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Sall2 knockdown exacerbates palmitic acid induced dysfunction and apoptosis of pancreatic NIT-1 beta cells

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ABSTRACT

Spalt-like (Sall) proteins are a class of transcription factors. The role of Sall2 in beta cells remain poorly understood. Here, we aimed to explore whether Sall2 involved in lipotoxicity-mediated dysfunction and apoptosis in pancreatic NIT-1 beta cells. Our results showed that high concentrations of palmitic acid (PA) led to impaired cell viability and decreased Sall2 expression in NIT-1 cells. Knocking down of Sall2 in NIT-1 cells resulted in increased sensitivity to lipotoxicity and caused higher rates of cell apoptosis following PA treatment. Additionally, Sall2 Knockdown impaired insulin synthesis and secretion in response to glucose. Further research indicated Sall2 knockdown attenuate antioxidant capacity and decreased expression level of Peroxiredoxin 2 in NIT-1 cells. These finding implicate that Sall2 may play a significant role in NIT-1 cell function and cell apoptosis under lipotoxic conditions. Therefore, the study of Sall2 in NIT-1 cells provided a new perspective for molecular mechanism of lipotoxicity mediating dysfunction and apoptosis of beta cells.

1. Introduction

The prevalence of diabetes increased dramatically in recent years as the number of overweight and obese individuals has grown [1]. According to epidemiological data published in 2015, it was estimated that there were 415 million diabetic patients and 5 million people died of diabetes [2]. Excessive reduction in quantity of islet beta cells induced by apoptosis is a pivotal cause of the development of type 2 diabetes mellitus [3]. Beta cell apoptosis caused by a variety of stimuli, such as chronic hyperglycemia, hyperlipidemia, inflammatory cytokines, hypoxia [4]. Moreover, free fatty acids (FFA) have been shown to have deleterious effects (termed “lipotoxicity”) on beta cells in vitro [5,6]. Oxidative stress, endoplasmic reticulum stress and other pathways have been shown to contribute to lipo-apoptosis; however, the specific molecular mechanism mediating this process are still unclear.

Spalt-like (SALL) proteins are a class of transcription factors characterized by presence of zinc finger domains throughout the entire sequence [7]. Four paralogs of the *SALL* gene family (*SALL* 1-4) are evolutionarily conserved from Caenorhabditis elegans to human, all of which are involved in embryonic development and genetic disorders [7]. As the least analogous, SALL2 (also known as Sal-2, p150(Sal2)) have a special “SAL box” [8]. Human *SALL2* gene is located in chromosome 14q11.1-12 [8]. Previous research has shown that SALL2 is expressed in brain, heart, lung, kidney, ovary, prostate and pancreas [9,10]. The function of Sall2 had been related with neurogenesis and kidney development [7]. Sall2 also play a pivotal role in eye morphogenesis, and loss of function of the gene causes ocular coloboma in humans and mice [11].

Expression of SALL2 in human pancreatic beta cells has been analyzed [12]. However, the biological functions of Sall2 in pancreatic beta cells have not been reported. Intracellular expression of Sall2 is altered when cells under certain stress such as DNA damage caused by genotoxic stress [13,14]. Further studies have shown that Sall2 is required for full apoptotic response to doxorubicin [13], indicating that Sall2 expression is correlated with cellular apoptosis.

NIT-1 cells, a mouse pancreatic beta-cell line established from a transgenic NOD/Lt mouse, whose response to stimuli is similar to islet cells cultured in vitro [15]. As a kind of non-esterified fatty acid,

Abbreviations: PA, palmitic acid; NEFA, non-esterified fatty acid; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; shRNA, short hairpin RNA; CCK8, cell counting assay kit-8; Pdx-1, pancreatic and duodenal homeobox factor 1; MafA, V-maf musculoaponeurotic fibrosarcoma oncogene homologue A; Sod1, superoxide dismutase-1; Prdx2, peroxiredoxin-2; HRP, horseradish peroxidase

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palmitic acid (PA) has been widely used to induce oxidative stress, endoplasmic reticulum stress and apoptosis in pancreatic beta cells [16–18]. Our previous experiments have shown that Sall2 expression is significantly decreased in NIT-1 cells treated with PA (unpublished data). Accordingly, we hypothesized that Sall2 may involved in lipotoxicity-mediated beta cell dysfunction. To test this hypothesis, we investigated the role of Sall2 in pancreatic NIT-1 beta cells.

2. Methods and materials

2.1. Cell culture and treatment

Pancreatic NIT-1 beta cells were purchased from the Cell Bank of Chinese Academy of Sciences. NIT-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% ((v/v) fetal bovine serum, 1% glutamine and 1% penicillin/streptomycin in humidified 5% CO2, 95% air at 37 ℃. Palmitic acid (Sigma-Aldrich) was conjugated with fatty-acid-free BSA before addition to cell culture. PA was dissolved in 99% ethanol and then mixed with 10% BSA in serum-free DMEM to make a 50 mM PA stock solution.

2.2. Plasmid construction, transfection and stable cell line establishment

Short hairpin RNAs (shRNAs) were designed and synthesized by GENECHEM (Shanghai, China). Short-hairpin sequences against the *Sall2* gene (NM_015772)or the scrambled shRNA sequences were cloned into the GFP-labeled plasmid vector GV102. The target sequences selected were as follows: Sall2–sh1, 5′-CACTCTTCACTTGTGT CTT-3′; Sall2–sh2, 5′-CTTCCCTTATGTGCTAGAA-3′; Sall2–sh3, 5′-GCA ATTTGCGCGCACATTT-3′ and scrambled shRNA, 5′- TTCTCCGAACGT GTCACGT-3′. Sall2 mRNA expression levels were determined by RT-PCR, and protein expression was confirmed by western blot analysis.

