ORIGINAL ARTICLE

DPP-4 Inhibitor Linagliptin is Neuroprotective in Hyperglycemic Mice with Stroke *via* the AKT/mTOR Pathway and Anti-apoptotic Effects

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Abstract Dipeptidyl peptidase 4 (DPP-4) inhibitors have been shown to have neuroprotective effects in diabetic patients suffering from stroke, but less research has focused on patients with mild hyperglycemia below the threshold for a diagnosis of diabetes. In this investigation, a hyperglycemic mouse model was generated by intraperitoneal injection of streptozotocin and then subjected to focal cerebral ischemia. We demonstrated that the DPP-4 inhibitor linagliptin significantly decreased the infarct volume, reduced neuronal cell death, decreased inflammation, and improved neurological deficit compared with control mice. Linagliptin up-regulated the expression of p-Akt and p-mTOR and regulated the apoptosis factors Bcl-2, Bax, and caspase 9. Taken together, these results suggest that linagliptin exerts a neuroprotective action likely through activation of the Akt/mTOR pathway along with anti-apoptotic and anti-inflammatory mechanisms. Therefore, linagliptin may be considered as a therapeutic treatment for stroke patients with mild hyperglycemia.

Keywords Cerebral ischemia · Hyperglycemia · DPP-4 inhibitor · Linagliptin · Neuroprotection

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Introduction

Ischemic stroke is a global health concern that leads to lifelong disability or death [1]. In the USA, approximately 795,000 people experience acute first and recurrent ischemic strokes each year [2]. Despite declining stroke mortality rates, the global burden of stroke is increasing. As of 2010, the total expenditure on the disease was $53 billion/year in the USA and is expected to double by 2030 [3]. Over the past 20 years, the understanding of the pathophysiology, diagnosis, and treatment of acute ischemic stroke has improved significantly [4]. Many causes of stroke have been identified, such as high blood pressure, cardiovascular disease, high cholesterol, diabetes, cigarette smoking or exposure to secondhand smoke, and a personal or family history of stroke. More specifically, the prognosis of acute ischemic stroke is affected by multiple factors such as the patient's glucose level on admission. Retrospective clinical studies have shown that hyperglycemia, an independent risk factor for stroke, is closely associated with the progression, treatment, and prognosis of stroke [5, 6]. Higher admission blood glucose levels are associated with increased morbidity and mortality [7, 8]. Studies have also shown that hyperglycemia increases the area of cerebral infarction in animal models [9–11]. This may occur because hyperglycemia contributes to damaging processes such as increased anaerobic metabolism, hyperosmolarity, lactic acidosis, and focal toxicity of ischemic vascular disease [12–15]. For these reasons, the management of blood glucose levels is especially important in patients with stroke and hyperglycemia.

Hyperglycemia is recognized as an important risk factor for cerebrovascular disease, especially ischemic stroke [16, 17]. Patients with hyperglycemia carry a 1.5–3 times higher risk of stroke compared with the general population

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[18]. Stroke mortality is also higher and post-stroke outcomes are poorer in patients with hyperglycemia [19]. Most notably, however, clinical studies have found that effective control of blood glucose effectively improves the prognosis of patients with diabetes complicated with stroke.

Inhibitors of dipeptidyl peptidase 4 (DPP-4) are widely used in clinical settings to treat type 2 diabetes [20, 21] due to their ability to effectively reduce blood sugar levels in diabetics. The effect of DPP-4 inhibitors on reducing stroke incidence tends to vary widely, with some studies suggesting no effect on stroke incidence [22–24] and others suggesting reduced incidence after DPP-4 inhibitor treatment [25]. Despite this, there is evidence that DPP-4 inhibitor treatment promotes neuroprotective effects in patients with diabetes complicated with stroke [26, 27], suggesting that DPP-4 inhibitors may be useful for stroke recovery in patients with conditions such as hyperglycemia. Thus, this investigation focused on markers of neuroprotection such as cell death and sensorimotor deficit, rather than rates of stroke incidence and death that tend to be the focus of clinical trials [28]. In addition, previous studies failed to recognize patients with hyperglycemia below the threshold for a diagnosis of diabetes. Whether DPP-4 inhibitors also promote neuroprotection in the patients that suffer a stroke is not clear. In this study, we established a hyperglycemic mouse model with stroke to determine the potential efficacy of the DPP-4 inhibitor linagliptin. We further investigated its neuroprotective mechanism.

Materials and Methods

Animals

Adult male C57BL/6 mice were housed in standard cages in 12-h light/12-h dark cycle in the Emory University animal facility where the room temperature was kept at 22°C ± 1°C. All experimental procedures were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Emory University Institutional Animal Care and Use Committee.

Hyperglycemic Mouse Model

All animal experiments and surgical procedures were approved by the Institutional Animal Care and Use Committee at Emory University and met NIH standards. Hyperglycemia was induced in adult male C57BL/6 mice 10–12 weeks old weighing ∼ 25 g. Mice were given an intraperitoneal (i.p.) injection of streptozotocin (STZ)

dissolved in citrate buffer. All solutions were prepared immediately before use. Controls received i.p. vehicle. After the injections, mice were housed for 2–3 weeks and given food and water *ad libitum*. Every week, blood glucose levels were measured after a 4-h fast using samples from the tail vein with the FreeStyle glucose meter.

Focal Ischemic Stroke Model

The focal cerebral ischemic stroke model in adult male mice was created as previously described [29]. In summary, mice were anesthetized with 4% chloral hydrate. The distal branches of the right middle cerebral artery were permanently ligated, followed by bilateral occlusion of the common carotid arteries and subsequent reperfusion. During surgery and recovery, body temperature was maintained at 37.0 °C ± 0.5° C using a temperature-controlling ventilator and heating pads.

Quantification of Infarct Volume Using 2,3,5-triphenyltetrazolium Chloride (TTC) Staining

Infarct volume was assessed 3 days after ischemia *via* TTC staining. Brains were removed and cut into 1-mm coronal slices using a brain matrix. The slices were incubated in 2% TTC (Sigma) at 37°C for 5 min, then stored in 10% buffered formalin for 24 h. A flatbed scanner was then used to capture digital images of the caudal aspect of each slice. Ipsilateral and contralateral hemispheres and infarct areas were measured using ImageJ software (NIH, Bethesda, MD, USA) and infarct volume was calculated using the indirect method [30].

