

Role of the TGF β /p65 pathway in tanshinone IIA-treated HBZY-1 cells

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Abstract. Tanshinone IIA (TIIA) is widely used for the treatment of a number human diseases, including diabetic nephropathy (DN) (1). The present study was performed to examine the role of the transforming growth factor β (TGF β)/p65 pathway under TIIA treatment in a glomerular mesangial cell model of DN. Firstly, it was identified that TIIA inhibited the proliferation of HBZY-1 cells, while simultaneously suppressing the expression of TGF β and p65. In addition, glucose-induced HBZY-1 cells were treated with TIIA, si-TGF β and si-p65. The results revealed that si-TGF β or si-p65 were able to inhibit the proliferation of HBZY-1 cells as well. Finally, the expression of TGF β and p65 in a rat model of DN treated with TIIA was detected. The results demonstrated that renal hypertrophy and 24 h urinary protein excretion were ameliorated in TIIA-treated rats with DN. Furthermore, it was revealed that the protein levels of TGF β and p65 were decreased in the DN rats following TIIA treatment. In conclusion, the present study demonstrated that TGF β and p65 were activated by TIIA in HBZY-1 cells. In addition, the expression of TGF β and of p65 was downregulated in rats with DN treated with TIIA.

Introduction

Diabetic nephropathy (DN) is characterized functionally by glomerular hyperfiltration and proteinuria, and histologically by the expansion of the glomerular mesangium, which is associated with the loss of renal function. The pathological changes in the kidney include increased glomerular basement membrane

thickness, microaneurysm formation and mesangial nodule formation (Kimmelsteil-Wilson bodies) (1-4). A total of >30% of diabetes mellitus patients develop clinically evident DN, which becomes a major cause of end-stage renal disease (ESRD) worldwide (1,3). Its molecular pathogenesis is therefore becoming the target of a growing number of studies. Numerous factors have been reported to be associated with the development of DN and a large number of investigations are involved in therapeutic studies of the condition (4-7). One study revealed that treatment with vildagliptin + telmisartan controlled blood pressure, renovascular structural and biochemical parameters in diabetic neuropathy rats (4). Endo *et al* (8) performed a randomized, open trial on 162 type 2 diabetic patients and found that probucol suppressed the progression of DN and renal dysfunction events.

Tanshinone IIA (TIIA) is a major phenanthrene-quinone isolated from *Salvia miltiorrhiza* Bunge, which is a Traditional Chinese Medicine (TCM) that is commonly used in various diseases and types of cancer (9-12). More importantly, a study by Kim *et al* (13) demonstrated that TIIA has protective effects during the progression of DN and may be a potential drug candidate for the prevention of DN. As a multi-functional agent in TCM, TIIA has a fundamental role in cellular responses to extracellular stimuli. For example, TIIA protects cardiomyocytes in part through B-cell lymphoma 2/B-cell lymphoma 2-associated X protein and Akt-signaling pathways (14-15), is able to produce an inhibitory effect on the sterol regulatory element-binding transcription factor 1 pathway through the phosphoinositide 3-kinase/Akt signaling pathway (16) and significantly inhibits the expressions of numerous factors, including transforming growth factor β (TGF β), in a rat model of DN (13). However, only the primary action of TIIA on DN has been studied, and the mechanisms underlying how TIIA ameliorates DN require further elucidation.

There is an increasing amount of evidence that TGF β induces and promotes inflammatory responses via activation of nuclear factor (NF)- κ B in various diseases. In one study, TGF β induced p65 acetylation to enhance bacteria-induced NF- κ B activation (17). Several studies have also indicated that TGF β -Smad signaling mediates the activation of NF- κ B in human airway epithelial cells (18-19). In addition, the clinical and pathological aspects of diabetic neuropathies are associ-

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ated with inflammatory phenomena (20-21). Therefore, the underlying mechanism of the activity of TIIA on DN may proceed via the TGF β /p65 pathway. It was hypothesized that TIIA may ameliorate DN by regulating TGF- β and p65. The present study aimed to investigate the functions of TGF- β and p65 during TIIA therapy of DN using glucose-induced HBZY-1 cells.

