Silencing of TRB3 Ameliorates Diabetic Tubule Interstitial Nephropathy via PI3K/AKT Signaling in Rats

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Background: Nephropathy, a chronic progressive kidney disease often characterized by glomeruli scarring and sclerosis, is a major complication of diabetes mellitus. Development of nephropathologic lesions has been shown to be associated with suppressed AKT phosphorylation and elevated level of apoptosis. Moreover, it has been established that the TRB3 gene is capable of inhibiting AKT phosphorylation and promoting apoptosis.

Material/Methods: In this study, we injected TRB3 siRNA into Wistar rats with type 1 diabetes, and monitored development of nephropathy in the rats. Urinary albumin excretion and serum creatinine were used as primary indicators, and nephritic histology was also examined. We also measured the serum level of pro-inflammatory cytokines collagen expression, and phosphorylation of PI3K and AKT proteins in the kidneys.

Results: By silencing the TRB3 gene with siRNA, diabetic-induced nephropathy symptoms were alleviated, such as increased serum creatinine level and urinary albumin secretion. Additionally, histological examination showed lower levels of nephropathic lesions, and samples of the kidneys showed less accumulation of collagen proteins. Levels of serum cytokines, including TNF-α, IL-1β, and IL-6, were also lowered, whereas phosphorylation levels of PI3K and AKT were increased. In summary, TRB3 silencing in diabetic rats had a significant ameliorative effect on their nephropathy.

Conclusions: Silencing of TRB3 has a significant ameliorative effect on diabetic nephropathy in rats.

MeSH Keywords: Collagen • Diabetes Insipidus, Nephrogenic • Phosphatidylinositol 3-Kinases • Proto-Oncogene Proteins c-akt • RNA, Small Interfering

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Background

Diabetes mellitus, a metabolic disease characterized by persistent high blood sugar levels, is now considered a pandemic disease; it is found in over 400 million adults (International Diabetes Federation, 2016) and is responsible for more than 3.7 million deaths (WHO, 2013). In the diabetic population, long-term high blood sugar damages small blood vessels of organs, including the eyes, kidneys, and nerves [1]. Diabetic nephropathy is most common cause of terminal renal disease in many countries [2], and can manifest as early mesangial and glomerular hypertrophy, microalbuminuria, and late-stage accumulation of extracellular matrix (ECM) components secreted by the mesangial cells [3,4]. Pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, are often elevated in diabetic nephropathy, affecting functions of transporters and ion channels from the nephron, and could be partially responsible for development of renal fibrosis and glomerulosclerosis. Glomerulosclerosis, in particular, has been found to be characterized by loss of functional glomerular cells and excessive accumulation of ECM proteins, especially interstitial collagens, including collagens I, III, and IV [5]. Collagen I is most commonly found at the accumulation site [6], but collagens III and IV has also been implicated in glucose-induced ECM over-production [7].

TRB3 is a mammalian homolog of Drosophila tribbles, and has been found to be induced by insulin resistance and an inhibitor of AKT activation in the liver [8]. A previous study also showed the role of TRB3 in the inhibitory effect of fenofibrate against the proliferation of glomerular mesangial cell induced by high glucose and its critical molecular roles in homocysteine-mediated cell-cycle arrest in endothelial cells [9]. It also negatively modulates AKT signaling in vitro, thereby regulating muscle cell differentiation in a skeletal muscle cell line [10]. The mechanism by which TRB3 plays a role is not completely known, but it is likely by promoting apoptosis, as shown in human monocyte-derived macrophage or in vivo renal tubular cells [11,12]. TRB3 plays a profound role in the complex networks of glucose homeostasis and cellular proliferation. For example, elevated TRB3 has been shown to be correlated to increased glucose intolerance and repressed insulin signaling via the IRS-1/phosphatidylinositol 3-kinase (PI3K)/AKT pathway, and db/db mice have been reported to have remarkably higher TRB3 levels [13].

