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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

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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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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 calculated 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
$\mathrm{HbA_{1c}}$ (%)	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

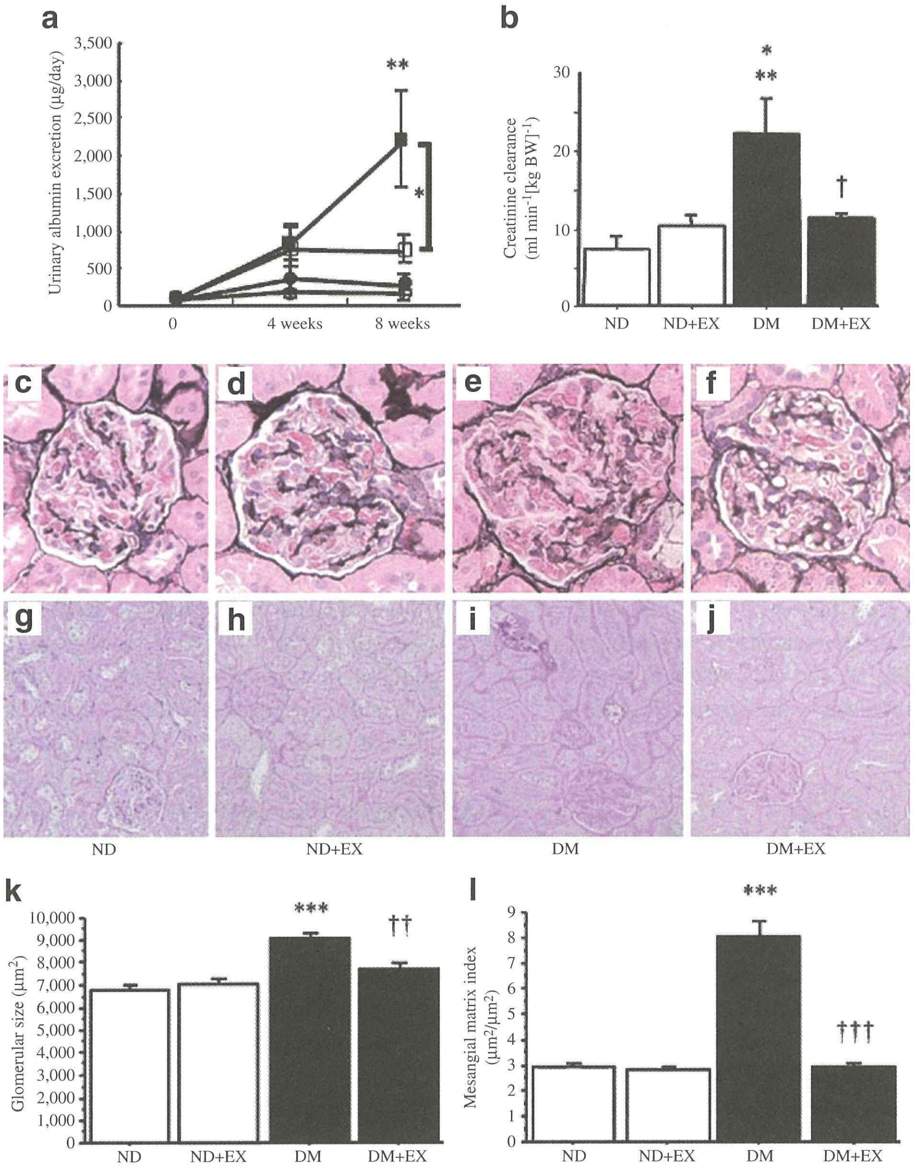

Fig. 1 **a** Time course of 24 h urinary albumin excretion. Urinary albumin excretion increased gradually over 8 weeks in the diabetic group. Exendin-4 resulted in a significantly lower level of urinary albumin excretion at 8 weeks than in the untreated diabetes group. *$p < 0.05$; **$p<0.01$ vs non-diabetic 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.

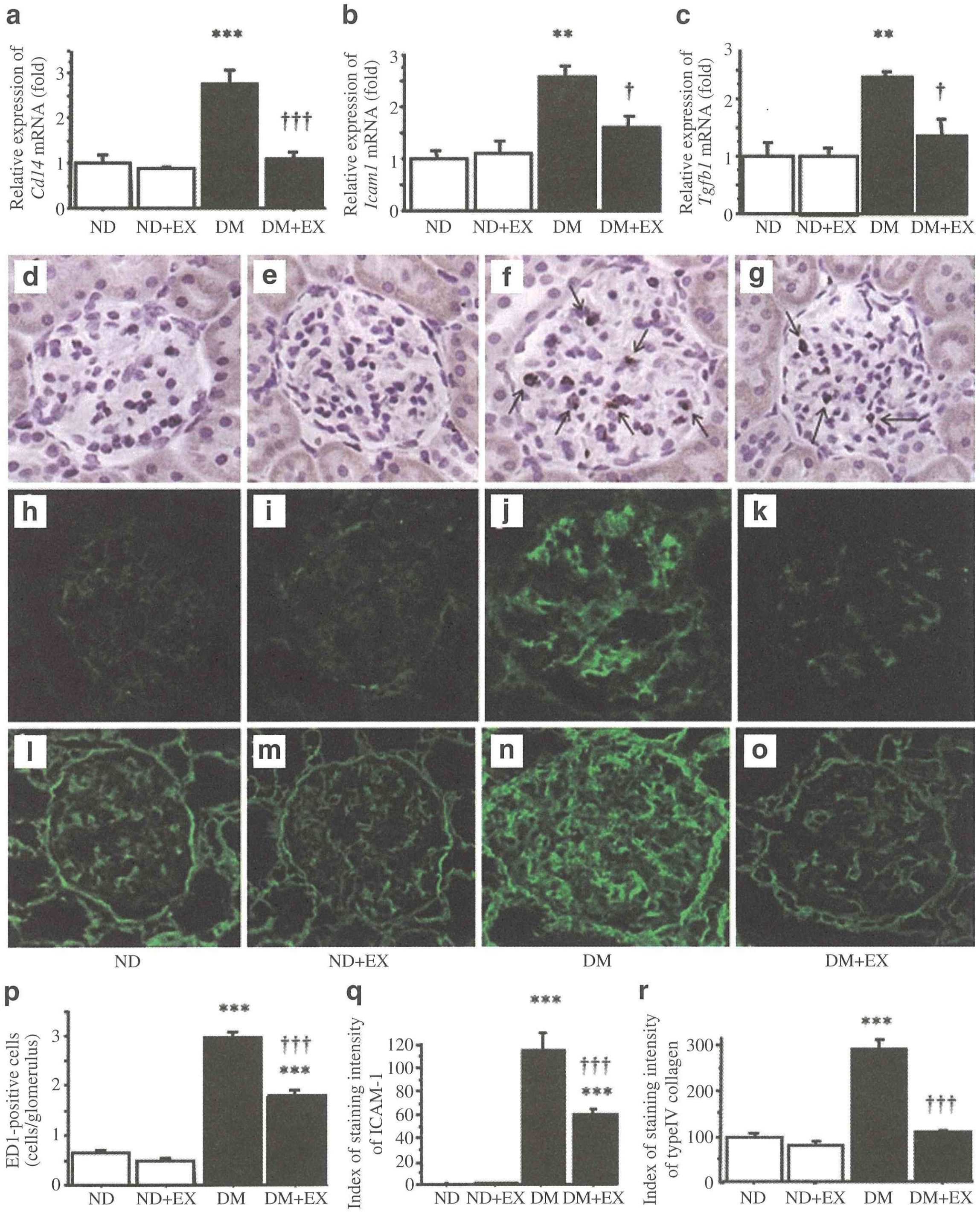

Fig. 2 Exendin-4 treatment suppressed the inflammatory axis in the kidney. **a** Quantification of *Cd14*, (**b**) *Icam1* and (**c**) *Tgfb1* gene expression by real-time RT-PCR in the renal cortex. All three genes were significantly downregulated by exendin-4 treatment. Values (means ± SEM) are presented 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).