Cell Death Assay Using Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick-End Labeling (TUNEL)

A TUNEL assay kit (DeadEnd Fluorometric TUNEL system; Promega, Madison, WI, USA) was used to assess cell death in 10-μm brain sections according to manufacturer's instructions. Briefly, sections were fixed in 10% buffered formalin for 10 min and then permeabilized in 0.2% Triton-X 100 after fixation for 5 min in ethanol:acetic acid (2:1). They were incubated in equilibration buffer for 10 min and then incubated in the dark at 37°C for 60 min in recombinant terminal deoxynucleotidyl transferase and nucleotide mixtures. The reaction was stopped by 2× SSC solution for 15 min, and nuclei were then counterstained with DAPI for 5 min.

Immunohistochemical Staining and Cell Counts

Brain cryosections 10 μm thick were dried on a slide warmer for 30 min, fixed in 10% buffered formalin for 10 min, washed with −20°C ethanol:acetic acid (2:1) for 10 min, permeabilized with 0.2% Triton-X 100 and phosphate-buffered saline (PBS) for 5 min, blocked with 1% fish gel (Sigma) and PBS for 60 min, and incubated with the following primary antibodies overnight at 4°C: mouse anti-NeuN (1:400; Chemicon, Temecula, CA) for mature neurons, rabbit anti-glucose transporter 1 (GLUT-1) (1:400, Chemicon, Millipore) for vessels, and rabbit anti-ionized calcium binding adaptor molecule-1(Iba-1) (1:200; Biocare Medical, CA). After rinsing with PBS, the sections were then exposed for 1 h at room temperature to the relevant secondary antibodies: secondary antibody Alexa Fluor 488 anti-rabbit, anti-goat, anti-mouse IgG (1:200; Invitrogen, Carlsbad, CA), cyanine-3 (Cy3)-conjugated anti-rat, anti-rabbit IgG (1:1000; Invitrogen), or Cy5-conjugated anti-goat IgG (1:400; Invitrogen). DAPI was applied at 1:25,000 for 5 min and washed with PBS. Slides were then mounted with Vectashield (Vector Laboratory, Burlingame, CA) for fluorescence microscopy analysis and imaging.

For systematic random sampling in design-based stereological cell counting, 9 sections, each 90 μm apart, in the region of interest were used. For multi-stage random sampling, 6 fields per section were randomly selected in the penumbra [31] at a magnification of 400× under a fluorescence microscope. This was repeated in 6 separate sections per brain.

Western Blotting

The peri-infarct region was defined by a 500-mm boundary extending from the edge of the infarct core, medial and lateral to the infarct, as previously described [32]. Peri-infarct tissue samples of cortex were collected from each group 3 days after ischemia. Proteins were extracted from the tissue by homogenization in protein lysis buffer (in mmol/L: 25 Tris-HCl [pH 7.6], 150 NaCl, 5 EDTA, 2 sodium orthovanadate, 100 NaF, 0.1% SDS, 1% Triton, leupeptin, aprotinin, and pepstatin). Protein from each sample was loaded into the gradient gel and run at a constant current until the protein markers were sufficiently separated. Protein was transferred onto polyvinyl difluoride membranes that were then probed using a standard protocol [33]. The membranes were then blocked with 5% bovine serum albumin for 1 h and incubated overnight at 4°C with primary antibodies. The primary antibodies used and the dilutions for each were mouse β-actin (1: 5000; Sigma-Aldrich, St. Louis, MO), rabbit mTOR (1:500; Santa Cruz Biotech, Inc., Dallas, TX), rabbit AKT (1:1000; Santa Cruz), rabbit TNF-α (1:1000; Cell Signaling, Danvers, MA), rabbit IL-1β (1:1000; Cell Signaling), rabbit IL-6 (1:1000; Cell Signaling), rabbit Bcl-2 (1:1000; Cell Signaling), rabbit Bax (1:1000; Cell Signaling), cleaved caspase-3 (1:1000; Cell Signaling), p-mTOR (1:1000; Santa Cruz) and p-AKT (1:1000; Santa Cruz). After washing in TBST, the membranes were incubated with ammonium persulfate (AP)-conjugated secondary antibodies (GE Healthcare, Piscataway, NJ) for 1 h at room temperature. The signal was developed using nitro-blue tetrazolium and 5-bromo-4-chloro-3'indolyphophate solution. The band intensities were analyzed using ImageJ. The expression of each target protein was then normalized to β-actin.

Local Cerebral Blood Flow (LCBF) Measurement

Laser scanning imaging was used to measure LCBF at 0 day for baseline and at 21 days after ischemia as previously described. Mice were anesthetized and an incision was made to expose the skull above the right middle cerebral artery. The laser was centered on the right coronal suture. Unlike conventional laser Doppler probes which measure small blood flow points, the scanner method used a laser Doppler perfusion imaging system (PeriFlux System 5000-PF5010 LDPM unit, Perimed, Stockholm, Sweden) to measure a square area of 2.4 mm × 2.4 mm [2]. This measurement largely avoided inaccuracies caused by inconsistent positioning of the laser. Laser scanning imaging measurements and analyses of the area were performed using the PeriScans system and LDPIWin programs (Perimed).

Adhesive Removal Test

The adhesive removal test was used to assess sensorimotor deficits after focal cerebral ischemia in mice. In summary, a small adhesive dot was placed on one forepaw, and the number of times to contact and remove the adhesive dot were recorded. Mice were trained in three sessions before surgery to ensure normal sensorimotor function. A recording was discontinued after 120 s of no contact with the adhesive dot. Three trials were performed by the mice in different groups at 3, 7, and 14 days after ischemia and the mean time was calculated for data analysis.

