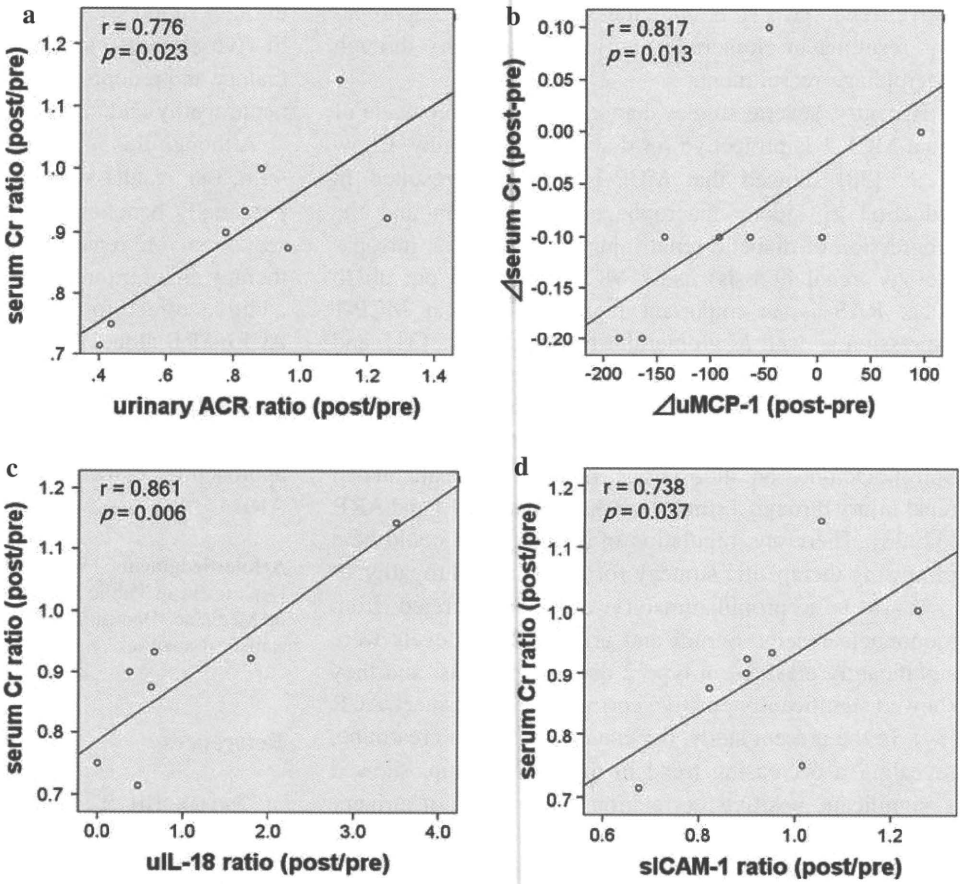

Fig. 3 Correlation between the change in serum creatinine and urinary ACR (**a**), urinary MCP-1 (**b**), urinary IL-18 (**c**) and serum levels of soluble ICAM-1 (**d**) in the CAM group

Table 2 Independent associations between urinary ACR and proinflammatory markers from multiple linear regression analysis [$r^2 = 0.705$ ($P = 0.043$)]

	B	SE	*β*	*t*	*P*
uACR					
sMCP-1	−0.311	0.270	−0.521	−1.151	0.302
uMCP-1	0.290	0.096	0.584	3.028	0.029
Soluble ICAM-1	−1.056	0.245	−1.350	−4.316	0.008
sIL-18	1.030	0.496	1.208	2.078	0.092
uIL-18	1.805	1.386	0.309	1.303	0.240
hs-CRP	−0.005	0.020	−0.060	−0.264	0.802

Dependent variable: urinary ACR at baseline. Independent variables: sMCP-1, uMCP-1, soluble ICAM-1, sIL-18, uIL-18 and hs-CRP

the changes of serum creatinine showed a significant positive correlation with those of urinary ACR, urinary MCP-1, urinary IL-18 and serum levels of soluble ICAM-1 in patients treated with clarithromycin.

Recent studies have shown the involvement of inflammatory mechanisms in the pathogenesis of diabetic nephropathy. We previously demonstrated that ICAM-1 is expressed in the renal tissues and promotes the infiltration of macrophages into the kidney in diabetic patients and animal models of diabetes [17–19]. Moreover, we showed that ICAM-1 deficiency resulted in suppression of macrophage infiltration into the renal tissues, transforming growth factor-β (TGF-β) and type IV collagen expression using ICAM-1 knockout mice induced diabetes [20]. In addition to these, several studies suggest the possibility of inflammatory mechanisms as a new therapeutic target for diabetic nephropathy. It was reported that statin or immunosuppressing agents such as methotrexate (MTX) and mycophenolate mofetil (MMF) attenuated renal damage through suppression of macrophage infiltration in animal models of diabetes [21–24]. Kikuchi et al. [25] also reported that mizoribine prevented renal injury via inhibition of renal MCP-1 expression and macrophage accumulation in non-insulin-dependent diabetic rats.

MCP-1 is one of the chemokines inducing macrophage migration to the lesion, and it is upregulated in diabetic nephropathy [10]. Renal MCP-1 is synthesized in the tubular epithelial cells and mesangial cells, and urinary MCP-1 levels reflect renal MCP-1 production [11]. Gruden et al. [26] reported that mechanical stretch induces MCP-1 via the NF-κB-dependent pathway in human mesangial cells. It is also reported that high glucose directly induces MCP-1 expression though activation of NF-κB in mesangial cells [27–29]. Via several pathways as described

above, renal MCP-1 is upregulated in diabetic nephropathy, resulting in glomerular and interstitial injury through macrophage recruitment.

Recently, several studies demonstrated that blockade of renal MCP-1 is protective for diabetic nephropathy. Chow et al. [30] showed that MCP-1 deficiency resulted in reduction of kidney macrophage accumulation and the progression of diabetic renal injury (albuminuria, histopathology, renal fibrosis) using MCP-1 knocked out db/db mice. RAS is an important regulator of local MCP-1 expression as well as glomerular hemodynamics [31], and ACEI and ARB are reported to suppress renal MCP-1 expression and macrophage infiltration, resulting in diminished proteinuria in experimental diabetic rats [13]. Spironolactone, an aldosterone blocker, also attenuated renal injury through a similar mechanism to ACEI and ARB [32, 33]. Therefore, regulation of local MCP-1 could be a promising therapeutic strategy for diabetic nephropathy.

IL-18 is a proinflammatory cytokine secreted from mononuclear cells. Serum and urinary IL-18 levels were significantly elevated in type 2 diabetic patients, and they showed significantly positive correlation with urinary ACR [34]. In the present study, the changes of serum creatinine, revealing a decreasing trend in the CAM group, showed a significant positive correlation with those of urinary MCP-1 and IL-18 in the patients treated with clarithromycin. This might suggest the possibility that clarithromycin showed renoprotective effects via modulation of macrophage-mediated microinflammation.

On the other hand, it is well known that 14-membered ring macrolide antibiotics have anti-inflammatory effects as well as antibacterial effects, and low-dose and long-term administration of macrolides is in common clinical use for COPD, DPB and chronic sinusitis. Recently, Li et al. [35] showed that macrolides inhibited the expression of adhesion molecules such as ICAM-1 in the lung tissues and attenuated the migration of inflammatory cells into air spaces, especially of neutrophils and macrophages, using bleomycin-induced lung injury mice. It is also reported that macrolides have anti-inflammatory effects both on vascular endothelial cells and monocytes/macrophages [36–39]. In addition to these, azithromycin reduced MCP-1 production in human umbilical vein endothelial cells (HUVECs) stimulated with tumor necrosis factor-α (TNF-α) and significantly inhibited transendothelial migration of neutrophils and monocytes [40].

Based on these findings, we previously demonstrated that erythromycin, one of the macrolide antibiotics, ameliorated renal injury through suppression of renal NF-κB activation and macrophage recruitment into the renal tissues in experimental diabetic rats [16]. Yamabe et al. [41] also showed that roxithromycin inhibited TGF-β and type IV collagen production in cultured human mesangial cells through suppression of NF-κB activation. These results of in vivo and vitro studies strongly suggest the anti-inflammatory and renoprotective effect of macrolides for diabetic nephropathy and support our present study.

Although this is a pilot study with small size and short-term, our results suggest that clarithromycin might have potentially beneficial effects for diabetic nephropathy via reduction of renal MCP-1. Furthermore, combination therapy of clarithromycin and ACEI/ARB may exert an additive effect for diabetic nephropathy compared with ACEI/ARB alone, because the therapy with clarithromycin may have the potential to enhance the renal MCP-1-reducing effect of ACEI/ARB. Thus, modulation of microinflammation with clarithromycin may provide a new approach for diabetic nephropathy, together with ACEI and ARB.

