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Dipeptidyl peptidase-4 deficiency protects against experimental diabetic nephropathy partly by blocking the advanced glycation end products-receptor axis

Takanori Matsui[1], Sae Nakashima[1], Yuri Nishino[1], Ayako Ojima[1], Nobutaka Nakamura[1], Kazunari Arima[2], Kei Fukami[3], Seiya Okuda[3] and Sho-ichi Yamagishi[1]

Advanced glycation end products (AGEs) and their receptor (RAGE) have a role in diabetic nephropathy. We have recently found that linagliptin, an inhibitor of dipeptidyl peptidase-4 (DPP-4), could inhibit renal damage in type 1 diabetic rats by suppressing the AGE-RAGE axis. However, it remains unclear whether DPP-4 deficiency could also have beneficial effects on experimental diabetic nephropathy. To address the issue, we rendered wild-type F344/NSlc and DPP-4-deficient F344/DuCrl/Crlj rats diabetic by injection of streptozotocin, and then investigated whether DPP-4 deficiency could block the activation of AGE-RAGE axis in the diabetic kidneys and resultantly ameliorate renal injury in streptozotocin-induced diabetic rats. Compared with control rats at 9 and 11 weeks old, body weight and heart rates were significantly lower, while fasting blood glucose was higher in wild-type and DPP-4-deficient diabetic rats at the same age. There was no significant difference of body weight, fasting blood glucose and lipid parameters between the two diabetic rat strains. AGEs, 8-hydroxy-2′-deoxyguanosine (8-OHdG) and nitrotyrosine levels in the kidney, renal gene expression of RAGE and intercellular adhesion molecule-1, glomerular area, urinary excretion of 8-OHdG and albumin, and the ratio of renal to body weight were increased in wild-type diabetic rats at 9 and/or 11 weeks old compared with age-matched control rats, all of which except for urinary 8-OHdG levels at 11 weeks old were significantly suppressed in DPP-4-deficient diabetic rats. Our present study suggests that DPP-4 deficiency could exert beneficial actions on type 1 diabetic nephropathy partly by blocking the AGE-RAGE axis. DPP-4 might be a novel therapeutic target for preventing diabetic nephropathy.
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Sugars, including glucose and fructose, can react non-enzymatically with the amino groups of proteins, lipids and nucleic acids to form reversible Schiff bases, and then Amadori products.[1–3] These early glycation products undergo further complex reactions such as rearrangement, dehydration and condensation to become irreversibly cross-linked, heterogeneous fluorescent derivatives called 'advanced glycation end products (AGEs)'.[1–3] The process of non-enzymatic glycation is also known as Maillard reaction, and formation and accumulation of AGEs in various tissues have progressed at a physiological aging and at an extremely accelerated rate under diabetes.[1–3] There is a growing body of evidence that AGEs and their receptor (RAGE) interaction evoke oxidative stress generation and inflammatory and fibrotic reactions, thereby causing progressive alteration in renal architecture and loss of renal function in diabetes.[4–8] Furthermore, RAGE-overexpressing diabetic mice have been shown to exhibit progressive glomerulosclerosis with renal dysfunction, compared with diabetic littermates lacking the RAGE transgene.[9] Diabetic homozygous RAGE null mice displayed diminished albuminuria and glomerulosclerosis and failed to develop significantly increased mesangial matrix expansion or thickening of the glomerular basement membrane.[10] These observations suggest that the AGE-RAGE-induced oxidative stress generation in the kidneys may be a therapeutic target for diabetic nephropathy.

Incretins such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are gut hormones secreted from L and K cells in the intestine in response to food intake, respectively.[11,12] GLP-1 and GIP are

[1]Department of Pathophysiology and Therapeutics of Diabetic Vascular Complications, Kurume University School of Medicine, Kurume, Japan; [2]Department of Chemistry and Bioscience, Faculty of Science, Kagoshima University Graduate School of Science and Engineering, Kagoshima, Japan and [3]Department of Medicine, Kurume University School of Medicine, Kurume, Japan
Correspondence: Professor S-i Yamagishi, MD, PhD, Department of Pathophysiology and Therapeutics of Diabetic Vascular Complications, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan.
E-mail: shoichi@med.kurume-u.ac.jp

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target proteins of dipeptidyl peptidase-4 (DPP-4) and rapidly degraded and inactivated by this proteolytic enzyme.[13,14] Since GLP-1 and GIP augment glucose-induced insulin release from pancreatic β-cells, suppress glucagon secretion and slow gastric emptying,[11,12] inhibition of DPP-4 has been proposed as a potential therapeutic target for the treatment of patients with type 2 diabetes.