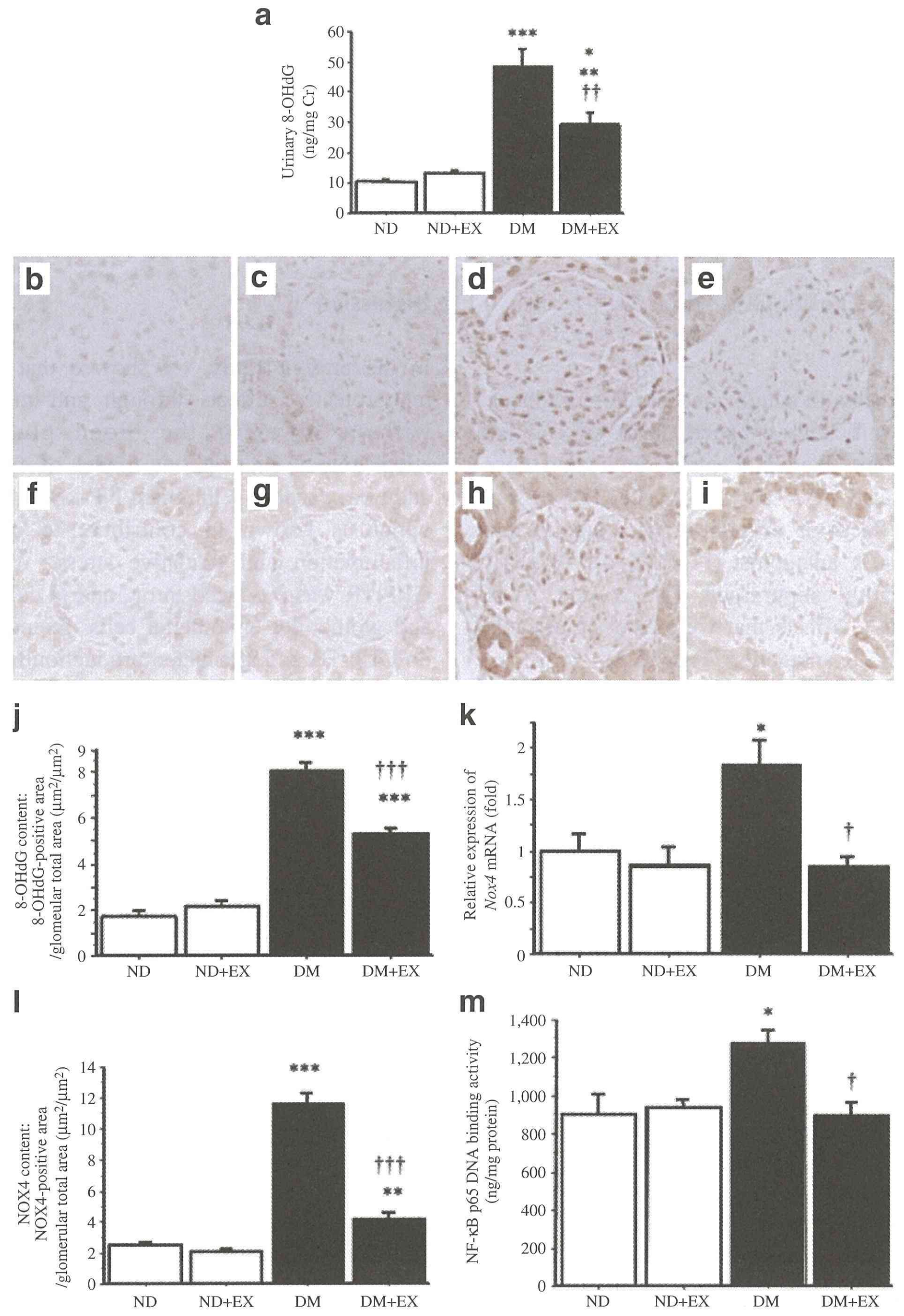

Fig. 3 Exendin-4 treatment suppressed oxidative stress and NF-κB activation. **a** Urinary 8-OHdG concentration in a 24 h urine collection. Urinary 8-OHdG excretion was significantly increased in the diabetic groups (DM). However, exendin-4 treatment (EX) significantly decreased urinary 8-OHdG excretion; $n=5$ 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; $^{††}p<0.01$ 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; $^{†††}p<0.001$ 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; $^{†}p<0.05$ 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; $^{†††}p<0.001$ 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; $^{†}p<0.05$ 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-

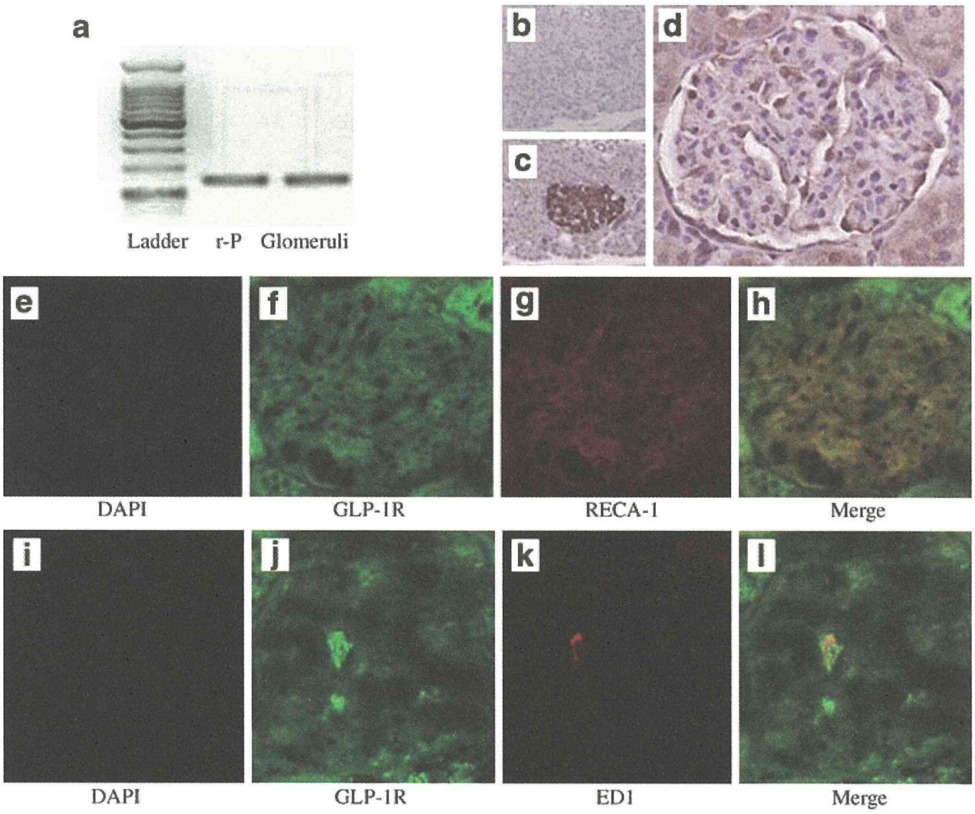

Fig. 4 The production of GLP-1R in rat glomeruli. **a** *Glp1r* gene expression in rat glomeruli. r-P: rat positive control (RIN-5F: rat islet-cell tumour). **b** Immunoperoxidase staining for GLP-1R, with negative control in rat pancreas islet cells, (**c**) positive control in rat pancreas islet cells 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

