The calcium-sensing receptor participates in testicular damage in streptozotocin-induced diabetic rats

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Male infertility caused by testicular damage is one of the complications of diabetes mellitus. The calcium-sensing receptor (CaSR) is expressed in testicular tissues and plays a pivotal role in calcium homeostasis by activating cellular signaling pathways, but its role in testicular damage induced by diabetes remains unclear. A diabetic model was established by a single intraperitoneal injection of streptozotocin (STZ, 40 mg kg⁻¹) in Wistar rats. Animals then received GdCl₄ (an agonist of CaSR, 8.67 mg kg⁻¹), NPS-2390 (an antagonist of CaSR, 0.20 g kg⁻¹), or a combination of both 2 months after STZ injection. Diabetic rats had significantly lower testes weights and serum levels of testosterone compared to healthy rats, indicating testicular damage and dysfunction in STZ-induced diabetic rats. Compared with healthy controls, the testicular tissues of diabetic rats overexpressed the CaSR protein and had higher levels of malondialdehyde (MDA), lower superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity, and higher numbers of apoptotic germ cells. The testicular tissues from diabetic rats also expressed lower levels of Bcl-2 and higher levels of Bax and cleaved caspase-3 in addition to higher phosphorylation rates of c-Jun NH₂-terminal protein kinase (JNK), p38, and extracellular signaling-regulated kinase (ERK) 1/2. The above parameters could be further increased or aggravated by the administration of GdCl₄, but could be attenuated by injection of NPS-2390. In conclusion, the present results indicate that CaSR activation participates in diabetes-induced testicular damage, implying CaSR may be a potential target for protective strategies against diabetes-induced testicular damage and could help to prevent infertility in diabetic men.

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INTRODUCTION

Diabetes mellitus is a prominent public health problem and is rapidly increasing in prevalence worldwide.¹ Although the adverse effects of diabetes on male fertility have long been controversial, it is now accepted that diabetes can cause testicular damage by inducing various subtle molecular changes that are important for sperm quality and function; thus, diabetes is considered to be one of the causes of male infertility.²,³ However, the mechanism underlying the diabetes-induced testicular damage remains unclear. Oxidative stress and apoptosis of germ cells are two major events involved in testicular damage induced by diabetes,⁴,⁵ and calcium homeostasis contributes to these mechanisms.⁶

The calcium-sensing receptor (CaSR), a member of the G-protein coupled receptors family, plays a pivotal role in disorders of calcium homeostasis by activating many signaling pathways.⁷ Pancreatic insulin secreting β-cells express extracellular CaSR, and the activation of CaSR leads to a transient increase in insulin secretion followed by the inhibition of basal and nutrient-stimulated insulin secretion at supra-physiological levels of Ca²⁺.⁸ L-histidine-induced CaSR activation inhibits glucose-induced insulin secretion in β-cells through spatial rearrangement of the CaSR and L-type voltage-dependent calcium channels⁹ indicating that CaSR may participate in the pathogenesis of diabetes.

CaSR has been found in the parathyroid,¹⁰ heart,¹¹ kidney,¹² and bone.¹³ Functional CaSR has also been found to be expressed in testicular tissues and sperm cells, which can be activated by calcimimetics. Localization of CaSR is an important contributor to the regulation of cell proliferation and apoptosis.¹⁴ It was recently reported that CaSR activation plays a critical role in the apoptosis of cardiomyocytes in diabetes-induced cardiac injury in rats by inducing calcium overload and subsequent activation of mitochondrial and mitogen-activated protein kinase (MAPK) pathways.¹⁵ Therefore, we conducted the present study to investigate whether CaSR activation participates in diabetes-induced testicular damage using experimental streptozotocin-induced diabetic rats.

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MATERIALS AND METHODS

Experimental animals
Fifty-eight male Wistar rats weighing between 200 and 220 g were supplied by the Experimental Animal Center of Harbin Medical University, China. The animals were given free access to rodent chow and water under humane care and were housed in special polycarbonate cages in a controlled environment (22–24°C, 55%–70% humidity, 12 h light/12 h dark cycle). All experimental protocols were performed in accordance with the guidelines of the Experimental Animal Ethic Committee of Harbin Medical University, China (Animal Experimental Ethical Inspection Protocol No. 2009104).

