Exendin-4 Inhibits Matrix Metalloproteinase-9 Activation and Reduces Infarct Growth After Focal Cerebral Ischemia in Hyperglycemic Mice

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Background and Purpose—Admission hyperglycemia is an independent risk factor for poor outcome of ischemic stroke. Amelioration of hyperglycemia by insulin has not been shown to improve the poststroke outcome. Glucagon-like peptide 1 receptor agonists, which modulate glucose levels by stimulating insulin secretion, have been shown to exert cytoprotective effects by inhibiting inflammation and oxidative stress. This study aimed to evaluate whether the glucagon-like peptide 1 receptor agonist exendin-4 could reduce glucose levels and exert protective effects after acute focal ischemia in hyperglycemic mice.

Methods—Hyperglycemia was induced by intraperitoneal injection of dextrose 15 minutes before transient middle cerebral artery occlusion was performed for 60 minutes using an intraluminal thread. We assessed 4 groups: (1) normal glucose (vehicle control), (2) induced hyperglycemia, (3) induced hyperglycemia with insulin treatment, and (4) induced hyperglycemia with exendin-4 treatment. Neurovascular injuries in brains from each group were evaluated 24 hours and 7 days post ischemia.

Results—Hyperglycemia significantly increased infarct volume (36.3±1.20 versus 26.9±1.28; P<0.001), brain edema (P<0.05), and hemorrhagic transformation compared with control (P<0.001). This increase in infarct volume was associated with increased blood–brain barrier disruption and matrix metalloproteinase-9 activation. Exendin-4, but not insulin, attenuated matrix metalloproteinase-9 activation, proinflammatory cytokine (tumor necrosis factor-α) release, and biomarkers of oxidative stress and showed significant inhibition of infarct growth at 24 hours (23.6±0.97 versus 36.3±1.20; P<0.001) and at 7 days after ischemia (21.0±0.92 versus 29.3±1.41; P<0.001).

Conclusions—Treatment with exendin-4 could be a potentially useful therapeutic option for treatment of acute ischemic stroke with transient hyperglycemia. (Stroke. 2016;47:1328-1335. DOI: 10.1161/STROKEAHA.116.012934.)

Key Words: brain ischemia ■ glucagon-like peptide 1 ■ hyperglycemia ■ insulin ■ matrix metalloproteinase 9 ■ tumor necrosis factor-alpha
glucagon-like peptide-1 receptor agonists could be potential therapeutic agents for various neurodegenerative disorders, including stroke.20,21 Several previous studies, including ours, have demonstrated the neuroprotective role of Ex-4 in nonhyperglycemic models of stroke22–24; however, the effect of Ex-4 treatment on infarct growth and BBB disruption with acute hyperglycemia remains unclear. Therefore, in this study, we analyzed the neurovascular protective effect of Ex-4 in transient hyperglycemic mice after focal ischemia.

Materials and Methods

Experimental Protocol

Animal procedures were approved by the Animal Care Committee of the Juntendo University. Adult 10-week-old male C57BL/6 mice weighing 20 to 25 g were used in this study. They were housed under controlled lighting and provided with food and water ad libitum. Mice were anesthetized with 4.0% isoflurane (Abbott Japan Co., Ltd, Tokyo, Japan) and maintained on 1.0% to 1.5% isoflurane in 70% N2O and 30% O2 using a small-animal anesthesia system. Mice were randomly divided into 4 groups:

1. The vehicle control group. These mice received an intraocular injection of 0.9% saline 15 minutes before left middle cerebral artery occlusion (MCAO) was performed using an intraluminal thread for 60 minutes as described previously.25

2. High glucose group. This group received 50% dextrose (0.6 mL/kg IP) 15 minutes before MCAO.

3. High glucose with insulin group. These mice received 50% dextrose (0.6 mL/kg IP) 15 minutes before MCAO and insulin intraperitoneally 60 minutes after ischemia.26 To adjust the blood glucose level, the dose of insulin administered was determined according to blood glucose level (301–400 mg/dL; 1.0 IU/kg, 401–500 mg/dL; 1.5 IU/kg, >501 mg/dL; 2.0 IU/kg).

