Original Paper

Role of Calcium Sensing Receptor in Streptozotocin-Induced Diabetic Rats Exposed to Renal Ischemia Reperfusion Injury

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Key Words

Calcium-sensing receptor • Calmodulin • P47phox • R-568 • NPS-2143 • Renal ischemia/reperfusion injury • Diabetes mellitus

Abstract

Background/Aims: Renal ischemia/reperfusion (I/R) injury (RI/RI) is a common complication of diabetes, and it may be involved in altering intracellular calcium concentrations at its onset, which can result in inflammation, abnormal lipid metabolism, the production of reactive oxygen species (ROS), and nitroso-redox imbalance. The calcium-sensing receptor (CaSR) is a G-protein coupled receptor, however, the functional involvement of CaSR in diabetic RI/RI remains unclear. The present study was intended to investigate the role of CaSR on RI/RI in diabetes mellitus (DM). **Methods:** The bilateral renal arteries and veins of streptozotocin (STZ)-induced diabetic rats were subjected to 45-min ischemia followed by 2-h reperfusion with or without R-568 (agonist of CaSR) and NPS-2143 (antagonist of CaSR) at the beginning of I/R procedure. DM without renal I/R rats served as control group. The expressions of CaSR, calmodulin (CaM), and p47phox in the renal tissue were analyzed by qRT-PCR and Western blot. The renal pathomorphology, renal function, oxidative stress, inflammatory response, and calcium disorder were evaluated by detection of a series of indices by hematoxylin-eosin (HE) staining, transmission electron microscope (TEM), commercial kits, enzyme-linked immunosorbent assay (ELISA), and spectrophotofluorometry, respectively. **Results:** Results showed that the expressions of CaSR, CaM, and p47phox in I/R group were significantly up-regulated as compared with those in DM group, which were accompanied by renal tissue injury, increased calcium, oxidative stress, inflammation, and nitroso-redox imbalance. **Conclusion:** These results suggest that activation of CaSR is involved in the induction of damage of renal

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tubular epithelial cell during diabetic RI/RI, resulting in lipid peroxidation, inflammatory response, nitroso-redox imbalance, and apoptosis.

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Published by S. Karger AG, Basel

Introduction

The kidney is a vital organ for eliminating toxic substances from the blood and regulating water, electrolyte and acid-base balance in the body. In other respects, however, the kidney is vulnerable to damage by many disease processes given such as renal ischemia/reperfusion (I/R) injury (RI/RI) during the perioperative period [1, 2]. Renal sensitivity to I/R injury is found to be increased in streptozotocin (STZ)-induced diabetic rats [3], and in fact, RI/RI frequently occurs in diabetic patients [4] and often emerges as one of the dangerous complications of this procedure [5, 6]. In such a condition, patients may need renal transplantation in their later life when diabetic nephropathy occurs [7]. It is assumed that in diabetic rats, a comparatively short ischemia of 30 minutes is a predisposing factor contributing to the reversible acute kidney injury (AKI), causing a progressive injury with early-stage renal failure [4, 8, 9]. Clarification of the underlying mechanism is pivotal for determining efficacious management for diabetic RI/RI. Mounting studies have previously demonstrated that the underlying mechanism of I/R injury, including RI/RI mainly involves inflammatory cascade, reactive oxygen species (ROS), abnormal lipid metabolism, nitroso-redox imbalance, and calcium overload from ischemic tissues during reperfusion of ischemic tissues that may act indirectly in redox signaling to turn on apoptosis [10, 11].

The calcium-sensing receptor (CaSR), a G-protein coupled receptor with a large extracellular domain (ECD) in the N-terminal portion of the receptor, a seven-transmembrane region, and an intracellular carboxyl-terminal tail, is primarily expressed in the parathyroid gland and the renal tubules of the kidney [12-15]. Intriguingly, abundant data have demonstrated that activation of CaSR can exacerbate RI/RI [16] and is implicated in the pathogenesis of diabetes mellitus (DM) [17-19]. Intracellular calcium can be released through the binding of calcium to the ECD of CaSR activated G_q proteins, stimulated phospholipase C activity, and an increase in intracellular inositol triphosphate 3 level [20, 21]. The change in intracellular calcium is involved in many cell activities, such as cell proliferation and apoptosis. Ample evidence shows that extracellular calcium can be absorbed and transported into mitochondria during I/R, leading to changes of mitochondria membrane and promoting the activation of oxidative stress and apoptosis [22-24].

