Brain Research Bulletin 111 (2015) 27–35

Contents lists available at ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Research report

The dynamic changes of endoplasmic reticulum stress pathway markers GRP78 and CHOP in the hippocampus of diabetic mice

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ARTICLE INFO

Article history:
Received 1 September 2014
Received in revised form
30 November 2014
Accepted 10 December 2014
Available online 19 December 2014

Keywords:
Diabetic encephalopathy
Endoplasmic reticulum stress
GRP78
CHOP
Hippocampus
Apoptosis

ABSTRACT

Diabetic encephalopathy has recently been recognized late complication of diabetes resulting in progressive cognitive deficits. Emerging evidence has indicated that endoplasmic reticulum (ER) stress-mediated apoptosis is involved in the pathogenesis of diabetic eye and kidney as well as non-diabetic neurodegeneration. However, there was little direct evidence for the involvement of ER stress in diabetic encephalopathy up to now. In the present work, we investigated the role of ER stress in the pathogenesis of diabetic encephalopathy. Our results have demonstrated the existence of ER stress in the hippocampus of streptozotocin (STZ)-induced diabetic mice. STZ injection i.p. rapidly induced up-regulation of the ER stress marker, the prosurvival chaperone glucose-regulated protein 78 (GRP78), as early as 6–24 h and persisted at least for up to 72 h in the hippocampus of mice, indicating the UPR activation soon after STZ administration. The increased expression of GRP78 in hippocampal cells is to relieve the ER stress. With the development of diabetes, the expression of GRP78 decreases while the expression of UPR-associated proapoptotic transcriptional regulator C/EBP homologous protein (CHOP) increases significantly in the hippocampal neurons of diabetic mice from 1 week after STZ administration to 12 weeks/the end of the study. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-positive cells in the hippocampus of diabetic mice were largely colocalized with NeuN- and CHOP-positive cells, indicating that the up-regulation of CHOP in hippocampal neurons of diabetic mice may promote neuronal apoptosis and account for the damaged learning and memory ability of diabetic mice. Therefore, our study provides evidence that ER stress may play an important role in the pathogenesis of neuronal degeneration and may contribute to cognitive dysfunction of diabetic encephalopathy.

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1. Introduction

Endoplasmic reticulum (ER) is one of the major compartments for the biosynthesis of lipids as well as the proper synthesis, maturation and folding of proteins. Perturbation of ER homeostasis such as imbalance in ER calcium levels, accumulation of misfolded proteins in ER, glucose and energy deprivation, hypoxia can all cause ER stress (Rao et al., 2004). ER stress triggers an evolutionarily conserved cellular stress response termed the unfolded protein response (UPR) which intended to protect the cell against the toxic aggregated proteins. The activation of UPR results in an overall decrease in translation, increase clearance of misfolded proteins in ER lumen and increased levels of ER chaperones, including glucose-regulated protein 78 (GRP78), which consequently increases the protein folding capacity of ER (Kim et al., 2008; Xu et al., 2005). In general, cells may return to normal ER homeostasis under ER stress. However, cells may also continue toward apoptosis under prolonged ER stress if homeostasis is not restored. This apoptotic event is mediated by induced the expression of the UPR-associated proapoptotic transcriptional regulator C/EBP homologous protein (CHOP) and others, which participate in the control of cellular redox status and cell death (Oyadomari and Mori, 2004; Paz Gavilán et al., 2006). GRP78 and CHOP are the markers when ER stress occurred. The balance between the prosurvival chaperone GRP78 and CHOP drives the cell destiny following ER stress.

Abbreviations: Aβ, beta-amyloid; CHOP, C/EBP homologous protein; DAPI, 4′,6-diamidino-2-phenylindole; DM, diabetes mellitus; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; UPR, unfolded protein response; STZ, streptozotocin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; NeuN, neuron-specific nuclear protein.

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http://dx.doi.org/10.1016/j.brainresbull.2014.12.006
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Diabetic encephalopathy is now accepted complications of diabetes mellitus (DM). The patients of diabetes have impairment of cognitive function (Sima, 2010; Umegaki et al., 2013). In type 1 diabetes, perturbed cognitive function involves intelligence, attention, processing speed, spatial learning, and long-term memory, whereas learning abilities per se appear to be spared (Brands et al., 2005). In type 2 diabetes, neuropsychological deficits tend to involve verbal and recent memory and information processing (Awad et al., 2004). Studies in streptozotocin (STZ)-induced diabetes in rats and mice have demonstrated neurobehavioral deficits using the Morris water maze paradigm, associated with impaired hippocampal long-term potentiation (Biessels et al., 1996; Gispen and Biessels, 2000; Zhao et al., 2003). STZ-rat was reported loss of neocortical neurons (Jakobsen et al., 1987). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive neurons as well as nucleosomal DNA fragmentation were increased in the hippocampus and frontal cortex of 8-month diabetic rats, in association with significant neuronal loss in hippocampal CA1 and CA2 (Li et al., 2002; Sima and Li, 2005), suggesting increased apoptotic activity and neuronal death with duration of diabetes. However, the precise mechanism(s) for the onset and progression of diabetes encephalopathy has still not been determined. Our previous study showed that the Ser199/Ser202 sites of microtubule-associated tau protein were hyperphosphorylated and the expression of beta-amyloid (Aβ) was increased in the hippocampus of STZ-diabetic mice, indicating that many proteins metabolism was disturbed in the brain of diabetic mice (Zhao et al., 2003; Shuli et al., 2001). Aβ and phospho-tau accumulation is also found in type 1 diabetic BB/Wor rats and type 2 diabetic BBZDR/Wor rats as well as type 2 db/db mouse models (Li et al., 2007; Kim et al., 2009), indicating that abnormal metabolism of proteins exists in the ER of the brain of diabetic mice which may disrupt ER function. ER becomes swollen in the diabetic brains under ultrastructural examination (Ai et al., 2010; Dheen et al., 1994), suggesting the disorder of the ER under diabetic condition. ER-stress mediated cell death may play an important role in the pathogenesis of diabetic encephalopathy. Emerging evidence has indicated that ER stress-mediated apoptosis is involved in the pathogenesis of diabetic eye and kidney as well as non-diabetic neurodegeneration, such as Alzheimer's disease and Parkinson's disease (Oyadomari and Mori, 2004; Cornejo and Hetz, 2013; Abisambra et al., 2013). However, there was little direct evidence for the involvement of ER stress in diabetic encephalopathy up to now.

