Original Paper

Activation of Nrf2/HO-1 Pathway by Glycogen Synthase Kinase-3 β Inhibition Attenuates Renal Ischemia/Reperfusion Injury in Diabetic Rats

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Key Words

TDZD-8 • Nrf2/HO-1 • Bcl-2 • NF- κ B • Ischemia/reperfusion • Diabetes mellitus

Abstract

Background/Aims: Diabetes mellitus can exacerbate renal ischemia-reperfusion (I/R) injury (RI/RI). The aim of the present study was to evaluate the protective effect of GSK-3 β inhibition (TDZD-8) on I/R-induced renal injury through the Nrf2/HO-1 pathway in a streptozocin (STZ)-induced diabetic rat model. **Methods:** STZ-induced diabetic rats preconditioned with TDZD-8 and ZnPP were subjected to renal I/R. The extent of renal morphologic lesions. Renal function was assessed from blood urea nitrogen (BUN) and serum creatinine (Scr), as determined utilizing commercial kits. Oxidative stress and inflammatory activity in the kidney tissue was estimated from levels of malondialdehyde (MDA), interleukin-10 (IL-10), tumor necrosis factor- α (TNF- α), and nitric oxide (NO), as well as the activities of superoxide dismutase (SOD) and glutathione (GSH) using qRT-PCR and ELISA. The expressions of Nrf2, HO-1, Bcl-2 and NF- κ B in the renal tissue were measured by qRT-PCR and western blotting. **Results:** I/R-induced renal inflammation was reduced significantly by TDZD-8 pretreatment. Preconditioning with TDZD-8 suppressed NF- κ B expression and enhanced Bcl-2 expression in the renal tissue. The upregulated level of malondialdehyde (MDA), and reduced activities of superoxide dismutase (SOD) and glutathione (GSH) in I/R-shocked rats were markedly restored by TDZD-8 pretreatment. Furthermore, pretreatment with TDZD-8 enhanced activation of the Nrf2/

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HO-1 pathway in the renal tissue of diabetic RI/RI rats. **Conclusion:** These findings suggest that preconditioning with TDZD-8 may protect the kidney from I/R-induced damage via the activation of the Nrf2/HO-1 pathway in STZ-induced diabetic rats. Further detailed studies are needed to further clarify the underlying mechanisms.

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Introduction

The incidence of diabetes mellitus (DM) has increased significantly in recent years worldwide, which brings substantial burdens to both patients and healthcare systems [1]. The primary complications of DM mainly involve the kidneys, eyes, and nerves due to damage to small blood vessels [2]. DM-induced damage to the kidney, also known as diabetic nephropathy, can result in renal tissue damage, urine protein loss and eventually chronic kidney disease (CKD), which may in turn exacerbate renal ischemia/reperfusion (I/R) injury (RI/RI) [2-4]. RI/RI remains an unresolved problem that has immediate and deleterious effects in both native and transplanted kidneys. Despite new therapeutic progress in renal protection over years, management of RI/RI after DM remains a clinical challenge [5].

Nuclear factor (erythroid-derived 2)-like 2, also known as NFE2L2 or Nrf2, is a transcriptional factor encoded by the Nrf2 gene in humans [6]. Nrf2 is a basic leucine zipper (bZIP) protein that regulates the expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation [7]. Under normal conditions, Nrf2 is kept in the cytoplasm by Kelch like-ECH-associated protein 1 (Keap-1) [8, 9]. Oxidative stress or electrophilic stress disrupts critical cysteine residues in Keap-1, translocating Nrf2 into the nucleus, where Nrf2 binds to the antioxidant response element (ARE) in the upstream promoter region of many antioxidative genes and initiates their transcription [10]. Activation of Nrf2 could induce many cytoprotective proteins such as heme oxygenase-1 (HO-1), an enzyme that catalyzes the degradation of heme into the antioxidant biliverdin, the anti-inflammatory agent carbon monoxide, and ferrous iron [11-13]. HO-1 is a highly inducible isoform in response to stress such as oxidative stress, I/R, hypoxia, heavy metals, cytokines, and nitric oxide [14-16].