Previous studies showed that CaSR expressed in myocardial tissues played an important role in increased intracellular calcium during I/R injury [13, 21, 25, 26]. However, little is known about whether changes of CaSR expression induce renal injury during diabetic RI/RI. Herein, we took advantage of a conventional STZ-induced diabetic rat model of bilateral clamping of renal pedicles to investigate the impact of CaSR activation on diabetic RI/RI.

Materials and Methods

Animal models and treatment protocols

Sprague-Dawley rats (200-220 g) were obtained from the Laboratory Animal Unit of Jiaxing University, and were housed with a 12-/12-h light/dark cycle with *ad libitum* access to standard rodent chow and water. All experimental procedures were approved by the Ethics Committee for Animal Care and Use at the Jiaxing University (No. JUMC2015-029). The STZ-induced diabetic rat model was generated with i.p. injection of STZ dissolved in 0.1 M citrate buffer (PH 4.5, 50 mg/kg body weight [bw]). Rats with serum glucose levels ≥ 16.7 mmol/L in three consecutive measurements (three consecutive days) were considered to be a successful diabetic rat model [27].

Forty STZ-induced diabetic rats were housed for 5 days and generally anesthetized by i.p. injection with 2 % (w/w) pentobarbital sodium (3.0 ml/kg bw) [28, 29]. Then they were equally randomized into

four groups: (1) STZ-induced diabetic rat (DM group), wherein the diabetic rats were treated only with separating the bilateral renal arteries and veins; (2) DM + renal I/R injury (I/R group), wherein the diabetic rats were treated with ischemia by clamping the bilateral renal arteries and veins for 45 min and subsequent 2-h reperfusion; (3) DM + renal I/R injury + R-568 group (R group), wherein the R-568 (an agonist of CaSR, 2.5 mg/kg bw, dissolved in 10% dimethyl sulphoxide) was administered by i.p. injection to diabetic rats at 30 min prior to the I/R procedure; and (4) DM + renal I/R injury + NPS-2143 group (NPS group), wherein the NPS-2143 (an antagonist of CaSR, 30 mg/kg bw, dissolved in 15% aqueous solution of 2-hydroxypropyl- β -cyclodextrin) was administered by i.p. injection to diabetic rats at 30 min prior to the I/R procedure.

All animals were terminated under general anesthesia, blood and kidneys were collected and snap-frozen for biochemical and histological analysis. Blood samples were obtained from the abdominal aorta after reperfusion. Blood was washed away from the resected kidney with ultrapure water. All the resected left kidneys per group were used for evaluating renal histopathologic alterations and ultrastructural changes of renal epithelial cells of proximal tubules. The resected right kidneys per group were homogenized in ice-cold normal saline and then centrifuged at 4 °C at 3,600× *g* for 15 min to harvest the supernatant.

Detection of renal function and assessment of oxidative stress and inflammation in the renal tissue

The serum levels of blood urea nitrogen (BUN) and serum creatinine (Scr) and the levels of superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione (GSH) in the rat renal tissue were measured with the Clinical Automatic Biochemistry Analyzer 7600 (Hitachi, Japan) employing a kit provided by Roche Diagnostics (Mannheim, Germany). The levels of interleukin-10 (IL-10), IL-6, and nitric oxide (NO) in the rat renal tissue were measured by the double antibody sandwich enzyme-linked immunosorbent assay (ELISA) kits (Roche) according to the manufacturer's instructions.

Histopathological evaluation of renal damages

The left renal tissue was fixed in 10% neutral formalin solution, paraffin-embedded, sliced into 4- μ m sections, stained with hematoxylin-eosin (HE) staining. One whole deep coronal section was examined under the optical microscope (Leica, Germany) 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 renal tissue was graded using the following parameters: tubular cell necrosis, apoptosis, 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 [9, 30].

