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Streptozotocin induces endoplasmic reticulum stress and apoptosis via disruption of calcium homeostasis in mouse pancreas

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ABSTRACT

Calcium homeostasis refers to the regulation of calcium ion concentration in the body. This concentration is tightly controlled by a stabilizing system consisting of calcium channels and calcium buffering proteins. Calcium homeostasis is crucial for cell survival. Various forms of cell death (e.g., necrosis and apoptosis) also share calcium signaling pathways and molecular effectors. Calcium acts not only as a ubiquitous second messenger involved in apoptosis along with various cell death inducers but also a regulator for the synthesis of enzymes/hormones such as insulin. We hypothesized that streptozotocin disrupts calcium homeostasis and the altered intracellular calcium levels may induce cell death. After streptozotocin administration, blood glucose level was increased while insulin levels decreased. The expression of insulin response markers also decreased relative to the vehicle group. L-type voltage-gated calcium channel expression and sarcoplasmic reticulum Ca^{2+} ATPase were increased by streptozotocin. Calcium buffering protein calbindin-$_D$9k and calmodulin family members were also increased. The expression of genes involved in transporting calcium ions to the endoplasmic reticulum (ER) was decrease while the expression of those affecting the removal of calcium from the ER was increased. Depletion of calcium from the ER leads to ER-stress and can induce apoptosis. In the streptozotocin-treatment group, apoptosis markers were increased. Taken together, these results imply that the disruption of calcium homeostasis by streptozotocin induces ER-stress and leads to the apoptosis of pancreatic cells. Additionally, findings from this study suggest that imbalances in calcium homeostasis could promote pancreatic beta cell death and result in type I diabetes.

1. Introduction

Pancreatic β-cells secrete insulin into the bloodstream. Insulin binds to receptors in target tissues to lower blood glucose and promote the storage of energy in the form of glycogen, proteins, or lipids (Wiederkehr and Wollheim, 2012). Diabetes mellitus is characterized by hyperglycemia. One of the most crucial factors of hyperglycemia is the dysfunction of pancreatic β-cells (Herchuelz et al., 2012). The two main types of diabetes are type 1 and type 2. In cases of type 1 diabetes mellitus, programmed cell death (apoptosis) is thought to be the main mechanism of β-cell death while type 2 diabetes is a complex disease characterized by both insulin resistance and β-cell dysfunction (Mandrup-Poulsen, 2003). Streptozotocin, a reagent used to establish experimental type 1 diabetes models, is a glucosamine–nitrosourea compound derived from *Streptomyces achromogenes* (Kang et al., 2014). Streptozotocin causes unique alkylation cytotoxicity typical of nitrosourea compounds and also elicits a proinflammatory response. Under proinflammatory conditions, inflammatory mediators and cytokines may alter calcium homeostasis (Xu et al., 2014; Zhang and Kaufman, 2008).

The endoplasmic reticulum (ER) is an intracellular organelle that is present in all eukaryotic cells. The ER is mainly responsible for the biosynthesis and modification of numerous proteins. The manufactured proteins are folded into functional three-dimensional structures before being released to various organelles or undergoing exocytosis. Physiological states that increase the demand for protein folding or stimuli that disrupt the reactions that control protein folding create an imbalance between the protein-folding load and ER capacity, and result in unfolded or misfolded proteins. This physiological condition produces ER stress.

Recently, a set of intracellular pathways that signal the presence of cellular stress was identified (Bertolotti et al., 2000). These pathways are collectively known as the unfolded protein response

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(UPR). The main UPR signaling cascades are initiated by three ER-localized protein sensors: inositol-requiring 1α (IRE1α), double-stranded RNA-dependent protein kinase (PKR)-like ER kinase (PERK), and activating transcription factor 6 (ATF6). In resting cells, all three ER stress sensors are maintained in an inactive state through association with the abundant ER chaperone immunoglobulin heavy chain-binding protein (BiP) (Hetz, 2012). In the presence of ER stress, BiP is sequestered through binding to unfolded or misfolded polypeptide chains, thereby leading to the release and subsequent activation of the ER stress sensors (Bertolotti et al., 2000).

Aside from IRE1α, PERK, and ATF6, several transcriptional factors and genes are also known as markers for ER stress. Protein disulfideisomerases (PDI), ER oxidoreductin 1 (ERO1), and C/EBP-homologous protein (CHOP) are representative markers of ER stress and the UPR. PDI and ERO1 are known as ER resident enzymes. The formation of disulfide bonds of proteins in the ER is driven by PDI and ERO1. Reduced PDI can mediate the decrease of mispaired —SH groups in oxidized protein-folding substrates and functions as an isomerase. Because ERO1 activity is modulated by the amount of FAD in the ER, disulfide bond formation is linked to the nutritional and/or metabolic status of the cell (Uehara et al., 2006). Apoptosis induced by ER stress is mediated by CHOP. However, the mechanism by which CHOP promotes apoptosis remains unclear.

