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Glycogen synthase kinase-3β inhibitor ameliorates imbalance of connexin 43 in an acute kidney injury model

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ABSTRACT

This study was designed to evaluate whether glycogen synthase kinase-3β (GSK-3β) inhibitor, 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8) induced the the expression of connexin 43 (Cx43) to protect against renal ischemia–reperfusion (I/R) injury (RI/RI) in rats. Rats were subjected to 45 min ischemia followed 2 h reperfusion with TDZD-8 (1 mg/kg) for 5 min prior to reperfusion. The results indicated that TDZD-8 improved the recovery of renal function, reduced oxidative stress and inflammation injury, and upregulated the expression of (Cx43) as compared to I/R group. Therefore, our study demonstrated that TDZD-8 provided a protection to the kidney against I/R injury in rats through inducing the expression of (Cx43).

1. Introduction

Ischemia–reperfusion (I/R)-induced injury is a primary reason of acute organ dysfunction and that usually occurs in association with pathophysiological conditions, including primary surgery, trauma, large area burns, and acute hypovolemia due to heavy fluid and blood loss [1]. Although, reperfusion after ischemia generally improves the recovery of organ dysfunction caused by ischemia, in some instances, reperfusion may aggravate the damage associated with ischemia [2,3].

Renal ischemia–reperfusion injury (RI/RI) is mediated by multiple pathophysiological mechanisms of which free radical damage and inflammatory factor are two key events [4,5]. During RI/RI, free radicals and inflammatory factor are both increased, resulting in extensive damage and apoptosis of tubular epithelial cells [6,7]. Among these factors, connexin 43 (Cx43) is involved in the pathophysiology of damage and apoptosis through multiple roles [8].

Glycogen synthase kinase-3β (GSK-3β) is a well-conserved, ubiquitously expressed serine–threonine protein kinase originally characterized as one that phosphorylates glycogen synthase and regulates glucose metabolism [9]. Subsequently, studies demonstrate that GSK-3β positively participated in a multitude of cellular processes, including prolifeation, apoptosis–necrosis, plays an important role in the pathophysiology of a number of diseases, including kidney diseases. And more recent evidence suggest that GSK3-β plays dirty in acute kidney injury [10]. GSK-3β inhibitors strikingly prevent acute renal histological injury induced by ischemia reperfusion [11–13]. Previous studies [14,15] have showed that GSK-3 beta inhibitor (TDZD-8) reduces enteral and hepatic ischemia–reperfusion injury through inhibiting the apoptosis. And literature have showed that TDZD-8 can prevents NSAID-induced acute kidney injury [11]. Cx43 is an important material in cell connection communication, new research find that Cx43 plays an important role in drug preconditioning and ischemia postconditioning [16]. In the current study, Cx43 may be important for exchanging small molecules in the glomerular apparatus and tubular cells in the kidney, necessary for keeping a normal renal function [17]. It has demonstrated that the glomerular of Cx43 expression can reduce RI/RI in rats [8].

Thus, TDZD-8 may give play to protective effects against ischemia–reperfusion injury in many organs, including the kidney. Also, in different ischemia–reperfusion models, the role of Cx43 varied. However, to date, no studies have inspected the effects of TDZD-8 on renal ischemia–reperfusion injury and Cx43 expression. Therefore, in this preliminary study, we inquire into possible

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protective effects of TDZD-8 on renal ischemia–reperfusion injury in rats. We also inspect the expression of renal Cx43 in these rats.

2. Materials and methods

2.1. Animals and reagents

Sprague-Dawley (SD) rats (180–220 g) were supplied by the Animal Research Center of Shantou University Medical College, Shantou, China. All the procedures and care administered to the animals had been approved by the institutional ethic committee. All reagents and drugs were purchased from Sigma.

2.2. Animal experimental design

In the experiments 30 rats were randomly assigned to three groups (each group had 10 rats): (1) sham + vehicle: 10% dimethyl sulfoxide (DMSO, 1 ml/kg, i.v.) and subjected to the surgical procedure; (2) I/R + vehicle: 45 min of ischemia followed by 2 h reperfusion with DMSO (1 ml/kg, i.v.); (3) I/R + TDZD-8:45 min of ischemia followed by 2 h reperfusion with TDZD-8 (1 mg/kg, i.v., dissolved in DMSO) for 5 min prior to reperfusion. The doses were selected based on previous reports [15,18].

2.3. Surgical procedures of I/R

The methods had been described previously [4]. Rats were anesthetized with 1% pentobarbital sodium (50 mg/kg) through injecting in enterocoelia. The enterocoelia was opened and the bilateral renal artery–vein were separated. The artery–vein were occluded for 45 min by two artery clamps, and the success was confirmed by observing the color changing of kidney. The artery clamp was removed 45 min later and renal artery and vein were allowed reperfusion for 2 h. The blood samples were collected via abdominal aorta, and centrifuged at 3600 × *g* for 15 min to harvest the sera. The left kidneys of animals were immediately removed and stored at −20 °C for the analyses below, respectively.

