The Neuroprotective Effect of Liraglutide is Mediated by Glucagon-Like Peptide 1 Receptor-Mediated Activation of cAMP/PKA/CREB Pathway

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Key Words
GLP-1 • Liraglutide • AGEs • Astrocytes • cAMP

Abstract
Background/Aims: Glucagon-like peptide-1 (GLP-1)-based drugs are being used to achieve better glucose control in patients with type 2 diabetes, and GLP-1 mimetics such as liraglutide have shown therapeutic potential in preventing diabetes-related microvascular and macrovascular complications as well as comorbidities such as neurodegenerative disorders. In the present study, we investigated the effects of liraglutide on primary rat cortical astrocytes treated with advanced glycation end-products (AGEs).

Methods: Gene and protein expression was analyzed by quantitative real time PCR, western blots, and enzyme-linked immunosorbent assay. The caspase-3 activity was assessed using a caspase-3 colorimetric assay kit. The ROS production was measured with CM-H2DCFDA staining. The cell viability of rat astrocytes was determined using MTT assay.

Results: Liraglutide ameliorated AGEs-induced oxidative stress, inflammatory cytokine secretion, caspase activation, and cell death in astrocytes, and reversed the AGEs mediated reduction in intracellular cyclic AMP (cAMP) levels, protein kinase A (PKA) activity, and the phosphorylation of the cAMP response element-binding (CREB) protein. The protective effects of liraglutide against AGEs-mediated toxicity were abolished by GLP-1R knockdown or pretreatment of cells with the adenylyl cyclase inhibitor SQ22536 or the PKA inhibitor Rp-cAMP.

Conclusions: Liraglutide exerts its neuroprotective effects via the GLP-1R-mediated activation of the cAMP/PKA/CREB pathway. The results of the present study support the therapeutic potential of liraglutide for the treatment of neurodegenerative disorders.

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Introduction

Patients with type 2 diabetes mellitus (T2DM) have a significantly increased risk of developing Alzheimer's disease (AD) [1]. A growing body of evidence suggests that insulin resistance, inflammation, and oxidative stress in the brain are key factors linking T2DM to AD [2, 3]. Long standing hyperglycemia in T2DM patients accelerates the formation of advanced glycation end products (AGEs), which increase inflammation and oxidative stress in many cell types and contribute to the pathogenesis of diabetes-related diseases [4]. AGEs are detected in neurons and astrocytes in the brains of patients with AD [5, 6]. Serum AGEs from diabetic patients on hemodialysis induce neuronal cell death in cultured cortical neurons [7]. AGEs have been shown to play a role in neurodegenerative diseases including AD and Parkinson’s disease [8].

Glucagon-like peptide-1 (GLP-1) is a gut hormone secreted by intestinal L cells in response to food intake. GLP-1 regulates blood glucose levels by enhancing glucose-dependent insulin secretion, inhibiting glucagon secretion, delaying gastric emptying, and reducing food intake [9]. Additionally, GLP-1 prevents β cell loss in diabetes [10]. Binding of GLP-1 to the GLP-1 receptor (GLP-1R) activates adenylyl cyclase (AC) to produce cAMP, which in turn activates protein kinase A (PKA) to phosphorylate and activate cAMP response element-binding protein (CREB), a constitutively expressed nuclear transcription factor that regulates the expression of genes involved in neuronal survival and function [11, 12]. GLP-1R-mediated activation of the cAMP/PKA pathway has been shown to mediate various functions of GLP-1 such as stimulation of glucose-dependent insulin secretion. Because of its antidiabetic properties, GLP-1 is a highly effective therapeutic molecule in the management of T2DM. GLP-1-based therapies including GLP-1 receptor (GLP-1R) agonists and dipeptidyl peptidase-4 inhibitors have been approved by the FDA and EMA for the treatment of T2DM [13], and GLP-1 mimetics have shown beneficial effects in various neurodegenerative diseases including AD [14]. The GLP-1 analog liraglutide has anti-inflammatory and neuroprotective effects and was shown to cross the blood-brain barrier; it reduces amyloid plaque deposition in a mouse model of AD and is currently being tested in clinical trials in AD patients [15-17].