Statistical Analysis

The mice were randomly separated into sham, stroke, hyperglycemia stroke, and linagliptin treatment + hyperglycemia groups. The behavioral tests and data analyses were done double-blind by 2–3 people without knowledge of grouping. GraphPad Prism 6 (GraphPad Software, San

Diego, CA) was used for statistical analysis and graphic presentation, and sample sizes were tested with G-Power Analysis (University of Dusseldorf, Dusseldorf, Germany) to yield sufficient statistical power. Student's *t*-test was used to compare two groups. Multiple comparisons were done using one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. One-way analysis followed by Bonferroni selective comparisons was performed for pairwise comparisons. Data with $P \leq 0.05$ were considered significantly different. Data are expressed as the mean ± SEM.

Results

### Assessment of the Hyperglycemic Mouse Model

A hyperglycemic mouse model was created in adult male mice by injection of STZ. Two weeks after the injection, animals exhibited significantly elevated levels of blood glucose with respect to age-matched vehicle control mice (Fig. 1A). This hyperglycemic state lasted for at least 3 weeks before we performed ischemic surgery. The results showed that linagliptin treatment (10 mg/kg by gavage) decreased the fasting blood glucose levels (Fig. 1B). However, we did not find that the blood glucose levels were related to the body weight, and linagliptin treatment did not affect the body weight (Fig. 1C and D).

Linagliptin Reduces Infarct Volume after Ischemic Stroke in Hyperglycemic Mice

Compared with animals with stroke and normal blood glucose, the hyperglycemic stroke mice showed a significantly larger brain infarct volume (Fig. 2A–C). Linagliptin treatment significantly reduced the infarct volume compared with the stroke control group (Fig. 2A–C).

Linagliptin Reduces Neuronal Cell Death in Hyperglycemic Mice

To understand the protective mechanism of linagliptin at the cellular level, TUNEL staining in brain tissues harvested 3 days after ischemia was used to determine whether linagliptin treatment affected cell death. Compared with the normal blood glucose group, the hyperglycemic group showed significantly increased numbers of TUNEL-positive cells and TUNEL +/NeuN + co-labeled cells in the penumbra. However, linagliptin treatment significantly reduced the neuronal cell death, as evidenced by a decrease in the number of TUNEL-positive cells double-stained with NeuN, compared with the hyperglycemic stroke control group ($P < 0.05$) (Fig. 3A–C). In addition, we assessed the protein expression of cleaved caspase-3 in the penumbra 3 days after stroke. The data showed that cleaved caspase-3 significantly increased after stroke. Linagliptin treatment decreased the caspase-3 activation ($P < 0.001$) (Fig. 3D).
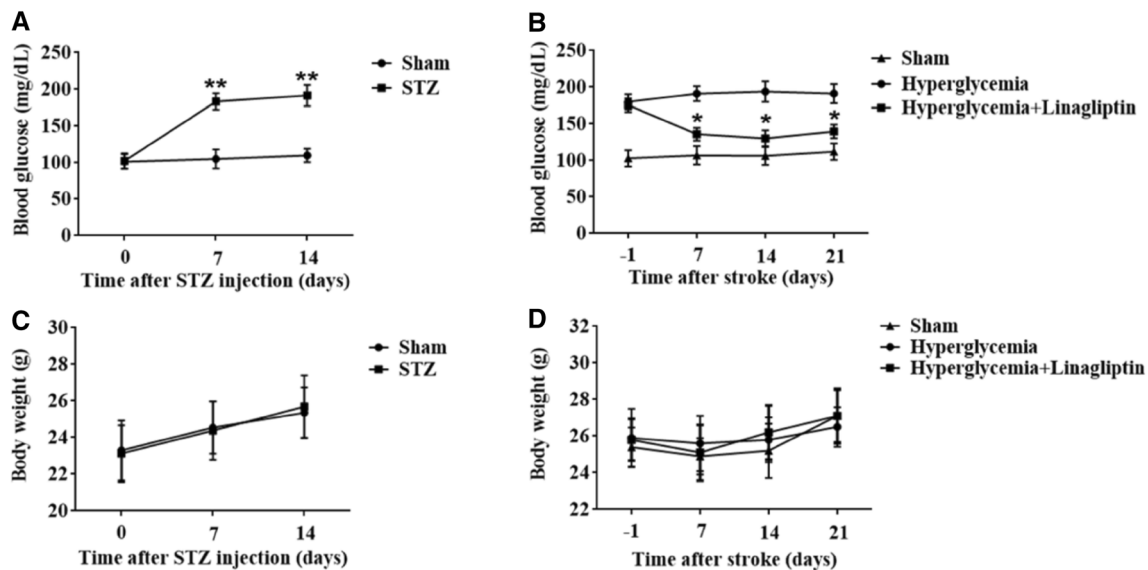

