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# GLUCAGON-LIKE PEPTIDE-1 PROTECTS HIPPOCAMPAL NEURONS AGAINST ADVANCED GLYCATION END PRODUCT-INDUCED TAU HYPERPHOSPHORYLATION

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**Abstract—We have previously demonstrated that glucagon-like peptide-1 (GLP-1) receptor agonist ameliorated neurodegenerative changes in rat models of diabetes-related Alzheimer's disease (AD), and protected neurons from glucose toxicity *in vitro*. Herein, we investigated the effects of GLP-1 receptor mediates on cell toxicity and tau hyperphosphorylation induced by advanced glycation end products (AGEs), which are associated with glucose toxicity, and the molecular mechanism in PC12 cells and the primary hippocampal neurons. Our study demonstrated that the similar protection effects of GLP-1 existed in PC12 cells treated with glucose–bovine serum albumin (BSA) in hyperglycemic conditions or with glycoaldehyde–BSA alone. Additionally, glucose–BSA alone did not induce significant cytotoxicity in PC12 cells, but resulted in tau hyperphosphorylation in primary hippocampal neurons in 24 h. And we found that GLP-1 could reduce cell tau phosphorylation induced by high glucose or glucose–BSA. Furthermore, our data in the present study suggested that GLP-1 regulated tau phosphorylation induced by AGEs through a signaling pathway involving glycogen synthase kinase 3β (GSK-3β), similarly to the GSK-3β inhibitor, lithium chloride. Our findings suggest that GLP-1 can protect neurons from diabetes-associated AGE insults *in vitro*, and provide new evidence for a potential therapeutic value of GLP-1 receptor agonist in the treatment of AD especially diabetes-related AD.**



**Key words: diabetes-related Alzheimer's disease, glucagon-like peptide-1, advanced glycation end products, high glucose, tau, glycogen synthase kinase 3β.**

## INTRODUCTION

There is a growing body of evidence suggesting that type 2 diabetes mellitus (DM) is associated with changes in Alzheimer's disease (AD), even called type 3 diabetes. Ott et al. (1999) reported that having type 2 diabetes doubled the risk of developing dementia and the risk increased fourfold in patients who needed insulin treatment in an initial Rotterdam study. Furthermore, in the studies of Janson et al. (2004), it was shown that patients with AD were also more vulnerable to type 2 diabetes, and 81% of cases of AD had either type 2 diabetes or impaired fasting glucose (IFG). Several physiopathological features of diabetes including hyperglycemia, oxidative stress and dysfunctional insulin signaling affect several organs including the brain, and increase the risk of central nervous system (CNS) disease including AD.

Numerous studies are of great importance in the field dealing with the beneficial effects of glucagon-like peptide-1 receptor (GLP-1R) activation in neurodegenerative diseases. In the initial study, protective results have been reported in PC12 cells, showing the effects of glucagon-like peptide-1 (GLP-1) and its long-acting analog exendin-4 (Ex-4) on β-amyloid, glutamate and growth factor challenge (Perry et al., 2002, 2003). Furthermore, in our previous study, we have reported similar protective results against high glucose- as well as oxidative stress (hydrogen peroxide)-induced PC12 cell injury (Chen et al., 2012). Several animal model studies have also shown the potential efficacy of Ex-4 therapy in kinds of nervous system diseases. GLP-1R stimulation improved functional outcomes in well-characterized cellular and animal models of both stroke and Parkinson's disease (Li et al., 2009). Li et al. (2010) has also reported that Ex-4 proved to be highly effective on Alzheimer transgenic mice with and without STZ-induced diabetes. In a mouse model of Huntington's disease, it was confirmed that Ex-4 treatment could improve survival

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*Abbreviations:* Aβ, amyloid β-peptide; AD, Alzheimer's disease; AGEs, advanced glycation end products; APP, amyloid precursor protein; BSA, bovine serum albumin; DM, diabetes mellitus; DMSO, dimethyl sulfoxide; DPP-IV, dipeptidyl peptidase IV; Ex-4, exendin-4; FBS, fetal bovine serum; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor; GLP-1RA, glucagon-like peptide-1 receptor agonist; GSK-3β, glycogen synthase kinase-3β; HG, high glucose; ICV-STZ, intracerebroventricular-streptozotocin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; NFTs, neurofibrillary tangles; PBS, phosphate buffer saline; PHF, paired helical filament; PI3-K, phosphatidyl inositol 3 kinase; RAGE, receptor for advanced glycation end products.

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(Martin et al., 2009). *In vivo* (SOD1 G93A mutant mice) models of amyotrophic lateral sclerosis (ALS) mice treated with Ex-4 showed normalization of behavior compared to the control, and Ex-4 proved to be neuroprotective in cell culture (NSC-19 neuroblastoma cells) models of ALS (Li et al., 2012a).

