

RESEARCH PAPER

Sildenafil, a phosphodiesterase type 5 inhibitor, attenuates diabetic nephropathy in non-insulin-dependent Otsuka Long-Evans Tokushima Fatty rats

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BACKGROUND

It is well established that the pathogenesis of diabetic nephropathy is associated with abnormalities of renal nitric oxide (NO) generation. Many of the biological actions of NO are mediated by cGMP, which is rapidly degraded by phosphodiesterases. In this study, we evaluated the renoprotective effects of sildenafil (SIL), an inhibitor of phosphodiesterase-5, in type 2 diabetic rats.

METHODS

Male Otsuka Long-Evans Tokushima Fatty (OLETF) rats, a non-insulin-dependent diabetes model, and Long-Evans Tokushima Otsuka rats, a non-diabetic control, were treated with either SIL (2.5 mg·kg⁻¹ in drinking water) or undosed water for 28 weeks, starting at 30 weeks of age.

RESULTS

Sildenafil treatment significantly decreased albuminuria, attenuated glomerular hyperfiltration and resulted in a decrease in glomerular hypertrophy, in addition to a reduced glomerulosclerosis score and a dramatic decrease in the number of glomerular and tubulointerstitial proliferating cell nuclear antigen-positive cells in OLETF rats. This was accompanied by a significant reduction in renal cortical mRNA levels of collagen types I and III. The increased mRNA levels of matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitors of MMPs (TIMP)-1 and TIMP-2 in the OLETF rats were significantly or partially attenuated by SIL treatment.

CONCLUSIONS

This study suggests that SIL attenuated diabetic nephropathy due to its potent antiproliferative effects and its regulatory effects on extracellular matrix. This latter effect is thought to be a result of its ability to affect the balance between MMPs and their inhibitors.

Abbreviations

BUN, blood urea nitrogen; BW, body weight; Ccr, creatinine clearance; Cr, creatinine; DAB, diaminobenzidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LETO, Long-Evans Tokushima Otsuka; L-NAME, N-nitro-L-arginine methyl ester; MAPK, mitogen-activated protein kinases; MEK, MAPK kinase; MMP, matrix metalloproteinase; NO, nitric oxide; OLETF, Otsuka Long-Evans Tokushima Fatty; PAS, periodic acid Schiff; PDE, phosphodiesterase; Silver, periodic acid-methenamine silver; STZ, streptozotocin; TIMP, tissue inhibitors of MMPs

Introduction

Nitric oxide (NO) is an important mediator in a variety of physiological and pathophysiological processes. Many of the biological actions of NO are mediated by cGMP, which is rapidly degraded by phosphodiesterases (PDEs). Sildenafil is an inhibitor of PDE type 5, the enzyme that selectively hydrolyses cGMP, and it has been widely used to treat erectile dysfunction (Jeremy *et al.*, 1997; Rosen and Kostis, 2003). Its pharmacological action is due to its ability to prolong the signalling actions of NO by raising the available cGMP pool in penile smooth muscle. The safety and high tolerability of sildenafil make it an attractive tool to investigate diverse therapeutic effects beyond erectile dysfunction, even for indications in which chronic administration is necessary. The approval of sildenafil for the treatment of pulmonary hypertension in 2005 was a notable success in this area of research (Galie *et al.*, 2005). In the heart, sildenafil has cardioprotective effects against ischaemia-reperfusion and anthracycline toxicity, blunts acute adrenergic contractile stimulation, and can suppress chronic hypertrophy and dysfunction attributable to pressure overload (Kass *et al.*, 2007). A number of other potential new indications are currently in various phases of preclinical research and development (Kass *et al.*, 2007; Sandner *et al.*, 2007).

It is well established that the pathogenesis of diabetic nephropathy is associated with abnormalities of renal NO generation (Komers and Anderson, 2003; Prabhakar, 2004). Several studies have suggested that diabetic nephropathy is associated with a defect in NO production (Reyes *et al.*, 1993; Pieper *et al.*, 1997). The administration of L-arginine, a precursor of NO, has been found to prevent hyperfiltration and ameliorate proteinuria in streptozotocin (STZ)-induced diabetic rats (Pieper *et al.*, 1997). In addition, long-term dietary supplementation of arginine, which increases NO production, prevented the changes in renal function and structure normally found in STZ-induced diabetic rats (Reyes *et al.*, 1993). On the other hand, Komers *et al.* (1994) demonstrated that, in STZ-induced diabetic rats, urinary NO metabolites (NO₂ + NO₃) were increased, and inhibition of NO production by N-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, prevented the increase in glomerular filtration rate that normally characterizes diabetes in these models. Also, Kamijo *et al.* (2006) demonstrated that administration of L-arginine exacerbated proteinuria in type 2 diabetic rats. Thus, conflicting evidence continues to exist in this field. Cell culture studies using glomerular endothelial cells or mouse kidney slices have also shown that high glucose levels rapidly up-regulate eNOS protein expression but decrease NO synthesis in glomerular endothelial cells through superoxide overproduction and protein kinase C activation (Hoshiyama *et al.*, 2003; Chu and Bohlen, 2004). Furthermore, it has been shown that NO release and NO-dependent cGMP production declines progressively in glomeruli isolated from diabetic rats (Craven *et al.*, 1994; 1995).

All these results indicate that pharmacological inhibition of the cGMP-specific PDE-5 has the potential to ameliorate diabetic nephropathy. Therefore, the present study was designed to determine whether sildenafil, a selective PDE type 5 inhibitor, might have renoprotective effects in type 2 diabetic rats.

Methods

Experimental protocol

The experimental protocol for this study was reviewed and approved by the Animal Care Committee of Showa University in Tokyo. Male Otsuka Long-Evans Tokushima Fatty (OLETF) rats ($n = 14$) at 30 weeks of age, a type 2 (non-insulin-dependent) diabetic model, and age-matched Long-Evans Tokushima Otsuka (LETO) rats (LETO group) ($n = 6$), a non-diabetic control, were obtained from Otsuka Pharmaceutical Tokushima Research Institute (Tokushima, Japan) (Kawano *et al.*, 1994; Hirashima *et al.*, 1995). These inbred rats were isolated from an outbred colony of Long-Evans rats that had been purchased in 1982 from Charles River Canada (St Constant, Canada). The rats were given free access to tap water and standard rat chow. OLETF rats were administered either sildenafil (OLETF-SIL group) ($n = 7$) (2.5 mg·kg⁻¹ in drinking water) or undosed water (OLETF-CON group) ($n = 7$) from 30 weeks of age for 28 weeks. At the end of the study, the rats were anaesthetized, blood was collected by cardiac puncture and the kidneys were removed. Renal tissue was divided; some portions were snap-frozen in liquid nitrogen, and some portions were fixed in 2% paraformaldehyde/PBS for later use.

Metabolic data

Body weight (BW) was recorded periodically for each rat. Systolic and diastolic blood pressure was measured every month by the tail cuff method; at least three readings for each rat were averaged. Plasma glucose levels were measured every month using a glucose oxidase-based method. Serum creatinine (Cr), blood urea nitrogen (BUN), total cholesterol, triglyceride and urinary Cr were measured by standard methods using an automatic analyser (Hitachi 7170, Hitachi, Tokyo, Japan). For the analysis of albuminuria, rats were housed individually in metabolic cages for 24 h urine collection. Urine samples were collected once a month. Urinary albumin was determined using a Nephrot kit (Exocell, Philadelphia, PA, USA). The creatinine clearance (Ccr) was calculated using a standard formula. Urinary cGMP excretion was measured using commercially available assay kits following the manufacturer's instructions (Direct cGMP Enzyme Immunoassay Kit, Sigma-Aldrich, St. Louis, MO, USA).