Detection of ultrastructural changes of renal proximal tubular endothelial cells

The renal cortical tissue was sliced into small size (1 × 1 × 1 mm), fixed in 4% (v/v) glutaraldehyde at room temperature overnight, and then post-fixed in 1% (v/v) osmium tetroxide for 2 h. The fixed tissue was dehydrated using ascending grades of ethanol, epoxy resin-embedded, sliced into 60-70 nm ultrathin sections, placed on 300 mesh copper grids covered by carbon formvar, stained with uranyl acetate and lead citrate, and observed under transmission electron microscopy (TEM) (Hitachi, Japan) operating at 80 kV. The section was assessed by an electron microscopy technician who was blinded to the treatments.

Calcium determination in the renal tissue

The renal cortical interstitial tissue was severed and thoroughly rinsed off the residual blood with ice-cold ultrapure water produced by a Milli-QRG water purification system (Millipore, France). The kidney proximal tubule was mechanically isolated under a microscope. Further, the samples (ca. 200 mg) were placed directly in the decontaminated microwave digestion vessels. Next, they were digested with 3.0 mL of concentrated (65 % w/w) HNO₃ and 1.0 mL of (30% v/v) H₂O₂ solution (Yongda, Tianjin, China) in an intelligence microwave digestion/extraction instrument (Xintuo, Shanghai, China), under a pressure (MPa)/time (min) program: 20/1, 0/2, 10/2, 20/2, and 10/3. After cooling, the mixtures were made up to 10 mL with ultrapure water and stored in closed propylene tubes at 4 °C for use. Ca quantifications were performed by inductively coupled plasma atomic emission spectrophotometry (ICP-AES) (Bohui Innovation Technology, Beijing, China) as described previously [31-33]. Briefly, the aliquots of the mixtures were mineralized with nitric/sulfuric/perchloric acid (3:1:1, v/v) in closed Teflon vessels in order to eliminate

interference from the organic matrixes. The achromatous and homogeneous diluted digestions, which were diluted with ultrapure water to reduce the nitric acid level, were nebulized in argon plasma. The reference wavelength was 193.091 nm (the atomic line of carbon emission) for monitoring the remaining undigested organic materials.

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). The primers sequences were shown in Table 1. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to the expression of GAPDH.

Western blot analysis

The renal tissue homogenate was electrophoretically separated in 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Aspecific binding of antibodies was blocked using 5% skimmed milk in Tris-buffered saline-0.1% Tween-20 (TBST) for 1 hour. The membrane was incubated with primary antibodies (against active CaSR, calcium-modulated protein (calmodulin, CaM), and neutrophil cytosol factor 1 (p47phox)) overnight at 4°C and subsequently with the alkaline phosphatase-conjugated secondary antibodies. The protein level was normalized with respect to β -actin band density. The antigen-antibody product was measured by Thermo Scientific Super Signal West Pico Chemiluminescent Substrate (Thermo, USA) and analyzed with a Fluor Chem system (Alpha, USA).

Statistical analysis

All experiments were repeated in triplicate. Statistical analysis was carried out using SPSS (v19.0) software, and the processed data were reported in the form of mean $\pm$ standard deviation (SD). Mean comparison between multiple groups was realized using single-factor analysis of variance (ANOVA) with posthoc Dunn's multiple comparison tests. A $p < 0.05$ or 0.01 was considered to represent statistical significance.

Results

Changes of the renal function in different groups

Compared with DM group, the serum levels of BUN and Scr were significantly increased in I/R group. They were further elevated by R-568 pretreatment, and decreased by NPS-2143 preconditioning ($p < 0.01$) (Fig. 1). These results suggest that R-568 and NPS-2143 may regulate I/R-induced renal damage in diabetic rats.