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ARTICLE

Glucagon-like peptide-1 receptor agonist ameliorates renal injury through its anti-inflammatory action without lowering blood glucose level in a rat model of type 1 diabetes

R. Kodera · K. Shikata · H. U. Kataoka · T. Takatsuka · S. Miyamoto · M. Sasaki · N. Kajitani · S. Nishishita · K. Sarai · D. Hirota · C. Sato · D. Ogawa · H. Makino

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Abstract

Aims/hypothesis Glucagon-like peptide-1 (GLP-1) has various extra-pancreatic actions, in addition to its enhancement of insulin secretion from pancreatic beta cells. The GLP-1 receptor is produced in kidney tissue. However, the direct effect of GLP-1 on diabetic nephropathy remains unclear. Here we demonstrate that a GLP-1 receptor agonist, exendin-4, exerts renoprotective effects through its anti-inflammatory action via the GLP-1 receptor without lowering blood glucose.

Methods We administered exendin-4 at 10 μg/kg body weight daily for 8 weeks to a streptozotocin-induced rat model of type 1 diabetes and evaluated their urinary albumin excretion, metabolic data, histology and morphometry. We also examined the direct effects of exendin-4 on glomerular endothelial cells and macrophages in vitro.

Results Exendin-4 ameliorated albuminuria, glomerular hyperfiltration, glomerular hypertrophy and mesangial matrix expansion in the diabetic rats without changing blood pressure or body weight. Exendin-4 also prevented macrophage infiltration, and decreased protein levels of intercellular adhesion molecule-1 (ICAM-1) and type IV collagen, as well as decreasing oxidative stress and nuclear factor-κB activation in kidney tissue. In addition, we found that the GLP-1 receptor was produced on monocytes/macrophages and glomerular endothelial cells. We demonstrated that in vitro exendin-4 acted directly on the GLP-1 receptor, and attenuated release of pro-inflammatory cytokines from macrophages and ICAM-1 production on glomerular endothelial cells.

Conclusions/interpretation These results indicate that GLP-1 receptor agonists may prevent disease progression in the early stage of diabetic nephropathy through direct effects on the GLP-1 receptor in kidney tissue.

Keywords Anti-inflammatory effect · Diabetic nephropathy · Exendin-4 · Glomerular endothelial cells · GLP-1 receptor agonist · Intercellular adhesion molecule-1 · Macrophage · Nuclear factor-κB · Type 1 diabetic rats

Abbreviations

GLP-1	Glucagon-like peptide-1
GLP-1R	Glucagon-like peptide-1 receptor
hGECs	Human glomerular microvascular endothelial cells
ICAM-1	Intercellular adhesion molecule-1
NOX4	NADPH oxidase 4
NF-κB	Nuclear factor-κB
8-OHdG	8-Hydroxydeoxyguanosine

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R. Kodera · K. Shikata (✉) · H. U. Kataoka · T. Takatsuka · S. Miyamoto · M. Sasaki · N. Kajitani · S. Nishishita · K. Sarai · D. Hirota · C. Sato · H. Makino
Department of Medicine and Clinical Science,
Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences,
2-5-1 Shikata-cho,
Okayama 700-8558, Japan
e-mail: shikata@md.okayama-u.ac.jp

C. Sato · D. Ogawa
Department of Diabetic Nephropathy, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences,
Okayama, Japan

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Introduction

The number of patients with diabetes is increasing dramatically throughout the world, while diabetic nephropathy is the leading cause of end-stage renal disease in developed countries. In addition, chronic kidney disease contributes to development of cardiovascular disease and leads to an increase in all-cause mortality rates [1, 2]. Therefore, prevention of renal insufficiency improves the prognosis of diabetic patients.

Numerous factors contribute to the development of diabetic nephropathy, such as glomerular hyperfiltration [3], which is mainly observed in early-stage nephropathy, oxidative stress [4], accumulation of AGEs [5], activation of protein kinase C [6], acceleration of the polyol pathway and overexpression of TGF-β [7]. Accumulating evidence also points to the critical role of the inflammatory process in the development of diabetic vascular complications, suggesting that microinflammation is a common mechanism in pathogenesis of diabetic nephropathy [8, 9]. Furuta et al. [10] reported that infiltration of mononuclear cells was prominent in the glomeruli of patients with diabetic nephropathy. Our group has also reported similar results, as well as observing an increase in the production of cell adhesion molecules in the kidney of diabetic patients [11]. We found that intercellular adhesion molecule-1 (ICAM-1) played a key role in promoting macrophage infiltration in glomeruli from a rat model of diabetes [12], and using mice deficient in ICAM-1, we also showed that blockade of ICAM-1 activation ameliorated diabetic nephropathy [13]. Additionally, we showed that methotrexate, an immunosuppressant, ameliorated diabetic nephropathy and that anti-inflammatory agents also had a beneficial effect on diabetic nephropathy [14]. Modulation of the inflammatory process prevents renal injury in animal models of diabetes, suggesting that microinflammation is a potential therapeutic target in diabetic nephropathy [14–17].

Glucagon-like peptide-1 (GLP-1) is a gut incretin hormone and currently considered an attractive agent for treatment of type 2 diabetes. It has various beneficial effects on pancreatic beta cells, such as enhancement of glucose-dependent insulin secretion [18], acceleration of beta cell proliferation and inhibition of beta cell apoptosis [19]. In the gut and hypothalamus, GLP-1 inhibits motility, gastric emptying [20] and central regulation of feeding [21], resulting in body weight loss [18]. However, native GLP-1 is rapidly degraded in the circulation by dipeptidylpeptidase-IV [22]. Today, dipeptidylpeptidase-IV-resistant, long-acting GLP-1 receptor (GLP-1R) agonists such as exendin-4 and liraglutide are available for type 2 diabetic patients. Previous reports have shown that GLP-1R is produced not only in the pancreas, gut and hypothalamus, but also in the kidney [23–25]. With respect to the effects of GLP-1 on the kidney, it has been reported that exendin-4 ameliorated hypertension by regulating sodium excretion in tubular cells [26] and attenuated renal injury by improving metabolic anomalies in a mouse model of type 2 diabetes [25]. From these results, the amelioration of hypertension and metabolic anomalies by GLP-1 would seem to have a beneficial effect on diabetic nephropathy. In the present study, we focused on the direct effect of GLP-1 through GLP-1R in the kidney, independently of the numerous other effects of GLP-1, including its glucose-lowering action.

Methods

Animals

Male Sprague–Dawley rats (5 weeks old; Charles River, Yokohama, Japan) were divided into the following groups: (1) non-diabetes (n=5); (2) non-diabetes treated with exendin-4 (n=6); (3) diabetes (n=6); and (4) diabetes treated with exendin-4 (n=6). At the age of 5 weeks, the groups allocated to be made diabetic received intravenous injections of streptozotocin (Sigma-Aldrich, St Louis, MO, USA) at 65 mg/kg body weight in citrate buffer (pH 4.5). We included only rats with blood glucose concentrations >16 mmol/l at 3 and 7 days after streptozotocin injection in the diabetes groups. The non-diabetic groups received injections of citrate buffer alone. The two groups treated with exendin-4 were given exendin-4 (Bachem, Bubendorf, Switzerland) intraperitoneally at 10 μg/kg body weight daily for 8 weeks, starting at 1 week after the streptozotocin or citrate buffer injections. The placebo groups were given water alone using the same schedule as in the exendin-4 treatment groups. All rats had free access to standard chow and tap water. All procedures were performed according to the Guidelines for Animal Experiments at Okayama University Medical School, the Japanese Government Animal Protection and Management Law and the Japanese Government Notification on Feeding and Safekeeping of Animals. All rats were killed at 9 weeks after induction of diabetes in the diabetes groups, and the kidneys were weighed and fixed in 10% (vol./vol.) formalin, or frozen in acetone cooled on dry ice.

Metabolic variables

Systolic BP was measured by tail-cuff plethysmography (Softron, Tokyo, Japan). HbA_{1c} was measured by the HPLC method. Serum creatinine was measured by the 3-hydroxy-2,4,6-triiodobenzoic acid method. Food intake was calculat-

ed as the average over 3 days. Insulin concentration was measured by a rat insulin RIA kit (LincoResearch, St Charles, MO, USA). Urine samples were collected over a 24 h period in individual metabolism cages. Urinary albumin excretion was measured by nephelometry using anti-rat albumin antibody (ICN Pharmaceuticals, Aurora, OH, USA). Creatinine clearance (ml min^{-1} kg^{-1}) was calculated as described previously [15].

Light microscopy

The glomerular tuft area and mesangial matrix index (ratio of the mesangial matrix area/glomerular tuft area) were measured using a software package (Lumina Vision; Mitani, Fukui, Japan) as described previously [13]. Periodic acid–Schiff's reagent staining was used to observe the interstitium of the kidney. Quantitative analysis for all staining was performed in a blinded manner.