Light microscopic study

Tissues fixed in 2% paraformaldehyde/PBS were embedded in paraffin using routine protocols. Paraffin-embedded materials were sectioned at 4 µm for routine staining with periodic acid Schiff and Masson trichrome. Two-micrometer-thick sections were used for periodic acid-methenamine silver stains (silver). The glomerular tuft area was quantified in 50 full-sized glomeruli (periodic acid Schiff stain) using WinROOF image processing software (Mitani Corp., Tokyo, Japan). Glomerulosclerosis was assessed in 50 glomeruli on silver-stained sections using a semiquantitative score from 0 to 4: 0, no sclerosis; 1, sclerosis up to 25% of glomeruli; 2, sclerosis from 25% to 50% of glomeruli; 3, sclerosis from 50% to 75% of glomeruli; and 4, sclerosis more than 75% of glomeruli. For evaluating tubulointerstitial fibrosis, 10 fields for each section (Masson trichrome stain) were evaluated at 200× magnifica-

tion using WinROOF image processing software (Mitani Corp.). The extent of tubulointerstitial damage was evaluated by counting the percentage of areas with tubular dilatation, interstitial infiltration and fibrosis per field of cortex. Scores from 0 to 5 were used: 0, normal interstitium; 1, <10% of areas injured; 2, 11–25% of areas injured; 3, 26–50% of areas injured; 4, 51–75% of areas injured; and 5, >76% of areas injured. All histological analyses were performed by two investigators without knowledge of the origin of the slides, and the mean values were calculated.

Immunohistochemistry

The monoclonal antibody against mouse anti-proliferating cell nuclear antigen (PCNA) was purchased from Progen (Heidelberg, Germany), and polyclonal antibody against goat anti-human collagen type IV was purchased from Southern Biotechnology (Birmingham, AL, USA). Biotinylated rabbit anti-mouse IgG, biotinylated rabbit anti-goat IgG and peroxidase-conjugated streptavidin (LSAB 2 kit/HRP) were purchased from Dako (Glostrup, Denmark). Immunohistochemical staining for PCNA and collagen type IV was performed as follows. The paraffin sections of renal tissues were dewaxed, washed in PBS, drained and incubated with the anti-PCNA and collagen type IV antibodies as the primary antibodies overnight at 4°C. In collagen type IV staining, the sections were autoclave-heated at 121°C for 10 min before using primary antibody. They were then washed three times in PBS and incubated with a secondary antibody. After endogenous peroxidase was inactivated by incubation with 0.3% H₂O₂ in methanol, the sections were incubated with LSAB 2 kit/HRP and developed using diaminobenzidine (DAB) (Dako) as the substrate to produce a brown stain. The number of PCNA-positive cells in glomeruli was assessed by counting stained cell nuclei in 50 random glomerular cross-sections. The quantification of PCNA-positive cells in the tubulointerstitium was performed by counting stained cell nuclei in 10 consecutive renal cortical fields at 200× magnification and expressed as number of cells per 0.5 mm². The percentage of glomerular tuft area occupied by collagen type IV-stained matrix was quantified in 20 full-sized glomeruli using WinROOF image processing software (Mitani Corp.).

Real-time reverse transcriptase polymerase chain reaction

Gene expressions of rat collagen type I, collagen type III, fibronectin, MMP-2, MMP-9, TIMP-1, TIMP-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were analysed using real-time reverse transcriptase polymerase chain reaction (RT-PCR). Briefly, kidney tissues (cortex) were homogenized using a TissueLyser (QIAGEN, Hilden, Germany), and total RNA was isolated using an RNeasy Fibrous Tissue Mini kit (QIAGEN), in accordance with the manufacturer's instructions. cDNA synthesis was carried out using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). Predesigned TaqMan probe sets for the targets indicated above were purchased from Applied Biosystems (Foster City, CA, USA). Each probe had a fluorescent reporter dye (FAM) linked to its 5' end and a downstream quencher dye (TAMRA) linked to its 3' end. Each reaction consisted of 25 µL containing 2 × Universal

Master Mix (Applied Biosystems), primers, labelled probes and cDNA. The amplification conditions consisted of 40 cycles at 95°C for 15 s and 60°C for 1 min after incubation at 95°C for 10 min. Amplification and fluorescence measurements were performed using the MicroAmp Optical 96-Well Reaction Plate on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). mRNA expressions were normalized using GAPDH as an endogenous control to correct for the differences in the amount of total RNA added to each reaction.

Homogenization of kidney tissues

Kidney tissues (cortex) were homogenized with T-PER Mammalian Protein Extraction Reagent (20 mL·g⁻¹ renal tissues) (Pierce Biotechnology, Rockford, IL, USA) containing 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich) using a TissueLyser (QIAGEN, Hilden, Germany). Harvested lysates were then centrifuged for 10 min at 4°C to remove the cellular debris. The supernatants were collected and stored at -80°C. Protein concentration was measured using the BCA protein assay reagent kit (Pierce Biotechnology).

Western blot analysis

Ten micrograms of protein in kidney tissue homogenate from each sample was separated on a 4–20% gradient gel (Invitrogen) using SDS-PAGE and transferred to PVDF membrane. The blots were blocked with TBST buffer [20 mM Tris-HCl (pH 7.4), 140 mM NaCl and 0.05% Tween 20] containing 5% skimmed milk at room temperature for 1 h, washed three times in TBST buffer and incubated with primary antibody [MMP-2 and MMP-9 (Daiichi Fine Chemical Co., Ltd, Toyama, Japan, 1:100)] overnight at 4°C. The membranes were then incubated with secondary antibody [HRP-conjugated anti-mouse IgG antibody (Cell Signaling Technology, 1:3000)] at room temperature for 1 h. The reaction products were detected using the enhanced chemiluminescence detection system. After stripping, GAPDH was used as the loading control.

Zymography

Renal cortical tissue samples were homogenized, and protein concentration was measured using the BCA protein assay reagent kit (Pierce Biotechnology). The homogenized samples were loaded onto a 10% SDS acrylamide gel containing 1 mg·mL⁻¹ gelatin (Bio-Rad). After electrophoresis, the gel was incubated in renaturing buffer (Invitrogen) and activated in developing buffer (Invitrogen), and gelatinase activity was visualized by staining with Coomassie blue.

Measurement of transforming growth factor-β1 protein level in kidney tissue homogenate

Transforming growth factor (TGF)-β1 protein level was measured in kidney tissue homogenate from each sample by a commercially available TGF-β1 ELISA kit (R&D Systems, Abingdon, Oxfordshire, UK) following the manufacturer's instructions. To control for the difference between samples, the concentration was corrected based on the amount of total tissue protein.