Fig. 1 Changes in body weight and fasting blood glucose. **A** Levels of fasting blood glucose after STZ injection. **B** Levels of fasting blood glucose after daily linagliptin administration after stroke. **C** Body weight after STZ injection. **D** Body weight after daily linagliptin administration after stroke. Values are expressed as the mean ± SEM. *$P < 0.05$, **$P < 0.01$.
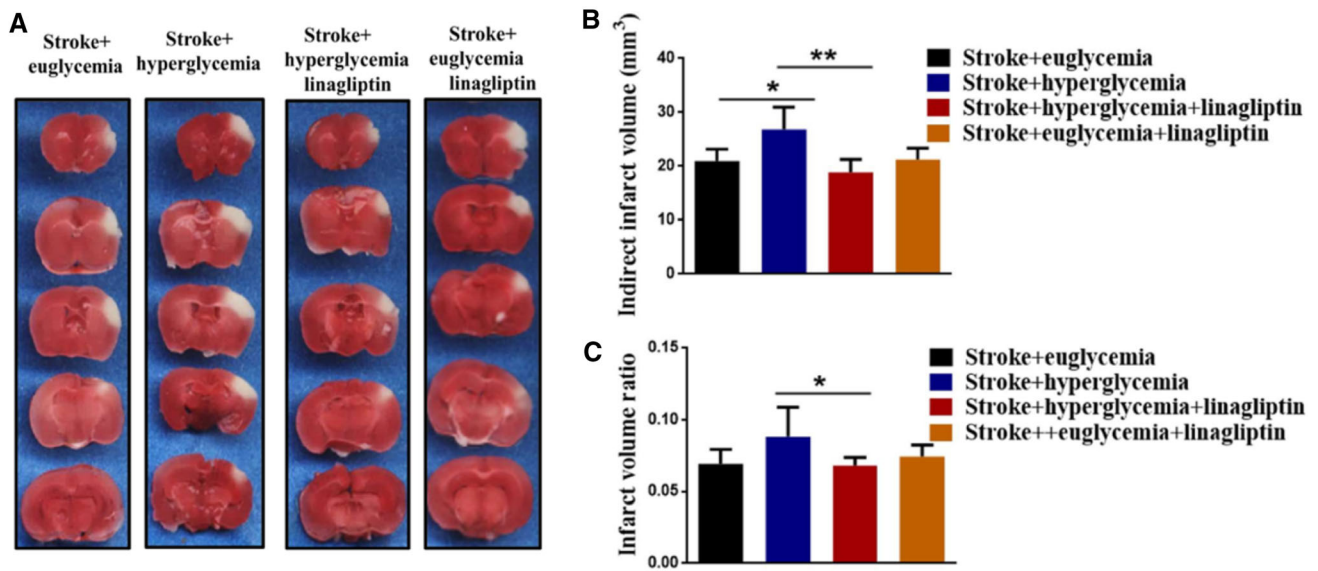
Fig. 2 Linagliptin reduces infarct volume after ischemic stroke in hyperglycemic mice. **A** TTC staining 3 days after stroke to evaluate infarct formation after stroke. **B** Linagliptin treatment significantly reduced the indirect infarct volume. **C** Linagliptin treatment significantly reduced the indirect infarct ratio. Values are expressed as the mean ± SEM. $*P < 0.05$, $**P < 0.01$.
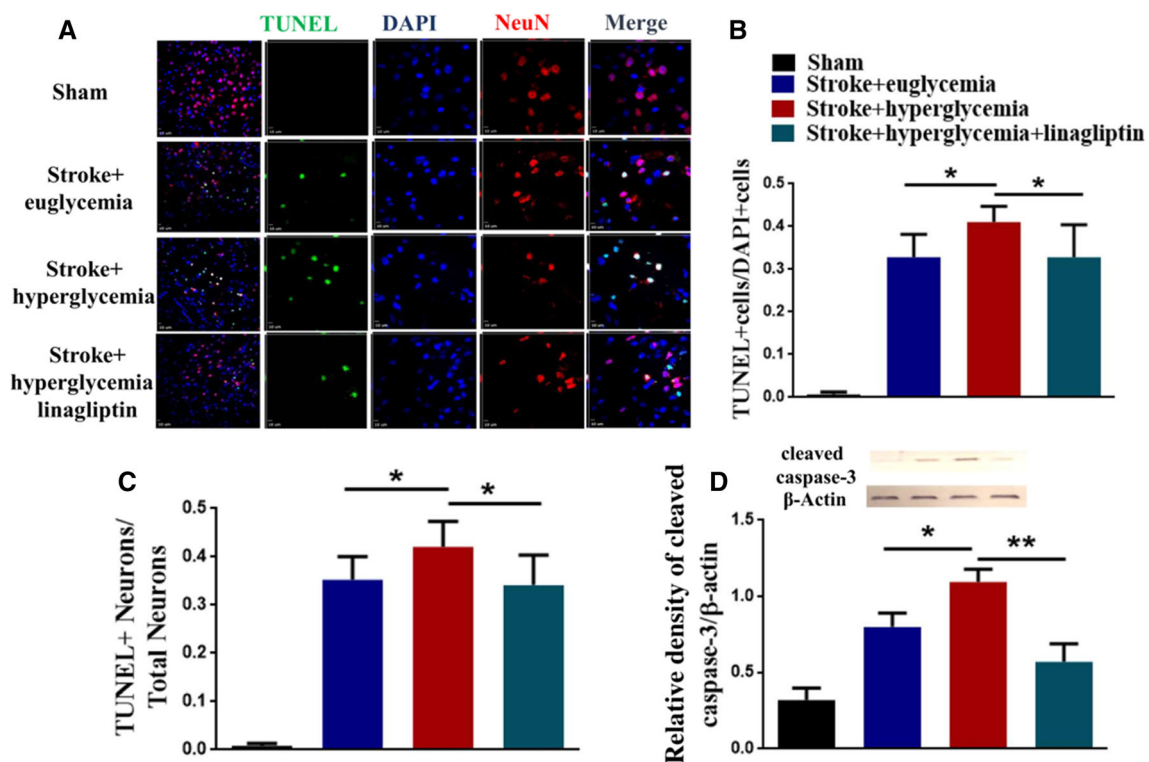

Fig. 3 Linagliptin reduces neuronal cell death in hyperglycemic mice. **A** TUNEL (green), DAPI (blue), and the neuronal marker NeuN (red) staining 3 days after stroke. TUNEL +/NeuN + co-labeled cells indicate dead neurons; TUNEL +/DAPI + co-labeled cells indicate dead cells. **B** The ratio of TUNEL-positive cells to DAPI-positive (blue) cells. **C** The ratio of TUNEL +/NeuN + co-labeled cells to total NeuN + cells. **D** Representative western blots of the protein expression of cleaved caspase-3 in the penumbra 3 days after stroke. Values are expressed as the mean ± SEM. $*P < 0.05$, $**P < 0.01$.