Immunoperoxidase staining

Immunoperoxidase staining was performed as described [12, 27]. Primary antibodies were macrophages mouse antibody (ED1, 1:50; Serotec, Oxford, UK), 8-hydroxydeoxyguanosin (8-OHdG) mouse antibody (1:10; JalCA, Shizuoka, Japan), NADPH oxidase 4 (NOX4) rabbit antibody (1:300; Novus Biologicals, Littleton, CO, USA) or GLP-1R rabbit antibody (ab39072, 1:200; Abcam, Tokyo, Japan), all of which were applied for 12 h at 4°C. Secondary antibodies were biotin-labelled anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA), which were applied for 60 min at room temperature. The average number of ED1-positive cells per glomerulus was used for the estimation. The ratio of the area stained positive with each of the above antibodies to the glomerular tuft area was calculated with a software package (Lumina Vision).

Immunofluorescence staining

Immunofluorescence staining was performed as described [12]. The primary antibodies were ICAM-1 mouse antibody (1:25; Abcam) or type IV collagen rabbit antibody (1:200; LSL, Tokyo, Japan), which were applied for 60 min at room temperature. Secondary antibodies were FITC-conjugated anti-mouse or anti-rabbit IgG (1:150; Zymed Laboratories, San Francisco, CA, USA), which were applied for 30 min at room temperature. Micrographic fluorescence photos were obtained with a laser-scanning confocal microscope (LSM-510; Carl Zeiss, Jena, Germany). The ICAM-1 and type IV collagen indexes were calculated as described [15].

Double immunofluorescence staining

The primary antibodies were GLP-1R rabbit antibody (1:200; Abcam) and rat endothelial cell antigen (RECA-1, 1:40; Monosan, Uden, the Netherlands), macrophages (ED1, 1:50) mouse antibody, or NOX4 rabbit antibody (1:300), which were applied for 12 h at 4°C. The secondary antibodies were Alexa-Fluor 488-labelled anti-rabbit and 546-labelled anti-mouse IgG (1:400; Invitrogen, Carlsbad, CA, USA), which were applied for 30 min at room temperature. Nuclei were stained with DAPI (Millipore, Tokyo, Japan). The sections were observed under a fluorescence microscope (BZ-800; Keyence, Osaka, Japan).

Quantitative real-time RT-PCR and gene expression

Total RNA was extracted from each sample (the rat renal cortex, glomeruli isolated by a previously reported mechanical sieving technique [28] and cultured cells) using a kit (RNeasy plus Mini; Qiagen, Valencia, CA, USA). Single-strand cDNA was synthesised from the individual samples of total RNA at 1 μg using a kit (GeneAmp RNA PCR-

Table 1 Metabolic variables of four rat groups at 8 weeks

Variable	Non-diabetic		Diabetic	
	Placebo	Exendin-4	Placebo	Exendin-4
Body weight (g)	471±23.5[a]	397±8.3[b]	256±30.5	246±22.9
Food intake (g/day)	27.6±0.9[c,d]	19.3±3.1[a]	44.6±12.5	38.6±0.5
HbA_{1c} (%)	3.7±0.1[a]	3.9±0.1[a]	10.5±0.5	10.0±0.8
Fasting blood glucose (mmol/l)	4.0±0.2[a]	4.9±0.3[a]	26.6±0.9	26.6±2.4
Systolic BP (mmHg)	115±2.6	114±1.5	120±1.3	123±2.6
Relative kidney weight (g/kg)	5.9±0.2[a]	6.1±0.1[a]	11.6±0.7	12.0±0.7

Values are the means ± SEM; n=5 animals in the non-diabetic placebo group; n=6 animals per group in the three other groups

[a] $p<0.001$ vs diabetes and diabetes + exendin-4 groups; [b] $p<0.01$ vs diabetes and diabetes + exendin-4 groups; [c] $p<0.001$ vs diabetes placebo group; [d] $p<0.05$ vs diabetes plus exendin-4 group

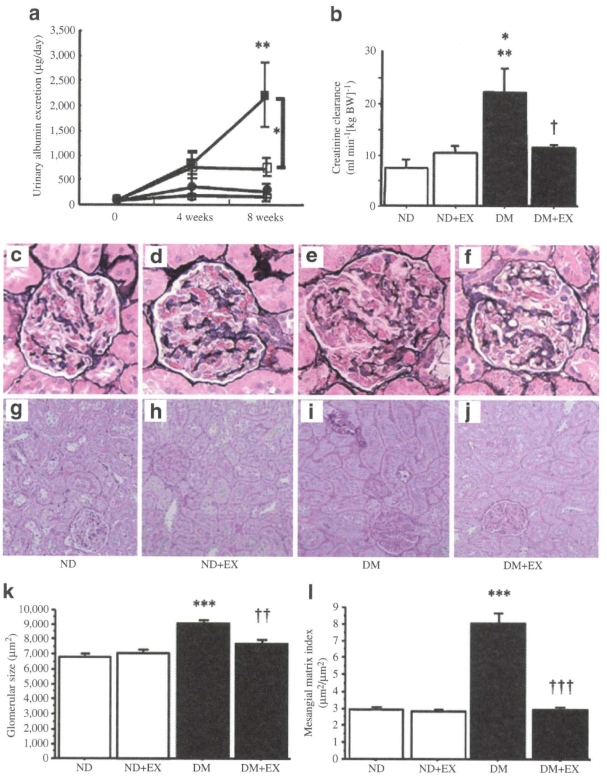

Fig. 1 **a** Time course of 24 h urinary albumin excretion. Urinary albumin excretion increased gradually over 8 weeks in the diabetic group. Exendin-4 resulted in a significantly lower level of urinary albumin excretion at 8 weeks than in the untreated diabetes group. *p <0.05; **p<0.01 vs non-diabetic and non-diabetic + exendin-4 groups. Black circles, non-diabetic group; white circles, non-diabetic + exendin-4; black squares, streptozotocin-induced diabetes group; white squares, diabetes + exendin-4 group. **b** Creatinine clearance. Hyperfiltration in the diabetic (DM) nephropathy group was significantly decreased by exendin-4 treatment (DM+EX) at 8 weeks. *p< 0.05 vs non-diabetic + exendin-4 (ND+EX); **p<0.01 vs ND; $^{\dagger}p$< 0.05 vs DM. **c–f** Periodic acid–methenamine-silver (PAM) staining in glomeruli (original magnification ×200). **g–j** Periodic acid–Schiff's reagent staining in the kidney (×100). **k** Glomerular size (tuft area). Glomerular hypertrophy was significantly greater in the DM group than in the ND groups. Exendin-4 treatment significantly suppressed glomerular hypertrophy. ***p<0.001 vs ND and ND+EX; $^{\dagger\dagger}p$<0.01 vs DM. **l** Mesangial matrix index, calculated by the PAM-positive area in the tuft area, was significantly increased in the DM group. Exendin-4 treatment significantly reduced mesangial matrix expansion. ***p< 0.001 vs ND and ND+EX; $^{\dagger\dagger\dagger}p$<0.001 vs DM. n=5 animals in the untreated ND group; n=6 animals per group in the three other groups. **k**, **l** Glomeruli: n=30 from each rat kidney; n=4 per group. Values are the means ± SEM

Core kit; Applied Biosystems, Foster City, CA, USA). After addition of each set of primers (final concentration 0.4 μmol/l) and template DNA to the master mix, quantitative real-time RT-PCR was performed with a LightCycler (Roche Diagnostics, Tokyo, Japan) and SYBR Premix-Ex-Taq (Takara Bio, Shiga, Japan). The PCR protocol was as follows: initial denaturation (95°C for 30 s), followed by 40 cycles of denaturation (95°C for 5 s), and annealing and extension (60°C for 20 s). The specific oligonucleotide primer sequences are shown in

Electronic supplementary material (ESM) Table 1. To visualise gene expression, individual DNA fragments were electrophoresed on a 2% (wt./vol) agarose gel (Sigma-Aldrich) and treated with ethidium bromide. cDNAs of the human pancreas (Takara Bio) and of rat islet-cell tumour cells (RIN-5F; DS Pharma Biomedical, Osaka, Japan) were used as positive controls.

Urinary 8-OHdG excretion

8-OHdG is a marker of oxidative DNA damage [29]. Urinary 8-OHdG concentration in a 24 h urine collection was measured with a kit (8-OHdG ELISA; JalCA) according to the manufacturer's instructions.

Nuclear factor-κB activation

Nuclear proteins of kidney tissues were extracted by a nuclear extract kit and nuclear factor-κB (NF-κB) p65 activity determined by ELISA using reagents (Active-Motif; Carlsbad, CA, USA) according to the manufacturer's instructions. Absorbance was normalised to milligram cell protein.