In order to investigate the molecular basis of diabetic encephalopathy, we have studied whether ER stress is involved in the mechanism underlying the neurons death in the brain of diabetic mice induced by STZ. We have examined the cognitive performance of diabetic mice and the expression of ER stress markers GRP78 and CHOP in the hippocampus of diabetic mice sequentially in different time, as well as the correlation between CHOP expression and hippocampal neuronal apoptosis, by using immunohistochemisty, double immunofluorescence, Western blotting analysis and TUNEL staining, so as to understand the mechanism underlying diabetic encephalopathy.

2. Material and methods

2.1. Experimental animals and creation of animal model

All animal experiments were approved by the Institutional Animal Care and Use Committee of Xuanwu Hospital of Capital Medical University, Beijing, and were in accordance with the principles outlined in the NIH Guide for the Care and Use of Laboratory Animals. Male Kunming mice weighing from 32 to 37 g were supplied by the Beijing Vital River laboratory animal technology Co. Ltd and housed five or six per cage under a 12/12-h dark/light cycle and standard pathogen free condition. All efforts were made to minimize both the suffering and the number of animals used.

After 1 week acclimatization to the home cage, mice were randomly divided into two groups: a normal control group (C, $n = 88$) and a diabetic group (DM, $n = 88$). STZ (Sigma, St. Louis, MO, USA) was freshly prepared before each use at 20 mg/ml, with 0.1 M pH 4.4 citrate buffer and was injected at 200 mg/kg, i.p., into mice which had been fasted for 12 h prior to receiving the injection (Zhao et al., 2003; Shuli et al., 2001). Three days later, non-fasting blood glucose in a tail-vein sample was determined by a glucose analyzer; a value >15 mM/L was accepted as a successfully created diabetic model. C group mice received only citrate buffer injection. Each group was divided further into eight subgroups according to the different living time after STZ or citrate buffer administration, i.e. 2 h, 6 h, 24 h, 72 h, 1 week, 4 weeks, 8 weeks and 12 weeks subgroups.

2.2. Morris water maze test

The Morris water maze test was performed as described at 4, 8 and 12 weeks after STZ or citrate buffer was given (Zhang et al., 2006). This process of Morris water maze test consisted of 5 days learning-memory training. The animals were trained in a circular pool (120 cm in diameter) located in a lit room with visual cues. An escape platform (9.5 cm in diameter) was submerged 1.0 cm below the surface of the pool water, which was maintained at $23 \pm 2\,^{\circ}C$. The location of the platform remained in the center of northwest quadrant throughout the test. On each day, mouse was trained for one morning block and one afternoon block. Each block consisted of two trials, and each trial lasted for 120 s or ended soon if the mouse reached the submerged platform, thus escaping from the water maze. Before the first trail, each mouse was put on the platform for 15 s, followed with a 60 s free swim and then was assisted to the platform where it remained for another 15 s rest. Then the test began: it was released into the water facing pool's wall, in turn of north, south, east and west for each trial. Whether a mouse found or failed to find the platform within 120 s, it was placed on the platform for 15 s. The escape latencies from the water maze (finding the submerged escape platform) were recorded and calculated by a computerized video system. Learning-memory ability was assessed by the escape latencies taken by the mice to find the platform.

2.3. Immunohistochemical analysis

After the mice were anesthetized with chloral hydrate injection (4 ml/kg, i.p.), the chest was cut open. A fast injection of normal saline was given through a catheter placed in the left ventricle; at the same time the right auricle was cut open. When the liver turned white from bleeding, 4% paraformaldehyde was infused until rigor mortis supervened. The brain was removed and fixed in a solution containing 30% sucrose and 4% paraformaldehyde. After the brain had sunk to the bottom, 40 μm cryostat sections were prepared on a freezing microtome (CM1900, Leica, Germany). Immunohistochemistry were performed as previously described (Zhao et al., 2003; Shuli et al., 2001). The primary antibodies used were rabbit anti-GRP78 polyclonal antibody (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and rabbit anti-CHOP polyclonal antibody (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). SP immunohistochemical stain kit was from Zymed Laboratories Co. (San Francisco, CA, USA). The tests were performed according to the instruction sheet. Negative controls were treated with normal serum instead of the primary antibody.

For quantitative analysis of immunohistochemistry, images were captured by using Nikon 80i microscope. Four brains for each experimental subgroup were measured. Every 3rd section was selected (total = 6 sections per brain). Three fields

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