博士論文

Anti-inflammatory role of DPP-4 inhibitors in a non-diabetic model of glomerular injury.

（非糖尿病性糸球体腎炎モデルにおける DPP-4 阻害薬の腎保護効果に関する検討）

東島　佳毅

Abstract

Dipeptidyl peptidase-4 (DPP-4) is an enzyme that cleaves and inactivates incretin hormones capable of stimulating insulin secretion from pancreatic β cells. DPP-4 inhibitors are now widely used for the treatment of type 2 diabetes. Experimental studies have suggested a renoprotective role of DPP-4 inhibitors in various models of diabetic kidney disease (DKD), which may be independent of lowering blood glucose levels. In the present study, we examined the effect of DPP-4 inhibitors in the rat Thy-1 glomerulonephritis model, a non-diabetic glomerular injury model. Rats were injected with OX-7 and treated with the DPP-4 inhibitor alogliptin or vehicle for 7 days, orally by gavage. Alogliptin significantly reduced the number of CD68-positive inflammatory macrophages in the kidney, which was associated with a non-significant tendency to ameliorate glomerular injury and reduce proteinuria. Another DPP-4 inhibitor, anagliptin and a glucagon-like peptide-1 receptor agonist, exendin-4 similarly reduced CD68-positive macrophage infiltration to the kidney. Furthermore, ex vivo transmigration assays using peritoneal macrophages revealed that exendin-4, but not alogliptin, dose-dependently reduced monocyte chemotactic protein-1-stimulated macrophage infiltration. These data suggest that DPP-4 inhibitors reduced macrophage infiltration directly via GLP-1-dependent signaling in the rat Thy-1 nephritis model, and they indicate that the control of inflammation by DPP-4 inhibitors are useful for the treatment of non-DKD models.

Introduction

Glucagon-like peptide-1 (GLP-1) and Glucose-dependent insulinotropic polypeptide (GIP) are incretin hormones secreted from intestinal endocrine cells. In response to food intake, GLP-1 and GIP are released respectively from intestinal L and K cells, and GLP-1 promotes insulin secretion from pancreatic β cells. GLP-1 is also related to suppressing glucagon secretion, delaying gastric motility, and exerting anorexic effects. On the other hand, inslinotropic effects of GIP are less effective in type 2 diabetes patients (34), and GIP increase glucagon secretion, facilitates fat accumulation, and promotes obesity (41). Under normal conditions, GLP-1 and GIP are rapidly degraded by the enzyme dipeptidyl peptidase-4 (DPP-4) (Figure 1).

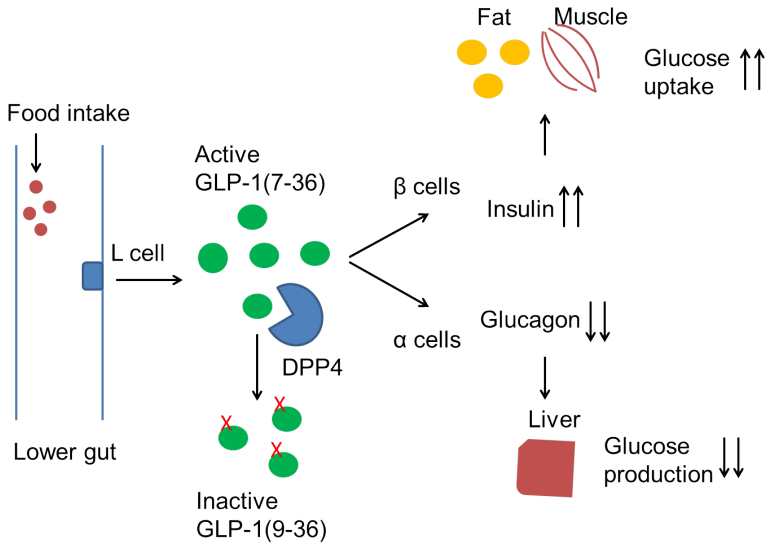


Figure 1. Glucose-lowering actions of glucagon-like peptide-1 (GLP-1)

In response to food intake, GLP-1 is secreted from intestinal L cells. GLP-1 stimulates insulin secretion and reduces glucagon secretion which leads to reduction in blood glucose levels. In normal condition, GLP-1 is rapidly degraded by the enzyme dipeptidyl peptidase-4 (DPP-4).

In pancreatic β cells, GLP-1 binds to its G protein-coupled receptor (GLP-1R) and stimulates the adenylyl cyclase pathway, resulting in protein kinase A-mediated and Epac2-mediated insulin secretions in a glucose-dependent manner (Figure 2).

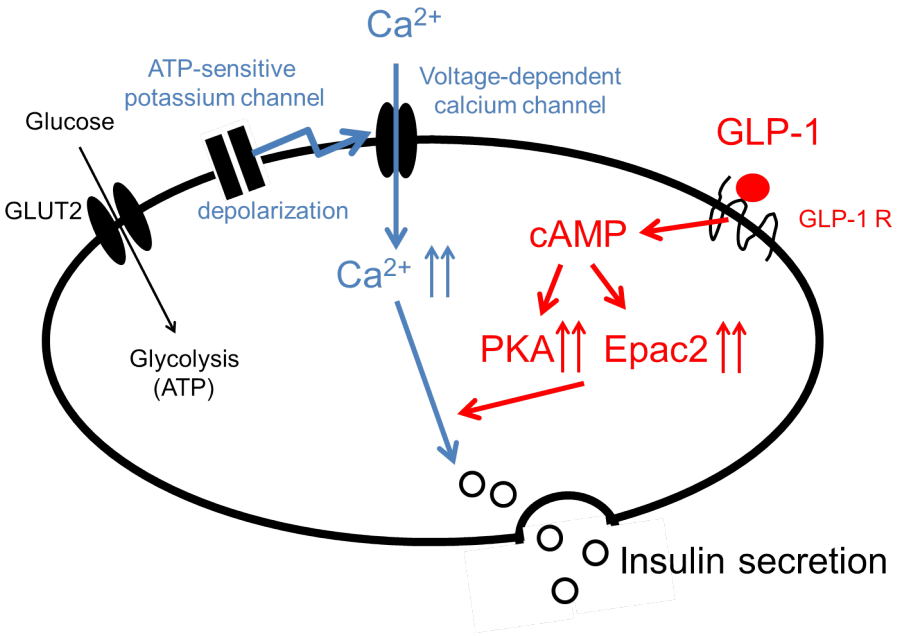


Figure 2. Molecular mechanism of GLP-1-mediated insulinotropic action in pancreatic β cells

Glucose enters pancreatic β cells through glucose transporter 2 (GLUT2). Increased ATPs via glycolysis closes ATP-sensitive potassium channels, which results in membrane depolarization, leading to opening of voltage-dependent calcium channel and allowing Ca^{2+} influx. The resultant raise in free cytosolic Ca^{2+} triggers the exocytosis of insulin. GLP-1 binds to its G protein-coupled receptor (GLP-1R) and activates the adenylyl cyclase pathway, which increases intracellular levels of cAMP. cAMP activates both protein kinase A (PKA) and Epac2 to potentiate insulin secretion.

Currently, DPP-4 resistant GLP-1R agonists and DPP-4 inhibitors are widely used as novel glucose-lowering agents for the treatment of type 2 diabetes. In experimental models of diabetes, GLP-1R stimulation not only reduced blood glucose but improved pancreatic islet

function through proliferative and anti-apoptotic effects on β cells (8, 14, 28). The potential to improve pancreatic islet function has also been demonstrated in human studies (4, 54).

Importantly, GLP-1R expression is not only limited to pancreatic β cells but is also observed in multiple organs such as the gut, lungs, heart, kidney, and central nervous system (46). This widespread distribution of GLP-1R has raised our expectations regarding its functionality, particularly its protective roles in extrapancreatic tissue, which could be mediated by GLP-1R agonists and DPP-4 inhibitors currently available. The roles of GLP-1 signaling beyond glycemic control have been extensively explored in cardiovascular systems, (46, 52). For example, GLP-1R agonist exhibited a marked reduction in infract size in heart ischemia reperfusion injury models(2), and DPP-4 deficient rats exhibited less left ventricular systolic dysfunction, ventricular fibrosis, and cardiomyocyte hypertrophy, which is associated with increased plasma GLP-1 levels(25). In addition to cardiovascular systems, GLP-1R agonist promotes central nerve cell survival and function in animal models of neuro-degeneration(3, 31), and the use of DPP-4 inhibitor is also associated with peripheral neuro-protection in the streptozotocin (STZ) induced rat diabetic model(19).

In the kidney, the protective effects of GLP-1R agonists and DPP-4 inhibitors have been well documented in diabetic kidney disease (DKD) models (33, 50). However, only a few studies have examined the effects of GLP-1R agonists and DPP-4 inhibitors in non-DKD models, such as on cisplatin-induced nephrotoxicity, ischemia-reperfusion injury (10, 22), and

the remnant kidney (20) (Table 1).

Table 1. Animal studies of GLP-1R agonists and DPP-4 inhibitors in diabetic kidney disease (DKD) and non DKD models

Category	Model	Drug	Observed effects	Refs
DKD (Type 2)	db/db mice	exendin-4	Decreases in albuminuria and glomerular injury, which was associated with reduction in oxidative stress markers	(37)
	db/db mice	linagliptin	Reduced albuminuria and glomerular injury which was related to attenuation of podocyte injury and inhibition of myofibroblast transformation	(42)
	KK/Ta-Akita mice	liraglutide	Reduced albumin excretion and mesangial expansion, which was associated with anti-oxidative effects via cAMP-PKA pathway	(9)
	Zucker diabetic fatty rats	sitagliptin	Improvements of glomerular and tubulointerstitial injury, which was related to reduced lipid peroxidation	(32)
DKD (Type 1)	STZ-induced type 1 diabetic rats	liraglutide	Reduced albumin secretion via PKA-mediated inhibition of renal NAD(P)H oxidase	(12)
	STZ-induced type 1 diabetic rats	exendin-4	Improvements of albuminuria and glomerular injury via anti-inflammatory effects through GLP-1 signaling	(23)
	STZ-induced type 1 diabetic rats	PKF275-055 (DPP-4 inhibitor)	Reduced albuminuria and glomerular injury via anti-inflammatory through GLP-1 signaling	(24)
	STZ-induced type 1 diabetic rats	vidagliptin	Improvements of albuminuria, creatinine clearance, and glomerular injury, which was associated with reduced oxidative DNA damage and apoptosis	(29)
	STZ-induced	exendin-4	Decreases in albumin secretion and	(35)

	type 1 diabetic rats		glomerular injury, which was associated with inhibition of AGE-RAGE axis	
	STZ-induced type 1 diabetic CD-1 mice	linaglitpin	Reduced renal fibrosis and plasma cystatin C levels, which was related to inhibition of endothelial to mesenchymal transition	(43)
AKI	Ischemia-reperfusion injury (rats)	exendin-4	Decreases in serum creatinine levels and tissue injury, which was associated with induction of HO-1 protein	(53)
	Ischemia-reperfusion injury (rats)	vildagliptin	Dose –dependent decrease in serum creatinine, which was related to anti-apoptotic, immunological, and anti-oxidative changes.	(10)
	Cisplatin-induced renal injury (mice)	alogliptin and exendin-4	Decreases in histological damage and serum creatinine levels, which was associated with anti-apoptotic effects	(22)
CKD	Remnant kidney (rats)	sitagliptin	Decreases in histological damage and serum creatinine levels, which was related to reduction of apoptosis and inflammation.	(20)
	Dahl salt-sensitive hypertensive rats	saxagliptin and sitagliptin	Reduced albuminuria and glomerular injury without altering blood glucose levels and systolic blood pressure	(39)

Abbreviations: AGE, advanced glycation end products; cAMP, cyclic adenosine monophosphate; DKD, diabetic kidney disease; DPP-4, dipeptidyl peptidase-4; GLP-1, glucagon-like pepteide-1; GLP-1R, G protein-coupled receptor; HO-1, heme oxygenase-1; NAD(P)H, nicotinamide adenine dinucleotide phosphate-oxidase; PKA, protein kinase A; RAGE, receptor for AGE; STZ, streptozotocin.