Western blotting

Cells were lysed with cell lysis buffer containing 10 mmol/l TRIS (pH 7.4), 1% (vol./vol.) Triton X-100, 0.5% (vol./vol.) Nonidet P-40 and phosphatase inhibitor cocktail, 150 mmol/l NaCl, 1 mmol/l EDTA, 0.2 mmol/l EGTA, vanadate and phenylmethanesulfonyl fluoride. The cell lysates were subjected to 7.5% SDS-PAGE (Bio-Rad Japan, Tokyo, Japan). The separated proteins were transferred to polyvinylidene fluoride membranes (Bio-Rad) by electrotransfer. The blots were subsequently blocked with 5% (vol./vol.) skimmed milk (Nacalai Tesque, Kyoto, Japan) and then incubated with GLP-1R rabbit antibody (1:500; Abcam) and ICAM-1 mouse antibody (1:100; Abcam) for 12 h at 4°C, or with β-actin rabbit antibody (1:1,000; Sigma-Aldrich) for 1 h at room temperature. The membrane was incubated with horseradish-peroxidase-linked donkey anti-rabbit or anti-mouse IgG (1:5,000; GE Healthcare Japan, Tokyo, Japan) at room temperature for 2 h. The blots were then visualised with a western blotting detection system (ECL plus; GE Healthcare).

Culture

Human glomerular microvascular endothelial cells (hGECs) (ACBRI, Kirkland, WA, USA) were cultured in EGM-MV2 medium (Cambrex, East Rutherford, NJ, USA) supplemented with 19.4 mmol/l D-glucose, 10% (vol./vol.) FCS and growth factor within a gelatin-precoated flask in a 5% CO_2 incubator at 37°C.

THP-1 cells (a human monocytic cell line; JCRB, Tokyo, Japan) were cultured in RPMI 1640 supplemented with 10 mmol/l D-glucose, 10% FCS and growth factor in a 5% CO_2 incubator at 37°C.

Human circulating monocytes

The human circulating monocytes were extracted using lymphocyte separation medium (MP Biomedicals, Tokyo, Japan) according to the manufacturer's instructions. After incubation in RPMI 1640 with 50 ng/ml phorbol myristate acetate (Sigma-Aldrich) for 24 h, total RNA was collected from the attaching cells as described above.

The effects of GLP-1 in THP-1 cells

THP-1 cells (1×10^6 cells/ml) were incubated for 24 h in six-well plates in RPMI 1640 medium supplemented with 1% FCS and 5.5 mmol/l D-glucose. THP-1 cells were exposed to the following conditions: (1) 5.5 mmol/l D-glucose (normal glucose); (2) 5.5 mmol/l D-glucose with 9.5 mmol/l mannitol (osmotic control); (3) 15 mmol/l D-glucose (high glucose); (4) high glucose with 2.5 nmol/l exendin-4; (5) high glucose with 10 nmol/l exendin-4; (6) high glucose with 100 nmol/l exendin-4; and (7) high glucose with 100 nmol/l exendin-4 and 1000 nmol/l GLP-1R antagonist (9-39) (Bachem). After incubation for 72 h, total RNA and supernatant fractions were collected from the cells. The supernatant fractions were measured using a human TNF-α and IL-1β immunoassay (Quantikine; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

The effect of GLP-1 in hGECs

After starvation for 12 h, hGECs were exposed to the following conditions: (1) no TNF-α stimulation (control); (2) 100 pg/ml TNF-α alone; (3) TNF-α with 2.5 nmol/l exendin-4; (4) TNF-α with 10 nmol/l exendin-4; (5) TNF-α with 100 nmol/l exendin-4; and (6) TNF-α with 100 nmol/l exendin-4 and 1000 nmol/l GLP-1R antagonist (9-39). After incubation for 6 h, total RNA and protein were collected from cells as described above. Recombinant human TNF-α was purchased from R&D Systems.

Statistical analysis

All values are expressed as the means ± SEM. Differences between groups were examined for statistical significance using the Mann–Whitney test or one-way ANOVA followed by Scheffe's test. Values of $p<0.05$ were considered to indicate statistically significant differences.

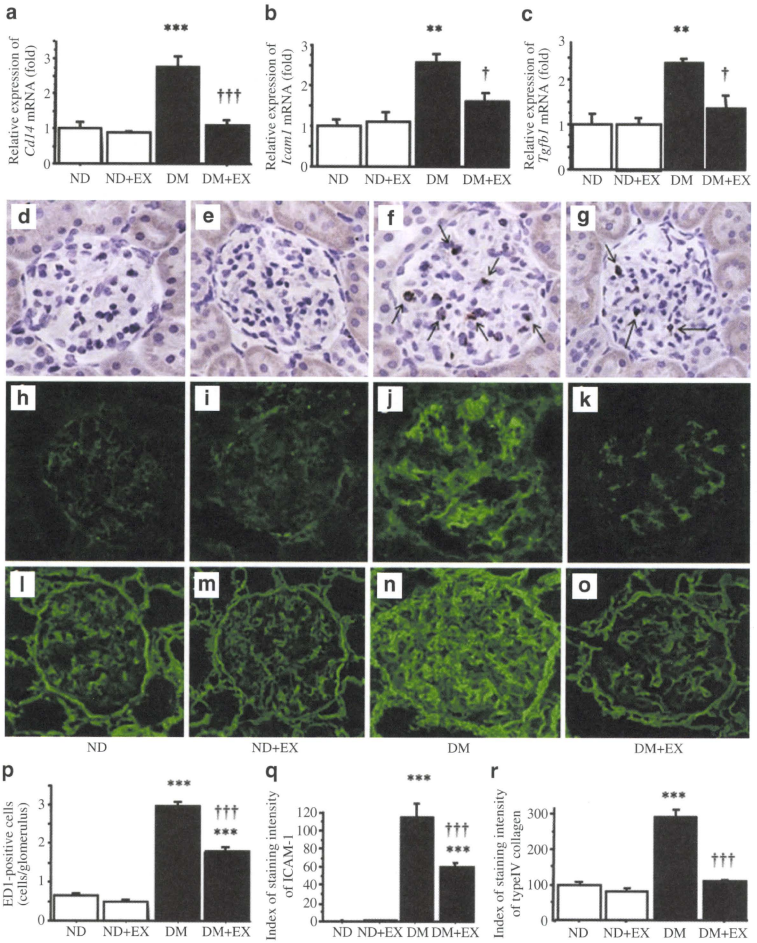

Fig. 2 Exendin-4 treatment suppressed the inflammatory axis in the kidney. **a** Quantification of *Cd14*, (**b**) *Icam1* and (**c**) *Tgfb1* gene expression by real-time RT-PCR in the renal cortex. All three genes were significantly downregulated by exendin-4 treatment. Values (means ± SEM) are presented as fold relative to *Actb* and expressed as 1 in ND; $n=4$ per group. Each experiment was performed three times. $^{**}p<0.01$ and $^{***}p<0.001$ vs non-diabetic (ND) and ND + exendin-4 (EX); $^{\dagger}p<0.05$ and $^{\dagger\dagger\dagger}p<0.001$ vs diabetes (DM). **d–g** Immunoperoxidase staining for macrophages (ED1-positive cells), indicated by arrows. **h–k** Immunofluorescence staining for ICAM-1 and (**l–o**) for type IV collagen. Magnification, all images ×200. **p** Quantification of the number of macrophages per glomerulus, which was significantly increased in the diabetic groups. Exendin-4 treatment significantly prevented glomerular macrophage infiltration in diabetes. $^{***}p<0.001$ vs ND and ND+EX; $^{\dagger\dagger\dagger}p<0.001$ vs DM. **q** Quantification of glomerular ICAM-1 staining per glomerulus, which was significantly increased in the diabetic groups and significantly reduced vs DM by exendin-4 treatment. $^{***}p<0.001$ vs ND and ND+EX; $^{\dagger\dagger\dagger}p<0.001$ vs DM. **r** Quantification of type IV collagen staining per glomerulus. Type IV collagen was significantly increased in the DM group and significantly reduced by exendin-4 treatment. $^{***}p<0.001$ vs ND and ND+EX; $^{\dagger\dagger\dagger}p<0.001$ vs DM. **p–r** Values are the means ± SEM; $n=20$ glomeruli from each rat kidney; $n=4$ per group. Each experiment was repeated three times

Results

Metabolic variables and urinary albumin excretion

Body, organ weights and systolic BP As seen in Table 1, body weights of the diabetic groups at 8 weeks after initiation of exendin-4 treatment were significantly lower than those of the non-diabetic groups. The kidney weights per body weight of the diabetic groups were significantly higher than those of the non-diabetic groups. There were no significant differences among the diabetic groups. Systolic BP was similar in all groups.

Food intake, HbA_{1c} and insulin Food intake and HbA_{1c} were significantly elevated in the diabetic groups. However, there were no significant differences among the diabetic groups. Although GLP-1 has beta cell-protective effects, serum insulin concentration was not detectable in the diabetic groups in spite of the high blood glucose levels (data not shown).

Urinary albumin excretion and creatinine clearance Urinary albumin excretion, which is a characteristic feature of the early stage of diabetic nephropathy, increased progressively in the diabetic groups during the study. Exendin-4 treatment significantly reduced urinary albumin excretion compared with that of the diabetes group at 8 weeks (Fig. 1a). In addition, exendin-4 treatment prevented diabetes-induced hyperfiltration (Fig. 1b).