Pathological alterations in the kidneys of model rats viewed by light microscopy and TEM

Under the optical microscope, several hemorrhagic lesions were observed. The renal proximal tubules showed varying degrees of vacuolar degeneration and apoptosis in I/R group. These histopathological alterations were markedly increased in R-568 group (sporadic necrosis of epithelial cells and infiltration of inflammatory cells) and reduced in NPS-2143 group (Fig. 1). We detected ultrastructural abnormalities in proximal tubular endothelial cells of

Table 1. The primer used for qRT-PCR

Primer name	Primer sequence
casr-F	5'-GTCTGAAGGCTGGGCAGATT-3'
casr-R	5'-TTCTCACGAAGGTGTCCACG-3'
p47phox-F	5'-GGCACAAAGGGTGAGCG-3'
p47phox-R	5'-CGCGGAGTCGATGGATTGTC-3'
calm-F	5'-CCTGGTGTGCTAACCCTCT-3'
calm-R	5'-TTCCAGGGTCGCACATCTTC-3'
gapdh-F	5'-GGTGAAGGTCGGAGTCAACG-3'
gapdh-R	5'-CAAAGTTGTTCATGGATGACC-3'

the kidneys in diabetic RI/RI rats by TEM, such as swelling of the cytoplasm, endoplasmic reticulum changes, swelling of the mitochondria with the loss of cristae, nuclear chromatin margination, and karyopyknosis. The chromatin margination, and mitochondrion swell and vacuole change were observed in I/R group. The ultrastructural damages were aggravated by the agonist of CaSR R-568 pretreatment and limited by the antagonist of CaSR NPS-2143 preconditioning (Fig. 1). These abnormalities suggest that the tubular endothelial cells undergo a stage of asphyxia and start dying from the dead mitochondria and nuclei after I/R treatment. CaSR may exert a critical role in this pathological process.

SOD activity and the levels of MDA and GSH in the renal tissue

To evaluate the role of CaSR in renal I/R-induced oxidative stress in diabetic rats, SOD activity and the levels of MDA and GSH were detected in the renal tissue. As shown in Fig. 2, as compared with I/R group, SOD activity and GSH level in R-568 group and MDA level in NPS-2143 group were both significantly decreased, while SOD activity and GSH level in NPS-2143 group and MDA level in R-568 group were significantly increased ($p < 0.01$). The results indicate that CaSR participates in oxidative stress of diabetic RI/RI, which can be modulated by its regulators.

The mRNA and protein expression of CaSR in the renal tissue

CaSR is substantially expressed within the renal tissue and has been implicated in a variety of functions in kidneys. In our study, the expression of CaSR in the renal tissue was detected by qRT-PCR and Western blot. The expressions of CaSR in I/R and R-568 preconditioning groups were up-regulated compared to DM group. NPS-2143 could inhibit the expressions ($p < 0.01$) (Fig. 3). The results show that the expression of CaSR is increased in diabetic RI/RI, and R-568 can up-regulate the expression of CaSR, while NPS-2143 can down-regulate the expression of CaSR.

Fig. 1. Histopathological and ultrastructure alterations of kidneys and renal function. Note: Under the light microscope (HE, $\times 200$), the renal proximal tubules show vacuolar degeneration and apoptosis in I/R group (arrow), while these alterations were markedly increased in R-568 group (arrow) and reduced in NPS-2143 group. Under the TEM (the scale bar is 2 μ m), The nuclear chromatin margination (arrow) are observed in I/R group. The damages are aggravated by the agonist of CaSR R-568 pretreatment (karyopyknosis, arrow) and limited by the antagonist of CaSR NPS-2143 preconditioning. (A) Injury scores of the kidney; (B) Renal function. * $p < 0.001$ vs. DM group; ** $p < 0.01$ vs. I/R group.

Fig. 2. Antioxidant activation of the renal tissue. Note: * $p < 0.001$ vs. DM group; ** $p < 0.01$ vs. I/R group.

Fig. 3. The expression of CaSR and Ca level in the renal tissue. Note: (A) Protein expression of CaSR, (B) Relative mRNA expression of CaSR, (C) Ca level, (D) Protein expression of CaM and p47phox, (E) Relative mRNA expression of CaM and p47phox. * $p < 0.001$ vs. DM group; ** $p < 0.01$ vs. I/R group.