In this study, we investigated the potential role of DPP-4 inhibitors in the rat Thy-1 glomerulonephritis model. DPP-4 inhibitors reduced macrophage infiltration to the injured kidney, glomerular injury, and proteinuria. The number of classically activated, inflammatory, M1-like macrophages was reduced, whereas that of alternatively activated, tissue repairing,

M2-like macrophages was not affected by the treatment with DPP-4 inhibitors. These findings suggest that the protective effect of DPP-4 inhibitors was mainly mediated by its anti-inflammatory action.

Materials and Methods

Animals

All animal studies were conducted in accordance with the guidelines of the Committee on Ethical Animal Care and Use at the University of Tokyo (approval numbers P14-025 and P14-041). Male Sprague-Dawley rats aged 6 weeks and male C57BL6/J mice aged 9 weeks were purchased from CLEA Japan (Shizuoka, Japan). The animals were housed in individual cages in a temperature- and light-controlled environment and had ad libitum access to chow and water.

Drug administration

Anti-Thy-1 glomerulonephritis was induced in rats by a single intravenous injection of IgG (OX-7) mouse monoclonal anti-Thy-1.1 antibody (1.2 mg/kg). DPP-4 inhibitors, alogliptin and anagliptin, were generously provided by Takeda Pharmaceutical Co., Ltd. (Osaka, Japan) and Sanwa Kagaku Kenkyusho (Aichi, Japan), respectively. In the first experiment, rats were treated with alogliptin (10 mg/kg, twice a day) or vehicle, orally by gavage for 7 days after Thy-1 nephritis induction. Rats were divided into four groups (control: n = 9, control alogliptin: n = 9, Thy-1: n = 14, and Thy-1 alogliptin: n = 15). In the second experiment, rats were treated with anagliptin (300 mg/kg/day mixed with food) or vehicle and were divided into four groups (control: n = 3, control anagliptin: n = 3, Thy-1: n = 5, and Thy-1 anagliptin:

n = 5). In the third experiments, rats were treated with the GLP-1R agonist exendin-4 (5 μg/kg, twice a day; AstraZeneca, Osaka, Japan) via subcutaneous injection for 7 days and were divided into four groups (control: n = 4, control exendin-4: n = 4, Thy-1: n = 6, and Thy-1 exendin-4: n = 6). Rats were housed in metabolic cages for overnight collection of urine from day 6 to day 7 and then killed for tissue analysis.

Renal histologic analyses

Tissues were fixed in formalin and embedded in paraffin. Three-micrometer sections were stained with periodic acid–Schiff (PAS) reagent and counterstained with hematoxylin. Quantification of renal histology was performed as described previously (6). Briefly, 30 glomeruli per section were scored using the following system: 0 = normal appearance; 1 = mesangial expansion and/or hyper cellularity; 2 = microaneurysms, necrosis, capsular hemorrhage, or matrix or cellular crescents. Quantitative analyses were performed in a blinded manner.

Immunohistochemistry

Formalin-fixed or methyl-Carnoy-fixed and paraffin-embedded tissues were sectioned at 3 μm for immunohistochemistry. The following primary antibodies were used: polyclonal goat anti-superoxide dismutase 1 (SOD1) (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal

rabbit anti-nitrotyrosine (Sigma–Aldrich, St. Louis, MO), monoclonal mouse anti-CD68 (Merck Millipore, Billerica, MA), monoclonal mouse anti-CD163 (AbD serotec, Kidlington, UK), and monoclonal rabbit anti-CD206 (abcam, Cambridge, MA). As a secondary antibody, biotinylated horse anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA), goat anti-rabbit IgG antibody (Vector Laboratories), or biotinylated rabbit anti-goat IgG antibody (Dako Japan, Tokyo, Japan) was used as appropriate. Horseradish peroxidase-labeled avidin D (Vector Laboratories) was used as a third antibody and the reaction product was visualized by treatment with 3, 3′-diaminobenzidine tetrahydrochloride. CD68- and CD163-positive cells were counted in 5 randomly selected cortical fields at ×100 magnification.

Measurement of DPP-4 activity and its substrates

DPP-4 activity in plasma and homogenates of the renal cortex were measured using an assay kit (Enzo Life Sciences Inc., Farmingdale, NY). Renal DPP-4 activity was normalized according to the weight of the homogenized tissue sample. Active GLP-1 levels in plasma were measured using an EIA kit (Merck Millipore).

Real-Time PCR

RNA was isolated using RNAiso Plus (Takara, Shiga, Japan) and reverse-transcribed with PrimeScript RT Master Mix (Perfect Real Time; Takara). One-twentieth (v/v) of the

synthesized cDNA was used as a template for PCR quantification. PCR was performed on CFX96 (Bio-Rad, Hercules, CA) with the THUNDERBIRD^{TM} SYBR® qPCR mix (Toyobo, Osaka, Japan) or KAPA SYBR® Fast universal 2× qPCR Master Mix (Kapa Biosystems, Wilmington, MA). Relative expression levels were calculated using β-actin mRNA as reference. The primers for quantification are listed in Table 2.

Table 2. A list of primers for real-time PCR

Target gene	Species	Strand	Sequence (5′ end)
CD68	Rat	Forward	TGCTGGTACTGCTTGTAGCC
		Reverse	GTGGCAGCCTTTTTGTGAGG
CD163	Rat	Forward	CCAACGGCTTACAGTTTCCTC
		Reverse	TAGCTGGCTGTCATGTCAAGG
MCP-1	Rat	Forward	GACAGAGGCCAGCCCAGAAACC
		Reverse	CAACAGGCCCAGAAGCGTGACA
RANTES	Rat	Forward	CTGCATCCCTCACCGTCATC
		Reverse	TCCTTCGAGTGACAAAGACGA
CCR2	Rat	Forward	CTTAGACCAGGCCATGCAGGTG
		Reverse	ATGTTGAGCTCACTGGTCTGC
CCR5	Rat	Forward	AACTCTGGCTCTTGCAGGATGG
		Reverse	GAGATGGCCAGGTTGAACAGGT
IL-1β	Rat	Forward	CACCTCTCAAGCAGAGCACAG
		Reverse	GGGTTCCATGGTGAAGTCAAC
TNF-α	Rat	Forward	CCAGGAGAAAGTCAGCCTCCT
		Reverse	TCATACCAGGGCTTGAGCTCA
IL-6	Rat	Forward	CACTTCACAAGTCGGAGGCT
		Reverse	TGCCATTGCACAACTCTTTTCT
β-actin	Rat	Forward	CTTTCTACAATGAGCTGCGTG
		Reverse	TCATGAGGTAGTCTGTCAGG

Cell preparation and RT-PCR

Mouse leukaemic monocyte/macrophage cell line, RAW264 was purchased from RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Cells were grown in DMEM (Nissui Seiyaku, Tokyo, Japan) supplemented with antibiotics and 10% fetal bovine serum. Peritoneal macrophage was prepared by injecting 2.5 ml of 4% thioglycollate medium (Sigma) intraperitoneally to C57BL/6J mice. After 84 h, the peritoneal cavity was washed twice with 5 ml of phosphate-buffered saline and macrophages were collected. The macrophages were centrifuged and suspended in RPMI 1640 (Nissui Seiyaku) supplemented with antibiotics and 10% fetal bovine serum. RNA isolation and cDNA synthesis from RAW264 and peritoneal macrophage were performed as described above. GLP-1R and DPP-4 mRNA expressions were checked using the following primers: forward (5′-AAA GAT GCT GCC CTC AAG TGG ATG TAT AG-3′) and reverse (5′-CTC GTC CTC ATA GAG ATA CTT GAC AAT C-3′) for GLP-1R and forward (5′-CAG AAG AGA AGA TTC CAA ATA ATA CAC A-3′) and reverse (5′-CAT CAG AAT AGA AGG AGT ATT CAA TGA G-3′) for DPP-4. The PCR products were visualized by agarose gel electrophoresis and a UV transilluminator.

Chemotaxis migration assay

A migration assay was performed as described previously (48, 57). Briefly, peritoneal

macrophages (2×10^5 cells in 300 μl of RPMI 1640) were seeded in the 24-well upper chamber of a cell culture insert (Becton Dickinson, Franklin Lakes, NJ) having an 8-μm pore size membrane and were pre-incubated with various concentrations of exendin-4 (from 1 nM to 100 nM) or alogliptin (from 10 nM to 1000 nM) for 30 min. Then, 800 μl of RPMI containing the monocyte chemotactic protein (MCP-1) (100 ng/ml) was added to the lower chamber. Cells were allowed to migrate for 6 h and then fixed and stained. The number of migrated cells in 5 random microscopic fields per well was counted at ×200 magnification.

Statistical analysis

Data are expressed as mean ± SE, unless otherwise indicated. Statistical differences were analyzed by Tukey–Kramer test for multiple comparisons. Nonparametric data were analyzed with the Mann–Whitney test when appropriate. Differences with *P* values of <0.05 were considered statistically significant.

Results

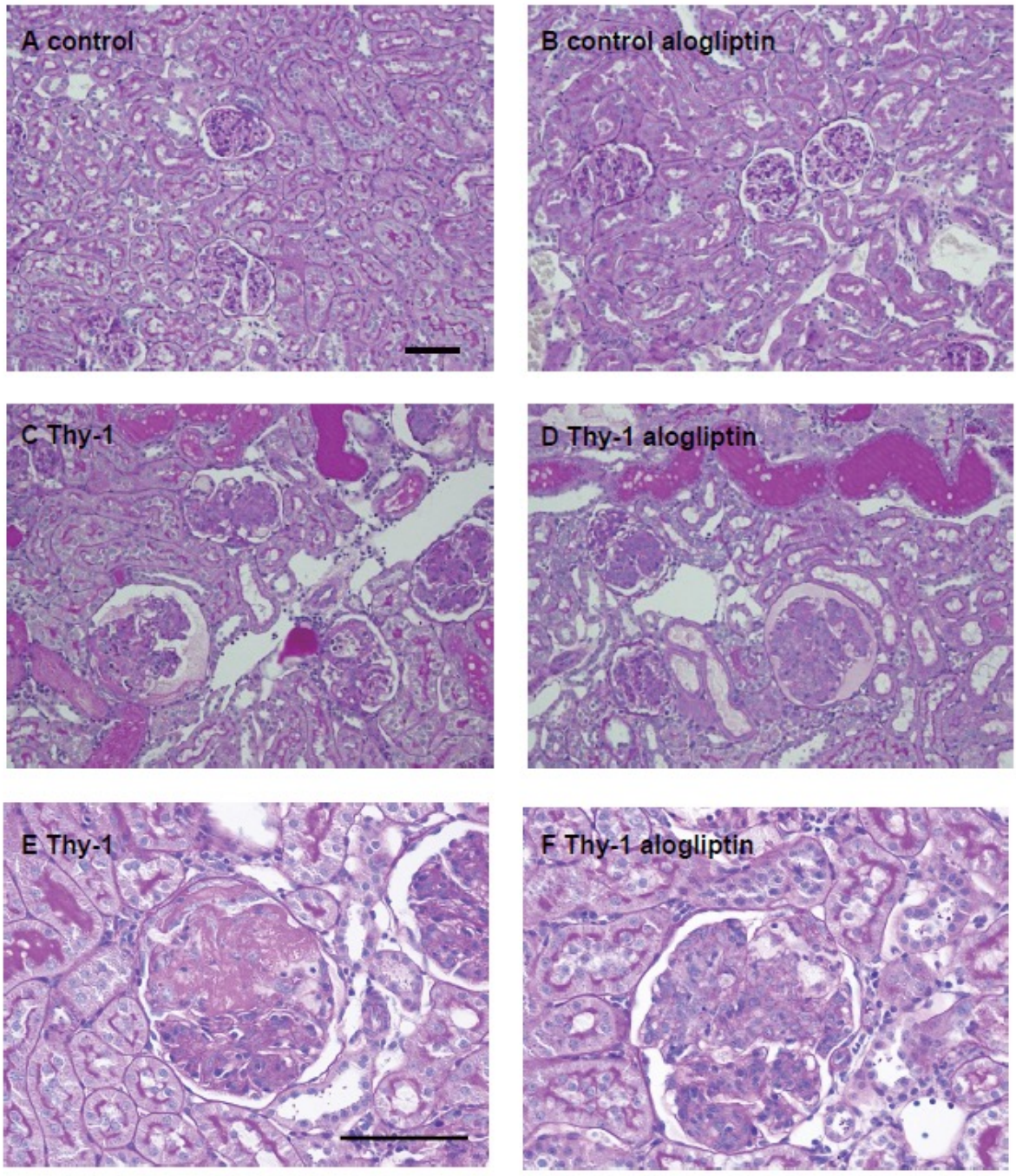
Effect of DPP-4 inhibitors on glomerular injury and proteinuria in the rat Thy-1 nephritis model

DPP-4 inhibitors are anti-diabetic drugs acting on incretins, which exert glucose-lowering effects only in hyperglycemia. Therefore, they are not likely to induce hypoglycemia in young, non-diabetic animals. In a pilot study, we confirmed that alogliptin did not affect the fasting blood glucose level (control: 79.8 ± 4.1 mg/dl, control alogliptin: 73.4 ± 3.3 mg/dl, Thy-1: 74.2 ± 2.9 mg/dl, Thy-1 alogliptin: 74.5 ± 1.9 mg/dl) or systolic blood pressure (control: 120.7 ± 6.7 mmHg, control alogliptin: 122.7 ± 10.5 mmHg, Thy-1: 116.4 ± 2.4 mmHg, Thy-1 alogliptin: 119.3 ± 1.3 mmHg).