Linagliptin Treatment Decreases Apoptotic Cell Death *via* the Akt/mTOR Pathway

In Western blotting analysis, there were no significant differences in the protein levels of Akt and mTOR between the normal blood glucose and hyperglycemic stroke groups (Fig. 4A–C). The phosphorylated Akt (p-Akt) and phosphorylated mTOR (p-mTOR) proteins, however, were lower in the hyperglycemic stroke group. After treatment with linagliptin, the protein levels were significantly higher (Fig. 4D–F). Compared with the normal blood glucose with stroke group, the Bcl-2 expression in the hyperglycemic group was decreased, and this was prevented by linagliptin treatment ($P < 0.05$) (Fig. 4G–I). On the other hand, compared with the normal blood glucose and stroke group, a higher level of the pro-apoptotic Bax protein was seen in the hyperglycemic group ($P < 0.05$). Consistent with an anti-apoptotic action, the Bax expression was suppressed by linagliptin treatment ($P < 0.05$) (Fig. 4G–I).
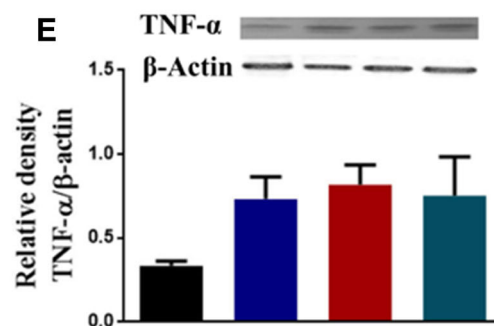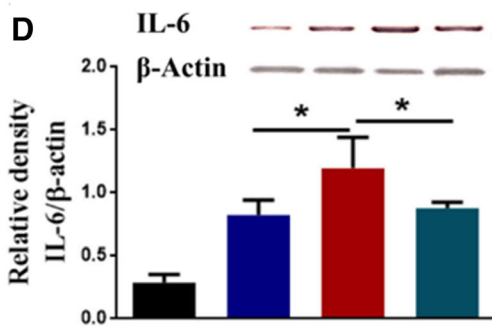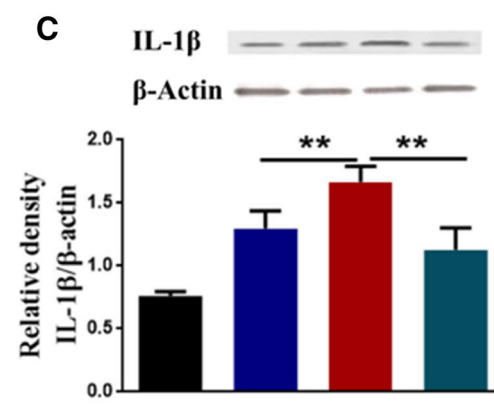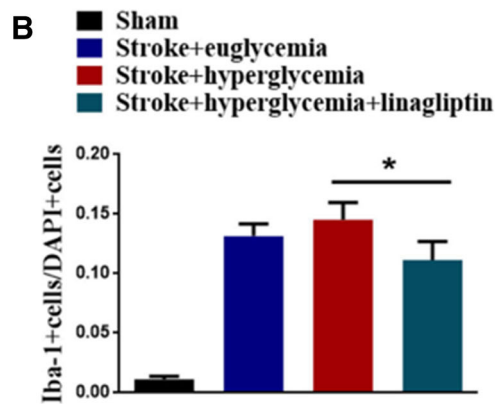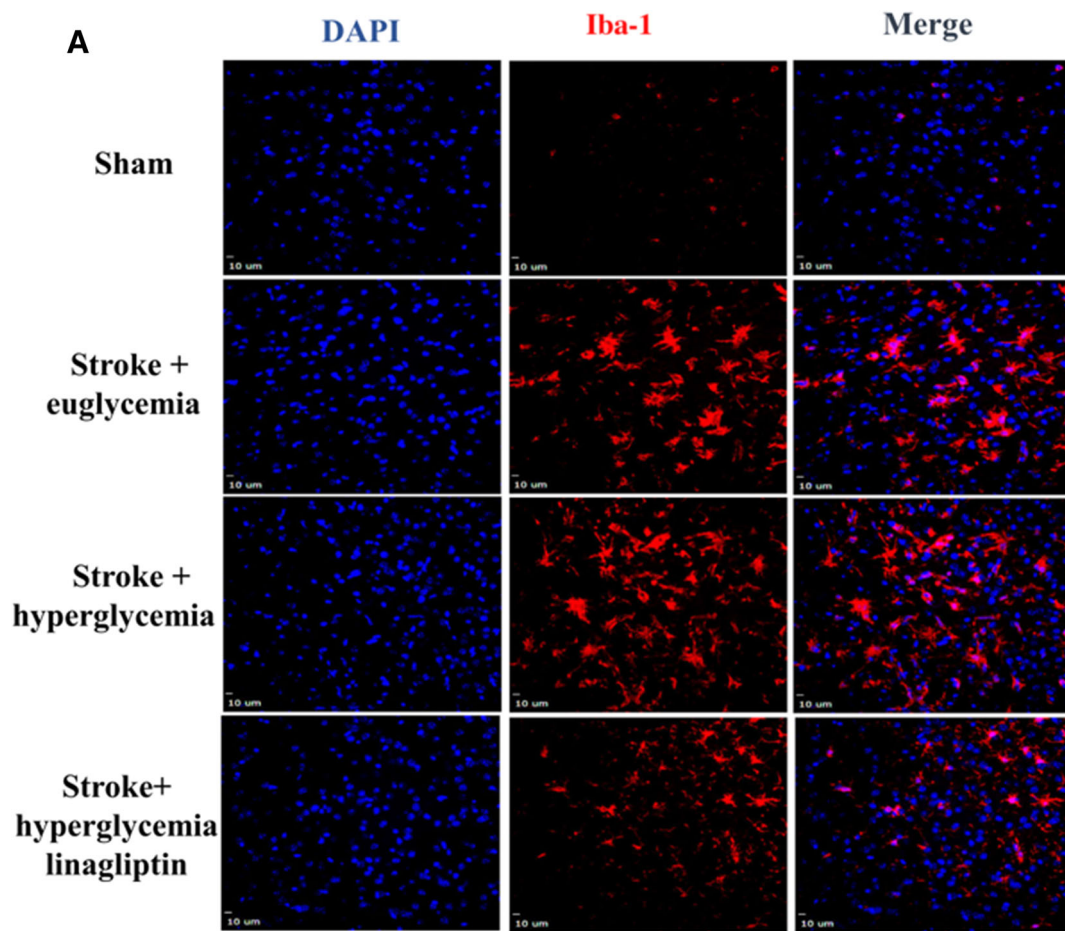
Linagliptin Decreases Inflammation in Hyperglycemic Mice