4. High glucose with Ex-4 (Sigma-Aldrich, St. Louis, MO) group. This group received 50% dextrose (0.6 mL/kg IP) 15 minutes before MCAO and Ex-4 (1.0 μg/mouse IP) 60 minutes after ischemia.

During the procedure, body temperature was maintained at 37.0±0.5°C using a heating pad. Regional cerebral blood flow was measured in a double-blind fashion under anesthesia using laser-Doppler flowmetry before, during, and after MCAO as well as before the mice were euthanized. The regional cerebral blood flow signal was then obtained from the same place throughout the entire experiment. To measure plasma glucose and insulin, blood (200 μL) was collected from the ophthalmic venous plexus before MCAO and at 0 (immediately), 30 minutes, 60 minutes, 90 minutes, 120 minutes, 180 minutes, and 24 hours after reperfusion (Figure 1A). Measurements were performed using a blood glucose meter (Johnson&Johnson, New Brunswick, NJ) and an insulin ELISA (Ultra Sensitive Mouse Insulin ELISA Kit; Morinaga, Yokohama, Japan). The neurological severity score was a composite of motor (muscle status and abnormal movement), sensory (visual, tactile, and proprioceptive), and reflex test scores, similar to contralateral neglect described in humans.27 The neurological severity was graded on a scale score of 0 to 14 (normal score: 0; maximal deficit score: 14). One point was awarded for the inability to perform, abnormal task performance, or the absence of a tested reflex. At 24 hours and 7 days after reperfusion, mice were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg). We included mice in which the reduction in regional cerebral blood flow in the laser Doppler signal was below 60% compared with the preischemic state. Furthermore, we excluded mice in which a blood glucose level of 300 mg/dL was not achieved despite intraperitoneal administration of glucose. PBS and 4% paraformaldehyde were perfused through the heart, and the mice were decapitated. The 7-day survival rate for each treatment group (n=10–15) was determined using Kaplan–Meier analysis. Of these, 5 mice from each group were used for histological and immuno histochemical analysis at 7 days after ischemia.

Histological Analysis

At 24 hours and 7 days after reperfusion, the brains (n=5 from each group for each time point) were carefully removed and fixed in 4% paraformaldehyde for at least 2 days at 4°C and then placed in 30% sucrose overnight. Nine consecutive coronal cryostat brain slices (20 μm) from the forebrain of each mouse were used for staining. To evaluate infarct area and volume and brain edema, brain slices were stained with cresyl violet, scanned using Axio-Vision software (Carl Zeiss, Jena, Germany), and evaluated using the ImageJ program (National Institutes of Health; http://rsb.info.nih.gov/ij/imagej).28 We also evaluated the brain edema volume ([contralateral volume/ipsilateral volume]−1)×100 as previously reported.29 To assess hemorrhagic changes, the brains were removed (n=10 in each group) and sliced into 2-mm-thick cross sections using a mouse brain matrix (RWD Life Science, Shenzhen, China). Brain sections were incubated in 2% triphenyl tetrazolium chloride solution (Sigma) at 37°C for 20 minutes. In accordance with a previous study,26 the grade of hemorrhagic transformation was classified into 5 groups: (1) non-hemorrhage; (2) hemorrhagic infarction type 1, defined as small petechiae generally along the boundary of the infarct; (3) hemorrhagic infarction type 2 with more confluent petechiae within the damaged area; (4) parenchymal hemorrhage type 1 characterized by blood clots in 30% of the injured parenchyma; and (5) parenchymal hemorrhage type 2 with clots in 30% of the infarct.

Double Immunofluorescence Immunohistochemistry

Double immunofluorescence staining was performed by simultaneous incubation of the sections with DyLight 594-labeled Lycopersicon Esculentum (Tomato) Lectin (Vector Laboratories, Burlingame, CA), rat anti-neutrophil (dilution 1:100; Abcam, Mayo, MN), and anti-Iba-1 (dilution 1:500; Abcam) antibodies. For double labeling, the primary antibodies were detected with rhodamine- or fluorescein isothiocyanate–conjugated secondary antibody (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA) after incubation for 1 hour at room temperature. Subsequently, the slides were covered with VECTASHIELD mounting medium with 4,6-diamidino-2-phenylindole (Vector Laboratories).