In the present study, we investigated the effects of liraglutide on AGEs induced toxicity in primary rat cortical astrocytes. Astrocytes were selected because they express GLP-1 and GLP-1R, and because they play a role in inflammatory responses through the production of pro-inflammatory cytokines such as TNFα, contributing to neuronal injury and the formation of the hallmark lesions of AD [18], and AGEs increase oxidative stress and cytokine production in cultured astrocytes [19, 20]. Our results showed that liraglutide reversed the AGEs-mediated production of reactive oxygen species (ROS), inflammatory cytokine secretion, caspase activation, and cell death through a mechanism involving GLP-1R signaling via cAMP/PKA. Taken together with previous reports, our findings support the therapeutic potential of liraglutide for the treatment of neurodegenerative disorders.

Materials and Methods

Preparation of AGEs

AGEs were prepared following previously described procedures [21, 22]. Briefly, 50 mg/ml BSA in phosphate buffer saline (PBS, pH 7.4) containing 0.5 M D-glucose was incubated at 37°C in the dark under sterile conditions for 90 days. After the incubation was completed, the mixture was extensively dialyzed against 0.01 M PBS buffer (72 h, 4°C) to remove the remaining free glucose. The purified AGEs was stored at -60°C. The sugar-free BSA solution used in control experiments underwent the same incubation and dialysis treatment.

Isolation and culture of primary rat astrocytes

Rat astrocytes were isolated from the cerebral tissue of 1- to 2-day-old postnatal Wistar rat pups as described previously [23, 24]. Briefly, cells collected from disrupted rat cerebral tissue were seeded in 75-
cm² poly-L-lysine-coated culture flasks at an initial density of 2×10⁵ cells/cm² and cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS, PAA laboratories GmbH, Austria), 50 μg/ml streptomycin, and 50 U penicillin at 37°C, 5% CO₂ in a humidified incubator. The culture medium was changed twice a week. After 11 – 14 days, the culture flasks were shaken at 200 rpm overnight to remove the microglia and oligodendrocytes. The astrocytes that remained attached to the flask were harvested by trypsinization and subcultured in the same medium. Astrocytes prepared this way were routinely more than 95% pure when assessed by glial fibrillary acidic protein (GFAP) immunostaining. All animal care and use protocols were in compliance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of Changzheng Hospital, Second Military Medical University.

**Cell treatment**

Astrocytes were seeded at 2.5×10⁴ cells/ml into 24-well plates and cultured for 2–3 days. Cells were then pre-treated for 4 h with indicated concentrations of Liraglutide. Medium was removed and the cells were exposed to various concentrations of sugar-free BSA or AGEs for different time points.

**Construction and transfection of shRNAs**

Two short hairpin RNAs (shRNAs) directed against GLP-1R (GLP-1R shRNA1, GTTCCGCTGCTGTTCGTTATC; GLP-1R shRNA2, TGAGGGTCTCTGCTACATAA) and a scrambled shRNA (control shRNA, CCATTCTACTGTTCACCTGAT) were synthesized at Genepharma (Shanghai, P. R. China). Astrocytes were transfected with shRNAs using Lipofectamine 2000 (Invitrogen, USA) following manufacturer’s instructions. GLP-1R expression was determined 2 days post transfection.

**Quantitative real time PCR**

Total RNA of cultured astrocytes was extracted using TRIZOL reagent (Invitrogen) following manufacturer’s instructions. cDNA synthesis was carried out using oligo (dT) primers (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen). Real-time quantitative PCR (qRT-PCR) was performed using a SYBR green qPCR kit (TaKaRa, Japan) on a StepOneTM Real-Time PCR System (Applied Biosystems). Sequences of primers used for amplifications were as follows:

- **GLP-1R:**
  - forward: 5’-AGTGCGAAGAGTCCAAGCAA-3’
  - reverse: 5’-TTGAGGGCAGCGTCTTTGAT-3’
- **β-actin:**
  - forward: 5’-CATCCAGGCTGTGTTGTCCC-3’
  - reverse: 5’-CACGCACGATTTCCCTCTCA-3’

Data were normalized to β-actin.