Inflammation and microglial activation play important roles in the pathogenesis of stroke. To measure whether linagliptin influenced the activation of microglia/macrophages, we detected the expression of Iba-1 in the penumbra 3 days after stroke. Compared with the sham group, microglia were significantly activated in the stroke groups. In addition, the number of Iba-1-positive cells was significantly higher in the hyperglycemic group. After treatment with linagliptin, the number of Iba-1-positive cells was significantly decreased (Fig. 5A, B). Some studies have shown that inflammatory factor precursors are released by activated microglia in stroke [34, 35], so we measured the protein expression of inflammatory factors in the penumbra extra 3 days after stroke using western blot. The results showed that the protein levels of the inflammatory factors IL-1β, IL-6, and TNF-α were significantly
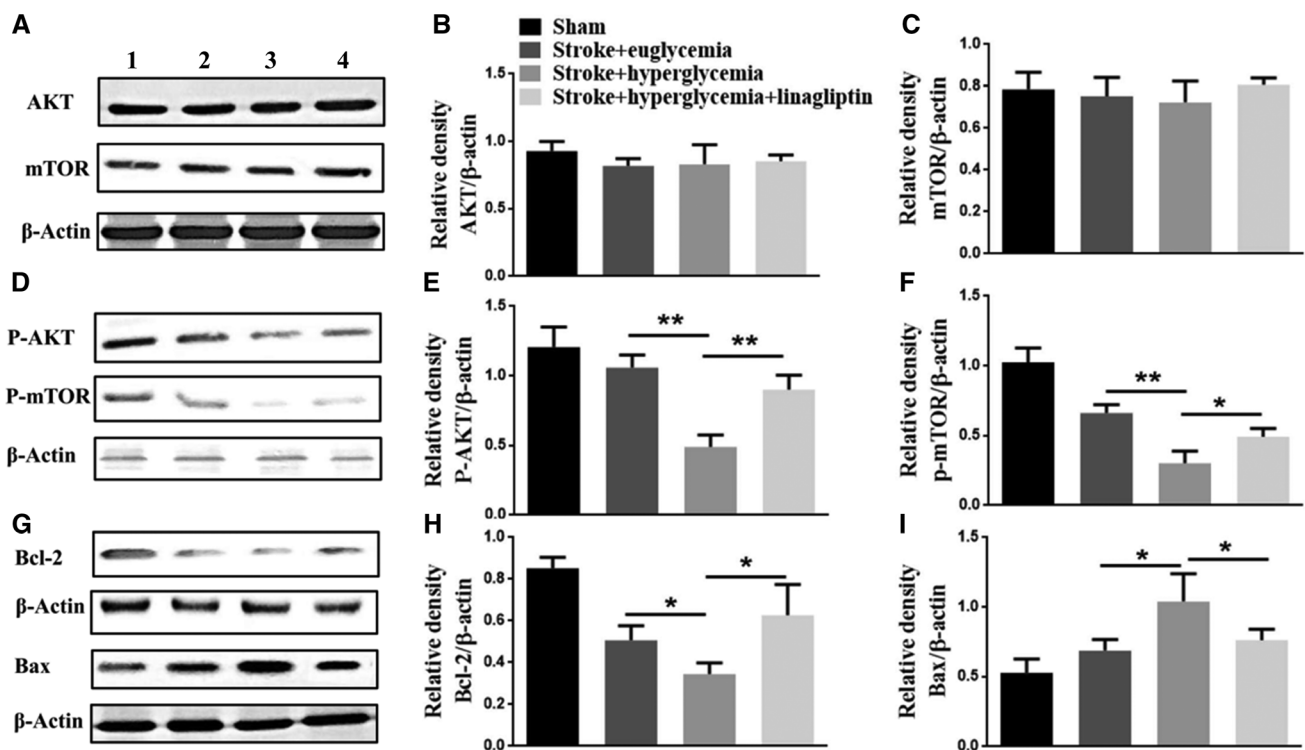
Fig. 4 Linagliptin treatment affects apoptotic cell death *via* the Akt/mTOR pathway. **A–C** The protein expression levels of Akt and mTOR did not significantly differ among groups. **D–F** Representative western blot images and quantification of the protein expression of p-Akt and p-mTOR in the penumbra region. The levels of p-Akt and p-mTOR significantly increased after treatment with linagliptin. **G–I** Representative western blot images and quantification of the protein expression of Bcl-2 and Bax. Bcl-2 levels significantly increased and Bax significantly decreased after treatment with linagliptin. In **A**, **D** and **G**, lane 1, sham group; lane 2, stroke + euglycemia group; lane 3, stroke + hyperglycemia group; and lane 4, stroke + hyperglycemia + linagliptin group. Values are expressed as the mean ± SEM. *$P < 0.05$, **$P < 0.01$.

higher in the stroke groups than in the sham group. After
treatment with linagliptin, the IL-1β and IL-6 levels were
increased compared with the hyperglycemia group
($P < 0.05$) (Fig. 5C–E).

Linagliptin Promotes Recovery of LCBF after Stroke in Hyperglycemic Mice

Recovery of local blood flow is important for cell survival
and tissue repair after stroke. To demonstrate that
linagliptin promoted angiogenesis and the production of
functional blood vessels, we measured the LCBF using a
laser Doppler scanner 21 days after stroke. Scanning
imaging showed that mice with stroke that received
linagliptin treatment had significantly higher LCBF than
the stroke-saline group. At the same time, we found that
LCBF in the hyperglycemic group was relatively lower
than that of the normal blood glucose group ($P < 0.01$)
(Fig. 6A, B).