Materials and methods

TIIA treatment. TIIA was purchased from Nanjing Zelang Medical Technological Co. Ltd (Jiangsu, China). The rat mesangial HBZY-1 cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in RPMI-1640 (Gibco-BRL, Carlsbad, CA, USA) medium containing 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) under standard conditions (37°C, 5% CO₂). The HBZY-1 cells were seeded in 96-well plates at 2.0x10⁴ cells/well, and were cultured in 100 μ l Dulbecco's modified Eagle's medium (DMEM) with or without high glucose (HG) and TIIA. To determine the safe dosages of TIIA to be used in further experiments in this study, the cells were randomly divided into six groups: i) Control; ii) high glucose (HG, 30 mmol/l); iii) HG + TIIA (10 μ M); iv) HG + TIIA (20 μ M); v) HG + TIIA (40 μ M) and vi) HG + TIIA (80 μ M). At 48 h following treatment with TIIA, the cell viability was examined by the MTT assay.

Small interfering (si)RNA transfection. HBZY-1 cells were plated onto six-well plates (1x10⁵ cells/well), maintained in DMEM containing 10% FBS for 12 h and transfected with a mixture containing Opti-MEM, 8 μ l/well LipofectamineTM 2000 (Invitrogen, San Diego, CA, USA) and either 0.5 μ g/well scrambled siRNA (mock) or siRNAs (smart pool; Guangzhou RiboBio Co., Ltd., Guangzhou, Guangdong, China) for 6 h. The sequences of these siRNAs are listed in Table I. To detect the function of TGF β and p65 in the HBZY-1 cells induced by HG, the cells were randomly divided into four groups: i) Control, ii) HG + mock; iii) HG + si-TGF β ; and iv) HG + si-p65. Following 48 h, the cell viability was examined by the MTT assay.

MTT assay. The HBZY-1 cells treated with or without HG, siRNA or TIIA were collected and resuspended in 100 μ l DMEM. A total of 20 μ l MTT (5 mg/ml) was added, followed by incubation for 4 h at 37°C. Finally, the samples were recorded at 490 nm using a microplate reader. The wells with only MTT served as blank controls. At least three independent experiments were performed.

Animals. Male Sprague Dawley (SD) rats (6-8 weeks old; weighing, 180-200 g) were obtained from the Guangdong Medical Laboratory Animal Center (Guangdong, China). The rats were divided into three groups: Normal control rats (n=8), DN rats (n=8) and TIIA rats (n=8). DN and TIIA rats were injected intraperitoneally with 45 mg/kg streptozotocin (STZ; Sigma, St. Louis, MO, USA), while the normal control rats received physiological saline (PS). Following seven weeks, the TIIA rats were administered an intramuscular injection, at a dose of 8 mg/kg, of TIIA daily for another three weeks, whereas the other rats received PS only. All of the animals were fed with high-fat food. After the animals were sacrificed,

fresh kidney samples were stored in formaldehyde solution for histopathological observation. The remaining kidneys were stored at -80°C for later analysis. The experimental procedure was approved by the Animal Experimentation Ethics Committee at The Guangzhou University of Chinese Medicine (Guangzhou, Guangdong, China).

Urine analyses. All of the animals were housed in metabolic cages for 24 h to obtain urine at seven weeks (one day prior to TIIA treatment) and 10 weeks (one day prior to sacrifice) for the measurement of urine protein. The 24 h urinary protein excretion was assessed by the Coomassie brilliant blue method.

Histopathology. The cell morphology was examined in formalin-fixed, paraffin-embedded kidney sections (5 μ m) stained with hematoxylin-eosin (H&E) (22). The histopathological scores were examined by glomerular mesangial expansion, mesangial matrix increase, interstitial mononuclear cells and extracellular matrix accumulation. Histological analysis was performed using a light microscope (Leica DM400 B, Bannockburn, IL, USA).

