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PROTECTIVE EFFECT OF LITHIUM CHLORIDE AGAINST HYPOGLYCEMIA-INDUCED APOPTOSIS IN NEURONAL PC12 CELL

YUZHEN XU,[a] QIAN WANG,[b] DONGSHENG LI,[c] ZHENGHUA WU,[c] DAWEI LI,[c] KAILI LU,[a] YUWU ZHAO[a]* AND YONGNING SUN[d]

[a] *Department of Neurology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China*

[b] *Department of Central Laboratory, The Central Hospital of Tai'an, Taishan Medical College, Tai'an, Shandong Province, China*

[c] *School of Pharmacy, Shanghai Jiao Tong University, Shanghai, China*

[d] *Department of Traditional Chinese Medicine, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China*

Abstract—Hypoglycemia is defined by an arbitrary plasma glucose level lower than 3.9 mmol/L and is a most common and feared adverse effect of treatment of diabetes mellitus. Emerging evidences demonstrated that hypoglycemia could induce enhanced apoptosis. Lithium chloride (LiCl), a FDA approved drug clinically used for treatment of bipolar disorders, is recently proven having neuroprotection against various stresses in the cellular and animal models of neural disorders. Here, we have established a hypoglycemia model *in vitro* and assessed the neuroprotective efficacy of LiCl against hypoglycemia-induced apoptosis and the underlying cellular and molecular mechanisms. Our studies showed that LiCl protects against hypoglycemia-induced neurotoxicity *in vitro*. Exposure to hypoglycemia results in enhanced apoptosis and the underlying cellular and molecular mechanisms involved inhibition of the canonical Wnt signaling pathway by decreasing wnt3a levels, β-catenin levels and increasing GSK-3β levels, which was confirmed by the use of Wnt-specific activator LiCl. Hypoglycemia-induced apoptosis were significantly reversed by LiCl, leading to increased cell survival. LiCl also alters the expression/levels of the Wnt pathway genes/proteins, which were reduced due to exposed to hypoglycemia. Overall, our results conclude that LiCl provides neuroprotection against hypoglycemia-induced apoptosis via activation of the canonical Wnt signaling pathway.

Key words: hypoglycemia, apoptosis, lithium chloride, wnt signaling pathway.

INTRODUCTION

Hypoglycemia is defined by an arbitrary plasma glucose level lower than 3.9 mmol/L and is a most common and feared adverse effect of treatment of diabetes mellitus with insulin and sulphonylureas (Hompesch et al., 2015). The incidence of serious hypoglycemia was 2.76 episodes per 100 person-years in those treated with insulin and 1.23 episodes per 100 person-years for patients treated with sulfonylureas (Abdelhafiz et al., 2015). Episodes of hypoglycemia are particularly associated with an increased risk of mortality, impairments in cognitive function, and a decreased quality of life in diabetic patients (Morales and Schneider, 2014). Nowadays diabetes is one of the leading metabolic disorders for public health with the rapid growth of diabetic patients all over the world. It is estimated that there are 382 million people living with diabetes mellitus worldwide, and the number is about to increase by over 50% in the next 20 years (Kleinberger and Pollin, 2015). Increased diabetic population and attempts made at intensive glycemic control invariably increases the risk of hypoglycemia, which has become a major barrier to better glycemic control and a tremendous economic and social burden (Nathan, 2015). The pathological process of hypoglycemia-induced neurological damage is complex, thus searching for potential mechanisms and developing prophylaxis and treatments are top priorities.

Lithium chloride (LiCl) is a US Food and Drug Administration (FDA) approved drug extensively used as a mood stabilizer in the manic depressive disorders treatment for over half a century (Hao et al., 2015). LiCl can also induce the accumulation of β-catenin and enhance the activation of canonical Wnt pathway, which first identified in 1982 plays an important role in cells differentiation, communication, survival, proliferation and apoptosis (Kahn, 2014). A host of studies indicate that LiCl can modulate diverse biologic processes, such as tumorigenesis, gene expression and inflammation (Marchetti and Pluchino, 2013). Although the beneficial effects of LiCl therapy have been known for decades, the mechanism remains poorly understood. Moreover, Lithium has recently emerged as a robust neuroprotective agent that prevents apoptosis of neurons under various stresses (Meffre et al., 2014). However, whether LiCl can protect neurons from apoptosis under hypoglycemia condition is yet unclear.

*Corresponding author. Address: Department of Neurology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, 600 Yishan Road, Xuhui District, Shanghai, China. Tel: +86-18930177606. fax: +86-021 64701361.

E-mail address: zhao_yuwu2005@126.com (Y. Zhao).

Abbreviations: AD, Alzheimer's disease; AMBMP, 2-amino-4-(3,4-(methylenedioxy)benzylamino)-6-(3-methoxyphenyl)pyrimidine; DMEM, Dulbecco's Modified Eagle Medium; LDH, lactate dehydrogenase; LiCl, Lithium chloride; NGF, nerve growth factor; PBS, phosphate buffered saline; PVDF, polyvinylidene difluoride.