The rat Thy-1 nephritis model is characterized by early mesangiolysis (by day 2-3), followed by subsequent mesangial cell proliferation, matrix expansion, and crescent formation that peaks on day 7. To see the potential effects of alogliptin at early phase in this model, we performed pathological analysis, but reduction of mesangiolysis was not observed by alogliptin treatment at day3 (data not shown). In addition, it was difficult to see significant difference in proteinuria because proteinuria at day 3 was even milder than that of day 7 (control: 2.29 ± 3.3 mg, control alogliptin: 2.42 ± 0.22 mg, Thy-1: 6.77 ± 1.41 mg, and Thy-1 alogliptin: 4.73 ± 0.93 mg). Thus, we next evaluated glomerular pathology on day 7.

Alogliptin treatment non-significantly reduced the total injury score of glomeruli (Thy-1: 35.1 ± 1.3, Thy-1 alogliptin: 31.4 ± 1.7; $P = 0.37$) (Figure 3A–G). The number of glomeruli categorized as score 0 (no injury) was slightly increased from 1.9 ± 1.1 to 7.6 ± 2.9 and that belonging to score 2 (severe injury) was moderately decreased from 19.1 ± 3.7 to 12.4 ± 3.5, albeit the results were not significant (Figure 3H). Consistent with pathological analyses, a non-significant trend toward a reduction in proteinuria was observed (Figure 3I). Serum creatinine levels were not altered in this model (control: 0.33 ± 0.03 mg/dl, control alogliptin: 0.30 ± 0.01 mg/dl, Thy-1: 0.36 ± 0.02 mg/dl, and Thy-1 alogliptin: 0.34 ± 0.07 mg/dl).

Figure 3


A control
B control alogliptin
C Thy-1
D Thy-1 alogliptin
E Thy-1
F Thy-1 alogliptin

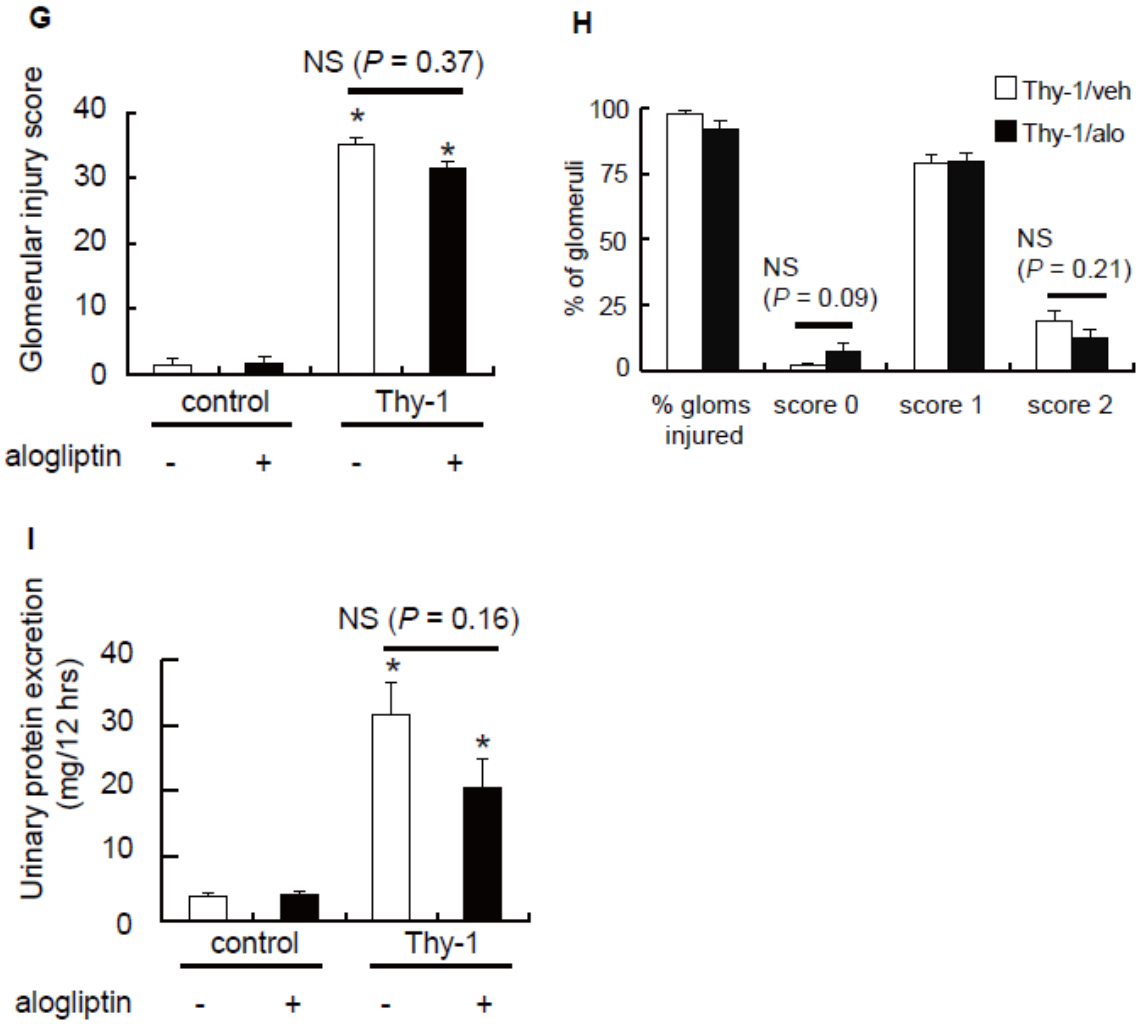


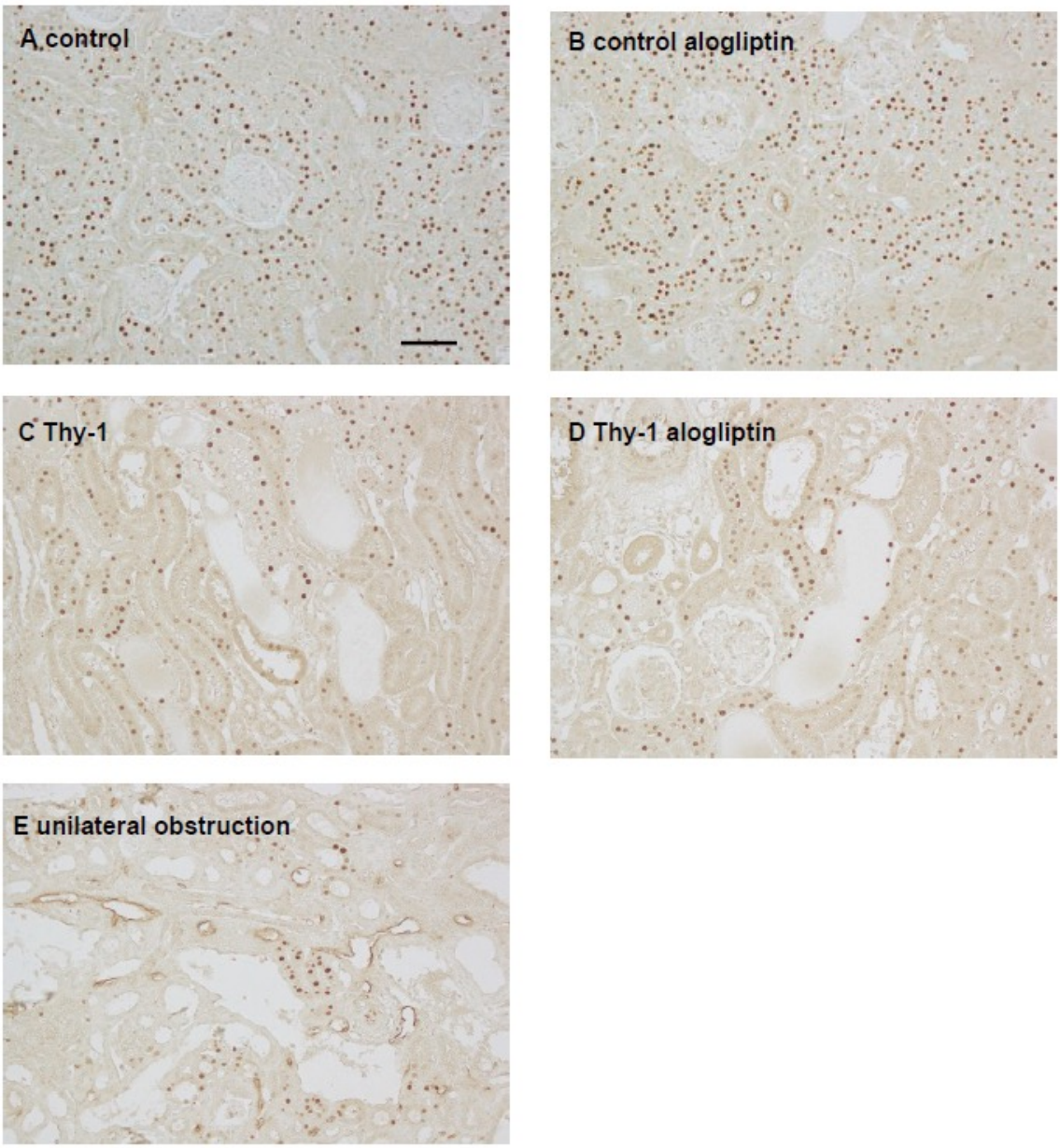
Figure 3. Effects of a dipeptidyl peptidase-4 (DPP-4) inhibitor, alogliptin, on renal histology and urinary protein excretion

Representative periodic acid–Schiff (PAS) staining at low magnification of control, control alogliptin, Thy-1, and Thy-1 alogliptin (A, B, C and D) and at high magnification of Thy-1 and Thy-1 alogliptin (E and F) are shown. Bars = 500 μm (low magnification, ×40) and 100 μm (high magnification, ×400). The right panels show the total glomerular injury scores (G) and the distribution of scores (H). The bar graph shows urinary protein excretion levels in the control and Thy-1 groups with and without alogliptin (I). Data are expressed as means ± SE. *$P < 0.05$ compared with the control group.

Equivocal impact of the DPP-4 inhibitor on oxidative stress

Previous studies reported that DPP-4 inhibitors exhibit renoprotective effects through anti-oxidative stress in experimental DKD models (12, 29, 35). Thus, we examined the effect of alogliptin on oxidative stress by immunohistological analyses. The expression levels of the anti-oxidant enzyme SOD1 in the renal cortex were decreased by disease-induction but were not affected by alogliptin (Figure 4A–E). In addition, an oxidative stress marker, nitrotyrosine was not detected in this model (Figure 4F–J), making it less likely that the anti-oxidant property was the major determinant of injury.