Quantitative polymerase chain reaction (qPCR). Total RNA was extracted from the frozen kidney samples using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. The mRNA expression of TGF β and p65, and β -actin was detected by qPCR utilizing a 7500 detector (ABI Prism 7500; Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. PCR was performed with 100 ng total RNA using one-step SYBR PrimeScript RT-PCR kit (TakaRa, Tokyo, Japan) as follows: 42°C for 5 min, 94°C for 10 sec followed by 40 cycles at 94°C for 5 sec, 60°C for 35 sec, with a final extension at 72°C for 5 min. The primers used in the present study were as follows: TGF β forward 5'-TGCTCTTGTGACAGCAAAGATAA-3' and reverse 5'-CTCTGTGGAGCTGAAGCAATAGT-3'; p65 forward 5'-CACCAAAGACCCACCTCACC-3' and reverse 5'-GGACCGCATTCAAGTCATAGTC-3'; β -actin forward 5'-GACAGGATGCAGAAGGAGATTACT-3' and reverse 5'-TGATCCACATCTGCTGGAAGGT-3'. The gene expression of interest was determined using the 2^{- $\Delta\Delta$} CT method (23). The expression levels of all the transcripts were normalized to that of the housekeeping gene β -actin in the same tissue.

Western blot analysis. The proteins extracted from kidney tissues were examined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed by western blot analysis. Antibodies used in the present study included: Rabbit polyclonal TGF- β (sc-146, diluted by 1:1,000), p65 (sc-101749, diluted by 1:2,000) and β -actin (sc-130657, diluted by 1:5,000). All antibodies were purchased from Santa Cruz (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Statistical analysis. The presented data were analyzed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used to analyze the intergroup differences. The differences between the groups were compared using a t-test. P<0.05 was considered to indicate a statistically significant difference.

Table I. RNA oligos of sense and anti-sense strands of small interfering RNA.

Gene	Strand name	RNA oligos
NF- κ B p65	p65 sense strand A	GAAGAAGAGUCCUUUCAAUtt
	p65 anti-sense strand A	AUUGAAAGGACUCUUCUUCtt
	p65 sense strand B	CCAUCAACUUUGAUGAGUUtt
	p65 anti-sense strand B	AACUCAUCAAAAGUUGAUGGtt
	p65 sense strand C	GCAUUAACUUCCCUGAAGUtt
	p65 anti-sense strand C	ACUUCAGGGAAGUUAUUGCtt
TGF β	TGF β sense strand A	CUGCUCUUGUGACAGCAAAGAtt
	TGF β anti-sense strand A	UCUUUGCUGUCACAAGAGCAGtt
	TGF β sense strand B	UUCAGCUCCACAGAGAAGAACtt
	TGF β anti-sense strand B	GUUCUUCUCUGUGGAGCUGAAtt

NF- κ B, nuclear factor- κ B; TGF β , transforming growth factor β .

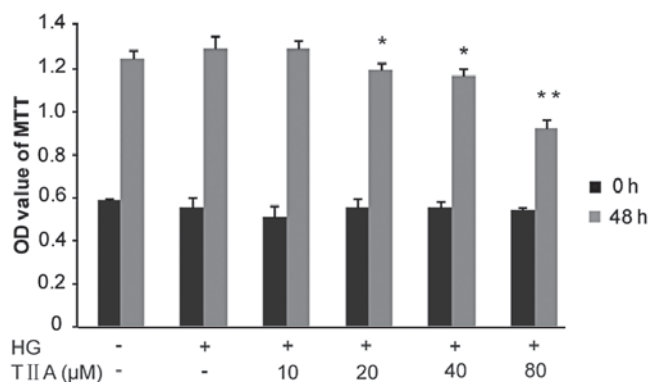


Figure 1. Proliferation inhibitory effect of TIIA on HBZY-1 cells. HBZY-1 cells were cultured in normal Dulbecco's modified Eagle's medium with or without HG (30 mmol/l). The dose-dependent effect of TIIA (10, 20, 40, 80 μ M) on cell viability was evaluated by the MTT assay. * P <0.05 vs. the (HG+TIIA) control; ** P <0.01 vs. the (HG+TIIA) control. OD, optical density; TIIA, tanshinone IIA; HG, high glucose.