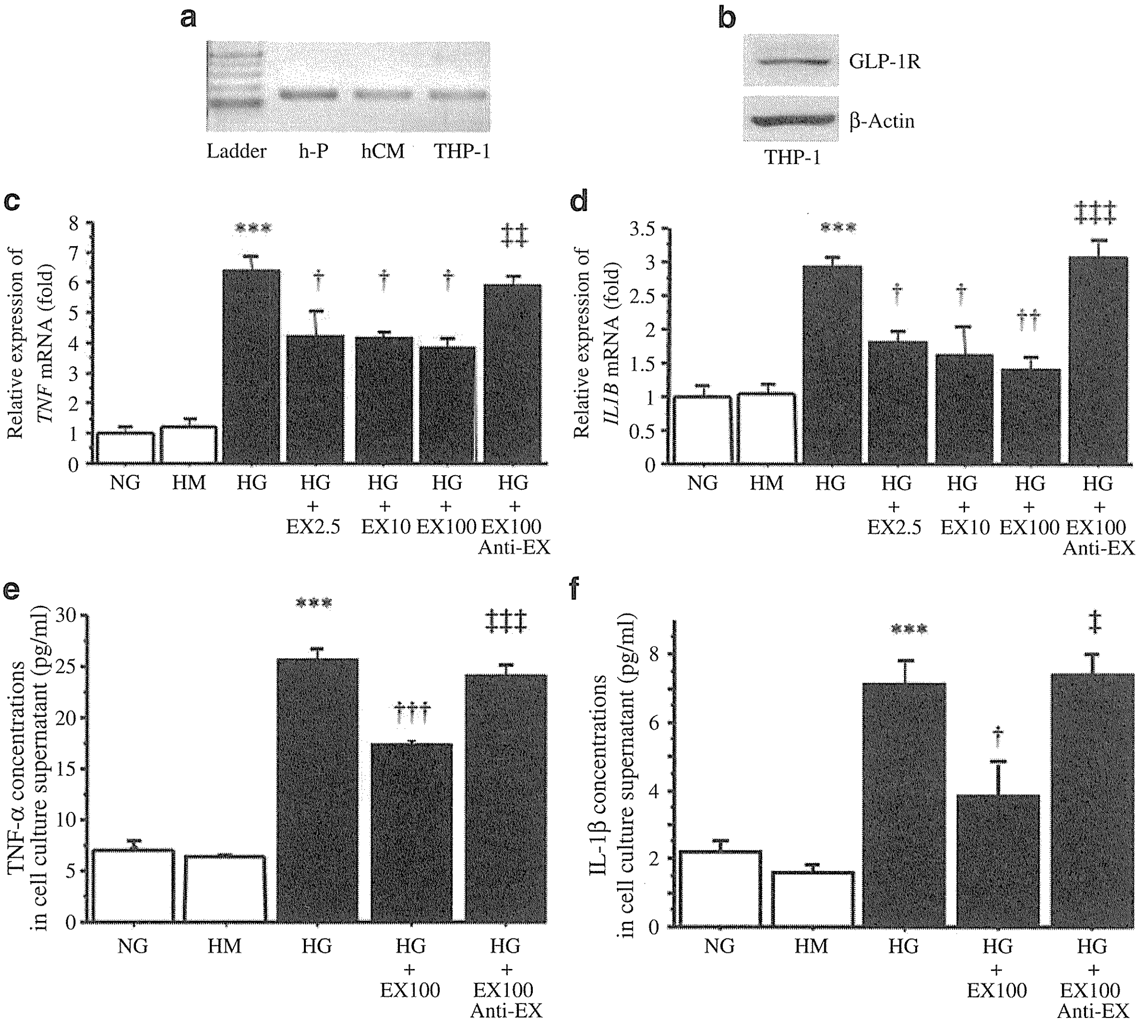

Fig. 5 The direct effects of exendin-4 on THP-1 cells. **a** *GLP1R* gene expression in human circulating monocytes (hCM) and THP-1 cells. h-P, human positive control (human pancreas). **b** GLP-1R protein production in THP-1 cells by western blotting. **c** Quantification of *TNF* and (**d**) *IL1B* 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-

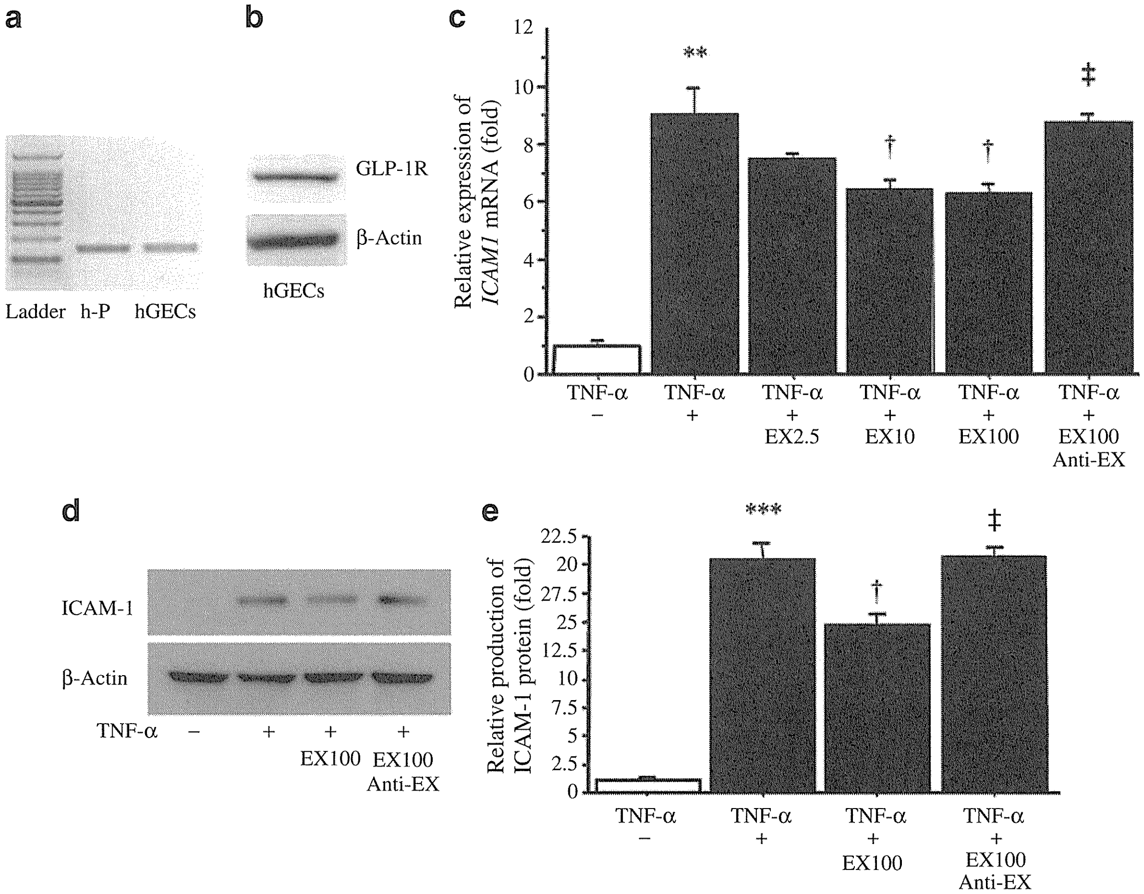

Fig. 6 The direct effects of exendin-4 on hGECs. **a** *GLP1R* gene expression in hGECs. h-P, human positive control (human pancreas). **b** GLP-1R protein production in hGECs by western blotting. **c** Quantification of *ICAM1* expression in hGECs by real-time RT-PCR. hGECs stimulated with TNF-α (100 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.