Experimental findings and preliminary clinical data have suggested that DPP-4 inhibition, in addition to improving metabolic control, have the potential to interfere with the onset and progression of diabetic nephropathy.[15–17] Indeed, linagliptin, an inhibitor of DPP-4, ameliorated kidney fibrosis in type 1 diabetic mice by blocking the endothelial-to-mesenchymal transition.[16] Linagliptin administered in addition to stable renin-angiotensin-aldosterone system inhibitors led to a significant reduction in albuminuria in patients with type 2 diabetes and renal dysfunction.[17] Furthermore, we have recently found that linagliptin could inhibit renal damage in streptozotocin (STZ)-induced diabetic rats (STZ rats) by suppressing the AGE-RAGE axis.[18] Since linagliptin did not improve hyperglycemia in STZ rats, our findings suggest that inhibition of DPP-4 might have glucose-lowering-independent, AGE-RAGE-axis suppressive pleiotropic actions on diabetic nephropathy. However, pharmacological inhibitors are not absolutely specific to DPP-4, and therefore, it remains unclear whether DPP-4 itself could be directly involved in diabetic nephropathy. To address the issue, we rendered wild-type F344/NSlc and DPP-4-deficient F344/DuCrl/Crlj rats diabetic by injection of STZ, and then investigated whether DPP-4 deficiency could block the activation of AGE-RAGE axis in the diabetic kidneys and resultantly ameliorate renal injury in STZ rats.

MATERIALS AND METHODS

Animal Experiments

Seven-week-old male DPP-4-deficient F344/DuCrl/Crlj rats and wild-type F344/NSlc rats were purchased from Charles River Japan (Yokohama, Japan) and Japan SLC, Inc. (Shizuoka, Japan), respectively. DPP-4-deficient and wild-type rats received single 35 mg/kg intraperitoneal injection of STZ (Sigma, St Louis, MO, USA) in 10 mM citrate buffer (pH 4.5). Non-diabetic DPP-4-deficient rats and wild-type rats (Control rats) received citrate buffer alone. Animals with blood glucose levels greater than 250 mg/dl 48 h later were considered as diabetic. At baseline and after 2 and 4 weeks, animals were housed in metabolic cages to collect urine for measurement of urinary excretion levels of albumin and 8-hydroxy-2′-deoxyguanosine (8-OHdG), and then body weight (BW), heart rates (HR), fasting blood glucose (FBG) and blood biochemistry were measured. Albuminuria and urinary excretion levels of 8-OHdG were determined with commercially available enzyme-linked immunosorbent assay (ELISA) kits (Shibayagi Co., Ltd., Gunma, Japan for albuminuria and Japan Institute for the Control of Aging NIKKEN SEIL Co., Ltd., Shizuoka, Japan for urinary 8-OHdG, respectively). Other biochemistry was determined as described previously.[19] Then, the rats were killed and the kidneys were excised for measuring the ratio of renal to BW and performing real-time reverse transcription-PCRs (RT-PCR), dot and western blot, immunohistochemical and morphological analyses. All experimental procedures were conducted in accordance with the National Institutes Health Guide for Care and Use of Laboratory Animals and were approved by the ethnical committee of Kurume University School of Medicine.

Dot Blot Experiments

Accumulation levels of AGEs in the kidneys were evaluated by dot blot technique using Bio-Dot SF Microfiltration Apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the supplier's recommendations. In brief, 50 μl of each sample containing total protein amounts of 5 μg was applied to the wells and transferred onto nitrocellulose membranes. Membranes were probed with 1:3000 dilution of polyclonal antibodies raised against AGE-modified bovine serum albumin, and then immune complexes were visualized with an enhanced chemiluminescence detection system (Amersham Bioscience, Buckinghamshire, UK) as described previously.[20] Color intensity was analyzed by microcomputer-assisted image J.

Western Blot Analysis

Twenty micrograms of proteins were extracted from the kidneys of Control, DPP-4-deficient, STZ or DPP-4-deficient STZ rats with lysis buffer, and then separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes as described previously.[19] Membranes were probed with antibodies raised against AGEs or α-tubulin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and then immune complexes were visualized with an enhanced chemiluminescence detection system (Amersham Bioscience) as described previously.[19] Data were normalized by the intensity of α-tubulin-derived signals and related to the value of 9-week-old Control rats.

Real-Time RT-PCR

Total RNAs were extracted from the kidneys of Control, DPP-4-deficient, STZ or DPP-4-deficient STZ rats with RNAqueous-4PCR kit (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed using Assay-on-Demand and TaqMan 5 fluorogenic nuclease chemistry (Applied Biosystems, Foster City, CA, USA) according to the supplier's recommendation. IDs of primers for rat RAGE, intercellular adhesion molecule-1 (ICAM-1) and β-actin gene were Rn00584249_m1, Rn00564227_m1 and Rn00667869_m1, respectively.