The level of TRB3 can be modulated by many different genetic factors, including NF-kB, and ER stress marker CHOP [14]. It has also been reported to be induced by glucose deprivation [15] and genotoxic or ER stress signals in vitro [16]. On the other hand, TRB3 expression is repressed by intense physical activity in ob/ob and diet-induced obese (DIO) mouse livers [17,18]. Additionally, Hua et al. reported that TRB3 may interact with Smad3 to participate in the positive regulation of TGF-β-SMAD-mediated cellular biological functions, indicating TRB3 may interact with a broader range of cellular signals [19]. According to Tejada et al., db/db mice develop early diabetic nephropathy and albuminuria due to lower levels of AKT phosphorylation, resulting in their podocytes being more susceptible to apoptosis [20]. Previous results showed that silencing of TRB3 attenuates albumin-induced apoptosis in a rat tubular cell line [12], and alleviates diabetic cardiomyopathy in rats with type 2 diabetes [21], by at least partially restoring AKT phosphorylation. In a different study, TRB3 silencing also protected photoreceptors against ER stress in a retinal detachment rat model [22]. Despite these efforts, the in vivo effect of TRB3 silencing in the kidneys has not been investigated in depth.

In this study, siRNA was used to silence TRB3 in a Wistar rat model, and TRB3 silencing ameliorated diabetic-induced weight loss, blood glucose increase, and accumulation of serum creatinine and urinary albumin. Further investigation showed alleviation of nephropathy, lower collagen I and III protein levels in the kidney, as well as lower serum cytokine levels, including TNF-α, IL-1β, and IL-6 in the TRB3-siRNA-treated rats. To elucidate the mechanism by which TRB3 silencing affects the nephritic pathology, we then performed Western blot analysis of kidney samples, and discovered that phosphorylation of PI3K and AKT in the rat kidneys was reverted to various degrees. These results show that silencing of the TRB3 gene can lead to amelioration of diabetic nephropathy, likely by improving PI3K/AKT activation.

Material and Methods

Animals

Thirty male specific pathogen-free (SPF) Wistar rats were obtained from the Medical Experimental Animal Center of Guangdong at 8 weeks of age. They were housed in a well-ventilated, quiet, SPF facility at 22–24°C, 50–60% relative humidity, and diet was provided ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC).

Induction of type 1 diabetes

The rats were acclimated for 1 week after arriving at our facility. After 1 week, 10 rats were randomly selected to be the Normal group. The 20 remaining rats were fasted for 12 h, then intraperitoneally injected 60 mg/kg streptozotocin (STZ; Sigma, St. Louis, USA). STZ was dissolved at 1% in 0.1 M citrate buffer, pH 4.5. All rats were injected within 30 min. In the Normal group, rats were injected an equal volume of citrate buffer. Twenty-four hours after injection, blood sugar level was monitored daily from the tail vein for 3 days, and all STZ-injected rats had blood sugar levels of greater than 16.7 mM, indicating development of renal fibrosis and glomerulosclerosis. Glomerulosclerosis, in particular, has been found to be induced by insulin resistance and an inhibitor of AKT phosphorylation, resulting in their podocytes being more susceptible to apoptosis [20]. Previous results showed that silencing of TRB3 attenuates albumin-induced apoptosis in a rat tubular cell line [12], and alleviates diabetic cardiomyopathy in rats with type 2 diabetes [21], by at least partially restoring AKT phosphorylation. In a different study, TRB3 silencing also protected photoreceptors against ER stress in a retinal detachment rat model [22]. Despite these efforts, the in vivo effect of TRB3 silencing in the kidneys has not been investigated in depth.

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Table 1. Body weight, blood glucose, urinary albumin excretion and serum creatinine of the Normal, Control and TRB3 group rats at 16 weeks.

|          | Normal          | Control          | TRB3            |
|----------|-----------------|------------------|-----------------|
| BW (g)   | 471.92±19.21    | 207.32±12.46**   | 221.35±10.37**  |
| BG (mmol/L) | 5.23±0.89      | 23.26±4.03**     | 16.42±2.38**    |
| UAE (mg/d) | 0.03±0.01      | 14.79±2.30**     | 8.69±1.63**     |
| Scr (µmol/L) | 80.02±5.41     | 154.81±11.38**   | 107.68±8.25*    |

BW – body weight; BG – blood glucose; UAE – urinary albumin excretion; Scr – serum creatinine. * P<0.05; ** P<0.01 compared to the Normal group. * P<0.05; ** P<0.01 compared to the Control group.

