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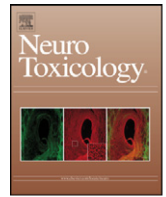

Contribution of single-minded 2 to hyperglycaemia-induced neurotoxicity

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ABSTRACT

Diabetes mellitus is associated to central nervous system damage, which results in impairment of brain functions and cognitive deficits and decline in memory. However, the mechanisms mediating the actions of glucose on the neurons remained elusive. Single-minded 2 (Sim2), a basic helix-loop-helix (bHLH)-PAS transcriptional repressor, is thought to be involved in some symptoms of Down syndrome. We hypothesized that Sim2 mediated hyperglycaemia-induced neuronal injury and impairment of learning and memory. It was found that expression of Sim2 protein in cortical neurons was increased in streptozotocin-induced diabetes mellitus rat model. Drebrin, down-regulated by Sim2, was subsequently decreased as detected by confocal laser scanning microscopy and Western blot analysis. The expression pattern of Sim2 and Drebrin correspond to 50 mmol/L glucose (hyperglycaemia) was also found in primary cultured neurons. Curcumin, one neuroprotective agent, inhibited hyperglycaemia-induced neurotoxicity. Moreover, curcumin alleviated Sim2 expression, and reversely raised Drebrin expression in neurons treated with hyperglycaemia. Finally, we found that silencing Sim2 expression decreased hyperglycaemia-induced neuronal injury. In conclusion, Sim2 may mediate neurotoxicity during hyperglycaemia and thereby play a critical role in the development of hyperglycaemia-induced cognitive deficits.

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1. Introduction

Diabetes mellitus (DM) is a major endocrine disorder and a growing health problem in most countries. A growing body of literature describes a series of neuropathological and neurobehavioral changes in diabetic subjects, such as cognitive dysfunction and decline in memory. Diabetes-related cognitive dysfunction is mainly a consequence of changes within the central nervous systems (CNS) that are secondary to chronic hyperglycaemia (Malone et al., 2008). Prolonged exposure to chronic hyperglycaemia leads to various complications in both the peripheral and CNS. Previous studies have also shown that spatial learning and memory are impaired in diabetic animals. Learning and memory deficits have been partly associated with the structural and functional deficits in certain brain regions such as the hippocampus and cerebral cortex (Artola, 2008; Hou et al., 2012). In humans, DM is associated with moderate impairments in cognitive function and patients present a high risk of affective disorders, dementia and Alzheimer disease (Biessels et al., 2006; Gaudieri et al., 2008). However, the molecular mechanisms underling learning and memory deficits in DM need to be further studied.

Besides DM, there are also a few of diseases which impair learning and memory such as Down syndrome (DS) and Alzheimer's disease. Accumulating data suggest that they may have common mechanisms (Costa, 2012; Kodiha and Stochaj, 2011). It has been shown that an overdose of Sim2 in Down syndrome, a member of the basic helix-loop-helix-PAS transcription factor gene family protein, contributes to the symptoms proposed on the basis of the quantitative analysis of Sim2 expression in mouse models of DS. An anxiety-related/reduced exploratory behavior was observed in mice overexpressing Sim2 in the open field test and in zero-maze (Chatterjee et al., 2011; Chrast et al., 2000). Moreover, it has been reported that expression of Sim2 was also increased in cortex in Alzheimer's disease (Julien et al., 2008). These results suggest that Sim2 plays an important role in diseases related with learning and memory deficits. However, expression of Sim2 and its role in DM related neuron dysfunction and decline in memory is still unknown.

Given the important role of Sim2 in learning and memory deficits, the present study hypothesized that high glucose may induce neuron injury through increasing the expression of Sim2, ultimately leading to neuron dysfunction and subsequent learning and memory deficits. To test this hypothesis, we first used

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streptozotocin (STZ) induced DM rat model to examine the expression of Sim2 in cortex and the expression of Drebrin, a dendritic spine protein, which is negatively regulated by Sim2. Then, we also determined their expression in primary culture neurons treated with high glucose. Furthermore, we detected whether curcumin regulates the expression of Sim2 and Drebrin, and then alleviated neuronal injury. At last, the direct role of Sim2 in hyperglycaemia-induced injury was explored by silencing Sim2 expression with lentivirus.

2. Materials and methods

2.1. Ethics statement

All animal experimental procedures carried out in this study were approved by the Laboratory Animal of the Ethics Committee of Huazhong University of Science and Technology, and were in compliance with the guidelines for animal care set forth by this Committee.

2.2. Induction of DM in rats

Adult (250–280 g) male Sprague–Dawley rats were housed individually and kept on a reversed light-dark 12–12 h cycle, food and water were available *ad libitum*. The rats were randomly divided into two groups: control rats and STZ-induced diabetic rats. Diabetes was induced by a 60 mg/kg intraperitoneal injection of streptozocin (STZ, 60 mg/kg, Sigma, St. Louis, MO, USA) dissolved in sterile sodium citrate buffer solution (0.1 mol/L citric acid and 0.2 mol/L sodium phosphate, pH 4.5). The control animals received saline injection. Age-matched control rats were injected with an equivalent volume of citrate buffer solution. Three days after STZ administration, blood glucose levels were determined with a blood glucometer (OneTouch II; Johnson). Animals were considered diabetic if plasma glucose levels exceeded 16.7 mmol/L (Olukman et al., 2010), and the diabetic state was reconfirmed after 4 weeks prior to sacrifice.

