Exendin-4 inhibits MMP-9 activation and reduces infarct growth after focal cerebral ischemia in hyperglycemic mice

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Abstract

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 post-stroke outcome. Glucagon-like peptide 1 receptor (GLP-1R) 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 GLP-1R 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 min before transient middle cerebral artery occlusion (MCAO) was performed for 60 min using an intraluminal thread. We assessed four groups: 1) normal glucose (vehicle control), 2) induced hyperglycemia (IH), 3) IH with insulin treatment, and 4) IH with exendin-4 treatment. Neurovascular injuries in brains from each group were evaluated 24 h and 7 days post-ischemia.

Results—Hyperglycemia significantly increased infarct volume (36.3±1.20 vs. 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 MMP-9 activation. Exendin-4, but not insulin, attenuated MMP-9 activation, pro-inflammatory cytokine (TNF-α) release, and biomarkers of oxidative stress, and showed significant inhibition of infarct growth at 24 h (23.6±0.97 vs. 36.3±1.20, P<0.001) and at 7 days after ischemia (21.0±0.92 vs. 29.3±1.41, P<0.001).

Conclusion—Treatment with exendin-4 could be a potentially useful therapeutic option for treatment of acute ischemic stroke with transient hyperglycemia.
Introduction

Admission hyperglycemia, which has been reported in up to 30-40% 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.\(^3\) Furthermore, an elevated glucose level is associated with increased infarct volume and neurological deterioration in patients without diabetes, but not in patients with diabetes.\(^4\) These findings indicate that acute hyperglycemia may be more harmful in non-diabetic cases.

In animal models of ischemic stroke, induction of hyperglycemia before stroke was significantly associated with increased infarct volume\(^5,6\) 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 prognosis\(^9-11\) 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 (GLP-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 GLP-1 receptor agonist that mitigates hyperglycemia in diabetes,\(^16\) 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, GLP-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 non-hyperglycemic models of stroke\(^22-24\), 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 1.0–1.5% isoflurane in 70% N₂O and 30% O₂ using a small-animal anesthesia system. Mice were randomly divided into four groups:

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

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

3. High glucose with insulin group: These mice received 50% dextrose (0.6 mL/kg) IP 15 min before MCAO and insulin IP 60 min 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, over 501 mg/dl; 2.0 IU/kg).

4. High glucose with Ex-4 (Sigma-Aldrich, St. Louis, MO, U.S.A) group: This group received 50% dextrose (0.6 mL/kg) IP 15 min before MCAO and Ex-4 (1.0 μg/mouse) IP 60 min 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 sacrificed. 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 min, 60 min, 90 min, 120 min, 180 min, and 24 h after reperfusion (Figure 1A). Measurements were performed using a blood glucose meter (Johnson & Johnson, New Brunswick, NJ, U.S.A) and an insulin enzyme-linked immunosorbent assay (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 testing described in humans. The neurological severity was graded on a scale of 0–14 (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 h and 7 days after reperfusion, mice were anesthetized with an IP injection of pentobarbital (50 mg/kg). We included mice in which the reduction of regional cerebral blood flow in the laser Doppler signal was below 60% compared to the pre-ischemic state. Furthermore, we excluded mice in which a blood glucose level of 300 mg/dl was not achieved despite intraperitoneal administration of glucose. Phosphate-buffered saline (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 immunohistochemical analysis at 7 days after ischemia.

**Histological Analysis**

At 24 h 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 Image J program (NIH, http://rsb.info.nih.gov/nih-image/). We also evaluated the brain edema volume [(contralateral volume/ipsilateral volume)-1]×100 as previously reported. 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 (TTC) solution (Sigma) at 37 °C for 20 min. In accordance with a previous study, 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, U.S.A), rat anti-neutrophil (dilution 1:100, Abcam, Mayo, MN, U.S.A), 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, U.S.A) after incubation for 1 h at room temperature. Subsequently, the slides were covered with VECTASHIELD mounting medium with DAPI (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 visualized using the avidin–biotin complex method (Vectastain ABC kit, dilution 1:400, Vector Laboratories) or fluorescence-conjugated streptavidin.

