Streptozotocin-Induced Diabetes Increases Apoptosis through JNK Phosphorylation and Bax Activation in Rat Testes

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ABSTRACT. Diabetic disease is known to suppress male reproductive activity in laboratory animals and humans. The present study was designed to evaluate whether streptozotocin-induced diabetes increases apoptotic cell death in rat testes through activation of the JNK and Bax pathway. Diabetes was induced by a single intravenous injection of streptozotocin (40 mg/kg) and testis samples were collected after 3 months. Compared with controls, body weight and testicular weight were lower in the diabetic group, and the apoptotic index in testicular germ cells was significantly increased. Expression of phospho-JNK and Bax was significantly increased in the diabetic group, and the level of activated caspase-3 was also increased, compared to that of controls. Our findings suggest that streptozotocin-induced diabetes increases apoptotic cell death in rat testes through phosphorylation of JNK and activation of Bax.

KEY WORDS: Bax, diabetic, Testes.

Sexual dysfunction is frequently associated with diabetes in men and experimental animals, and it is accepted that infertility is a common complication in diabetic men [2, 5, 10, 16]. Diabetic rats exhibit decreased testicular weight, sperm count, sperm motility and testosterone levels, and increased frequency of abnormal spermatogenesis [6, 12, 13, 17]. Furthermore, diabetes increases apoptosis in testicular germ cells and causes dysfunction of testes in mice [1, 11].

The c-Jun NH2-terminal kinase (JNK) is a classic stress-activated protein kinase involved in apoptotic signal transduction in response to various stimuli, including UV radiation and heat [3]. JNK is a critical factor involved in the release of mitochondrial pro-apoptotic molecules such as cytochrome c in response to apoptotic stimuli [19]. Activation of JNK plays an essential role in apoptosis that is mediated by promoting accumulation of active Bax at the mitochondria [7, 19]. This stimulates the release of cytochrome c, which in turn triggers activation of the caspase cascade and induces apoptotic cell death [4, 7, 19]. Previous studies demonstrated that diabetes increases apoptotic cell death in testicular germ cells [1, 11]. However, few data are available regarding expression of apoptosis-related proteins in testes of diabetic animals. We propose that streptozotocin-induced diabetic rats enhance apoptosis in testis through the regulation of the apoptosis-related proteins including JNK, bax, and caspase-3. Therefore, the present study was performed to provide this information.

Adult male Sprague-Dawley rats (320–340 g, n=30) were purchased from Samtako Co. (Animal Breeding Center, Korea), randomly divided into control group and diabetic group (n=15 per group). All animal experiments were performed in accordance with the NIH Guidelines for Use of Laboratory Animals. Animals were maintained under controlled temperature (25°C) and lighting (14L:10D), and allowed free access to food and water. Diabetes was induced by a single intravenous injection of streptozotocin (STZ, Sigma, St. Louis, MO, U.S.A.) in 0.1 M citrate buffer (pH 4.0) at a dose of 40 mg/kg body weight [12]. The control animals received an equal volume of citrate buffer. Blood glucose and body weight levels were monitored regularly. Diabetes was confirmed by measurement of blood glucose concentrations and defined as blood glucose above 300 mg/dl. Animals from each group were sacrificed after 3 months. Testes were removed and rapidly weighed. And then, the right testes were frozen in liquid nitrogen and were stored at −70°C until use. The left testes were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) and embedded in paraffin.

TUNEL histochemistry was performed using the DNA Fragmentation Detection Kit (Oncogene Research Products, Cambrige, MA, U.S.A.). Sections were incubated proteinase K (20 µg/ml) at room temperature for 5 min and then washed in PBS. Sections were incubated with a mixture of terminal deoxynucleotidyl transferase and digoxigenin-labelled dideoxy nucleotide in a humidified chamber at 37°C for 1 hr. Immuno-staining was carried out according to the manufacture’s protocol. The color reaction was performed with DAB (Sigma, St. Louis, MO, U.S.A.) solution with 0.03% hydrogen peroxidase for 3 min. Sections were counterstained with hematoxylin. Slides were observed under microscope, and then photographed. To quantify the incidence of apoptosis, the seminiferous tubules containing three or more apoptotic cells by TUNEL stain were calculated. The apoptosis index was calculated by the ratio of the positive seminiferous tubules of apoptosis to the total number.
number of seminiferous tubules in cross sections.

Tissues samples were homogenized in lysis buffer (1% Triton X-100, 1 mM EDTA in PBS) containing 10 μM leupeptin and 200 μM phenylmethylsulfonyl fluoride. The protein concentration of each lysate was determined using the bicinchoninic acid kit (Pierce, Rockford, IL, U.S.A.) according to the manufacturer’s protocol. Thirty micrograms of total protein was applied to each lane on 10% SDS-polyacrylamide gels. After gel electrophoresis and immunoblotting, the poly-vinylidene fluoride membranes (Millipore, Billerica, MA, U.S.A.) were washed in Tris-buffered saline containing 0.1% Tween-20 and then incubated with anti-phospho-JNK (pJNK), anti-Bax, anti-caspase-3 antibodies (diluted 1:1000, Cell Signaling, Beverly, MA, U.S.A.) as primary antibody. And the membranes were incubated with the secondary antibody (1:5,000, Pierce) and the ECL Western blot analysis system (Amer sham Pharmacia Biotech, Piscataway, NJ, U.S.A.) according to the manufacturer’s protocol was used for detection. The intensity analysis was carried out using SigmaGel 1.0 (Jandel Scientific, San Rafael, CA, U.S.A.).