Table 1

Effect of sildenafil treatment on blood glucose, body weight and blood pressure in the study groups

	Duration of treatment			
	0 week	8 weeks	20 weeks	28 weeks
Blood glucose (mg·L ⁻¹)				
LETO	1168.30 ± 28.30	1333.30 ± 61.20	1075.00 ± 51.00	1105.00 ± 63.10
OLETF-CON	1388.70 ± 101.60	1726.20 ± 169.60	1571.20 ± 80.40***	1445.00 ± 75.70**
OLETF-SIL	1318.80 ± 60.20	1722.50 ± 89.30	1465.00 ± 44.80	1567.50 ± 155.80
Body weight (g)				
LETO	493.13 ± 3.05	493.01 ± 4.79	522.91 ± 6.12	536.26 ± 8.29
OLETF-CON	594.03 ± 12.64***	578.41 ± 24.27*	639.85 ± 22.92**	653.37 ± 26.69**
OLETF-SIL	605.78 ± 15.09	590.75 ± 16.62	660.23 ± 16.53	657.18 ± 22.68
Systolic blood pressure (mmHg)				
LETO	110.38 ± 6.37	118.61 ± 6.84	115.45 ± 5.76	115.16 ± 7.35
OLETF-CON	138.14 ± 9.71*	168.51 ± 6.12***	146.91 ± 8.51*	159.77 ± 6.68***
OLETF-SIL	134.51 ± 3.47	148.50 ± 6.43#	141.46 ± 5.36	148.87 ± 3.44
Diastolic blood pressure (mmHg)				
LETO	84.71 ± 5.59	94.76 ± 5.25	90.23 ± 4.52	91.90 ± 8.28
OLETF-CON	108.80 ± 4.82**	132.17 ± 4.51***	115.85 ± 7.53*	126.51 ± 4.43**
OLETF-SIL	103.47 ± 4.97	114.17 ± 4.95#	110.87 ± 6.62	116.73 ± 3.34

P* < 0.05, *P* < 0.01, ****P* < 0.001 vs. LETO.

#*P* < 0.05 vs. OLETF-CON.

LETO, Long-Evans Tokushima Otsuka; OLETF, Otsuka Long-Evans Tokushima Fatty.

Table 2

Clinical parameters of the study groups at the time of death (58 weeks of age)

	LETO	OLETF-CON	OLETF-SIL
Total kidney weight (g)	2.61 ± 0.04	3.61 ± 0.09***	3.71 ± 0.12
Kidney weight/body weight	4.94 ± 0.07	5.58 ± 0.14**	5.60 ± 0.15
Blood urea nitrogen (mg·L ⁻¹)	302.30 ± 4.20	217.90 ± 7.50***	283.10 ± 9.20##
Serum creatinine (mg·L ⁻¹)	4.10 ± 0.20	2.70 ± 0.10***	3.10 ± 0.20#
Creatinine clearance (mL·min ⁻¹)	2.08 ± 0.17	2.79 ± 0.20*	2.54 ± 0.14
Total cholesterol (mg·L ⁻¹)	1296.70 ± 28.80	1562.50 ± 93.60*	1666.30 ± 104.10
Triglycerides (mg·L ⁻¹)	60.83 ± 10.68	293.38 ± 44.00***	270.25 ± 66.60
Urine cGMP			
nmol·24 h ⁻¹	30.45 ± 1.99	37.12 ± 1.89**	50.29 ± 5.56#
nmol·mg ⁻¹ creatinine	1.80 ± 1.05	2.45 ± 0.65***	3.15 ± 0.26#

Data are mean ± SEM.

P* < 0.05, *P* < 0.01, ****P* < 0.001 vs. LETO.

#*P* < 0.05, ##*P* < 0.001 vs. OLETF-CON.

LETO, Long-Evans Tokushima Otsuka; OLETF, Otsuka Long-Evans Tokushima Fatty.

Statistical analysis

Data were recorded as means \pm SEM. The Mann–Whitney test was performed, and values of $P < 0.05$ were considered statistically significant.

Results

Effects of sildenafil on biochemical parameters in OLETF rats

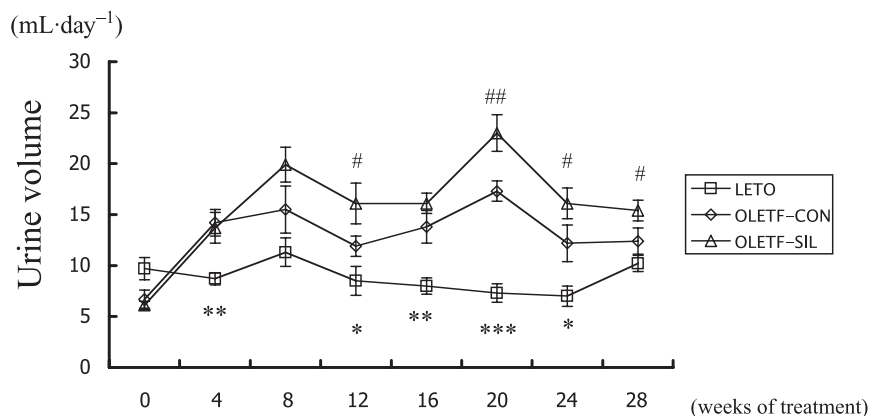
The OLETF-CON and OLETF-SIL rats had higher plasma glucose concentrations, BW, and systolic and diastolic blood pressure than the LETO rats throughout the study period. There were no significant changes in plasma glucose concentrations and BW in the OLETF-CON and OLETF-SIL rats throughout the study period. Sildenafil treatment induced significant changes in systolic and diastolic blood pressure at 8 weeks of treatment (Table 1). Table 2 shows the biochemical results for each group. Kidney weight was significantly higher in OLETF-CON rats than in LETO rats ($P < 0.001$). Sildenafil treatment did not affect kidney weight in

OLETF rats. Serum Cr ($P < 0.001$) and BUN ($P < 0.001$) levels were significantly lower, and Ccr ($P < 0.05$) was significantly higher in OLETF-CON rats than in LETO rats, reflecting glomerular hyperfiltration in diabetic kidneys. Sildenafil treatment significantly attenuated these changes in serum Cr ($P < 0.05$) and BUN ($P < 0.001$) levels in OLETF rats. The increase of Ccr in OLETF rats tended to be less with sildenafil treatment, but this trend was not statistically significant. Total cholesterol ($P < 0.001$) and triglyceride ($P < 0.05$) levels were significantly higher in OLETF-CON rats than in LETO rats, and sildenafil treatment did not affect these parameters. To verify the effect of sildenafil treatment on NO production, urinary cGMP excretion was measured. Sildenafil treatment significantly increased urinary cGMP excretion in OLETF rats ($P < 0.05$).