Terminal Deoxynucleotidyl Transferase (TdT)-Mediated dUTP-Biotin Nick-End Labeling (TUNEL) Staining

TUNEL 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 TUNEL reaction mixture for 60 min at 37°C in the dark.

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis and Immunoblotting

In each animal, a brain sample was harvested from the ischemic region comprising the cortex on the operated side at 24 h after reperfusion (n = 5 in each group). Protein extraction and electrophoresis were performed as described previously. After performing electrophoresis and transferring to polyvinylidene difluoride membranes, the membranes were blocked in Brockace (Dainichi-Seiyaku, Gifu, Japan) for 60 min at room temperature. Membranes were then incubated overnight at 4°C with primary antibodies against anti-tumor necrosis factor-α (TNF-α) (dilution 1:500, Abcam), anti-dinitrophenol (DNP) (dilution 1:500, Millipore, Billerica, MA, USA) 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, U.S.A) and electrophoresed on 8% SDS-polyacrylamide gels containing 1 mg/mL gelatin as the protease substrate. Following electrophoresis, gels were placed in 2.7% Triton X-100 for 1 h to remove SDS, and then incubated for 40 h 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% Briji 35; Invitrogen, Carlsbad, CA, U.S.A) on a rotary shaker. After incubation, gels were stained in 30% methanol, 10% acetic acid, and 0.5% w/v Coomassie brilliant blue for 1 h followed by de-staining. 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 NeuN-positive and NeuN-negative brain areas (transition area)³¹ (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 $\alpha = 0.05$ and $\beta = 0.8$ 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 ± standard error of the mean. A two-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 TTC staining, among the groups. Wilcoxon rank sum test was used to determine the significant differences in neurological severity score and hemorrhagic transformation. P values less than 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

Firstly, we examined the different doses of Ex-4 (0.1 μg, 1.0 μg, 10 μg/mice) in induced hyperglycemic mice to determine the effective dose for attenuation of hyperglycemia. Ex-4 doses of 1.0 μg/mice 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 min after ischemia and gradually decreased to within the normal range 24 h after ischemia (Figure 1C). Treatment with Ex-4 as well as insulin decreased blood glucose levels significantly 2 h after ischemia compared to levels in the 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).

Exendin-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 brain edema volume compared to that in the vehicle control at 24 h 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 to 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 h after ischemia. This beneficial effect of Ex-4 treatment was associated with significant improvement in neurologic 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 of TUNEL-positive cells in the ischemic boundary zone compared to that in the IH or IH+insulin groups (Figure 2D).

Effects of Exendin-4 on Matrix MetalloProteinase-9 (MMP-9) Activation and BBB Permeability

Hyperglycemic mice showed a significantly higher grade of hemorrhagic transformation compared to mice in the other three groups (Figure 3A). Hyperglycemia significantly increased IgG leakage 24 h after ischemia, but Ex-4 treatment resulted in significant reduction in IgG leakage compared to 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 to 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 compared with that in the other three groups.
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 Exendin-4 on Migration and Activation of Microglial or Neutrophil cells, Expression of Pro-inflammatory 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 to that in the other three groups (Figure 4A). Ex-4 treatment resulted in significantly less neutrophil infiltration compared to 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 to the IH and IH+insulin groups (Figure 4B). Hyperglycemia significantly increased the level of pro-inflammatory cytokine TNF-α, which is cytotoxic in the acute stage of brain ischemia. Ex-4 treatment significantly decreased the level of TNF-α compared to 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 DNP compared to 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 post-ischemic BBB regulation and excessive brain
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 to the hyperglycemia group. Ex-4 also reduced the pro-inflammatory 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 or in atherosclerotic intimal lesions of diabetic apolipoprotein E-deficient (apoE-/-) mice. However, it is still controversial whether insulin-mediated reduction in blood glucose in acute hyperglycemia during ischemic stroke represents an effective intervention. Moreover, caution must be used when attempting to achieve euglycemic control with insulin treatment because of the risk of hypoglycemia. 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. In fact, Ex-4 increased the serum insulin level as much as insulin treatment, resulting in a significant reduction of blood glucose levels. Furthermore, Ex-4 independently attenuates MMP-9 mRNA levels in a kidney ischemia/reperfusion model. Moreover, Ex-4 treatment has also been shown to suppress the levels of TNF-α and monocyte chemoattractant protein-1 (MCP-1) in LPS-induced inflammation. 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 post-ischemic 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.\textsuperscript{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 (tMCAO) model in this study. Although this model is highly reproducible and widely used in basic stroke research, it is not comparable to naturally occurring clinical strokes.\textsuperscript{42} Other stroke models such as permanent MCAO, the thromboembolic clot model, or using aged animals should be studied in future experiments.\textsuperscript{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 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