Antibodies and reagents
The antibodies (Abs) against CaSR, extracellular signaling-regulated kinase (ERK), JNK, and p38 were obtained from Cell Signaling Technology (Shanghai, China). The Abs against caspase-3, Bcl-2, Bax, and GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Gadolinium (III) chloride (GdCl₃, an agonist of CaSR), STZ, and NPS-2390 (an antagonist of CaSR) were from Sigma-Aldrich (Beijing, China).

Animal experiments
Forty rats were given a single intraperitoneal injection of 40 mg kg⁻¹ STZ which was dissolved in fresh citrate buffer (pH 4.5) at a concentration of 0.1 mol L⁻¹. The fasting blood glucose levels were monitored by a blood glucose analyzer (OneTouch Ultra, Johnson and Johnson, China) 3 days after STZ injection. Thirty rats with blood glucose levels no <16.7 mmol L⁻¹ measured three consecutive times were considered diabetic. We used 30 diabetic rats and 18 healthy rats in the study.

Two months after STZ injection, six diabetic rats, and six healthy rats were euthanized, bilateral testes were removed and weighed, and the sera was harvested as described below. The remaining diabetic rats were randomly assigned into four groups (n = 6): untreated, GdCl₃, NPS-2390, and GdCl₃ + NPS-2390. The diabetic rats in the untreated group received daily intraperitoneal injections of 1 ml of citrate buffer. GdCl₃ or NPS-2390 was intraperitoneally injected daily at a dose of 8.67 mg kg⁻¹ or 0.20 g kg⁻¹, in the GdCl₃ or NPS-2390 groups, respectively. In the GdCl₃ + NPS-2390 group, NPS-2390 was given 2 h prior to the GdCl₃ injection. Two groups (n = 6) of healthy rats were given daily intraperitoneal injections of either 1 ml of citrate buffer or GdCl₃, at a dose of 8.67 mg kg⁻¹. One week later, rats were euthanized by intraperitoneal injection of an overdose of pentobarbital sodium (60 mg kg⁻¹). Blood samples were collected from the vena cava, and sera were harvested and kept at ~ 80°C. Bilateral testes were removed rapidly, weighed, and then frozen and stored in liquid nitrogen.

Measurement of serum testosterone
Serum levels of testosterone were measured using a testosterone ELISA Kit (ab108666) provided by Abcam Trading Co., Ltd., (Shanghai, China) according to the manufacturer’s instruction.

Measurements of MDA, SOD, and GSH-Px in testicular tissues
The level of malondialdehyde (MDA) and the enzymatic activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were measured using commercial kits from Nanjing Jiancheng Bioengineering Institute, China. Briefly, testicular tissues were weighed and homogenized in ice-cold phosphate buffered saline. The protein content of testicular homogenates was measured using a Coomassie Protein Assay Kit (BioWorld Technology, Inc., Nanjing, China). The level of MDA was estimated by evaluating the thioarbituric acid (TBA) reacting substances at the wavelength of 532 nm. The activity of SOD was quantified as the rate of inhibition of nucleotide oxidation at a wavelength of 412 nm. The activity of GSH was determined by the ratio of the reduced glutathione to the oxidized glutathione at a wavelength of 550 nm. The results for MDA, SOD, and GSH-Px were defined as nmol mg⁻¹ protein, U mg⁻¹ protein, and U mg⁻¹ protein, respectively. The experiments were repeated 3 times.

Measurement of apoptotic germ cells in the testes
Testicular sections (5 μm) were prepared and stained with the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) (Roche, Shanghai, China). The nuclei were counterstained by hematoxylin. The stained sections were examined by light microscopy. The nuclei of TUNEL-positive apoptotic germ cells were stained brown, and the nuclei of normal germ cells were blue. The TUNEL-positive germ cells were counted in a double-blind manner and 10 seminiferous tubule cross-sections per slide were randomly selected. The apoptosis index was calculated by dividing the numbers of positive staining nuclei by the total number of nuclei in the cells.

Western blot analysis
Testicular tissues were homogenized in protein lystate buffer, and total protein was evaluated as above. Homogenates were resolved on polyacrylamide SDS gels and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dried milk for 2 h, incubated overnight at 4°C with primary Abs, and then subsequently incubated with alkaline phosphatase-conjugated secondary Abs. They were developed by 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Tiangen Biotech Co., Ltd., Beijing, China). The blots were measured by the gray analysis with Quantity One software (Bio-Rad Laboratories, Shanghai, China).