Immunohistochemical Analysis

The kidneys were fixed in 4% paraformaldehyde and embedded in paraffin, sectioned at 4-μm intervals and

mounted on glass slides. After blocking endogenous peroxidase activity, the sections were incubated overnight at 4 °C with anti-8-OHdG antibodies (Japan Institute for the Control of Aging NIKKEN SEIL Co., Ltd.) or anti-nitrotyrosine antibodies (StressMarq Biosciences, Inc., Victoria, Canada). Then, the reactions were visualized with a Histofine Simple Stain Rat MAX-POMULTI kit (Nichirei Co., Tokyo, Japan) as described previously.[19] Immunoreactivity of each sample was measured by microcomputer-assisted image J.

Histopathological Examinations

The kidneys were fixed in 4% paraformaldehyde and embedded in paraffin, sectioned at 4-μm intervals and mounted on glass slides. The sections were stained with hematoxylin and eosin for light microscopic analysis. Glomerular area delimited by the internal edge of the Bowman's capsule was measured using Image J software as described previously.[19,21]

Statistical Analysis

All values were presented as means ± standard error (s.e.). Student's *t*-test was performed for statistical comparisons; $P < 0.05$ was considered as significant.

RESULTS

Clinical Characteristics of Animals

As shown in Table 1, compared with Control rats at 9 and 11 weeks old, BW and HR were significantly lower, while FBG, the ratio of renal to BW and urinary excretion levels of 8-OHdG were higher in both wild-type and DPP-4-deficient diabetic rats at the same age. Although there was no significant difference of BW, FBG and lipid parameters between the two diabetic rat strains, HR in DPP-4-deficient STZ rats at 9 weeks old was significantly higher than those in STZ rats at the same age. Further, urinary 8-OHdG levels of STZ rats at 9 weeks old and increased ratio of renal to BW of STZ rats at 9 and 11 weeks old were significantly reduced in age-matched DPP-4-deficient STZ rats. HR of 9-week-old DPP-4 deficient rats was significantly higher than those of age-matched Control rats, while HR, FBG and serum creatinine levels of 11-week-old DPP-4 deficient rats were lower compared with Control rats at the same age.

Effects of DPP-4 Deficiency on the AGE-RAGE Axis

We investigated the effects of DPP-4 deficiency on the AGE-RAGE axis in the kidneys of diabetic rats. As shown in Figure 1a and b, dot and western blot analyses revealed that renal AGE-modified protein levels in 9-week-old STZ rats were increased compared with age-matched Control rats. The enhanced accumulation of AGEs in the diabetic kidneys was significantly suppressed in DDP-4-deficient STZ rats at the same age. Furthermore, renal RAGE gene expression in 9-week-old STZ rats was significantly enhanced compared with age-matched Control rats, which was also reduced in age-matched DDP-4-deficient STZ rats.

Effects of DPP-4 Deficiency on Oxidative Stress Levels in the Kidneys

We further studied the effects of DPP-4 deficiency on oxidative stress levels in the diabetic kidneys. As shown in Figure 2a, a marker of oxidative stress, nitrotyrosine levels in the kidneys of STZ rats at 9 and 11 weeks old, was increased compared with age-matched Control rats. Moreover, 8-OHdG levels, another marker of oxidative stress in the kidneys of STZ rats at 11 weeks old was also enhanced compared with Control rats at the same age (Figure 2b). All of the changes in the diabetic kidneys were significantly suppressed in age-matched DDP-4-deficient STZ rats (Figure 2a and b).

Effects of DPP-4 Deficiency on Inflammatory Reaction, Expansion of Glomerular Area and Albuminuria

AGE-RAGE-induced oxidative stress generation evokes inflammatory reactions in diabetic nephropathy.[4–8] So, we further examined whether DPP-4 deficiency could affect ICAM-1 gene expression in the kidneys and expansion of glomerular area of STZ rats. As shown in Figure 3a and b, renal ICAM-1 gene expression and glomerular area expansion were increased in STZ rats at 9 weeks old compared with age-matched Control rats, both of which were significantly blocked in DPP-4-deficient STZ rats at the same age.

Moreover, albuminuria was significantly increased in STZ rats at 11 weeks old, which was prevented in age-matched DPP-4-deficient diabetic rats (Figure 4). Compared with Control rats, albuminuria in non-diabetic DPP-4-deficient rats was also suppressed.

DISCUSSION

We have previously shown that (1) AGEs stimulate DPP-4 release from endothelial cells (ECs), (2) DPP-4 elicits oxidative stress generation and subsequently increases RAGE and ICAM-1 gene expression in ECs and (3) linagliptin, an inhibitor of DPP4 blocks the AGE-RAGE-induced reactive oxygen species generation and upregulation of ICAM-1 mRNA levels in ECs.[22] In addition, linagliptin treatment was found to decrease renal levels of AGEs and 8-OHdG levels, suppresses RAGE and ICAM-1 gene expression in the kidneys and reduces albuminuria in STZ rats, although it did not affect blood glucose levels in the animals.[18] Further, serum DPP-4 levels were significantly increased in STZ rats,[18] and AGEs levels were independently correlated with circulating levels of DPP-4 in humans.[23] These observations could indicate the pathological crosstalk between the AGE-RAGE axis and DPP-4 in vascular injury of diabetes, thus suggesting that inhibition of DPP-4 by linagliptin might have glucose-lowering-independent, beneficial actions on experimental diabetic nephropathy. However, it remains unclear whether DPP-4 deficiency could also exert pleiotropic effects on diabetic nephropathy of STZ rats, a model of type 1 diabetic animal.

