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The dipeptidyl peptidase-4 inhibitor sitagliptin ameliorates renal injury in type 1 diabetic mice *via* inhibiting the TGF-β/Smad signal pathway

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Diabetic nephropathy (DN) is a common cause of end-stage kidney disease (ESKD) all over the world. Sitagliptin, an inhibitor of DPP-IV plays a beneficial role in type 2 diabetic nephropathy. The purpose of this study was to explore the effect and mechanism of sitagliptin on renal injury in type 1 diabetic mice. Streptozotocin (STZ) induced type 1 diabetic mice were treated with oral administration of sitagliptin (15 mg/kg/ day) for 4 weeks. The results showed that sitagliptin treatment did not change the levels of blood glucose in STZ induced type 1 diabetic mice. Sitagliptin attenuates diabetic nephropathy by significantly inhibiting 24 h proteinuria, renal injury and fibrosis. Sitagliptin can inhibit the expression level of TGF-β1 and the other related fibrosis factors in renal tissue of type 1 diabetic mice while delaying the progression of type 1 diabetic nephropathy. These results indicated that sitagliptin treatment is potentially a new strategy for treating type 1 diabetic nephropathy.

1. Introduction

Type 1 diabetes mellitus (T1DM) is a progressive disease caused by the autoimmune destruction of β-cells, which result in hyperglycemia (Smith et al. 2017; Gao et al. 2018). Diabetic nephropathy (DN), a common microvascular complication of diabetes, is a leading cause of end-stage kidney disease (ESKD) or kidney failure (Iwai et al. 2018). The progress of DN is characterized by proteinuria, renal tubular atrophy and interstitial fibrosis, which ultimately leads to a steady decline in renal function (An et al. 2015).

Although the mechanism of DN remains unclear, there is growing evidence that transforming growth factor-β1 (TGF-β1) plays a decisive effect in the kidney pathophysiology development of DN (Singh et al. 2018). Previous studies have demonstrated that TGF-β1 promotes renal fibrosis in the vessels by increasing extracellular matrix (ECM) protein deposition, including α-smooth muscle actin (α-SMA) and fibronectin, leading to glomerulosclerosis and tubulointerstitial fibrosis (Pan et al. 2013). The TGF-β1/Smad signaling pathway is a classical pathway for the production and transdifferentiation of renal tubular epithelial cells into fibroblasts ECM in DN (Park et al. 2018).

Dipeptidyl peptidase-4 (DPP-4) inhibitors are a class of antidiabetic drugs, including sitagliptin (SIT), saxagliptin, vildagliptin, linagliptin and alogliptin (Messori et al. 2014; Bae 2016). They are stable, orally effective and virtually devoid of any hypoglycemic effects, while inhibiting the DPP-4 enzyme and increase the active concentration and duration of action of glucagon-like peptide-1 (GLP-1). GLP-1 is the decisive factor in determining glucose homeostasis (Cho et al. 2014). Sitagliptin has been widely used as a therapeutic option for the treatment of type 2 diabetic patients (Yang 2012), and may also offer cardiovascular and neuropathic protection (Zhou et al. 2018). Previous research indicated that SIT treatment is able to attenuate renal damage in type 2 diabetic nephropathy (Wang et al. 2018). However, it is uncertain whether SIT has a beneficial effect on kidney function in T1DM patients.

Therefore, our purpose was to evaluate the effects of a DPP-4 inhibitor, sitagliptin, on proteinuria, renal fibrosis and pathological changes in type 1 diabetic mice. We also investigated whether SIT ameliorates renal injury *via* inhibiting the TGF-β/Smad signal pathway in type 1 diabetic mice.

Table: The effects of sitagliptin on BW, BG, 24h UmAlb, Scr and BUN in diabetic mice

Group	BW (g)	BG (mM)	24h UmAlb (mg/24h)	Scr (μM)	BUN (mM)
CON	38.00±7.90	8.28±1.04	0.82±0.34	192.24±28.66	8.65±1.15
DM	21.38±6.39**	30.18±4.04***	11.96±3.36***	352.58±47.09**	26.08±4.65***
SIT	29.58±3.89$^{\#}$	32.56±2.09	2.46±0.97$^{\#\#\#}$	238.51±53.03$^{\#\#}$	10.40±2.47$^{\#\#\#}$