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PC12 is a clonal cell line of rat pheochromocytoma cells, which responses to nerve growth factor (NGF) and then acquires the appearance of neurons (Westerink and Ewing, 2008). It is a useful model for studying neural differentiation, cell signaling and neurochemistry (Greene, 2013). Thus, a well-defined cell system for *in vitro* studies of glucose neurotoxicity can be provided by the PC12 cell line. In our present study, to produce a hypoglycemic insult, PC12 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with no glucose to mimic the hypoglycemia, which is similar to the severe hypoglycemic episodes most frequently occurred in a patient under antidiabetic therapies.

Various studies demonstrated that hypoglycemia could induce increased apoptosis (Languren et al., 2013). Interestingly, the association between canonical Wnt pathway and apoptosis-dependent cellular death was also widely reported. In addition, LiCl, one of canonical Wnt pathway agonists, has emerged as a neuroprotective agent efficacious in preventing diverse stresses. We hypothesized that LiCl might attenuate hypoglycemia-induced apoptosis in PC12 cells via canonical Wnt pathway. The aim of this study was to test the hypothesis and LiCl may be a potential therapy for reducing neurological damage caused by hypoglycemia.

EXPERIMENTAL PROCEDURES

Cell culture and drug treatment

The neuronal differentiated PC12 cells was kindly donated from Professor Weilin Jin (Shanghai Jiao Tong University, Shanghai, China) and cultivated in DMEM 0 (no glucose DMEM, catalog No. 11966025) or DMEM 25 (25 mM glucose DMEM, catalog No. 11965092) (Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 50 ng/mL NGF (Sigma, St Louis, MO, USA), 1% glutamine (Invitrogen, Carlsbad, CA, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin (Invitrogen) in a humidified atmosphere of 5% CO_2 in air at 37 °C. PC12 cells were firstly cultivated in DMEM 25 to logarithmic phase and then incubated for 6 h continuously varying exposures: (a) DMEM 25 (normal glucose group, control); (b) DMEM 0, mimic the hypoglycemia (low glucose group, LG); (c) DMEM 0 treated with LiCl (Sigma, St Louis, MO, USA) at 0.1–50 μM concentrations (LiCl treatment group, LG + LiCl). (d) DMEM 0 treated with 10 μM Wnt agonist (2-amino-4-(3,4-(methylenedioxy)benzylamino)-6-(3-methoxyphenyl)pyrimidine) (AMBMP) (Sigma, St Louis, MO, USA) (Wnt agonist treatment group, LG + Ag). (e) DMEM 0 treated with 10 μM LiCl and 100 ng/ml Dickkopf-1 (Dkk1), an antagonist of canonical Wnt signaling pathway (Sigma, St Louis, MO, USA) (Wnt antagonist treatment group, LG + LiCl + Antag). Mannitol (25 mmol/L) (Sigma, St Louis, MO, USA) was also used as negative control for osmolarity. After 6 h of continuous exposures exponentially growing PC12 cells were used as indicated for all experiments immediately, and every experiment was repeated three times. PC12 cells were plated at an appropriate density according to each experimental scale and were fed every other day and passed twice a week.

MTT assay

Cell viability was measured using MTT assay (Beyotime Biotech, Jiangsu, China) as previously described (Rizak et al., 2013). Briefly, PC12 cells were seeded into 96-well plates with 5000 cells in each well, allowed to adhere and incubated overnight. After incubation, PC12 cells were divided into different groups and exposed to accordingly stresses for 6 h, then treated with the 0.5 mg/ml MTT solution for 4 h at 37 °C. After removal of the MTT solution, PC12 cells were treated with 150-μl dimethylsulfoxide (DMSO) to dissolve the dark blue formazan crystals and the plates were shaken for 10 min. Absorption was measured using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 570 nm. Survival of the control groups was defined as 100%, and the viability was calculated as the mean OD of one group/mean OD of the control.

Lactate dehydrogenase (LDH) release assay

After PC12 cells incubated overnight and exposed to hypoglycemia in the presence or absence of LiCl at the various concentrations for 6 h, the medium was collected and LDH activity was determined using a commercially available assay kit (Beyotime Biotech, Jiangsu, China) according to the manufacturer's protocol (Wani et al., 2014). In brief, at the end of the drug treatment, the medium was collected and 100 μl of it was added to 1 mL of LDH reagent. The damage of the PC12 cells was assessed by measuring the release of LDH into culture medium. The absorbance of sample was measured at 490 nm. LDH release of the control groups was defined as 100%, and the cytotoxicity in the treated groups was expressed as a percentage of the control groups.

Real-time PCR analysis

Total RNA was extracted from cultured PC12 cells using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Synthesis of cDNA was performed using 1 μg of total RNA using ReverTra Ace qPCR RT Master Mix (Toyobo Co., Osaka, Japan). Five hundred nanograms of cDNA were amplified by a 20-μl Mastermix (Toyobo Co., Osaka, Japan) containing dNTPs, MgCl2 and the SYBR Green reporter dye along with specific primers (Table 1). Real-time PCR was conducted using an ABI StepOnePlus Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). The relative expression levels of target genes were calculated as $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct_{targetgene} - Ct_{\beta\text{-actin}}$ and $\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{control}$.

Western blotting analysis

Protein was extracted from cultured PC12 cells with RIPA lysisbuffer (containing 0.1% PMSF) (Beyotime Biotech, Jiangsu, China) based on the manufacturer's