**Western blot analysis**

Protein concentration of cell lysates was determined using the BCA Protein Assay kit (Beyotime Institute of Biotechnology, China). Total proteins (20 μg) were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes (Amersham International, UK). After blocking in 3% BSA for 2 h at 37°C, the membranes were incubated with antibodies of GLP-1R (1:200), full-length caspase 3 (1:1000), cleaved caspase-3 (1:1000), phospho-PKA (Thr197) (1:500), PKA (1:1000), phospho-CREB (S133) (1:500), CREB (1:500), and β-actin (1:1000), respectively. All primary antibodies were purchased from Abcam (Cambridge, MA, USA). After washing, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase. Protein expression was determined using enhanced chemiluminescence reagents (Amersham International) and normalized to β-actin.

**Measurement of ROS production**

After treatment, cells were washed twice with PBS and incubated with 5 mM 5- and 6-chloromethyl-20, 70-dichlorodihydrofluorescein diacetate (CM-H2DCFDA, Molecular Probes, USA) in serum-free medium at 37°C for 30 min. After, cells were washed twice with PBS to remove the remaining dyes and subjected to examination under a Leica fluorescent microscope (DMI 3000B; Leica Microsystems Inc., USA). Fluorescence images were obtained with excitation and emission wavelengths at 485 nm and 535 nm, respectively.
To determine the intracellular level of reactive oxygen species (ROS), the cells were subjected to flow cytometric analysis with the same excitation and emission wavelengths on a FACSCanto II flow cytometer (BD Biosciences, USA).

**Determination of cell viability**

The cell viability was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were incubated with 0.2 mM MTT in fresh medium at 37°C for 4 h. The medium was carefully removed and 500 μl DMSO was added to dissolve the purple formazan crystals. The color formation was determined by absorbance at 570 nm on a Benchmark Microplate Reader (Bio-Rad Laboratories, USA). The absorbance at 650 nm was used to correct for nonspecific background values.

**Caspase-3 activity assay**

The cellular caspase-3 activity was determined using a caspase-3 colorimetric assay kit (BioVision Inc., USA) following manufacturer's instructions. Briefly, cells were lysed and the protein concentration was determined using the BCA Protein Assay Kit (Pierce Biotechnology, USA). The lysates (50 μl, ~ 200 μg total protein) were mixed with 2x reaction buffer containing 10 μM DTT and the colorimetric substrate Ac-DEVD-pNA. After 2 h incubation at 37°C, the absorbance at 405 nm was recorded on a Benchmark Microplate Reader (Bio-Rad Laboratories). The caspase-3 activity was calculated using a calibration curve prepared with defined pNA solutions.

**ELISA assay**

The levels of TNF-α and IL-1β released into the cell culture medium by the astrocytes were determined using ELISA assay kits following manufacturer's protocols. The TNF-α and IL-1β ELISA kits were from IBL-America (Minneapolis, USA). The absorbance at 450 nm was recorded on a Benchmark Microplate Reader (Bio-Rad Laboratories) within 30 minutes after adding STOP solution.

**Intracellular cAMP measurement**

After treatment, cells were washed with Krebs-Ringer Bicarbonate (KRB) buffer. Intracellular cAMP levels were determined using a cAMP ELISA kit (IBL-America) as previously described [25]. Data were normalized to the protein concentration in samples.

**PKA activity assay**

After treatment, cells were collected and placed in PBS buffer containing protease (1:500, Sigma-Aldrich, St Louis, MO, USA) and phosphatase inhibitor (1:200, Sigma-Aldrich) cocktails. Cells were then subjected to sonication to disrupt the cell membrane, and cytosolic proteins were harvested by centrifugation. The PKA activity was determined utilizing the Kemptide phosphorylation assay as previously described [26].