2.4. Measurement of serum BUN and Scr

The methods had been described previously [4]. Briefly, the blood of abdominal aorta was collected after reperfusion 2 h and centrifuged at 3600 × *g* for 15 min to harvest the sera. The BUN and Scr of serum were measured by *O*-phthalaldehyde–picric acid method.

2.5. Measurement of SOD activity–MDA content

The levels of SOD–MDA in tissues were measured to assess lipid peroxidation as described previously [4]. The kidney was collected after reperfusion 2 h and blood was washed from the tissue, homogenized (100 mg) in ice normal saline and made from 10% homogenate, then centrifuged at 3600 × *g* for 15 min to harvest the supernatant. The SOD activity and MDA content were measured by xanthine oxidase–thiobarbituric acid method. The absorbance was measured at 550–532 nm. The level of lipid peroxides was expressed as U of SOD/mg prot and nmol of MDA/mg tissue.

2.6. Measurement of IL-10 content

The levels of IL-10 in tissues were measured to assess anti-inflammatory as described previously [19]. The samping process was the same as above and the IL-10 content was measured by double antibody sandwich ELISA method. The absorbance was measured at 450 nm. The level of anti-inflammatory was expressed as ng of IL-10/g tissue.

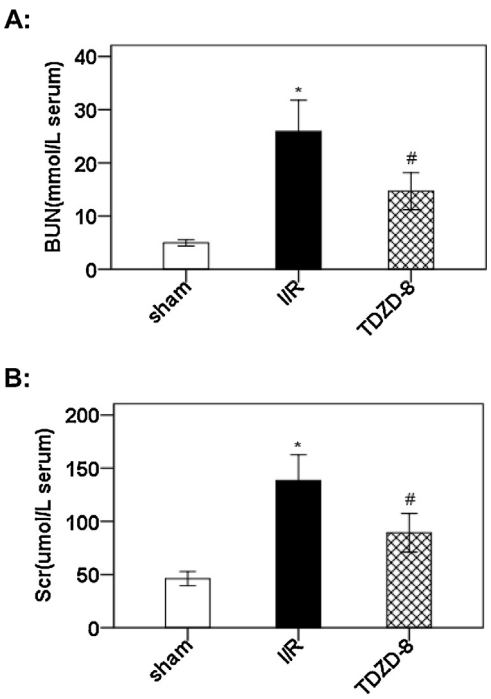

Fig. 1. Serum levels of BUN and Scr. The blood of sham, I/R, I/R + TDZD-8 groups rats was collected 2 h after reperfusion and the serum levels of BUN and Scr measured. Results expressed as mean ± SD. (A) A significant increase from the sham group was denoted by ($p < 0.01$), a significant decrease from the I/R group, by ($p < 0.01$); (B) a significant increase from the sham group was denoted by ($p < 0.01$), a significant decrease from the I/R group, by ($p < 0.01$).

2.7. Western blot analysis

The methodology has been described previously [8]. Briefly, renal tissues were homogenized in protein lysate buffer. The homogenates were resolved on polyacrylamide SDS gels, and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with 3% BSA, incubated with primary Abs against active Cx43 and subsequently, with alkaline phosphatase conjugated secondary Abs. They were developed by 5-b-romo-4-chloro-3-indolyl phosphate–nitro blue tetrazolium. Blots were stained with anti-β-actin Ab, and the levels of proteins were normalized with respect to β-actin band density.

2.8. Statistical analysis

All the data were expressed as mean values ± standard deviation (SD). Statistical analysis between groups was carried out using ANOVA with post-hoc testing. A value of less than 0.01 ($P < 0.01$) was used for statistical significance.

3. Results

3.1. Serum level of BUN and Scr

The serum levels of BUN and Scr I/R rats were significantly higher than that in Sham group ($p < 0.01$). The levels of BUN ranged from 4.87 to 5.13 mmol (4.97 ± 0.10 mmol) in Sham group, whereas the level of BUN reached 25.89 ± 0.98 mmol in I/R group. Administration of I/R + TDZD-8 significantly, reduced the levels of BUN (14.67 ± 0.58 mmol) compared with I/R group ($p < 0.01$) (Fig. 1A); The levels of Scr ranged from 44.89 to 47.78 μmol