BW = body weight, BG = blood glucose, UmAlb =urinary microalbumin, Scr =serum creatinine concentration, BUN =blood urea nitrogen, mM= mmol/L, μM= μmol/L. All values are mean±SD; n = 7 mice in all groups. ** $P < 0.01$ vs. CON group; *** $P < 0.001$ vs. CON group; $^{\#}$ $P < 0.05$ vs. DM group; $^{\#\#}$ $P < 0.01$ vs. DM group; $^{\#\#\#}$ $P < 0.001$ vs. DM group

2. Investigations and results

2.1. Effects of sitagliptin on body weight, blood glucose, 24-h urinary albumin and serum biochemical profiles in STZ-induced diabetic mice

As shown in the Table, after the whole experimental period, the body weight was significantly decreased and blood glucose was significantly elevated in the DM group compared with control ($P < 0.001$). In contrast, body weight was obviously increased and blood glucose was not significantly changed in the SIT group compared with those in the DM group ($P < 0.05$). The levels of 24-h urinary albumin, Scr and BUN were markedly increased in the DM group compared to control ($P < 0.01$). Treatment with SIT obviously decreased the levels of 24-h urinary albumin, Scr and BUN in mice, compared with animals in the DM group ($P < 0.01$).

2.2. Effects of sitagliptin on renal morphologic changes in STZ-induced diabetic mice

As shown in Fig. 1, H&E staining revealed that glomerulosclerosis, glomerular basement membrane thickening, extracellular matrix increase, renal tubular atrophy, focal tubular dilation and interstitial fibrosis were observed in the DM group. PAS staining showed increased glycogen accumulation in kidney in the DM group. In contrast, treatment with SIT significantly inhibited these histological damages. Sirius red staining showed that the red-stained collagen deposition was increased considerably in the DM group compared with in the SIT group. The results indicate that SIT attenuates renal damage and fibrosis.

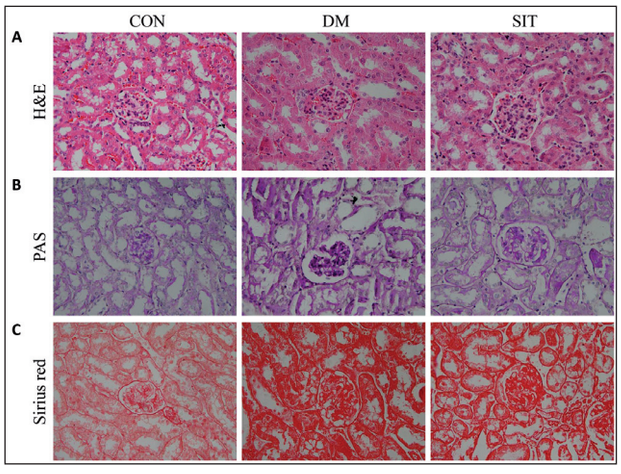

Fig. 1: Histological morphology of the nephric lesions of mice. (A) H&E, (B) PAS and (C) Sirius red staining (magnification x200).

2.3. Effects of sitagliptin on renal mRNA expression levels of fibrosis-associated markers in STZ-induced diabetic mice

It was evaluated whether sitagliptin modulates the renal mRNA expression levels of fibrosis-associated markers in STZ-induced diabetic mice. As shown in Fig. 2, RT-qPCR revealed that TGF-β1, α-SMA, CTGF and fibronectin mRNA levels were significantly elevated in the DM mice compared with control animals ($P < 0.01$). In contrast, treatment with SIT significantly decreased the mRNA expression levels of fibrosis-associated markers compared with those in the DM group ($P < 0.01$).

2.4. Effects of sitagliptin on TGF-β/Smad signal pathway in the kidney of STZ-induced diabetic mice

In order to explore the effect of sitagliptin on the TGF-β/Smad signal pathway in the kidney of STZ-induced diabetic mice, western blotting and immunohistochemical staining were performed. As shown

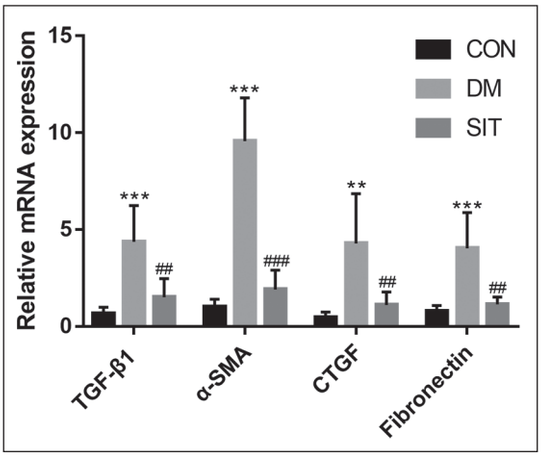

Fig. 2: The ratio of TGF-β1, α-SMA, CTGF and fibronectin mRNA expression in each group. Data represent mean±SD of at least three independent experiments. ** $P < 0.01$ vs. CON group; *** $P < 0.001$ vs. CON group; ## $P < 0.01$ vs. DM group; ### $P < 0.001$ vs. DM group.

