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

Takuma Kuroki, MD; Ryota Tanaka, MD, PhD; Yoshiaki Shimada, MD, PhD; Kazuo Yamashiro, MD, PhD; Yuji Ueno, MD, PhD; Hideki Shimura, MD, PhD; Takao Urabe, MD, PhD; Nobutaka Hattori, MD, PhD

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

Admission hyperglycemia, which has been reported in up to 30% to 40% of acute ischemic stroke cases, exacerbates acute ischemic stroke–induced brain damage.1,2 Hyperglycemia is associated with high risk of short-term mortality in ischemic stroke patients without diabetes mellitus.3 Furthermore, an elevated glucose level is associated with increased infarct volume and neurological deterioration in patients without diabetes mellitus, but not in patients with diabetes mellitus.4 These findings indicate that acute hyperglycemia may be more harmful in nondiabetic cases. In animal models of ischemic stroke, induction of hyperglycemia before stroke was significantly associated with increased infarct volume1,6 and blood–brain barrier (BBB) permeability and hemorrhagic transformation with or without thrombolysis.7,8 In human acute ischemic stroke, euglycemic control by insulin treatment failed to improve the short-term prognosis9-11 and conferred significant risk of hypoglycemia.12,13 Hypoglycemia has also been shown to exacerbate brain ischemic damage.14 Because few treatment strategies exist for hyperglycemia management in acute ischemic stroke, alternative treatments are needed.

Glucagon-like peptide-1, a hormone secreted by the small intestine in response to eating, facilitates glucose-dependent insulin secretion.15 Exendin-4 (Ex-4) is a stable glucagon-like peptide-1 receptor agonist that mitigates hyperglycemia in diabetes mellitus,16 with low risk of hypoglycemia.17 If hypoglycemia does occur, it is usually associated with concomitant insulin or insulin secretagogue use.16 Ex-4 has also exhibited beneficial effects on endothelial dysfunction, oxidative stress, and inflammation in human studies.18,19 Thus,
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., 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 intraperitoneal 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. 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 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).

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).26 We also evaluated the brain edema volume ([contralateral volume ipsilateral volume /]−1)×100 as previously reported.27 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,28 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=3 in each group). Protein extraction and electrophoresis were performed as described previously.29 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 1:10,000; 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 CuCl₂, and 0.2% 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 area²⁵; 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.
(Figure 1B). 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.32 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,33 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.10 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.34 Hyperglycemia increases oxidative stress and MMP-9 activation after focal...
ischemia/reperfusion, and these changes play a critical role in postischemic BBB regulation and excessive brain inflammation. 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

In conclusion, we demonstrated hyperglycemia-induced treatment with low hypoglycemic risk, could be a strong candidate for neurovascular protective treatment of ischemic stroke with hyperglycemia.

Acknowledgments
We gratefully acknowledge the assistance of Risa Nonaka of Juntendo University Research Institute for Diseases of Old Age for her histopathologic evaluations.

Sources of Funding
This study was partly supported by a grant from the Research Institute for Diseases of Old Age, Juntendo University School of Medicine, Tokyo, and by Japan Society for the Promotion of Science KAKENHI Grant Number, Dr. Shimura; 24500423, Dr Urabe; 25430053, Dr. Tanaka; 25430053. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the article.

Disclosures
None.

References
2001;189:105–111.


Exendin-4 Inhibits Matrix Metalloproteinase-9 Activation and Reduces Infarct Growth After Focal Cerebral Ischemia in Hyperglycemic Mice
Takuma Kuroki, Ryota Tanaka, Yoshiaki Shimada, Kazuo Yamashiro, Yuji Ueno, Hideki Shimura, Takao Urabe and Nobutaka Hattori

Stroke. 2016;47:1328-1335; originally published online March 15, 2016; doi: 10.1161/STROKEAHA.116.012934
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/47/5/1328

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2017/07/10/STROKEAHA.116.012934.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/
exendin-4は高血糖マウスにおける局所脳虚血後のマトリックスメタプロテアーゼ-9の活性化を阻害し梗塞成の拡大を抑制する

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

Takuma Kuroki, MD1; Ryota Tanaka, MD, PhD1; Yoshiaki Shimada, MD, PhD2, et al.

1Department of Neurology, Juntendo University, Tokyo, Japan; and 2Department of Neurology, Juntendo University Urayasu Hospital, Chiba, Japan