Kidney morphology

The level of glomerular hypertrophy was significantly higher in the diabetes group than in non-diabetic groups. In contrast, exendin-4 treatment inhibited glomerular hypertrophy (Fig. 1c–f, k) in diabetes. Quantitative analysis showed that mesangial matrix index, which was used as an index of mesangial expansion, was significantly increased in the diabetes group. However, exendin-4 treatment significantly reduced mesangial matrix expansion (Fig. 1l). The renal interstitium showed a significantly higher level of tubular hypertrophy in the diabetic groups than in non-diabetic groups. However, there was no remarkable difference among the diabetic groups. In addition, no histological change of fibrosis in the renal interstitium was seen in any of the groups (Fig. 1g–j).

Microinflammation in the kidney

To evaluate the anti-inflammatory effect of exendin-4 in the kidney, we examined gene expression of *Cd14*, which is regarded as a cell surface marker of macrophages, as well as expression of *Icam1* and *Tgfb1* in the cortex. *Cd14*, *Icam1* and *Tgfb1* were significantly upregulated in the diabetes group and significantly downregulated by exendin-4 treatment (Fig. 2a–c). Regarding the glomeruli, we evaluated macrophage infiltration, and ICAM-1 and type IV collagen levels in glomeruli. The number of macrophages in glomeruli was significantly elevated in the diabetic compared with the non-diabetic groups. In contrast, exendin-4 treatment significantly prevented glomerular macrophage infiltration (Fig. 2d–g, p) in diabetes. The ICAM-1 level was significantly increased in the diabetic groups, but was significantly reduced by exendin-4 treatment (Fig. 2h–k, q). The type IV collagen level, which is an important component in the mesangial matrix, was significantly increased in the diabetes group and significantly reduced by exendin-4 treatment (Fig. 2l–o, r).

Influence of exendin-4 on oxidative stress

To evaluate oxidative stress, we focused on 8-OHdG and NOX4. Urinary excretion of 8-OHdG was significantly increased in the diabetic groups compared with the non-diabetic groups. Exendin-4 treatment significantly decreased urinary excretion of 8-OHdG in diabetes (Fig. 3a). Immunoperoxidase staining for 8-OHdG revealed a significant abundance of 8-OHdG in glomeruli in the diabetic groups, which was significantly reduced by exendin-4 treatment (Fig. 3b–e, j). *Nox4* gene expression in the cortex was significantly upregulated in the diabetes group and significantly downregulated by exendin-4 treatment (Fig. 3k). We demonstrated the presence of NOX4 in glomerular endothelial cells in the rat kidney (ESM Fig. 1h–k). Immunoperoxidase staining for NOX4 revealed a significant abundance of NOX4 in the diabetic kidney. However, exendin-4 treatment significantly reduced the level of NOX4 in diabetes (Fig. 3f–i, l).

NF-κB activation in the kidney

The activation of NF-κB p65 DNA-binding activity was significantly enhanced in the diabetes compared with the non-diabetic groups. Exendin-4 treatment significantly inhibited NF-κB p65 DNA-binding activity in diabetes (Fig. 3m).

GLP-1R in rat glomeruli

We demonstrated the existence of GLP-1R in rat glomeruli (Fig. 4a, d). Double immunofluorescence staining revealed production of GLP-1R on glomerular endothelial cells (Fig. 4e–h). In addition, we ascertained that GLP-1R was produced on macrophages in rat glomeruli (Fig. 4i–l). The GLP-1R levels in glomeruli were not significantly different among the groups (ESM Fig. 1a–g).

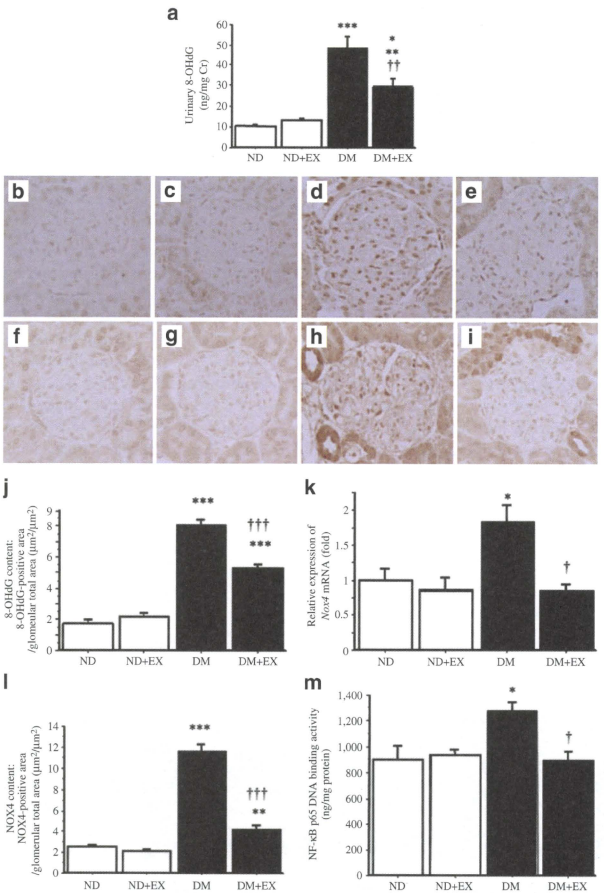

Fig. 3 Exendin-4 treatment suppressed oxidative stress and NF-κB activation. **a** Urinary 8-OHdG concentration in a 24 h urine collection. Urinary 8-OHdG excretion was significantly increased in the diabetic groups (DM). However, exendin-4 treatment (EX) significantly decreased urinary 8-OHdG excretion; $n=5$ per group. The experiment was repeated twice. *$p<0.05$ vs non-diabetic (ND)+EX; **$p<0.01$ vs ND; ***$p<0.001$ vs ND and ND+EX; $^{\dagger\dagger}p<0.01$ vs DM. **b–e** Immunoperoxidase staining for 8-OHdG and (**f–i**) NOX4 in glomeruli. Magnification, all images ×200. **j** Quantification of 8-OHdG content ($\mu m^2/\mu m^2$) as staining per glomerulus. Glomerular 8-OHdG content was significantly increased in the diabetic groups and significantly reduced by exendin-4 treatment. Values are means ± SEM; $n=20$ glomeruli from each rat kidney; $n=4$ per group. ***$p<0.001$ vs ND and ND+EX; $^{\dagger\dagger\dagger}p<0.001$ vs DM. **k** Quantification of *Nox4* by real-time RT-PCR in the renal cortex. *Nox4* expression was significantly downregulated by exendin-4 treatment. Values (means ± SEM) are presented as fold relative to *Actb* and expressed as 1 in ND; $n=4$ per group. Each experiment was repeated three times. *$p<0.05$ vs ND and ND+EX; $^{\dagger}p<0.05$ vs DM. **l** Quantification of NOX4 content ($\mu m^2/\mu m^2$) as staining per glomerulus. NOX4 content was significantly increased in the diabetic groups and significantly suppressed by exendin-4 treatment. Values are means ± SEM; $n=20$ glomeruli from each rat kidney; $n=4$ per group. **$p<0.01$ and ***$p<0.001$ vs ND and ND+EX; $^{\dagger\dagger\dagger}p<0.001$ vs DM. **m** NF-kB p65 DNA-binding activity. NF-κB p65DNA-binding activity was significantly increased in the DM group. Exendin-4 treatment significantly decreased NF-κB p65 DNA-binding activity. Values are the means ± SEM; $n=5$ per group. The experiment was repeated twice. *$p<0.05$ vs ND and ND+EX; $^{\dagger}p<0.05$ vs DM

GLP-1R in human macrophages and hGECs

We identified the existence of GLP-1R in THP-1 cells and hGECs (Figs 5a, b and 6a, b). In addition, we examined *GLP1R* gene expression in human circulating monocytes. We demonstrated that the *GLP-1R* gene was not only expressed in the THP-1 cell line, but also in human circulating monocytes (Fig. 5a).

The effects of GLP-1 through GLP-1R on THP-1 cells and hGECs

THP-1 cells stimulated with a high concentration of glucose for 72 h showed significantly enhanced levels of *TNF* and *IL1B* gene expression. Exendin-4 significantly and dose-dependently attenuated *TNF* and *IL1B* gene expression. Additionally, the effects of exendin-4 were significantly blocked by a GLP-1R antagonist (Fig. 5c, d). Similarly, exendin-4 significantly suppressed TNF-α and IL-1β secretion from THP-1, effects that were also significantly blocked by the GLP-1R antagonist (Fig. 5e, f).

hGECs stimulated with TNF-α for 6 h showed significantly enhanced *ICAM1* gene expression. Exendin-4 significantly and dose-dependently attenuated *ICAM1* gene expression. In addition, the effect of exendin-4 was significantly blocked by the GLP-1R antagonist (Fig. 6c). Likewise, exendin-4 significantly suppressed TNF-α-induced ICAM-1 production on hGECs, an effect that, again, was also significantly blocked by the GLP-1R antagonist (Fig. 6d, e).