Effects of sildenafil on urine volume and urinary albumin excretion in OLETF rats

Urine volume was significantly higher in OLETF-CON rats than in LETO rats at 4 ($P < 0.01$), 12 ($P < 0.05$), 16 ($P < 0.01$), 20 ($P < 0.001$) and 24 ($P < 0.05$) weeks, but not at 8 and 28

A Urine volume



B Urine albumin

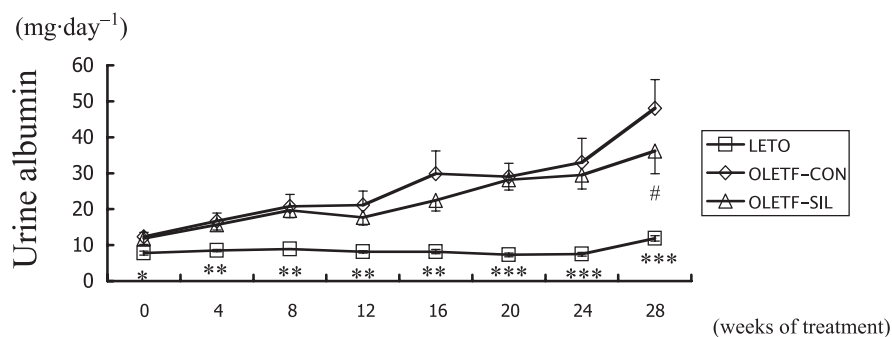


Figure 1

Urine volume and urinary albumin excretion in the study groups. Changes in (A) urine volume and (B) urinary albumin excretion of LETO rats and OLETF rats treated with either sildenafil (OLETF-SIL) or vehicle (OLETF-CON) are shown. Data are means \pm SEM. Mann–Whitney test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: LETO vs. OLETF-CON; # $P < 0.05$, ## $P < 0.01$: OLETF-SIL vs. OLETF-CON. LETO, Long-Evans Tokushima Otsuka; OLETF, Otsuka Long-Evans Tokushima Fatty.

weeks. Sildenafil treatment significantly increased urine volume in OLETF rats at 12 ($P < 0.05$), 20 ($P < 0.01$), 24 ($P < 0.05$) and 28 ($P < 0.05$) weeks, but not at 4, 8 and 16 weeks (Figure 1A). Urine albumin excretion was significantly higher in OLETF-CON rats than in LETO rats throughout the study period. The increased urinary albumin excretion in OLETF rats was significantly reduced by sildenafil treatment at the end of the study ($P < 0.05$) (Figure 1B).

Effects of sildenafil on renal histological findings in OLETF rats

Figure 2 shows the renal histological findings of the study groups. Glomerular lesions in OLETF rats were characterized by hypertrophy, mesangial matrix expansion, sclerotic lesions and hyalinosis. The quantitative analysis of the glomerular tuft area is presented in Table 3. Sildenafil treatment significantly reduced glomerular hypertrophy in OLETF rats ($P <$

0.05). The glomerular sclerosis score increased significantly in OLETF-CON rats compared with in LETO rats ($P < 0.001$), and this increase was significantly reduced by sildenafil treatment ($P < 0.05$) (Table 3). Although the tubulointerstitial damage in OLETF rats was faint, the tubulointerstitial damage score was greater in OLETF-CON rats than in LETO rats ($P < 0.001$). Sildenafil treatment did not significantly improve the tubulointerstitial damage in OLETF rats (Table 3).

Effects of sildenafil on cell proliferation in OLETF rats

Quantitative evaluation of glomerular cell proliferation and tubulointerstitial cell proliferation by measurement of PCNA-staining cells (Figure 3) in glomeruli and the tubulointerstitium showed a twofold and a threefold increase in PCNA-positive cells in OLETF-CON rats compared with LETO rats respectively. These increases in PCNA-positive cells in

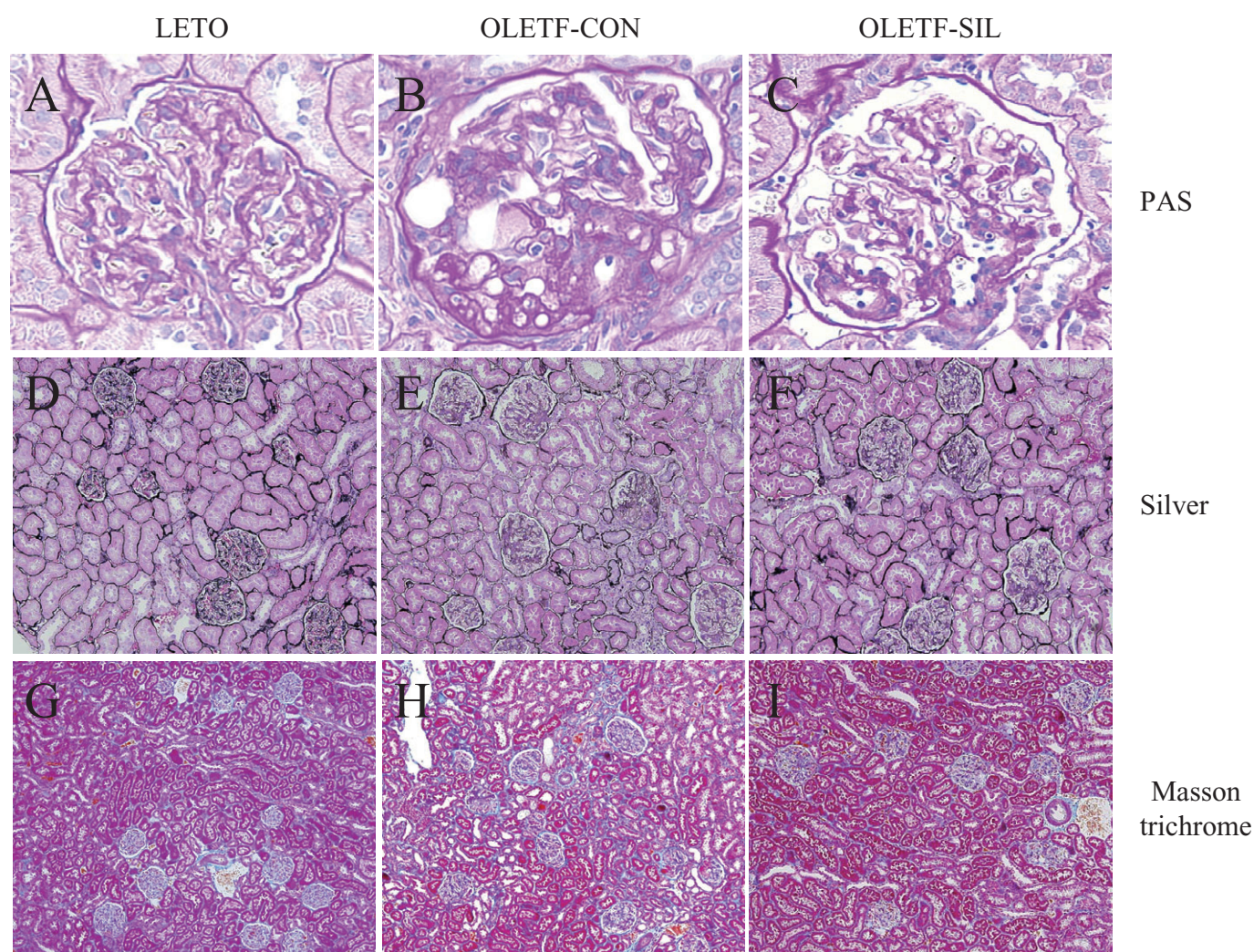


Figure 2

Light microscopic findings in the study groups. Representative pictures stained with periodic acid Schiff (PAS; A–C), silver (D–F) and Masson trichrome (G–I) in a LETO rat (A, D, G), an OLETF-CON rat (B, E, H) and an OLETF-SIL rat (C, F, I). LETO, Long-Evans Tokushima Otsuka; OLETF, Otsuka Long-Evans Tokushima Fatty. Original magnifications, $\times 400$ (A–C), $\times 100$ (D–F) and $\times 40$ (G–I).