References

1. de Zeeuw D, Parving HH, Henning RH (2006) Microalbuminuria as an early marker for cardiovascular disease. J Am Soc Nephrol 17:2100–2105
2. Go AS, Chertow GM, Fan D, McCulloch CE, Hsu CY (2004) Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. N Engl J Med 351:1296–1305
3. Magee GM, Bilous RW, Cardwell CR, Hunter SJ, Kee F, Fogarty DG (2009) Is hyperfiltration associated with the future risk of developing diabetic nephropathy? A meta-analysis. Diabetologia 52:691–697

4. Baynes JW (1991) Role of oxidative stress in development of complications in diabetes. Diabetes 40:405–412
5. Brownlee M, Cerami A, Vlassara H (1988) Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. N Engl J Med 318:1315–1321
6. Koya D, Jirousek MR, Lin YW, Ishii H, Kuboki K, King GL (1997) Characterization of protein kinase C beta isoform activation on the gene expression of transforming growth factor-beta, extracellular matrix components, and prostanoids in the glomeruli of diabetic rats. J Clin Invest 100:115–126
7. Ziyadeh FN, Sharma K, Ericksen M, Wolf G (1994) Stimulation of collagen gene expression and protein synthesis in murine mesangial cells by high glucose is mediated by autocrine activation of transforming growth factor-beta. J Clin Invest 93:536–542
8. Nelson CL, Karschimkus CS, Dragicevic G et al (2005) Systemic and vascular inflammation is elevated in early IgA and type 1 diabetic nephropathies and relates to vascular disease risk factors and renal function. Nephrol Dial Transplant 20:2420–2426
9. Saraheimo M, Teppo AM, Forsblom C, Fagerudd J, Groop PH (2003) Diabetic nephropathy is associated with low-grade inflammation in type 1 diabetic patients. Diabetologia 46:1402–1407
10. Furuta T, Saito T, Ootaka T et al (1993) The role of macrophages in diabetic glomerulosclerosis. Am J Kidney Dis 21:480–485
11. Hirata K, Shikata K, Matsuda M et al (1998) Increased expression of selectins in kidneys of patients with diabetic nephropathy. Diabetologia 41:185–192
12. Sugimoto H, Shikata K, Hirata K et al (1997) Increased expression of intercellular adhesion molecule-1 (ICAM-1) in diabetic rat glomeruli: glomerular hyperfiltration is a potential mechanism of ICAM-1 upregulation. Diabetes 46:2075–2081
13. Okada S, Shikata K, Matsuda M et al (2003) Intercellular adhesion molecule-1-deficient mice are resistant against renal injury after induction of diabetes. Diabetes 52:2586–2593
14. Yozai K, Shikata K, Sasaki M et al (2005) Methotrexate prevents renal injury in experimental diabetic rats via anti-inflammatory actions. J Am Soc Nephrol 16:3326–3338
15. Tone A, Shikata K, Sasaki M et al (2005) Erythromycin ameliorates renal injury via anti-inflammatory effects in experimental diabetic rats. Diabetologia 48:2402–2411
16. Ohga S, Shikata K, Yozai K et al (2007) Thiazolidinedione ameliorates renal injury in experimental diabetic rats through anti-inflammatory effects mediated by inhibition of NF-kappaB activation. Am J Physiol Renal Physiol 292:F1141–F1150
17. Usui H, Shikata K, Matsuda M et al (2003) HMG-CoA reductase inhibitor ameliorates diabetic nephropathy by its pleiotropic effects in rats. Nephrol Dial Transplant 18:265–272
18. Baggio LL, Drucker DJ (2007) Biology of incretins: GLP-1 and GIP. Gastroenterology 132:2131–2157
19. Farilla L, Hui H, Bertolotto C et al (2002) Glucagon-like peptide-1 promotes islet cell growth and inhibits apoptosis in Zucker diabetic rats. Endocrinology 143:4397–4408
20. Nauck MA, Niedereichholz U, Ettler R et al (1997) Glucagon-like peptide 1 inhibition of gastric emptying outweighs its insulinotropic effects in healthy humans. Am J Physiol 273:E981–E988
21. Turton MD, O'Shea D, Gunn I et al (1996) A role for glucagon-like peptide-1 in the central regulation of feeding. Nature 379:69–72
22. Orskov C, Wettergren A, Holst JJ (1993) Biological effects and metabolic rates of glucagon like peptide-1 7-36 amide and glucagon like peptide-1 7-37 in healthy subjects are indistinguishable. Diabetes 42:658–661
23. Bullock BP, Heller RS, Habener JF (1996) Tissue distribution of messenger ribonucleic acid encoding the rat glucagon-like peptide-1 receptor. Endocrinology 137:2968–2978
24. Schlatter P, Beglinger C, Drewe J, Gutmann H (2007) Glucagon-like peptide 1 receptor expression in primary porcine proximal tubular cells. Regul Pept 141:120–128
25. Park CW, Kim HW, Ko SH et al (2007) Long-term treatment of glucagon-like peptide-1 analog exendin-4 ameliorates diabetic nephropathy through improving metabolic anomalies in db/db mice. J Am Soc Nephrol 18:1227–1238
26. Hirata K, Kume S, Araki S et al (2009) Exendin-4 has an anti-hypertensive effect in salt-sensitive mice model. Biochem Biophys Res Commun 380:44–49
27. Etoh T, Inoguchi T, Kakimoto M et al (2003) Increased expression of NAD(P)H oxidase subunits, NOX4 and p22phox, in the kidney of streptozotocin-induced diabetic rats and its reversibity by interventive insulin treatment. Diabetologia 46:1428–1437
28. Fong JS, Drummond KN (1968) Method for preparation of glomeruli for metabolic studies. J Lab Clin Med 71:1034–1039
29. Shigenaga MK, Gimeno CJ, Ames BN (1989) Urinary 8-hydroxy-2′-deoxyguanosine as a biological marker of in vivo oxidative DNA damage. Proc Natl Acad Sci U S A 86:9697–9701
30. Liu H, Dear AE, Knudsen LB, Simpson RW (2009) A long-acting glucagon-like peptide-1 analogue attenuates induction of plasminogen activator inhibitor type-1 and vascular adhesion molecules. J Endocrinol 201:59–66
31. Pawluczyk IZ, Harris KP (1997) Macrophages promote prosclerotic responses in cultured rat mesangial cells: a mechanism for the initiation of glomerulosclerosis. J Am Soc Nephrol 8:1525–1536
32. Leonarduzzi G, Scavazza A, Biasi F et al (1997) The lipid peroxidation end product 4-hydroxy-2,3-nonenal up-regulates transforming growth factor beta1 expression in the macrophage lineage: a link between oxidative injury and fibrosclerosis. Faseb J 11:851–857
33. Ziyadeh FN, Hoffman BB, Han DC et al (2000) Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal antitransforming growth factor-beta antibody in *db/db* diabetic mice. Proc Natl Acad Sci U S A 97:8015–8020
34. Sharma K, Ziyadeh FN (1995) Hyperglycemia and diabetic kidney disease. The case for transforming growth factor-beta as a key mediator. Diabetes 44:1139–1146
35. Guijarro C, Egido J (2001) Transcription factor-kappa B (NF-kappa B) and renal disease. Kidney Int 59:415–424
36. Roebuck KA, Finnegan A (1999) Regulation of intercellular adhesion molecule-1 (CD54) gene expression. J Leukoc Biol 66:876–888
37. Arakawa M, Mita T, Azuma K et al (2010) Inhibition of monocyte adhesion to endothelial cells and attenuation of atherosclerotic lesion by a glucagon-like peptide-1 receptor agonist, exendin-4. Diabetes 59:1030–1037
38. Gorin Y, Block K, Hernandez J et al (2005) Nox4 NAD(P)H oxidase mediates hypertrophy and fibronectin expression in the diabetic kidney. J Biol Chem 280:39616–39626
39. Frey RS, Ushio-Fukai M, Malik AB (2009) NADPH oxidase-dependent signaling in endothelial cells: role in physiology and pathophysiology. Antioxid Redox Signal 11:791–810
40. Sharma K, Ramachandrarao S, Qiu G et al (2008) Adiponectin regulates albuminuria and podocyte function in mice. J Clin Invest 118:1645–1656
41. Doyle ME, Egan JM (2007) Mechanisms of action of glucagon-like peptide 1 in the pancreas. Pharmacol Ther 113:546–593
42. Ollivier V, Parry GC, Cobb RR, de Prost D, Mackman N (1996) Elevated cyclic AMP inhibits NF-kappaB-mediated transcription in human monocytic cells and endothelial cells. J Biol Chem 271:20828–20835
43. Kim JS, Diebold BA, Babior BM, Knaus UG, Bokoch GM (2007) Regulation of Nox1 activity via protein kinase A-mediated