In our previous study, we have demonstrated that glucagon-like peptide-1 receptor agonist (GLP-1RA) can protect neurons from high glucose or hydrogen peroxide insults *in vitro* and from intracerebroventricular-streptozotocin (ICV-STZ) insult *in vivo* (Chen et al., 2012). Advanced glycation end products (AGEs) are formed by a series of nonenzymatic modification reactions between amino groups on proteins with glucose in the diabetics, and the effect of hyperglycemia on cell dysfunction is additionally mediated by accelerated formation of AGEs after long-term exposure. Recently, Li et al. (2012b) have demonstrated in their study that AGEs can induce tau hyperphosphorylation through receptor for advanced glycation end product (RAGE)-mediated glycogen synthase kinase 3 (GSK-3) activation and targeting RAGE/GSK-3 pathway can improve AD-like changes. And we have already demonstrated that GLP-1RA reversed ICV-STZ-induced tau hyperphosphorylation through down-regulation of GSK-3β activity *in vivo* (Chen et al., 2012). However, until now, it is not known whether GLP-1R mediates the down-regulation effects on AGE-induced tau hyperphosphorylation and the molecular mechanism remains poorly understood. In the present experiments, we studied the effects of GLP-1/Ex-4 on neuronal cell toxicity induced by glycoaldehyde–bovine serum albumin (BSA) or glucose–BSA *in vitro*, and the effects of GLP-1RA on tau hyperphosphorylation induced by high glucose or AGEs as well as the molecular mechanisms involved.

## EXPERIMENTAL PROCEDURES

### Preparation of AGEs

AGEs were prepared by dissolving 50 mg/ml BSA with 0.5 mol/L D-glucose in phosphate buffer saline (PBS, pH 7.4) under sterile conditions, and the mixture was incubated at 37 °C in darkness for 90 days (Makita et al., 1992; Li et al., 2012b). The control BSA was incubated under the same conditions but without sugar. After incubation, the free glucose was removed by dialysis against 0.01 mol/L PBS (pH 7.4) at 4 °C for 72 h and then stored at −60 °C.

### Cell culture

PC12 cells were cultured and treated as described by Qian et al. (2008). Briefly, PC12 cells were maintained in Dulbecco's modified Eagle's medium (Gibco Life Technologies, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin at 37 °C in a water-saturated 5% $CO_2$ atmosphere.

The primary hippocampal neurons were prepared and cultured from day E18 embryos of Wistar rats. After mechanical dissociation, cells were seeded on 6-well plates coated with poly-D-lysine (50 μg/ml) at a density of $9 \times 10^5$ cells/well in Dulbecco's modified Eagle's medium supplemented with 10% FBS. After 4 h, the medium was then changed to Neurobasal/B27 medium (Gibco Life Technologies) containing L-glutamine (0.5 mM). Cell cultures were incubated at 37 °C, 5% $CO_2$ for 7 days and half of the medium was replaced every 2–3 days. At 8 days, the primary hippocampal neurons were treated with BSA, glucose–BSA, glucose–BSA plus Ex-4 or GLP-1 (100 nM; TASH Biotechnology Co., Ltd., Shanghai, China), or glucose–BSA plus LiCl (4 mM; Sigma, St. Louis, MO, USA) for 24 h. For studying the mechanism of Ex-4/GLP-1 effects and involvement of the related signaling pathways, the primary hippocampal neurons were pretreated with wortmannin (20 nM; Beyotime Institute of Biotechnology, Shanghai, China) for 30 min.

### Cell viability assay

Cell viability was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma) assay. Briefly, PC12 cells were seeded on flat-bottomed 96-well plates in indicated medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin. After overnight incubation, cells were treated with glycoaldehyde–BSA (Merck, Darmstadt, Germany)/glucose–BSA or high glucose, or both, in the absence and presence of Ex-4 or GLP-1. At the 24-h time point post-treatment, culture medium was removed and MTT was added to the wells at a concentration of 5 mg/ml dissolved in PBS. After incubation at 37 °C for 4 h, the MTT solution was removed and 150 μl of dimethyl sulfoxide (DMSO) was added to each well to solubilize the reduced dye. Absorbance was measured at the wavelength of 570 nm with 630 nm as reference wavelength.

### Western blot analysis

Protein concentrations of the extracts of PC12 cells or the primary hippocampal neurons were determined with the BCA Protein Assay kit (Beyotime Institute of Biotechnology). The protein samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to Nitrocellulose Membranes (Amersham International, Buckinghamshire, UK). After preincubating for 2 h at 37 °C in 3% BSA blocking buffer, the membranes were incubated with thr205-phosphorylated tau antibody (Invitrogen, California, USA), thr181-phosphorylated tau-, β-actin-specific antibodies (Santa Cruz Biotechnology, California, USA), total tau-, total GSK-3β-, and ser9-phosphorylated GSK-3β-specific antibodies (Cell Signaling Technology, Beverly, MA, USA). Immunoreactive bands were then detected by incubation with conjugates of secondary antibody with horseradish peroxidase and enhanced chemiluminescence reagents (Amersham International) at room temperature.