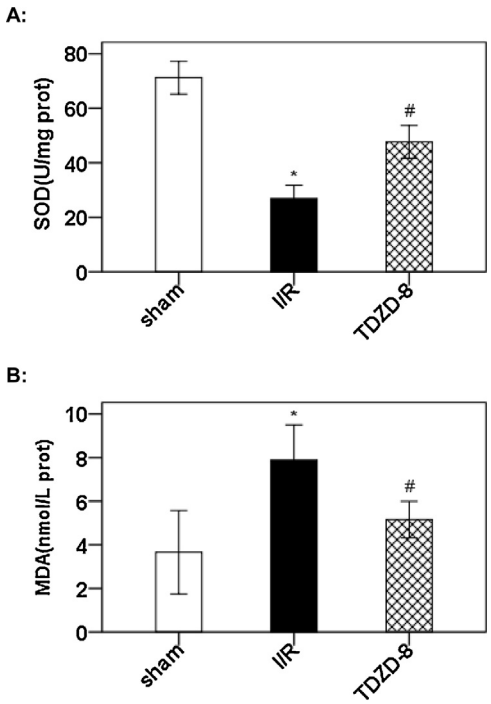

Fig. 2. The activity of SOD and the level of MDA in renal tissues by I/R group induced. The renal tissues of sham, I/R, I/R + TDZD-8 groups rats were collected 2 h after reperfusion and the activity of SOD and the level of MDA measured. Results expreed as mean ± SD. (A) A significant decrease from the sham group was denoted by ($p < 0.01$), a significant increase from the I/R group, by ($p < 0.01$); (B) a significant increase from the sham group was denoted by ($p < 0.01$), a significant decrease from the I/R group, by ($p < 0.01$).

(46.13 ± 1.11 μmol) in Sham group, whereas the level of Scr reached 138.32 ± 4.03 μmol in I/R group. Administration of I/R + TDZD-8 significantly reduced the levels of Scr (89.24 ± 3.03 μmol) compared with I/R group ($p < 0.01$) (Fig. 1B).

3.2. The activity of SOD and the level of MDA in renal tissues

The activity of SOD in renal tissues which was induced by I/R was significantly less and than that in Sham group ($p < 0.01$). The activity of SOD in renal tissues reached 71.21 ± 1.00 U in Sham group, whereas the activity of SOD in renal tissues reached 26.87 ± 0.82 U by I/R group induced. Administration of I/R + TDZD-8 significantly increased the activity of SOD (46.67 ± 1.01 U) in renal tissues, compared with the activity of SOD in renal tissues by I/R group induced ($p < 0.01$) (Fig. 2A); The level of MDA in renal tissues which was induced by I/R was significantly higher than that in sham group ($p < 0.01$). The level of MDA in renal tissues reached 3.66 ± 0.32 nmol in Sham group, whereas the level of MDA in renal tissues reached 7.88 ± 0.27 nmol by I/R group induced. Administration of I/R + TDZD-8 reduced the level of MDA (5.16 ± 0.14 nmol) in renal tissues, compared with the level of MDA in renal tissues by I/R group induced ($p < 0.01$) (Fig. 2B).

3.3. The level of IL-10 in renal tissues

The level of IL-10 in renal tissues which was induced by I/R was moderately higher than that in Sham group ($p < 0.05$). The level of IL-10 in renal tissues reached 38.28 ± 1.44 ng in Sham group, whereas the level of IL-10 in renal tissues reached 57.84 ± 0.76 ng by I/R group induced. Administration of I/R + TDZD-8 significantly increased the level of IL-10 (82.07 ± 1.29 ng) in renal tissues, compared with the level of IL-10 in renal tissues by I/R group induced ($p < 0.01$) (Fig. 3).

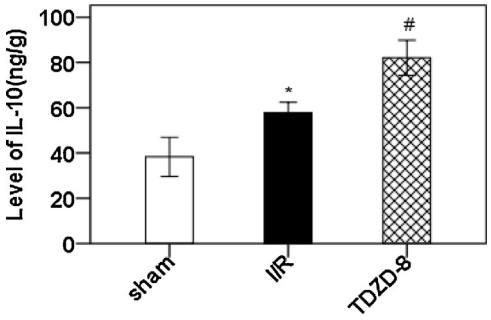

Fig. 3. The level of IL-10 in renal tissues by I/R group induced. The renal tissues of sham, I/R, I/R + TDZD-8 groups rats were collected 2 h after reperfusion and the level of IL-10 measured. Results expreed as mean ± SD. A moderate increase from the sham group was denoted by ($p < 0.05$), a significant increase from the I/R group, by ($p < 0.01$).

