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High-glucose induces tau hyperphosphorylation through activation of TLR9-P38MAPK pathway

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ABSTRACT

Diabetic encephalopathy (DE) is one of the most common complications of diabetes. The major pathological variations include neurofibrillary tangles (NFTs), which are caused by tau hyperphosphorylation, and senile plaques (SPs) consisting of amyloid β- protein(Aβ) deposits. In recent years, DE research studies have focused on exploring the activation of the inflammatory signaling pathway in immune cells. Toll-like receptor 9 (TLR9) is well known to regulate the inflammatory reactions in immune processes. During the tau hyperphosphorylation process, TLR9 in microglia plays bidirectional roles. However, no studies have clearly demonstrated the relationship between TLR9 and tau hyperphosphorylation in neurons. Based on our experiments, we found significant increase in TLR9 expression in neurons and an increase in tau hyperphosphorylation in high-glucose media. However, these alterations can be reversed by TLR9 inhibitor. Furthermore, we specifically inhibited the activation of P38mitogenactivated protein kinase(P38MAPK) and found an effective decrease in tau hyperphosphorylation. This effect is likely related to Unc93b1. Meanwhile, High glucose levels can induce neuronal apoptosis via the TLR9 signaling pathway.

Our studies are the first to reveal that high glucose can regulate tau hyperphosphorylation and neuronal apoptosis via TLR9-P38MAPK signaling pathway. These findings provide a new method for studying the mechanism underlying DE.

1. Introduction

Diabetes mellitus (DM) is one of the most common metabolic disorders and has become the third most prevalent non-infectious disease after tumors and cardiovascular diseases. Many studies have shown that diabetic complications not only include infections, cardiovascular injury, and nephropathy but also include lesions in the central nervous system, i.e., Diabetic encephalopathy (DE). Cognitive dysfunction, which is unrelated to the type of diabetes and the educational level of the patients, is the most significant clinical symptom of DE. Previous studies have demonstrated that diabetes significantly increases the incidence of dementia [1]. Because learning and memory impairments are the main features of cognitive dysfunction, a great number of the studies have focused on the correlation between DE and Alzheimer's disease (AD) [2]. The clinical and molecular biological findings have demonstrated that the pathophysiological changes that occur in diabetes are very similar to those that occur in AD in terms of biochemistry, ultrastructure, electrophysiology and praxeology. Despite these findings, the mechanism underlying DE remains unclear. Currently, the mechanisms underlying DE are thought to involve tau hyperphosphorylation, amyloid β-protein (Aβ) deposition and cerebrovascular atherosclerosis [3].

Based on our experiments, the hyperphosphorylation of the tau protein is hypothesized to be a leading cause of the cognitive impairment in diabetic patients. The tau protein, which is a microtubule-associated protein (MAP), plays a role in the regulation of the microtubule assembly, stabilization of cell structure and promotion of axonal transport. Once tau proteins are hyperphosphorylated, they no longer possess the ability to bind microtubules and begin to abnormally accumulate. Eventually, the abnormal accumulation of tau proteins generates neurofibrillary tangles (NFTs). This phenomenon occurs not only in DE but also occurs in AD [4,5]. Recent studies on brain functioning have shown that Aβ is a trigger of AD, and the abnormal

Abbreviations: DM, diabetes mellitus; DE, diabetic encephalopathy; AD, Alzheimer's disease; NFTs, neurofibrillary tangles; SPs, senile plaques; Aβ, amyloid β-protein; TLR9, toll-like receptor 9; P38MAPK, P38mitogen-activated protein kinase; MAP, microtubule-associated protein; MEM, minimum essential medium; FBS, fetal bovine serum; ECL, enhanced chemiluminescence; rTdT, terminal deoxynucleotidyl transferase recombinant enzyme; mtDNA, mitochondrial DNA; MCP-1, monocyte chemotactic protein-1; ER, endoplasmic reticulum

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modification of tau proteins is more closely associated with the clinical cognitive decline [6]. Although there is no direct evidence implicating the NFTs as the cause of the neurodegeneration, the NFTs can impair cognition by inducing neuronal apoptosis [7]. An abnormal phosphorylation of tau proteins is found in the brains of mice with DM. However, compared with the control group, tau knockout mice do not display NFTs or a loss of learning and memory after an injection of streptozotocin [8]. These results have proven that the abnormal phosphorylation of tau proteins is critical in the cognitive function decline in patients with DM. Therefore, further investigations regarding the mechanism underlying the hyperphosphorylation of tau proteins can lead to a better understanding and treatment of DE.