Figure 1. (A–D) Time curve of physiological parameters of the Normal and diabetic rats. At 2–4 weeks after the STZ injection, the diabetic rats started to show signs of type 1 diabetes, as indicated by body weight loss, and elevated blood glucose, urinary albumin excretion, and serum creatinine levels. N=10 for Normal rats, N=20 for diabetic rats. * P<0.05; ** P<0.01 compared to the Normal group.

Adenovirus vector injection

Twelve weeks after the injection, the 20 diabetic rats were randomly divided into 2 groups, the Control group and the TRB3 group, and subjected to intravenous adenovirus injection (Sinogenomax, Beijing, China). The rats were injected in the tail vein, with 0.1 ml PBS containing 3×10^8 PFU empty virus vector (Control group) or TRB3-siRNA virus group (TRB3 group) while they were restrained. The intravenous virus injections were repeated after 2 weeks. The sequences used in the TRB3-siRNA were:

Sense 5’-GGCACAGAGUACACCUGCATT-3’,
Anti-sense 5’-UGCAGGUGUACUCUGUGCCTT-3’.

level in the urine, and serum creatinine level were measured at 2, 4, 8, and 12 weeks after injection.
Sample collection

The rats were euthanized 4 weeks after virus injection. Prior to euthanasia, the rats were placed in metabolic cages to collect 24-h urine. For euthanasia, rats were weighed and then intraperitoneally injected with 3.5ml/kg 10% chloral hydrate. Blood was collected by cardiac puncture. The abdominal aorta superior to the renal artery and the superior mesenteric artery were quickly ligated, and the kidneys were irrigated in situ with 4°C saline. The kidneys were then cut off and the renal capsules were removed. The left kidney was longitudinally cut in 2 halves and preserved in 4% neutralized formalin for later pathological analysis. The right kidney was cut into small pieces with scissors, and snap-frozen by liquid nitrogen in cryotubes for further molecular biological evaluation. The blood and urine samples were centrifuged at 2000 rpm for 10 min, and the supernatant was collected for assays of serum creatinine and 24-h total urinary albumin excretion.

Histopathology

Kidney tissue was fixed in 4% neutralized formalin for 24 h, then dehydrated by alcohol gradient, paraffin embedded, sectioned at 5 micrometers, and stained with HE. The appearances of the kidney, tubules, and interstitium were observed under an optical microscope (Olympus BX51, Olympus, Japan).

Figure 2. HE strain of representative kidneys of the Normal (A), Control (B), and TRB3 (C) groups. Tubulointerstitial nephropathy was apparent in the Control rats, but treatment of TRB3-siRNA had an ameliorative effect. Magnification: 200×.
Serum levels of interleukins, TNF-α, IL-1β, and IL-6 were measured by ELISA (Boster, Wuhan, China). Procedures were carried out according to the manufacturer’s protocol.

**Western blotting**

Protein was extracted from the collected kidney tissues and quantified by the BCA method (Pierce, Waltham, USA). Twenty micrograms (20 µg) of proteins were loaded to run an SDS-PAGE gel, and then transferred to a PVDF membrane. The membrane was then blocked with 5% nonfat milk for 1.5 h, and incubated with 1: 500 primary antibody (diluted in 5% BSA) at 4°C overnight. The membrane was then washed with TBS-T (TBS with 0.1% Tween-20) 3 times for 6 min each time, and incubated with 1: 5000 secondary antibody at room temperature for 2 h. Color development was by dark-room enhanced chemiluminescence. Rabbit anti-rat collagen I and collagen III polyclonal antibodies were from Abcam (Cambridge, UK), rabbit anti-rat TRB3 polyclonal antibody was from Calbiochem (San Diego, USA), and rabbit anti-rat AKT, p-AKT, PI3K, p-PI3K polyclonal antibodies were from Cell Signaling (Danvers, USA). HRP-conjugated goat anti-rabbit secondary antibody was from Boster (Wuhan, China). Beta actin (β-actin) was used as a protein loading control, and ImageJ (NIH, Bethesda, USA) software was used to quantify the band intensity.