Discussion

In the present study, we showed that exendin-4 exerted renoprotective effects through anti-inflammatory actions without lowering the blood glucose level in a streptozotocin-induced rat model of type 1 diabetes. In addition, exendin-4 inhibited NF-κB activity in the kidney, which is known to contribute to cross-talk between inflammation and oxidative stress. We also found that GLP-1R was produced in rat, and in cultured macrophages and glomerular endothelial cells. Exendin-4 acted directly on GLP-1R and attenuated production of pro-inflammatory cytokines and ICAM-1 in vitro. This is the first report of a GLP-1R agonist directly contributing, via its anti-

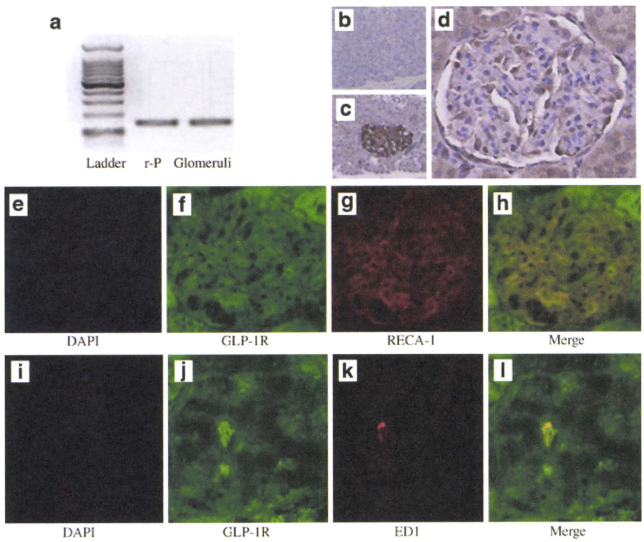

Fig. 4 The production of GLP-1R in rat glomeruli. **a** *Glp1r* gene expression in rat glomeruli. r-P: rat positive control (RIN-5F: rat islet-cell tumour). **b** Immunoperoxidase staining for GLP-1R, with negative control in rat pancreas islet cells, (**c**) positive control in rat pancreas islet cells and (**d**) rat glomeruli. **e–h** Double immunofluorescence staining for GLP-1R and glomerular endothelial cells as labelled. RECA-1, rat endothelial cell antigen. **i–l** Double immunofluorescence staining for GLP-1R and macrophages

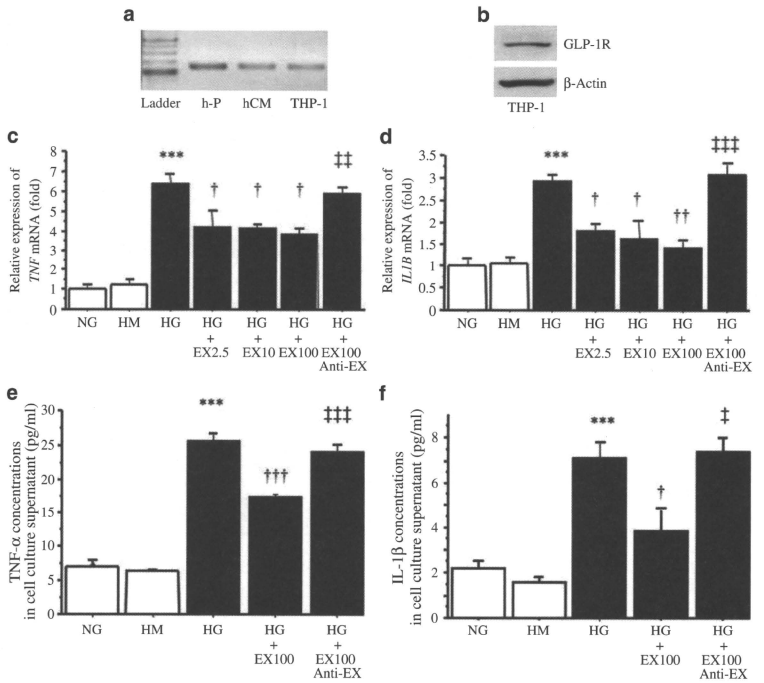

Fig. 5 The direct effects of exendin-4 on THP-1 cells. **a** *GLP1R* gene expression in human circulating monocytes (hCM) and THP-1 cells. h-P, human positive control (human pancreas). **b** GLP-1R protein production in THP-1 cells by western blotting. **c** Quantification of *TNF* and (**d**) *IL1B* mRNA expression in THP-1 by real-time RT-PCR. THP-1 cells stimulated with 15 mmol/l high glucose (HG; 15.0 mmol/l D-glucose) for 72 h showed significantly enhanced *TNF* and *IL1B* expression. Exendin-4 (EX) significantly and dose-dependently suppressed *TNF* and *IL1B* gene expression. GLP-1R antagonist (anti-EX; 1,000 nmol/l) significantly inhibited the suppressive effects of exendin-4 (100 nmol/l) on *TNF* and *IL1B* expression. Values (means ± SEM) are presented as fold relative to *GAPDH* and expressed as 1 in normal glucose (NG; 5.5 mmol/l D-glucose), $n=5$ per group. The experiment was repeated three times. $^{***}p<0.001$ vs NG and 5.5 mmol/l D-glucose with 9.5 mmol/l mannitol (HM); $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$ vs HG; $^{\ddagger\ddagger}p<0.01$ and $^{\ddagger\ddagger\ddagger}p<0.001$ vs HG+EX (100 nmol/l; EX100). **e** Quantification of TNF-α and (**f**) IL-1β secretion (pg/ml) from THP-1 by ELISA. Stimulation of THP-1 cells with HG for 72 h significantly promoted TNF-α and IL-1β secretion. Exendin-4 significantly suppressed TNF-α and IL-1β secretion. GLP-1R antagonist (anti-EX; 1,000 nmol/l) significantly inhibited the suppressive effects of exendin-4 (100 nmol/l) on TNF-α and IL-1β secretion. Values are the means ± SEM; $n=5$ per group. The experiment was repeated twice. $^{***}p<0.001$ vs NG and HM; $^{\dagger}p<0.05$ and $^{\dagger\dagger\dagger}p<0.001$ vs HG; $^{\ddagger}p<0.05$ and $^{\ddagger\ddagger\ddagger}p<0.001$ vs HG+EX100. EX2.5, 2.5 nmol/l exendin-4; EX10, 10 nmol/l exendin-4

inflammatory effects, to amelioration of characteristic features of diabetic nephropathy, such as increased urinary albumin excretion, glomerular hypertrophy and mesangial matrix expansion.

The current results suggest that exendin-4 alleviated the above-mentioned features by suppressing: (1) ICAM-1 production; (2) macrophage infiltration; (3) NF-κB activation; (4) oxidative stress; and (5) *Tgfb1* mRNA expression and type IV collagen accumulation in the kidney.

An increase in the level of ICAM-1 on glomerular endothelial cells promotes macrophage infiltration into glomeruli [12, 14]. In our study, exendin-4 prevented macrophage infiltration into glomeruli. The mechanism underlying this effect was thought to be the suppression of ICAM-1 production on glomerular endothelial cells and direct inhibition of cytokine release from macrophages, which breaks the vicious cycle between macrophages and glomerular endothelial cells that gives rise to microinflam-