**Statistical analysis**

Data are presented as the mean ± SE (standard error). Each experiment was conducted at least three times. Statistical differences between two groups were compared using a two-tailed Student's t-test. ANOVA (one-way analysis of variance or repeated measures) was used with the post hoc Bonferroni's test for the comparison of data from more than two groups. Differences with a P value less than 0.05 were considered statistically significant.

**Results**

**AGES induce ROS production and inhibit cell proliferation in astrocytes**

The primary rat cortical astrocytes isolated from the cerebral tissue of Wistar rat pups exhibited typical morphological characteristics of astrocytes. The cultures were routinely more than 95% pure when assessed by GFAP immunostaining (Fig. 1A). Previous studies have shown that AGEs can induce oxidative stress and decrease antioxidant activities in astrocytes [19, 20]. In the present study, ROS production was monitored in astrocytes
treated with sugar-free BSA (100 mg/l) or AGEs at doses of 50 – 400 mg/l for 24 h by CM-H2DCFDA staining, a fluorescent ROS indicator. Flow cytometric analysis showed that AGEs significantly increased intracellular ROS levels in a dose and time-dependent manner (Fig. 1B, 1C). Moreover, AGEs significantly decreased cell viability in astrocytes in a dose and time-dependent manner when assessed by the MTT assay (Fig. 1D, 1E). Based on these results, a dose of AGEs of 200 mg/l for 24 h were the conditions used for subsequent experiments.
Bao et al.: Relation of Liraglutide and GLP-1R in Neuroprotective Effect

Liraglutide ameliorates AGES-induced ROS production and cell death in astrocytes

Previous in vitro studies have demonstrated that GLP-1 prevents high glucose-induced cell death and AGES-induced tau hyperphosphorylation in neurons [15]. We speculated that the GLP-1 mimetic liraglutide might have similar cytoprotective effects in AGES-treated astrocytes. To test this hypothesis, we treated primary rat cortical astrocytes with AGES (200 mg/l) in the absence or presence of liraglutide (10–1000 nM). After 24 h of incubation, the intracellular ROS level and cell viability were determined by flow cytometric analysis and the MTT assay, respectively. Liraglutide significantly reduced AGES-induced ROS production (Fig. 2A) and promoted cell viability (Fig. 2B) in astrocytes in a dose-dependent manner, demonstrating the neuroprotective effects of GLP-1 stimulation.

The inhibitory effects of liraglutide on AGES-induced ROS production, cell death, and inflammatory cytokine secretion in astrocytes are GLP-1R-dependent

To investigate the mechanisms underlying the beneficial effects of liraglutide in AGES-treated astrocytes, we studied the effects of GLP-1R knockdown with shRNA. Transfection of astrocytes with GLP-1R shRNA1 or GLP-1R shRNA2 led to over 50% decrease in both mRNA and protein expression of GLP-1R (Fig. 3A, 3B). Liraglutide ameliorated AGES-induced ROS production and cell death in control shRNA-transfected but not GLP-1R shRNA-transfected astrocytes (Fig. 3C, 3D), indicating that these beneficial effects of liraglutide were mediated by GLP-1R-dependent signaling. Additionally, AGES increased the caspase-3 activity and the protein level of cleaved caspase-3 (Fig. 3E, 3F) as well as TNF-α and IL-1β secretion (Fig. 3G, 3H) in astrocytes, and AGES-induced caspase activation and inflammatory cytokine secretion were reduced by coincubation with liraglutide in control shRNA-transfected but not in GLP-1R shRNA-transfected astrocytes (Fig. 3E–3H). Taken together, our results demonstrate that liraglutide ameliorates AGES-induced ROS production, inflammatory cytokine secretion, caspase activation, and cell death in astrocytes via GLP-1R-dependent mechanisms.