Results

TIIA inhibits HBZY-1 cell proliferation. The effects of TIIA on HBZY-1 cell proliferation were investigated using the MTT assay. Firstly, it was identified that the proliferative ability of the HBZY-1 cells treated with HG was slightly increased compared with that of the control group (although P >0.05). TIIA inhibited the HG-induced HBZY-1 proliferation in a concentration-dependent manner. The data revealed that, following 48 h, TIIA at 20 and 40 μ M (P <0.05) as well as 80 μ M (P <0.01) had a significant growth-inhibitory effect on the HBZY-1 cells (Fig. 1).

TGF β and p65 are suppressed by TIIA in HBZY-1 cells. Previous studies demonstrated that TGF β and NF- κ B increased in DN and had a key role in cell proliferation (24). Therefore, next, the effects of TIIA on TGF β and NF- κ B in HBZY-1 cells were examined using qPCR. As demonstrated in Fig. 2, TGF β and p65 were significantly upregulated in the HG-induced group compared with the control cells. However, TIIA significantly inhibited the increase in TGF β and p65 in the HBZY-1 cells induced by high glucose. This result may indicate that TGF β

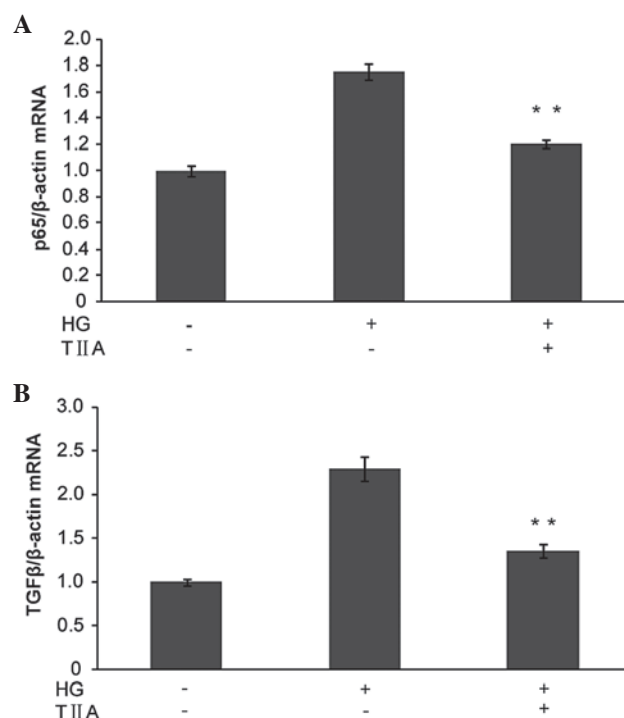


Figure 2. TIIA treatment suppressed the expression of p65 and TGF β in HG-induced HBZY-1 cells. (A) qPCR analysis of p65 mRNA expression. (B) qPCR analysis of TGF β mRNA expression. HBZY-1 cells were cultured in normal Dulbecco's modified Eagle's medium with or without HG and TIIA for 48 h. The p65 and TGF β expression measured by qPCR were normalized with β -actin. ** P <0.01 vs. the (HG+TIIA) control. HG, high glucose; TIIA, tanshinone IIA; TGF β , transforming growth factor β ; qPCR, quantitative polymerase chain reaction.

and p65 were involved in the process by which TIIA inhibited HBZY-1 cell proliferation.

si-TGF β and si-p65 inhibit the proliferation of glucose induced HBZY-1 cells. To determine whether TGF β and p65 were able to inhibit HBZY-1 cell proliferation, their functions were investigated using RNA interference and subsequent MTT assay. qPCR data demonstrated that TGF β and p65 were significantly inhibited by the designed si-TGF β and si-p65 separately (Fig. 3A).