Toll-like receptor 9 (TLR9) belongs to the TLR family and is critically involved in the regulation of the innate immunity. TLR9 primarily senses the unmethylated CpG motif. The structure of this type of motif exists in bacterial DNA and eukaryotic mitochondrial DNA (mtDNA). Previous studies have found that mtDNA can activate TLR9 to amplify inflammation, aggravate heart failure, cause sepsis and induce adaptive immune disorders that are related to the circadian rhythm [9]. In addition to its participation in the regulation of immune inflammatory reactions, TLR9 can adjust the metabolism of non-immune cells. For example, it has been previously reported that mature neurons can reduce their cell energy metabolism to survive by up regulating the TLR9-AMPK signaling pathway in the presence of acute hypoxia [10,11]. This finding was the first to demonstrate that non-immune cells can regulate and control TLR9 in response to acute destructive stimuli but not through the inflammatory signaling pathway. Thus far, no study has been carried out to explore whether chronic hyperglycemia can modify the hyperphosphorylation of tau proteins and induce neuronal apoptosis by activating the TLR9 signaling pathway. To explore this scientific question, we cultivated mature neuronal cells in a high-glucose medium and detected the level of tau protein phosphorylation and the expression of potential biological molecules that are involved in the TLR9 signaling pathways. Based on the results, we found a high-glucose medium up regulates the expression of TLR9 in neurons, which is accompanied by an accumulation of tau hyperphosphorylation. This process is dependent on P38 mitogen-activated protein kinase (P38MAPK).

2. Experimental procedures

2.1. Cell culture

All animal experimental procedures were approved following the laboratory animal ethical review and complied with the Guide for the Care and Use of Laboratory Animals, NRC, 2011. The laboratory mice were purchased from the Animal Experimental Center of Chongqing Medical University on postnatal day 1. The experiments were approved by the ethics committee of Chongqing Medical University.

The culture method for the primary hippocampal neurons followed a previously described protocol with some modifications [12]. The hippocampus was dissected in a pre-cooling D-Hank's solution. Then, the hippocampus was digested in papain at 37 °C for 30 min and triturated with 1-mL pipette every 10 min. A minimum essential medium (MEM, Gibcon BRL Co, Ltd., USA) with 5% fetal bovine serum (FBS, Gibcon BRL Co, Ltd., USA) and 0.6% glucose (Sigma-Aldrich Co, Ltd., Darmstadt, Germany) was added to stop the cell dissociation. After the centrifugation, the cells were resuspended and plated onto a poly-L-lysine-coated(0.1 mg/mL, Sigma-Aldrich Co, Ltd., Darmstadt, Germany) 6-well culture plate. The proper cell density was 2×10^5 cells per well. After 4 h, the medium was replaced with the maintenance medium. The composition of the maintenance medium includes a neurobasal-A medium (Gibco BRL Co, Ltd., USA) supplemented with B27(2%, Gibco BRL Co, Ltd., USA), glutamine(0.5 mM) and Hepes. Then, half of the solution was replaced every 3 days. After 10 days, the neuronal cells were collected for the subsequent analyses.