phosphorylation of NoxA1 and 14-3-3 binding. J Biol Chem 282:34787–34800

44. Gutzwiller JP, Tschopp S, Bock A et al (2004) Glucagon-like peptide 1 induces natriuresis in healthy subjects and in insulin-resistant obese men. J Clin Endocrinol Metab 89:3055–3061
45. Xu H, Barnes GT, Yang Q et al (2003) Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest 112:1821–1830
46. Ross R (1999) Atherosclerosis—an inflammatory disease. N Engl J Med 340:115–126
47. Bongartz LG, Cramer MJ, Doevendans PA, Joles JA, Braam B (2005) The severe cardiorenal syndrome: 'Guyton revisited'. Eur Heart J 26:11–17
48. Nakamura A, Shikata K, Hiramatsu M et al (2005) Serum interleukin-18 levels are associated with nephropathy and atherosclerosis in Japanese patients with type 2 diabetes. Diabetes Care 28:2890–2895

INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Relation between the Estimated Glomerular Filtration Rate and Pulse Wave Velocity in Japanese

Nobuyuki Miyatake[1], Kenichi Shikata[2], Hirofumi Makino[2] and Takeyuki Numata[3]

Abstract

Objective We investigated the link between renal function as evaluated by estimated glomerular filtration rate (eGFR) and pulse wave velocity (PWV) in Japanese without medications.

Methods A total of 1,244 Japanese subjects, aged 20-79 years, were recruited in a cross-sectional clinical investigation study. They received no medications. eGFR was calculated using serum creatinine (Cr), age and sex. Peripheral arterial stiffness was evaluated by brachial-ankle PWV (baPWV).

Results eGFR and baPWV were significantly correlated with age. eGFR was negatively correlated with baPWV (men: $r=-0.308$, $p<0.0001$, women: $r=-0.293$, $p<0.0001$). Twenty-six men (5.6%) and 35 women (4.5%) were diagnosed as reduced eGFR (eGFR <60 mL/min/1.73 m^2). We compared clinical parameters between subjects with reduced eGFR (Group R) and without such reduction (Group N). baPWV in Group R was significantly higher than that in Group N even after adjusting for age. In women, systolic blood pressure in Group R was also significantly higher than that in Group N.

Conclusion eGFR was closely associated with peripheral arterial stiffness in Japanese.

Key words: estimated glomerular filtration rate (eGFR), brachial-ankle pulse wave velocity (baPWV), peripheral arterial stiffness, creatinine

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Introduction

Chronic kidney disease (CKD) (1) has become a public health challenge in Japan (2). For example, 18.7% of adults have CKD, which is defined as kidney damage or a glomerular filtration rate (GFR) <60 mL/min/1.73 m^2 for at least three months regardless of cause, and 4.1% have moderate or severe CKD (2). We have also previously reported in a cross-sectional study that estimated glomerular filtration rate (eGFR) (3) in men with abdominal obesity and in women with hypertension was significantly lower than that without such components (unpublished data). CKD is associated with an increased risk of cardiovascular disease (CVD) outcomes after adjustment for traditional risk factors (4, 5).

Arterial stiffness represents one of the major hemodynamic factors determining pulse pressure even at an early stage of disease and its changes have been shown to be an independent predictor of hard endpoints in patients with a high cardiovascular risk. Pulse pressure and heart rate constitute other outcomes that may be useful as additional factors in risk assessment (6). Pulse wave velocity (PWV) is measured from the initial upstroke of pressure wave and constitutes an established index of arterial stiffness. It is directly related to arterial compliance, arterial distensibility and other factors describing arterial stiffness (7). PWV is not only a good tool for assessing vascular damage, but also an independent predictor of all-cause and cardiovascular mortality (8). Therefore, evaluation of the relationship between eGFR and PWV may provide quite useful data for preventing future diseases in the general population.

In this study, we evaluated the link between eGFR and brachial-ankle PWV (baPWV) and compared clinical pa-

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Table 1. Clinical Profiles of Subjects

	Men(n=464)					Women(n=780)				
	Mean	±	SD	Minimum	Maximum	Mean	±	SD	Minimum	Maximum
Age	44.7	±	12.7	20	79	45.0	±	12.6	20	79
Height (cm)	169.7	±	6.1	143.7	187.5	157.1	±	5.4	141.4	175.1
Body weight (kg)	69.8	±	11.3	39.1	164.8	55.0	±	8.5	36.8	113.9
Body mass index (kg/m^2)	24.2	±	3.5	13.6	52.9	22.3	±	3.3	15.2	44.9
Abdominal circumference (cm)	84.3	±	9.6	62.0	150.0	75.8	±	9.8	55.1	120.0
Hip circumference (cm)	94.2	±	6.5	77.4	153.5	91.8	±	6.1	75.5	127.5
Heart rate (beat/min)	65.2	±	10.7	39.0	110.0	65.0	±	9.8	35.0	104.0
Systolic blood pressure (mmHg)	126.2	±	13.6	94.0	191.0	116.5	±	16.4	87.0	193.0
Diastolic blood pressure (mmHg)	75.9	±	10.1	52.0	112.0	68.5	±	10.9	43.0	111.0
baPWV (right)	1338.0	±	207.5	693.0	2375.0	1223.8	±	227.4	838.0	2449.0
baPWV (left)	1341.7	±	211.5	677.0	2476.0	1244.3	±	224.6	840.0	2369.0
baPWV (mean)	1339.9	±	206.8	685.0	2425.5	1234.1	±	224.2	841.5	2409.0
ABI (right)	1.14	±	0.08	0.88	1.43	1.11	±	0.09	0.75	1.69
ABI (left)	1.12	±	0.09	0.87	1.72	1.09	±	0.08	0.73	1.46
ABI (mean)	1.13	±	0.08	0.88	1.38	1.10	±	0.75	0.75	1.36
Creatinine (mg/dL)	0.85	±	0.12	0.51	1.20	0.62	±	0.09	0.29	0.98
eGFR (mL/min/1.73m^2)	81.2	±	14.5	50.2	138.6	84.3	±	16.9	45.7	173.2

baPWV: brachial-ankle pulse wave velocity
ABI: ankle brachial index
eGFR : estimated glomerular filtration rate

rameters between subjects with reduced eGFR and without such reduction in Japanese without medications.

