Pancreatic Changes in Nerve Growth Factor/TrkA Associated with Insulin Secretion in Cerebral Ischemia

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Insulin Secretion in Cerebral Ischemia

Regulation of blood glucose levels as a therapeutic strategy for cerebral ischemia plays an important role in suppressing neuronal damage. In particular, suppression of post-ischemic glucose intolerance improves cerebral ischemia. We have reported that cerebral ischemia induces glucose intolerance and an increase in plasma insulin levels. However, the mechanism of insulin secretion after cerebral ischemia is unclear. Nerve growth factor (NGF), a member of the neurotrophin family, has high affinity for tropomyosin-related kinase A (TrkA). NGF/TrkA signaling is associated with neuronal survival, differentiation, and function. Recently, NGF/TrkA signaling has been reported to be associated with insulin synthesis and secretion. In the present study, we evaluated the insulin content and expression of NGF/TrkA by immunofluorescence and Western blotting after middle cerebral artery occlusion (MCAO) as a cerebral ischemia model. At 6, 12, and 24 h after MCAO, insulin contents were increased in MCAO mice. The expression of NGF was increased at 6, 12, and 24 h, whereas the expression of TrkA tended to decrease in pancreas after MCAO. These results suggest that NGF/TrkA signaling is an important factor in cerebral ischemia-induced insulin synthesis and secretion in the pancreas.

Key words cerebro ischemia; insulin; pancreas; nerve growth factor; tropomyosin-related kinase A

Diabetes and hyperglycemia are important risk factors in the development of cerebral ischemia, including stroke and elevated neuronal damage.\(^1,2\) In the clinical field, cerebral ischemia induces hyperglycemia, including glucose intolerance.\(^3,4\) The maintenance of normal blood glucose levels after cerebral ischemia improves prognoses for neuronal damage and death rate.\(^2\) In our previous studies, post-ischemic glucose intolerance was induced by focal cerebral ischemia, mediated by cerebral ischemia-induced insulin resistance via a decrease in hepatic insulin receptors and accompanied by an increase in gluconeogenic enzymes.\(^5\) We have clearly confirmed that plasma insulin levels are drastically increased by cerebral ischemia.\(^6\) However, the mechanisms of insulin regulation under ischemic conditions remain unknown.

Insulin is synthesized and secreted in the pancreas. Hyperglycemia and/or diabetes increase insulin contents and its synthesis in the pancreas.\(^7\) Interestingly, insulin itself has a neuroprotective effect and improves cerebral ischemia by reducing blood glucose levels following systemic administration.\(^8,9\)

In general, neurotrophins, such as nerve growth factor (NGF) and its receptor, tropomyosin-related kinase A (TrkA), a member of the tropomyosin-related kinase family (Trk), are involved in neuronal development, differentiation, and function.\(^9\) Recently, it has been reported that NGF/TrkA signaling is associated with systemic glucose metabolism.\(^10\) NGF is also synthesized and TrkA is expressed in \(\beta\) cells in the pancreas.\(^1\) The activation of NGF/TrkA signaling increased insulin synthesis, and its secretion is promoted by glucose stimulation.\(^1,12\) Under hyperglycemic conditions, NGF mRNA is increased in the pancreas.\(^5\) In contrast, increased insulin secretion under hyperglycemic conditions induces the up-regulation of NGF in the pancreas.\(^13\) It is thus possible that pancreatic NGF/TrkA signaling is associated with systemic glucose metabolism.

In the present study, we investigated the effect of insulin conditions in the pancreas on changes in the expression of NGF/TrkA after cerebral ischemia.

MATERIALS AND METHODS

Ethics Approval of the Study Protocol All procedures were in accordance with the Guiding Principles for the Care and Use of Laboratory Animals adopted by the Japanese Pharmacological Society. The study protocol was approved by the Animal Ethics Committee of Kobe Gakuin University, Kobe, Japan (approval number: A14–21).

