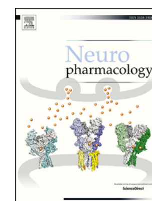
Neuropharmacology 131 (2018) 316–325

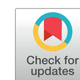
Contents lists available at ScienceDirect

Neuropharmacology

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



Liraglutide ameliorates cognitive decline by promoting autophagy via the AMP-activated protein kinase/mammalian target of rapamycin pathway in a streptozotocin-induced mouse model of diabetes



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ARTICLE INFO

Article history:
Received 9 August 2017
Received in revised form
22 December 2017
Accepted 2 January 2018
Available online 3 January 2018

Keywords:
Liraglutide
Autophagy
Diabetes
Neuronal apoptosis
Cognitive decline

ABSTRACT

Diabetic cognitive dysfunction has gained widespread attention for its deleterious impact on individuals with diabetes. However, few clinical interventions are available to prevent the disorder. The glucagon-like peptide-1 analog liraglutide exerts neuroprotective effects in several models of neurodegenerative diseases. We investigated the effect of liraglutide pretreatment on diabetes-induced cognitive decline and explored the underlying mechanisms *in vivo* and *in vitro*. Liraglutide pretreatment prevented diabetes-induced cognitive impairment as assessed by the Morris Water Maze test, and alleviated neuronal injuries and ultrastructural damage to synapses in the hippocampal CA1 region. Furthermore, liraglutide promoted autophagy as indicated by enhanced expression of the autophagy markers Microtubule-associated protein 1 light chain 3 (LC3)-II and Beclin 1, decreased expression of p62, and increased formation of autophagic vacuoles and LC3-II aggregates. *In vitro*, liraglutide treatment elevated phosphorylated (p)-AMP-activated protein kinase (AMPK) levels and reduced p-mammalian target of rapamycin (p-mTOR) expression. Additionally, the AMPK inhibitor Compound C exhibited an inhibitory effect on liraglutide-induced increased LC3-II expression and p62 degradation. Liraglutide exhibits neuroprotective effects against diabetes-induced hippocampal neuronal injuries and cognitive impairment by promoting autophagy via the AMPK/mTOR pathway.

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1. Introduction

Diabetes mellitus (DM), a common metabolic disorder attributable to aberrant insulin secretion and/or action, is characterized by hyperglycemia often accompanied by glycosuria, polydipsia, and polyuria (2014). Prevention of DM is performed worldwide, but its incidence continues to rise. DM is linked to chronic complications including nephropathy, angiopathy, retinopathy, and peripheral neuropathy.

Cumulative evidence has also indicated that both Type-1 (T1) and Type-2 (T2) DM, in different degrees, impair cognitive function, including learning and memory, psychomotor efficiency and attention (Biessels et al., 1994; Gispen and Biessels, 2000; Yates et al., 2012), and enhance the vulnerability to depression and anxiety (Dutheil et al., 2016; Zemdegs et al., 2016). Moreover, it has been determined that DM increases the risk of Alzheimer's disease (AD), vascular dementia, and any other type of dementia (Biessels et al., 2006), and aggravates learning and memory deficits in AD and vascular dementia (Ascher-Svanum et al., 2015; Kwon et al., 2015). DM-associated cognitive decline is gaining increasing attention for its deleterious impact on patients with DM. In addition to affecting daily functioning and quality of life, it is associated with an increased incidence of related complications and mortality (Sinclair et al., 2000). Studies in both experimental models and humans have confirmed the phenomenon and investigated its underlying mechanisms. However, few clinical interventions are available to prevent the disorder.

Glucagon-like peptide-1 (GLP-1) is a 30-amino acid endogenous incretin hormone, which is secreted by small-intestinal L cells in response to food ingestion and executes a variety of physiological roles related to the control of cell metabolism by binding to GLP-1

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https://doi.org/10.1016/j.neuropharm.2018.01.001
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receptors (GLP-1R) (Campbell and Drucker, 2013). GLP-1R stimulation not only enhances glucose-dependent insulin secretion and insulin gene expression in pancreatic β-cells, but also induces β-cells proliferation and inhibits β-cells apoptosis (Farilla et al., 2002; Salehi et al., 2010). Hence, novel long-acting GLP-1 analogues, such as exenatide and liraglutide, are currently used to treat T2DM (Drucker and Nauck, 2006; Lovshin and Drucker, 2009). Beyond the improvement in glycemic control, it has been demonstrated that GLP-1 and its analogues exert neurotrophic and neuroprotective actions in neurodegeneration and neurogenesis (Kamble et al., 2016; Gault and Holscher, 2008; Holscher, 2014; Hunter and Holscher, 2012; McClean et al., 2010; Solmaz et al., 2015). In addition to pancreas tissue, GLP-1 receptors are found on neurons in the brain, especially on pyramidal neurons in the hippocampus (Hamilton and Holscher, 2009; Larsen et al., 1997; Wei and Mojsov, 1995), a structure with considerable plasticity crucial for learning and memory (Kandel, 2001). Liraglutide, a long-acting analog of GLP-1, can cross the blood-brain barrier and bind to GLP-1R in the brain to exhibit neuroprotive effects (Hunter and Holscher, 2012). Reportedly, liraglutide administered peripherally stimulates neurogenesis, promotes cell proliferation, reduces cell apoptosis, and exerts beneficial effects on cognitive function and hippocampal synaptic plasticity in *vivo* and *vitro* studies (Li et al., 2015a,b; McClean et al., 2010; Porter et al., 2010).