Phosphorylation of a protein by glycogen synthase kinase-3 β (GSK-3 β) usually inhibits the activity of its downstream target [17]. GSK-3 β is active in a number of central intracellular signaling pathways, including cellular proliferation, migration, glucose regulation, and apoptosis [17, 18]. The 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8) is one of GSK-3 β competitive inhibitors that has been reported to alleviate enteral and hepatic I/R injury through inhibiting apoptosis [19, 20]. TDZD-8 can increase glucose tolerance in diabetic rats [21]. A previous study reported that TDZD-8 could exert its protective effect against RI/RI by regulating the Nrf2/TrxR2 signaling pathway in the renal tissue in an RI/RI rat model [5]. However, the role of TDZD-8 in regulating the Nrf2/HO-1 signaling pathway in diabetic RI/RI remains unknown. The aim of the present study was to evaluate the protective effect of GSK-3 β inhibition by TDZD-8 on I/R-induced renal injury through the Nrf2/HO-1 pathway in a streptozotocin (STZ)-induced diabetic rat model.

Materials and Methods

Establishment of Animal models

Sprague-Dawley (SD) male rats (200-220g) were obtained from the Animal Research Center of Jiaying University Medical College (Jiaying, China). A single dose of streptozotocin (STZ) dissolved in citrate buffer (PH 4.5, 50 mg/kg body weight [bw]) was injected into the rat abdominal cavity to generate the STZ-induced diabetic rat model. Rats were housed under climate-controlled conditions with a 12-h light/dark cycle and provided with standard food and water for 2 weeks. Rats with a serum glucose level of 16.7-25.6 mmol/L were used in the following experiments. Forty STZ-induced diabetic rats were equally randomized

into four groups: 1) a sham-operated group (sham group), where the rats were treated only by separating the bilateral renal arteries and veins and 10% dimethyl sulfoxide (DMSO, 1 ml/kg bw, i.v.); 2) a renal I/R group (I/R group), where the rats were treated with ischemia by clamping the bilateral renal arteries and veins for 45 min and subsequent 24-h reperfusion with DMSO (1 ml/kg bw, i.v.); 3) an I/R+TDZD-8 group (TDZD-8 group), where the rats were pretreated with TDZD-8 (dissolved in DMSO, 1 mg/kg bw) via a single-dose injection into the abdominal cavity 30 min prior to the I/R procedure; and 4) an I/R+TDZD-8+ZnPP group (ZnPP group), where the rats were administered intraperitoneally with zinc protoporphyrin (ZnPP, 20 mg/kg bw) 24 h prior to the I/R procedure, and TDZD-8 was administered into animals based on TDZD-8 group. The animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Jiaxing University Medical College. The study protocol was approved by the Committee on the Ethics of Animal Experiments of the said University.

Sample processing

Blood samples were collected via the abdominal aorta after 24-h reperfusion and centrifuged at $3600 \times g$ at 4 °C for 15 min to harvest the serum. All the resected left kidneys per group were used for histopathologic evaluation, and the resected right kidneys per group were homogenized in ice-cold normal saline and centrifuged at $3600 \times g$ at 4 °C for 15 min to harvest the supernatant.

Histopathological changes

The left renal tissue was harvested longitudinally, fixed in 4% paraformaldehyde, paraffin-embedded, sliced into 4- μ m sections, stained with hematoxylin-eosin (HE) staining, and observed with an optical microscope (Leica, Germany). One whole deep coronal section was examined under the microscope and graded according to the degree of damage based on the percentage of involvement of the kidney. The damage quantification from ten areas corresponding to the kidney proximal tubules was graded using the following parameters: tubular cell necrosis, cytoplasmic vacuole formation, hemorrhage, and tubular dilatation based on a five-score system (1, histopathological changes <10%; 2, = 10-25%; 3, =25-50%; 4, =50-75%; and 5, =75-100%). The mean score for each parameter was calculated and subjected to statistical analysis [5].

Renal function assessment

The renal function was evaluated through measurement of blood urea nitrogen (BUN) and serum creatinine (Scr) levels using Hitachi automatic biochemical analyzer 7060 c (Japan) and Roche Diagnostics kits (Mannheim, Germany) by the picric acid method according to the manufacturer's instructions [22].