Figure 4


A control
B control alogliptin
C Thy-1
D Thy-1 alogliptin
E unilateral obstruction

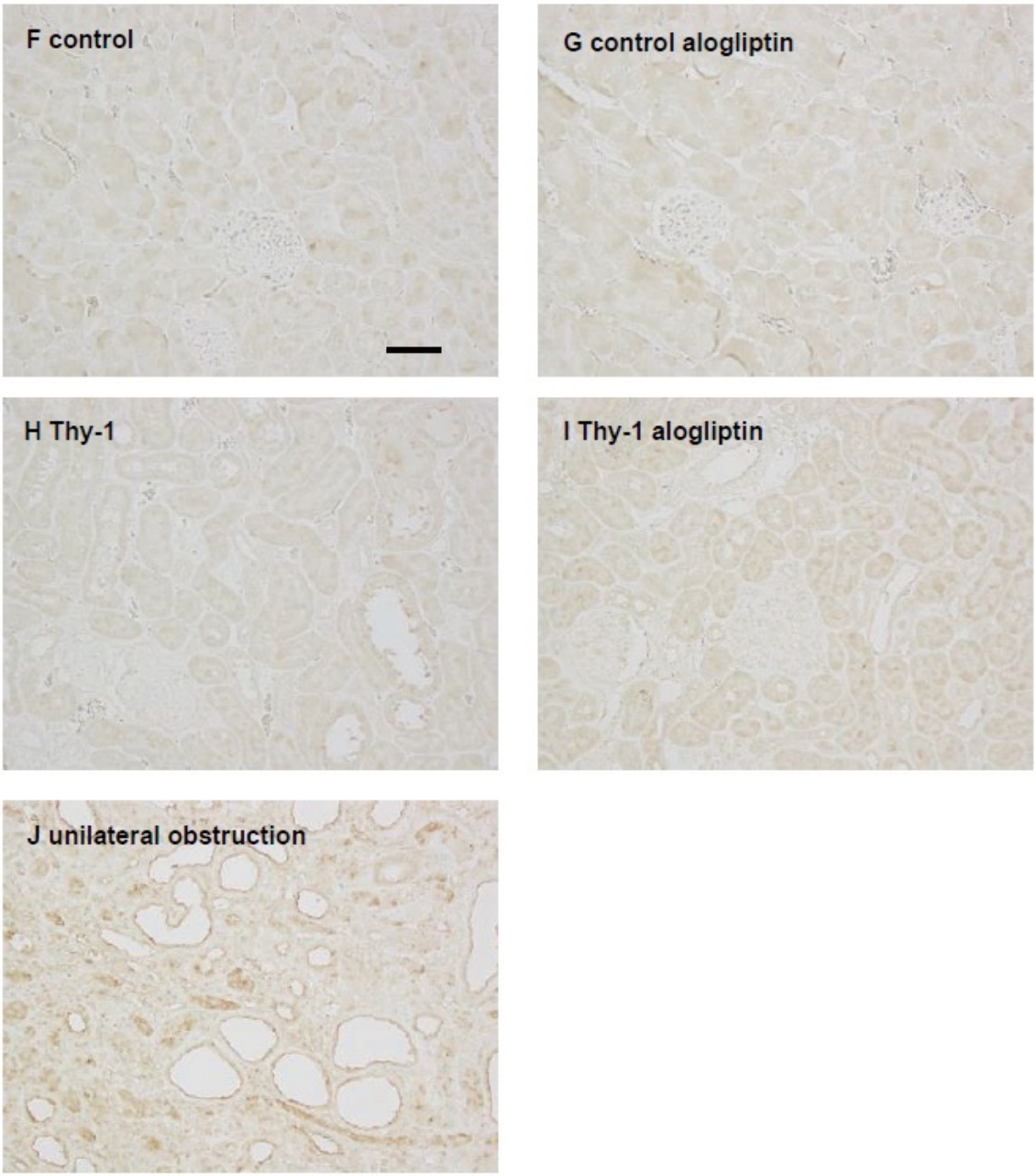


Figure 4. Effects of alogliptin on renal oxidative stress

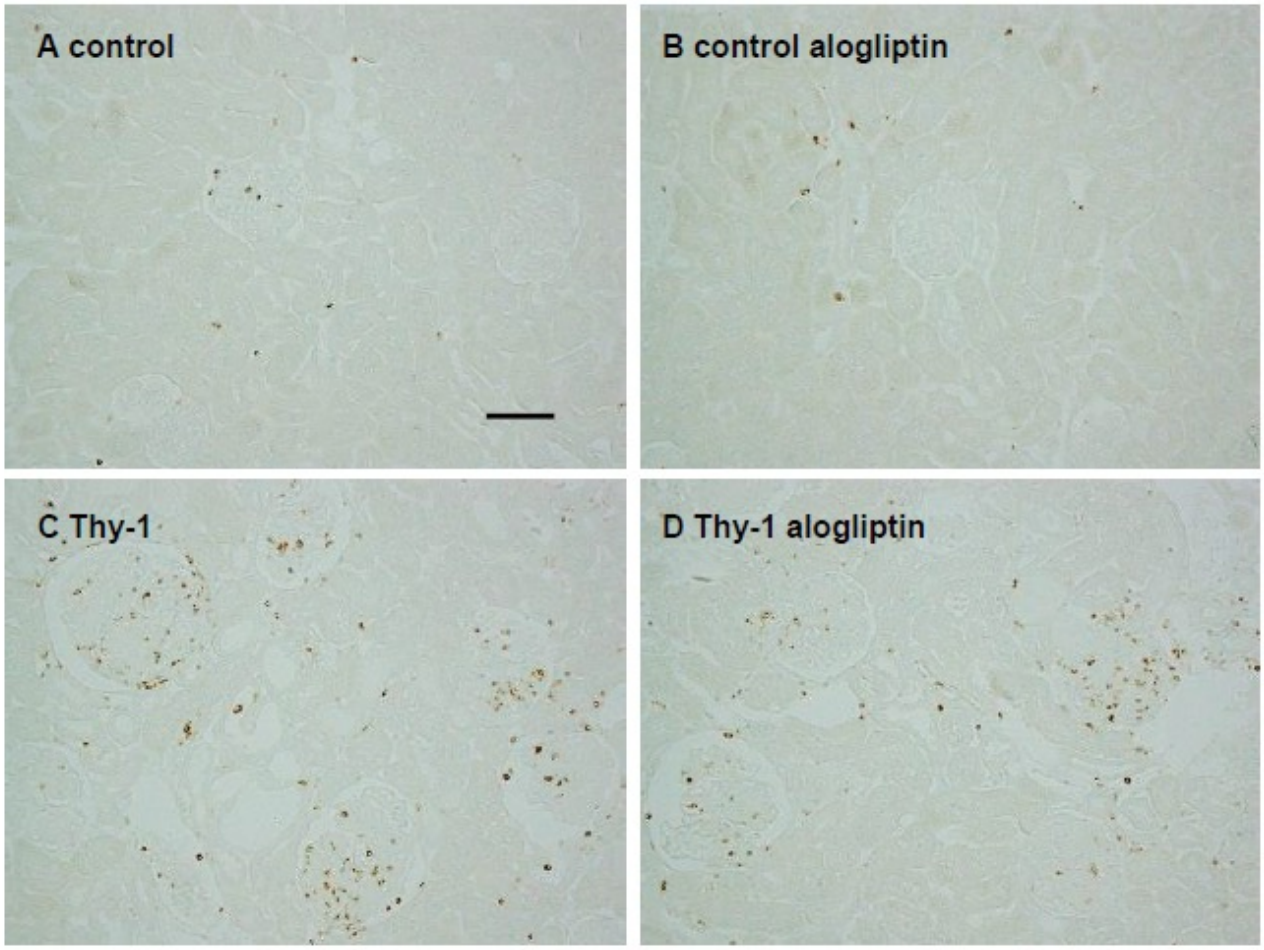
Oxidative stress was estimated by the immunohistochemistry of superoxide dismutase 1 (SOD1) (A–E) and nitrotyrosine (F–J). Representative staining of control (A and F), control alogliptin (B and G), Thy-1 (C and H), Thy-1 alogliptin (D and I), and unilateral obstruction (used as the positive control; E and J) are shown. Bars = 100 μm (×200).

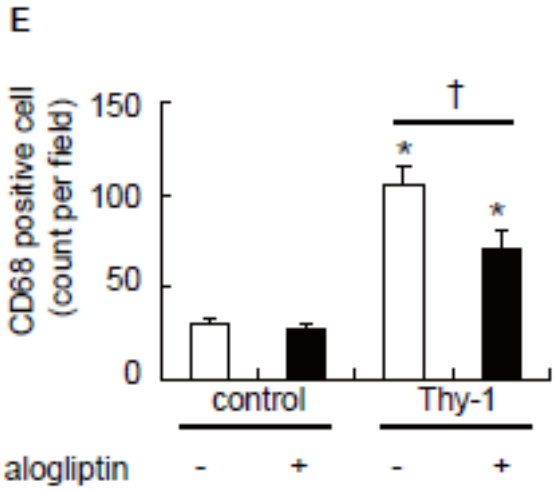
DPP-4 inhibitor reduces macrophage infiltration in the rat Thy-1 model

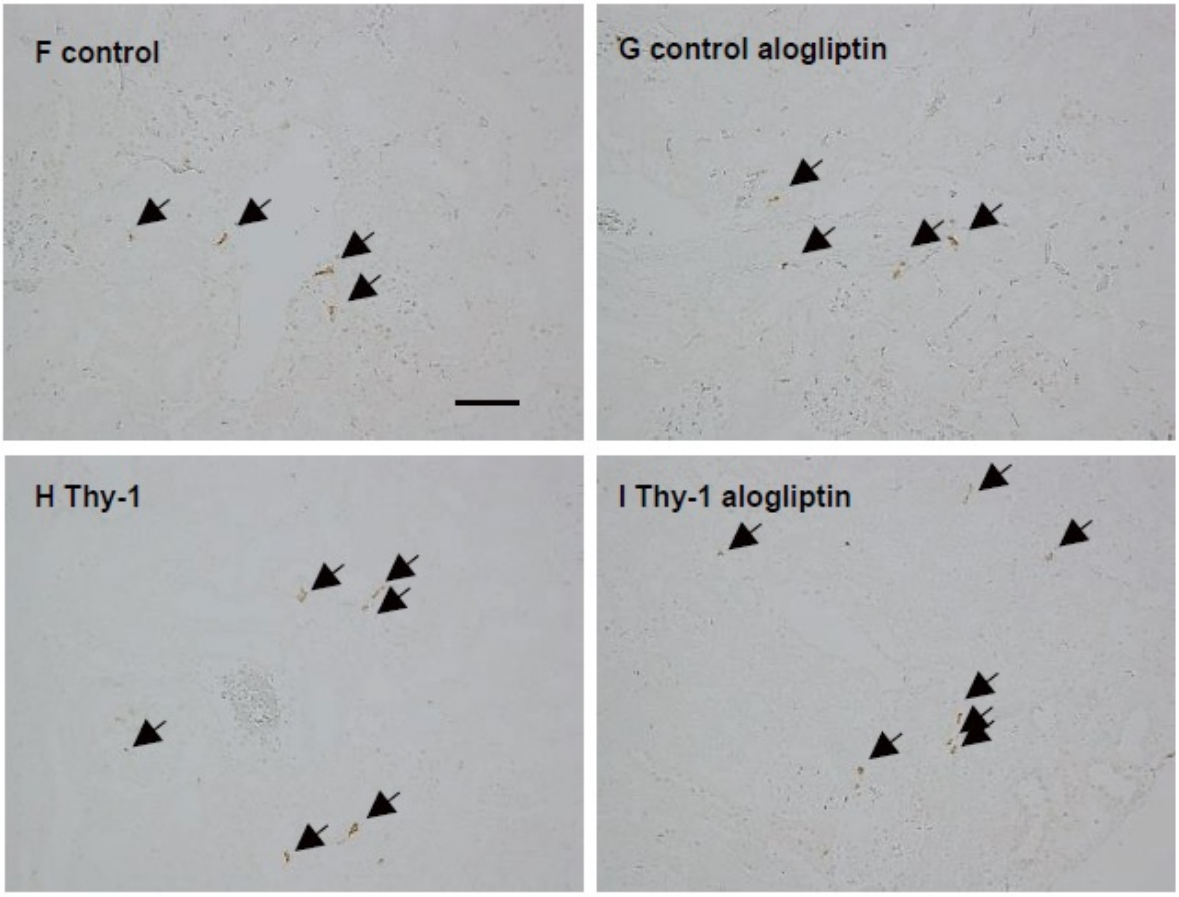
Conversely, reduction in albuminuria and glomerular mesangial matrix expansion by DPP-4 inhibition has been reported in streptozotocin-induced type 1 diabetes, which was attributed to the anti-inflammatory action via the GLP-1 signaling (24). We followed this model and then examined the possible effects of DPP-4 inhibitors on inflammation. Immunohistochemical analysis demonstrated that alogliptin significantly reduced the number of CD68-positive macrophages in the tubulointerstitial area (Figure 5A–E). Another DPP-4 inhibitor, anagliptin similarly, albeit non-significantly (P = 0.77), reduced the number of CD68-positive cells (control: 37.3 ± 4.9, control anagliptin: 31.9 ± 1.6, Thy-1: 154.4 ± 31.3, and Thy-1 anagliptin: 120.6 ± 28.8). Nevertheless, results of two independent DPP-4 inhibitors suggest that the suppression of infiltrating macrophages is a class effect. Next, we focused on subsets of macrophages generically known as M1 and M2 macrophages. Because we were unable to reproduce the stain of CD169-positive macrophages by immunohistochemistry, we evaluated the number of CD163-positive macrophages infiltrating the kidney, which did not change (Figure 5F–J). Similar results were observed in CD206-positive macrophages (data not shown). This indicated that the M2 subset was not significantly altered by alogliptin. Several inflammatory mediators such as chemokines and cytokines are known to play crucial roles in macrophage infiltration. We thus analyzed mRNA expression of representative inflammatory mediators in the renal cortex using quantitative RT-PCR. Treatment with alogliptin did not