Table 3

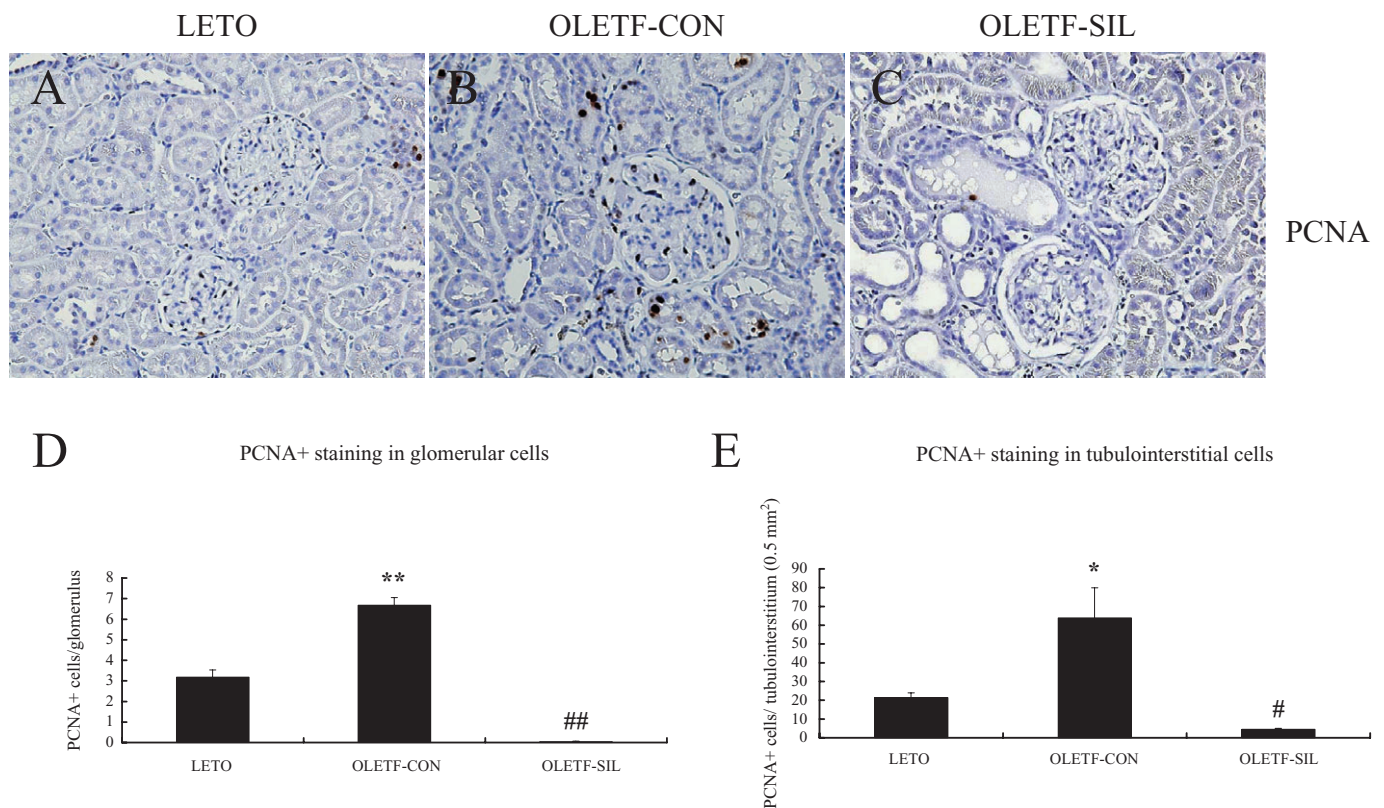
Morphological evaluation of glomerular size, glomerular sclerosis and tubulointerstitial damage at the end of study (58 weeks of age)

	LETO	OETF-CON	OETF-SIL
Glomerular size (μm^2)	9207.80 \pm 199.18	10 856.60 \pm 206.65*	9921.71 \pm 266.60#
Glomerular sclerosis score (0–4)	0.12 \pm 0.02	1.42 \pm 0.13*	0.89 \pm 0.13#
Tubulointerstitial damage score (0–5)	0	0.43 \pm 0.19*	0.29 \pm 0.17

Morphometric analysis of kidney sections from the LETO rats, OETF-CON rats and OETF-SIL rats.

Data are mean \pm SEM.* $P < 0.001$ vs. LETO rats.# $P < 0.05$ vs. OETF-CON rats.

LETO, Long-Evans Tokushima Otsuka; OETF, Otsuka Long-Evans Tokushima Fatty.

**Figure 3**

Immunohistochemistry for proliferating cell nuclear antigen (PCNA) in the study groups. (A–C) Representative pictures stained by immunohistochemistry for PCNA of a LETO rat (A), an OETF-CON rat (B) and an OETF-SIL rat (C). Original magnifications, $\times 200$. (D, E) Quantitative score of immunohistochemical analysis for PCNA in glomerular cells (D) and tubulointerstitial cells (E) in the study groups. Data are expressed as means \pm SEM. Mann–Whitney test: * $P < 0.01$, ** $P < 0.001$: LETO vs. OETF-CON; # $P < 0.001$, ## $P < 0.0001$: OETF-SIL vs. OETF-CON. LETO, Long-Evans Tokushima Otsuka; OETF, Otsuka Long-Evans Tokushima Fatty.

OETF rats were dramatically reduced to -99% and -93% in glomeruli and in the tubulointerstitium, respectively, by sildenafil treatment (Figure 3).

Effects of sildenafil on profibrogenic genes in OETF rats

The gene expression level of collagen type I was identical in OETF-CON and LETO rats, as assessed by real-time RT-PCR.

Sildenafil treatment significantly decreased collagen type I gene expression in OETF rats (Figure 4A). There was an increase in collagen type III gene expression in OETF-CON rats compared with LETO rats. Attenuated collagen type III gene expression was found in the kidneys of OETF rats treated with sildenafil (Figure 4B). As shown in Figure 4C, fibronectin mRNA was higher in OETF-CON than in LETO rats. Sildenafil treatment did not affect this parameter in