Statistical analysis
All data were presented as the mean ± standard error of mean (s.e.m.). Comparisons were performed with a one-way analysis of variance followed by Student-Newman-Keuls test using an SPSS (Statistical Package for the Social Sciences) 18.0 software (IBM Corporation, NY, USA). P < 0.05 was considered to be statistically significant.

RESULTS

Damaged testicular tissues express higher levels of the calcium-sensing receptor in diabetic rats
Diabetic rats had significantly lower body weights (Figure 1a) and testicular weights (Figure 1b) 2 months after STZ injection compared to healthy rats. Serum levels of glucose were significantly higher in diabetic rats compared to healthy rats (Figure 1c), but serum levels of testosterone were significantly lower in diabetic rats compared to healthy rats (Figure 1d). The above results indicate that STZ-induced diabetes was successfully established in rats as evidenced by testicular damage and dysfunction. We next demonstrated that the level of CaSR protein expression was significantly higher in testicular tissues from diabetic rats compared to healthy rats (Figure 1e), indicating that CaSR may participate in the testicular damage in STZ-induced diabetic rats.

CaSR affects lipid peroxidation and antioxidant activity in testicular tissues
We investigated whether the activity of CaSR was associated with the level of MDA and the activities of SOD and GSH-Px, which are key factors involved in testicular damage induced by STZ in diabetic rats. As shown in Figure 2a–2c, the level of MDA (Figure 2a) was significantly higher in testicular tissues of diabetic rats than that
of healthy rats, while the testicular activities of SOD (Figure 2b) and GSH-Px (Figure 2c) were significantly lower in diabetic rats compared to healthy rats. Administration of GdCl$_3$, an agonist of CaSR, further increased the level of MDA and reduced the activities of SOD and GSH-Px. However, injection of NPS-2390, an antagonist of CaSR, reduced the level of MDA and increased the activities of SOD and GSH-Px.

CaSR affects germ cell apoptosis in testicular tissues
Injection of STZ led to the apoptosis of germ cells in diabetic rats, thus contributing to the testicular dysfunction. Here, we demonstrated that there were more apoptotic germ cells in testicular tissues from diabetic rats compared to healthy controls (Figure 3a and 3b). Administration of GdCl$_3$, further increased the apoptotic index, while injection of NPS-2390 reduced the apoptotic index and also attenuated the increased apoptotic index induced by GdCl$_3$ in diabetic rats (Figure 3b–3g). Administration of GdCl$_3$ increased the apoptotic index in healthy rats (Figure 3a, 3f, and 3g).

CaSR affects the expression of Bcl-2, Bax, and caspase-3 proteins in testicular tissues of diabetic rats
We further investigated the alteration of apoptosis-related proteins by Western blotting analysis of testicular homogenates with Abs against rat Bcl-2, Bax, and cleaved caspase-3. The expression of Bcl-2 was downregulated in testicular tissues from diabetic rats compared to healthy controls (Figure 4a). The expression of Bax (Figure 4b) and cleaved caspase-3 (Figure 4c) was upregulated in testicular tissues from diabetic rats compared to healthy controls. Administration of GdCl$_3$ further reduced the expression of Bcl-2 (Figure 4a) and increased the expression of Bax (Figure 4b) and cleaved caspase-3 (Figure 4c) in testicular tissues from diabetic rats. However, simultaneous injection of NPS-2390 attenuated the alterations in Bcl-2, Bax, and cleaved caspase-3 expression induced by GdCl$_3$ in diabetic rats (Figure 4a–4c).

CaSR affects the phosphorylation of JNK, p38, and ERK proteins in testicular tissues of diabetic rats
c-Jun NH$_2$-terminal protein kinase (JNK), p38, and extracellular
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signal-regulated protein kinase (ERK) act as vital regulators of cell apoptosis and proliferation signaling pathways. Therefore, we investigated the phosphorylation of these proteins by Western blotting analysis. The phosphorylation rates of JNK (Figure 5a), p38 (Figure 5b) and ERK (Figure 5c) were all significantly increased in testicular tissues from diabetic rats compared to healthy controls. Administration of GdCl₃ further increased their phosphorylation, while simultaneous injection of NPS-2390 attenuated the increased phosphorylation of these proteins induced by GdCl₃ in diabetic rats (Figure 5a–5c).