Animals Male ddY mice (5 weeks old; Japan SLC, Inc., Shizuoka, Japan) were housed in an animal room maintained at 24°C and 55±5% humidity on a 12-h light–dark cycle (light phase, 08:00–20:00). Mice were provided with food and water ad libitum.

Animal Model of Focal Cerebral Ischemia The experimental transient focal ischemia mouse model was produced by middle cerebral artery occlusion (MCAO) and reperfusion as described previously.\(^14\) In briefly, the MCAO was performed under isoflurane anesthesia. The common carotid artery (CCA) and external carotid artery were first ligated, followed by isolation of the internal carotid artery. The left middle carotid artery was occluded for 2h by the insertion of an 8–0 nylon monofilament with a thin silicon coat (Provil\(^\text{® novo}\) Medium; Heraeus Kulzer, Hanau, Germany) through the CCA followed by reperfusion. Sham-operated mice underwent the same surgical procedure without suture insertion.

Measurement of Infarct Volume Brain was stained by 2,3,5-triphenyltetrazoliumchloride (TTC; Sigma, St. Louis, MO, U.S.A.) as described previously.\(^14\) At 24 h after MCAO, mice were killed, and their brains were immediately dissected.

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Brains were cut into 2-mm-thick coronal slices (0, +2, and +4 mm from the bregma) using a brain slicer. Brain slices were then incubated in normal saline containing 2% (w/v) TTC for 10 min at 37°C. The stained slices were then fixed with 4% paraformaldehyde (Sigma).

**Immunofluorescence** The experiments were performed as described previously.15) Mice were perfused transcardially with saline, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS). The pancreas was extracted, incubated, and fixed in PBS containing 4% PFA for 3 h and then dehydrated in 10 and 20% sucrose for 3 h followed on overnight. Tissues were then embedded and frozen in the Tissue-Tek optimal cutting temperature compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan). Frozen blocks were sliced with a cryostat (Leica CM1850; Microsystems GmbH, Wetzlar, HE, Germany), and on a MAS-coated glass slide (Matsunami Glass Ind., Osaka, Japan). Slices were post-fixed in 10% formaldehyde for 15 min and then washed with PBS containing 0.1% Tween 20 (PBS-T). Slices were exposed to 3% bovine serum albumin (BSA) for insulin in PBS-T for 1 h and then incubated overnight at 4°C with the first antibody, chicken anti-insulin (1:2000, Abcam, Tokyo, Japan). The secondary anti-insulin antibody, Alexa Fluor 594 goat polyclonal anti-chicken (1:200, Life Technologies, Carlsbad, U.S.A.) in

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**Fig. 1. Changes in Insulin Contents after Cerebral Ischemia in the Pancreas**

Insulin contents after cerebral ischemia were analyzed by immunofluorescence staining. (A) Infarction area after 24 h was stained by TTC. (B–G) Immunofluorescence staining and results. (B, D, F) Insulin, left panel; sham, right panel; MCAO. Scale bar; 100 µm. (C, E, G) Quantitative of insulin contents at each time. (B, C) Sham; n=3, MCAO; n=3. (D, E) Sham; n=3, MCAO; n=3. (F, G) Sham; n=3, MCAO; n=3. Each column represents the mean±S.E.M. **p<0.01, *p<0.05 vs. sham, unpaired Student’s t-test.
1% BSA, was incubated with slices for 2 h at room temperature.

