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GLUCAGON-LIKE PEPTIDE-1 REGULATES MITOCHONDRIAL BIOGENESIS AND TAU PHOSPHORYLATION AGAINST ADVANCED GLYCATION END PRODUCT-INDUCED NEURONAL INSULT: STUDIES *IN VIVO* AND *IN VITRO*

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Abstract—Our previous study has proved that glucagon-like peptide-1 (GLP-1), which is developed to treat type 2 diabetes, has a significant effect on neuroprotection against advanced glycation end product (AGE)-induced neuronal insult *in vitro* models of diabetes-related Alzheimer's disease (AD). However, the molecular mechanisms remain to be elucidated and it is not clear whether GLP-1 receptor mediates the down-regulation effects on AGE-induced AD-like changes *in vivo*. This study aims to explore the effect and mechanisms of GLP-1 receptor agonists (GLP-1RA) against the AGE-dependent signaling pathway both *in vitro* and *in vivo*. In this study, we demonstrated that GLP-1RA could inhibit oxidative stress and repair mitochondrial damage in addition to decreasing tau hyperphosphorylation in PC12 cells treated with AGEs. Importantly, we first observed AGEs in the circulatory system could induce tau hyperphosphorylation after we injected AGEs (1 μg/kg bodyweight) into the mice tail vein. We found GLP-1RA could promote mitochondrial biogenesis and antioxidant system via regulating peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) signaling pathway *in vivo* besides down-regulating the activity of glycogen synthase kinase 3β (GSK-3β) to reverse tau hyperphosphorylation directly. Collectively, our results suggest that GLP-1RA protects neurons against AGE-induced tau hyperphosphorylation via regulating GSK-3β and PGC-1α two cooperative signaling pathways.

Key words: glucagon-like peptide-1, advanced glycation end products, tau pathology, reactive oxygen species, Mitochondrial biogenesis.

INTRODUCTION

Alzheimer's disease (AD) and diabetes mellitus (DM) are two of the most prevalent degenerative diseases. Accumulating evidence implies a close biological relationship between type 2 diabetes and AD (Frisardi et al., 2010; Profenno et al., 2010; Takeda et al., 2010). AD has been even referred to by some as type 3 diabetes (Kroner, 2009). In a carefully controlled community study of Janson et al. it was found that more than 80% of an unselected group of AD patients had either type 2 diabetes or dysglycemia (Janson et al., 2004). Both AD and DM are associated with increased oxidative stress (Cooper, 2011) and production of the advanced glycation end products (AGEs). AGEs, as an important toxicity moiety of disturbed glucose metabolism, have been found in retinal vessels, peripheral nerves, kidneys, and the CNS of diabetics (Yamagishi et al., 2007). AGEs are also known to modify amyloid plaques and neurofibrillary tangles (NFTs), both implicated in AD (Valente et al., 2010). Substantial evidence supports the hypothesis that AGEs play a crucial role in linking diabetes with AD. AGEs can enhance amyloid β-peptide (Aβ) expression, which formed amyloid plaques (Ko et al., 2010). However, the link between AGEs and NFTs which are composed of the hyperphosphorylated tau remains elusive.

A growing body of evidence suggests that glucagon-like peptide-1 (GLP-1) and its long-acting analog exendin-4 (Ex-4) which have been developed to treat type 2 diabetes play an important role in neuroprotection. A study showed that chronic administration of the GLP-1 analog completely blocked the synaptic degradation that normally occured in a rat model of AD and rescued synaptic plasticity (McClean et al., 2010). Li et al. found that Ex-4 down-regulated the STZ-induced Aβ and AβPP elevations evident to normal levels of 3xTg-AD female mice brain, and showed a trend toward lowering brain Aβ (25% decrease) in

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Abbreviations: Aβ, amyloid β protein; AD, Alzheimer's disease; AGEs, advanced glycation end products; BSA, bovine serum albumin; CAT, catalase; DM, diabetes mellitus; Ex-4, exendin-4; GLP-1, glucagon-like peptide-1; GLP-1RA, glucagon-like peptide-1 receptor agonist; GSH-Px, glutathion peroxidase; GSK-3β, glycogen synthase kinase 3β; HRP, horseradish peroxidase; Mn-SOD, manganese superoxide dismutase; NFTs, neurofibrillary tangles; NRF-1, nuclear respiratory factor 1; PBS, Phosphate- buffered saline; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1α; PP2A, protein phosphatase-2A; RAGE, the receptor for AGEs; ROS, reactive oxygen species; TEM, transmission electron microscopy; Tfam, mitochondrial transcription factor A.