Ca level in the renal tissue

Ca^{2+} plays an important role in regulating nuclear functions, including cell growth and death. Results showed that Ca level was increased significantly in I/R (160.47 ± 15.24 nM) and R-568 groups (187.82 ± 18.76 nM) as compared with that in DM group (82.59 ± 8.48 nM) ($p < 0.01$). Ca concentration was decreased significantly in NPS-2143 group (143.19 ± 11.27 nM) as compared with that in I/R group ($p < 0.01$) (Fig. 3). These results indicate that diabetic I/R-induced renal injury can up-regulate CaSR expression that triggers a feedforward signaling mechanism in which elevates Ca level.

The mRNA and protein expressions of CaM and p47phox in the renal tissue

CaM, a multifunctional intermediate calcium-binding messenger protein, acts as part of a calcium signal transduction pathway by modifying its interactions with various target proteins such as kinases or phosphatases [34]. p47phox is vital to the activation of NADPH oxidase for producing superoxide anion [35]. We found that the mRNA expressions of CaM and p47phox were up-regulated by 4.48- to 8.02-fold after I/R and R-568 preconditioning. NPS-2143 could inhibit the mRNA expressions of them ($p < 0.01$). Correspondingly, the protein expressions of CaM and p47phox were increased significantly in I/R group and R-568 preconditioning group as compared with those in DM group ($p < 0.01$). They were decreased significantly in NPS-2143 preconditioning group ($p < 0.01$) (Fig. 3).

Fig. 4. The levels of IL-10, IL-6, and NO in the renal tissue. Note: (A) IL-10, (B) IL-6, and (C) NO. * $p < 0.001$ vs. DM group; ** $p < 0.01$ vs. I/R group.

The levels of IL-10, IL-6, and NO in the renal tissue

NO is biosynthesized endogenously from L-arginine, oxygen, and NADPH by various NOS enzymes, playing a role in a variety of biological processes [36]. To evaluate the inflammatory reactions, the levels of IL-10 (anti-inflammatory factor) and IL-6 (pro-inflammatory factor) in the renal tissue were also measured. The mean levels of IL-10, IL-6, and NO were significantly increased in I/R group as compared with those in DM group ($p < 0.01$). Preconditioning with the agonist of CaSR R-568 further enhanced significantly the levels of IL-6 and NO, and decreased markedly IL-10 level ($p < 0.01$) (Fig. 4). These results indicate that CaSR acts a vital role in diabetic RI/RI, which involves in the increase of $[Ca^{2+}]$, oxidative stress, inflammation, and apoptosis.

Discussion

It is well documented that the clinical mortality in diabetic patients with acute kidney injury (AKI) is often increased as a result of multi-organ dysfunction and progression of MOF [32, 37, 38]. To explore the underlying molecular mechanisms on diabetic RI/RI, the establishment of a reliable animal model is critical. In the present study, single dose of STZ-induced diabetes mellitus offered a very cost-effective and expeditious technique. The surveillance of serum glucose levels, impaired renal function, and pathomorphological injuries indicated the successful establishment of the diabetic RI/RI model.