2.3. Primary neuronal cell culture and treatment

Pregnant Sprague–Dawley rats were used for the neuronal culture. Neuronal cultures were prepared from embryonic day 17–18 rat cortices as described previously (Meng et al., 2012). Cortical tissue was disrupted into a cell suspension by gentle trituration and seeded in 60-mm dishes at a density of 2×10^6 cells/dish. Neurons were grown on poly-l-lysine-coated tissue culture dishes in neurobasal medium (Invitrogen Corporation, Grand Island, NY, USA) consisting of 2% B-27 Supplement, 1 mmol/L L-glutamine. The purity of neuronal cells was detected by double fluorescent labeling of NeuN and 4′, 6-diamidino-2-phenylindole (DAPI). Neurobasal medium has 25 mmol/L glucose, which is optimal for neuron survival and growth. We referred this glucose concentration as normal and used in control group. Additional 25 mmol/L glucose was added to the medium as high glucose. Mannitol 25 mmol/L was also used here to create a high osmotic pressure mimicking the high glucose condition. Cortical neurons were cultured for about 5 d (DIV 5) before drug treatment. Cells were cultured in the presence or absence of different concentrations of curcumin (0.1 and 1 μmol/L) for 4 h before adding glucose at final concentrations 50 mmol/L. Curcumin were bought from Tauto Biotech (Shanghai, China).

2.4. Transduction of cortical neurons with lentiviral vectors

The lentiviral vectors express green fluorescent protein (GFP) and rat Sim2 shRNA were constructed by Genechem Co. Ltd. (Shanghai, China). The shRNA sequence that targets the rat Sim2 sequence (GenBank No. NM_001107108) was designed as follows: 5′-GCT CAC AGG CAA CAG TAT T-3′. Lentiviral infection was performed by adding virus solution to cells at the desired multiplicity of infection. The randomly chosen nonsense sequence 5′-TTC TCG CAA CGT ATG CGC TGA-3′ was used as scramble control. After transfection for 5 days, green fluorescence protein (GFP) fluorescence was observed using an excitation filter of 490 nm and an emission filter of 520 nm in a Nikon Diaphot inverted microscope equipped with a 75 W Xenon lamp and a Nikon 40X, 1.3 numerical aperture, epifluorescence oil immersion objective. Transfection efficiency was calculated by counting the number of fluorescein-positive cells *vs.* the total number of cells in nine randomly selected regions from three independent experiments.

2.5. Quantitative reverse transcription PCR analysis of mRNA levels of Drebrin

After 24 h incubation at different experimental conditions, neurons were processed by quantitative reverse transcription PCR analysis. Total RNA was isolated from neurons by TRIzol (Invitrogen, Grand Island, NY, USA). The concentration of the total RNA was quantified by determination of optical density at 260 nm (OD_{260}). Then cDNA was generated by 2 μg RNA using a RT-Kit (Fermentas, EU) with oligo (dT) primer. The mRNAs for Drebrin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (internal control) were amplified and quantified with primers listed below. The synthetic oligonucleotide primer sequences for Drebrin, and GAPDH were as follows: Drebrin 5′- CTCCAATG-GACTGGCTCG-3′ (sense) and 5′- GCATCCGTCTTCTGGTAGGT -3′ (antisense); GAPDH 5′-GGC ACA GTC AAG GCT GAG AAT G-3′ (sense) and 5′-ATG GTG GTG AAG ACG CCA GTA-3′ (antisense). Quantitative PCR was performed using SYBR-Green dye (Applied Biosystems) and Applied Biosystems hardware and software (7500 RT-PCR System). Expression value of the targeted gene in a given sample was normalized to the corresponding expression of GAPDH. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative expression of the targeted genes.

2.6. Immunofluorescent staining

Double-immunofluorescent staining was performed using frozen slides from rat cortices. After fixation, the slides were incubated with goat anti-Sim2 (Catalog No. sc-8716, Santa Cruz Biotechnology Inc, CA, USA, 1:200), rabbit anti-Drebrin (Catalog No. 10260-1-AP, Proteintech Group, Wuhan, China, 1:200), or mouse anti-NeuN (Catalog No. MAB377, Millipore Corporation, MA, USA, 1:50) for overnight at 4 °C. After washing, the slides were incubated with different fluorescein-labeled secondary antibodies. Finally, the slides were mounted and subjected to examinations using a confocal laser scanning microscope (Fluoview FV1000, Olympus, Japan).

2.7. Western blot analysis

Western blot analyses were performed as previously described (Zhang et al., 2011). Briefly, equal amounts of protein were fractionated on sodium dodecyl sulfate polyacrylamide gels and transferred electrophoretically to a polyvinylidene difluoride membrane. The membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.05% Tween 20 for 1 h at room temperature. Then, the membrane was probed with primary antibodies of anti-Sim2 (1:200), anti-Drebrin (1:200) or anti-β-actin (1:5,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C followed by incubation with horseradish

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