Subjects and Methods

Subjects

We used data of 1,244 Japanese, aged 20-79 years in a cross-sectional study (Table 1). All subjects met the following criteria: 1) they had undergone an annual health checkup from April 2006 to May 2008 at Okayama Southern Institute of Health; 2) they had received creatinine, baPWV and anthropometric measurements as part of their annual health checkup; 3) received no medications for diabetes, hypertension, and/or dyslipidemia and 4) they provided informed consent. Ethical approval for the study was obtained from the Ethical Committee of Okayama Health Foundation.

Anthropometric measurements

The anthropometric parameters were evaluated by using the following respective parameters such as height, body weight, body mass index (BMI), abdominal circumference, hip circumference. BMI was calculated by weight/[height]^2 (kg/m^2). The abdominal circumference was measured at the umbilical level and the hip was measured at the widest circumference over the trochanter in standing subjects after normal expiration (9).

Blood sampling and assays

We measured overnight fasting serum levels of creatinine (Cr) (enzymatic method). eGFR was calculated using the following equation: eGFR (mL/min/1.73 m^2) $=194\times Cr^{-1.094}\times Age^{-0.287}\times 0.739$ (if women) (3). Reduced eGFR was defined as an eGFR <60 mL/min/1.73 m^2.

PWV measurements

The baPWV and ankle brachial index (ABI) were measured using a form PWV/ABI (Colin, Co., Ltd., Komaki, Japan) after resting at least 15 minutes as described previously (10). This instrument records PWV, blood pressure, electrocardiogram and heart sounds simultaneously. The subjects were examined in the spine position after at least 5 minutes rest, with electrocardiogram electrodes placed on both wrists, a microphone for detecting heart sounds placed on the left edge of the sternum, and cuffs wrapped on both the brachia and ankles. The cuffs were connected to a plethymographic sensor that determines volume pulse form and an oscillometric pressure sensor that measures blood pressure. Volume waveforms for the brachium and ankle were stored, and the sampling time was 10s with automatic gain analysis and quality adjustment.

Statistical analysis

Data are expressed as means ± standard deviation (SD) values. A comparison of parameters between the 2 groups was made using an unpaired t test, covariance analysis and stepwise multiple regression analysis. Simple correlation analysis was performed as well to test for the significance of the linear relationship among continuous variables; $p<0.05$ was considered to indicate statistical significance.

Results

Clinical profiles are summarized in Table 1. eGFR was 81.2±14.5 mL/min/1.73 m^2 in men and 84.3±16.9 mL/min/1.73 m^2 in women. Mean baPWV was 1339.9±206.8 cm/min in men and 1234.1±224.2 cm/min in women.

We evaluated the age-related changes in baPWV (Fig. 1) and eGFR (Fig. 2). baPWV was positively correlated with age (men: $r=0.519$, $p<0.0001$, women: $r=0.651$, $p<0.0001$)

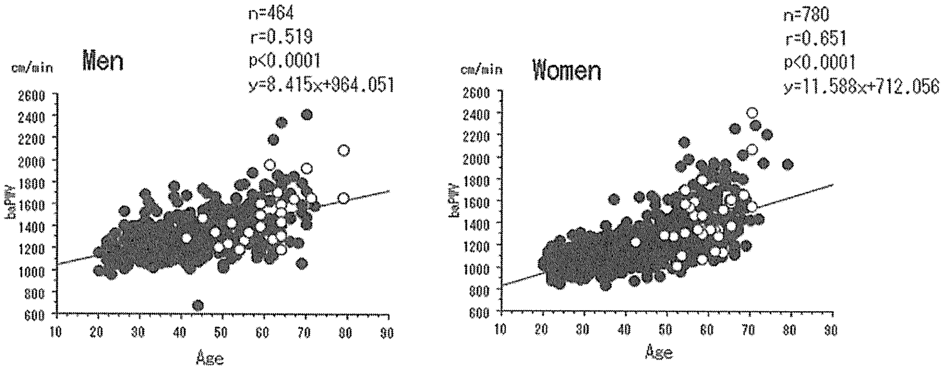

Figure 1. Simple correlation analysis between baPWV and age. baPWV: brachial-ankle pulse wave velocity. ● : eGFR (estimated glomerular filtration rate) ≧ 60mL/min/1.73m^2, ○ : eGFR < 60mL/min/1.73m^2

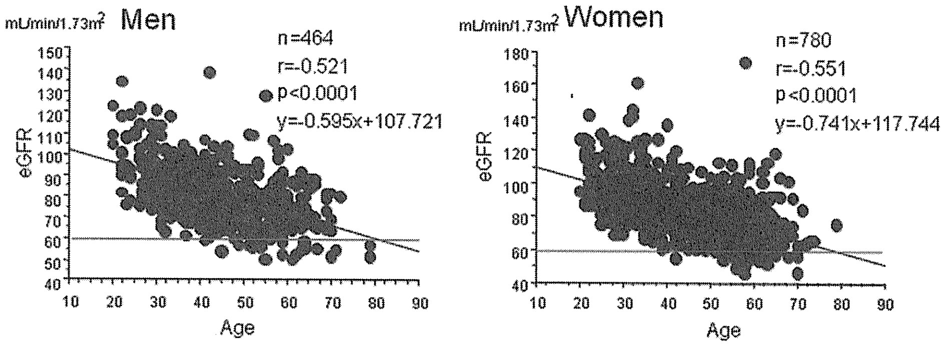

Figure 2. Simple correlation analysis between eGFR and age. Red line is the level of 60 mL/min/1.73m^2 in eGFR. eGFR: estimated glomerular filtration rate

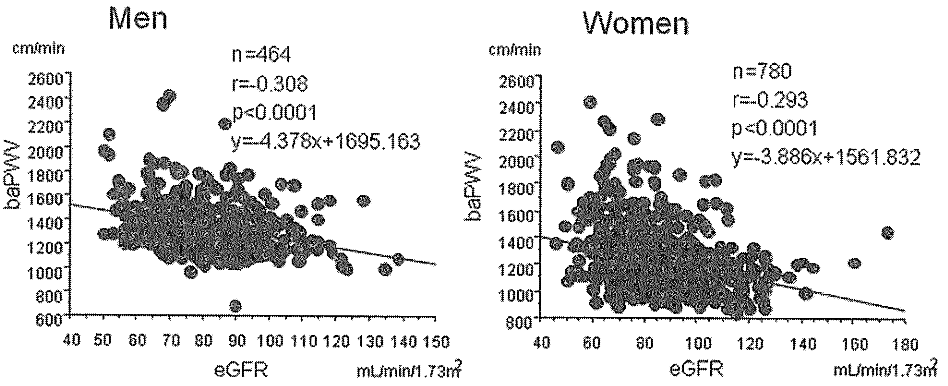

Figure 3. Simple correlation analysis between baPWV and eGFR. baPWV: brachial-ankle pulse wave velocity, eGFR: estimated glomerular filtration rate

and eGFR was negatively correlated with age (men: r=-0.521, p<0.0001, women: r=-0.551, p<0.0001). We also evaluated the relationship between baPWV and eGFR (Fig. 3). baPWV was weakly correlated with eGFR (men: r=-0.308, p<0.0001, women: r=-0.293, p<0.0001).