Liraglutide protects astrocytes from AGES-induced detrimental effects via activation of the cAMP/PKA/CREB pathway

To clarify the mechanisms underlying the effect of liraglutide on reversing AGES-induced damage, the activation status of the cAMP/PKA/CREB pathway was examined in astrocytes.
Fig. 3. Liraglutide protects astrocytes against AGEs-induced ROS production and cell death and reduces AGE-induced TNF-α and IL-1β secretion via GLP-1R-dependent signaling. (A and B) GLP-1R expression in primary rat cortical astrocytes transfected with control shRNA (Scr), GLP-1R shRNA1 (sh-1), or GLP-1R shRNA2 (sh-2) for 48 h. (A) GLP-1R mRNA expression by qRT-PCR. Data were normalized to β-actin. (B) GLP-1R protein expression by western blot analysis. Data were normalized to β-actin. **p<0.01 vs. untransfected cells (NC). (C – H) Primary rat cortical astrocytes were transfected with Scr, sh-1, or sh-2 for 48 h and treated with AGEs (200 mg/l) in the absence or presence of 100 nM liraglutide (Lira) for 24 h. (C) ROS production by flow cytometric analysis with CM-H2DCFDA staining. *p<0.05, **p<0.01 vs. Scr-Ctrl. #p<0.05. (D) Cell viability by the MTT assay. Cell viability was calculated as the percent of that in Scr-Ctrl. (E) Caspase-3 activity by the colorimetric assay. Data were normalized to Scr-Ctrl. (F) Protein levels of full-length caspase-3 and cleaved caspase-3 by western blot analysis. Data are presented as the cleaved/full-length caspase-3 ratio. (G) TNF-α and (H) IL-1β levels in the culture medium by ELISA. *p<0.05, **p<0.01 vs. Scr-Ctrl. *p<0.05.
AGEs significantly decreased the intracellular cAMP level (Fig. 4A), PKA activity (Fig. 4B), PKA level (Fig. 4C) and CREB phosphorylation (Fig. 4D) in primary rat cortical astrocytes. These AGEs-induced changes in cAMP/PKA/CREB pathway were reversed by coincubation with liraglutide in control shRNA-transfected but not GLP-1R shRNA1-transfected astrocytes (Fig. 4A–4D). Further, the protective effects of liraglutide against AGEs-induced detrimental effects including ROS production, TNF-α and IL-1β secretion, caspase-3 activation, and cell death in astrocytes were abolished by pretreatment of cells with the adenylyl cyclase inhibitor SQ22536 or the PKA inhibitor Rp-cAMP (Fig. 5A–5F). Collectively, these data suggest that liraglutide protects astrocytes against AGEs-induced damages via GLP-1R-mediated activation of the cAMP/PKA/CREB pathway.

**Discussion**

Desensitization of insulin receptors in the brains of patients with AD has been suggested as a contributing factor to the increased risk of AD associated with T2DM [27]. Therefore,
strategies to normalize insulin signaling in the brain have attracted attention. Among them, GLP-1 has been investigated as a treatment for AD because of its ability to enhance glucose-dependent insulin secretion and lower blood glucose in T2DM patients, and because it plays important roles in the brain, where it acts as a neurotransmitter [28-30]. Furthermore, the beneficial effects of GLP-1 on diabetes-related diseases have been shown to be mediated by mechanisms beyond the regulation of glucose homeostasis [31, 32]. GLP-1 receptor agonists have neuroprotective effects in animal models of AD, and their ability to cross the blood-brain barrier has made them attractive therapeutic options for the treatment of neurodegenerative disorders [15, 33, 34]. The GLP-1 agonists exendin-4, liraglutide and lixisenatide have been approved for the treatment of T2DM. Here, we investigated the neuroprotective properties of liraglutide by assessing its effect on AGEs-induced ROS production, decreased cell viability, apoptosis and inflammatory cytokine secretion, and examined the underlying mechanisms.