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from the prepared renal samples using TRIzol reagent (Invitrogen, USA). cDNA was synthesized by reverse transcription following the manufacturer's protocols (MBI Fermentas, Lithuania). qRT-PCR was performed with a standard SYBR-green PCR kit (Toyobo, Japan), and gene-specific PCR amplification was performed using the ABI 7300 (Applied Biosystems, Germany). Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to the mRNA level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Assessment of antioxidative activity and inflammatory injury in the renal tissue

The antioxidative activity and the magnitude of inflammatory injury were detected with the levels of superoxide dismutase (SOD), malondialdehyde (MDA), glutathione (GSH), tumor necrosis factor- α (TNF- α), interleukin-10 (IL-10), nitric oxide (NO), and HO-1. All measurements were performed using the double antibody sandwich enzyme-linked immunosorbent assay (ELISA) kits (Roche, Germany) according to the manufacturer's instructions.

Western blot

Western blot analysis was performed as previously described [23, 24]. Briefly, protein aliquots 150 μ g from the supernatant of the renal tissue was run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After blocking with 3% bull serum albumin in Tris-buffered saline containing 0.2-0.4% Tween-20 (TBST) for 1 h, the membranes were incubated with the primary antibodies (against

active Nrf2, HO-1, Bcl-2, and NF- κ B) (Sigma-Aldrich, USA) at 4 °C overnight, and subsequently with alkaline phosphatase-conjugated secondary antibodies. The membranes were developed by 5-b-romo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium. Blots were stained with an anti- β -actin antibody, and the protein levels were normalized with respect to β -actin band density. The antigen-antibody products were measured by Thermo Scientific Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, USA). The results were analyzed with a Fluor Chem system (Alpha Innotech, USA).

Table 1. Serum levels of BUN and Scr

Groups	BUN (mmol/L)	Scr (μ mol/L)
Sham	5.98 $\pm$ 0.93	29.16 $\pm$ 3.62
I/R	34.50 $\pm$ 5.19*	120.83 $\pm$ 10.75*
TDZD-8	22.59 $\pm$ 4.61#	64.19 $\pm$ 7.24#
ZnPP	27.51 $\pm$ 4.25**	72.59 $\pm$ 8.48**

Note: BUN, blood urea nitrogen; Scr, serum creatinine. Data are presented as the mean $\pm$ standard deviation (n=10). *P<0.01 vs. Sham group; #P<0.01 vs. I/R group; **P<0.05 vs. TDZD-8 group

Fig. 1. Histopathological alterations of kidneys. Note: HE, $\times$ 200. Data are presented as the mean $\pm$ standard deviation (n=10). The stars indicate the injury zone. *P<0.01 vs. Sham group; #P<0.01 vs. I/R group; **P<0.05 vs. TDZD-8 group.

Statistical analysis

Data were expressed as the mean $\pm$ standard deviation (SD) and performed by Student's *t*-test and ANOVA analyses with a further test of posthoc Dunn's multiple comparisons. Microsoft Excel 2013 database and the SPSS 19.0 software for windows were used to record and analyze data respectively. The level of significance was set at P<0.05.

Results

TDZD-8 preconditioning attenuated I/R-induced renal damages

Tubular cell necrosis, hemorrhage, protein cast, and tubular dilatation were observed in the I/R group. These histopathological alterations were markedly reduced in the TDZD-8 group and moderately reduced in ZnPP group (Fig. 1). The serum levels of BUN and Scr were significantly increased by 4-6-fold after I/R and reduced by TDZD-8 pretreatment (P<0.01) (Table 1). These results suggest that TDZD-8 may attenuate I/R-induced renal damage.

TDZD-8 inhibited I/R-induced oxidative stress and inflammation in the renal tissue

Seven genes were selected for validation (as shown in Table 2). The mRNA expressions of IL-10 and TNF- α were up-regulated by 3.28 to 4.53-fold after I/R in our model (P<0.01

vs. sham group). Preconditioning with TDZD-8 enhanced the mRNA expression of SOD and IL-10 ($P < 0.01$ vs. I/R group) (Fig. 2 and 3).

The SOD activity and GSH level in the renal tissue of I/R group were reduced significantly as compared with that of sham group (11.85 ± 2.42 vs. 44.15 ± 5.37 U/mg for SOD, 4.25 ± 0.53 vs. 7.89 ± 0.75 nmol/mg for GSH, $P < 0.01$). Both of them in TDZD-8 group (30.30 ± 5.68 U/mg for SOD, 9.45 ± 0.78 nmol/mg for GSH) was increased significantly as compared with those in the I/R group, and they were decreased in ZnPP group (23.04 ± 3.16 U/mg for SOD, 4.91 ± 0.68 nmol/mg for GSH, $P < 0.05$). Correspondingly, the MDA level in I/R group was significantly higher than that in the sham group (17.33 ± 1.70 vs. 4.18 ± 0.68 nmol/mg, $P < 0.01$). The MDA level in the renal tissue was decreased significantly after administration of a single dose of TDZD-8 (10.49 ± 1.22 vs. 17.33 ± 1.70 nmol/mg, $P < 0.01$) (Fig. 2).