We further investigated the difference of clinical parameters between subjects who had different levels of eGFR [Group R: eGFR <60 mL/min/1.73 m^2, Group N: eGFR ≧ 60 mL/min/1.73 m^2] (Table 2). baPWV was also positively correlated with age in subjects with CKD (men: r=0.649, p=0.0003, women: r=0523, p=0.0013). The slope of regression line in subjects CKD was higher than that in all subjects in either sex. There were significant differences in diastolic blood pressure, baPWV and ABI between the two groups in both sexes. In women, abdominal circumference and systolic blood pressure in Group R were significantly higher than

Table 2. Comparison of Parameters between Group R and Group N

	Group R	Group N	p	p (After adjusting for age)
Men				
Number of subjects	26	438		
Age	59.8 ± 9.4	43.8 ± 12.4	<0.0001	
Body weight (kg)	70.0 ± 8.3	69.8 ± 11.5	0.9273	0.2260
Body mass index (kg/m^2)	24.5 ± 2.2	24.2 ± 3.6	0.6971	0.4238
Abdominal circumference (cm)	86.9 ± 7.5	84.1 ± 9.7	0.1522	0.2940
Hip circumference (cm)	93.7 ± 5.0	94.3 ± 6.5	0.6484	0.1134
Heart rate (beat/min)	64.0 ± 10.0	65.3 ± 10.8	0.5351	0.5255
Systolic blood pressure (mmHg)	128.2 ± 13.1	126.1 ± 13.7	0.4349	0.4346
Diastolic blood pressure (mmHg)	80.8 ± 8.1	75.6 ± 10.1	0.0114	0.1018
baPWV (right)	1468.5 ± 231.1	1330.2 ± 203.6	0.0009	0.0415
baPWV (left)	1500.8 ± 273.3	1332.2 ± 203.8	<0.0001	0.0141
baPWV (mean)	1484.6 ± 249.2	1331.3 ± 201.1	0.0002	0.0222
ABI (right)	1.20 ± 0.07	1.13 ± 0.08	0.0001	0.1458
ABI (left)	1.16 ± 0.07	1.12 ± 0.09	0.0174	0.4252
ABI (mean)	1.18 ± 0.06	1.13 ± 0.08	0.0006	0.2086
Women				
Number of subjects	35	745		
Age	58.9 ± 6.2	44.4 ± 12.4	<0.0001	
Body weight (kg)	55.9 ± 6.5	55.0 ± 8.6	0.5084	0.4327
Body mass index (kg/m^2)	22.8 ± 2.7	22.3 ± 3.4	0.3702	0.1200
Abdominal circumference (cm)	80.4 ± 9.2	75.6 ± 9.8	0.0045	0.3307
Hip circumference (cm)	91.8 ± 4.2	91.8 ± 6.2	0.9688	0.4599
Heart rate (beat/min)	63.6 ± 10.2	65.1 ± 9.8	0.3912	0.3018
Systolic blood pressure (mmHg)	124.3 ± 20.2	116.1 ± 16.1	0.0038	0.0178
Diastolic blood pressure (mmHg)	72.8 ± 11.8	68.3 ± 10.8	0.0162	0.3262
baPWV (right)	1418.3 ± 284.1	1214.7 ± 220.4	<0.0001	0.0126
baPWV (left)	1444.5 ± 277.3	1234.9 ± 217.5	<0.0001	0.0221
baPWV (mean)	1431.4 ± 278.9	1224.8 ± 217.1	<0.0001	0.0152
ABI (right)	1.16 ± 0.08	1.11 ± 0.09	0.0013	0.9763
ABI (left)	1.15 ± 0.09	1.09 ± 0.08	<0.0001	0.5987
ABI (mean)	1.15 ± 0.08	1.10 ± 0.07	<0.0001	0.7638

Group R: eGFR<60mL/min/1.73m^2
Group N: eGFR≧60mL/min/1.73m^2
baPWV: brachial-ankle pulse wave velocity
ABI: ankle brachial index
eGFR : estimated glomerular filtration rate

those in Group N. However, significant difference in age was also noted between the two groups. To avoid the influence of age on clinical parameters, we used age as a covariate and compared clinical parameters using covariance analysis. After adjusting for age, baPWV in Group R was significantly higher than that in Group N in both sexes. In women, systolic blood pressure in Group R was also significantly higher than that in Group N. The differences in other clinical parameters between the two groups did not show statistical significance after adjusting for age.

We also used stepwise multiple regression analysis to evaluate the effect of clinical parameters *i.e.* abdominal circumference, systolic blood pressure, diastolic blood pressure and eGFR on baPWV (mean), and found that systolic blood pressure, diastolic blood pressure and eGFR were significant (men: baPWV (mean) =511.906+4.150 (systolic blood pressure) +6.580 (diastolic blood pressure) -2.407 (eGFR), r^2= 0.388, p<0.0001, women: baPWV (mean) =264.104+7.562 (systolic blood pressure) +3.598 (diastolic blood pressure) -1.868 (eGFR), r^2=0.567, p<0.0001). Age is also thought to be a strong predictor of baPWV and we further analyzed stepwise multiple regression analysis by also using age and found that age, systolic blood pressure, diastolic blood pressure and eGFR were significant in women (baPWV (mean) = -58.089+7.593 (age) +6.090 (systolic blood pressure) +2.530 (diastolic blood pressure) +0.797 (eGFR), r^2=0.671, p<0.0001). However, in men, clinical impact of eGFR on baPWV was attenuated (baPWV (mean) =102.719+6.717 (age) +5.959 (systolic blood pressure) +2.438 (diastolic blood pressure), r^2=0.498, p<0.0001).

Discussion

It is well known that baPWV increases with age (11, 12), and eGFR also decreases with age (13). El Feghali et al have reported a significant correlation between PWV and age (r=0.59, p<0.001) by a cross-sectional multi-center study performed in 46 healthcare centers, from 14 countries (11). Ni et al also reported that PWV was positively related to age (r=0.531, p=0.001) in 3156 Chinese (12). According to the link between eGFR and age, by the large sample of Japanese cohort, the decline rate of eGFR was 0.36 mL/min/1.73 m^2/year (13). In this study, we also found that baPWV was positively correlated with age and eGFR was negatively correlated with age by cross-sectional analysis. The decline rate of eGFR in this study was 0.595 mL/min/1.73 m^2/year in men and 0.741 mL/min/1.73 m^2/year in women. This study was cross-sectional and the enrolled subjects in this study were younger than those in the previous report. Therefore, the rate of decline in eGFR in this study might differ from the previous report.