**Statistical analysis**

SPSS 19.0 was used for statistical analysis. All results are shown as mean ±SEM. Statistical significance was set at \( P < 0.05 \).

**Results**

**Effect of TRB3-siRNA injection**

As shown in Table 1, at 16 weeks, compared to the Normal group, both the Control group and TRB3 group had significantly lower body weight (\( P < 0.01 \)), but the TRB3 group had higher body weight than the Control group, although the difference was not statistically significant. Both TRB3 and Control groups had significantly higher blood sugar levels (\( P < 0.01 \)), and TRB3 rats had significantly lower levels, than the Control group (\( P < 0.05 \)). The TRB3 and Control groups also had significantly higher urinary

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**Figure 3.** Serum levels of TNF-α (A), IL-1β (B), and IL-6 (C) in the Normal, Control, and TRB3 rats. The cytokines levels in the Control group were significantly higher than in the Normal group, whereas the levels in the TRB3 group were significantly lower than in the Control group. * \( P < 0.05 \); ** \( P < 0.01 \) compared to the Normal group; # \( P < 0.05 \); ## \( P < 0.01 \) compared to the Control group.
albumin levels \((P<0.05)\), and the TRB3 group had significantly lower levels than the Control group \((P<0.05)\). Furthermore, the Control group had significantly higher serum creatinine \((P<0.01)\), whereas the TRB3 rats had significantly lower levels than the Control group \((P<0.05)\). The TRB3 serum creatinine level was not significantly different from the Normal rats \((P>0.05)\).

**Determination of biochemical parameters**

The body weight, blood sugar, urinary albumin excretion, and serum creatinine of rats were affected after STZ injection. The body weight of the rats kept increasing in the Normal group, whereas after STZ injection, the body weight of the diabetic rats (Control group and TRB3 group) was largely unchanged throughout the study (Figure 1A), and was significantly lower than the Normal group \((P<0.05)\). The Normal rats also had low and stable blood sugar levels, whereas the diabetic rats had increased blood sugar, and remained high \((P<0.01;\) Figure 1B). The 24-h urinary albumin excretion of the Normal rats remained low, and within 4 weeks after STZ injection, the diabetic rats also maintained a low level of urinary albumin level. Starting from 8 weeks after STZ injection, however, the urinary albumin increased to levels significantly different from the Normal rats \((P<0.01;\) Figure 1C). The serum creatinine followed the same pattern \((P<0.01;\) Figure 1D).

**Histopathology**

The HE stains of the Normal rat kidneys show normal size and morphology of glomeruli and tubules, a small amount of urinary cast in the tubules, and no tubule expansion, interstitial infiltration of inflammatory cells, or fibroplastic proliferation (Figure 2A). In the Control tissue, glomerular size increase was apparent, and segmented glomerulosclerosis was also observed in some glomeruli. Urinary cast and tubular atrophy were visible in the tubules (Figure 2B). Compared to the Control group, the TRB3 tissue had less increase in glomerular size, less interstitial infiltration of inflammatory cells, less tubular atrophy, fewer signs of lesions, and more signs of recovery (Figure 2C).

**Serum levels of TNF-\(\alpha\), IL-1\(\beta\), and IL-6**

The serum levels of TNF-\(\alpha\), IL-1\(\beta\), and IL-6 were assayed by ELISA. Compared to the Normal group, the Control rats had
significantly higher serum TNF-α, IL-1β, and IL-6 levels (P<0.05 or P<0.01, see Figure 3), whereas the TRB3 serum TNF-α and IL-1β levels were not significantly different from the Normal rats (Figure 3A, 3B). Serum IL-6 was higher in the TRB3 rats than in the Normal rats (Figure 3C). Moreover, the serum levels of TNF-α, IL-1β, and IL-6 in the TRB3 rats were significantly lower than in the Control rats, confirming the ameliorative effect of TRB3-siRNA.