To address the issue, we used DPP-4-deficient F344/DuCrl/Crlj rats in the present experiments because (1) the rats

Table 1 Characteristics of animals

Characteristics	*7 weeks old*		*9 weeks old*				*11 weeks old*			
	Control rats (n = 6)	*DPP-4-deficient rats (n = 6)*	*Control rats (n = 8)*	*DPP-4-deficient rats (n = 8)*	*STZ rats (n = 11)*	*DPP-4-deficient STZ rats (n = 7)*	*Control rats (n = 4)*	*DPP-4-deficient rats (n = 4)*	*STZ rats (n = 6)*	*DPP-4-deficient STZ rats (n = 4)*
Body weight (BW) (g)	140 ± 2.40	131 ± 3.61	179 ± 2.45	171 ± 3.82	125 ± 3.71[a]	124 ± 2.25[a]	203 ± 6.59	193 ± 9.60	134 ± 10.23[d]	129 ± 3.03[d]
Heart rates (beats/min)	394 ± 2.52	391 ± 3.23	417 ± 2.72	430 ± 3.43[a]	365 ± 4.30[a]	399 ± 3.22[a, b]	435 ± 2.48	395 ± 3.47[d]	349 ± 7.00[d]	347 ± 5.81[d]
Fasting blood glucose (mg/dl)	79 ± 2.19	75 ± 2.14	73 ± 2.70	69 ± 2.01	281 ± 26.59[a]	226 ± 24.74[a]	75 ± 2.47	64 ± 1.32[d]	375 ± 53.30[d]	318 ± 34.53[d]
Creatinine (mg/dl)	0.26 ± 0.04	0.20 ± 0.00	0.18 ± 0.02	0.25 ± 0.03	0.16 ± 0.03	0.13 ± 0.02	0.28 ± 0.03	0.20 ± 0.00[c]	0.25 ± 0.06	0.33 ± 0.09
Total cholesterol (mg/dl)	76.0 ± 3.5	81.2 ± 3.3	54.0 ± 1.0	51.0 ± 2.8	47.0 ± 4.5	49.3 ± 5.4	48.8 ± 7.4	59.5 ± 3.0	67.4 ± 11.9	59.5 ± 1.8
Triglycerides (mg/dl)	92.9 ± 16.9	101.3 ± 15.6	73.2 ± 6.5	64.4 ± 5.5	103.0 ± 25.6	38.5 ± 7.4[a]	100.2 ± 11.7	62.5 ± 11.3	64.2 ± 12.5	114.6 ± 23.7
Renal weight (g)/BW (g) (%)	0.49 ± 0.01	0.50 ± 0.01	0.44 ± 0.02	0.44 ± 0.01	0.66 ± 0.02[a]	0.62 ± 0.03[a, b]	0.43 ± 0.00	0.42 ± 0.01	0.70 ± 0.05[d]	0.67 ± 0.04[d,e]
Urinary 8-OHdG levels (μg/g creatinine)	—	—	8.65 ± 0.53	11.66 ± 0.70[a]	29.04 ± 3.22[a]	20.34 ± 2.52[a, b]	7.58 ± 0.70	8.17 ± 0.42	19.11 ± 0.72[d]	20.98 ± 0.99[d]

Data are presented as means ± standard error.
[a]$P < 0.01$ compared with Control rats at 9 weeks old.
[b]$P < 0.01$ compared with STZ rats at 9 weeks old.
[c] and [d]$P < 0.05$ and $P < 0.01$ compared with Control rats at 11 weeks old, respectively.
[e]$P < 0.01$ compared with STZ rats at 11 weeks old.

express DPP-4 mRNA but lack the enzymatic activity of DPP-4 due to point mutation in the active site[24] and (2) we have previously shown that pro-oxidative and pro-inflammatory property of DPP-4 is totally dependent on its intrinsic enzymatic activity.[22] In this study, we found first that accumulation levels of AGEs, RAGE gene expression and oxidative stress markers, nitrotyrosine and 8-OHdG levels in the kidneys were increased in STZ rats compared with Control rats, all of which were significantly suppressed in DPP-4-deficient STZ rats. DPP-4 deficiency also inhibited the increase in renal ICAM-1 mRNA levels, glomerular area and albuminuria in STZ rats. There was no significant difference of metabolic parameters, including FBG between the two diabetic strains. The inhibitory effects of DPP-4 deficiency on the AGE-RAGE oxidative stress axis and albuminuria in STZ rats were completely similar to those of linagliptin.[18] Moreover, we have recently found that a DPP-4 inhibitor, alogliptin suppresses the AGE-RAGE axis and resultantly reduces albuminuria in type 2 diabetes patients.[25] In addition, vildagliptin has been shown to prevent the increase in AGEs, RAGE, 8-OHdG levels and inflammatory reactions in the thoracic aorta of obese and type 2 diabetic rats.[26] Taken together, the present findings suggest that inhibition of DPP-4 could protect against diabetic nephropathy partly by blocking the AGE-RAGE axis. Suppression of DPP-4 enzymatic activity might be clinically relevant for preventing vascular complications in diabetes. In support of this speculation, several papers have reported the glucose-independent renoprotective effects of DPP-4 inhibition in animal models of diabetic nephropathy.[15,16,18,27]

