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Blockade of store-operated calcium entry alleviates high glucose-induced neurotoxicity via inhibiting apoptosis in rat neurons

Zhenkuan Xu, Wenzhe Xu, Yan Song, Bin Zhang, Feng Li, Yuguang Liu*

Department of Neurosurgery, Qilu Hospital of Shandong University, Brain Science Research Institute of Shandong University, Jinan, Shandong Province 250012, PR China

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ABSTRACT

Altered store-operated calcium entry (SOCE) has been suggested to be involved in many diabetic complications. However, the association of altered SOCE and diabetic neuronal damage remains unclear. This study aimed to investigate the effects of altered SOCE on primary cultured rat neuron injury induced by high glucose. Our data demonstrated that high glucose increased rat neuron injury and upregulated the expression of store-operated calcium channel (SOC). Inhibition of SOCE by a pharmacological inhibitor and siRNA knockdown of stromal interaction molecule 1 weakened the intracellular calcium overload, restored mitochondrial membrane potential, downregulated cytochrome C release and inhibited cell apoptosis. As well, treatment with the calcium chelator BAPTA-AM prevented cell apoptosis by ameliorating the high glucose-increased intracellular calcium level. These findings suggest that SOCE blockade may alleviate high glucose-induced neuronal damage by inhibiting apoptosis. SOCE might be a promising therapeutic target in diabetic neurotoxicity.

1. Introduction

Diabetes is a common metabolic disorder characterized by hyperglycemia. Chronic hyperglycemia can lead to microvascular and macrovascular complications [51]. Despite the attention paid to end-organ disease affecting the kidney, eyes, and peripheral nervous system, the central nervous system is also affected [55]. Accumulating evidence suggests that diabetes is an independent risk factor for cognitive dysfunction [12]. Substantial evidence supports that neuronal damage to the hippocampus, the major area associated with learning and memory, is involved in diabetic cognitive dysfunction [33,47]. Understanding the pathophysiological mechanisms of diabetes-related damage to hippocampus neurons is of crucial importance in developing treatments to prevent cognitive dysfunction and reduce the burden of cognitive dysfunction in a diabetic population [5].

Calcium is a universal second messenger involved in various cellular processes, such as proliferation, transcription, contraction, exocytosis, apoptosis, the immune response, and neurotransmission [19,50]. Store-operated calcium entry (SOCE), an essential calcium entry mechanism in both excitable and non-excitable cells, is mediated by the store-operated calcium channel (SOC), which is activated by depletion of internal calcium stores [31,44,52]. High throughput RNAi screening identified stromal interaction molecule (STIM) as a required component of SOCE [17,34]. STIM1 is a single-pass transmembrane protein located in the endoplasmic reticulum (ER) membrane that functions as an ER calcium sensor to sense ER luminal calcium concentration [17,48]. Over the past decade, a growing body of evidence has demonstrated that many diabetic complications involve altered SOCE and its signaling pathways [13,67]. However, the association of SOCE and neuronal damage to the hippocampus in diabetes remains obscure.

In this study, we aimed to investigate the role of the SOC in neuron injury induced by high glucose and the effectiveness of SOC inhibition in alleviating high glucose-induced apoptosis in primary cultured neurons, to find a new target for clinical treatment of diabetic cognitive dysfunction.

Abbreviations: SOCE, store-operated calcium entry; SOC, store-operated calcium channel; MMP, mitochondrial membrane potential; MPT, mitochondrial permeability transition; STIM1, stromal interaction molecule 1; ER, endoplasmic reticulum; MTT, 3-[4,5-Dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide; DMSO, dimethyl sulphoxide; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid.

* Corresponding author.
E-mail addresses: kuankuancui@163.com (Z. Xu), xuwenzhe2008@126.com (W. Xu), super326@126.com (Y. Song), zhangbin747@126.com (B. Zhang), feng.li@hotmail.com (F. Li), ns3000@126.com (Y. Liu).

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2. Materials and methods

2.1. Reagents

3-[4,5-Dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulphoxide (DMSO), 1,2-bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid (BAPTA-AM) and lanthanides 3+ (La3+) were from Sigma Aldrich (St. Louis, MO, USA). Neurobasal medium, fetal bovine serum, B27, Hanks' Balanced Salt Solution (HBSS, pH 7.4) and trypsin–EDTA solution were from Gibco (Invitrogen, Grand Island, NY, USA). Fluo-3/acetoxymethylester (Fluo-3/AM) and pluronic F-127 were from Biotium (Hayward, CA, USA). Rhodamine 123 (Rh123) was from Sigma Aldrich (St. Louis, MO, USA). All antibodies were from Cell Signaling (Danvers, MA, USA). Other reagents were from Sigma Aldrich (St. Louis, MO, USA) unless otherwise indicated.