8. Won SJ, Tang XN, Suh SW, Yenari MA, Swanson RA. Hyperglycemia promotes tissue plasminogen activator-induced hemorrhage by increasing superoxide production. *Ann Neurol.* 2011;70:583-590


Figure Legends

Figure 1 Physiological parameters

(A) Experimental protocol. (B) Temporal changes in rCBF until 24 h after ischemia. Data are mean ± s.e.m. of five mice in each group. (C) Changes in plasma glucose and (D) insulin levels in each groups. Data are mean ± s.e.m. of 5 mice (panels B, C, and D) in each group. * P < 0.05, ** P < 0.01, # P < 0.001; Ex-4, exendin-4; rCBF, regional cerebral blood flow; IS, ischemia.

Figure 2 Attenuation of infarct growth, brain edema, and cell death by Ex-4 in hyperglycemia

(A) Infarct volume and brain edema at 24 h and 7 d after reperfusion in each group. Bar = 2 mm. (B) Neurologic severity score in each group. (C) Survival rate in each group until 7 d after ischemia. (D) TUNEL staining in each group at 24 h after reperfusion. Data are mean ± s.e.m. of 5 mice (panels A, B, and D) and 10-15 mice (panel C) in each group. * P < 0.05, ** P < 0.01, # P < 0.001; Ex-4, exendin-4; IS, ischemia; IH, induced hyperglycemia; a, vehicle control group at 24 h; b, induced hyperglycemia group at 24 h; c, induced hyperglycemia with insulin treatment group at 24 h; d, induced hyperglycemia with Ex-4 treatment group at 24 h. e, vehicle control group at 7 d; f, induced hyperglycemia group at 7 d; g, induced hyperglycemia with insulin treatment group at 7 d; h, induced hyperglycemia with Ex-4 treatment group at 7 d.

Figure 3 Effects of Ex-4 on blood–brain–barrier integrity and permeability, and expression of MMP-9.

(A) Protective effect of Ex-4 against ischemia/reperfusion on macroscopic hemorrhages. Hemorrhages were classified by type and extension into five groups: (grade 1) non
hemorrhage; (grade 2) hemorrhagic infarction type 1 defined as small petechiae, generally along the boundary of the infarct; (grade 3) hemorrhagic infarction type 2 with more confluent petechiae within the damaged area; (grade 4) parenchymal hemorrhage type 1 characterized by blood clots in 30% of the injured parenchyma; and (grade 5) parenchymal hemorrhage type 2 with clots in 30% of the infarct. Each animal was assigned to 1 group according to the severity of bleeding. Data are expressed as percent of animals showing signs of bleeding. (B) IgG staining in each group at 24 h after reperfusion. Bar = 2 mm. (C) Zymographic analysis of MMP-9 and densitometric analysis of MMP-9 protein. (D) The expression of MMP-9 in cerebrovascular endothelium and semiquantitation of the percentage of MMP-9/lectin merged area in 0.25 mm² of the ischemic boundary zone. Arrows indicate MMP-9 positive cells. Bar = 50 µm. Data are mean ± s.e.m. of 10 (panel A) and 5 mice (panels B, C and D).

* P < 0.05, ** P < 0.01, # P < 0.001; Ex-4, exendin-4; IS, ischemia; IH, induced hyperglycemia; a, vehicle control group; b, induced hyperglycemia group; c, induced hyperglycemia with insulin treatment group; d, induced hyperglycemia with Ex-4 treatment group.

Figure 4 Effect of Ex-4 on inflammatory response.