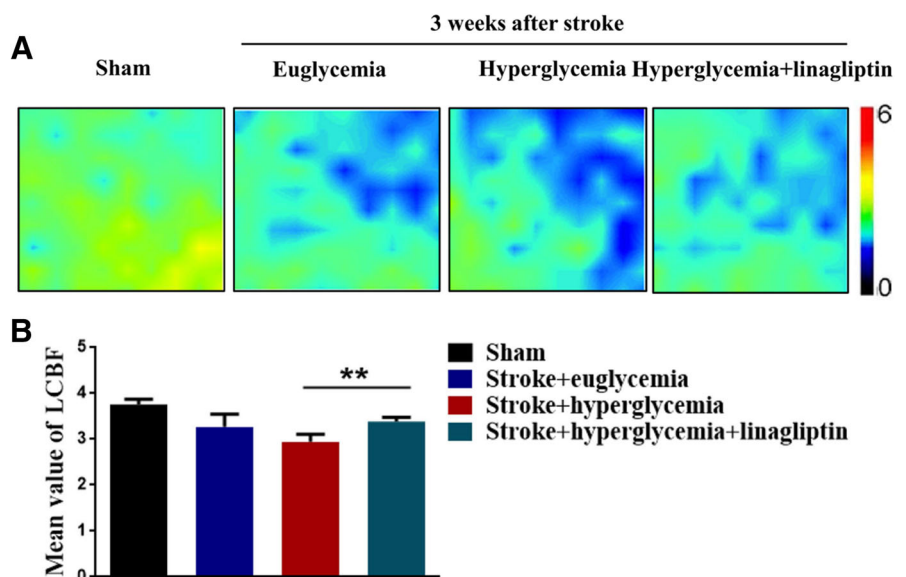### Linagliptin Promotes Functional Recovery after Stroke in Hyperglycemia Mice

We also evaluated whether linagliptin can improve func-
tional deficits after acute stroke. To specifically assess

sensorimotor function, we used the adhesive removal test at
0, 3, 7, and 14 days after stroke. The results showed that
both normal blood glucose and hyperglycemic mice took
significantly more time to detect and remove sticky dots
attached to the left paw after ischemic damage to the
sensorimotor cortex in the right hemisphere. Linagliptin-
treated animals took less time to detect the dot than stroke
control hyperglycemic mice at 7 and 14 days after stroke
($P < 0.05$) (Fig. 7A). Linagliptin-treated animals were
significantly faster in removing the sticky dot than stroke
control hyperglycemic animals at 7 and 14 days after
stroke ($P < 0.05$) (Fig. 7B). In addition, there was no
significant difference in right paw function.

Discussion

In this study, we investigated whether DPP-4 inhibitors are
neuroprotective in a rodent stroke model with hyper-
glycemia. Although previous large studies have shown that
DPP-4 inhibitor treatment does not reduce the incidence of
stroke in diabetic patients [22–24], studying factors that
promote functional recovery after stroke remains directly
relevant to clinical treatment and therapy. Our study
demonstrated the neuroprotective effects of acute linaglip-
tin treatment, explored possible mediating mechanisms *via*
anti-apoptotic and anti-inflammatory actions, and demon-
strated the ability of linagliptin to improve functional
sensorimotor recovery in a hyperglycemic stroke mouse
model. Previous studies have suggested that DPP-4
inhibitors such as alogliptin and linagliptin have beneficial
effects after stroke in normal mice [36, 37] and diabetic
patients who suffer strokes [26, 27]. However, we inves-
tigated the neuroprotective effects of linagliptin on less-

Fig. 6 Linagliptin promotes
LCBF recovery after stroke in
hyperglycemic mice. **A** Local
cerebral blood flow (LCBF) in
the penumbra 21 days after
stroke measured by laser Dop-
pler in each group. **B** Quantified
data showing that animals with
stroke that received linagliptin
exhibited better LCBF recovery
than stroke hyperglycemic ani-
mals that did not receive lina-
gliptin. Values are expressed as
the mean ± SEM. **$P < 0.01$.

Fig. 7 Linagliptin promotes functional recovery after stroke in hyperglycemic mice. **A** Linagliptin-treated animals took less time to detect the adhesive than stroke hyperglycemic mice that did not receive linagliptin at 7 and 14 days in the adhesive removal test. **B** Linagliptin-treated animals were significantly faster in removing the sticky dot than stroke hyperglycemic animals that did not receive linagliptin at 7 and 14 days. Values are expressed as the mean ± SEM. *$P < 0.05$.
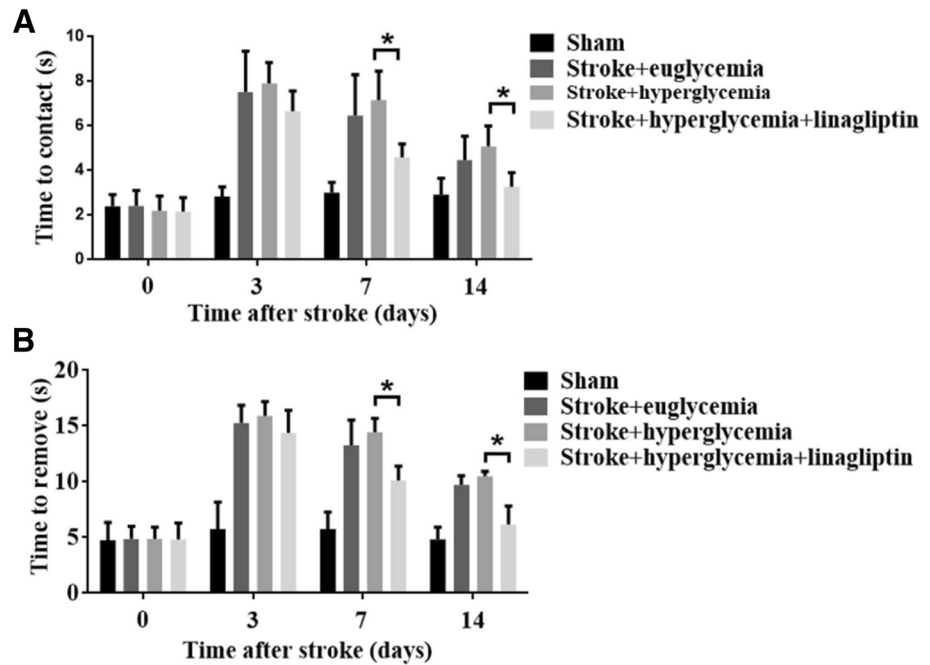
studied hyperglycemic mice and explored the unclear mechanism of the Akt/mTOR pathway.