IgG Staining

After paraformaldehyde fixation, 20-μm-thick brain sections were incubated in 3% H2O2 followed by blocking with 10% bovine serum albumin (Sigma) in PBS. Then, the sections were incubated overnight at 4°C with donkey anti-mouse IgG 1:300 (Vector Laboratories). Immunoreactivity visualization using the avidin–biotin complex method (Vectastain ABC kit, dilution 1:400; Vector Laboratories) or fluorescein-conjugated streptavidin.

Terminal Deoxynucleotidyl Transferase–Mediated dUTP-Biotin Nick-End Labeling Staining

Terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick-end labeling staining was performed according to the manufacturer’s protocols (In Situ Cell Death Detection kit; Roche Diagnostics, Mannheim, Germany) on the 20-μm-thick coronal sections. After incubation in 0.1% sodium citrate in 0.1% PBS containing 0.1% Triton X-100, the sections were incubated with the terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick-end labeling reaction mixture for 60 minutes at 37°C in the dark.

SDS-PAGE and Immunoblotting

In each animal, a brain sample was harvested from the ischemic region comprising the cortex on the operated side at 24 hours after reperfusion (n=5 in each group). Protein extraction and electrophoresis were performed as described previously.30 After performing electrophoresis and transferring to polyvinylidene difluoride membranes,
the membranes were blocked in Brockace (Dainichi-Seiyaku, Gifu, Japan) for 60 minutes at room temperature. Membranes were then incubated overnight at 4°C with primary antibodies against anti–tumor necrosis factor-α (dilution 1:500; Abcam), anti-dinitrophenol (dilution 1:500; Millipore, Billerica, MA) and mouse anti-actin (dilution 1:100,000; Millipore) antibodies, followed by incubation with peroxidase-conjugated secondary antibodies and visualization by enhanced chemiluminescence (GE healthcare UK, Little Chalfont, Buckinghamshire, England).

Gelatin Zymography

The collected brain samples were concentrated, and then each sample was mixed with equal amounts of SDS sample buffer (Thermo Fisher Scientific Inc., Waltham, MA) and electrophoresed on 8% SDS-PAGE containing 1 mg/mL gelatin as the protease substrate. After electrophoresis, gels were placed in 2.7% Triton X-100 for 1 hour to remove SDS and then incubated for 40 hours at 37°C in developing buffer (50 mmol/L Tris base, 40 mmol/L HCl, 200 mmol/L NaCl, 5 mmol/L CaCl₂, and 0.2% Brij 35; Invitrogen, Carlsbad, CA) on a rotary shaker. After incubation, gels were stained in 30% methanol, 10% acetic acid, and 0.5% wt/vol Coomassie brilliant blue for 1 hour followed by destaining. Human matrix metalloproteinase-9 (MMP-9) standards (Chemicon, Heule, Kortrijk, Belgium) were used as positive controls.

Cell Count and Statistical Analysis

For immunohistochemical analysis, positively stained cells in the ischemic boundary area of neuronal nuclei-positive and neuronal nuclei-negative brain areas (transition area⁶; 0.25 mm²) were counted in 3 sections from each of the 5 mice using ZEN software (Carl Zeiss). This counting was performed by an investigator who was blinded to the experimental groups. For MMP-9/lectin staining, cell count was performed semiquantitatively by determining the percentage of MMP-9/lectin merged area in 0.25 mm² of the ischemic boundary zone. Power estimates were calculated based on α=0.05 and β=0.8 to obtain group sizes appropriate for detecting effect sizes in the range of 30% to 50% for in vivo models. All values in this study are expressed as mean±SEM. A 2-way ANOVA followed by post hoc Fisher-protected least significant difference test was used to determine the significant differences in various indices, except for the neurological severity score and triphenyl tetrazolium chloride staining, among the groups. Wilcoxon rank-sum test was used to determine the significant differences in neurological severity score and hemorrhagic transformation. P values <0.05 indicate statistical significance. All experiments and measurements including behavior outcome assessment, infarct volume measurement, and histological analysis were performed in a blinded and randomized manner.