Oxidative stress plays an important role in the progression of neurodegenerative diseases including AD and Parkinson’s disease [35]. The vulnerability of the central nervous system to oxidative stress mediated neurodegeneration is due in part to its high metabolic rate and restricted cell renewal. Despite the fact that oxidative stress is a known causative...
factor in neurodegenerative disorders, a mechanism directly linking ROS production and neuronal cell death remains to be elucidated [36]. However, ROS-mediated signaling pathways inducing inflammatory responses have provided a link between oxidative stress and inflammation in neurodegeneration [37]. GLP-1 has been shown to protect against oxidative injury in the brain [38], and the neuroprotective effect of GLP-1 was shown to be mediated by the reduction of oxidative stress in human neuroblastoma cells in vitro [39, 40]. These studies suggest that GLP-1 agonists are of value for the treatment of oxidative stress-related neurodegeneration. This was supported by the results of the present study, which showed that liraglutide restored AGEs-induced ROS production and cell death. The present study used a model of AGEs-induced toxicity in cultured astrocytes. Future studies should focus on hippocampal or cortical neurons, which are the affected areas in AD and may provide more conclusive results.

The detrimental effects of inflammation in the brain have been demonstrated extensively and many neurodegenerative diseases including AD and Parkinson's disease are associated with chronic inflammation [41, 42]. Furthermore, immune cells in the brain, such as microglia, release pro-inflammatory cytokines and free radicals such as nitric oxide, which is neurotoxic [43]. Therefore, research efforts have focused on the identification of anti-inflammatory drugs for the treatment of neurodegenerative diseases. In the present study, we showed that liraglutide inhibited the AGEs induced secretion of the proinflammatory cytokines TNF-α and IL-1β. The anti-inflammatory effects of liraglutide were shown previously in a mouse model of chronic inflammation induced by irradiation, in which liraglutide reduced the levels of IL-6, IL-12p70, and IL-1β [16]. IL-1β, which is induced in response to brain insult and is expressed in neurodegenerative disorders, plays an important role in the activation of microglia; it enhances neuronal excitation and is found in areas of focal neuronal loss, indicating that reducing its production may have neuroprotective effects [44, 45]. GLP-1 treatment prevents lipopolysaccharide induced release of IL-1β by astrocytes [46]. Although the exact mechanism underlying the effect of GLP-1 analogues on decreasing inflammation is not known, it could be mediated by their effect on the activation of cAMP/PKA/CREB signaling [17]. CREB suppresses the expression of the proinflammatory cytokines TNF-α and IL-1β [47], suggesting a potential mechanism underlying the effect of liraglutide on decreasing inflammation mediated by the inhibition of CREB responsive genes. The present results support this hypothesis, as we showed that the effect of liraglutide on reversing AGEs-induced ROS production, pro-inflammatory cytokine secretion, and cell viability were mediated by the activation of cAMP/PKA signaling. Liraglutide was previously shown to upregulate the prohormone converting enzymes PC1/3, which process proinsulin to insulin in pancreatic β-cells, in vitro and in vivo through the activation of the GLP-1R/cAMP/PKA pathway [48]. In a mouse model of diabetic nephropathy, liraglutide showed a protective role against renal oxidative stress triggered by hyperglycemia mediated by cAMP/PKA activation [49]. The role of the cAMP/PKA/CREB pathway in the neuroprotective activity of liraglutide was shown in neuronal cultures and in a mouse model of traumatic brain injury (TBI), where liraglutide rescued neuronal cells from oxidative stress and glutamate excitotoxicity-induced cell death and ameliorated memory impairment in mice caused by TBI via cAMP/PKA signaling [50]. These studies together with the findings of the present study support the neurotrophic and neuroprotective role of GLP-1R stimulation. Given the association between T2DM and neurodegenerative disorders, our findings confirm the value of GLP-1-based antidiabetic drugs for the treatment of neurological disorders, which are increasing in incidence in our aging population.

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Disclosure Statement

All authors are in agreement with the content of this manuscript. The authors declare no conflict of interest.

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