**TRB3, Col I, and Col III protein expression in the kidneys**

The protein levels of TRB3 and type I and type III collagen were detected by Western blot and are shown in Figure 4. The Control rats had significantly higher TRB3 (Figure 4A), Col I (Figure 4B), and Col III (Figure 4C) protein levels than the Normal rats (P<0.01). On the other hand, the levels of these 3 proteins in TRB3 rats were significantly lower than in the Control rats, and they were not significantly different from the Normal rats.

**PI3K, p-PI3K, AKT, and p-AKT protein expression in the kidneys**

Figure 5 shows the protein levels of PI3K, phosphor-PI3K (p-PI3K), AKT, and phosphor-AKT (p-AKT). The Control rats had significantly lower PI3K and AKT phosphorylation (as calculated by p-PI3K/PI3K and p-AKT/AKT ratios) than the Normal rats (P<0.01). On the other hand, the phosphorylation levels of these 2 proteins in TRB3 rats were significantly higher than in the Control rats, and they were not significantly different from the Normal rats.

**Discussion**

Due to its prevalence, treatment and management of diabetes mellitus is clinically and economically critical. In combating its wide spectrum of subsequent complications, it is important to control diabetic nephropathy, one of the primary complications of diabetes. Although some argue that diabetic nephropathy has
become less prevalent due to better glycemic control, it still accounts for approximately 30% of diabetic deaths [23]. Diabetic nephropathy is typically manifested as glomerulosclerosis, microalbuminuria, and ECM protein accumulation, and can lead to kidney failure and death. With diabetes mellitus becoming increasingly prevalent, it is imperative to reduce the incidence of clinical nephropathy, in combination with its associated increase in cardiomyopathy. To improve overall prognosis of diabetic nephropathy, it is essential to stop glomerulosclerosis as early as possible, in addition to conventional measures of glycemic control and blood sugar level reduction. The recently found AKT signaling inhibitor TRB3 [8] has been shown to regulate expression of nephritic collagen and is therefore a potential therapeutic target, and some studies have reported that knocking down TRB3 slows albumin-induced tubular cell apoptosis and alleviates diabetic cardiomyopathy [12,21]. However, how TRB3 silencing affects diabetic nephropathy in vivo and how the different types of collagen molecules are differentially regulated by TRB3 are not yet fully understood [24].

In this study, the results demonstrated that TRB3 gene silencing is capable of improving nephropathic symptoms in diabetic rats by lowering urinary protein excretion and serum creatinine. In fact, TRB3 silencing even reversed body weight loss and blood glucose increase in the T2D-induced type 1 diabetic rats to different degrees, although the body weight change was not enough to cause a significant difference between the TRB3 rats and Control rats. Additionally, TRB3 silencing also significantly repressed expression of pro-inflammatory factors, including TNF-α, IL-1β, and IL-6, thereby slowing tissue damage. Moreover, it clearly slowed the accumulation of 2 ECM proteins, type I and type III collagen, in the kidney. The discovery that type III collagen follows a similar pattern as type I collagen in response to TZD injection and TRB3 silencing is novel, and requires further investigation. Moreover, TRB3 silencing has an ameliorative effect against diabetes-induced tubular interstitial lesions, likely by promoting phosphorylation of PI3K and AKT and increasing the signaling activity of the PI3K/AKT pathway. This may have further implications, since the PI3K/AKT signaling pathway is an important and highly regulated pathway that has a cascade of downstream effects, including cellular functions such as cell growth, proliferation, differentiation, motility, survival, and intracellular trafficking.

Conclusions

Based on the data presented in this study, we report that TRB3 silencing has a protective effect in rat kidneys, and may shed some light on a new direction towards treatment and management of diabetic nephropathy.

Statement

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