(A) Infiltrated neutrophils in 0.25 mm² of the ischemic boundary zone. Bar = 50 µm. (B) Immunofluorescence of Iba-1 in the ischemic boundary zone at 24 h after ischemia, and semiquantitation of the percentage of Iba-1-positive cells in 0.25 mm² of the ischemic boundary zone. Bar = 50 µm. (C) Immunoblot analysis of TNF-α. (D) Immunoblot analysis of DNP. Equal protein loading was confirmed by measuring β-actin level. Data are mean ± s.e.m. of 5 mice (panels A, B, C, and D).

* P < 0.05, ** P < 0.01, # P < 0.001; Ex-4, exendin-4; IS, ischemia; IH, induced hyperglycemia; a, vehicle control group; b, induced hyperglycemia group; c, induced hyperglycemia with insulin treatment group; d, induced hyperglycemia with Ex-4 treatment group.
hyperglycemia; a, vehicle control group; b, induced hyperglycemia group; c, induced hyperglycemia with insulin treatment group; d, induced hyperglycemia with Ex-4 treatment group.
**Experimental protocol**

**B: Temporal changes in rCBF until 24h after reperfusion**

**C: Changes in plasma glucose level**

**D: Changes in plasma insulin level**

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**B: Blood before dextrose injection**

- **B: IS**
  - 30min after IS
  - 90min after IS
  - 180min after IS
  - 24h after IS

**Dextrose injection**

- 60 min ischemia

**Ex-4 or insulin injection**

- 24h after ischemia
- 48h after ischemia
- 7days after ischemia

**Changes in plasma glucose level**

- **Plasma glucose (mg/dl)**
  - **vehicle**
  - **IH**
  - **IH+insulin**
  - **IH+Ex-4**

**Changes in plasma insulin level**

- **Plasma insulin (ng/ml)**
  - **vehicle**
  - **IH**
  - **IH+insulin**
  - **IH+Ex-4**

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**B: Blood before dextrose injection**

- **Blood after IS**
  - 30min after IS
  - 90min after IS
  - 180min after IS
  - 24h after IS

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**D: Changes in plasma insulin level**

- **Blood before dextrose injection**
  - 30min after IS
  - 60min after IS
  - 90min after IS
  - 120min after IS
  - 180min after IS
  - 24h after IS

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**C: Changes in plasma glucose level**

- **Blood glucose (mg/dl)**
  - **vehicle**
  - **IH**
  - **IH+insulin**
  - **IH+Ex-4**

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**D: Changes in plasma insulin level**

- **Plasma insulin (ng/ml)**
  - **vehicle**
  - **IH**
  - **IH+insulin**
  - **IH+Ex-4**

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**B: Temporal changes in rCBF until 24h after reperfusion**

- **rCBF/% rCBF (pre operation)**
  - Before operation
  - During operation
  - 24h after IS

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**D: Changes in plasma insulin level**

- **Blood before dextrose injection**
  - 30min after IS
  - 60min after IS
  - 90min after IS
  - 120min after IS
  - 180min after IS
  - 24h after IS

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**C: Changes in plasma glucose level**

- **Blood glucose (mg/dl)**
  - **vehicle**
  - **IH**
  - **IH+insulin**
  - **IH+Ex-4**

---

**D: Changes in plasma insulin level**

- **Blood before dextrose injection**
  - 30min after IS
  - 60min after IS
  - 90min after IS
  - 120min after IS
  - 180min after IS
  - 24h after IS

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**B: Blood before dextrose injection**

- **Blood after IS**
  - 30min after IS
  - 90min after IS
  - 180min after IS
  - 24h after IS

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**D: Changes in plasma insulin level**

- **Blood before dextrose injection**
  - 30min after IS
  - 60min after IS
  - 90min after IS
  - 120min after IS
  - 180min after IS
  - 24h after IS
A Infarct volume and brain swelling

B Neurological severity score

C Survival rate of each groups until 7d after ischemia

D TUNEL staining
A macroscopic hemorrhages

B IgG staining

C Zymographic analysis of MMP-9

D The expression of MMP-9 on cerebro-vascular endothelial