Fig. 2. The concentration of SOD, MDA, GSH, IL-10, TNF- α , and NO and the mRNA expression of SOD, IL-10, and TNF- α in the renal tissue. Note: (A) SOD; (B) MDA; (C) GSH; (D) IL-10; (E) TNF- α ; (F) NO. The level of SOD, MDA, GSH, IL-10, TNF- α , and NO in the renal tissue, as measured by ELISA. (G) Relative mRNA expressions of SOD, IL-10, and TNF- α , as determined by qRT-PCR. Data are presented as the mean $\pm$ standard deviation of three repeat experiments ($n = 10$). * $P < 0.01$ vs. Sham group; # $P < 0.01$ vs. I/R group; ** $P < 0.05$ vs. TDZD-8 group.

Table 2. Primer used for qRT-PCR

Primer name	Primer sequence
sod1	F: 5'-AGGGCGTCATTCACCTTCGAG-3' R: 5'-CCTCTCTTCATCCGCTGGAC-3'
il-10	F: 5'-CCTGCTCTTACTGGCTGGAG-3' R: 5'-CCTGGGGCATCACTTCTACC-3'
tnfa	F: 5'-ATGGGCTCCCTCTCATCAGT-3' R: 5'-CAAGGGCTCTTGATGGCAGA-3'
nrf2	F: 5'-CACAGTGCTCCTATGCGTGA-3' R: 5'-TTCTGGGCGGCGACTTTATT-3'
hmox1	F: 5'-TTAAGCTGGTGATGGCCTCC-3' R: 5'-GTGGGGCATAGACTGGGTTC-3'
nfkb1	F: 5'-TACGATGGGACGACACCTCT-3' R: 5'-TCTCGGAGCTCATCTCATAGT-3'
bcl2	F: 5'-ACTCTTCAGGGATGGGGTGA-3' R: 5'-TGACATCTCCCTGTTGACGC-3'
gapdh	F: 5'-GGTGAAGGTCGGAGTCAACG-3' R: 5'-CAAAGTTGTTCATGGATGACC-3'

Fig. 3. The expression of Nrf2, NF-κB, Bcl-2, and HO-1 and concentration of HO-1 in the renal tissue. Note: (A) The protein expression of Nrf2, NF-κB, Bcl-2, and HO-1, as examined by western blotting, and proteins level Nrf2, NF-κB, and Bcl-2 in the renal tissue. (B) The mRNA expression of Nrf2, NF-κB, and Bcl-2, as determined by qRT-PCR. (C) The protein level of HO-1 in the renal tissue. (D) The mRNA expression of HO-1 (qRT-PCR). (E) The concentration of HO-1 in the renal tissue, as measured by ELISA. Data are presented as the mean ± standard deviation of three repeat experiments (n=10). **P*<0.01 vs. Sham group; #*P*<0.01 vs. I/R group; ***P*<0.05 vs. TDZD-8 group.

After I/R, the levels of IL-10 (anti-inflammatory factor), TNF-α (pro-inflammatory factor), NO, and HO-1 were significantly higher than those in the sham group (37.85±6.26 vs. 19.90±3.17 pg/g for IL-10, 0.89±0.11 vs. 0.47±0.07 ng/g for TNF-α, 128.25±10.53 vs. 67.89±7.75 μmol/mg for NO, 0.59±0.04 vs. 0.24±0.03 pg/g for HO-1, *P*<0.01). After TDZD-8 administration, the levels of IL-10 (53.27±6.53 pg/g) and HO-1 (0.86±0.07 pg/g) was increased, and TNF-α (0.62±0.09 ng/g) and NO (89.45±8.78 μmol/mg) were decreased as compared with those in I/R group (*P*<0.01). Administration of ZnPP displayed an inhibitory effect on TDZD-8 (Fig. 2 and 3). These results clearly indicate that preconditioning with TDZD-8 may effectively inhibit I/R-induced renal inflammation and oxidative stress.