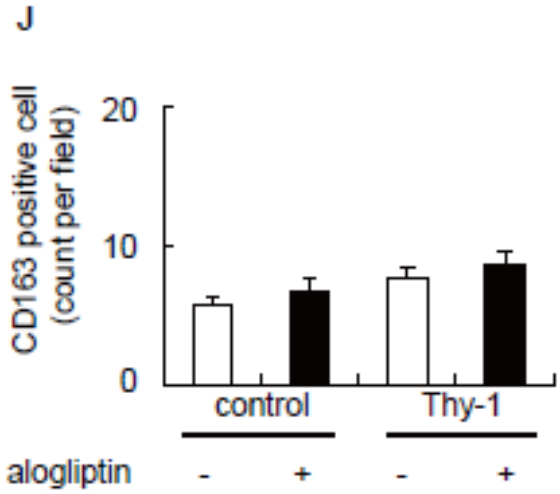
affect the mRNA levels of MCP-1 and regulated on activation, normal T cell expressed and secreted (RANTES), and tumor necrosis factor-alpha but showed a non-significant trend toward decreases in chemokine (C-C motif) receptor 2 (CCR2), chemokine (C-C motif) receptor 5 (CCR5), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) (Figure 5K–S) Complement activation and subsequent inflammation occur early in this model. However, we only analyzed kidney tissues at day7 in this gene expression study and timing of assessing might not be appropriate. Thus, when we analyzed kidney tissue at earlier point, we might be able to see positive effects of alogliptin against inflammatory chemokine and cytokine.

Figure 5


A control
B control alogliptin
C Thy-1
D Thy-1 alogliptin


E
CD68 positive cell (count per field)
150
100
50
0
†
*
*
control
Thy-1
alogliptin
-
+
-
+


F control
G control alogliptin
H Thy-1
I Thy-1 alogliptin


J
CD163 positive cell
(count per field)
20
10
0
control
Thy-1
alogliptin
-
+
-
+

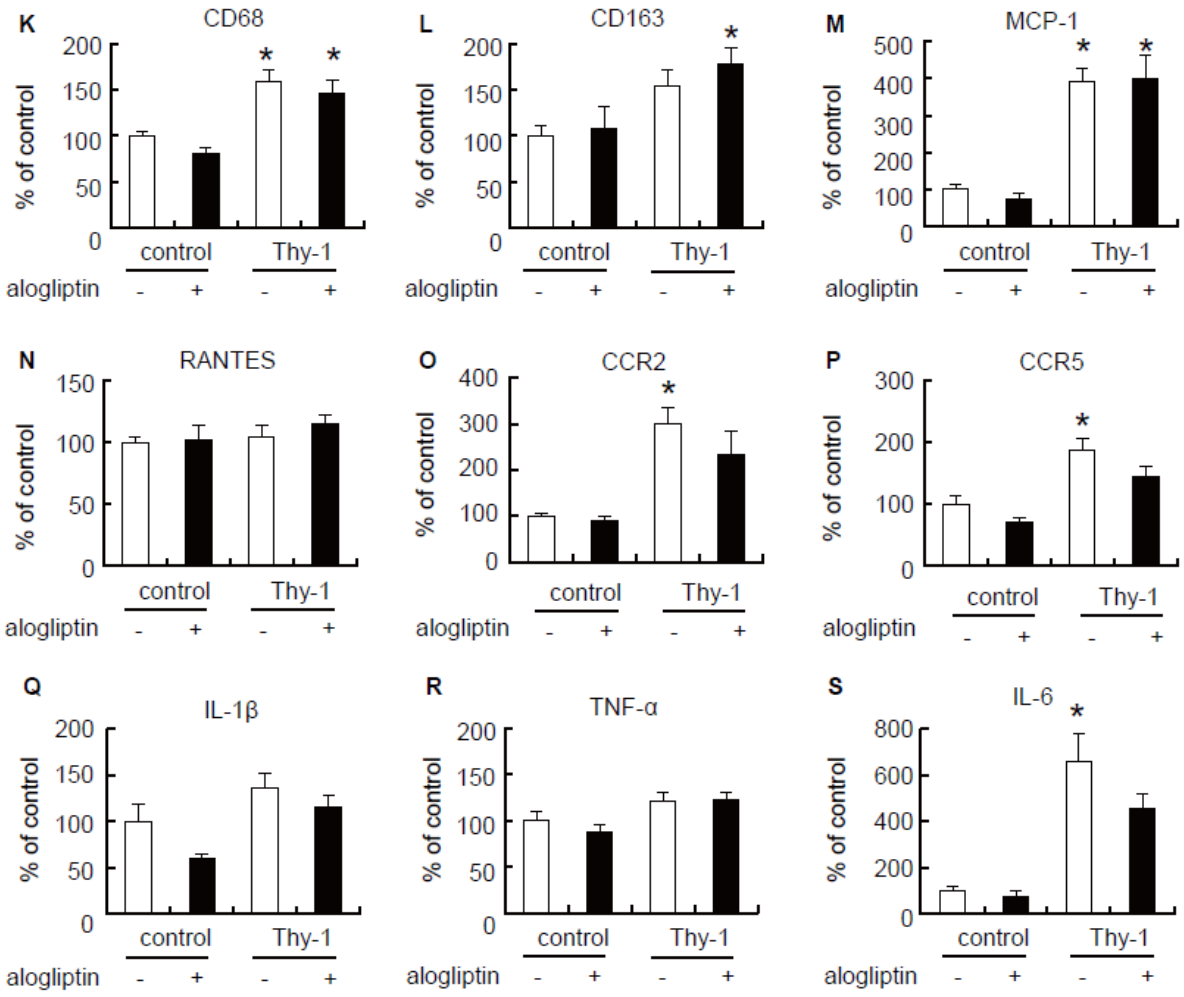


Figure 5. Effects of alogliptin on renal inflammation

Renal macrophage infiltration was assessed by the immunohistochemistry of macrophage markers, CD68 and CD163. Representative images of CD68 (A–D) and CD163 (F–I) stains are shown. Bars = 100 μm (×200). CD68- and CD163-positive cells were counted and expressed as a count per field (E and J). The mRNA expression of inflammation-related genes in the renal cortex was quantified by real-time PCR (K–S). Data are expressed as means ± SE. *$P < 0.05$ compared with the control group. † $P < 0.05$ compared with the Thy-1 group.

Plasma and renal DPP-4 activity in rat Thy-1 model

In an effort to elucidate the mechanisms underlying these observations, we examined DPP-4 activity in plasma and kidney homogenates and found that it was significantly reduced after the induction of Thy-1 nephritis. Alogliptin further significantly reduced both plasma and renal DPP-4 activity, as expected (Figure 6A and B). Consistently, plasma GLP-1 levels were significantly increased again by treatment with alogliptin (Figure 6C).