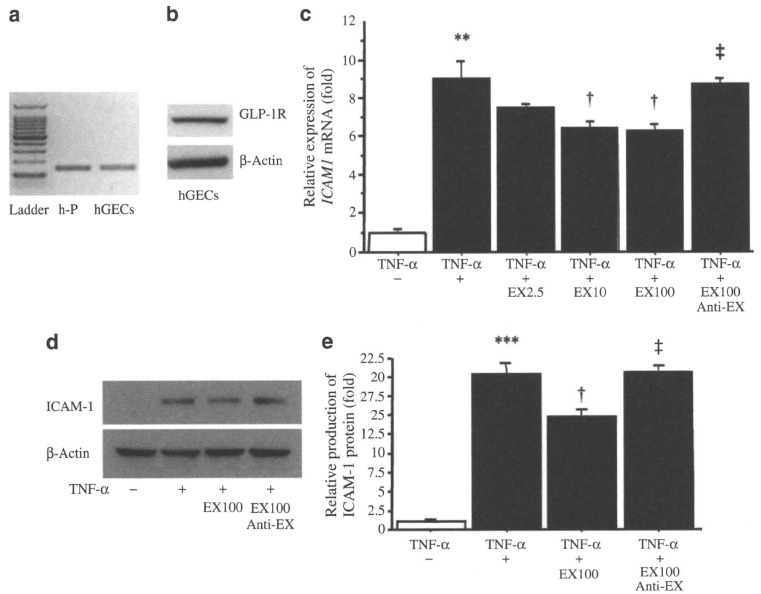

Fig. 6 The direct effects of exendin-4 on hGECs. **a** *GLP1R* gene expression in hGECs. h-P, human positive control (human pancreas). **b** GLP-1R protein production in hGECs by western blotting. **c** Quantification of *ICAM1* expression in hGECs by real-time RT-PCR. hGECs stimulated with TNF-α (100 pg/ml) for 6 h showed significantly enhanced *ICAM1* expression. Exendin-4 (EX) significantly and dose-dependently suppressed *ICAM1* gene expression. GLP-1R antagonist (1,000 nmol/l; anti-EX) significantly inhibited the suppressive effect of 100 nmol/l exendin-4 (EX100) on *ICAM1* expression. Values (means ± SEM) are presented as fold relative to *ACTB* and expressed as 1 in control (no TNF-α stimulation); $n=5$ per group. The experiment was repeated three times. $^{**}p<0.01$ vs control; $^{\dagger}p<0.05$ vs TNF-α stimulation; $^{\ddagger}p<0.05$ vs TNF-α + 100 nmol/l EX (EX100). **d** ICAM-1 production in hGECs by western blotting analysis, with (**e**) quantification. hGECs stimulated with TNF-α (100 pg/ml) for 6 h significantly promoted ICAM-1 production. Exendin-4 significantly suppressed ICAM-1 production. Anti-EX significantly inhibited the suppressive effect of EX100 on ICAM-1 production. Values (means ± SEM) are presented as fold relative to β-actin and expressed as 1 in control (no TNF-α stimulation); $n=5$ per group. The experiment was repeated twice. $^{***}p<0.01$ vs control; $^{\dagger}p<0.05$ vs TNF-α stimulation; $^{\ddagger}p<0.05$ vs TNF-α + EX100. EX2.5, 2.5 nmol/l exendin-4; EX10, 10 nmol/l exendin-4

mation. In HUVECs, treatment with liraglutide, a long-acting GLP-1 analogue, has also been shown to inhibit TNF-α or hyperglycaemia-mediated induction of ICAM-1 gene and protein [30]. These reports support our present findings. In our study, high glucose (15 mmol/l) stimulation for a period of 24 to 72 h did not significantly enhance *ICAM1* gene expression in hGECs (data not shown).

Macrophages play a critical role in the development of diabetic nephropathy. In vitro, the culture supernatant fraction of macrophages has been shown to stimulate mesangial cells to produce fibronectin [31], while macrophages directly secrete TGF-β [32]. Both of these processes play a central role in the enhancement of glomerular extracellular matrix production in diabetic nephropathy [33, 34]. Based on these previous and our present findings, we conclude that the inhibition of macrophage infiltration by exendin-4 has a beneficial effect on suppressing progression of diabetic nephropathy.

In the diabetic state, many factors contribute to elevated NF-κB activation [35]. NF-κB is also the most important transcription factor regulating ICAM-1 production [36]. Arakawa et al. [37] reported that exendin-4 suppressed NF-κB activation of lipopolysaccharide-induced macrophages, suggesting that exendin-4 reduced direct NF-κB activation in macrophages. The reduction of NF-κB activity by exendin-4 may lead to inhibition of ICAM-1 levels and suppression of pro-inflammatory cytokines derived from macrophages.

Oxidative stress and inflammation are closely related to each other and create a vicious cycle in the diabetic state. Gorin et al. [38] showed that NADPH oxidase, and especially the NOX4 component of NADPH in the kidney, is important as the major source of oxidative stress in streptozotocin-induced diabetic nephropathy. Although many stimuli activate NOX4 production, cytokines and shear stress are important factors in the diabetic state [39]. NOX4 has been reported to be produced on epithelial cells [40] and mesangial cells [27], and was confirmed to be produced on endothelial cells in this study. In our study, exendin-4 suppressed NOX4 levels in the kidney. We speculate that reducing the release of pro-inflammatory cytokines from macrophages and normalising hyperfiltration by exendin-4 treatment may have contributed to the suppression of NOX4 production. Etoh et al. [27] reported that localisation and levels of NOX4 were in parallel with those of 8-OHdG. Therefore, the reduction of NOX4 level by exendin-4 treatment would contribute to a decrease in 8-OHdG production in glomeruli. Park et al. [25] also reported similar results in regard to 8-OHdG reduction by exendin-4 in a mouse model of type 2 diabetes. We speculate that exendin-4 contributes to an attenuation of oxidative stress and that this helps ameliorate diabetic vascular complications.

It is well known that GLP-1 signalling through GLP-1R enhances cyclic AMP as a second messenger [41]. Previous reports have revealed that an increase in activity of the cyclic AMP/protein kinase A pathway suppresses NF-κB activity in THP-1 cells and HUVECs [42], and inhibits NADPH oxidase [43]. These findings support our finding that exendin-4 modulated the inflammatory vicious cycle in the kidney.

In our model, exendin-4 did not affect blood glucose levels, blood pressure, food intake or body weight as it has been shown to do in models of type 2 diabetes. To determine that the effects of exendin-4 occurred without lowering of blood glucose, we started exendin-4 treatment at 1 week after the streptozotocin injections and confirmed that exendin-4 did not restore insulin secretion in our model. A much higher dose than that used in our study would have been necessary to reduce blood pressure in diabetic rats [26]. GLP-1 inhibits food intake and results in weight loss [18, 20, 21]. In the present study, the non-diabetic group treated with exendin-4 had decreased food intake and weight loss compared with the control group, but there were no significant differences. It is difficult to differentiate the effect of exendin-4 from the significant weight reduction that is generally seen in the model of type 1 diabetes.

In this study, the ratio of kidney weight to body weight in the diabetic groups was significantly increased in diabetic rats compared with the non-diabetic groups. However, exendin-4 treatment did not affect them. As periodic acid–Schiff's reagent staining revealed, exendin-4 did not ameliorate tubular hypertrophy. Tubular hypertrophy may be the main factor contributing to kidney weight, and we need to investigate a longer period to appreciate the effect of exendin-4 on tubular hypertrophy and interstitial fibrosis. Additionally, exendin-4 prevented diabetes-induced hyperfiltration. Previous reports have revealed that hyperfiltration was improved by exendin-4 treatment in obese diabetic patients [44] and the *db/db* mouse model [25]. There has been no report that exendin-4 affects creatinine clearance in a later stage of diabetic nephropathy.

The inflammatory process is involved in the mechanism of obesity-related insulin resistance [45] and in the pathogenesis of atherosclerosis [46]. Moreover, there is also a close relationship between chronic renal insufficiency and the cardiorenal syndrome, through several pathways including inflammation [47, 48]. GLP-1R agonists might be beneficial for these diseases through their anti-inflammatory effects. Recently, Arakawa et al. [37] reported an anti-inflammatory effect of exendin-4 in an animal model of atherosclerosis. Their report also pointed out the importance of exendin-4 as a potential therapeutic agent for cardiovascular disease in diabetes.

In conclusion, we have shown that exendin-4 exerts renoprotective effects through anti-inflammatory actions without lowering blood glucose in a streptozotocin-induced rat model of type 1 diabetes. Furthermore, exendin-4 directly acted on GLP-1R and suppressed production of pro-inflammatory cytokines and ICAM-1. This study may provide the first evidence that GLP-1R agonists directly contribute to the prevention of diabetic nephropathy via an anti-inflammatory effect.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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Microinflammationの関与

Microinflammation in diabetic microvascular complications

梶谷展生[1]　四方賢一[2]

Key words：細胞接着分子，ケモカイン，サイトカイン，VEGF，マクロファージ

はじめに

糖尿病性腎症の腎組織では，マクロファージを主体とする炎症細胞浸潤，細胞接着分子やケモカインの発現および炎症性サイトカインの増加など，炎症と共通した特徴が認められる．また，糖尿病網膜症の網膜組織においても白血球の集積や細胞接着分子の発現増加などが認められ，糖尿病性細小血管症の病態に炎症が関与していることが示唆される．

糖尿病性細小血管症の病態形成における‘炎症’とは，関節リウマチなどの炎症性疾患にみられる‘発熱，発赤，腫脹，疼痛，機能障害’を主徴とする従来の炎症の概念とは異なっている．すなわち高感度測定系で検出されるCRP(高感度CRP)の軽度の上昇などに特徴づけられる，血管を首座とする軽度の炎症を意味しており，‘microinflammation’や‘low grade inflammation’などと呼ばれている．慢性高血糖状態では，ポリオール代謝異常，プロテインキナーゼC(PKC)活性化，糖化反応(グリケーション)や酸化ストレスの亢進，レニン・アンジオテンシン系の活性化などが認められ，これらの因子が複合的に関与して炎症を惹起し糖尿病性細小血管症の病態に関与するものと考えられている．

本稿では，糖尿病性細小血管症におけるmicroinflammationの役割について概説する．

1. 網膜症におけるmicroinflammation

糖尿病網膜症は，網膜血管障害が機転となり血管透過性の亢進や毛細血管閉塞から網膜の虚血が惹起され，脆弱な網膜新生血管の形成を経て増殖網膜症へ至る．

糖尿病網膜症を有する患者は網膜症を有さない患者に比べて血清中のCRPやICAM-1が有意に高値であると報告されている[1]．また糖尿病モデルラットの網膜では血管内皮細胞におけるICAM-1発現が増加し，網膜血管に多くの白血球が接着している[2]．ICAM-1およびその白血球側のリガンドであるCD18を欠損したマウスでは糖尿病状態における網膜血管の白血球増加，血管透過性の亢進や網膜血管の変性が抑制されたとの報告もあり，これらは糖尿病網膜症の発症進展におけるmicroinflammationの関与を示唆するものと考えられる．