In the present study, we could not clarify the underlying mechanism for the suppression of AGE-RAGE axis in DPP-4-deficient STZ rats. However, it is unlikely that DPP-4 deficiency decreased renal AGEs levels by improving glycemic or lipid parameters because there was no significant difference of these parameters between DPP-4-deficient STZ rats and wild-type STZ rats. We have previously found that GLP-1 or GIP limits endothelial and mesangial cells' susceptibility toward pro-oxidative and pro-inflammatory effects of AGEs by suppressing RAGE gene expression and oxidative stress generation through the elevation of cyclic AMP, whose effect could be augmented by the addition of DPP-4 inhibitor.[28–31] In addition, the AGE-RAGE axis evokes oxidative stress generation in various cell types *via* NADPH oxidase activity, which is blocked by cAMP-elevating agents.[3,32,33] Aortic AGEs and oxidative stress levels have been shown to decrease in diabetic RAGE and apolipoprotein E double knockout

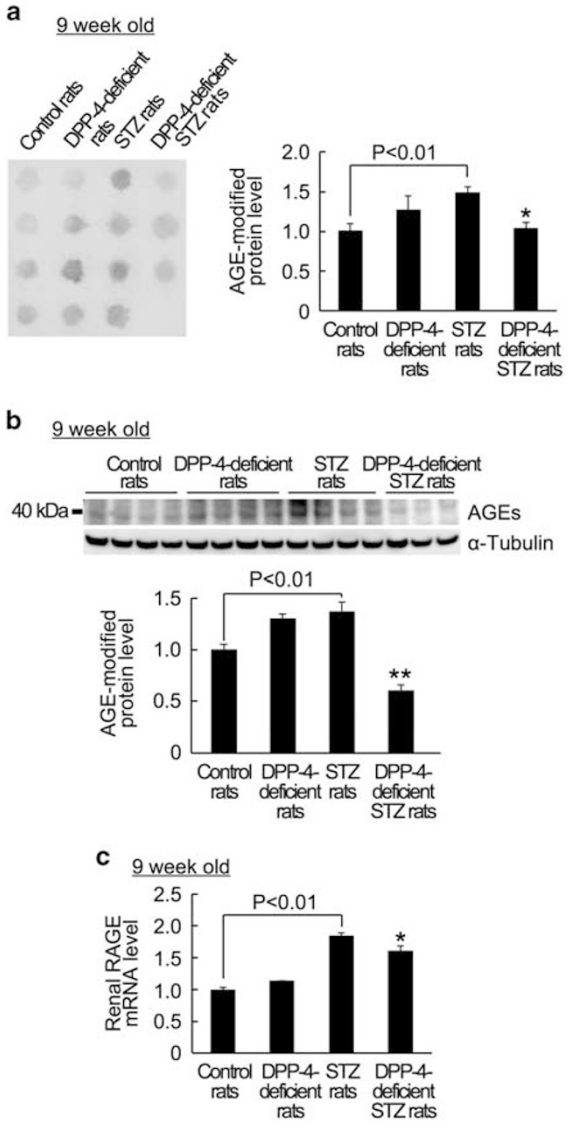

Figure 1 AGE-modified protein levels (**a** and **b**) and RAGE gene expression (**c**) in the kidneys of Control, DPP-4-deficient, STZ or DPP-4-deficient STZ rats at 9 weeks old. (**a**) Accumulation levels of AGE-modified protein levels in the kidneys were evaluated by dot blot technique using Bio-Dot SF Microfiltration Apparatus according to the supplier's recommendations. In brief, 50 μl of each sample containing total protein amounts of 5 μg was applied to the wells and transferred onto nitrocellulose membranes. Membranes were probed with 1:3000 dilution of polyclonal antibodies raised against AGE-modified bovine serum albumin, and then immune complexes were visualized with an enhanced chemiluminescence detection system. Color intensity was analyzed by microcomputer-assisted image J. (**b**) Twenty micrograms of proteins were extracted from the kidneys of Control, DPP-4-deficient, STZ or DPP-4-deficient STZ rats with lysis buffer, and then separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were probed with 1:3000 dilution of antibodies raised against AGEs or 1:1000 dilution of antibodies against α-tubulin, and then immune complexes were visualized with an enhanced chemiluminescence detection system. Data were normalized by the intensity of α-tubulin-derived signals and related to the value of 9-week-old Control rats. * and **$P<0.05$ and $P<0.01$ compared to the value with STZ rats, respectively. $N=4$ for Control rats. $N=4$ for DPP-4-deficient rats. $N=4$ for STZ rats. $N=3$ for DPP-4-deficient STZ rats. (**c**) Total RNAs were extracted from the kidneys of Control, DPP-4-deficient, STZ or DPP-4-deficient STZ rats with RNAqueous-4PCR kit. Quantitative real-time RT-PCR was performed. Data were normalized by the intensity of β-actin mRNA-derived signals. *$P<0.05$ compared to the value with STZ rats. $N=3$ for Control rats. $N=4$ for DPP-4-deficient rats. $N=4$ for STZ rats. $N=3$ for DPP-4-deficient STZ rats.