Figure 6

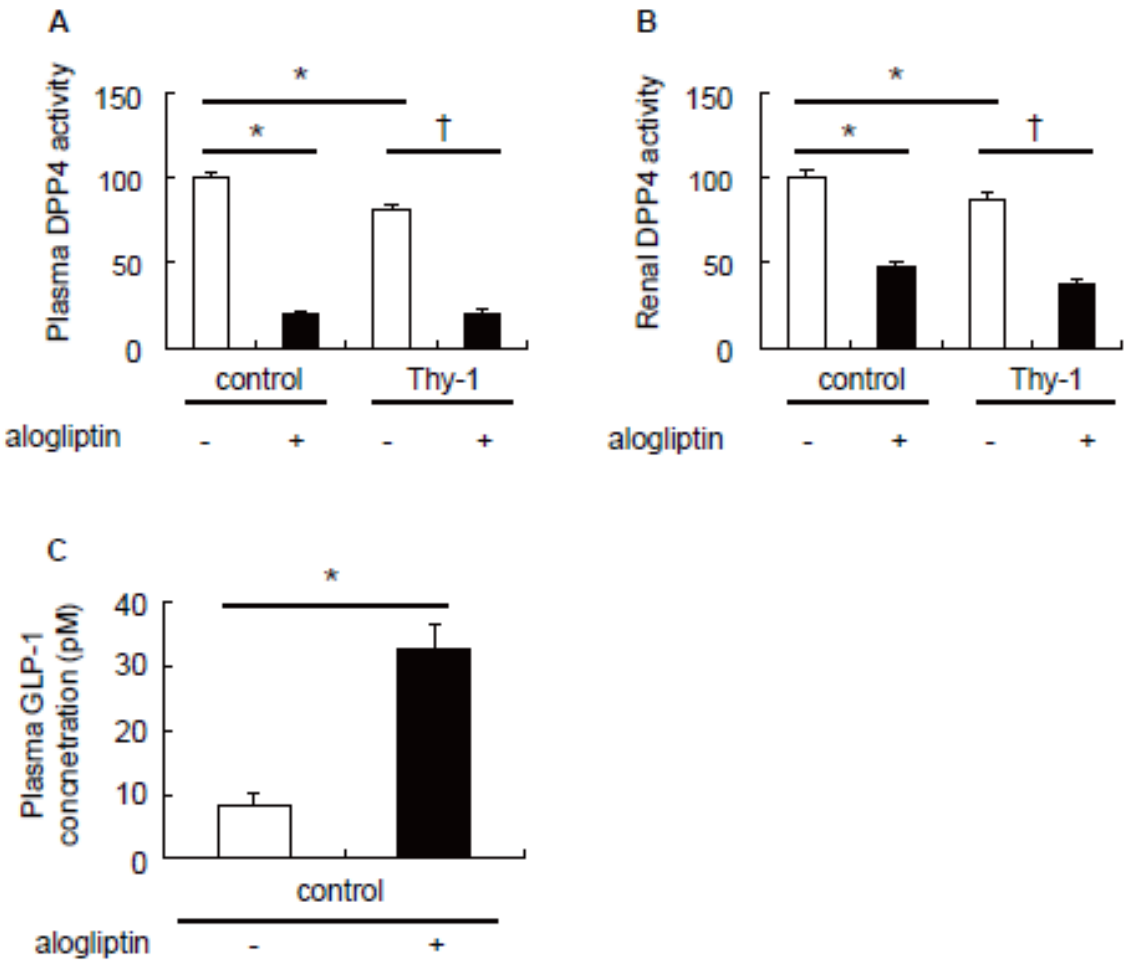


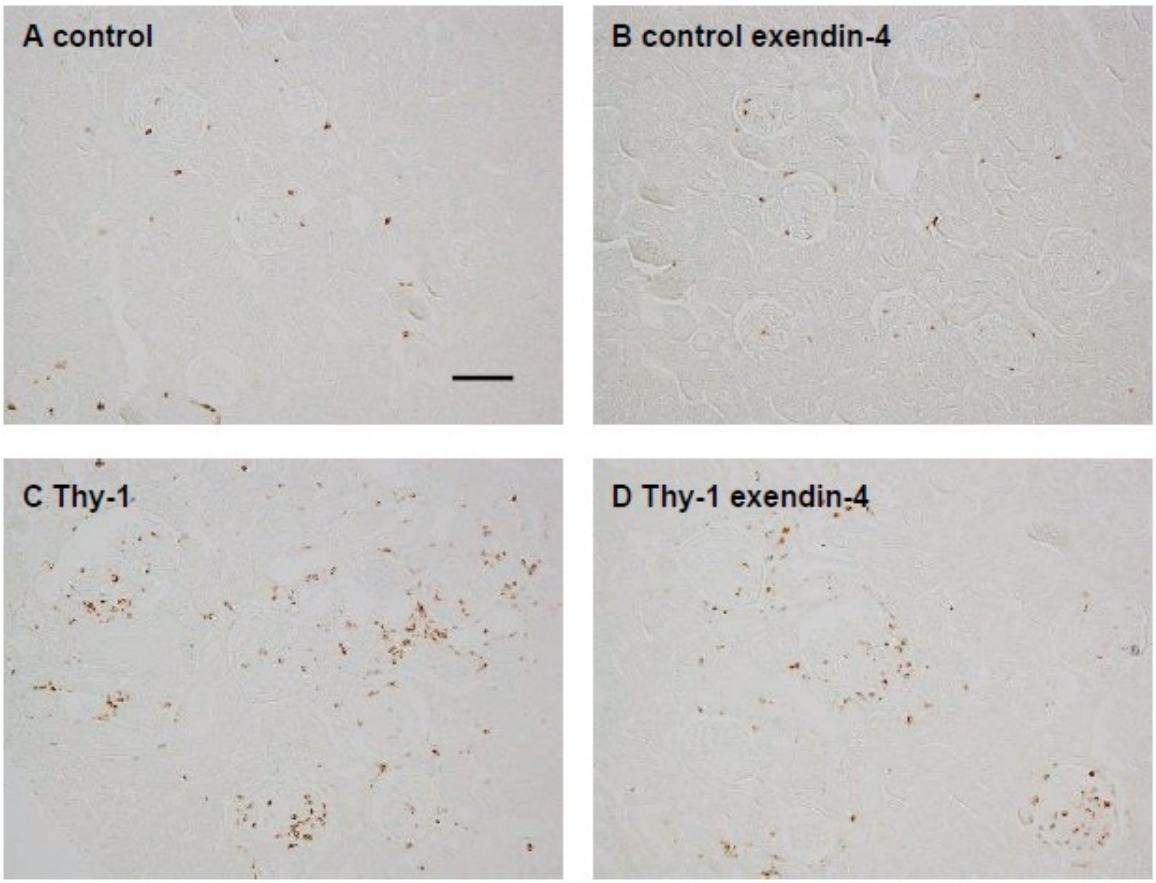
Figure 6. Plasma and renal DPP-4 activity and plasma glucagon-like peptide-1 (GLP-1) levels

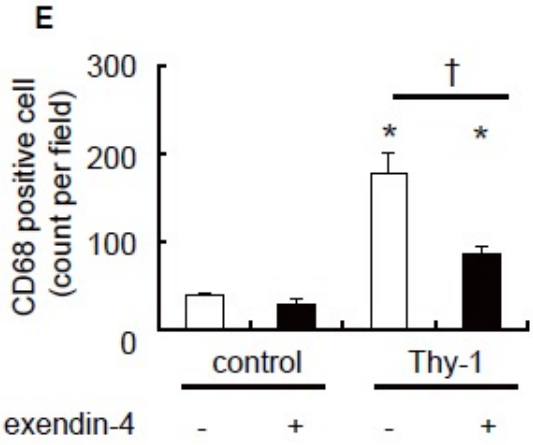
Plasma (A) and renal (B) DPP-4 activities were measured using a fluorescent assay kit. DPP-4 activity is expressed as a percentage of the mean value in the control group. Plasma GLP-1 levels were measured using an ELISA kit (control: n = 5 and control alogliptin: n = 4) (C). Data are expressed as means ± SE. **P* < 0.05 compared with the control group. † *P* < 0.05 compared with the Thy-1 group.

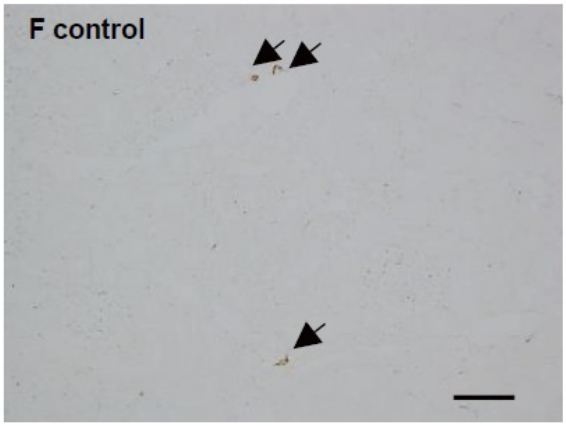
Reduction in infiltrating macrophages by the GLP-1R agonist

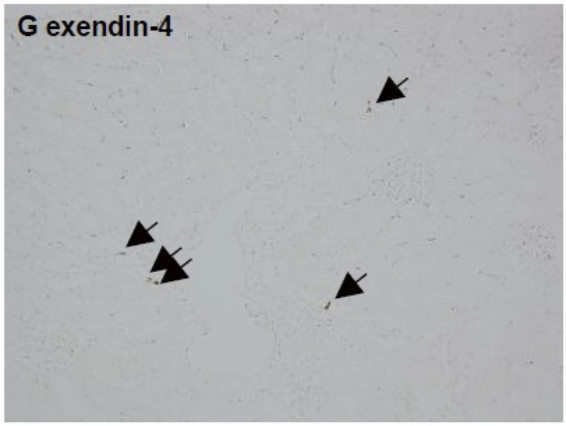
GLP-1 is the most clearly established substrate of DPP-4 in vivo, and most of the protective effects of DPP-4 inhibitors are phenocopied by GLP-1R agonists in DKD models. Thus, we reasoned that GLP-1R signaling might be responsible for the suppression of macrophage infiltration and used exendin-4 to examine the effects of GLP-1R agonist in the rat Thy-1 model. Although exendin-4 did not reduce glomerular injury (Thy-1: 35.4 ± 0.7, Thy-1 exendin-4: 37.2 ± 1.5) and proteinuria (Thy-1: 29.0 ± 3.4 mg/12 h and Thy-1 exendin-4: 38.4 ± 6.4 mg/12 h), the number of CD68-positive macrophages infiltrating the kidney was significantly reduced by exendin-4 treatment (Figure 7A–E), with a parallel increase in the CD163-positive subpopulation (Figure 7F–J). Treatment with exendin-4 reduced mRNA expression of tumor necrosis factor-alpha in the kidney, and this decrease was associated with non-significant decreases in RANTES, CCR2, CCR5, IL-1β, and IL-6 mRNA expressions (Figure 7K–S).

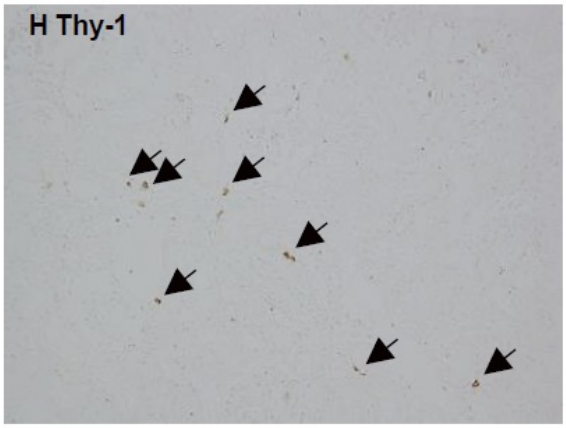
Figure 7

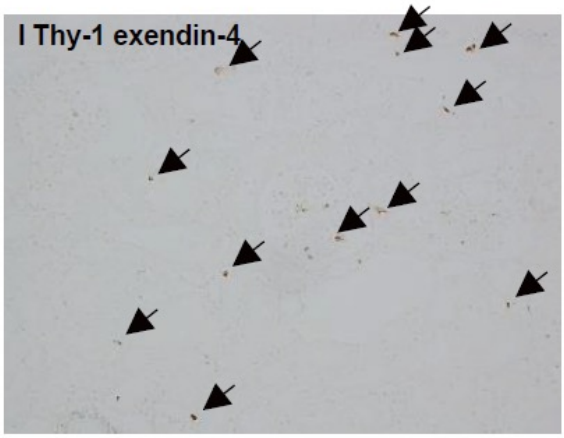

A control
B control exendin-4
C Thy-1
D Thy-1 exendin-4

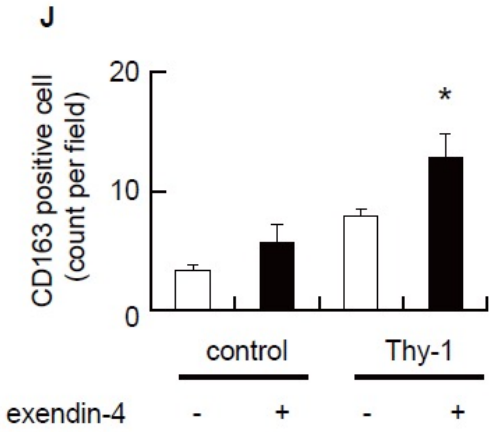

E
CD68 positive cell (count per field)
300
200
100
0
†
*
*
control
Thy-1
exendin-4
-
+
-
+


F control


G exendin-4


H Thy-1


I Thy-1 exendin-4


J
CD163 positive cell (count per field)
20
10
0
*
control
Thy-1
exendin-4
-
+
-
+