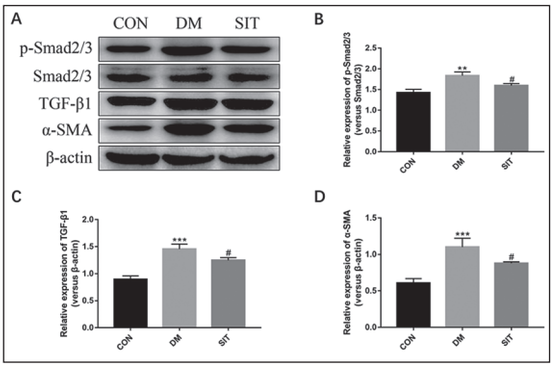

Fig. 3: The effects of sitagliptin on the protein expression in each group. (A) Western blot analysis of p-Smad2/3, Smad2/3, TGF-β1 and α-SMA protein levels. (B-D) Semi-quantitative data from densitometric analysis of p-Smad2/3, TGF-β1 and α-SMA. Data represent mean±SD of three independent experiments. ** $P < 0.01$ vs. CON group; *** $P < 0.001$ vs. CON group; # $P < 0.05$ vs. DM group.

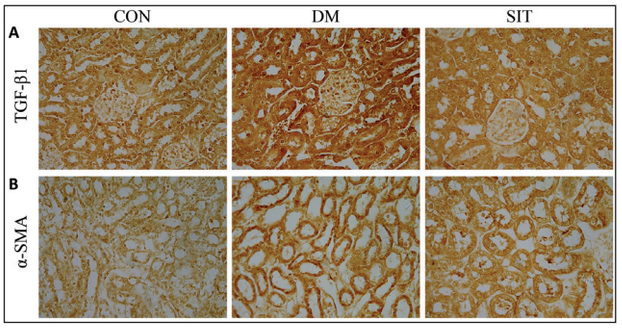

Fig. 4: The photomicrographs of immunohistochemical staining sections of the kidney. (A) TGF-β1, (B) α-SMA (magnification x200).

in Fig. 3, compared with the NC group, the content of TGF-β1, α-SMA and p-Smad2/3 protein in the renal tissue of the DM group increased significantly ($P < 0.01$). However, compared with DM, the TGF-β1, α-SMA and p-Smad2/3 protein content in the renal tissue of the SIT mice decreased significantly, the difference was statistically significant ($P < 0.05$). Immunohistochemistry analysis of TGF-β1, α-SMA in kidney tissue of STZ-induced diabetic mice have shown that, compared with the NC group, mice in the DM group showed severe immunopositivity for TGF-β1, and α-SMA (Fig. 4). However, SIT treatments diminished immunopositivity for TGF-β1, α-SMA.

All these findings indicated that sitagliptin could effectively improve renal function in diabetic mice by inhibiting the TGF-β/Smad signaling pathway.

3. Discussion

Among all the pathogenic factors of DN, TGF-β has attracted much attention. In the current study, we found that SIT treatment can ameliorate DN in type 1 diabetic mice. In this work, we show that SIT significantly inhibited STZ induced renal fibrosis and significantly reduced proteinuria. The inhibitory effect of SIT on diabetic nephropathy is related to blocking the TGF-β/Smad signal pathway. It is widely believed that TGF-β is a key factor in the development of diabetic nephropathy (Wolf 2003). TGF-β can promote basement membrane thickening, glomerular hypertrophy, extracellular matrix deposition and collagen fiber formation, leading to renal interstitial fibrosis. Therefore, TGF-β1 is an important mediator of glomerulosclerosis and interstitial fibrosis (Segarra-Medrano et al. 2017). TGF-β1 not only upregulated the expression of ECM protein, but also enhanced the expression of CTGF, further induced persistent fibrosis and aggravated ECM production (Sonnylal et al. 2010; Wang et al. 2017). Smads protein, an important transduction molecule of its downstream signal, plays a decisive role in the intracellular effect of TGF-β (Budi et al. 2017). There are studies showing that TGF-β1, TGF-βRI, TGF-βRII and Smad2/3 were very obvious in renal tissue when only microalbuminuria and light microscopy did not yet show renal tissue damage in the early stage of DKD (Okazaki et al. 2007). In human chronic kidney disease and animal models of renal fibrosis, the expression of Smad2/3 in the kidneys is significantly increased (Zhao et al. 2016; Johnson et al. 2017). The results of this experiment showed that PAS and Sirius Red staining of T1DM mice had renal fibrosis, glycogen and collagen deposition were significant. The expression of TGF-β1, p-Smad2/3 and other fibrotic factors increased.