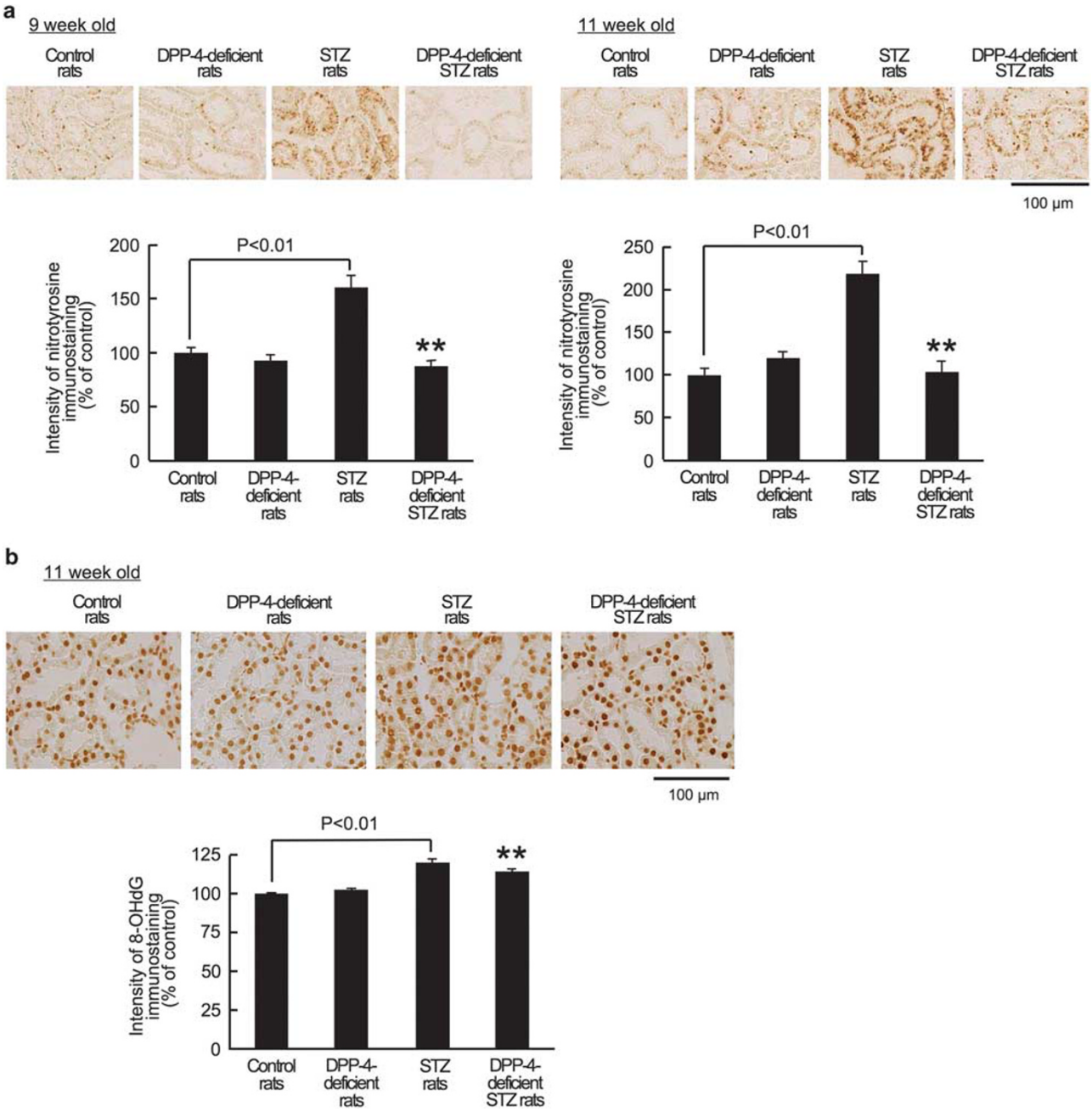

Figure 2 Nitrotyrosine (**a**) and 8-OHdG levels (**b**) in the kidneys of Control, DPP-4-deficient, STZ or DPP-4-deficient STZ rats at 9 (**a**) and 11 weeks old (**a** and **b**). The kidneys were fixed in 4% paraformaldehyde and embedded in paraffin, sectioned at 4-μm intervals and mounted on glass slides. Then, the sections were incubated overnight at 4 °C with anti-nitrotyrosine (**a**) or anti-8-OHdG antibodies (**b**), and the reactions were visualized with a Histofine Simple Stain Rat MAX-POMULTI kit. Immunoreactivity of each sample was measured by microcomputer-assisted image J. Five (**a**) or twenty different fields (**b**) in each sample were evaluated. Each upper panel shows the representative microphotographs. Each lower panel shows the quantitative data. $^{**}P<0.01$ compared to the value with STZ rats. $N=4$ for Control rats. $N=4$ for DPP-4-deficient rats. $N=4$ for STZ rats. $N=4$ for DPP-4-deficient STZ rats.

mice.[34] Further, the AGE-RAGE-induced oxidative stress generation could potentiate the harmful effects of AGEs *via* RAGE overexpression.[3,5,35] So, oxidative stress generation evoked by the AGE-RAGE axis could further stimulate the formation of AGEs and RAGE induction, forming a vicious cycle between the AGE-RAGE axis and oxidative stress generation. DPP-4 deficiency might enhance the inhibitory effects of incretins on the AGE-RAGE-oxidative stress axis by breaking the vicious cycle. This could lead to suppress the inflammatory reaction in the diabetic kidneys and subsequently reduce albuminuria in type 1 diabetic rats. We have previously shown that GLP-1 analog, exendin-4 treatment reduces renal RAGE and ICAM-1 gene expression and urinary excretion levels of 8-OHdG and albumin in STZ rats.[19]

