cells is the cause of hyperglycemia in Akita mice. DPP-4 inhibitor prevent degradation not only GLP-1 but also other DPP-4 substrate and maintain plasma incretin concentrations. Many studies reported the renal protective effects of DPP-4 inhibitors and GLP-1 receptor agonists in DKD, but the nephroprotective effects of incretins excepting GLP-1 are not clear. The GLP-1 receptor is expressed in the kidneys of many species and has been confirmed to be expressed in glomerular constituent cells, vascular endothelial cells, and proximal tubules[22, 23]. Since GIP receptors have not been identified in the kidney tissue, GLP-1 receptors are currently assumed to be the main target of incretin-based therapies focused on the kidney. This is the reason we focused on the effect of GLP-1 signaling in this study. Various molecular mechanisms have

been reported for the nephroprotective effects of GLP-1, including amelioration of inflammation via reduction of intercellular adhesion molecule-1 (ICAM) expression[22], anti-fibrotic effects via suppression of transforming growth factor-β (TGF-β) signaling[24] in glomerulus. Furthermore, some studies have shown DPP-4 inhibitor improved glomerular hyperfiltration, which is supportive our experiments. Difference in organ-protective effect between the physiological increase of GLP-1 by DPP-4 inhibitors and GLP-1 analogues are still unclear, future studies are expected to clarify the mechanism.

Our study has some limitations. First, the protective effects of DPP-4 inhibitor in endothelial cells were assumed to be mediated by GLP-1. However, we did not assess the direct effects of GLP-1 on endothelial cells in vivo. Second, BH4 levels and eNOS uncoupling were not examined in vivo and in vitro study. Previously we reported that maintenance of GTPCH I preserved the BH4 levels and improved eNOS uncoupling, contribute to decrease oxidative stress in glomerular endothelial cells[16]. Therefore, it was suggested that the maintenance of GTPCH I by linagliptin treatment increased the BH4 level and improved eNOS uncoupling, leading to reduction of oxidative stress in glomeruli.

In conclusion, the DPP-4 inhibitor linagliptin reduced albuminuria by improving endothelial function. Maintenance of plasma GLP-1 level was assumed to be a cause of albuminuria improvement. These findings suggest that the earlier administration of linagliptin in patients with diabetes mellitus may inhibit the onset and progression of nephropathy.

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