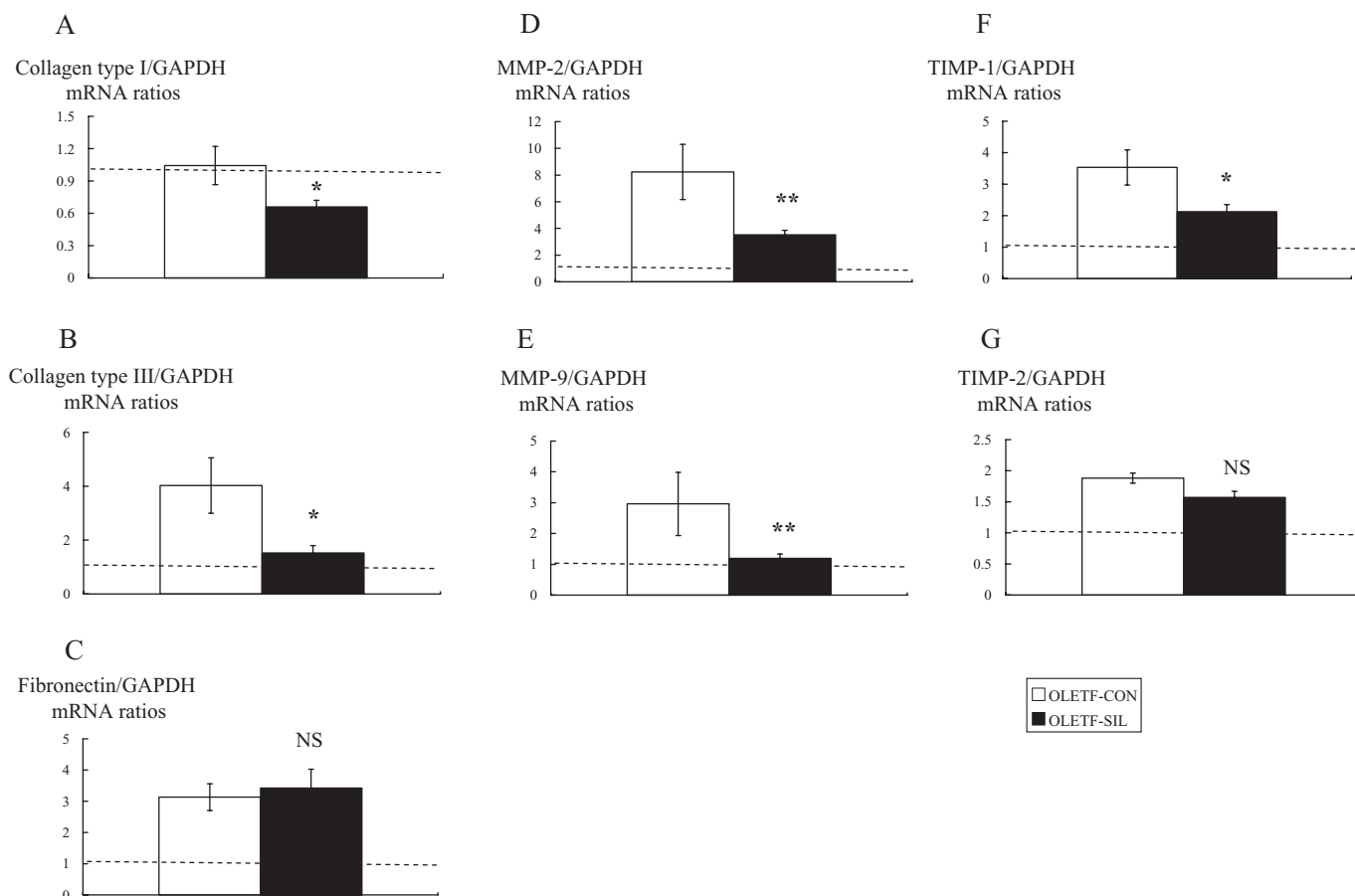


Figure 4

Effects of sildenafil on profibrogenic genes in the study groups. Real-time reverse transcriptase polymerase chain reaction for collagen type I (A), collagen type III (B), fibronectin (C), MMP-2 (D), MMP-9 (E), TIMP-1 (F) and TIMP-2 (G) in each group. The horizontal dotted lines show the expression levels in LETO rats. Data are expressed as means \pm SEM. The values were normalized to the GAPDH values and then expressed as relative quantity. Mann-Whitney test: * $P < 0.05$, ** $P < 0.01$ vs. OLETF-CON. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LETO, Long-Evans Tokushima Otsuka; MMP, matrix metalloproteinase; NS, not significant; OLETF, Otsuka Long-Evans Tokushima Fatty; TIMP, tissue inhibitors of MMPs.

OLETF rats. There was a significant increase in MMP-2 and MMP-9 gene expressions in diabetic kidneys compared with control kidneys and this was significantly reversed by sildenafil treatment (Figure 4D and E). To verify that the changes in mRNA expression are translated to protein and subsequently gelatinolytic activity, we used Western blot analysis and gelatin zymography methods, and showed that an almost similar pattern was obtained. Sildenafil treatment significantly reduced the protein levels of MMP-2 and MMP-9, and activity of MMP-2 but not MMP-9 (Figure 5A–C). A similar pattern was observed when TIMP-1 and TIMP-2 gene expressions were studied. There was a significant increase in the expression of these two inhibitors in diabetic kidneys compared with control kidneys. Sildenafil treatment reversed these changes, although the difference was only significant for TIMP-1 (Figure 4F and G).

Effects of sildenafil on glomerular extracellular matrix in OLETF rats

In order to verify the decrease in collagen gene expressions induced by sildenafil treatment, immunohistochemical staining for collagen type IV was performed. As shown in Figure 6, sildenafil treatment was associated with a significant decrease in the glomerular extracellular matrix as assessed by the percentage of glomerular tuft area occupied by the collagen type IV-stained matrix ($P < 0.05$).

Effects of sildenafil on TGF- β 1 protein synthesis in OLETF rats

To examine the effect of sildenafil on TGF- β 1 protein synthesis in OLETF rats, the amount of TGF- β 1 in kidney tissue homogenates was measured by use of a TGF- β 1 ELISA kit. The level of TGF- β 1 was significantly higher in OLETF-CON rats