Slices were exposed to 3% BSA for TrkA or 10% BSA for NGF in PBS-T for 1 h and then incubated overnight at 4°C with primary antibodies, rabbit anti-TrkA (1:500, Alomone, Jerusalem, Israel) or rat anti-NGF (1:50, Abcam). Slices were then incubated with the secondary antibody, Alexa Fluor 488 donkey polyclonal anti-rabbit (1:200, Life Technologies) against TrkA or Alexa Fluor 488 donkey polyclonal anti-rat against NGF (1:200, Life Technologies) for 2 h. After washing, slices were incubated for 10 min with 4',6-diamidino-2-phenylindole (DAPI) (Boster Biological Technology, Pleasanton, CA, U.S.A.). Slices were washed and then covered with Fluoromount/Plus (Thermo Shandon Inc., Pittsburgh, PA, U.S.A.). Immunoreactive signals were observed with a confocal laser scanning microscope (FV1000, OLYMPUS, Tokyo, Japan). Insulin contents were analyzed by insulin dividing positive intensity on area with Image J (National Institutes of Health, Bethesda, MD, U.S.A.).

Western Blotting These experiments were performed as described previously. In brief, the pancreas was homogenized in homogenization buffer and protein samples (40 µg) sodium dodecyl sulfate (SDS) polyacrylamide gels were prepared at 7.5% (w/v) for TrkA or 15% (w/v) for NGF. Protein was transferred onto nitrocellulose membranes (BioRad, Hercules, CA, U.S.A.). Blots were incubated overnight at 4°C with primary antibodies NGF antibody (1:1000, Abcam) in Tris-buffered saline (TBS), containing 0.1% (v/v) Tween 20 (TBS-T) and 5% (w/v) bovine serum albumin (Sigma), or TrkA antibody (1:200, Alomone) in TBS-T and blocking agent (GE Healthcare, Tokyo, Japan). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control and was detected using primary antibodies (1:20000, Chemicon, CA, U.S.A.). After washing, blots were incubated with horseradish peroxidase (HRP)-conjugated anti-rat immunoglobulin G (IgG) (1:1000; KPL, Guildford, U.K.) to NGF, anti-rabbit IgG (1:1000; KPL) against TrkA, and anti-mouse IgG (1:10000; KPL) against GAPDH for 1 h. All visualization of immunoreactive bands was performed using Light-Capture (AE-6981; ATTO, Tokyo, Japan) with an ECL™ Western blotting Analysis System (GE Healthcare). The signal intensities of immunoreactive bands were analyzed with a Cs-Analyzer (version 3.0, ATTO).

Statistical Analyses Data are mean ± standard error of the mean (S.E.M.). Statistical significance was assessed by unpaired Student’s t-test for single comparisons. A p value < 0.05 was considered significant.

Fig. 2. Changes in NGF in Pancreas after Cerebral Ischemia

The expression of NGF was observed in the pancreas as were changes in expression levels after MCAO. (A) Insulin, (B) NGF, (C) DAPI, and (D) merged images. Scale bars; 20 µm (E) on 6 h, (F) 12 h, (G) 24 h after MCAO, protein expression of NGF in the pancreas was analyzed by Western blotting. Lower panels represent Western blotting images of NGF (13kDa) or GAPDH (37kDa). Relative level of NGF was analyzed by the ratio NGF/GAPDH. (E) 6 h sham; n=3, MCAO; n=3. (F) 12 h sham; n=9, MCAO; n=9. (G) 24 h sham; n=6, MCAO; n=6. Each column represents the mean ± S.E.M. *p<0.05 vs. sham, unpaired Student’s t-test.
RESULTS

Changes in Insulin Contents after Cerebral Ischemia in the Pancreas

The infarction area was observed on day 1 after MCAO (Fig. 1A). Insulin content in the pancreas was significantly increased at 6 h (Figs. 1B, C), 12 h (Figs. 1D, E), and 24 h (Figs. 1F, G) after MCAO.

Changes in NGF in Pancreas after Cerebral Ischemia

Insulin (Fig. 2A), NGF (Fig. 2B), and DAPI (Fig. 2C) were merged (Fig. 2D) in native mice. At 6 h after MCAO, expression levels of NGF tended to increase as compared with the sham (Fig. 2E), and at 12 and 24 h, they were significantly increased in the pancreas (Figs. 2F, G).