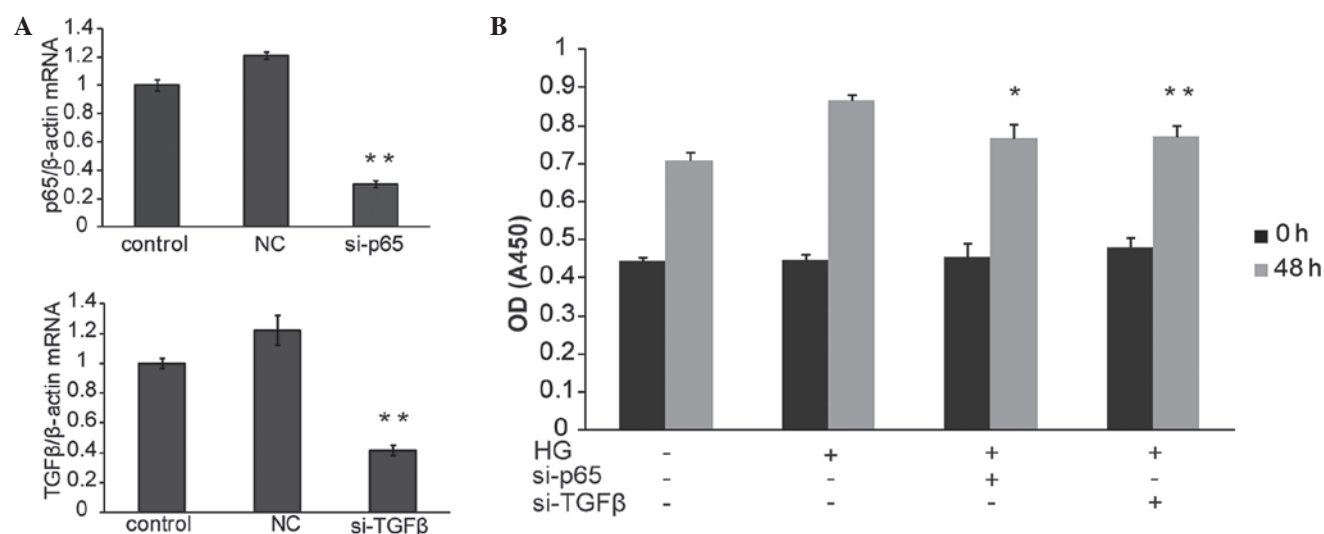


Figure 3. Delivery of si-p65 and si-TGF β inhibits proliferation of HBZY-1 cells. (A) Quantitative polymerase chain reaction analysis of p65 and TGF β mRNA expression. (B) Cell viability was evaluated by the MTT assay. Values are presented as the mean $\pm$ standard error of the mean for groups of three independent experiments. *P<0.05 vs. the (HG⁺) control; **P<0.01 vs. the (HG⁺) control. HG, high glucose; NC, negative control; OD, optical density; siRNA, small interfering RNA; si-p65, p65 siRNA; si-TGF β , TGF β siRNA; OD, optical density; TGF β , transforming growth factor β .