In the current study, we established a diabetic RI/RI model to assess the renal function, knowing that this model mimics the pathological conditions observed in humans where blood supply of both kidneys was normally reduced [39-41]. In recent years, considerable circumstantial evidence has indicated a role for diabetes-induced oxidative stress, which is routinely accompanied by diabetic renal I/R-induced renal damages. Oxidative stress contributes to increased cellular damage and death through protein oxidation, DNA damage, and peroxidation of membrane lipids [33]. High MDA and protein carbonyl levels are two major indices for oxidative stress [42]. It was found in our study that the activity of SOD and GSH level were decreased while the MDA level was increased in the renal tissue in diabetic RI/RI, demonstrating an oxidative injury. During the process of I/R injury, inflammatory reactions are activated, resulting in the formation of inflammatory cytokines, such as IL-6 and IL-10 [9, 27, 43, 44]. We also found that the expressions of IL-10 and IL-6 in the renal tissue were elevated in this diabetic RI/RI model. These results confirmed the inflammatory injury in kidneys due to renal I/R. Neutrophils are known as major cells for ROS production, and play a role in oxidative injury via the action of NADPH oxidase [5, 45, 46]. p47phox, an NADPH oxidase subunit, was significantly up-regulated in the present model. Another probable mechanism involved in ROS production might be associated with the NO system. It was found that DM could increase the activity and expression of renal and myocardial iNOS in STZ-induced diabetic rats, and NO level was elevated in the diabetic kidney in the early stage [30, 47]. CaM and Ca^{2+} can activate the NOS in the cytoplasm, which can catalyze the formation of NO. Likewise, we observed that NO level was increased in the renal tissue, as

accompanied by the increased Ca level and CaM expression, suggesting a disruption in nitro-redox homeostasis.

The overexpression of CaSR protein resulted in disturbance of calcium homeostasis, induced the alteration of a series of downstream signaling pathways, or directly injured cellular structure through mitochondrial and nuclear dysfunction. The results of HE staining and TEM showed varying degrees of vacuolar degeneration, mitochondrion swell, and apoptosis in renal proximal tubular endothelial cells of I/R group were markedly increased. In this study, R-568 and NPS-2143 (specific activator and inhibitor of CaSR) were used to investigate the role of CaSR in diabetic RI/RI. The results showed that CaSR and CaM were up-regulated, and calcium level was increased in the renal tissue during diabetic RI/RI. In addition, R-568 could significantly enhance levels of BUN, Scr, MDA, NO, and IL-6, and reduce SOD activity and the levels of GSH and IL-10, which may explain why the addition of R-568 increased the injury associated with renal dysfunction, lipid peroxidation, inflammatory response, nitroso-redox imbalance, and pathomorphological injury. The finding in this study that calcium level in R-568 group was significantly higher than that in DM and I/R groups indicates that R-568 activated CaSR, thus increasing Ca level and aggravating diabetic RI/RI. CaM, a key calcium receptor, is the main signal transduction molecule of the intracellular calcium-signaling pathway and mediates regulation of a series of physiological and biochemical reactions caused by Ca^{2+} . CaM usually regulates cell activities via Ca^{2+} /CaM compounds [48]. Excessively high endocellular calcium level will enhance CaM activity to regulate various metabolic enzymes via activating Ca^{2+} /CaM, such as NOS, causing cell metabolic disorders injuries [49]. In the current study, we found that CaSR agonist could further up-regulate the expression of CaM due to the increased intracellular calcium concentration in diabetic RI/RI, and aggravate the renal tissue damage.

This study has limitations. First, the study period of diabetic RI/RI model was only 3 days; therefore, the long-term effect of CaSR on diabetic renal I/R injury remains uncertain. Second, although plentiful data support that the overexpression of CaSR may regulate the level of calcium, causing inflammatory cascade, reactive oxygen species (ROS), abnormal lipid metabolism, and nitroso-redox imbalance from diabetic I/R renal tissues that may act indirectly in redox signaling to turn on apoptosis, the exact underlying mechanisms is still not completely clear in the present study.

Conclusion

The present study demonstrates that activation of CaSR could directly cause elevated calcium levels during diabetic RI/RI, inducing lipid peroxidation, inflammatory response, nitroso-redox imbalance, and triggering damage. CaSR plays a pivotal role in the diabetic RI/RI.

Disclosure Statement

The authors report no conflicts of interest.

Acknowledgements

The authors greatly appreciate the editors and the anonymous peer reviewers for their critical reading and insightful comments, which have improved our manuscript substantially. The current study was supported by grants from the Science and Technology Planning Project of Jiaying (2017AY33056, 2017AY33076), the Science and Technology Planning Project (Laboratory Animal Project) of Zhejiang Province (2015C37130). The sponsors of the study

were not involved in study design, data collection, data analysis, data interpretation, or manuscript written.

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