Changes in TrkA in Pancreas after Cerebral Ischemia

Insulin (Fig. 3A), TrkA (Fig. 3B), and DAPI (Fig. 3C) were merged (Fig. 3D) in native mice. At 6 h after MCAO, expression levels of TrkA were unchanged (Fig. 3E) but tended to decrease at 12 h (Fig. 3F). Expression levels of TrkA were significantly decreased in the pancreas 24 h after MCAO (Fig. 3G).

DISCUSSION

Insulin is important for systemic glucose metabolism, and cerebral ischemia causes the impairment of insulin function. In our previous findings, the administration of intermediate-acting insulin suppressed neuronal damage after cerebral ischemia, suggesting that not only the development of cerebral ischemia-induced insulin resistance alone but also an insufficiency of insulin secretion in response to elevated blood glucose may lead to hyperglycemia after cerebral ischemia. In addition, it should be determined whether the “protective effect” of insulin as a growth factor contributes to the regulation of post-ischemic hyperglycemic conditions. For this reason, it is desirable to investigate the mechanism of insulin secretion in the pancreas after cerebral ischemia. We have previously reported that post-ischemic hyperglycemia began to develop within 6 h after cerebral ischemia and was significantly increased at 12 and 24 h. In the present study, insulin contents were clearly increased 6 h after cerebral ischemia and before blood glucose levels were elevated. Sympathetic nerve activity is also induced by cerebral ischemia.
Considering other reports, it is clear that NGF is an important regulator of the synthesis and secretion of insulin by the pancreas. In the present study, NGF was dramatically increased from 6 to 24 h after cerebral ischemia. In previous studies, the sympathetic neurotransmitter norepinephrine induced the elevation of pancreatic NGF levels, which were dramatically inhibited by propranolol, a β-adrenergic receptor blocker. These results suggested that the activation of the sympathetic nervous system also increases NGF secretion from the pancreas, i.e., elevated NGF may be responsible for the cerebral ischemia-induced increase of insulin contents.

NGF is synthesized in β cells and promote the synthesis and secretion of NGF by autocrine/paracrine. In addition, expression levels of both NGF and TrkA are regulated by hyperglycemic conditions or diabetes. Thus, the activation of NGF/TrkA signaling may also promote insulin secretion after cerebral ischemia. In peripheral tissues, NGF is synthesized and promoted predominantly by the activation of sympathetic nerves. However, our findings showed that TrkA is decreased 24 h after cerebral ischemia. It may act via a negative feedback system by a continuous stimulation of early-elevated NGF. In contrast, the activation of NGF/p75 signaling reduces the expression levels of TrkA. The expression level of p75 is increased by hyperglycemic conditions or diabetes. Thus, the activation of NGF/TrkA signaling may also promote insulin secretion after cerebral ischemia. However, our findings showed that TrkA is decreased 24 h after cerebral ischemia. It may act via a negative feedback system by a continuous stimulation of early-elevated NGF. In contrast, the activation of NGF/p75 signaling reduces the expression levels of TrkA. The expression level of p75 is increased by hyperglycemic conditions or diabetes. Thus, the activation of NGF/TrkA signaling may also promote insulin secretion after cerebral ischemia. However, our findings showed that TrkA is decreased 24 h after cerebral ischemia. It may act via a negative feedback system by a continuous stimulation of early-elevated NGF. In contrast, the activation of NGF/p75 signaling reduces the expression levels of TrkA. The expression level of p75 is increased by hyperglycemic conditions or diabetes. Thus, the activation of NGF/TrkA signaling may also promote insulin secretion after cerebral ischemia.

In conclusion, the mechanism of increased insulin content and secretion after cerebral ischemia may be closely associated with the activation of NGF/TrkA signaling. Understanding of the insulin regulation system following cerebral ischemia may contribute to the development of a therapy for reducing the neuronal damage from cerebral ischemia.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES