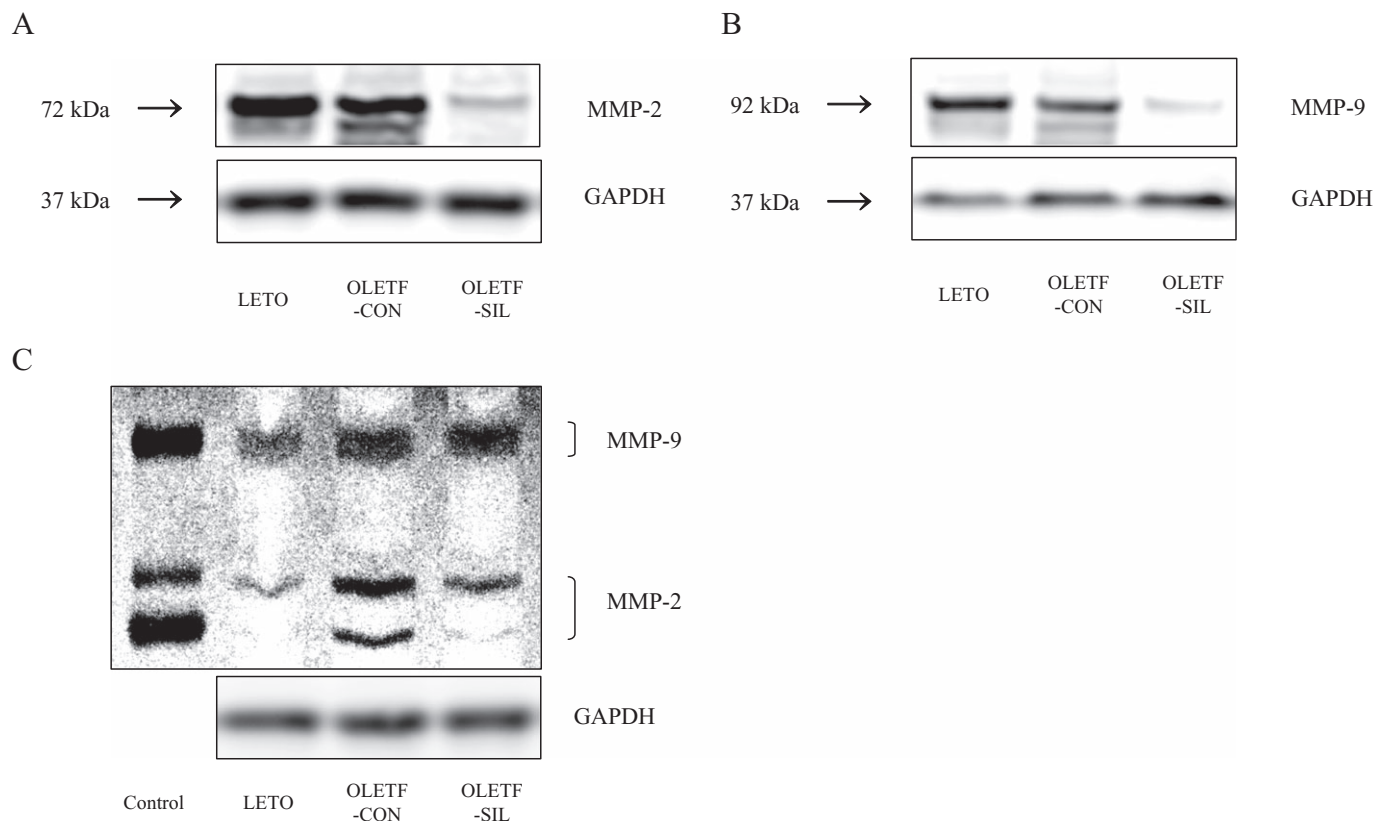


Figure 5

Effects of sildenafil on protein and gelatinolytic activity of MMP-2 and MMP-9 in the study groups. (A, B) Representative Western blot analysis for MMP-2, MMP-9 and GAPDH. (C) Representative gelatinolytic activity of MMP-2 and MMP-9, and Western blot analysis for GAPDH. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LETO, Long-Evans Tokushima Otsuka; MMP, matrix metalloproteinase; OLETf, Otsuka Long-Evans Tokushima Fatty.

than in LETO rats. Sildenafil treatment reduced the TGF- β 1 level by 15% ($P < 0.05$) in OLETf rats (Figure 7).

Discussion

The therapeutic benefit of sildenafil in animal models of kidney diseases, such as chronic renal failure (Rodriguez-Iturbe *et al.*, 2005), ischaemia-reperfusion injury (Choi *et al.*, 2009; Oruc *et al.*, 2010), type 1 diabetic nephropathy (Jeong *et al.*, 2009) and cisplatin-induced nephrotoxicity (Lee *et al.*, 2009), has been reported previously. In aggregate, these studies have shown that the beneficial effects of sildenafil treatment are the result of its inhibitory action on apoptosis (Rodriguez-Iturbe *et al.*, 2005; Choi *et al.*, 2009; Lee *et al.*, 2009), inflammation (Rodriguez-Iturbe *et al.*, 2005; Jeong *et al.*, 2009; Oruc *et al.*, 2010) and oxidative stress (Jeong *et al.*, 2009). In the present study, sildenafil treatment ameliorated progression of type 2 diabetic nephropathy in OLETf rats. The attenuation of glomerular hypertrophy, reduced glomerular extracellular matrix expansion and decreased glomerular sclerosis, along with suppression of albuminuria at the end of the study induced by sildenafil clearly show that it has renoprotective effects. Treatment of OLETf rats with

sildenafil did not appear to produce adverse effects during the study period. These findings provide the first *in vivo* evidence that inhibition of PDE type 5 protects against nephropathy in a non-insulin-dependent model of diabetes.

The most outstanding finding in the present study was the potent antiproliferative effect of sildenafil. Sildenafil treatment almost completely suppressed PCNA-positive glomerular cells ($\sim 99\%$), as well as tubulointerstitial cells ($\sim 93\%$). Indeed, the antiproliferative effects of PDE inhibitors have been documented in the acute phase of experimental mesangioproliferative glomerulonephritis (MSGN) (Tsuboi *et al.*, 1996; Hohenstein *et al.*, 2008). Tsuboi *et al.* (1996) reported that the selective PDE type 3 antagonist lixazine and the specific PDE type 4 antagonist rolipram decreased the activation and proliferation of mesangial cells, inhibited macrophage accumulation and prevented proteinuria in rat MSGN. Also, recently, Hohenstein *et al.* (2008) reported that vardenafil, a specific PDE type 5 inhibitor, markedly inhibited glomerular cell proliferation and extracellular matrix accumulation in rat MSGN. Several lines of evidence indicate that cGMP-increasing agents inhibit signal transduction from *Ras* by preventing *Ras*-dependent activation of *Raf-1*, thereby preventing the activity of the downstream kinases, including mitogen-activated protein kinases (MAPK) and MAPK kinase