All data are expressed as mean ± S.E.M. The results in each group were compared by unpaired Student’s t-test. The difference for comparison was considered significant at *P<0.05.

In streptozotocin-induced diabetic rats, blood glucose levels were markedly increased compared with controls, and body and testicular weight was significantly lower (Table 1). Apoptotic cells in testes of control and diabetic rats were identified by TUNEL staining. Only a few TUNEL-positive cells were observed in control animals (Fig. 1A). However, the number and signal density of positive cells significantly increased in diabetic rats (Fig. 1B). TUNEL-positive cells were detected in spermatogonia and spermatocytes. The apoptotic index was significantly increased in the diabetic group, compared to that of controls (Fig. 1C). The apoptotic indices were 3.4 ± 0.7% and 31.5 ± 6.8% in control and diabetic rats, respectively.

Western blot analysis showed that expression of pJNK was significantly increased in testes of diabetic rats compared with controls. The level of pJNK was 0.82 ± 0.18 in the control group and 1.19 ± 0.23 in diabetic rats (Fig. 2A). Similarly, the level of Bax was increased in the diabetic group, compared to controls. Levels of Bax were 0.79 ± 0.15 and 1.03 ± 0.21 in control and diabetic rats, respectively (Fig. 2B). Activated caspase-3 levels were 0.78 ± 0.13 and 1.06 ± 0.17 in control rats and diabetic rats, respectively (Fig. 2C).

Previous studies have demonstrated a decrease in testicular weight and atrophy of the seminiferous tubules in diabetic rats [1, 12]. Our results confirmed that both body weight and testicular weight are decreased and apoptotic cell death of spermatogonia and spermatocytes is increased in diabetic rats. In addition, diabetes induces a decrease of proliferation and differentiation in Leydig cells, and changes the pituitary-testicular axis [18]. Furthermore, diabetic animals exhibit decreased levels of testosterone that induce germ cell apoptosis and abnormal spermatogenesis [6, 11, 14, 15, 17].

To better understand the pathway leading to apoptosis in diabetes, we investigated the expression of apoptosis-related proteins in testes of rats with diabetes. We showed that diabetes increases the level of activated JNK, a mitogen-activated protein kinase that is associated with apoptosis and is activated by a variety of environmental stresses, including UV radiation and heat [3]. Activation of JNK is thought to be a cause of cell death in diabetic rat testes. Previous studies demonstrated that JNK is critical for increased Bax expression in response to stress [7, 19]. It is known that the translocation of Bax to the mitochondria stimulates the release of cytochrome c, which triggers activation of the caspase cascade and induces apoptosis [4]. Thus, Bax is

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**Table 1.** Blood glucose, body weight, and testicular weight of control and streptozotocin (STZ)-induced diabetic rats. Blood glucose and weights were evaluated 3 months after streptozotocin injection (n=15). *P<0.05 (vs. control).

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<thead>
<tr>
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<th>Control</th>
<th>STZ-diabetic</th>
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<tr>
<td>Blood glucose (mg/dL)</td>
<td>103 ± 5.16</td>
<td>435 ± 28.25*</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>395 ± 18.24</td>
<td>298 ± 22.05*</td>
</tr>
<tr>
<td>Testicular weight (g)</td>
<td>1.78 ± 0.07</td>
<td>1.58 ± 0.06*</td>
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**Fig. 1.** Representative photographs of TUNEL staining in testes of control (A) and streptozotocin (STZ)-induced diabetic rats (B). Positive cells of TUNEL staining were increased in STZ-diabetic rats. Arrows indicate positive cells. Scale bars = 100 μm. (C), Quantitative analysis of apoptosis in the testes. The apoptosis index was calculated as the ratio of apoptosis-positive seminiferous tubules to the total number of seminiferous tubules. *P<0.05 (vs. control).
Diabetes increases pJNK and BAX in testes.

Considered a pro-apoptotic protein. This study showed that Bax expression is elevated in diabetic rats in comparison with control rats. Furthermore, our results showed that the streptozotocin-induced diabetes increased the activation of caspase-3 in testes. This enhances apoptotic cell death through activation of caspase-activated DNase, which is integrally involved in DNA degradation [8]. Consequently, activated caspase-3 induces the morphological changes associated with apoptotic cell death [9]. In conclusion, our results indicate that streptozotocin-induced diabetes leads to apoptotic cell death in testes via activation of the JNK and Bax pathway.

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References