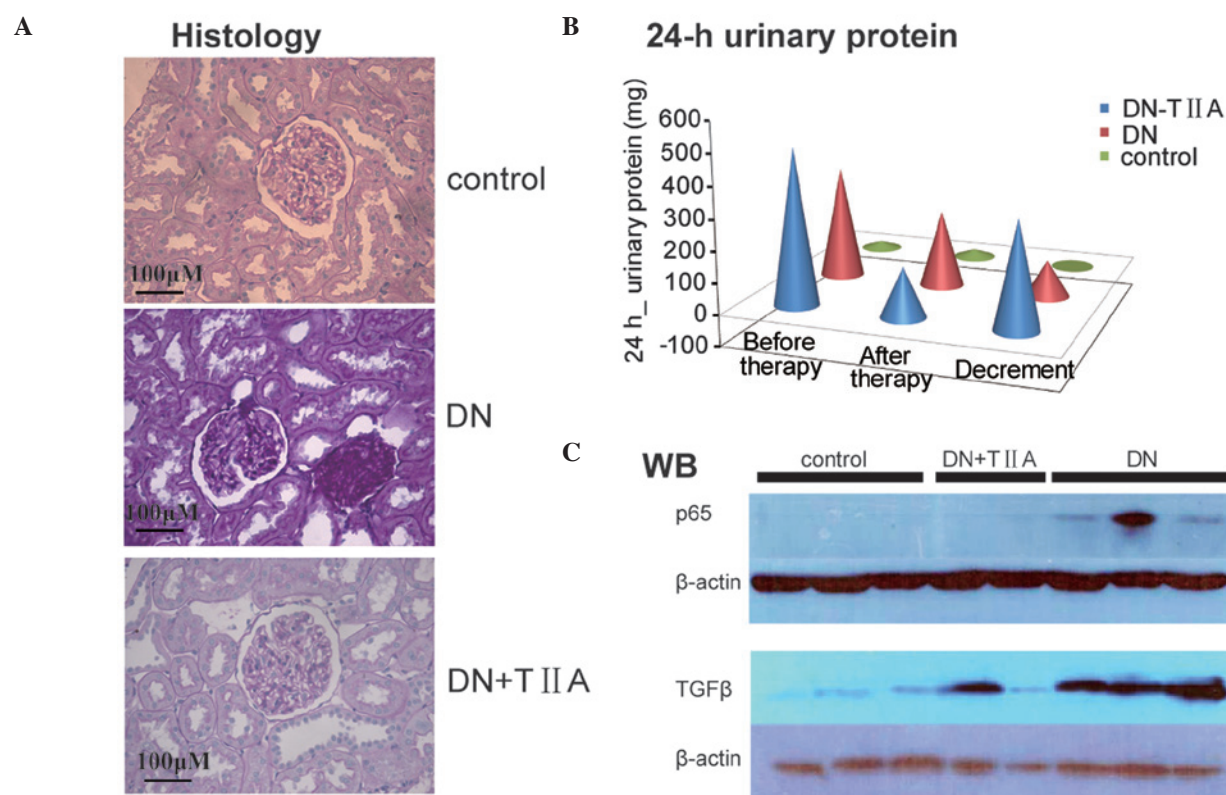


Figure 4. TIIA attenuates DN and inhibits p65 and TGF β protein expression in DN rats. (A) Histology of the kidney sections (stained with hematoxylin-eosin). (B) The 24 h urinary protein excretion was assessed by the Coomassie brilliant blue method. Values are presented as the mean $\pm$ standard error of the mean for groups of 4-5 rats. The decrement is the difference between the average amount of 24 h urinary protein excretion prior to therapy and that following therapy in each group. (C) Western blot analysis of p65 and TGF β in the rat kidney. Control, normal group; DN, DN group; DN-TIIA, DN group with TIIA therapy. DN, diabetic nephropathy; TIIA, tanshinone IIA; TGF β , transforming growth factor β ; WB, western blot.

Of note, the MTT assay revealed that the knockdown of either TGF β or p65 significantly inhibited HBZY-1 cell proliferation as compared with the HG-only treated group (Fig. 3B). The above data suggested that TGF β and p65 were necessary for HBZY-1 proliferation. TGF β and p65 may therefore be involved

in the intracellular mechanisms underlying the effect of TIIA on HBZY-1 cell proliferation.

Therapeutic effect of TIIA on the established DN rat. To determine the efficiency of TIIA therapy *in vivo*, DN rat models

induced by STZ were established. The 24 h urinary protein excretion of the DN rats and control rats was detected. In the DN rats group, the average 24 h urinary protein excretion reached 365 mg following STZ injection for seven weeks and then decreased to 247 mg during the subsequent physiological saline treatment over three weeks. However, in the TIIA therapy group, a more significant reduction in the average 24 h urinary protein excretion was identified, from 503 mg to 161 mg during the TIIA treatment over three weeks. This data demonstrated that TIIA contributed to a larger decrement of average 342 mg urinary protein compared with 118 mg in the rats that received PS (Fig. 4B). Furthermore, the morphological changes of the kidney were also examined. The kidney sections were stained with H&E for histochemical determination of the renal compartments. The DN rats developed renal lesions that consisted of an increasing glomerular mesangial matrix accumulation. In the TIIA treatment group, a moderate decrease in mesangial cellularity and hypertrophy of mesangial cells compared with control DN rats was observed (Fig. 4A).

TIIA inhibits the expression of TGF β and p65 in DN rats. Since TGF β and p65 are functional during the progression of DN in rats, next, whether they were involved in the beneficial effects of TIIA therapy was investigated. The effects of TIIA on the protein expression levels of TGF β and p65 in the rat kidneys were assessed by western blot analysis. As demonstrated in Fig. 4C, the western blot results indicated that both TGF β and p65 proteins were firstly upregulated in DN rats induced by STZ compared with normal control rats. However, following treatment of the DN rats with TIIA for three weeks, it was identified that TGF β and p65 levels in the kidney decreased compared with those in the DN rats treated with PS.