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non-diabetic 3xTg-AD mice (Li et al., 2010). Bomba et al. pointed that exenatide had different effects on cognitive performances in two AD transgenic mouse models: the PS1-KI mouse and the 3xTg-AD mouse. The positive cognitive effects were observed in exenatide-treated PS1-KI mice but not in 3xTg-AD mice (Bomba et al., 2013). The study of Bomba et al. is very novel because they confirmed exenatide may act as a cognitive enhancer and suggested exenatide was not so effective in 3xTg-AD mice, that more closely mimic the human disease. STZ is a drug used as a tool for creating experimental diabetes because of its selective toxicity for glucose transporter 2 (GLUT2)-expressing neuroendocrine cells. Based on different effects of Ex-4 in diabetic 3xTg-AD mice and 3xTg-AD mice, it suggests GLP-1RA may be of value particularly in diabetes-related AD. In our previous study, we have demonstrated that glucagon-like peptide-1 receptor agonist (GLP-1RA) had an effect on high glucose- or AGE-induced AD-like pathologies in PC12 cells or primary hippocampal neurons through a signaling pathway partly involving glycogen synthase kinase 3β (GSK-3β) (Chen et al., 2012, 2014), but the molecular mechanisms remain poorly understood, and it is not clear whether GLP-1 receptor mediates the down-regulation effects on AGE-induced AD-like changes *in vivo*.

Coupled with reactive oxygen species (ROS), AGEs cause oxidative stress (Nitti et al., 2005) and meanwhile oxidative stress would automatically cause AGEs, creating a vicious cycle (Sato et al., 2006). Since mitochondria are the major site of ROS production in the cell, they become the prime targets of oxidative damage, which formed oxidized damaged lipids, proteins and nucleic acids and lead to dysfunctional mitochondria (Cadenas and Davies, 2000). Accumulating evidence suggests that AD pathophysiology is closely associated with mitochondrial dysfunction (Takuma et al., 2005; Kopeikina et al., 2011). Takuma et al. reported that Aβ-binding dehydrogenase enhanced Aβ-induced cell stress via mitochondrial dysfunction (Takuma et al., 2005). Kopeikina et al. confirmed that tau accumulation caused mitochondrial distribution deficits in neurons (Kopeikina et al., 2011). Peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) has emerged as a master regulator of mitochondrial biogenesis (Sheng et al., 2012) and antioxidant defense system (Valle et al., 2005). Olson et al. reported that GSK-3β inhibition had been linked to PGC-1α stabilization and increased PGC-1α levels in primary neurons (Olson et al., 2008). This intriguing relationship between GSK-3β and mitochondrial biology points to a possibility to understand the molecular mechanisms of the effects of GLP-1 on AGE-induced AD pathology. Therefore in the present experiments, we studied the effects of GLP-1/Ex-4 on AGE-induced AD pathologies *in vitro* and *in vivo* as well as the molecular mechanisms involved.

EXPERIMENTAL PROCEDURES

Preparation of AGEs

AGEs were prepared as previously described (Chen et al., 2014). Briefly, 50 mg/mL bovine serum albumin (BSA) with 0.5 mol/L D-glucose were dissolved in phosphate-buffered saline (PBS, pH 7.4) under sterile conditions and incubated at 37 °C for 90 days in darkness. The control BSA was incubated without sugar under the same conditions. After incubation, the mixture was dialysed against 0.01 mol/L PBS (pH 7.4) at 4 °C for 72 h to remove the free glucose. Then, the remainder was stored at −80 °C.

Cell culture and treatments

PC12 cells were obtained from the Shanghai Institute of Cell Biology and cultured as previously described (Qian et al., 2008). Briefly, PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, NY, USA) containing 4.5 g/L D-glucose and supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies) and 1% penicillin–streptomycin at 37 °C in a water-saturated 5% CO_2 atmosphere. Cultured cells were treated with AGEs (500 μg/mL) in the absence and presence of Ex-4 or GLP-1 (100 nM; TASH Biotechnology Co., Ltd, Shanghai, China) for 48 h.

Animals and treatments

Male ICR mice (6 weeks, 30–33 g), supplied by Comparative Medicine Centre of the Yangzhou University, were randomly divided in the following groups ($n = 20$ in each group): control, AGEs and AGEs + Ex-4. AGEs (1 μg/kg bodyweight) were injected into the tail vein every 2 days and Ex-4 (25 nmol/kg bodyweight) was injected into the abdominal cavity twice daily. The saline solution was injected in the same way as the control. After the injection course, the mice were sacrificed and hippocampus samples were taken on the 16th day. In this study, surgical and animal care procedures were approved by the Institutional Animal Care and Use Committee in China Pharmaceutical University and performed by accepted veterinary standards.

Western blot and immunoprecipitation analysis

Protein content of the extracts of PC12 cells or the hippocampal tissues was analyzed using Western blots as previously described (Chen et al., 2012, 2014) with primary antibodies against thr205-phosphorylated tau-, ser396-phosphorylated tau-, β-actin-, total GSK-3β-, p-PP2A-, PP2Ac-, mitochondrial transcription factor A (Tfam)-specific antibodies (Santa Cruz Biotechnology, California, USA), thr181-phosphorylated tau-, ser9-phosphorylated GSK-3β-, total-tau-, nuclear respiratory factor 1 (NRF-1)-specific antibodies (Cell Signaling Technology, Beverly, MA, USA) and PGC-1α-specific antibody (Biovision, California, USA). Immunoreactive bands were treated with the appropriate species horseradish peroxidase (HRP)-conjugated secondary antibodies and immunological complexes were visualized by enhanced chemiluminescence reagents (ECL; Pierce, Rockford, IL, USA). For immunoprecipitation, protein content of the extracts of PC12 cells was incubated with primary antibody against GSK-3β at 4 °C for 1 h, followed