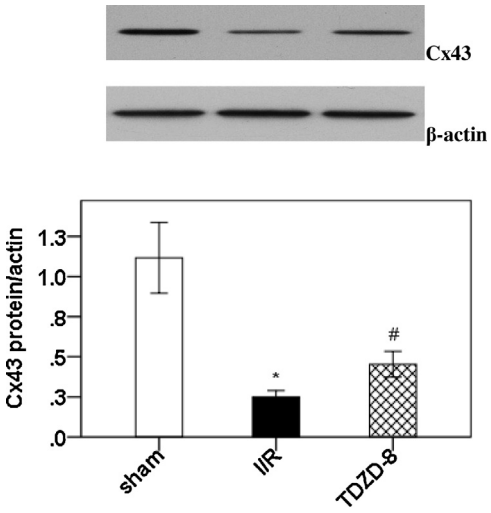

Fig. 4. The expression of Cx43 in renal tissues by I/R group induced. The renal tissues of sham, I/R, I/R + TDZD-8 groups rats were collected 2 h after reperfusion and the expression of Cx43 measured. Results expreed as mean ± SD. A significant decrease from the sham group was denoted by ($p < 0.01$), a significant increase from the I/R group, by ($p < 0.01$).

3.4. The expression of Cx43 in renal tissues

The expression of Cx43 in renal tissues which was induced by I/R was significantly less than that in Sham group ($p < 0.01$). Administration of I/R + TDZD-8 significantly upregulated the expression of Cx43 in renal tissues, compared with the expression of Cx43 in renal tissues by I/R group induced ($p < 0.01$) (Fig. 4).

4. Discussion

RI/RI is a common clinical pathophysiologic phenomena. This study examines the effect of GSK-3β inhibitor (TDZD-8, a selective GSK-3β inhibitor) on the expression of Cx43 exposed RI/RI in rats. The results of the present study demonstrate that TDZD-8 has protective effects on renal ischemia injury [13]. Glycogen synthase kinase-3β (GSK-3β) is a 47 kD serine–threonine kinase that is first observed to phosphorylate and inactivate glycogen synthase, a distal enzyme in the glycogen synthesis pathway [20]. GSK-3β is an ideal "survival" enzyme, because it controls several extra metabolic processes that are perturbed by ischemia, including cytoskeletal dynamics, gene expression, proliferation, and apoptosis [21–29]. However, in acute models of injury, GSK-3β promotes the systemic inflammatory response, increases the proinflammatory release of cytokines, induces apoptosis, and alters cell proliferation [30,31].

Substantially, evidence indicates that GSK-3β inhibitor (TDZD-8) preserves organ function after ischemia of the brain, gut [15,32]. Current literature shows that TDZD-8 inhibits ischemia-induced GSK-3β kinase activity and Bax and caspase-3 activation, reduces tissue injury, and improves organ function is consistent with this interpretation [17].

Gap junction (GJ) is a special structure on the adjacent cell membrane, it plays an important role in electricity and biochemical signals transmission between cells, and the function has a role in the metabolism of cells, homeostasis, differentiation and proliferation. In addition, (GJ) is closely related to the pathological processes of many diseases. (GJ) is a hollow cylinder water channel which is made up of special protein of adjacent cells-connexon, the channel diameter is about 1.5–2.0 nm and its hole diameter size depends on the types of connection protein(Cx), and it can permit the substances of molecular weight (<1KD) or small molecules (diameter <1.5 nm), such as cAMP, Ca^{2+}, IP3, amino acids, the second messenger, hormones, drugs and carcinogens to cross, and large molecules such as proteins and RNA material cannot pass; (GJ) not only has selectivity to the molecularsize, but also selectivity to the charge of molecules [33,34]. Current study indicates that intercellular gap junction communication is operated by a family of connexins in which connexin 43 (Cx43) may be critical for exchanging small molecules in the glomerular apparatus and tubular cells in the kidney, necessary for keeping a normal renal function. Decrements in expression of Cx43 imply impairs gap junctional communications among renal cells and play a role in abnormality of glomerular and tubular cells contributing to pathologies of DN [17].

In this study, I/R reduces Cx43 expression, renal function (BUN, Scr), antioxidant ability (SOD, MDA), and anti-inflammatory ability (IL-10), these results shows that there is a certain relationship between Cx43 and ischemia–reperfusion injury. The result is consistent with early myocardial ischemia reperfusion injury [35,36]. TDZD-8 can upregulate Cx43 expression and decrease renal injury. these hint that Cx43 is involved in TDZD-8 treatment in renal protection.

Previous study demonstrates that the activity of gene activator in Cx43 is reduced after reperfusion, reflow and oxygen supply, neutrophil infiltration, respiratory burst and release of inflammatory mediators (such as TNF-α, IL-10) [37–39]. In our study, Cx43 expression is higher than I/R group and TDZD-8 increases the level of IL-10, the result is consistent with literature.

To sum up, we find that TDZD-8 improves renal function in rats following I/R, and up regulates the expression of renal Cx43. Further studies are needed to explore whether the renal protective roles of TDZD-8 are due to this up regulated expression of Cx43 in the kidney.

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