網膜におけるmicroinflammationを惹起するkey moleculeの一つとしてvascular endothelial growth factor(VEGF)が考えられている．VEGFは網膜の内皮細胞，色素上皮細胞，ミュラー細胞や周皮細胞など種々の細胞に発現が認められ，主として虚血により誘導される．糖尿病においては虚血所見が明らかになる前からVEGFの発現亢進が認められるが，その誘引として高血糖やそれに引き続くPKC経路の活性化，糖化反

[1]Nobuo Kajitani: Medicine and Clinical Science, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Science　岡山大学大学院医歯薬学総合研究科 腎・免疫・内分泌代謝内科学　[2]Kenichi Shikata: Center for Innovative Clinical Medicine, Okayama University Hospital　岡山大学病院 新医療研究開発センター

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応(グリケーション)や酸化ストレスの亢進，レニン・アンジオテンシン系の活性化などが考えられている．VEGFは血管内皮細胞増殖作用と血管透過性亢進作用の両者を併せもつ唯一の増殖因子であることから，増殖網膜症や糖尿病黄斑浮腫の病態と深く関係していると考えられている．

2. 腎症における microinflammation

糖尿病性腎症患者の腎生検組織では糸球体や間質におけるマクロファージの数が増加しており，炎症性サイトカインの放出などを介して糸球体硬化や間質の線維化に関与していることが示唆される[3]．一般に，炎症巣への白血球の浸潤は細胞接着分子とケモカインにより誘導される．糖尿病性腎症患者の腎組織では糸球体や間質の小静脈にICAM-1とE-セレクチン，P-セレクチンの発現が亢進しており[3]，これらの接着分子が糸球体と間質へのマクロファージの浸潤を誘導していると考えられる．内皮細胞に発現する接着分子の多くは，細胞膜から外れて血液中に放出される性質をもっている(shedding)．炎症性疾患では，血管内皮細胞における発現亢進の結果としてICAM-1やVCAM-1の血液中濃度が上昇することが知られているが，糖尿病患者においても血中可溶性ICAM-1およびVCAM-1濃度が増加しており，腎症を有する患者では更に高値となる[4,5]．

ストレプトゾトシンで糖尿病を誘発したラットの腎臓では糖尿病誘発後に1週間以内にICAM-1の発現とマクロファージの浸潤の増加を認め，インスリン投与による血糖正常化によりICAM-1の発現とマクロファージの浸潤が抑制される．また抗ICAM-1抗体を投与することにより腎糸球体へのマクロファージの浸潤が抑制されることから，高血糖によるICAM-1の発現増加がマクロファージの浸潤を誘導していると考えられた[6]．

糖尿病性腎症の成因におけるマクロファージの関与を直接的に証明した研究は少ない．しかし，放射線照射で白血球を減少させたラットでは糖尿病発症後の腎症の進展が抑制されること[7]，免疫抑制薬でマクロファージの浸潤を減少させることにより腎症を抑制できる[8]などの報告があり，糖尿病性腎症の発症進展にマクロファージが重要な役割を果たしていることが強く示唆された．

著者らは糖尿病性腎症の成因におけるマクロファージの役割を明らかにするために，ICAM-1ノックアウト(KO)マウスにストレプトゾトシンで糖尿病を誘発し，6カ月後の腎症の変化を検討した．ICAM-1 KOマウスではアルブミン尿の増加の抑制，糸球体肥大やメサンギウム基質の増加，間質の線維化の抑制が認められ，腎臓におけるTGF-βとIV型コラーゲンの発現も低下していた[9]．著者らは1型糖尿病モデルを用いて上記の検討を行ったが，その後Chowらにより2型糖尿病モデルであるdb/dbマウスにおいてもICAM-1欠損により腎障害の進展が著しく抑制されることが明らかとなり[10]，2型糖尿病モデルにおいても著者らの結果が裏付けられた．

糖尿病性腎症の成因に関しては，これまでに多くのメカニズムが明らかにされてきた．上述してきたように，著者らは糖尿病性腎症の進展因子としてマクロファージを中心としたmicroinflammationが重要な役割を果たしていることを明らかにしてきた．糖尿病性腎症の成因におけるmicroinflammationの関与について，著者らは**図1**に示すような機序を考えている[11]．腎症の成因の最上流に位置するものは高血糖であるが，高血糖から腎組織障害に至る過程には様々な経路が想定されている．これらの因子が複合的に関与して血管内皮細胞機能障害を惹起し，ICAM-1をはじめとする接着分子やケモカインの発現が誘導すると考えられる．腎組織に浸潤したマクロファージはTGF-βなどのサイトカインを介してIV型コラーゲンなどの細胞外基質の産生を亢進させ，糖尿病性腎症の病変を形成するものと考えられる．

近年，糖尿病性腎症の重要な臨床的特徴であるアルブミン尿が心血管死と関連していることが報告され[12]，アルブミン尿は全身の血管内皮障害の指標となりうることが示された．著者ら

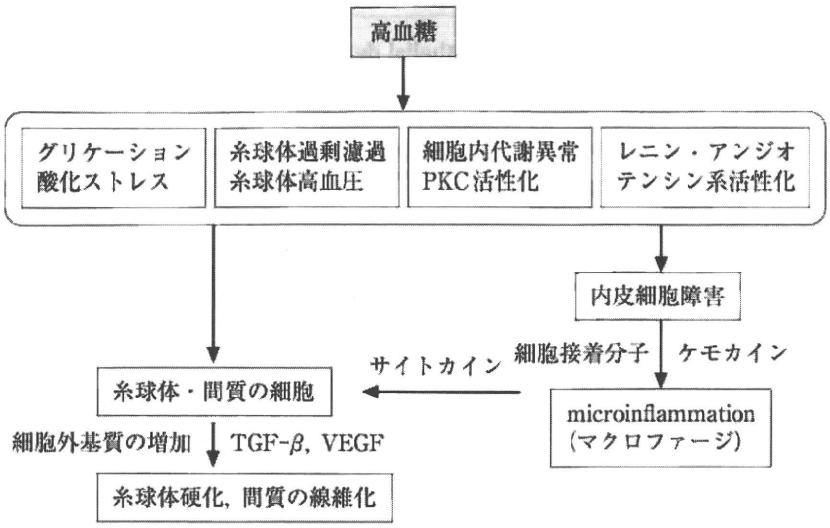

図1 糖尿病性腎症の成因における microinflammation の関与
(文献[11]より改変)

は2型糖尿病患者において炎症性サイトカインであるIL-18の血清濃度がアルブミン尿および動脈硬化の指標である脈波伝播速度(PWV)と相関し，更に血中および尿中のIL-18濃度が腎症の進展予測因子となりうることを見いだした[13]．また，腎症を有する2型糖尿病患者において種々の血清中炎症性サイトカイン，ケモカイン，細胞接着分子濃度がアルブミン尿や，PWV・頸動脈内膜中膜複合体厚(IMT)と相関することを報告した[14]．microinflammation は細小血管障害のみならず大血管障害の成因とも関連していることが示唆される．

3. 糖尿病性神経障害における microinflammation

糖尿病性神経障害における microinflammation の関与については網膜症や腎症ほどの報告はないものの，末梢神経の虚血・再灌流障害に伴う microinflammation の関与が報告されている．

高血糖に伴う血管内皮機能障害により神経栄養血管の循環障害が生じると，好中球・単球の浸潤を伴い急性炎症反応が生じる．糖尿病状態では神経細胞は虚血・再灌流による形態学的な脆弱性を認めており，正常神経では形態学的な変化を呈さない程度の虚血によっても病理学的な異常を呈することが報告されている[15]．その原因として，糖尿病状態では慢性高血糖に伴うポリオール代謝異常，グリケーション，PKC経路の活性化などにより神経細胞は既に酸化ストレスにさらされており，そこに虚血・再灌流による更なる酸化ストレスが加わることで炎症の増悪をきたすものと考えられている．

おわりに

糖尿病性細小血管症における microinflammation の役割について概説した．microinflammation は糖尿病性細小血管症の発症・進展に関与しており，microinflammation の制御は細小血管障害の治療ターゲットとなる可能性がある．糖尿病性腎症においては，免疫抑制薬であるメトトレキサートやミゾリビン，抗炎症作用を有するマクロライド系抗生物質(エリスロマイシン)などは，糖尿病ラットの腎臓における microinflammation を抑制することによって腎障害の進展を抑制することが報告されている．血管における炎症を制御する治療は，糖尿病性細小血管症に対する新しい治療戦略となることが期待される．