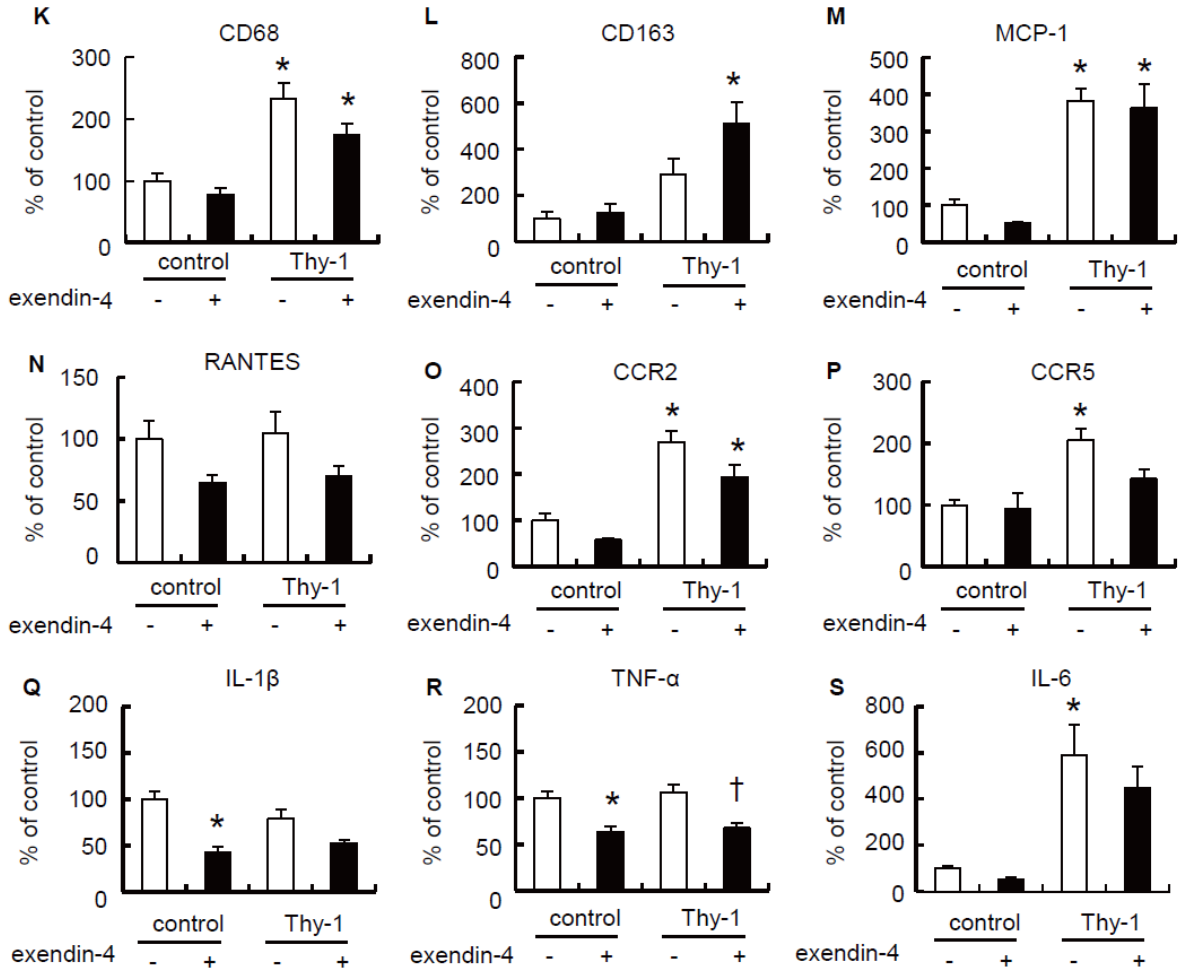


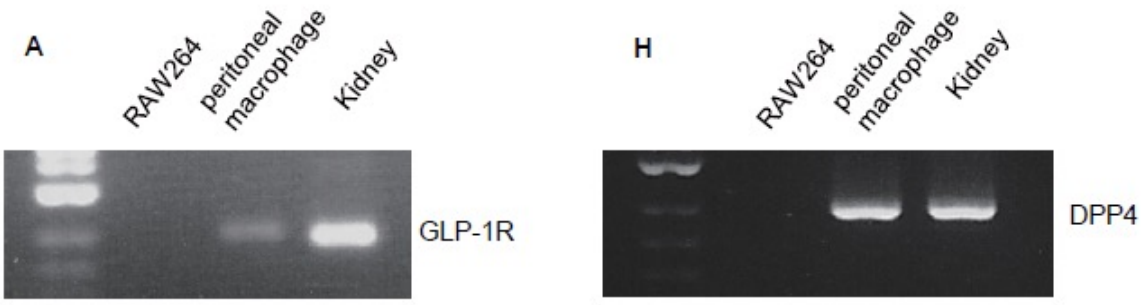
Figure 7. Effects of a GLP-1 receptor agonist, exendin-4, on renal macrophage infiltration

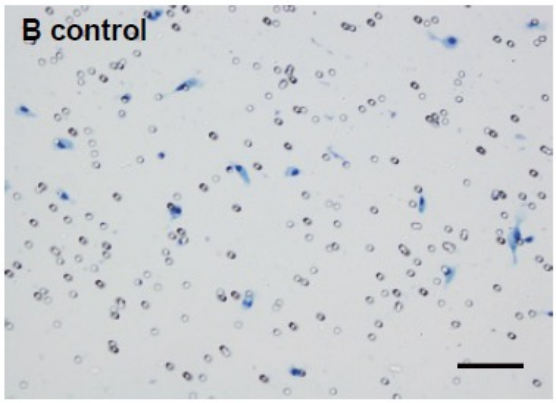
Representative images of CD68 (A–D) and CD163 (F–I) are shown. Bars = 100 μm (×200). The number of CD68- and CD163-positive cells is expressed as a count per field (E and J). The renal mRNA expression of inflammation-related genes was quantified by real-time PCR (K–S). Data are expressed as means ± SE. **P* < 0.05 compared with the control group. † *P* < 0.05 compared with the Thy-1 group.

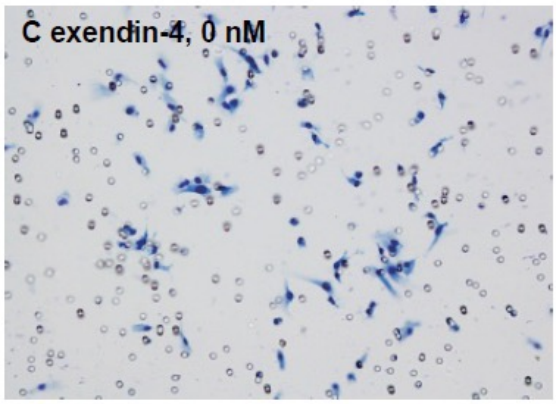
GLP-1R agonist, but not the DPP-4 inhibitor, reduces MCP-1-stimulated macrophage infiltration in vitro

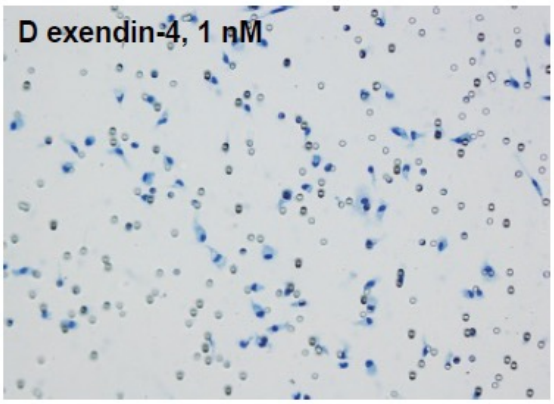
Finally, ex vivo transmigration assays were performed to examine the direct effect of DPP-4 inhibitor on macrophages. To address this, we checked mRNA expressions of GLP-1R and DPP-4 in macrophage cells. Although both GLP-1R and DPP-4 mRNAs were readily detectable in peritoneal macrophages, they were not detectable in a RAW264 cell line, within the amplification range (Figure 8A and H). Therefore, we performed migration assays using peritoneal macrophages in subsequent studies. Exendin-4 dose-dependently reduced MCP-1-stimulated macrophage infiltration to the lower chamber (Figure 8B–G). Conversely, alogliptin did not significantly reduce macrophage infiltration (Figure 8I–N), raising the possibility that the observed suppression of macrophage infiltration in vivo was mediated via GLP-1 dependent signaling subsequent to DPP-4 inhibition.

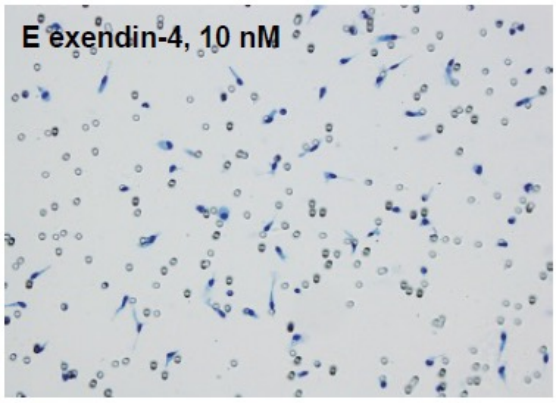
Figure8

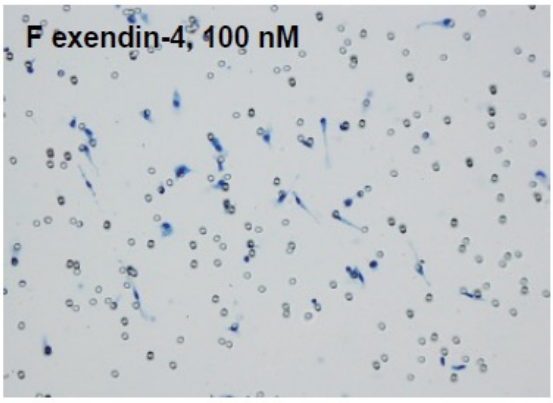


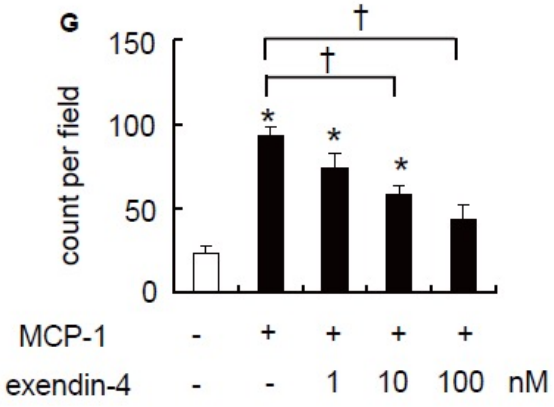

B control

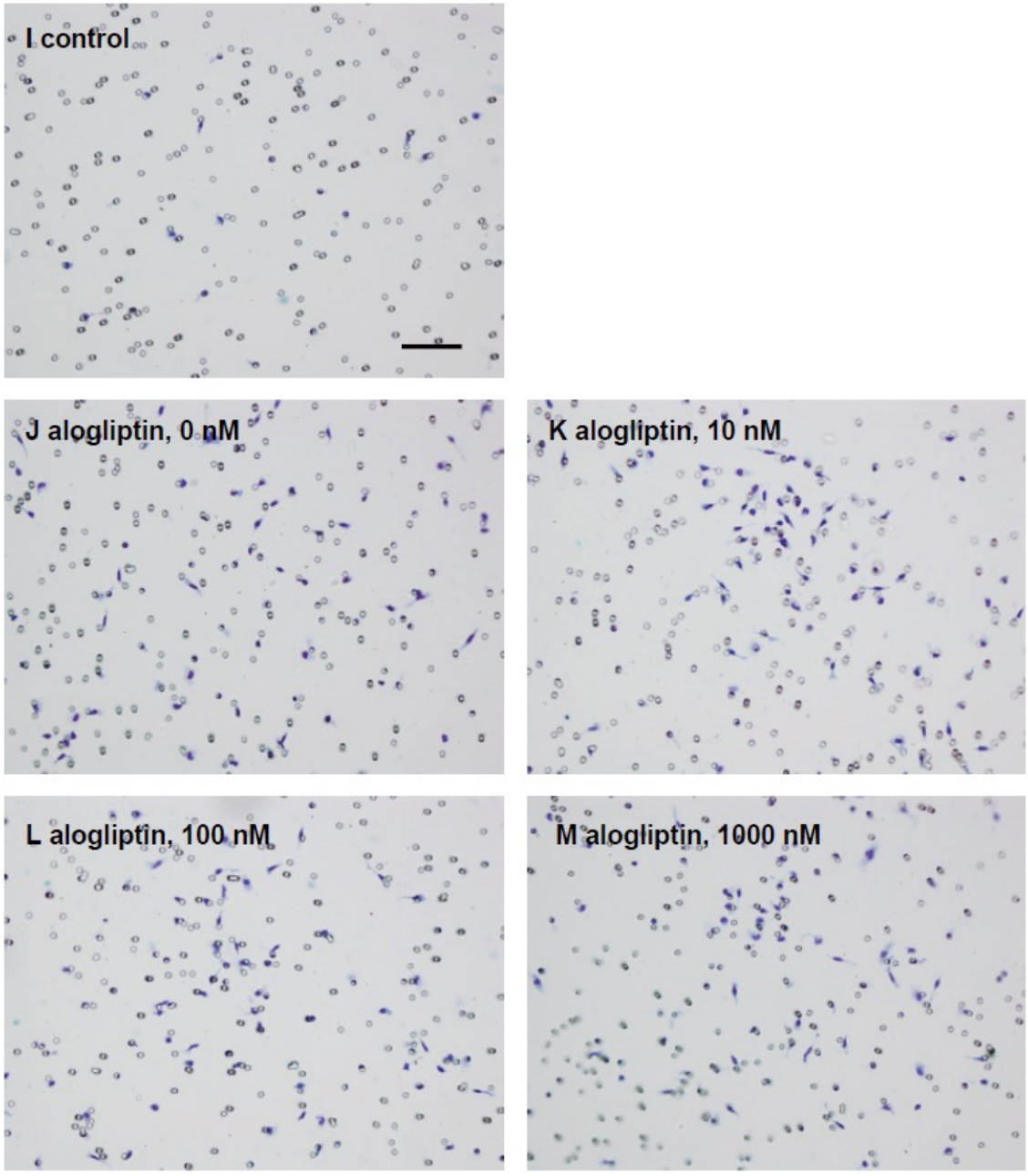

C exendin-4, 0 nM


D exendin-4, 1 nM


E exendin-4, 10 nM


F exendin-4, 100 nM


G
count per field
150
100
50
0
†
†
*
*
*
MCP-1 - + + + +
exendin-4 - - 1 10 100 nM