SIT is one of the many DPP-IV inhibitors and is also one of the most concerned new hypoglycemic drugs in the world. It combines with DPP-4 to form DPP-4 complex to inhibit the activity of the enzyme, delay the degradation of intestinal insulin GLP-1, prolong its action time, promote insulin secretion, stimulate islet beta cell proliferation and inhibit the pancreas. Glucagon secretion, thereby controlling blood sugar and protecting β-cell function (Marques et al. 2014; Fukui et al. 2015). In this experiment, after treatment with SIT, 24 h urinary albumin was significantly lower in the SIT group than in the DM group, and HE, PAS and Sirius Red staining showed that the degree of renal fibrosis in T1DM mice had improved in the SIT group. We detected the mRNA expression levels of TGF-β1, α-SMA, CTGF and fibronectin in the renal tissue of each group. The results show that all the fibrosis factors were upregulated in the DM group compared with the NC group, but compared with the DM group, the expression of fibrosis factors decreased after SIT treatment. The protein expression of TGF-β1, α-SMA and p-Smad2/3 in kidney of DM group was enhanced significantly, and the expression of related proteins decreased in the SIT group. Although SIT could not reduce the blood sugar of T1DM mice, suggesting that its protective effect on T1DM mice is not achieved by lowering blood sugar, but is mediated by the TGF-β/Smad pathway, indicating that SIT has a protective effect on the kidney that is independent of blood sugar.

In conclusion, our results indicate that SIT treatment can play a protective role in DN. Blocking renal fibrosis induced by TGF-β/Smad may be the mechanism of SIT in improving DN renal function. This study suggests that SIT may be a new therapeutic agent for the treatment of DN.

4. Experimental

4.1. Reagents and materials

Sitagliptin was purchased from the Merck & Co., Inc (Rahway, NJ, USA). Streptozotocin (STZ) was purchased from Sigma-Aldrich Company (Sigma, St. Louis, MO, USA). All of the primers were custom-synthesized by Sangon Biotech (Shanghai, China). Primary antibody against TGF-β1, α-SMA, phosphorylated (p)-Smad2/3 and Smad2/3 were purchased from Abcam (Cambridge, MA, USA). Primary antibody against β-actin was purchased from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies were purchased from Abcam (Cambridge, MA, USA). Diaminobenzidine (DAB) substrate kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China). The blood urea nitrogen (BUN) and serum creatinine (Scr) assay kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

4.2. Animals and experimental protocol

Animal experimentation was performed in compliance with the Mudanjiang Medical University Animal Care and Veterinary Services Committee (Mudanjiang, China). All experiments conformed to the Guidelines for the Care and Use of Laboratory Animals(2011). Male CD1 mice (animal code SCXK 2012-0004) weighing 25-30 g were obtained from the Academy of Military Medical Sciences (Beijing, China). During the experiment, the animals were maintained under standardized conditions (12 h light/dark cycle at 24 °C) and had free access to diet and water. A total of 21 mice were randomly separated into 3 groups with 7 mice in each: normal control (NC), DN model (DM) and sitagliptin intervention (SIT) groups. To induce type 1-like diabetes, mice in DM and SIT groups were induced by an intraperitoneal injection (i.p.) of STZ (150 mg/kg). Mice in the NC group received i.p. an equivalent volume of citrate buffer solution. After 7 days, mice were considered diabetic if they had a blood glucose level higher than 16.7 mmol/L. SIT group was then treated with 15 mg/kg/day sitagliptin via gavage for 4 weeks, while the other groups treated with a corresponding volume of distilled water via gavage for 4 weeks. During the experiment, mice were placed in metabolic cages to collect and record 24 h urine volume. All of the mice were sacrificed under anesthesia, blood samples were collected to measure BUN and Scr according to the manufacturer's protocol. Kidney samples were collected and weighed, one portion was immediately fixed in 10 % formalin for histological analyses, the other part was rapidly frozen and maintained at -80 °C until assessed.