Results

Experimental Design and Effects of Hyperglycemia on Physiological Parameters

First, we examined the different doses of Ex-4 (0.1, 1.0, and 10 μg/mice) in induced hyperglycemic mice to determine the effective dose for attenuation of hyperglycemia. Ex-4 doses of 1.0 and 10 μg/mice, but not 0.1 μg/mice, improved blood glucose levels (data not shown); therefore, we used the dose of 1.0 μg/mice for further experiments. There were no differences in regional cerebral blood flow between groups.
Blood glucose levels were over 450 mg/dL 30 minutes after ischemia and gradually decreased to within the normal range 24 hours after ischemia (Figure 1C). Treatment with Ex-4 as well as insulin decreased blood glucose levels significantly 2 hours after ischemia compared with levels in the induced hyperglycemia (IH) group (Figure 1C). No significant differences in blood glucose levels were observed between insulin and Ex-4 treatment groups at each time point. Serum insulin levels of the IH+insulin and IH+Ex-4 groups were significantly higher than that in the IH group 2 h after ischemia, and subsequently decreased in a time-dependent manner (Figure 1D).

**Ex-4, but Not Insulin, Attenuates Infarct Growth, Brain Edema, and Cell Death in the Ischemic Brain Under Hyperglycemic Conditions**

Hyperglycemia significantly increased either the infarct volume or the brain edema volume compared with that in the vehicle control at 24 hours after ischemia (Figure 2A). Although trends in infarct volume were observed at 7 days after ischemia, there were no significant differences in amount of brain edema between any of the groups (Figure 2A). Ex-4 treatment significantly attenuated growth of infarct volume compared with not only the IH group but also the IH+insulin group at each time point. A similar trend was also observed for brain edema at 24 hours after ischemia. This beneficial effect of Ex-4 treatment was associated with significant improvement in neurological scores (Figure 2B).

Hyperglycemia also decreased the survival rate 7 days after ischemia, and Ex-4 treatment improved the survival rate; this improvement was not observed in the IH+insulin group (Figure 2C). Ex-4 treatment also showed significant reduction in terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick-end labeling–positive cells in the ischemic boundary zone compared with that in the IH or IH+insulin groups (Figure 2D).

**Effects of Ex-4 on MMP-9 Activation and BBB Permeability**

Hyperglycemic mice showed a significantly higher grade of hemorrhagic transformation compared with mice in the other 3 groups (Figure 3A). Hyperglycemia significantly increased IgG leakage 24 hours after ischemia, but Ex-4 treatment...
resulted in significant reduction in IgG leakage compared with that in the IH group (Figure 3B). Activation of MMP-9 was significantly higher in the IH group than in the vehicle control group. However, treatment with Ex-4, but not insulin, attenuated the activation of MMP-9 compared with that in the IH group after ischemia (Figure 3C). The expression of MMP-9 was observed in tomato lectin-positive endothelial cells, and the number of MMP-9/tomato lectin double-positive cells was significantly higher in IH group than in the other 3 groups (Figure 3D). The number of double-positive cells in the IH+Ex-4 group was significantly lower than that in the IH or IH+insulin groups (Figure 3D).
Effects of Ex-4 on Migration and Activation of Microglial or Neutrophil Cells, Expression of Proinflammatory Cytokines, and Oxidative Stress

No migration of neutrophils in the contra lateral hemisphere was observed in each group (data not shown). Hyperglycemia increased the infiltration of neutrophils in the infarct area compared with that in the other 3 groups (Figure 4A). Ex-4 treatment resulted in significantly less neutrophil infiltration compared with the IH and IH+insulin groups (Figure 4A). Ex-4 treatment also significantly reduced the number of Iba-1–positive microglia/macrophages in the infarct area compared with the IH and IH+insulin groups (Figure 4B). Hyperglycemia significantly increased the level of proinflammatory cytokine tumor necrosis factor-α, which is cytotoxic in the acute stage of brain ischemia. Ex-4 treatment significantly decreased the level of tumor necrosis factor-α compared with that in the IH and IH+insulin groups (Figure 4C). Furthermore, protein oxidation, which is one of the biomarkers of oxidative stress, was also elevated in the IH group, but Ex-4 treatment resulted in significant attenuation of dinitrophenol compared with the IH group (Figure 4D).