In addition to serving as the site of protein folding, the ER acts as a cellular calcium storage organelle (Lee, 2014) and controls cellular calcium levels by responding to hormones or various cytokines (Botta et al., 2013). Disruption of intracellular calcium homeostasis can trigger apoptosis. Recently, it was found that overexpression of the calcium efflux channel plasma membrane Ca2+ ATPase (PMCA) depletes ER calcium storage, thus leading to ER stress and apoptosis (Jiang et al., 2010). In the present study, a murine streptozotocin-induced type 1 diabetes mellitus model was established to examine the relationship between calcium homeostasis disruption and ER stress-induced apoptosis.

2. Materials and methods

2.1. Animal experiments

Male ICR mice, weighing 25–30 g, 9 weeks of age, were obtained from Koatech (Pyeongtaek, Republic of Korea). All animals were housed in polycarbonate cages and acclimated in an environmentally controlled room (temperature: 23 ± 2 °C, relative humidity: 50 ± 10%, frequent ventilation, and a 12-h light/dark cycle). After approximately 1 week of acclimatization, we used the AMDCC (Animal Models of Diabetic Complications Consortium) protocol for induction of streptozotocin-induced insulin deficient mice, as previously described (Wu and Huan, 2008). Using this protocol, the animals were injected i.p. with a single dose of streptozotocin at 50 mg/kg body weight (BW) dissolved in 10 M citrate buffer (pH 4.5), and animals in the control group received injection with vehicle during 5 days. ICR mice were divided into two main groups: the vehicle (VE) group and the streptozotocin-induced type-1 diabetes model group (n = 7 mice per group, number of total mice use is 14). On day 6 after administration with streptozotocin, all mice were sacrificed using CO2 in a fume hood. Institutional Animal Care and Use Committee (IACUC) of Chungbuk National University approved all experimental procedures.

2.2. Collection and serological analysis of serum

Blood was collected from each mice, transferred to serum separator tubes (Microtainer tubes; Becton-Dickinson Co., NJ, USA), centrifuged at 400 × *g* for 15 min, and aliquoted as 200 μl. Serum glucose is analyzed by using the glucometer AccuChek® Active (Roche Diagnostics GmbH, Mannheim, Germany). The animals were fasted for 4 hours before performance of blood glucose measurements. Plasma insulin level was also determined by using the insulin ELISA kit (SHIBAYAGI, Japan) according to the manufacturer's instructions.

2.3. Total RNA extraction and quantitative real-time PCR

Mice were euthanized, and the pancreas were rapidly exercised and washed in cold, sterile NaCl (0.9%). Total RNA was prepared with TRIzol reagent (Invitrogen, Carlsbad, CA), and the concentration of RNA was determined by absorbance at 260 nm. Total RNA (1 mg) was reverse transcribed into first-strand cDNAs using Moloney murine leukemia virus (MMLV) reverse-transcriptase (iNtRON Bio, Sungnam, Gyeonggi, Korea) and random primers (9-mers; TaKaRa Bio., Inc., Otsu, Shiga, Japan). Two microliters of cDNA template was added to 10 μl of 2SYBR Premix Ex Taq (TaKaRa Bio) and 10 pmol of each specific primer. The reactions were carried out for 40 cycles according to the following parameters: denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. Fluorescence intensity was measured at the end of the extension phase of each cycle. The threshold value for the fluorescence intensity of all samples was set manually. The reaction cycle at which PCR products exceeded this fluorescence intensity threshold was identified as the threshold cycle in the exponential phase of the PCR amplification. The expressions of target genes were quantified against that of β-actin. Relative quantification was based on the comparison of CT at a constant fluorescent intensity. The amount of transcript is inversely related to the observed CT, and for every twofold dilution in the transcript, CT is expected to increase by 1. Relative expression was calculated using the equation $R = 2^{-(\Delta CTsample - \Delta CTcontrol)}$. To determine a normalized arbitrary value for target gene expression, its expression level was normalized to that of β-actin.

2.4. Western blot analysis

The pancreas of euthanized mice were rapidly excised and washed in cold sterile 0.9% NaCl solution. Protein was extracted with Pro-prep (InTron., Inc., Seoul, Korea) according to the manufacturer's instructions. Cytosolic protein (50 mg per lane) was separated on a10% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene fluoride transfer membrane (Perkin Elmer Co., Wellesley, MA) in a TransBlot Cell (TE-22, Hoefer Co., CA, USA) according to the manufacturer's protocol. The resulting blot was blocked in TBS-T containing 5% skim milk for 60 min, then incubated in primary antibody (1:1000) for 60 min at room temperature. After washing in buffer, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (anti-rabbit, 1:2000, Santa Cruz, or anti-mouse, 1:5000, Santa Cruz) for 1 hr at room temperature (RT). After washing, the blots were developed by incubation in ECL chemiluminescence reagent (Santa Cruz) and subsequently exposed to Biomax Light film (Kodak) for 1–5 min. Signal specificity was confirmed by blotting in the absence of primary antibody, and bands were normalized to β-actin immunoreactive bands visualized in the same membrane after stripping. Density measurements for each band were performed with NIH Image J software. Background samples from an area near each lane were subtracted from each band to obtain mean band density.

2.5. Statistical analysis

The results of all experiments are presented as the mean ± SD. Data were analyzed with a nonparametric one-way analysis of variance (ANOVA), using Tukey's test for multiple comparisons. Data were ranked according to these tests. All statistical analyses were

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