2.2. Cell culture and treatments

All experimental procedures were performed in accordance with the experimental standards of the Chinese Academy of Medical Sciences and were approved by the institutional review board of Qilu Hospital (Shandong, China). Primary cultures of hippocampus neurons from day 18 embryos of Sprague-Dawley rats were prepared as described [7,28] with some modification. Briefly, brains were removed from embryos and placed into HBSS at 4 °C. Hippocampi were dissected from brains, digested with 1.5% trypsin-EDTA for 10 min at 37 °C, and terminated with a mixture of DMEM/F-12 plus 10% fetal bovine serum. Neurons were placed in 6-well culture plates or 96-well culture plates precoated with 0.1 mg/mL poly-D-lysine in neurobasal medium, supplemented with 1x B27 and 0.05 mM glutamine. Neurons were maintained at 37 °C in a humidified 5% CO_2 incubator. After 7-day culture, cells were randomly divided into groups for treatment: control (normal glucose, 25 mM); high glucose (HG, 50 mM); HG + negative scramble inhibitor, HG + La3+ (1 μM, SOC inhibitor); HG + STIM1 siRNA, HG + BAPTA-AM (5 μM, calcium chelator). The osmolarity for all glucose exposures was 280–320 mOsm/kg, which was within the plasma osmolarity range and had no effect on the normal metabolism. Cells were treated for 48 h and prepared for experiments.

2.3. Measurement of cytoplasmic free Ca^{2+} concentration

The free cytosolic Ca^{2+} concentration was determined by flow cytometry with the fluorescent calcium indicator Fura 3-acetoxymethyl ester (Fura3-AM). Equal volumes of Fluo-3/AM and pluronic F127 were added into HBSS just before incubation as the Fluo-3 loading solution (final concentration of Fluo-3/AM and pluronic F127 was 5 μM and 0.1%, respectively). After cells were treated for 48 h, they were loaded with Fura3/AM for 45 min in the dark at 37 °C in a humidified incubator, washed 3 times in phosphate buffered saline (PBS), detached with trypsin, resuspended in HBSS, and directly introduced into a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) to detect green fluorescence with FL1. Data for 10,000 fluorescent events were obtained by use of Cell Quest software and analyzed with use of FCS Express 4.0 (De Novo Software, Los Angeles, CA, USA) and expressed as mean fluorescence intensity per 10,000 cells.

2.4. Transient transfection of primary cultured neurons

Small interfering RNA (siRNA) against STIM1 or scramble control siRNA was transiently transfected into primary cultured neurons by use of Lipofectamine and Plus reagent according to the manufacturer's (Invitrogen, Shanghai, China) protocols. The efficiency of transient transfection was confirmed by detecting STIM1 protein levels. After 48-h transfection, cells were treated with high glucose (50 mM) for 48 h. Then cells were collected.

2.5. Apoptosis analysis by Annexin-V FITC/PI staining

Cell apoptosis was evaluated by use of an Annexin V-FITC/PI kit (BD, Shanghai, China). Briefly, treated cells were collected and washed with PBS twice. In total, 400 μl binding buffer, 5 μl Annexin-V FITC and 5 μl PI were sequentially added to the cell suspension. After incubation in the dark for 15 min, cells were analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA). At least 3 independent experiments were performed.

2.6. Assessment of cell viability

The viability of primary cultured neurons was determined by standard MTT assay. Briefly, MTT solution (20 μl, 5 mg/ml) was added into each well for incubation at 37 °C for 4 h. The solution was removed by aspiration, and insoluble formazan crystals were dissolved in 150 μl/well DMSO, and absorbance at 490 nm was measured by use of a Varioskan Flash spectral scanning multimode reader (Thermo Electron, Vantaa, Finland). The spectrophotometer was calibrated to zero absorbance with culture medium without cells. The percentage cell survival was determined by comparing the average absorbance of treated cells to untreated cells. The experiment was repeated at least 3 times.

2.7. Western blot analysis

Cultured cells were lysed in RIPA buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1%Triton X-100, 1% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail with 1 mM phenylmethanesulfonyl fluoride. Protein concentration was determined by the BCA Protein Assay. Total protein (20 μg) was separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA), which were blocked with 5% non-fat milk, then incubated with primary antibodies against STIM1 (1:1000), cleaved caspase-3 (1:800), Bcl-2 (1:400), Bax (1:400) and β-actin (1:10,000) overnight at 4 °C. The membranes were rinsed and washed 3 times with TBS containing 0.1% Tween 20, then horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The membranes were visualized by use of the Immobilon Western Chemiluminescent HRP Substrate kit (Millipore, Bedford, MA, USA). Immunoreactive labeling was analyzed by use of ImageJ 1.44 (US National Institutes of Health, Bethesda, MD, USA) and standardized against β-actin protein level.

2.8. Total RNA isolation and real-time quantitative PCR (qPCR)

Total RNA from cells was isolated by the Trizol method (Invitrogen, Shanghai, China). cDNA was generated by reverse transcription of 1 μg total RNA with primer oligonucleotides. qPCR reactions were conducted in a 10 μl reaction volume with SYBR Green I, with a 1:25 dilution of the cDNA and 20 nM primers.

β-actin was used as a reference gene. The primers were designed by using Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA) on the basis of mRNA sequences from the GenBank database. Primer sequences were for STIM1 (sense: 5′-TCTGGCCAAGAAGACAATCC-3′; anti-sense: 5′-ATTCGTGTGTTTCGGCTACC-3′) and β-actin (sense: 5′-CGTTGACATCCGTAAAGACC-3′; anti-sense: 5′-TAGAGCCACCAATCCACACA-3′). The fold increase in expression compared with control cells was determined by the $2-\triangle\triangle CT$ method with β-actin as a normalizing