**DISCUSSION**

STZ is a well-known genotoxic agent and a potential source of oxidative stress that has been widely used to induce diabetes in experimental animals by causing damage or dysfunction in pancreatic insulin secreting β-cells. The number of germ cells and the ratio of germ cells/supporting cells are both reduced in the testes of diabetic rats. Oxidative stress, DNA damage, and mitochondrial dysfunction are involved in the damage to reproductive tissues in streptozotocin-induced diabetes. Accordingly, the present study has demonstrated that testicular tissues from STZ-injected rats had higher MDA content and lower SOD and GSH-Px activity. MDA is a product of lipid peroxidation and is used to measure the damage caused by free radicals in diabetic rats. SOD and GSH-Px have antioxidant properties against oxidative stress that help maintain the viable reproductive ability and protect testicular tissues from the damage caused by reactive oxygen species. Several antioxidants have been used to prevent testicular damage in diabetic animals.

Following the cloning and characterization of CaSR as an extracellular Ca²⁺-sensing receptor in 1993, it has become clear that CaSR has immense functional versatility by activating several different signaling pathways. CaSR plays crucial roles in both calcium homeostasis and calcium-independent disorders including diabetes and infertility. CaSR agonists, antagonists, and other drugs mediated through CaSR have been used in the management of many disorders. For the first time, the present study demonstrated that CaSR was upregulated in testicular tissues in diabetic rats and its activation is closely associated with testicular damage. Specific activation of CaSR by GdCl₃ aggravated, and specific inhibition of CaSR by NPS-2390 attenuated, testicular damage caused by STZ-induced diabetes as evidenced by alterations of oxidative markers, apoptosis of germ cells, and activation of the mitochondrial apoptotic pathways.

It is known that diabetes causes testicular dysfunction by inducing the apoptotic death of germ cells. Here, we could also show that STZ-induced apoptosis of germ cells by activating the mitochondrial pathway in accordance with previous reports. The mitochondrial pathway is a classic apoptotic pathway during which the permeability of the outer mitochondrial membrane increases resulting in the opening of the permeability transition pore causing the release of apoptogenic molecules from the intermembrane space into the cytoplasm. The opening of the permeability transition pore is regulated by Bcl-2 family members, which consist of antiapoptotic proteins like Bcl-2 and pro-apoptotic proteins like Bax. The main function of Bcl-2 is to stabilize mitochondrial membrane potential and inhibit Cyt-c release and caspase activation, while Bax plays a role that opposes Bcl-2.

The MAPK pathway is involved in maintaining normal spermatogenesis, ERK, JNK, and p38-MAPK, key members of the MAPK superfamily, play major roles in cell apoptosis and survival. The JNK-p38-MAPK pathway is closely associated with Bcl-2 expression and the caspase cascade in spermatogenesis, while the ERK signal pathway plays an important role in the activation of cellular processes involved in spermatogenesis including testicular heat shock and testicular torsion. Here, we have further confirmed that JNK, p38-MAPK, and ERK could be activated by CaSR in diabetic tissues from diabetic rats, in accordance with previous studies. Specific activation of CaSR by GdCl₃ further enhanced, while specific inhibition of GdCl₃ by NPS-2390 reduced the phosphorylation of the above three molecules. The results indicate that CaSR acts as an enhancer in the activation of the MAPK pathway.
In summary, the present study for the first time has demonstrated that CaSR, a well-known calcium-homeostasis regulator, participates in testicular damage in STZ-induced diabetes in rats. Diabetic rats had significantly lower testes weights and serum levels of testosterone compared to healthy controls. The testicular tissues of diabetic rats expressed higher levels of CaSR and had higher MDA content while having lower activities of SOD and GSH-Px and higher numbers of apoptotic germ cells. The diabetic testicular tissues also expressed lower levels of Bcl-2 and higher levels of Bax and cleaved caspase-3, which were associated with higher phosphorylation rates of JNK, p38, and ERK1/2. The above parameters could be further increased or aggravated by the administration of GdCl$_3$, a specific activator of CaSR, and attenuated by NPS-2390, a specific inhibitor of CaSR. There are indeed several drawbacks of the present study, and further investigations should be conducted in the future. For instance, we did not measure levels of LH (luteinizing hormone) and FSH (follicle-stimulating hormone), which have been demonstrated to have an impact on spermatogonial development and are involved in testicular function in insulin-dependent diabetes. More agonists or antagonists of CaSR should be applied for further investigation of the role of CaSR in diabetes-induced testicular damage. However, the preliminary results at least imply that CaSR may be a potential target for designing preventive or therapeutic strategies for protecting the testes from diabetes caused damage, thus preventing infertility in diabetic men.

COMPETING INTERESTS
The authors declare that they have no competing interests.

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