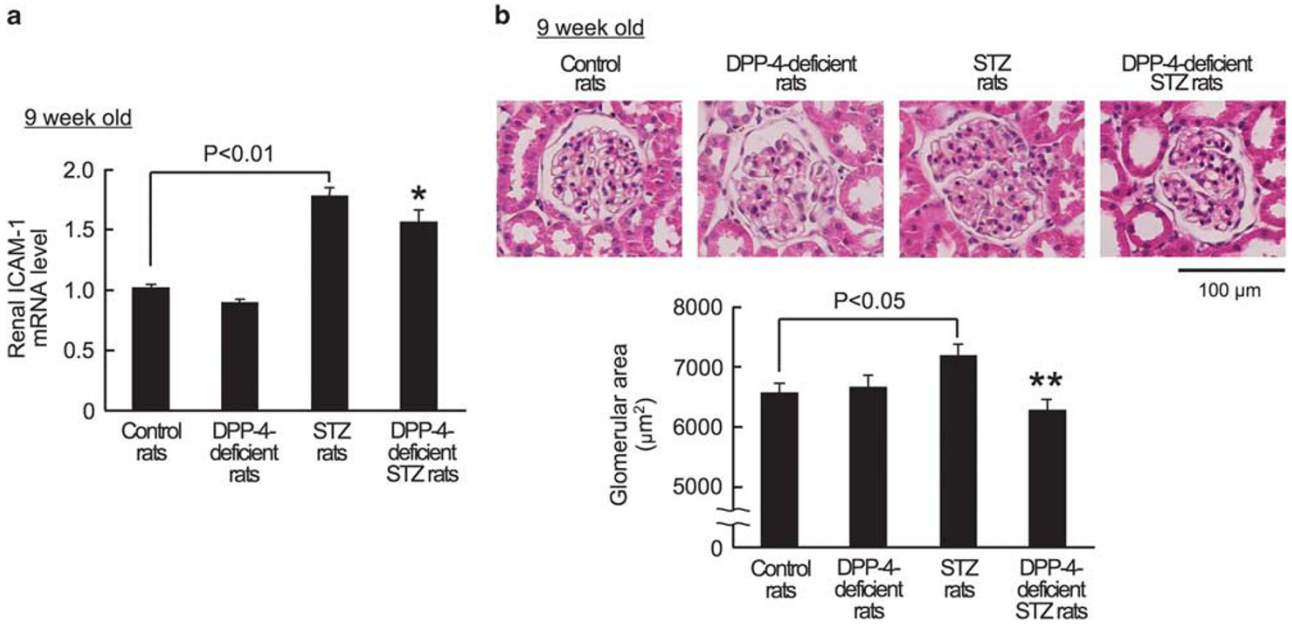

Figure 3 ICAM-1 gene expression in the kidneys (**a**) and glomerular area (**b**) of Control, DPP-4-deficient, STZ or DPP-4-deficient STZ rats at 9 weeks old. (**a**) Total RNAs were extracted from the kidneys of Control, DPP-4-deficient, STZ or DPP-4-deficient STZ rats with RNAqueous-4PCR kit. Quantitative real-time RT-PCR was performed. Data were normalized by the intensity of β-actin mRNA-derived signals. $^{*}P<0.05$ compared to the value with STZ rats. $N=3$ for Control rats. $N=4$ for DPP-4-deficient rats. $N=4$ for STZ rats. $N=3$ for DPP-4-deficient STZ rats. (**b**) The kidney sections were stained with hematoxylin and eosin for light microscopic analysis. Glomerular area was measured using Image J software. Fifteen glomeruli in each sample were evaluated. Upper panel shows the representative microphotographs. Lower panel shows the quantitative data. $^{**}P<0.01$ compared to the value with STZ rats. $N=4$ for Control rats. $N=4$ for DPP-4-deficient rats. $N=4$ for STZ rats. $N=3$ for DPP-4-deficient STZ rats.

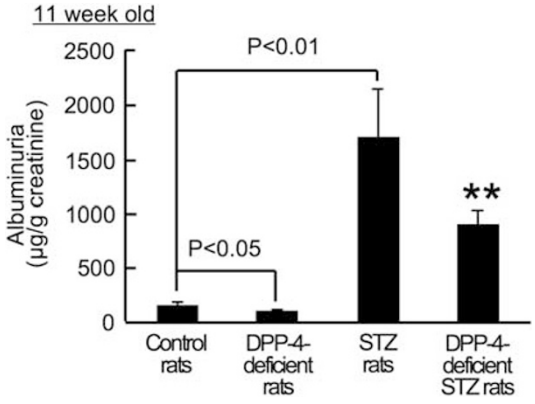

Figure 4 Albuminuria of Control, DPP-4-deficient, STZ or DPP-4-deficient STZ rats at 11 weeks old. Albuminuria was determined with an ELISA kit. $^{**}P<0.01$ compared to the value with STZ rats. $N=4$ for Control rats. $N=4$ for DPP-4-deficient rats. $N=4$ for STZ rats. $N=4$ for DPP-4-deficient STZ rats.

Exendin-4 improved glomerular area expansion in STZ rats as well.[19] These findings indicate that the effects of GLP-1 analog might be comparable to those of DPP-4 deficiency or DPP-4 inhibitors, in terms of oxidative stress and AGE-RAGE axis.

One early phase of diabetic nephropathy involves the recruitment and firm adhesion of inflammatory cells to mesangial areas, whose process is mediated by adhesion molecules such as ICAM-1.[36,37] Indeed, increased expression of ICAM-1 is associated with monocyte infiltration into both mesangial and tubulointerstitial lesions in diabetic nephropathy.[36,37] Urinary ICAM-1/creatinine ratio in type 2 diabetic patients with microalbuminuria was much higher than those in normal controls, and intensive insulin treatment significantly reduced urinary ICAM-1 and albumin excretions.[38] Since the AGE-RAGE-induced oxidative stress generation enhances ICAM-1 expression in various types of cells,[18,20,22,39,40] our present study suggests that DPP-4 deficiency could inhibit renal damage in STZ rats by suppressing ICAM-1 expression in the kidneys partly through the blockade of the deleterious effects of AGE-RAGE system.