I control
J alogliptin, 0 nM
K alogliptin, 10 nM
L alogliptin, 100 nM
M alogliptin, 1000 nM

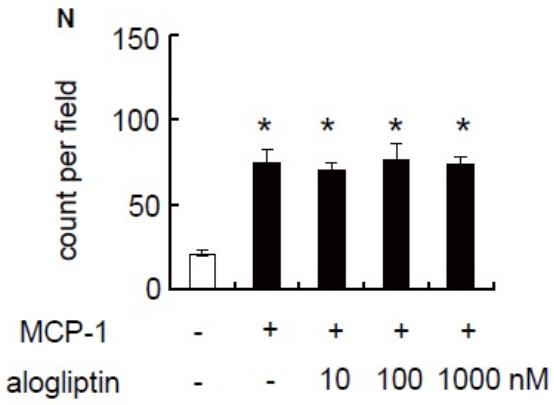

N
count per field
150
100
50
0
*
*
*
*
MCP-1 - + + + +
alogliptin - - 10 100 1000 nM

Figure 8. Effects of exendin-4 and alogliptin on monocyte chemotactic protein-1 (MCP-1)-induced chemotactic response

Expressions of GLP-1R and DPP-4 were examined in RAW264, peritoneal macrophages, and the kidney (used as the positive control) by PCR and agarose gel electrophoresis (A and H). Boyden chamber assays using peritoneal macrophages were performed and representative images are shown (exendin-4: B–F and alogliptin: I–M). Bars = 100 μm (×200). The number of migrating cells were counted and expressed as a count per field (G and N). Graphs are representative of three independent experiments. Data are expressed as means ± SD. **P* < 0.05 compared with the non-treated group. † *P* < 0.05 MCP-1-treated group.

Discussion

Therapeutic effects of GLP-1R agonists and DPP-4 inhibitors are currently being evaluated in DKD models. For example, previous studies have shown that the GLP-1R agonist and DPP-4 inhibitor ameliorated STZ-induced DKD model (12, 23, 24, 29, 35). In type 2 diabetes models, GLP-1R agonists also ameliorated kidney injury (9, 37). In contrast, only a few studies have examined the effects of GLP-1R agonists and DPP-4 inhibitors in non-DKD models. In AKI models, the GLP-1R agonist and DPP-4 inhibitor ameliorated cisplatin-induced nephrotoxicity and ischemia-reperfusion injury (10, 22). In a chronic kidney disease model, the DPP-4 inhibitor attenuated renal dysfunction and structural damage in the remnant kidney (20).

Shinosaki et al. previously reported that a monoclonal antibody against DPP-4 considerably reduced proteinuria and mesangial expansion via the suppression of the complement cascade in the rat Thy-1 model (44). Contrary to this report, we observed a non-significant trend toward reductions in glomerular injury and proteinuria in the present study. DPP-4 (CD26), on the one hand, is an important molecule that functions as a cleaving enzyme and also serves as a surface receptor and/or co-stimulatory protein, especially in immune response. For example, DPP-4/CD26 is a T cell activation antigen, but its enzymatic activity is not required for the signaling function in T cells (11). In addition, the expression of DPP-4/CD26, but not DPP-4 activity, is associated with the susceptibility of the human

immunodeficiency virus type-1 to CD4 T lymphocytes (36). In the rat Thy-1 model, Th1 cytokines produced by CD4 T lymphocytes contribute to the development of injury (16), and the suppression of cytokine production could be a valid target for therapy (15). Taken together, the results of the present study indicated that the expression of DPP-4, but not enzymatic activity, might be important for immune responses in CD4 T lymphocytes. Thus, the inhibition of DPP-4 activity only resulted in a non-significant reduction of glomerular injury and proteinuria in this model.

We focused on the anti-inflammatory effects of DPP-4 inhibitors. Two types of DPP-4 inhibitors, alogliptin and anagliptin, reduced macrophage infiltration to the injured kidney. This effect was associated by increased plasma GLP-1 levels. In addition, the GLP-1R agonist exendin-4 similarly reduced macrophage infiltration to the kidney. The mRNA levels of chemokines such as MCP-1 and RANTES in the renal cortex were not affected by the treatments with alogliptin and exendin-4. Furthermore, additional ex vivo transmigration assays revealed that exendin-4, but not alogliptin, dose-dependently reduced MCP-1-stimulated macrophage infiltration. These data indicated that DPP-4 inhibitors suppress macrophage infiltration via GLP-1-dependent signaling in the rat Thy-1 model.

Macrophages play an important role in the disease progression of glomerulonephritis (40). For example, MCP-1-neutralizing antibodies reduce macrophage infiltration and thereby ameliorate glomerular injury and proteinuria in a nephrotoxic-serum glomerular nephritis

model (30). In a lupus nephritis model, MCP-1 knockout MRL-Fas (lpr) mice exhibit less glomerular injury and reduced proteinuria, accompanied by a reduction in macrophage infiltration (51). In the Thy-1 model, Rampino et al. reported that neutralization of macrophage-stimulating proteins reduces macrophage infiltration and leads to attenuation of renal histology and proteinuria (38). In our study, treatment with alogliptin resulted in a non-significant trend toward reductions in glomerular injury and proteinuria. These trends were also associated with reduced macrophage infiltration, and thus, these results were consistent with those of Rampino et al. However, detailed molecular mechanisms involving DPP4 inhibitor suppression of macrophage infiltration merit further investigation.

Currently, macrophages can be broadly divided into classically-activated (M1-like) or alternatively-activated (M2-like) macrophages (45). However, whether M2-like macrophages contribute to the progression of kidney diseases remains controversial. In an ischemia reperfusion injury model, M2-like macrophages promote tubular cell proliferation and tissue repairing (27, 56). In contrast, in a polycystic kidney disease model, M2-like macrophages promote cyst cell proliferation, cyst growth, and fibrosis (47). Furthermore, in the Thy-1 model, prednisolone increased CD163 (M2-like) macrophages and exacerbated global glomerulosclerosis, whereas mizoribine reduced CD163-positive macrophages and alleviated mesangial expansion and glomerulosclerosis (17). In addition, epoetin β pegol (continuous erythropoietin receptor activator, CERA) ameliorated glomerular injury in the

Thy-1 model, with a reduction in the number of CD163-positive macrophages (1). These reports suggest that M2-like macrophages contribute to the progression of glomerular injury in the Thy-1 nephritis model. In our study, DPP-4 inhibitors, but not the GLP-1R agonist, exhibited the tendency of reducing proteinuria, whereas the number of CD163-positive macrophages was not affected by the treatment with the DPP-4 inhibitor, but it increased by the treatment with the GLP-1R agonist. We speculate that the polarity in changes of macrophages require potent signaling of GLP-1R, and that the increased number of M2-like macrophages by effects of the GLP-1R agonist might have negated the renoprotective effects observed with the DPP-4 inhibitor.

Contrary to many published papers, reducing renal macrophage infiltration did not result in significant reduction in glomerular injury and proteinuria in our study. Alogliptin was administered to rats in reference with a previous paper in which pharmacokinetics of alogliptin was closely examined and plasma DPP-4 inhibition in rats was observed through 12 h but not 24 h after single dose of 10 mg/kg alogliptin (26). To achieve constant inhibition of plasma DPP-4, we administered 10 mg/kg alogliptin twice a day and significant plasma DPP-4 inhibition and GLP-1 increase were confirmed but failed to reduce proteinuria significantly. As to exendin-4, we used the same dose as described in a previous study from another group (10 μg/kg/day), in which exendin-4 significantly reduced albuminuria in STZ-induced type1 diabetes model (23). In an additional experiment, rats in the Thy-1 group

were treated with exendin-4 (5 μg/kg twice a day) from day -7 to day 7 days, but proteinuria was not improved (data not shown). Based on these, we believe that the absent reduction in proteinuria was not due to insufficient DPP4 inhibition and GLP-1R stimulation, but rather because of the robust degree of renal injury in which reduction in macrophage infiltration alone was not sufficient to influence the disease course.

Takase et al. used eicosapentaenoic acid (EPA) in the same model (49). Treatment of EPA significantly reduced CD68 positive macrophage infiltration into tubulointerstitial area, which was associated with the prevention of tubulointerstitial injury. However, EPA did not reduce CD68 positive macrophage infiltration into glomeruli and failed to reduce proteinuria, suggesting that inhibition of macrophage infiltration in glomerulus is important for reducing proteinuria and glomerular injury in rat Thy-1 model. Indeed, many studies showed the reduction of proteinuria and glomerular injury was related to the reduction of macrophages in glomeruli (6, 38). In our study, the number of CD68 positive macrophages in glomeruli was not decreased by the treatment of alogliptin (data not shown). Thus, this may be another reason why the reduction of macrophage infiltration did not result in reduction in glomerular injury and proteinuria.

In the kidney, DPP-4 is expressed on the brush border membrane of proximal tubule, glomerular podocyte, vascular smooth muscle cells, and mesangial cells (18). On the other hand, exact localization of GLP-1R in the kidney remains controversial. In microdissected rat

kidney samples, GLP-1R mRNA was detected in gromeruli and proximal tubule (7). In contrast, recent in situ hybridization study using mice showed GLP-1R was localized in glomerular capillary walls and throughout vascular walls but not proximal tubule (9). Primary cause of Thy-1 nephritis is immune complex-mediated mesangial cell injury, which is partially overlapped with the expression pattern of DPP-4 in the kidney. Hence, the discrepancy in influence on proteinuria between DPP-4 inhibitor and GLP-1R agonist might be due to the difference in the localization between DPP-4 and GLP-1R.

Apart from GLP-1, DPP-4 can cleave multiple substrates such as the brain-derived natriuretic peptide, substance P, neuropeptide Y (NPY), peptide YY (PYY), high-mobility group protein B1, and stromal-derived factor (SDF)-1α Among these, protective effects of SDF-1α have been extensively examined. In a model of myocardial infraction, DPP-4 inhibition reduced the infract size and improved cardiac function via myocardial homing of circulating stem cells (13, 55). The DPP-4 inhibitor also recruits regenerated stem cells via SDF-1α signaling and improves lung ischemia reperfusion injury (21). Conversely, Katagiri et al. reported that the DPP-4 inhibitor ameliorated cisplatin-induced renal injury via GLP-1 but not SDF-1α signaling (22). In addition, another incretin hormone, GIP has also been reported to exhibit neuro-protective effects against the mouse model of Alzheimer's disease (5). Thus, multiple targets of DPP-4 appear to play a role in the protection against acute kidney injury, and substrates other than GLP-1 might have contributed to the tendency of proteinuria

reduction in our study.

In conclusion, DPP-4 inhibitors reduce macrophage infiltration directly via the GLP-1-dependent pathway and have a trend to ameliorate proteinuria in the rat Thy-1 nephritis model. Control of inflammation by DPP-4 inhibitors might have the potential to ameliorate the progression of non-diabetic kidney diseases. Currently, studies on the renoprotective effects of DPP-4 inhibitors in such contexts are limited, and further studies are warranted to understand the mechanisms through which DPP-4 inhibitors mediate beneficial actions.

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