Plasmids containing Sall2-shRNAs and scrambled shRNA were transfected into cells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Brief, cells were plated in 24-well plate at 70% confluence. For each well, 0.8 μg plasmids were added to 50 μl Opti-MEM medium, 1.6 μl of Lipofectamine 2000 to 50 μl Opti-MEM medium. The two solutions were then mixed, and the mixture was added to cells. The cells were incubated for 6 h, the medium was replaced, and the cells were incubated for an additional 48 h. Next, culture medium containing 400 μg/ml G418 (Biosharp, China) was used for selection and further characterization. After 2 weeks, stable cell lines with reduced Sall2 expression had been generated. The transfection efficiency of NIT-1 cells expressing Sall2-shRNA was assessed by RT–PCR and western blot analysis.

2.3. Assessment of cell viability

Cell viability was measured by Cell Counting Assay Kit-8 (CCK-8, Dojindo Molecular Technologies, Japan) according to the manufacturer's instructions. Cells were seeded in 96-well plates at a density of 5×10^3 cells/ml and cultured for 24 h, then treated with 0.5 mM PA for 48 h with concentration gradients. Equivalent BSA was used as vehicle control. CCK-8 reagent was added to each well and incubated for 2 h. The absorbance at 450 nm was measured using a microplate reader (Bio-Tek, USA). Cell viability was expressed as percentage of control (100%).

2.4. Determination of cell apoptosis

Annexin V-PE/7AAD apoptosis detection kit (Key-GEN, China) was used to analyze cell apoptosis according to the manufacturer's instructions. Cells were subjected to different treatments and collected after ethylenediaminetetraacetic acid-free trypsin digestion. Then cell pellets were collected via centrifugation for 6 min 1000 × g after two washes in cold phosphate-buffered saline. The cell density was adjusted to 5×10^5 cells/mL and resuspended in binding buffer, stained with 5 μl 7AAD for 15 min and then stained with1μl PE labeled Annexin V for 15 min away from light. Cells were examined by a flow cytometry FACSAria III (BD Biosciences, USA). Cells positive only for PE were early apoptotic and those which were positive only for 7-AAD were necrotic. Cells positive for both dyes were considered late apoptotic cells. The total ratio of apoptotic cells was calculated by the sum of cells in the early and late stages of apoptosis.

2.5. RNA isolation and real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesized from total RNA (1 μg) using a reverse transcription kit (Takara Bio, Japan) according to the manufacturer's instructions. The reaction was incubated at 42 for 2 min, then applied to 37 ℃ for 15 min, and finally 85 ℃ for 5 s. Real-time PCR was performed with CFX96 Real-Time PCR Detection System (Bio-rad, USA) by using SYBR Premix Ex Taq (Takara Bio). Relative abundance of mRNA expression was calculated by the well recognized $2^{-\triangle\triangle Ct}$ method [18]. The sequences of primers for PCR are listed in Supplemental Table 1.

2.6. Western blot analysis

Total soluble protein (100 μg) was resolved on 10% SDS–PAGE and transferred electrophoretically to polyvinylidene difluoride membranes. Blots were blocked with 5% skim milk, followed by incubation with antibodies separately. Primary antibodies used as follows: anti-GADPH (1: 10,000; Abcam, USA), anti-Sall2 (1:1000; Biorbyt, UK), anti-Bax (1: 2000; Abcam), anti-Cleaved caspase3 (1: 1000; CST, USA), anti-Pdx-1 (1: 500; Affinity Biosciences, China), anti-Peroxiredoxin 2 (1: 1000; Proteintech, China). The secondary antibodies were goat anti-rabbit conjugated with HRP (horseradish peroxidase) at a dilution of 1: 10,000 (KPL, USA). The immunoreactive bands were quantified using Image J (NIH, USA) and normalized to GAPDH expression.

2.7. Insulin secretion assay

On the day of experiment, NIT-1 cells were preincubated with DMEM without glucose for 30 min, then the cells were incubated for 2 h in HEPES-balanced Krebs-Ringer bicarbonate buffer (125 mM NaCl; 5.9 mM KCl; 1.2 mM $MgCl_2$; 1.3 mM $CaCl_2$; 25 mM HEPES, pH7.4) containing 0.1% BSA and different glucose concentrations (2.5 mM or 20 mM) [18]. At the end of incubation, insulin concentration was measured in the medium using a mouse insulin Elisa kit (Mercodia, Sweden).

2.8. Total antioxidant capacity and glutathione level measurement

Total antioxidant capacity(TOC) and glutathione (GSH) levels were determined using corresponding commercial kit (Nanjing Jiancheng Bioengineering Institute, China). In brief, cells were seeded in 6-well plates at a density of 2×10^5 cells/ml and then cultured with 48 h. The culture medium was collected and centrifuged at 1000 × *g* for 10 min, the pellet was discarded. On this basis, total antioxidant capacity and GSH levels was determined according to the procedure described in manufacturer's instructions.

2.9. Statistical analysis

Data are presented as mean ± SD. Statistical significance between two experimental conditions was analyzed by using Student's *t* test. A *p*-value < 0.05 was considered statistically significant.