Some studies have evaluated the link between baPWV and eGFR in Japanese (14-16). Kawamoto et al investigated 107 men, aged 68±9 years and 203 women, aged 67±7 years during their annual health examination in a single community, and eGFR [$eGFR=0.741\times175\times Cr^{-1.154}\times Age^{-0.203}\times 0.742$ (if female)] was significantly correlated with PWV ($r=-0.317$, $p<0.001$) (14). A reduced eGFR was significantly correlated with increased PWV in the heart-femoral in Japanese patients with type 2 diabetes ($r=-0.199$, $p<0.001$) (15). Ohya et al also showed that significant correlation between baPWV and creatinine clearance estimated by Cockcroft-Gault formula was noted in 3,387 subjects (mean age, 52 years) who attended a health checkup in Okinawa, Japan (16). In this study, we used newly developed equations for eGFR and the link between baPWV and eGFR in Japanese without medications. eGFR was negatively correlated with baPWV, and baPWV in Group R was significantly higher than that in Group N even after adjusting for age. Although Sengstock et al recently reported that the contribution of eGFR was not clinically meaningful when compared with traditional cardiovascular risk factors (17), eGFR especially in women, as well as systolic blood pressure, diastolic blood pressure and age, was good predictor for baPWV by stepwise multiple regression analysis. The present results are in agreement with the mild impairment of renal function might increase arterial stiffness even in Japanese without medications.

Potential limitations remain in our study. First, the cross-sectional study design and small sample size in subjects with reduced eGFR in our study make it difficult to infer association between baPWV and some parameters. Second, Insulin resistance is associated with high arterial stiffness and hemodynamic alterations in the common carotid artery (18). Insulin resistance causes diabetes, hypertension, metabolic syndrome and renal dysfunction through increased sympathetic stimulation, aldosterone activation, endothelial dysfunction as well as increased renal sodium absorption, advanced glycation end products, activation of the renin-angiotensin-aldosterone system and lipid peroxidation, resulting in vascular remodeling (19-22). Decreased renal function may increase PWV through insulin resistance. However, we could not prove the mechanism of the link between baPWV and eGFR. Third, Large 'central' artery stiffness predicts cardiovascular events in the general population and in end-stage renal disease (23, 24). However, we could not separately evaluate PWV of central and peripheral arteries. Therefore, our findings are applicable to clinical and public health practice settings. In conclusion, lower eGFR is associated with higher baPWV in Japanese. Further intervention studies are necessary to investigate the link between baPWV and eGFR.

References

1. National Kidney Foundation K/DOQI. Clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Am J Kidney Dis **39**: S1-S266, 2002.
2. Imai E, Horio M, Iseki K, et al. Prevalence of chronic kidney disease (CKD) in the Japanese general population predicted by the MDRD equation modified by a Japanese coefficient. Clin Exp Nephrol **11**: 156-163, 2007.
3. Matsuo S, Imai E, Horio M, et al. Collaborators developing the Japanese equation for estimated GFR: Revised equations for estimated GFR from serum creatinine in Japan. Am J Kidney Dis **53**: 982-992, 2009.
4. Weiner DE, Tighiouart H, Stark PC, et al. Kidney disease as a risk factor for recurrent cardiovascular disease and mortality. Am J Kidney Dis **44**: 198-206, 2004.
5. Meisinger C, Doring A, Lowel H; KORA Study Group. Chronic kidney disease and risk of incident myocardial infarction and all-cause and cardiovascular disease mortality in middle-aged men and women from the general population. Eur Heart J **27**: 1245-1250, 2006.
6. Asmar R, Darne B, Assaad M, Topouchian J. Assessment of outcomes other than systolic and diastolic blood pressure: pulse pressure, arterial stiffness and heart rate. Blood Press Monit **6**: 329-333, 2001.
7. Martyn CN, Greenwald SE. Pulse wave velocity as a marker of vascular disease. Lancet **348**: 1586, 1996.
8. Laurent S, Boutouyrie P, Asmar R, et al. Aortic stiffness is an independent predictor of all-cause and cardiovascular mortality in hypertensive patients. Hypertension **37**: 1236-1241, 2001.
9. Committee to Evaluate Diagnostic Standards for Metabolic Syndrome. Definition and the diagnostic standard for metabolic syndrome. Nippon Naika Gakkai Zasshi **94**: 794-809, 2005 (in Japanese).
10. Kohara K, Tabara Y, Tachibana R, Nakura J, Miki T. Microalbuminuria and arterial stiffness in a general population: the Shimanami Health Promoting Program (J-SHIPP) Study. Hypertens Res **27**: 471-477, 2004.
11. El Feghali R, Topouchian J, Pannier B, Asmar R. Ageing and blood pressure modulate the relationship between metabolic syndrome and aortic stiffness innever-treated essential hypertensive patients. A comparative study. Diabetes Metab **33**: 183-188, 2007.
12. Ni Y, Wang H, Hu D, Zhang W. The relationship between pulse wave velocity and pulse pressure in Chinese patients with essential hypertension. Hypertens Res **26**: 871-874, 2003.
13. Imai E, Horio M, Yamagata K, et al. Slower decline of glomerular filtration rate in the Japanese general population: a longitudinal 10-year follow-up study. Hypertens Res **31**: 433-441, 2008.
14. Kawamoto R, Kohara K, Tabara Y, et al. An association between decreased estimated glomerular filtration rate and arterial stiffness. Intern Med **47**: 593-598, 2008.
15. Kimoto E, Shoji T, Shinohara K, et al. Regional arterial stiffness in patients with type 2 diabetes and chronic kidney disease. J Am Soc Nephrol **17**: 2245-2252, 2006.
16. Ohya Y, Iseki K, Iseki C, Miyagi T, Kinjo K, Takishita S. Increased pulse wave velocity is associated with low creatinine clearance and proteinuria in a screened cohort. Am J Kidney Dis **45**: 790-797, 2006.
17. Sengstock D, Sands RL, Gillespie BW, et al. Dominance of traditional cardiovasucular risk factors over renal function in predicting arterial stiffness in subjects with chronic kidney disease. Nephrol Dial Transplant **25**: 853-861, 2010.
18. Watanabe S, Okura T, Kitami Y, Hiwada K. Carotid hemodynamic alterations in hypertensive patients with insulin resistance. Am J Hypertens **15**: 851-856, 2002.
19. Safar ME. Systolic hypertension in the elderly: arterial wall me-

chanical properties and renin-angiotensin-aldosterone system. J Hypertens **23**: 673-681, 2005.

20. Sarafidis PA, Lasaridis AN, Nilsson PM, et al. Ambulatory blood pressure reduction after rosiglitazone treatment in patients with type 2 diabetes and hypertension correlates with insulin sensitivity increase. J Hypertens **22**: 1769-1777, 2004.

21. Reaven GM, Lithell H, Landsberg L. Hypertension and associated metabolic abnormalities—the role of insulin resistance and the sympathoadrenal system. N Engl J Med **334**: 374-381, 1996.

22. Kadowaki T, Yamauchi T. Adiponectin and adiponectin receptors. Endocr Rev **26**: 439-451, 2005.

23. Boutouyrie P, Tropeano AI, Asmar R, et al. Aortic stiffness is an independent predictor of primary coronary events in hypertensive patients: a longitudinal study. Hypertension **39**: 10-15, 2002.

24. Blacher J, Guerin AP, Pannier B, Marchais SJ, Safar ME, London GM. Impact of aortic stiffness on survival in end-stage renal disease. Circulation **99**: 2434-2439, 1999.