Discussion

DN is a progressive kidney disease characterized by long-term damage to the kidneys as a result of long-term poorly controlled diabetes. It is the most common diabetes mellitus in industrialized countries and >20 million people suffer from DN with a marked range of clinical manifestations (20).

DN is considered to result from the interactions between a wide range of metabolic and haemodynamic factors that induce oxidative stress, polyol pathway flux, hexosamine flux and the accumulation of advanced glycosylated end-products (AGEs) (25,26). Therefore, therapeutic strategies that target DN have various mechanisms of action. The majority of previous treatment strategies have focused on the control of hyperglycemia. For example, prednisone was used at a dose of 0.75mg/kg/day, with daily control of glycaemia in patients (20). The major focus of newer treatments appears to be on newer targets and is associated with glucose-dependent pathways as a result of diabetes. For example, drugs that inhibit the formation of AGEs include aminoguanidine, ALT-946, pyridoxamine and OPB-9195. In other studies, prostaglandins are suggested to be involved in the development of nephropathy. Treatment with cyclooxygenase-2 inhibitors, prostacyclin analogues and thromboxane A₂ antagonists have also been demonstrated to be beneficial (6,27-29). The NF κ B inhibitor pyrrolidine dithiocarbamate has been studied and has been demonstrated to confer renoprotection (30,31). Miyata *et al* (32) suggested that recent approaches targeting

oxygen biology may offer novel treatments for DN. A study by Kim *et al* (13) demonstrated that TIIA may have protective effects during the progression of DN and may be a potential drug for the prevention of DN. The present study revealed that renal hypertrophy and 24 h urinary protein excretion were partly recovered in TIIA-treated rats, which further confirms that TIIA ameliorated the pathological changes in DN rats.

Various theories have proposed that DN is associated with oxygen biology, including hypoxia, oxidative stress and dyserythropoiesis (32). Previously, experimental studies demonstrated that a broad range of anomalies concerning the pathogenesis of DN, including proteinuria, genetics, race, hypoxia, ischemia and inflammation. Sustained inflammation may be the initiator to activate tissue fibrosis progression in DN (33,34). TGF- β has been demonstrated to have an essential role in fibrosis. Shaker and Sadik (35) demonstrated that serum TGF- β may also be a prognostic marker of DN. TGF- β s modulate the bodies' overall immune response by affecting different receptors and downstream signaling; for example, the inhibition of TGF- β s or the TGF- β -Smad signaling pathway has been demonstrated to exhibit anti-fibrotic effects (36-40). In addition, Smads also interact with other signaling pathways, including NF- κ B pathways (19,41). Liu *et al* (42) found that TGF- β /Smad-mediated renal fibrosis and NF- κ B-driven renal inflammation were involved in a mouse model of obstructive nephropathy. Ka *et al* (24) demonstrated that the TGF- β /Smad and NF- κ B signaling pathways were inhibited during therapy for type II DN. Pioglitazone attenuates DN by downregulating TGF- β and NF κ B in type II diabetic rats (43). Kim *et al* (13) identified that TGF- β was decreased in DN rat models treated with TIIA. In the present study, the results revealed that TGF β and p65 mRNA and protein expression were upregulated in DN rats and downregulated following treatment with TIIA. Furthermore, similar results were observed in HBZY-1 cells. It was identified that TGF β and p65 were suppressed by treatment with TIIA. To further examine the interaction between TGF β and p65 in this process, si-TGF β or si-p65 were transfected into cells induced by HG. The MTT assay revealed that the knockdown of either TGF β or p65 inhibited HBZY-1 cell proliferation, consistent with the effect of TIIA. In conclusion, the results demonstrated that TGF β and of p65 were downregulated by TIIA in HBZY-1 cells. The renoprotective effect of TIIA on DN may therefore proceed via the suppression of TGF β and p65. These results may provide valuable information for the development of novel therapeutic strategies for DN.

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