Additionally, intrahippocampal injection of liraglutide also protects against amyloid-β protein induced impairment of spatial learning and memory (Han et al., 2013). These results confirmed the neuroprotective activities of liraglutide. Therefore, we hypothesized that liraglutide would attenuate DM-induced cognitive decline in mice.

In this study, we explored the effect and its mechanism of liraglutide on DM-induced neuronal apoptosis and cognitive impairment in a streptozotocin (STZ)-induced mouse model of DM and primary cultured mouse hippocampal neurons.

2. Materials and methods

2.1. Animals and study design

All animals were treated in accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals* (United States National Institutes of Health, Bethesda, MD, USA). All animal experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of Zhejiang University (Zhejiang, China). All efforts were made to minimize the number of animals used and their suffering.

Six-week-old healthy male C57BL/6 mice weighing 18–20 g were purchased from Zhejiang Academy of Medical Sciences (Zhejiang, China) for use in this study. Animals were housed in a room with a consistent temperature (21 ± 2 °C), humidity (40–60%), and light cycle (12/12 h light/dark) and access to food and water *ad libitum*. Animals were allowed to acclimate for 1 week before the experiments. Subsequently, animals were randomly divided into two groups: the control (Con Group $n = 15$) and STZ-injected ($n = 35$) groups. Animals were fasted for 12 h prior to injection. To induce DM, a freshly prepared solution of 10 mg/ml STZ (Sigma-Aldrich Corp., St. Louis, MO, USA) in 0.1 M citrate buffer (pH 4.5) was injected intraperitoneally into each mouse at a dose of 150 mg/kg. The control group was injected with the same dose of sodium citrate buffer. Blood glucose levels of animals were monitored at 72 h post STZ injection in a tail-vein sample via a blood glucose meter (OneTouch Horizon, LifeScan, Johnson & Johnson, CA, USA). Animals with a non-fasting blood glucose level >16.7 mmol/l were considered diabetic (Li et al., 2015a,b) and used in the following experiments. Thirty mice became diabetic (30 of 35, 85.71%) and were randomly assigned to two groups: those injected intraperitoneally with liraglutide (GL Biochem Ltd., Shanghai, China; DM + LRG Group, $n = 15$; liraglutide intake was 200 μg/kg in 0.9% physiologic saline once daily for 8 weeks), and those injected intraperitoneally with vehicle alone (DM Group, $n = 15$). Body weight, food intake, and fluid intake were recorded once weekly, and non-fasting blood glucose was assessed every 2 weeks, always at 9:00–11:00 a.m. Eight weeks later, animals were subjected to behavioral studies to determine their cognitive function.

2.2. Cell culture and treatments

Hippocampal tissues from mouse embryos on embryonic day 18 were dissected, minced, and trypsinized with 0.05% trypsin. Digestion was then terminated using DMEM containing 10% heat-inactivated FBS (Kaech and Banker, 2006). Cells were seeded at a concentration of 1.5×10^5 cells/ml into six-well plates and maintained in serum-free Neurobasal® Medium (GIBCO, Carlsbad, CA, USA) supplemented with 2% B27 and 0.5 mM L-glutamine (GIBCO) at 37 °C in a humidified atmosphere containing 95% O_2 and 5% CO_2. Half of the medium was replaced with fresh medium after 2–3 days. Cells were cultured until the sixth day, and then treated with high-glucose Neurobasal® Medium (containing 100 mM glucose) for 24 h in the high-glucose group (Glu Group) or high-glucose Neurobasal® Medium containing 100 nM liraglutide for 24 h in the high-glucose + liraglutide group (Glu + LRG Group). To further explore the molecular mechanisms underlying the protective effects of liraglutide, a chemical inhibitor of AMP-activated protein kinase (AMPK), Compound C (Selleck Chemicals, Houston, TX, USA) was used. Cells were pretreated with 25 μM Compound C for 1 h subsequent to the treatment mentioned above.

2.3. Behavioral studies

2.3.1. Open-field test

The open-field test was performed to detect emotional and locomotor impairments. Each mouse was released in the center of the arena. Activity was measured as the total distance (m) traveled and the number of central square crossings in 10 min. At the end of each test, the arena was carefully cleaned with 75% alcohol to eliminate olfactory cues.

2.3.2. Morris Water Maze test

The Morris Water Maze (MWM) test was performed as previously described (Ge et al., 2015; Kong et al., 2012, 2013). A round pool (diameter, 150 cm; depth, 50 cm) was filled with warm (24 °C) opaque water to a height of 1.5 cm over a movable clear platform 15 cm in diameter in the third quadrant. A video tracking system recorded the swimming motions of mice, and data were analyzed using motion-detection software (Actimetrics, Wilmette, IL, USA). After every trial, mice were wiped dry and kept warm before being returned to their cages.

Place trials were performed for 4 days to determine the ability of mice to obtain spatial information. A dark curtain surrounded the pool to prevent confounding visual cues. All mice underwent four trials per day in each quadrant of the pool. During each trial, mice were placed in a fixed position into the swimming pool facing the wall. They were allowed 60 s to find the platform in the third quadrant, on which they sat for 20 s before being removed from the pool. If a mouse did not find the platform within 60 s, it was gently guided to the platform and allowed to remain there for 20 s. For all training trials, swimming speed and time taken to reach the platform (escape latency) were recorded. The less time it took a mouse to reach the platform, the better its learning ability. We took the

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