2.2. Cell treatment

A 35 mM concentration of glucose can induce hyperglycemic stress, reactive oxygen species (ROS) generation and cell damage. The concentration of glucose in the neurobasal-A solution is 25 mM, which meets the requirement for the basic energy metabolism of neurons in vitro. We added 25 additional mM of glucose to the neurobasal-A solution to mimic hyperglycemia, which is defined as a ≥ 1.4-fold increase in the blood glucose concentration of a man diagnosed with diabetes [13]. After 8 days, half of the medium was replaced with a fresh medium, and the cells were incubated with 25 mM of glucose (yielding a total 50 mM glucose) or 25 mM mannitol (plus 25 mM glucose in the normal medium), which was used as an osmotic control for 3 days. To determine the roles of the TLR9/P38MAPK signaling pathway in the pathogenesis of DE, the cells were pretreated with 5 μM of ODN2088(ODN; InvivoGen, Thermo Fisher Scientific Inc., Waltham, USA) or 20 μM of SB203580 (SB; Millipore Co, Ltd., USA) for an additional 3 days. Then, the neurons were used for the biochemical analyses.

2.3. Western blot analysis

We followed a previously described western blot protocol with some modifications [14]. Briefly, the extracted proteins were washed 3 times with PBS and were then lysed on ice. The lysate was a RIPA buffer with an EDTA-free protease and a phosphatase inhibitor cocktail tablet (Roche Applied Co, Ltd., CH). The samples were centrifuged at 1.2×10^4 rpm for 20 min at 4 °C. According to the molecular weight of the target molecule, all proteins were separated on 10% SDS-PAGE gels and were transferred to the PVDF membrane. The blots were blocked and then incubated with the appropriate primary antibodies overnight at 4 °C. After 3 washing cycles with T-TBS, the membrane was incubated with the peroxidase-conjugated secondary antibody for 1 h at 37 °C. Then, the protein band intensities were evaluated using Scion Image software (Scion Corporation, Frederick, MD, USA). Actin was always used as an internal loading control. The primary antibodies included TLR9 (1:500, Imgenex, Novus, USA, IMG-305A), P38MAPK (1:1000, Cell Signaling Technology Co, Ltd., Massachusetts,US, 8690), Phospho-P38MAPK(1:1000, Cell Signaling Technology, Co, Ltd., Massachusetts,US, 9216), Tau-5(1:1000, Abcam Co, Ltd., Cambridge, UK, ab80579), AT8 (1:1000, Pierce Biotechnology, Thermo Fisher Scientific Inc., Waltham, USA, MN1020), PHF13 (1:1000, Cell Signaling Technology, Co, Ltd., Massachusetts, US, 9632), PHF1 (1:1000, Abcam Co, Ltd., Cambridge, UK, ab92676), UNC93B1 (1:200, Sigma-Aldrich Co, Ltd., Darmstadt, Germany, AV49979), IkBα (1:1000, Cell Signaling Technology, Co, Ltd., Massachusetts,US, 4814),NF-κB p65 (1:500, Abcam Co, Ltd., Cambridge, UK, ab32536), caspase3 (1:1000, Gene Tex,Inc., California, USA, GTX110543), and procaspase 3(1:500, Abcam Co, Ltd., Cambridge, UK, ab32150). The horseradish peroxidase labeled secondary antibodies(1:200, Abbkine Scientific Co., Ltd, California, USA) and an enhanced chemiluminescence (ECL) Kit were used.

2.4. Immunofluorescent staining

Cell immunostaining was performed as previously described [15]. Briefly, after the intervention, the neurons were washed softly with PBS 3 times and then fixed in 4% paraformaldehyde for 15 min at room temperature. After rinsing 3 times with PBS, the cells were incubated in a blocking solution (0.2% Triton-100) for 15 min at room temperature and then with primary antibodies against MAP2 (1:500, Sigma-Aldrich Co, Ltd., Darmstadt, Germany, M1406) and TLR9 (1:100, Imgenex, Novus, USA) overnight at 4 °C. After several washings with PBS, the cells were incubated for 1 h with different fluorescein-labeled secondary antibodies.

The TUNEL assay was carried out following the manufacturer's instructions (TUNEL FITC Apoptosis Detection Kit, Vazyme Biotech