TDZD-8 up-regulated the expressions of Nrf2/HO-1 pathway and Bcl-2 and down-regulated NF-κB expression in the renal tissue

The Nrf2/HO-1 pathway has a pivotal role in antioxidant reaction [25, 26]. To investigate whether it is associated with the protective effects of TDZD-8 on I/R-induced oxidative damage (oxidative stress may induce apoptosis) to the kidneys, the expression of Nrf2 and HO-1 were examined. As expected, the mRNA and protein expressions of NF-κB and HO-1 were increased significantly, while the expressions of Nrf2 and Bcl-2 were reduced significantly in I/R-shocked rats as compared with those in the sham group (*P*<0.01). Preconditioning with TDZD-8 significantly up-regulated the expressions of Nrf2, HO-1, and Bcl-2, and down-regulated NF-κB expression in the renal tissue as compared with those in I/R group (*P*<0.01). Pretreatment with ZnPP displayed an inhibitory effect on TDZD-8 (Fig. 3). These data indicate that TDZD-8 protects the kidney from I/R-induced oxidative injury, at least partially via activation of the Nrf2/HO-1 pathway.

Discussion

GSK-3 β is reported to be associated with a host of high-profile diseases because it involves many signaling pathways. There has been much research on the therapeutic effects of GSK-3 β inhibitors in ischemic diseases, DM, or even some forms of cancer. Several studies reported that TDZD-8 as a GSK-3 β inhibitor could reduce I/R injury of the intestines and brain due to its anti-inflammatory and anti-apoptosis activities. [19, 27, 28]. It was found in the present study that RI/RI resulted in renal tissue damage accompanied with elevation of serum BUN and Scr levels. Preconditioning with TDZD-8 attenuated the renal tissue damage in RI/RI rats. Previous studies have reported that RI/RI increased the production of ROS and dependent signaling cascades, reduced SOD activity, and increased the level of MDA [29-31]. NF- κ B plays a critical role in the pathophysiology of RI/RI in DM with inflammation and oxidative stress [32, 33]. Activation of NF- κ B expression is negatively related to the expression of IL-10 [34-36]. Our data indicate that TDZD-8 could improve the levels of SOD and IL-10 and decrease the level of MDA and the NF- κ B expression, thus attenuating renal oxidative injury. ZnPP as a TDZD-8 inhibitor could antagonize the effect of TDZD-8. These results verify the above injury mechanism in the RI/RI model of DM rats.

The ability of HO-1 to catabolize free heme and produce carbon monoxide (CO) gives its anti-inflammatory properties by up-regulating IL-10 [37]. Nrf2 is a key regulator of the antioxidative defense pathway and could be activated in DM and diabetic neuropathy [38-41]. It is generally accepted that apoptosis leads to renal dysfunction subsequent to ischemia in DM [42-45]. Bcl-2 is a pivotal factor that determines whether or not apoptosis occurs in cells exposed to injury, and increasing expression of Bcl-2 can promote cell survival [46-50]. A previous study shows that enhancement of the Nrf2/HO-1 pathway played an anti-apoptosis role, and that activation of Nrf2 could induce the expression of HO-1 to enhance the expression of Bcl-2 [51]. The results of the present study showed that the expressions of Nrf2 and Bcl-2 were down-regulated and the expressions of HO-1 and NF- κ B were up-regulated in the I/R group. TDZD-8 pretreatment enhanced the expression of Nrf2, HO-1 and Bcl-2, and suppressed the expression of NF- κ B, suggesting that the protective mechanism of TDZD-8 lies in its anti-inflammatory activity by inhibiting the release of pro-inflammatory factors, and its anti-oxidative activity by reducing the production of lipid peroxidation and anti-apoptotic activity, which probably depends on the regulation of activated IL-10, TNF- α , Bcl-2 and NF- κ B via Nrf2/HO-1 signaling pathway on RI/RI in DM.

Conclusion

The present study reported a renal protective role of TDZD-8 in RI/RI. In addition, activation of the Nrf2/HO-1 signaling pathway is a potential mechanism by which TDZD-8 preconditioning protects against I/R-induced renal injury in diabetic rats. Further detailed studies are needed to better clarify the underlying mechanisms.

Disclosure Statement

The authors declare that they have no competing interests and nothing to disclose.

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