4.3. RNA extraction and RT-qPCR analysis

Total RNA was extracted using HP Total RNA Kit (OMEGA, China) according to the manufacturer's protocol. Total RNA (1 μg) was reverse transcribed to cDNA using the First-strand cDNA synthesis kit (Roche Diagnostics) according to the manufacturer's instructions. Expression levels of gene were measured by real-time quantitative PCR (RT-qPCR) using the StepOne Real-time PCR system (Applied Biosystems). The primer sequences for real-time PCR analysis were as follows:

Rps16, forward 5′-CGTGCTTGTGCTCGGAGCTA-3′, reverse 5'- GCTCCTTG-CCCAGAAGCAAA -3';

TGF-β1, forward 5′-CTCCCGTGGCTTCTAGTGC-3, reverse 5′- GCCTTAGTTTG-GACAGGATCTG -3′;

α-smooth muscle actin (α-SMA), forward 5'-GTGGCCCCTGAAGAGCATC-3', reverse 5'-AATCTGGGTCATTTTCTCCCG-3′;

CTGF, forward 5′-GCTTGGCGATTTTAGGTGTC-3′, reverse 5′- CAGACTG-GAGAAGCAGAGCC -3′;

and fibronectin, forward 5′-CACAAGGTTCGGGAAGAGGT-3′, reverse 5′- GAGCT-TAAAGCCAGCGTCAG -3′.

Rps16 was used as an internal control and the relative mRNA expression levels were performed using the $2^{-\Delta\Delta Ct}$ method(Livak and Schmittgen 2001).

4.4. Histological analysis

The renal tissues were fixed in neutral buffered formalin for 24 h at room temperature and embedded in paraffin. Tissue sections (5 μm) were stained by hematoxylin and eosin (H&E), PAS staining (Abcam, Cambridge, MA, USA) and Sirius red staining (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions. The morphological alterations were studied by light microscopy (BX41, Olympus, Japan).

4.5. Western blot analysis

Protein was extracted from renal tissues using radioimmunoprecipitation assay buffer (P0013B; China) with protease inhibitor 1 mM phenylmethylsulfonyl fluoride. Protein concentrations were measured with a BCA protein assay reagent kit according to the manufacturer's protocol. The protein samples (50 μg) were electrophoresed through 10 % SDS-PAGE and were transferred to polyvinylidene difluoride membranes. After blocking with 5 % fat-free milk for 1 h at room temperature, the membranes were then incubated with primary antibodies against TGF-β1 (1:2000 dilution; ab25121; Abcam), α-SMA (1:1000 dilution; ab5694; Abcam), Smad2/3 (1:1000 dilution; ab217553; Abcam), phosphorylated (p)-Smad2/3 (1:1000 dilution; ab63399; Abcam) and β-actin (1:1000 dilution; #4970; Cell Signaling Technology) at 4 °C overnight, followed by repeated washing in TBS containing 0.1 % Tween. Horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000 dilution; ab97051; Abcam) antibody was used as secondary antibody for 1 h at room temperature. The 0.01 % DAB (Shanghai, China) chromogenic reagent was used to detect reactive proteins. The gray levels of each band were analyzed using Image J v1.42q software (NIH).

4.6. Immunohistochemical staining

Immunohistochemical analysis was performed on 5 μm renal sections. The sections were deparaffinized, hydrated and fixed with methanol-3 % H_2O_2 solution for 15 min at room temperature. Following pre-incubation with 3 % bovine serum albumin/PBS for 30 min to prevent nonspecific staining at room temperature, sections were incubatet with the diluted primary antibodies against TGF-β1 (1:200 dilution; ab25121; Abcam) and α-SMA (1:150 dilution; ab5694; Abcam) at 4 °C overnight. After three washes in PBS, the sections were incubated for 1 h at room temperature with the

secondary antibody horseradish peroxidase (HRP)-conjugated anti-rabbit (1:1000 dilution; ab97051; Abcam). Subsequently, the sections were briefly stained with DAB (Shanghai, China) in the dark for the chromogenic reaction. The morphological alterations were studied by light microscopy (BX41, Olympus, Japan).

4.7. Statistical analysis

Results from at least three independent experiments were expressed as mean±standard deviation (SD). Data were analyzed using a one-way ANOVA analysis of variance with Tukey's multiple comparison test using GraphPad Prism 7.0 software (GraphPad Software, CA, USA). $P<0.05$ was considered to be statistically significant.

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Conflicts of interest: None declared.

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