Limitations

In this study, we evaluated the effect of DPP-4 deficiency on diabetic nephropathy using a 4-week type 1 diabetic model. So, the animals were studied for a rather short duration. This might reduce the ability of the model to exhibit a prominent renal phenotype. Due to the short-term diabetes study, we did not measure glycated hemoglobin values of each animal. However, since there was no significant difference of fasting blood glucose levels between the two diabetic rat strains during the study periods, it is unlikely that effects of DPP-4 deficiency on blood glucose levels could confound the present findings.

In the present study, the changes in 8-OHdG used as oxidative stress were moderate and no changes were observed in 11 weeks urine of DDP-4-deficient STZ rats. Since age is an important factor in oxidative stress, we performed additional measurements of oxidative stress in the kidneys. As shown in

Figure 2a, renal nitrotyrosine levels were increased in 9- and 11-week-old STZ rats compared with age-matched Control rats, both of which were significantly reduced in DDP-4-deficient STZ rats. So, although we did not know the exact reason why urinary excretion levels of 8-OHdG were not suppressed in DDP-4-deficient STZ rats at 11 weeks old, DPP-4 deficiency might suppress the AGE-RAGE-induced oxidative stress generation in the kidneys during 4-week diabetic exposure, which could lead to reduction of albuminuria in STZ rats. Further, it would be helpful to examine the effects of DPP-4 deficiency on RAGE and ICAM-1 gene expression and glomerular area expansion of 11-week-old STZ rats.

Experimental animal model does not completely mimic human diabetic nephropathy. Therefore, further large-scale prospective study is needed to clarify whether DPP-4 inhibition could suppress the AGE-RAGE-oxidative stress axis in the diabetic kidneys and be superior to other oral equipotent hypoglycemic agents in protecting against the development and progression of nephropathy in both type 1 and type 2 diabetic patients.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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