DPP-4 inhibitors block the enzymatic action of dipeptidyl peptidase-4 and increase the levels of glucagon-like peptide-1 (GLP-1), a peptide hormone secreted by cells in the distal gut and central nervous system (CNS), by preventing the degradation of GLP-1 by DPP-4. GLP-1 increases blood sugar levels *via* direct stimulation of pancreatic β-cells to potentiate insulin secretion [38]. In addition, GLP-1 leads to the feeling of satiety by transmitting signals to the CNS *via* the gut-to-brain axis [39]. GLP-1 receptors have been found in pancreatic tissue and have also been discovered in tissues outside the pancreas, including the heart, kidneys, and brain [40]. They are widely distributed throughout the CNS, with expression in the thalamus, hypothalamus, cerebellum, hippocampus, cortex, and brainstem [41]. Interestingly, GLP-1 has also been identified as a neuropeptide which can cross the blood-brain barrier [42–44].

Recently, the neuroprotective effect of GLP-1 was reported in animal models and clinical work [45–47]. The anti-inflammatory effects of GLP-1 have been found in endothelial islets and pancreatic cells [48–50]. A previous study showed that stimulating GLP-1 receptors reduces the inflammatory response in a rat model of type 1 diabetes [51]. Here, we evaluated the expression of Iba-1 in the penumbra 3 days after stroke. Immunostaining revealed that the number of Iba-1-positive cells was significantly higher in the penumbra than in a sham group. Compared with the normal blood glucose group, the hyperglycemic group showed that Iba-1-positive cells were significantly

increased. However, linagliptin treatment significantly reduced the number of Iba-1-positive cells. In addition, we found that the expression of the inflammatory protein was lower in the linagliptin treatment group than in the hyperglycemic group. A previous study has also shown that GLP-1 might be a modulator of inflammation in the CNS by preventing LPS-induced IL-1β mRNA expression [52]. GLP-1 plays a neuroprotective role by alleviating the inflammatory response in neurodegenerative diseases such as Alzheimer's and Parkinson's Disease [45, 53]. Thus, it has been suggested that GLP-1 may be a therapeutic option for targeting inflammation in the brain, although further clinical research is necessary [54, 55]. In addition, we demonstrated that linagliptin improved the recovery of blood flow after stroke, suggesting the mediation of angiogenesis and the preservation of functional blood vessels by DPP-4 inhibitors. This is in line with previous studies demonstrating that linagliptin improves cerebrovascular function and reverses diabetes-mediated cerebral artery remodeling in diabetic rats [56, 57] and provides another important avenue for future investigations of the use of linagliptin treatment for hyperglycemic stroke patients.

In this study, we observed a significant reduction in neuronal apoptosis in the linagliptin-treated group, suggesting that linagliptin may be involved in regulating the apoptotic pathway. A previous study in a mouse model of stroke with diabetes showed significantly increased expression levels of GLP-1 in peripheral blood and significantly reduced ischemic area of brain injury after linagliptin treatment [58]. *In vitro* experiments revealed that GLP-1

promoted the survival and proliferation of neurons and prevented apoptosis and oxidative stress-related cell death in hippocampal cells and glia [59]. GLP-1 mediates neuronal proliferation through the activation of protein kinase A and phosphoinositide 3-kinase, and regulates the apoptotic signaling pathway through decreased Bax and caspase-3 and increased Bcl-2 to enhance cell survival [60–63]. In our study, we found that linagliptin reduced neuronal death in hyperglycemic mice. It is possible that linagliptin might play a neuroprotective role by suppressing serum GLP-1 degradation and activating GLP-1-induced Akt/mTOR signaling pathways. Previous studies have also reported that GLP-1 protects against amyloid-induced neuronal apoptosis *via* the cAMP signaling pathway and oxidative stress-induced apoptosis *via* the MAPK pathway [64, 65]. Our data showed that linagliptin upregulated the anti-apoptotic protein Bcl-2 and downregulated the pro-apoptotic protein Bax. Linagliptin had an inhibitory effect on the cleavage of caspase-3. The Bcl-2 family contains two classes of protein: pro-apoptotic (such as Bax, Bak, Bok, and BAD) and anti-apoptotic proteins (such as Bcl-w, Bcl-2, and Bcl-xL) [66]. The Bcl-2 protein family constitutes a complex of interactions involved in the regulation of apoptosis. The response of cells to apoptotic signals is determined by the relative expression levels of apoptosis-inducing genes and apoptosis-inhibiting genes. When the expression of Bcl-2 is excessive, apoptosis is inhibited; when Bax is overexpressed, apoptosis is promoted; thus, the ratio of Bcl-2 to Bax is very important. In general, a higher ratio of Bcl-2:Bax protein expression is essential for cell survival and the reverse is essential for apoptosis. For example, after cerebral ischemia, both Bax and Bcl-2 mRNA levels are increased in the penumbra. However, Bax is more abundant than Bcl-2, so the former is the key cause of ischemia-induced cell death [67]. Our further experiments demonstrated that the effects of linagliptin might be mediated through the upregulation of AKT/mTOR signal pathway proteins. Although mTOR is a key regulator of cell growth and proliferation [68, 69], it is also involved in the regulation of apoptosis in a variety of cells [70–72]. Many factors lead to apoptosis, such as oxidative stress. Previous studies by Holville and Green suggest that the oxidative stress-induced apoptosis pathways are the Fas, P53/MPTP, NF-kB, and JNK pathways [73, 74]. In this investigation, we simply explored a possible mechanism in one pathway, which leaves the remaining pathways as interesting targets for future investigations.

In summary, the DPP-4 inhibitor linagliptin reduced neuronal death in hyperglycemic mice, and this may be mediated *via* suppression of the degradation of serum GLP-1 and the activation of GLP-1-induced Akt/mTOR signaling pathways. We also demonstrated that linagliptin

improves tolerance to inflammatory processes. With regard to long-term recovery, linagliptin induced significantly higher LCBF and also promoted functional recovery after stroke in hyperglycemic mice compared to the stroke-saline group. As a result, the DPP-4 inhibitor linagliptin holds future translational potential in stroke patients with hyperglycemia by promoting functional recovery in stroke survivors.

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