Discussion

Transient hyperglycemia by dextrose infusion results in a 48% larger infarct volume in an experimental model of stroke. Furthermore, transient severe hyperglycemia, especially when introduced shortly after ischemia, leads to enhanced BBB disruption and promotes hemorrhagic transformation in a transient ischemia/reperfusion rat model. Hyperglycemia increases oxidative stress and MMP-9 activation after focal
ischemia/reperfusion, and these changes play a critical role in postischemic BBB regulation\(^3\) and excessive brain inflammation\(^,29^{–}33\). Our data indicate that insulin treatment significantly decreases plasma glucose levels to a similar range as that in vehicle control; however, this was not sufficient to ameliorate infarct growth and improve the functional severity score. On the other hand, Ex-4 treatment significantly attenuated MMP-9 activation and BBB permeability compared with the hyperglycemia group. Ex-4 also reduced the proinflammatory cytokines and biomarkers of oxidative stress, which might be associated with attenuation of infarct growth and functional severity. Insulin also exerted a mild protective effect on hemorrhagic transformation and protein oxidation. In this regard, insulin also has the potential to inhibit MMP-9 activity and protect endothelial cells in subacute arterial injury\(^36\) or in atherosclerotic intimal lesions of diabetic apolipoprotein E–deficient (apoE\(^{-/-}\)) mice.\(^37\) However, it is still controversial whether insulin-mediated reduction in blood glucose in acute hyperglycemia during ischemic stroke represents an effective intervention.\(^38\) Moreover, caution must be used when attempting to achieve euglycemic control with insulin treatment because of the risk of hypoglycemia.\(^12,13\)

On the other hand, Ex-4 treatment could reduce blood glucose levels in a safer manner than insulin treatment by stimulating insulin secretion in a glucose-dependent manner.\(^38,39\) In fact, Ex-4 increased the serum insulin level as much as insulin treatment, resulting in a significant reduction in blood glucose levels. Furthermore, Ex-4 independently attenuates MMP-9 mRNA levels in a kidney ischemia/reperfusion model.\(^40\) Moreover, Ex-4 treatment has also been shown to suppress the levels of tumor necrosis factor-\(\alpha\) and monocyte chemotactic protein-1 in lipopolysaccharide-induced inflammation.\(^41\) In addition to insulin secretion, these synergistic effects of Ex-4 might result in a more pronounced reduction of MMP-9 activity, BBB disruption, and inflammatory responses beyond the reduction of blood glucose in postischemic hyperglycemic mice. Thus, Ex-4 protects against ischemic neuronal cell death and neurovascular damage and could be potentially more useful than insulin for the treatment of acute ischemic stroke with hyperglycemia.

Although we demonstrated the importance of hyperglycemia treatment with Ex-4 in the acute phase of ischemic stroke, this study has several potential limitations. First, we used a transient severe hyperglycemic model. Previous reports indicate that sustained mild hyperglycemia also increases BBB disruption.\(^34\) Furthermore, blood glucose levels usually exceed 360 mg/mL in dextrose infusion models, and this level is usually greater than that typically encountered in clinical practice. Second, we used a transient MCAO model in this study. Although this model is highly reproducible and widely used in basic stroke research, it is not comparable with naturally occurring clinical strokes.\(^42\) Other stroke models such as permanent MCAO, the thromboembolic clot model, or using aged animals should be studied in future experiments.\(^43\)

In conclusion, we demonstrated hyperglycemia-induced increases in MMP-9 activity and BBB disruption that resulted in infarct growth and hemorrhagic transformation after focal ischemia/reperfusion in transient hyperglycemic mice. Ex-4, which is already used clinically for type 2 diabetes mellitus treatment with low hypoglycemic risk, could be a strong candidate for neurovascular protective treatment of ischemic stroke with hyperglycemia.

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**Disclosures**

None.

**References**


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