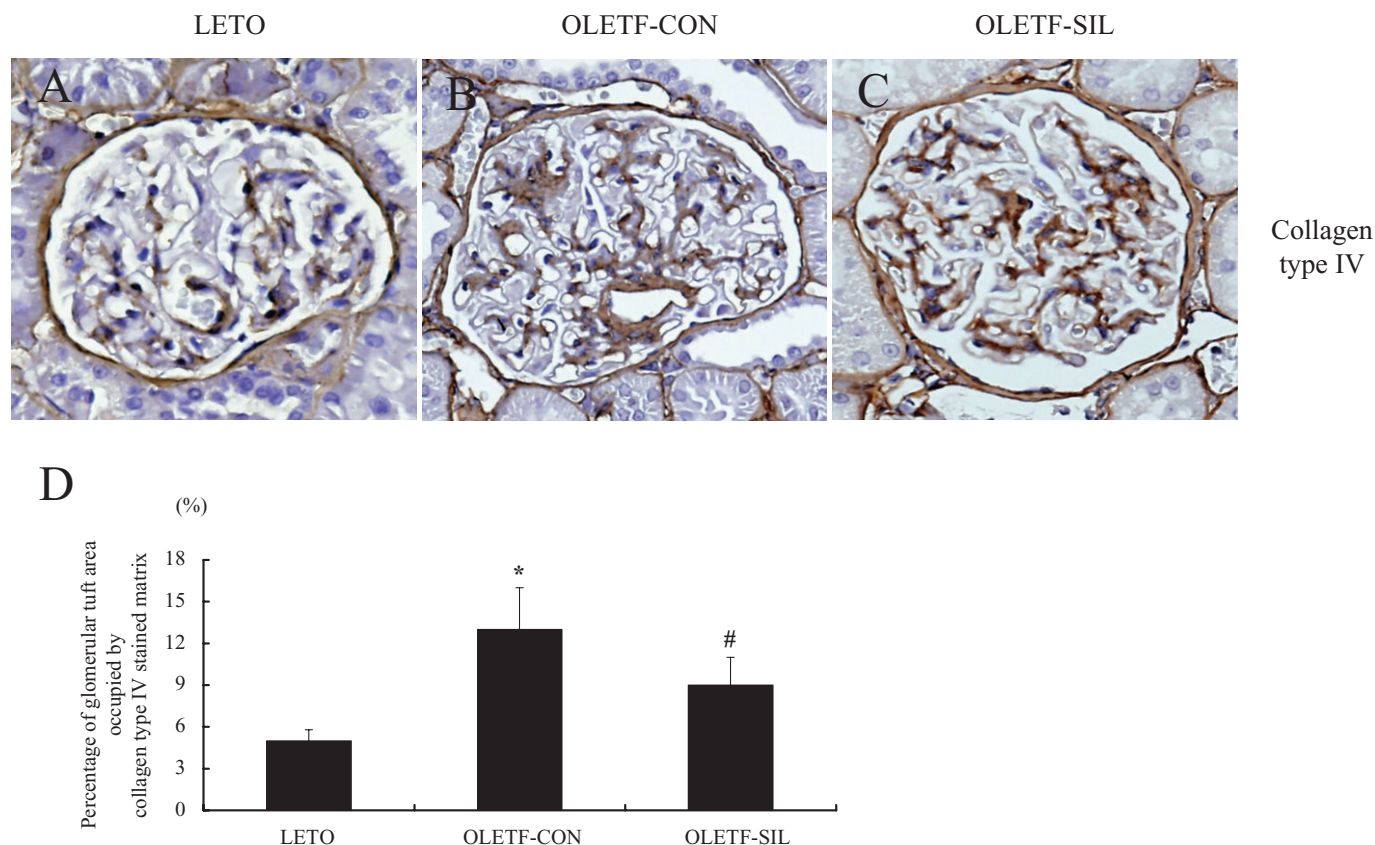


Figure 6

Immunohistochemistry for collagen type IV in the study groups. (A–C) Representative pictures stained with immunohistochemistry for collagen type IV of a LETO rat (A), an OLETF-CON rat (B) and an OLETF-SIL rat (C). Original magnifications, $\times 200$. (D) Percentage of glomerular tuft area occupied by collagen type IV-stained matrix in the study groups. Data are expressed as means \pm SEM. Mann–Whitney test: * $P < 0.01$: LETO vs. OLETF-CON; # $P < 0.01$: OLETF-SIL vs. OLETF-CON. LETO, Long-Evans Tokushima Otsuka; OLETF, Otsuka Long-Evans Tokushima Fatty.

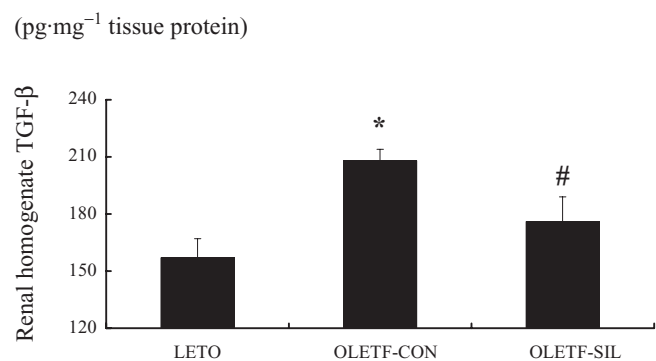


Figure 7

Effects of sildenafil on TGF- $\beta 1$ protein synthesis in OLETF rats. TGF- $\beta 1$ protein levels in renal cortical homogenate of each group measured by ELISA. Data are expressed as means \pm SEM. Mann–Whitney test: * $P < 0.01$: LETO vs. OLETF-CON; # $P < 0.001$: OLETF-SIL vs. OLETF-CON. LETO, Long-Evans Tokushima Otsuka; OLETF, Otsuka Long-Evans Tokushima Fatty; TGF, transforming growth factor.

(MEK) (Yu *et al.*, 1997; Li *et al.*, 2007). Thus, sildenafil has the potential to prevent the action of mitogenic stimuli that can activate glomerular or tubulointerstitial cell proliferation by inhibiting the signalling pathway at the *Raf-1* step, where different types of mitogenesis-activating signalling pathways converge, in this model of diabetic rats. The antiproliferative effects of sildenafil might, at least in part, contribute to subsequent inhibition of extracellular matrix accumulation and glomerular sclerosis in OLETF rats (Eng *et al.*, 1994; Johnson, 1994). Its potent inhibitory effect against cell proliferation resulted in a significant reduction of PCNA-positive cells in the OLETF-SIL rats compared with LETO rats. We speculate that the high dose of sildenafil used in the present study, which was 2.0- to 3.0-fold higher than the clinical dose, led to these results. However, this dose of sildenafil did not affect BW and other conditions in OLETF rats throughout the study period.

Sildenafil treatment significantly decreased collagen type I (–26%) and type III (–49%) gene expressions in OLETF rats. In the present study, glomerular fibronectin mRNA levels were increased in OLETF rats compared with LETO rats. Nevertheless, the increment of fibronectin mRNA levels was only marginally affected by sildenafil treatment (–9%). Thus, the antifibrotic effect of sildenafil is unlikely to be

associated with the suppression of fibronectin. The TGF- β 1 protein level in kidney tissue homogenate was significantly higher in OLETF-CON rats than in LETO rats, and this increase was significantly attenuated by sildenafil treatment.

In addition to increased synthesis, diabetes-related glomerulosclerosis is associated with a decrease in ECM degradation by the balance between MMPs and their inhibitors. Variable levels of MMP-2 and MMP-9 have been observed in the diabetic kidney, depending on the duration and model of diabetes. In early STZ-induced diabetes, a type 1 diabetes model, a decrease in MMP-2 and MMP-9 protein expressions and activity has been reported (McLennan *et al.*, 2002; Han *et al.*, 2006; Mankhey *et al.*, 2007); in longer-term STZ-induced diabetes, no changes in MMP-9 were observed (Dong *et al.*, 2004; Rodriguez *et al.*, 2006). On the other hand, MMP-2 protein has been shown to be increased in Goto-Kakizaki rats and MMP-9 mRNA and protein increased in Kkay mice, both type 2 diabetes models (Portik-Dobos *et al.*, 2006; Qing-Hua *et al.*, 2008). In the present study, OLETF-CON rats at 58 weeks of age had increased MMP-2 and MMP-9 gene expressions, and sildenafil treatment significantly reduced these levels by 48% and 60% respectively. The inhibitory effects of sildenafil on MMP-2 and MMP-9 protein levels and MMP-2 activity were confirmed by Western blot analysis and gelatine zymography. To the best of our knowledge, the MMP profiles in OLETF and LETO rats have not been reported previously. We are the first to show that sildenafil treatment exhibited renoprotective effects in diabetic nephropathy by regulating the expressions of MMPs.

One of the mechanisms by which MMP activity is regulated is by altering the expression of its inhibitors, TIMPs. Diabetic nephropathy in humans and experimental models is associated with increased TIMP-1 (McLennan *et al.*, 2002) and TIMP-2 (Han *et al.*, 2006) expressions, consistent with the findings of the present study. Although sildenafil treatment significantly attenuated the increase in TIMP-1 (–27% reduction), no significant effect on TIMP-2 expression (–13% reduction) was observed. Despite the inability of sildenafil to regulate TIMP-2 expression directly, its effects on other ECM regulatory pathways appear to be sufficient to effectively attenuate the progression of glomerulosclerosis in this model of diabetic nephropathy.

However, it should be mentioned here that while sildenafil treatment effectively attenuated glomerular sclerosis, its inhibitory effects on albuminuria or Ccr in OLETF rats were partial. Therefore, sildenafil could be more effective for the treatment of diabetic nephropathy when used in combination with conventional therapies, including renin-angiotensin system inhibitors that possess different modes of action.

In conclusion, sildenafil inhibited extracellular matrix accumulation partially by affecting the balance between MMPs and their inhibitors, exhibited potent antiproliferative effects, and thereby attenuated renal injury in an experimental model of diabetic nephropathy. Our study provides an encouraging example of the therapeutic potential of inhibiting PDE type 5 in diabetic nephropathy and is indicative of the potential success of sildenafil in the treatment of diabetic nephropathy.

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Conflicts of interest

The authors declare no conflicts of interest.

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